Handbook of the Protists

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Editors

Handbook of the Protists

Second Edition

With 383 Figures and 51 Tables

Springer

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2nd Edition of The Handbook of Protoctista. The Structure, Cultivation, Habitats, and Life Histories of the Eukaryotic Microorganisms and their Descendants Exclusive of Animals, Plants, and Fungi. A Guide to the Algae, Ciliates, Foraminifera, Sporozoa, Water Molds, Slime Molds, and Other Protoctists. Lynn Margulis, John O. Corliss, Michael Melkonian, David J. Chapman (eds.), Jones and Bartlett Publishers, Boston, 1990.

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In memory of Lynn Margulis (1938–2011),
tireless champion of the microbial biosphere

Foreword

Be it for their importance in the planet's food web, for their originality in carrying out their cellular processes, or for their Haeckelian elegance, one writing about the protists soon finds oneself composing a paean. As is true for any of the large groupings of living organisms, protists are varied, complex, and beautiful – only more so. They are a grab bag of hugely distinct organisms, but what a grab they are! Their endless fascination beguiles students of all aspects of life, those with orderly, as well as those with unruly minds.

Protist variety is easily told by the vast range of their dimensions. Even leaving aside the giant algae, readily visible with the naked eye, many (e.g., *Ostreococcus tauri*) are minimalists hovering at the one micrometer lower limit of eukaryotic size. Some have very large genomes – the claim has been made that the appropriately named amoeba *Polychaos dubium* has 670 billion base pairs! Others, such as the parasitic microsporidian *Encephalitozoon cuniculi*, manage with a minute 2.9 million base pairs.

Cellular complexity is carried to extremes by the protists. The ciliates and flagellates (mastigotes), for example, possess – to shamelessly use anthropomorphic terms – a mouth (some with fancy lips), a stomach, an anus, a bladder, multiple propulsion devices, and an armamentarium of weapons that allow them to feed on other microbes. And some dinoflagellates sport that most amazing of cell structures, the eye-like ocelloid. In multicellular organisms, such structures and their functions are assigned to specialized cells; in the protists, one cell does it all by itself. One wonders about the trade-off involved, in doing everything yourself versus employing a differentiated consortium to do the work. The very existence of protist complex body plans brings up the distinction between unicellularity and multicellularity, a blurry one at best, but one especially relevant to this field. Multicellularity has originated on multiple occasions within the protists, hence they are most likely to provide relevant answers to our conjectures.

The protists also make eccentric uses of chemicals, including some elements that are seldom employed in the biological world. Diatoms use silica for their shells (tests), which is exciting enough, but other planktonic organisms (the acantharia or "ray animalcules") make skeletons out of strontium sulfate (celestite)! Yet others form intracellular crystals of barium sulfate (barite). And many there are that make structures of imposing beauty. Some, such as the radiolarians, coccolithophorids,

and diatoms, are pure eye candy. Moreover, their skeletons have accumulated in formations of such massive dimensions that they have changed the Earth's geology.

Photosynthetic protists (algae) account for perhaps half the world's total photosynthesis and are indispensable for life on Earth. The others, the heterotrophic protists, make their living largely by eating other microbes and also play a huge role in the movement of nutrients on the planet. They graze on prokaryotes, which by and large they regard as packets of nutrients, and in the process regulate prokaryotic population densities in many natural environments. They are themselves eaten, often by other protists. One wonders how many layers there are to this.

Letting my mind wander. . .about what would happen if Nobel Prizes were given to organisms for having evolved important and readily ascertainable phenomena, *as well as* to the researchers who discovered such evolutionary achievements. Surely, *Tetrahymena* warrant multiple prizes for contributing the first known example of splicing and ribozymes, plus telomeres and telomerases. And *Paramecium* also comes to mind for cortical inheritance and other genetic marvels. Also, doesn't the invention of the macronucleus – that talented device that permits rapid growth of intricate cells – deserve a special accolade? Someday, as researchers continue to delve into this rich treasure trove, many more organisms will qualify. Possibly *Oxytrichia*, *Cyanidioschyzon*, *Ostreococcus* or, closer to immediate human concerns, *Giardia*, trypanosomes, and *Toxoplasma*. Make your own list of protists deserving to become Nobelists.

Whatever phylogenetic diagram you prefer, most of the eukaryotic lineages by far will be of protists. Their evolution started at the very emergence of the eukaryotic cell and has repeatedly involved the lateral transfer of whole genomes. In some instances, endosymbiotic events have occurred not just once, but twice and even three times in the same lineage. It is evident that evolution here did not proceed by timid jumps, but rather by bold leaps.

For these reasons and many others I have not brought up, protists are the basis for the understanding of all eukaryotic life in both time and space. So, the study of protists is not an obscure topic to be left to a few specialists. It is the concern of all biologists as well as planetary scientists and who knows who else?

I was asked to write this piece by the late Lynn Margulis, a friend of old. I tried to wiggle out of it, but those of you who reacted in such manner to a request of hers should be smiling by now. In all ways, scientific, intellectual, and personal, she was utterly irresistible. It worked out – I am glad to contribute to this work, a fitting tribute to her unique contribution to the field in which she started in science and which she never forsook.

Moselio Schaechter

Preface to the Second Edition

This *Handbook of the Protists* is the 2nd edition of the *Handbook of Protoctista*, which was published in 1990 and edited by Lynn Margulis, John O. Corliss, Michael Melkonian, and David J. Chapman. The subject is the biology, diversity, and evolution of eukaryotic (nucleus-containing) microbes and their descendants, exclusive of animals, land plants, and typical fungi. The new edition largely follows the structure of the original Handbook, but its content has changed dramatically to reflect 27 years of progress in many areas of life science research, including microscopy, microbial ecology, biochemistry, molecular biology, and phylogenetics.

The new Handbook contains some 44 chapters, each focused on a different group of protists. Taxonomic rank varies somewhat from chapter to chapter; each includes a Summary Classification of the lineages discussed therein. Many of the chapters are thorough updates of those appearing in the original Handbook, although a few are not retained in the new edition; in most cases these omissions reflect shifts in knowledge stemming from a more complete understanding of the large-scale phylogenetic structure of eukaryotic diversity. For example, Ellobiopsida ("Incertae Sedis" in the 1st Ed.) are no longer covered as a stand-alone chapter, as the group is now within Dinoflagellata. The fate of Phylum Myxozoa (original chapter published by the late Jiří Lom) reveals a stunning twist in the history of knowledge. Myxozoan parasites were usually seen as an enigmatic group of spore-forming "protozoa"; however, molecular phylogenetics confirmed that they are in fact a highly derived lineage of animals. Still other chapters have no counterpart in the original – they explore groups of protists that have gained substantially in importance over the past quarter century. And a handful of chapters address traditional protist assemblages based on morphology that do not correspond to evolutionarily unified groups; in these cases the different subgroups are clearly distinguished.

The opening chapter, entitled "Protist Diversity and Eukaryote Phylogeny," serves as a guide to the Handbook's overarching structure and content. It summarizes the biology of the currently recognized high-level protist lineages and provides references to the literature for those wishing to learn more about specific groups that are not covered in detail in this Handbook.

In writing and updating their chapters, authors were given the freedom to adhere to terms used in the original Handbook (e.g., "protoctists" = protists,

"undulipodia" = flagella) or not and encouraged to bring all relevant research to the table, including advances coming from molecular biology and molecular phylogenetics.

Key Features at a Glance

- Explores the ecological, medical, and economic importance of major groups of protists
- Covers the morphology, molecular biology, biochemistry, ecology, and fossil record of protists
- Collates work on an unparalleled breadth of eukaryotic microorganisms
- Is organized by current protist systematics, as informed by molecular phylogenetics and genomics

June 2017

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Alastair G. B. Simpson
Claudio H. Slamovits

List of First Edition Chapters and Contributors

I. Phyla in Which Members Lack Undulipodia at All Stages and Which Lack Complex Sexual Life Cycles

1. Rhizopoda, *F.L. Schuster*
2. Haplosporidia, *F.O. Perkins*
3. Paramyxea, *I. Desportes, F.O. Perkins*
4. Myxozoa, *J. Lom*
5. Microspora, *E.U. Canning*

II. Phyla in Which Members Lack Undulipodia at All Stages of Their Life Cycles and Which Display Complex Sexual Life Cycles

6. Acrasea, *R.L. Blanton*
7. Dictyostelida, *J.C. Cavender*
8. Rhodophyta, *P.W. Gabrielson, D.J. Garbary, M.R. Sommerfeld, R.A. Townsend, P.L. Tyler*
9. Conjugaphyta, *R.W. Hoshaw, R.M. McCourt, J.C. Wang*

III. Phyla in Which Members Display Reversible Formation of Undulipodia and Lack Complex Sexual Life Cycles

10. Xenophyophora, *Ø.S. Tendal*
11. Cryptophyta (Cryptomonads), *M. Gillott*
12. Glaucocystophyta, *L. Kies, B.P. Kremer*
13. Karyoblastea, *J.M. Whatley, C. Chapman-Andresen*
14. Zoomastigina

    Classes:

    a. Amebomastigota, *B.D. Dyer*
    b. Bicoecids, *B.D. Dyer*
    c. Choanomastigotes (Choanoflagellates), *K.R. Buck*
    d. Diplomonadida, *K. Vickerman*
    e. Pseudociliata, *J.O. Corliss*
    f. Kinetoplastida, *K. Vickerman*
    g. Opalinata, *J.O. Corliss*
    h. Proteromonadida, *G. Brugerolle, J.P. Mignot*
    i. Parabasalia, *B.D. Dyer*
    j. Retortamonadida, *G. Brugerolle, J.P. Mignot*
    k. Pyrsonymphida, *B.D. Dyer*

Acknowledgments

For us, this project began shortly after the unfortunate passing of Lynn Margulis. It has been a long and convoluted journey, and there are many people to thank for input along the way. First and foremost, we thank all the authors for their enthusiasm, patience, and hard work in producing authoritative chapters that reflect the tremendous advances that have taken place in so many different areas of protistology. And of course, for much of this work there is a huge debt owed to the authors of the original Handbook's chapters. These have served as the bedrock upon which the second edition is founded, even when the new chapters have been mostly or entirely rewritten to accommodate a quarter century of scientific advances. Michael Melkonian, David Chapman, and Yuemei Corliss are thanked for their help during the early stages of the project, and we greatly appreciate O. Roger Anderson for providing the initial link to Springer. We are grateful to Lars Koener, Associate Editor at Springer, for his interest and oversight in transitioning the Handbook into a Springer Reference Work. Susanne Friedrichsen, Neha Thapa, Flora Kenson, Monika Garg, Ankita Awasthi, Abhijit Baroi, and the rest of the team at Springer are thanked for their editorial expertise. We thank Marlena Dlutek for in-house assistance at Dalhousie University. Finally, we thank Jennifer Margulis for advice, assistance, and support. Jennifer felt strongly that Lynn would have wanted this book to serve as many students and professionals as possible, both within and beyond the protistological community. It is our sincere hope and belief that the *Handbook of the Protists* is up to the task.

June 2017

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About the Editors

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Alastair G. B. Simpson is a Professor of Biology at Dalhousie University and a Senior Fellow of the Canadian Institute for Advanced Research, Program in Integrated Microbial Biodiversity. He was an Associate Editor of the *Journal of Eukaryotic Microbiology* for more than a decade and is now a Monitoring Editor of *Protist*. He has served on the Systematics Committee of the International Society of Protistologists since 2004 (and is the current Chair) and received the Society's Seymour H. Hutner Young Investigator Prize in 2013. He has authored more than 100 scientific articles.

Claudio H. Slamovits is an Associate Professor in the Department of Biochemistry and Molecular Biology at Dalhousie University in Halifax, Nova Scotia. He is a Fellow of the Canadian Institute for Advanced Research, Program in Integrated Microbial Biodiversity. Dr. Slamovits is an active researcher with interests in the molecular biology and evolution of microbial eukaryotes. He holds board and committee memberships in the International Society for Evolutionary Protistology (ISEP) and the International Society of Protistologists (ISOP).

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Protist Diversity and Eukaryote Phylogeny

1

Alastair G. B. Simpson, Claudio H. Slamovits, and John M. Archibald

Abstract

The last quarter century has seen dramatic changes in our understanding of the phylogenetic relationships among protist groups and their evolutionary history. This is due in large part to the maturation of molecular phylogenetics, to genomics and transcriptomics becoming widely used tools, and to ongoing and accelerating progress in characterizing the major lineages of protists in the biosphere. As an introduction to the Handbook of the Protists, Second Edition, we provide a brief account of the diversity of protistan eukaryotes, set within the context of eukaryote phylogeny as currently understood. Most protist lineages can be assigned to one of a handful of major groupings ("supergroups"). These include Archaeplastida (which also includes land plants), Sar (including Stramenopiles/Heterokonta, Alveolata, and Rhizaria), Discoba, Metamonada, Amoebozoa, and Obazoa. This last group in turn contains Opisthokonta, the clade that includes both animals and fungi. Many, but not all, of the deeper-level phylogenetic relationships within these groups are now resolved. Additional well-known groups that are related to Archaeplastida and/or Sar include Cryptista (cryptophyte algae and their relatives), Haptophyta, and Centrohelida, among others. Another set of protist lineages are probably most closely related

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to Amoebozoa and Obazoa, including Ancyromonadida and perhaps Malawimonadidae (though the latter may well be more closely related to Metamonada). The bulk of the known diversity of protists is covered in the following 43 chapters of the Handbook of the Protists; here we also briefly introduce those lineages that are not covered in later chapters.

The Handbook is both a community resource and a guidebook for future research by scientists working in diverse areas, including protistology, phycology, microbial ecology, cell biology, and evolutionary genomics.

Keywords

Algae • Alveolata • Amoebozoa • Archaeplastida • Biodiversity • Discoba • Eukaryote • Metamonada • Obazoa • Opisthokonta • Phylogeny • Protist • Protozoa • Rhizaria • Sar • Stramenopiles

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Introduction

It has been more than 25 years since the publication of the Handbook of Protoctista (Margulis et al. eds. 1990). Since then, there have been tremendous advances in our understanding of the diversity and phylogeny of protists/protoctists (i.e., all eukaryotes other than the animals, land plants, and true fungi; we will use the term "protist"). Central to this progress has been the maturation of molecular phylogenetics as a tool for inferring evolutionary relationships, initially using single markers, such as small subunit ribosomal RNA gene sequences, and culminating in "phylogenomic analyses" that incorporate data from dozens or hundreds of genes (van de Peer and De Wachter 1997; Baldauf et al. 2000; Rodríguez-Ezpeleta et al. 2007; Burki et al. 2007; Burki 2014). Genome sequencing (of organellar genomes as well as nuclear genomes), together with transcriptomic surveys, has also greatly enhanced our understanding of the distribution of important cellular and molecular characteristics across the breadth of eukaryotic diversity (e.g., Lang et al. 1997; Ramesh et al. 2005; Hodges et al. 2010; de Mendoza et al. 2014; Wideman and Muñoz-Gómez 2016). At the same time, the discovery of new major lineages of protists (and reinvestigations of known "mystery taxa") has continued apace and even accelerated in recent years. This has resulted in dramatic changes to the catalogue of organisms that are important to consider when inferring the broadscale tree of eukaryote life (e.g., O'Kelly and Nerad 1999; Shalchian-Tabrizi et al. 2006; Not et al. 2007; Yabuki et al. 2010; Glücksman et al. 2011), on top of many important discoveries of novel diversity within major lineages (e.g., Moore et al. 2008; Massana et al. 2014; see numerous other examples below). There have also

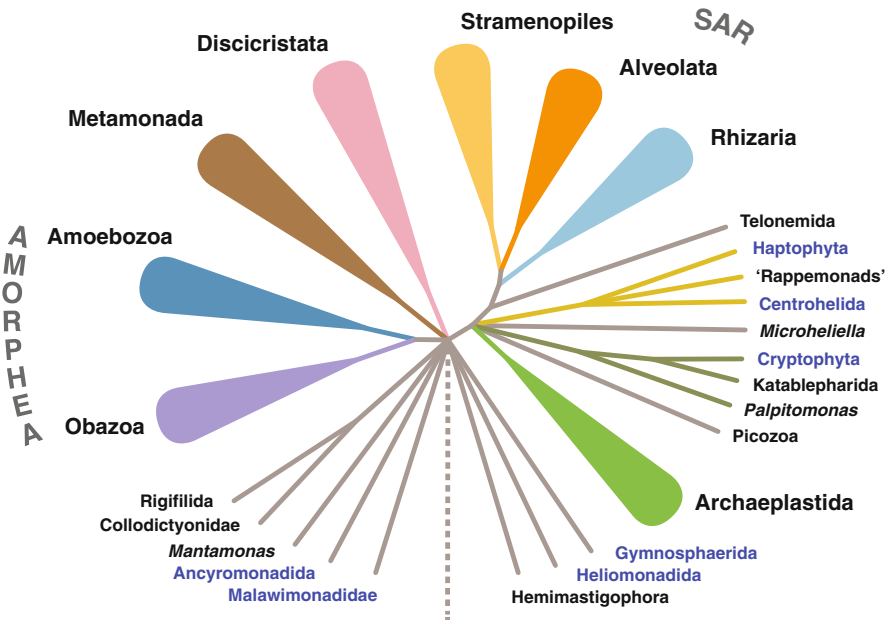

Fig. 1 Phylogeny of eukaryotes, based primarily on Brown et al. (2013), Cavalier-Smith et al. (2014), Kamikawa et al. (2014), Yabuki et al. (2014), Burki et al. (2016), and Leger et al. (2017). Groups with bulbous branches are examined in more detail in Figs. 2–5. Groups with narrow branches do not belong to well-established supergroups and are not illustrated separately; those covered in the Handbook are shown in *blue* and are as follows: ▶ Cryptophyta; ▶ Haptophyta; ▶ Centrohelida; ▶ Ancyromonadida; ▶ Malawimonadidae; ▶ Gymnosphaerida; ▶ Heliomonadida

been a number of important insights gained from electron microscopy studies, especially of the flagellar apparatus and cytoskeleton (e.g., Simpson 2003; Cavalier-Smith 2013; Heiss et al. 2013; Yubuki and Leander 2013).

The current picture of the tree of eukaryotic life can be characterized as largely resolved but with some major points of uncertainty. At present, it is common to divide the vast majority of known eukaryotic diversity into four to eight confirmed (or strongly suspected) monophyletic groups, usually referred to by the informal moniker "supergroups" (Fig. 1; Adl et al. 2012; Burki 2014; Worden et al. 2015; Simpson and Eglit 2016). The precise number and membership of the supergroups varies among accounts, reflecting not just personal taste but also the rapid pace with which important taxa are being added to broad molecular phylogenetic analyses. These supergroups are best thought of as standing well above the rank of "kingdom." For example, the animals and true fungi are generally each considered as a distinct kingdom but belong to the same supergroup (Obazoa, in our listing). The supergroups are often now amalgamated into as few as three or even two still more fundamental assemblages (Adl et al. 2012; Derelle et al. 2015), although this entails some bold assumptions about the position of the root of the tree (see below).

Our current understanding of eukaryote phylogeny confirms and extends a long-understood reality, namely, that most of the basic forms of protists identified by superficial morphology and physiology do not represent evolutionarily cohesive entities. Photosynthetic protists, or "algae," are found within most of the supergroups. In all of these groups (with the arguable exception of Archaeplastida – see below), the algae are intermingled with other kinds of protists, mainly "protozoa" (a term used to describe most heterotrophic protists, other than those that closely resemble fungi). Among the protozoa, "flagellates" (species which have eukaryotic flagella in the main feeding stage of their life history) are found across eukaryote diversity, reflecting the fact that all living eukaryotes descend from a heterotrophic, flagellum-bearing common ancestor. Amoebae, which lack flagella but produce one of the several distinct forms of pseudopodia, have evolved independently on multiple occasions, leading to a dozen or more major radiations of these lifeforms. Special forms of amoebae also have multiple origins. One example is the "heliozoa" – amoebae with many microtubule-supported pseudopodia radiating from a rounded cell body – which have at least three independent origins and likely more (Nikolaev et al. 2004; Bass et al. 2009). The "slime molds" are organisms that live mostly as amoebae (or as giant amoeboid plasmodia) but that also produce stalked structures bearing spores, either by differentiation of a single organism or by numerous amoebae aggregating together: collectively these strategies have evolved several times (Brown et al. 2012; Shadwick et al. 2009). Parasitic protozoa that are passed between hosts via infective spores have also evolved on numerous occasions. Other protists with more-or-less similarity to true fungi (e.g., they produce hyphae-like structures) are found in several different places within the eukaryote tree, mostly very distantly related to true fungi (Taylor and Berbee 2014).

As mentioned above, not all aspects of the deep-level phylogeny and evolutionary history of eukaryotes are well understood at present, which has consequences for any summary of protist diversity. Some important uncertainties and controversies revolve around particularly difficult problems in molecular phylogenetic inference. For example, it remains unclear what the relationships are among "excavate" lineages (Discoba, Metamonada, and Malawimonadidae), which include many groups with high overall rates of sequence evolution. The majority view at present is that they form two or more phylogenetically separate clades (Burki 2014). In a similar vein, phylogenomic analyses have yet to resolve whether the supergroup Archaeplastida truly represents a clade or whether other lineages (especially the Cryptista group) may belong inside it (Yabuki et al. 2014; Burki et al. 2016). The ongoing discovery of new lineages (discussed above) is itself a source of uncertainty, not least because it is unclear how many major lineages remain to be found and characterized. One of the most important open questions in eukaryote evolution concerns the precise history of plastids (chloroplasts). Most major lineages of photosynthetic eukaryotes actually have plastids that were obtained by symbiosis with eukaryotic algae, rather than by symbiosis with cyanobacteria; the number, sequence, and directions of these distinct eukaryote-eukaryote endosymbiotic events are all still unclear (Keeling 2013; Archibald 2015). Finally, one of the most difficult questions for eukaryote phylogeny is locating the "root" of the tree, that is,

identifying the very deepest division among the extant eukaryotes. Several mutually incompatible positions have been proposed in recent years, based mostly on sophisticated phylogenomic analyses or the distribution of particular genes across major groups of eukaryotes (e.g., Cavalier-Smith 2010; Derelle and Lang 2012, 2015; Katz et al. 2012; He et al. 2014).

An Overview of Protist Diversity

The remainder of this chapter gives a concise, up-to-date, and (in our view) appropriately cautious summary of the diversity and phylogeny of eukaryotes. The main aim is to provide a broad phylogenetic context for the various other chapters in the Handbook of the Protists, Second Edition (hereafter, "the Handbook"). The majority of these chapters cover a single phylogenetically coherent group and will then have a single placement within the account below. There are a few chapters that instead cover two or more unrelated groups that have historically been considered together (e.g., "heliozoa"); these chapters will be referenced more than once for this reason. Furthermore, we have sought to briefly introduce the important groups of protists that are not covered separately in the Handbook (for reasons of logistics alone; no perception of insignificance should be inferred in these cases). In some of these instances, we direct the reader to recent (2010–onward) publications that are reviews or are reasonably broad in scope. For the sake of brevity, single genera of uncertain phylogenetic position within eukaryotes are omitted (see Adl et al. 2012 for a partial listing), and most lineages known solely as environmental sequences are not discussed.

Archaeplastida (Fig. 2) The supergroup Archaeplastida (meaning "ancient plastids"; sometimes instead called Plantae) consists of the three principal photosynthetic groups with "primary" plastids, in other words eukaryotes whose plastids/chloroplasts were acquired directly through a symbiosis with a cyanobacterium. There is strong phylogenetic evidence, especially from the plastid genome and plastid-associated biochemical features (e.g., the protein import machinery), that true plastids stem from a single event of primary endosymbiosis and thus that all archaeplastids descend from a common primary plastid-containing ancestor (Reyes-Prieto et al. 2007; Price et al. 2012). ► Glaucophyta (also known as Glaucocystophyta) is the most obscure of the three archaeplastid lineages. Glaucophytes are rare freshwater algae that mostly associate with surfaces. ► Rhodophyta consists of several thousand described species of algae, most of which are marine. They range from a few unicellular species, to diverse filamentous forms, to complex red seaweeds. The third group, Chloroplastida (also known as Chlorobionta or Viridiplantae), includes both the green algae and the land plants. It is divided into two large clades, streptophytes and chlorophytes, with the former including land plants, as well as many green algae; streptophyte green algae are often referred to as "charophytes," and the best studied groups are the ► Zygnematophyta, which are unicellular or filamentous freshwater forms, and the ► Charophyceae (Charales), which are truly multicellular freshwater "plants." Despite

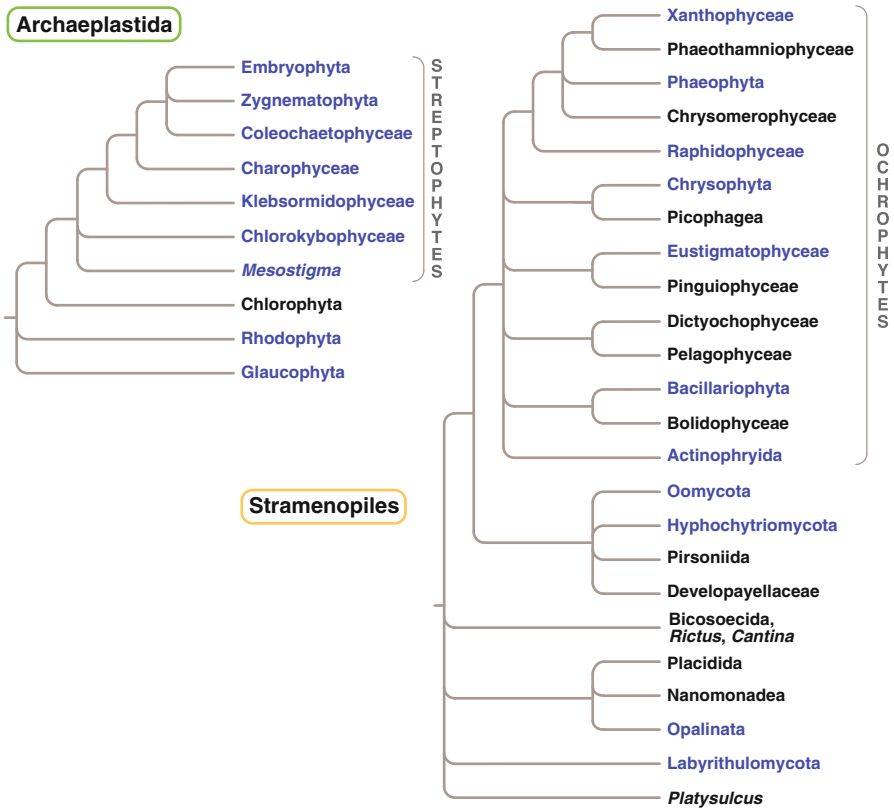

Fig. 2 Summary phylogenetic trees for Archaeplastida and Stramenopiles, based primarily on Leliaert et al. (2012) and Wickett et al. (2014) (Archaeplastida) and Riisberg et al. (2009), Cavalier-Smith and Scoble (2013), Yubuki et al. (2015), Shiratori et al. (2015), and Derelle et al. (2016) (Stramenopiles). Groups covered in Handbook chapters are shown in *blue* and are as follows: Archaeplastida: ▶ Glaucophyta; ▶ Rhodophyta; ▶ Zygnematophyta; ▶ Charophyceae; ▶ Chlorokybophyceae, Klebsormidiophyceae, Coleochaetophyceae, *Mesostigma*. Stramenopiles: ▶ Bacillariophyta; ▶ Phaeophyta; ▶ Raphidophyceae; ▶ Chrysophyta; ▶ Eustigmatophyceae; ▶ Xanthophyceae; ▶ Actinophryida; ▶ Hyphochytriomycota and Oomycota; ▶ Labyrinthulomycota; ▶ Opalinata. MAST clades without described representatives are not shown (see text)

the similarity in complexity between Charophyceae and land plants, recent phylogenetic evidence strongly indicates that land plants are more closely related to Zygnematophyta (Leliaert et al. 2012; Wickett et al. 2014). The remaining charophyte lineages, ▶ Chlorokybophyceae, Klebsormidiophyceae, Coleochaetophyceae, *Mesostigma*, are all discussed together. Chlorophytes include a wide diversity of unicellular flagellates (and some complex colonial forms), nonflagellated unicells and colonies, filamentous forms, and some more complex macroalgae, including green seaweeds. They are shown as a single branch in Fig. 2, but in reality, they are phylogenetically diverse. The best known subgroups include the Chlorophyceae (e.g., *Chlamydomonas*, *Volvox*), Ulvophyceae (marine macroalgae), and

Trebouxiophyceae. There are several additional distinct lineages, mostly of small flagellates, that collectively are referred to as "prasinophytes." The chlorophyte groups are not covered in the Handbook; the phylogeny and diversity of green algae, especially chlorophytes, is reviewed by Leliaert et al. (2012).

Sar; Stramenopiles (Fig. 2) The supergroup "Sar" (also known as SAR or Harosa) was identified through multigene/phylogenomic analyses (Burki et al. 2007; Hackett et al. 2007) and includes three lineages that are each hugely diverse and speciose in their own right: Stramenopiles, Alveolata, and Rhizaria (SAR is an acronym for these three groups). Stramenopiles, also known as Straminipila or Heterokonta, is distinguished by a characteristic form of rigid tubular flagellar hairs (the group name means "straw hairs"), although these have been lost in many species and several whole subgroups. Stramenopiles includes a wide range of photosynthetic forms as well as many heterotrophs (see Cavalier-Smith and Scoble 2013). Photosynthetic stramenopiles, also known as ochrophytes, have plastids derived ultimately from a red algal donor and form a monophyletic group (Cavalier-Smith and Scoble 2013; Derelle et al. 2016). The best known are the diatoms (► Bacillariophyta), which are unicellular/colonial forms with bipartite siliceous "cell walls" that are of huge ecological importance in the marine microplankton (for example), and the filamentous or genuinely multicellular ► Phaeophyta (Phaeophyceae), informally known as brown algae. As it happens, neither of these groups are flagellated in the vegetative state; the characteristic stramenopile flagellar hairs are seen only in (some) reproductive stages. Other ochrophyte groups include ► Raphidophyceae (Raphidophyta) and ► Chrysophyta, which are flagellates (though some famous chrysophytes are colonial and many are no longer photosynthetic), the mostly unicellular ► Eustigmatophyceae, and the ► Xanthophyceae, which are often filamentous and are among the closest relatives of the brown algae. Other, more obscure, groups of ochrophytes include Phaeothamniophyceae and Chrysomerophyceae (also related to brown algae) plus several groups of mostly unicellular marine forms: Bolidophyceae (the sister group to diatoms), Dictyochophyceae (including the well-known "silicoflagellates"), Pelagophyceae, Pinguiophyceae, and Picophagea (the latter being amoeboid and often non-photosynthetic): None of these are covered independently in the Handbook. Finally, ► Actinophryida, a small group of heterotrophic "heliozoan" organisms, belongs phylogenetically among ochrophytes (the exact placement is unresolved).

The heterotrophic stramenopiles are phylogenetically more diverse than the phototrophs and range from fungi-like organisms (most of which nonetheless produce flagellated dispersal stages) through to various kinds of "protozoa." ► Hyphochytriomycota and Oomycota are the most fungus-like stramenopiles: they produce (septate) hyphae with cell walls and generally parasitize plants or aquatic organisms. Oomycetes, in particular, cause several major diseases of agricultural crops (e.g., late blight in potatoes) and trees. ► Labyrinthulomycota produce non-walled extensions, with the best known, the labyrinthulids, existing as ectoplasmic networks containing numerous cell bodies. Pirsoniida (not covered) is a group of

parasitoids of algae that is related to oomycetes and hyphochytrids. Many groups of stramenopiles are heterotrophic flagellates with two flagella or sometimes one. The best known of these is Bicosoecida (sensu lato; also known as Bicosidia); others that are broadly similar but phylogenetically distinct include Placididea, *Cantina*, *Rictus*, *Platysulcus*, and Developayellaceae (the latter is also related to oomycetes and hyphochytrids; Cavalier-Smith and Scoble 2013; Yubuki et al. 2015; Shiratori et al. 2015). Furthermore, environmental sequencing studies have shown that the oceans contain a wide diversity of undescribed lineages of stramenopiles, collectively called "MASTs" (MArine STramenopiles; though some are also found in freshwater), which appear to be largely or entirely heterotrophic flagellates (Massana et al. 2014). In recent years, a couple of species that belong to one MAST lineage have been cultivated or reinvestigated (*Incisomonas* and *Solenicola*), and this group is now known as Nanomonadea (Cavalier-Smith and Scoble 2013). None of these various heterotrophic flagellate groups is covered in the Handbook; a summary of MAST diversity is given by Massana et al. (2014). Finally, the taxon ▶ Opalinata includes a range of inhabitants of animal intestinal tracts, including cells with two to four flagella, the multiflagellated opalinids, and the nonflagellated anaerobe *Blastocystis* (one of the most prevalent protists in the human gastrointestinal tract).

Sar; Alveolata (Fig. 3) Alveolata encompasses three of the most well-known groups of protists, Apicomplexa, Dinoflagellata, and Ciliophora, each represented by a chapter in the Handbook: ▶ Apicomplexa is quintessentially parasitic and includes species that are extremely harmful to humans and animals (e.g., *Plasmodium* spp., *Toxoplasma gondii*, *Cryptosporidium parvum*, etc.). The scope of the Handbook chapter has been extended to include the sister lineages to apicomplexan parasites, namely, colpodellids (which predate upon other protists or parasitize them) and the chromerid algae, which were only discovered this century (Moore et al. 2008). Colpodellids and chromerids are phylogenetically intermingled; recent analyses indicate they may be a clade, "chrompodellids" (Janouškovec et al. 2015). Research on these lineages has resulted in groundbreaking advances in our understanding of the evolution of apicomplexans and their relationships with dinoflagellates. Most notably, chromerids turned out to be the long-sought living descendants of the inferred photosynthetic ancestors of apicomplexans (most of which have non-photosynthetic plastids). ▶ Dinoflagellata includes numerous species that are conspicuous and important components of the marine microplankton, as autotrophs and/or grazers (many are mixotrophs and show both functions). Collectively, dinoflagellates are involved in several phenomena of great ecological importance, such as harmful algal blooms (e.g., *Karenia brevis*, *Alexandrium* spp.), symbioses with reef-forming corals (*Symbiodinium*), and important parasitic associations with animals or with other protists (e.g., *Hematodinium*, *Amoebophrya*). Dinoflagellates are closely related to Perkinsozoa, a small group of aquatic parasites with flagellated spores (not covered separately in the Handbook). While Apicomplexa-chrompodellids and Dinoflagellata-Perkinsozoa are closely related, there is still some uncertainty as to the position and evolutionary significance of several

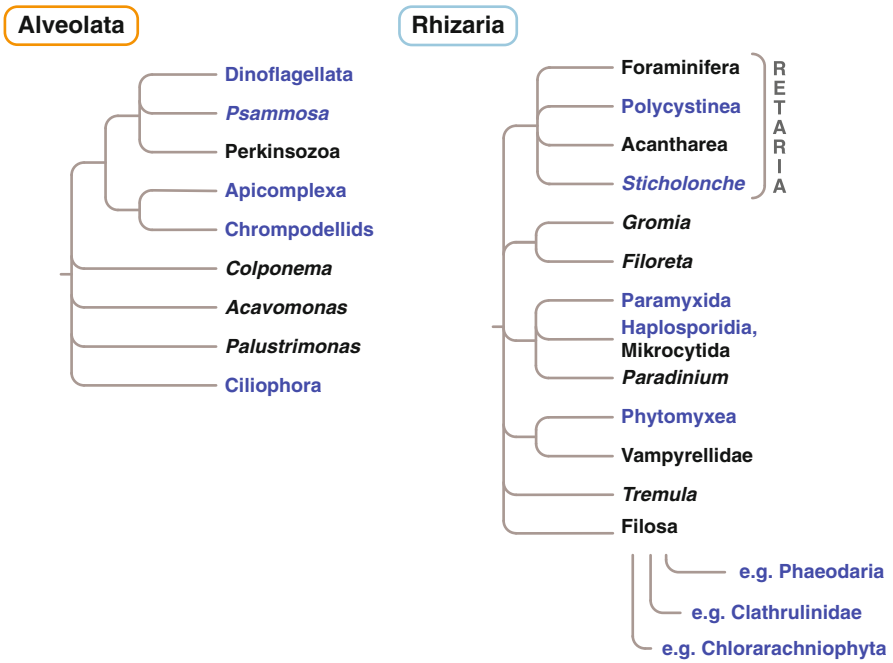

Fig. 3 Summary phylogenetic trees for Alveolata and Rhizaria, based primarily on Tikhonenkov et al. (2014), Janouškovec et al. (2015), Park and Simpson (2015), and Burki et al. (2016) (Alveolata) and Bass et al. (2009), Sierra et al. (2013, 2016), and Krabberod et al. (2017) (Rhizaria). Groups covered in Handbook chapters are shown in *blue* and are as follows: Alveolata: ▶ Apicomplexa (and "Chrompodellids"); ▶ Dinoflagellata (inc. *Psammosa*); ▶ Ciliophora. Rhizaria: ▶ Polycystinea; ▶ *Sticholonche*; ▶ Phaeodaria; ▶ Clathrulinidae; ▶ Chlorarachniophytes; ▶ Phytomyxea; ▶ Paramyxida; ▶ Haplosporidia. Note that Filosa contains many subgroups, and only those few subgroups covered in the Handbook are shown

heterotrophic flagellates lying at and near the split of these two groups (Fig. 3). Of these, *Psammosa* is often considered a basal dinoflagellate, while *Colponema*, *Acavomonas*, and *Palustrimonas* appear to represent one or more sister clades to the whole assemblage (none covered here; Tikhonenkov et al. 2014; Park and Simpson 2015). The third main group of alveolates, ▶ Ciliophora is extremely diverse and probably the most thoroughly studied group of (mostly) free-living heterotrophic protists. Most have large numbers of cilia (i.e., arrays of coordinated eukaryotic flagella), which in many species cover almost the entire cell, and they exhibit a characteristic form of nuclear dimorphism, with somatic macronuclei and germline micronuclei. In spite of the considerable wealth of knowledge on ciliates accumulated to date, the field of ciliate biodiversity is very active, and new environmental sequencing studies indicate that the full diversity of ciliates is far from uncovered.

Sar: Rhizaria (Fig. 3) One of the most morphologically diverse higher-order lineages, Rhizaria is comprised mostly of heterotrophic amoebae, flagellates, and amoeboflagellates, though it also includes some spore-forming parasites and unusual algae. No set of morphological features unites Rhizaria to the exclusion of other eukaryotes; they have emerged as a distinct taxon on the basis of molecular phylogenetic analyses (see Nikolaev et al. 2004). Reticulate or filose pseudopods are often present (thus the name "Rhizaria," referring to their often rootlike appearance), but these may be either actin- or microtubule-supported structures.

The most familiar rhizarians are foraminiferans (Foraminifera) and the radiolarians, most of which are large, often abundant, marine amoebae with microtubule-supported pseudopodia. Most foraminiferans inhabit multichambered tests that are constructed from calcium carbonate or assembled from agglutinated mineral particles; these have left an extensive fossil record extending back to the Cambrian. Foraminifera are not included in the Handbook (but see below). ▶ Radiolaria are subdivided into Polycystinea, usually with silica skeletons, and Acantharea, which have strontium sulfate skeletons (Acantharea are not covered in the Handbook). Foraminifera, Polycystinea, Acantharea, and the peculiar "rowing" radiolarian-like organism ▶ *Sticholonche* are related to one another (as Retaria), but their interrelationships are still unclear (see Sierra et al. 2013; Krabberød et al. 2017).

Much of rhizarian diversity falls within a clade called Filosa. This includes many free-living flagellates, which usually feed using some form of often-fine pseudopodia. The bulk of these flagellates associate with surfaces (e.g., most members of Cercomonadida, Glissomonadida, and Thaumatomonadida) but there are also some free-swimming forms (e.g., Ebriida). A few are parasites/parasitoids (e.g., *Pseudopirsonia*). Filosa also includes several groups of amoebae, the most famous being the filose testate amoebae (Euglyphida), although there is a greater diversity of naked forms (e.g., Bass et al. 2009). The Handbook has accounts of only a couple of groups of these organisms, namely, ▶ Phaeodaria, which are amoebae with siliceous skeletons that until relatively recently were considered to be radiolaria, and the "heliozoan" group ▶ Clathrulinidae. Filosa also includes two photosynthetic lineages, the mostly amoeboflagellate ▶ Chlorarachniophytes (e.g., *Bigelowiella* and *Lotharella*), which possess plastids of green algal secondary endosymbiotic origin, and *Paulinella chromatophora*, which is a euglyphid testate amoeba that harbors a cyanobacterium-derived photosynthetic "chromatophore" of separate origin than canonical plastids (Nowack 2014).

Most other rhizarian groups are various amoebae, including *Gromia*, *Filoreta*, and Vampyrellida (none covered in the Handbook), or are parasites. The latter include ▶ Phytomyxea, which are pathogens of plants and stramenopiles (e.g., *Plasmodiophora brassicae*, *Maullinia ectocarpi*), as well as ▶ Paramyxida and ▶ Haplosporidia, both of which parasitize marine invertebrates (though there are also freshwater haplosporidians). Other parasites infecting marine invertebrates (and not directly covered in the Handbook) include Mikrocytida, such as the oyster parasite *Mikrocytos* (which are very likely related to Haplosporidia or possibly descended from them), and *Paradinium*, which infects crustaceans. It is generally assumed that these parasites of invertebrates are all related, forming a taxon called

Ascetosporea, and this is incompletely supported by molecular phylogenies (e.g., Sierra et al. 2016). The flagellate *Tremula* (not covered) may represent the sister group to (other) Filosa (Howe et al. 2011).

Burki and Keeling (2014) provide a brief overview of the biology and evolution of rhizarian taxa, including some of the more important groups not covered in the Handbook. In addition, recent advances in the systematics of Foraminifera are treated by Pawlowski et al. (2013), and the current systematics of Euglyphida (and some other thecate amoebae within Filosa) was recently summarized by Kosakyan et al. (2016). The report by Howe et al. (2011) illustrates some of the range of free-living flagellates and small amoebae among Filosa. The diversity and phylogeny of Vampyrellida is examined and illustrated by Hess et al. (2012) and Berney et al. (2013). Hartikainen et al. (2014) give the first broad account of mikrocytids.

Other Archaeplastida- and Sar-Related Lineages (Fig. 1) A series of much smaller groups (in terms of the number of described species) are very likely related to Archaeplastida and/or Sar. The taxon Cryptista (sensu stricto) includes the well-known algal group ► Cryptophyta and two more obscure relatives, katablepharids and *Palpitomonas* (see Yabuki et al. 2014). Cryptophytes are mostly unicells with two flagella and with a plastid of red algal origin. Quite a few lack photosynthetic capabilities, including goniomonads, which are sister to other cryptophytes and may be ancestrally non-photosynthetic. Katablepharids and *Palpitomonas* are also biflagellated heterotrophs; neither is covered in the Handbook (Yabuki et al. 2010 and Nishimura et al. 2016 essentially summarize the published work on *Palpitomonas*). ► Haptophyta also known as Prymnesiophyta, is a major group of unicellular algae, especially in marine systems, where calcite-scale-producing coccolithophorid haptophytes are of regional and global significance in carbon cycling. They too have a plastid ultimately of red algal origin. Haptophyta are probably related to the recently discovered "rappemonads," which are uncultivated unicellular marine algae (not covered here; see Kim et al. 2011), and quite possibly to ► Centrohelida, a group of heterotrophic "heliozoan" amoebae with long, radiating microtubule-supported "axopodia" (another recently described heliozoan, *Microheliella*, is currently inferred to be a separate lineage from Centrohelida, but this has not been clearly resolved; Cavalier-Smith et al. 2015). Recent phylogenomic analyses suggest that cryptists are related to Archaeplastida, while haptophytes (and their relatives) are more closely related to Sar (Burki et al. 2016), but these relationships are not yet well resolved (e.g., Yabuki et al. 2014; Cavalier-Smith et al. 2015). Two further groups of obscure heterotrophic flagellates, Telonemida and Picozoa, are most likely related to Sar and Archaeplastida, respectively (Burki et al. 2016). Neither is covered in the Handbook; see Yabuki et al. (2013a), Seenivasan et al. (2013), and Moreira and Lopez-Garcia (2014) for recent publications on these groups.

Discoba (Fig. 4) This clade includes ► Jakobida, a group of heterotrophic flagellates best known for their particularly bacterial-like mitochondrial genomes, the recently discovered flagellate *Tsukubamonas*, and a clade called Discicristata which unites the well-known taxa Heterolobosea and Euglenozoa (Hampl et al.

2009; Yabuki et al. 2011; the name Discoba is a portmanteau of Discicristata and *Jakoba*). ▶ Heterolobosea are a collection of amoebae, flagellates, or "amoeboflagellates" with life cycles that include both cell types (the acrasids are also "slime molds" that produce sorocarps). Euglenozoa in turn includes three main subgroups: ▶ Euglenida encompasses many predatory species that glide over surfaces, as well as a large clade of algae with plastids of chlorophyte green algal origin (and very likely a group of specialist anaerobes – Symbiontida). ▶ Kinetoplastea includes a mix of free-living and parasitic flagellates, with the most famous being the trypanosomatids that cause sleeping sickness, Chagas' disease, and leishmaniases in humans. Diplonemids (Diplonemea) are the sister group to kinetoplastids. While previously obscure, diplonemids have recently been found to be extremely abundant and diverse in ocean waters (Flegontova et al. 2016). They also have remarkable mitochondrial gene expression systems, where genes are encoded as fragments on separate chromosomes and transcripts are trans-spliced together to produce functional mRNAs (and can be extensively edited too; Moreira et al. 2016). Diplonemids are not discussed further in the Handbook but are examined or reviewed in several recent publications (David and Archibald 2016; Flegontova et al. 2016; Gawryluk et al. 2016).

Metamonada (Fig. 4) The metamonads are a large group of anaerobic protozoa, almost all of which are flagellates. They are of contentious phylogenetic placement, with different molecular phylogenetic analyses favoring relationships with Discoba (see above) or Malawimonadidae (see below), this forming a part of the ongoing controversy about the phylogenetic coherence of the "excavates" (Hampl et al. 2009;

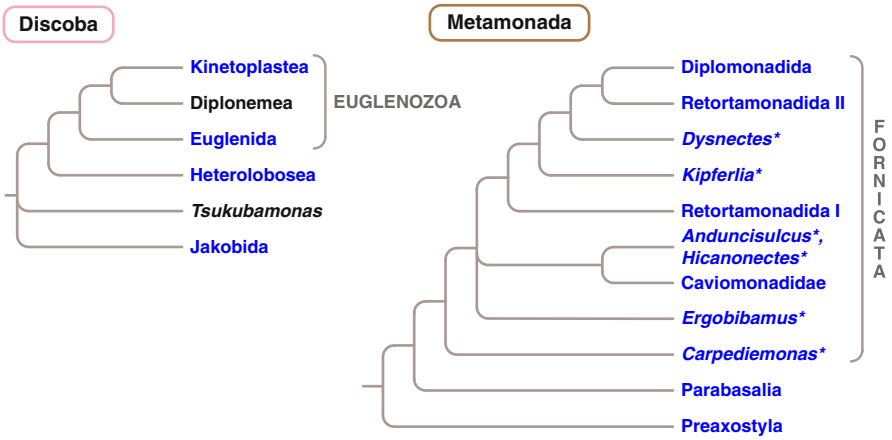

Fig. 4 Summary phylogenetic trees for Discoba and Metamonada, based primarily on Kamikawa et al. (2014) (Discoba) and Leger et al. (2017) and Yubuki et al. (2017) (Metamonada). Groups covered in Handbook chapters are shown in *blue* and are as follows: Discoba: ▶ Jakobida; ▶ Heterolobosea; ▶ Euglenida; ▶ Kinetoplastea. Metamonada: ▶ Preaxostyla; ▶ Parabasalia; ▶ Diplomonadida; ▶ Retortamonadida, Caviomonadidae, and *Carpediemonas*-like organisms (CLOs). CLOs are indicated by *asterisks*

Cavalier-Smith et al. 2014; Derelle et al. 2015). There are three main subgroups within Metamonada. ▶ Preaxostyla includes the oxymonads, which are gut commensals/symbionts, plus the free-living trimastigids. ▶ Parabasalia is a very diverse group (almost entirely) of endobiotic organisms. They range from small parasites (e.g., *Trichomonas vaginalis*, in humans) to giant multiflagellated cells of diverse kinds that are symbiotic in certain wood-eating termites and cockroaches. The third group, Fornicata, includes ▶ Diplomonadida, which are mostly "doubled" cells with two nuclei and flagellar apparatuses (e.g., *Giardia lamblia/intestinalis* in humans). The other members of Fornicata are the commensal/parasitic retortamonads and Caviomonadidae, as well as the various free-living "*Carpediemonas*-like organisms" (e.g., *Carpediemonas*, *Dysnectes*, *Kipferlia*); these are collectively a paraphyletic assemblage of relatives of diplomonads, but covered in a single chapter of the Handbook, ▶ Retortamonadida (with notes on *Carpediemonas*-Like Organisms and Caviomonadidae).

Amorphea; Amoebozoa (Fig. 5) The Amorphea assemblage unites two huge clades that are inferred to be related in most recent global analyses of eukaryotic phylogeny: (i) the animals, fungi, and their immediate protist relatives ("Obazoa"; see below) and (ii) the large grouping of heterotrophic protists called "Amoebozoa" (Adl et al. 2012; Burki 2014). As the name suggests, Amoebozoa mostly (though not entirely) consists of organisms that are amoebae for much or all of their life cycle. Many lineages are various kinds of "slime molds," which also produce a spore-releasing fruiting body. Due to this "fungus-like trait," these have often been studied separately from non-fruiting amoebae and are covered separately in the Handbook. Since "protosteloid" slime molds are phylogenetically intermingled with non-fruiting Amoebozoa, there is an imperfect fit between some chapters of the Handbook and the known phylogeny of the group (which is crystallizing rapidly as insights from phylogenomic analysis are integrated with those from taxon-rich ribosomal RNA gene phylogenies; e.g., Shadwick et al. 2009; Berney et al. 2015; Cavalier-Smith et al. 2016; Tice et al. 2016).

At present there are three reasonably well-accepted groupings that are largely or entirely composed of "lobose" amoebae with no other stages in the lifecycle – Tubulinea, Discosea, and the recently distinguished Cutosea (Cavalier-Smith et al. 2016). Tubulinea includes *Amoeba* itself and many other naked amoebae with thick pseudopodia, as well as the Arcellinida or lobose testate amoebae. Discosea tend to be flatter cells; examples include *Acanthamoeba*, *Vanella*, and *Thecamoeba*. Cutosea is a small group including particular scaled amoebae. The non-fruiting amoebae in these groups are covered in a single chapter, ▶ Amoebozoan Lobose Amoebae (Tubulinea, Flabellinea, and Others). A fourth group, Variosea, includes a few non-fruiting amoebae that often have filose or reticulate pseudopodia but also most of the "protosteloid" slime molds, some of which have flagellated stages as well as amoebae (Variosea also includes a couple of "flagellate-only" taxa, *Phalansterium* and *Multicilia*, that are not covered in the Handbook). A few of the amoebae are explicitly discussed in the lobose amoebae chapter (see above); the protosteloids are treated authoritatively in a separate chapter (▶ Protosteloid Amoebae). This latter

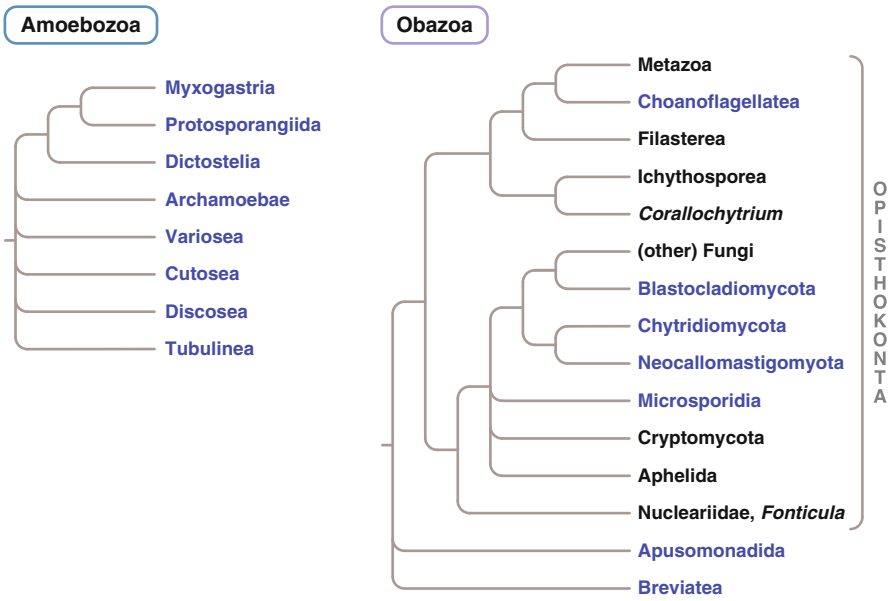

Fig. 5 Summary phylogenetic trees for Amoebozoa and Obazoa, based primarily on Berney et al. (2015), Cavalier-Smith et al. (2016), Tice et al. (2016), and M.W. Brown, pers. comm. (Amoebozoa) and Brown et al. (2013), Cavalier-Smith et al. (2014), Karpov et al. (2014), and Torruella et al. (2015) (Obazoa). Groups covered in Handbook chapters are shown in *blue* and are as follows: Amoebozoa: Many non-fruiting amoebae from Tubulinea, Discosea, Cutosea and Variosea are covered in ▶ Amoebozoan Lobose Amoebae (Tubulinea, Flabellinea, and Others); Protosteloid members of Variosea and Discosea, plus Protosporangiida, are covered in ▶ Protosteloid amoebae; ▶ Archamoebae; ▶ Myxomycetes; ▶ Dictyostelia. Obazoa: ▶ Choanoflagellatea; ▶ Blastocladiomycota; ▶ Chytridiomycota (and Neocallomastigota); ▶ Microsporidia; ▶ Apusomonadida (and Breviatea)

chapter also covers four small groups of fruiting amoebae that belong phylogenetically within Discosea (see above), as well as Protosporangiida, which are actually most closely related to Myxogastria (see below), and the couple of "protosteloid" members of Myxogastria.

The three remaining groups of Amoebozoa are each characterized by distinctive biological traits. Members of ▶ Archamoebae are amoeboid flagellates or amoebae (or cycle between these forms) that are anaerobic and have highly modified mitochondrial organelles (e.g., *Mastigamoeba*, *Pelomyxa*, *Entamoeba*). ▶ Myxomycetes or Myxogastria, is a well-known group of slime molds with complex life cycles that include small amoebae (and flagellates) but also multinucleate plasmodia that are macroscopic in size. It is the plasmodium stage that differentiates into the spore-bearing fruiting body. ▶ Dictyostelia are also slime molds, but unlike protosteloids or myxomycetes, they produce the fruiting body through aggregation of numerous unicellular amoebae. Myxogastria, Protosporangiida, and Dictyostelia are closely related, and they have sometimes been referred to collectively as Macromycetozoa.

Amorphea; Obazoa (Fig. 5) It is now well understood that animals (Metazoa) and Fungi are closely related to one another but also that they are specifically related to a heterogeneous assemblage of protist lineages, collectively forming a group called Opisthokonta (Cavalier-Smith 1987; Brown et al. 2009; Torruella et al. 2015). The protists most closely related to animals are the choanoflagellates (► Choanoflagellatea), which are unicellular or colonial flagellates that capture food using a characteristic "collar" of microvilli. Other close animal relatives include the Ichthyosporea (Mesomycetozoea), which are diverse parasites, mostly of aquatic animals and anurans, the isolated genus *Corallochytrium*, and Filasterea, the latter being a small group of free-living or parasitic forms mostly with fine pseudopodia. These groups are not discussed in detail in the Handbook; Ichthyosporea are reviewed by Glockling et al. (2013). Fungi are closely related to the nucleariid amoebae (Nucleariidae), with fine filose pseudopodia, and the slime mold-like organism *Fonticula* (not covered further). The phylogenetic and systematic distinction between Fungi and protists has perpetually been a gray area, and the Handbook contains accounts of groups that are usually considered the deepest branches among the fungi. These include ► Blastocladiomycota and ► Chytridiomycota with the latter chapter also including a brief account of Neocallimastigomycota; these organisms function as saprotrophs as well as parasites (or symbionts) and typically reproduce via uniflagellate zoospores. The other major taxa at the base of fungi include ► Microsporidia, an extensively studied and speciose group of spore-forming intracellular parasites, and the much more poorly known Cryptomycota (Rozellida) and Aphelida (not covered; see reviews by James and Berbee 2012; Karpov et al. 2014). Finally, two obscure groups of free-living heterotrophic flagellates, ► Apusomonadida and Breviatea, are now known to be sister taxa to the opisthokonts. It is this clade of all three taxa that is now known as Obazoa (Brown et al. 2013; OBA is an acronym for the three lineages).

Other Lineages (Fig. 1) There are a number of protist lineages that do not belong to any of the taxa listed above. Many are very poorly known groups for which there are very little data (e.g., limited or no electron microscopy data and no molecular sequence information). However, several lineages of free-living heterotrophs appear to be related to Obazoa and Amoebozoa (i.e., Amorphea) in phylogenetic/phylogenomic analyses (Zhao et al. 2012; Cavalier-Smith et al. 2014): Collodictyonidae (also known as diphylleids) are swimming flagellates, while their likely close relatives, Rigidifilida, are partly amoeboid cells without flagella. Ancyromonadida (Planomonadida) and *Mantamonas* are small gliding flagellates. Malawimonadidae is a small group of "excavate" flagellates that closely resemble Jakobida (in Discoba) and *Carpediemonas*-like organisms (in Metamonada) but do not branch with either Metamonada or Discoba in many phylogenomic analyses (though this is a topic of considerable contention; see above). Ancyromonadida is covered in a coda of the chapter on ► Apusomonadida and Malawimonadidae in the chapter on ► Jakobida Collodictyonidae, Rigidifilida, and *Mantamonas* are not covered in the Handbook, but recent publications on these protists include Zhao et al. (2012), Yabuki et al. (2013b), and Glücksman et al. (2011), respectively.

A different category of unplaced taxa consists of the few well-known groups of several species and genera for which there are substantial morphological data (albeit phylogenetically ambiguous) but no molecular data yet. One conspicuous example is Hemimastigophora, a taxon of multiflagellated cells that consume other smaller protists (Foissner and Foissner, 1993: not covered in the Handbook). We also highlight two groups of "heliozoa," Gymnosphaerida and Heliomonadida (formerly Dimorphida), each of which include several genera. One or both are sometimes placed tentatively within Rhizaria (Bass et al. 2009; Adl et al. 2012), but this awaits testing using molecular phylogenetics. Both are discussed briefly in the chapter on ► Centrohelida and Other Heliozoan-Like Protists.

Closing Remarks

Three decades ago, it would have been impossible to provide an accurate phylogenetic framework linking the many groups of protists covered in this edition of the Handbook. We are now able to present a reasonably comprehensive account of that framework, with confidence that most of it is essentially correct. Getting to this position has been a major achievement by the scientific community; this fact should not be lost amid concerns over the parts of the eukaryote tree that remain unresolved, contentious, or unknown. In a similar vein, while it has been necessary in this chapter to emphasize lineages that are not covered by the rest of the book, the Handbook is nonetheless an authoritative account of a substantial majority of known protist diversity. It represents an important collective effort by a large part of the protistology community and a major resource documenting the current state of knowledge on these organisms. We hope that this Handbook has a positive influence on the future direction of protistology, leading to greater depth and breadth in the understanding of our fascinating organisms.

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Glaucophyta

2

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Abstract

The Glaucophyta is by far the least species-rich phylum of the Archaeplastida comprising only four described genera, *Glaucocystis*, *Cyanophora*, *Gloeochaete*, and *Cyanoptyche*, and 15 species. However, recent molecular and morphological analyses reveal that glaucophytes are not as species poor as hitherto assumed with many novel lineages existing in natural environments. Glaucophytes are freshwater phototrophs of moderate to low abundance and retain many ancestral plastid traits derived from the cyanobacterial donor of this organelle, including the remnant peptidoglycan wall in their envelope. These plastids were originally named "cyanelles," which was later changed to "muroplasts" when their shared

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ancestry with other Archaeplastida was recognized. The model glaucophyte, *Cyanophora paradoxa*, is well studied with respect to biochemistry, proteomics, and the gene content of the nuclear and organelle genomes. Investigation of the biosynthesis of cytosolic starch led to a model for the transition from glycogen to starch storage during plastid endosymbiosis. The photosynthetic apparatus, including phycobilisome antennae, resembles that of cyanobacteria. However, the carbon-concentrating mechanism is algal in nature and based on pyrenoids. Studies on protein import into muroplasts revealed a primordial Toc/Tic translocon. The peptidoglycan wall was elucidated with respect to composition, biosynthesis, and involvement of nuclear genes. The muroplast genome is distinct, not due to the number of encoded genes but, rather, because of the presence of unique genes not present on other plastid genomes. The mosaic nature of the gene-rich (27,000) nuclear genome came as a surprise, considering the relatively small genomes of unicellular red algae.

Keywords

Archaeplastida • *Cyanophora paradoxa* • Muroplasts • Single primary endosymbiotic event • Phylogenomics • Carbon-concentrating mechanism • Eukaryotic peptidoglycan • Phycobilisomes

Contents

Summary Classification

●**Glaucophyta** (Skuja 1954) Glaucocystophyta (Kies and Kremer 1986)
●●**Glaucophyceae** Bohlin
●●●**Glaucocystales** Bessey
●●●●**Glaucocystaceae** G.S. West (*Gloeochaete*, *Cyanoptyche*, *Glaucocystis*)
●●●**Cyanophorales** Kies and Kremer
●●●●**Cyanophoraceae** Kies and Kremer (*Cyanophora*)

Introduction

General Characteristics

The phylum Glaucophyta Kies and Kremer 1986 (synonym: Glaucophyta Skuja 1954) contains a single class, the Glaucocystophyceae Schaffner 1922. It comprises a small group of unicellular mastigotes (monadoid members), unicellular and colonial organisms devoid of flagella with persistent contractile vacuoles (capsalean members), and unicellular and colonial organisms lacking any characters of mastigotes in the vegetative stage (coccoid members). Currently, four genera are known with at least 15 species. Glaucophytes live photoautotrophically with the aid of their unique plastids that are surrounded by a remnant peptidoglycan wall. These organelles were named cyanelles by Pascher (1929), a denomination which was later proven to be incorrect and thus was replaced by the more appropriate term "muroplast" coined by Schenk (1994) (Fig. 1). Muroplasts owe their origin to cyanobacteria, providing direct proof for the endosymbiotic theory of plastid evolution. The glaucophytes are thought to be the most ancient phylum of phototrophic eukaryotes although molecular data provide inconclusive data regarding this hypothesis (Martin et al. 1998; Reyes-Prieto and Bhattacharya 2007a; Price et al. 2012). Together with rhodophytes and chlorophytes/streptophytes, they constitute the Archaeplastida (Adl et al. 2005) that contain "primary" plastids surrounded by two envelope membranes. The major reason that we understand the evolutionary importance of glaucophytes is the excellent and meticulous ultrastructural studies conducted by Ludwig Kies as summarized in Kies (1992). The unifying characters of this phylum are the presence of muroplasts with peptidoglycan layers in their envelopes (Fig. 1) and a number of shared morphological features (see below). This grouping was later corroborated by phylogenetic analyses based on 16S (Helmchen et al. 1995) and 18S rRNA (Bhattacharya et al. 1995a; Marin et al. 1998) and concatenated protein sequences (Rodríguez-Ezpeleta et al. 2005).

Occurrence

Glaucophytes are relatively rare in nature, occupying niches. All members inhabit freshwater environments in the plankton or benthos of lakes, ponds, or ditches. Only

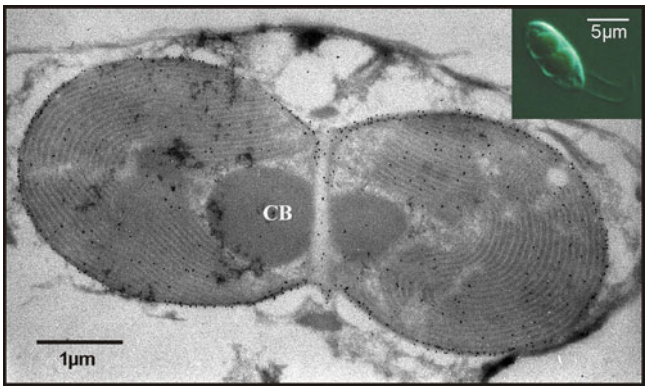

Fig. 1 *Cyanophora paradoxa* SAG 29.80. Immuno-EM of a dividing muroplast. Primary antibodies directed against peptidoglycan from *E. coli*. Gold particles mainly decorate the envelope and the newly formed septum. The division furrow neatly cleaves the RuBisCo-containing central body (CB), the genetic material surrounding it, and the concentric thylakoids into two halves destined for the daughter muroplasts. *Insert*: Interference contrast micrograph showing the ovoid cell, the flagella, and two muroplasts

four genera are maintained in culture collections, i.e., *Cyanophora*, *Gloeochaete*, *Cyanoptyche*, and *Glaucocystis* (Table 1), and thus are available for research. Almost all biochemical and molecular data acquired during the past 25 years (after the review by Kies and Kremer 1990) were obtained from *Cyanophora paradoxa*, which is the model organism for this phylum. A relatively fast growth rate, ease of cell lysis, and stable muroplasts account for its wide usage in research. Species that once were grouped together with the glaucophytes but were not deposited in an algal culture collection are not further dealt with here.

Literature and History of Knowledge

Kies and Kremer (1990) review the early literature, until the end of the 1980s, and explore the morphological criteria characteristic of glaucophytes. The excellent EM work of Ludwig Kies is presented in this chapter whenever possible. Bhattacharya and Schmidt (1997) review the phylogenetic analyses supporting the phylum Glaucophyta. Löffelhardt et al. (1997a) and Löffelhardt and Bohnert (2001) include the forthcoming molecular (muroplast genome sequence) and biochemical (fine structure of muroplast peptidoglycan) data until the end of the 1990s. The important issue of protein targeting to the muroplasts of *C. paradoxa* is dealt with in two reviews (Steiner and Löffelhardt 2002, 2005). Genomic data from *C. paradoxa* and *G. nostochinearum* and microarray data revealing CO_2-responsive genes and their involvement in the inorganic carbon-concentrating mechanism (CCM) are presented in Rodríguez-Ezpeleta et al. (2005) and Burey et al. (2007), respectively. The landmark paper describing the nuclear genome sequence of *C. paradoxa* (Price

Table 1 Strains of Glaucophyta available from culture collections of algae

Taxon	Culture collection and number	Isolator and year of isolation	Origin	Remarks
Cyanophora biloba	UTEX LB 2766	P. Kugrens 1997	USA	Ephemeral alpine pond
Cyanophora cuspidata T. Takahashi and Nozaki	NIES-3645	T.Takahashi and Nozaki	Japan	
	SAG 45.84	L. Kies 1967	Germany	1555 (Kies strain), axenic
	=CCAC 0091			
Cyanophora paradoxa Korsh	CCAP 981/1	G. Pringsheim 1943	England	Pringsheim strain, ovoid, axenic
	= UTEX LB 555			
	= SAG 29.80			
	= CCMP329			
	= NIES-547			
	= CCAC 0074			
Cyanophora kugrensii T. Takahashi and Nozaki	NIES-763	S. Suda 1991	Japan	Axenic
Cyanophora sudae T.Takahashi and Nozaki	NIES-764	S. Suda 1991	Japan	Broad bean shape, generally four plastids (2–8), axenic
Cyanophora tetracyanea[a]	Not available (NA)			Similar to *C. sudae*
Cyanoptyche gloeocystis	SAG 34.90	L. Kies 1984	Austria	2643 (Kies strain)
	SAG 4.97	O. Lourenco 1989	Portugal	ACOI 387 (Santos strain)
Cyanoptyche sp.	CCAC 2322 B	E. Kusel 1994	Austria	ASW 10005
Glaucocystis geitleri	SAG B 229–3 (= UTEX 1929?)	R. A. Lewin 1963		Designated *G.* cf. *nostochinearum* by Schnepf et al. (1966), G1 clade in Chong et al. (2014)
Glaucocystis geitleri	UTEX B 1929 (NA)	R. A. Lewin		Designated Lewin CY-11, G1 clade in Chong et al. (2014)

(*continued*)

Table 1 (continued)

Taxon	Culture collection and number	Isolator and year of isolation	Origin	Remarks
Glaucocystis geitleri	SAG 28.80	R.M. Brown 1975		G1 clade in Chong et al. (2014)
Glaucocystis nostochinearum Itzigs.	SAG 16.98	U. G. Schlösser 1997	Germany	G2 clade in Chong et al. (2014)
Glaucocystis nostochinearum Itzigs.	SAG 45.88	D. Mollenhauer 1983	Germany	Axenic, G2 clade in Chong et al. (2014)
Glaucocystis miyajii	NIES-1961	A. Kai 2003	Japan	G3 clade in Chong et al. (2014)
Glaucocystis oocystiformis	NIES-966	F. Kasai 1987	Japan	G4 clade in Chong et al. (2014)
Glaucocystis oocystiformis	NIES-1369	A. Kai 2003	Japan	G4 clade in Chong et al. (2014)
Glaucocystis bhattacharyae	SAG 27.80	Tassigny 1966	France	G5 clade in Chong et al. (2014)
Glaucocystis bhattacharyae	HS30 (NA)	H.S. Yoon 2010	USA	G5 clade in Chong et al. (2014)
Glaucocystis bhattacharyae	BBH (NA)	H.S. Yoon 2009	USA	G5 clade in Chong et al. (2014)
Glaucocystis incrassata	SAG 229–2	G. Pringsheim 1955	Denmark	Designated *G. incrassata* Lemmermann by Schnepf et al. (1966), G6 clade in Chong et al. (2014)
Glaucocystis incrassata	SAG B 229–1	E. A. George 1952	England	Designated *G. geitleri* nom. Prov. Pringsheim by Schnepf et al. (1966), G6 clade in Chong et al. (2014)
	= UTEX 64 (NA)			
	= NIES-2141			
	= CCAP 229/1			

Glaucocystis sp. (no molecular data)	CCAC 0088 B	B. Marin 1993	Germany	Axenic
	CCAC 2233 B	L. Kies 1980	Germany	2523 (Kies strain)
	CCAC 2234 B	L. Kies 1975	Germany	2343 (Kies strain)
	CCAC 2235 B	L. Kies 1977	Germany	2395 (Kies strain)
	CCAC 2323 B	E. Kusel 1993	Austria	ASW 10006
	CCAC 2877 B	M. Melkonian 2006	Russia	Collector A. Gontcharov
	CCAC 2994 B	M. Melkonian 2007	Germany	
	CCAC 3352 B	M. Melkonian 2007	Switzerland	
	CCAC 3353 B	M. Melkonian 2010	Switzerland	
Gloeochaete wittrockiana Lagerheim	SAG 46.84	L. Kies 1973	Germany	2323 (Kies strain)

UTEX The Culture Collection of Algae at the University of Texas at Austin, Texas 78,712, USA, *SAG* Sammlung von Algenkulturen, Pflanzenphysiologisches Institut der Universität, D-3400 Göttingen, Germany, *CCAC* Culture Collection of Algae at the University of Cologne, Cologne, Germany, *CCAP* Culture Collection of Algae and Protozoa, Scottish Marine Institute, Oban, UK, *NIES* Microbial Culture Collection at the National Institute for Environmental Studies, Tsukuba, Japan, *NA* currently not available

Data on strains of Glaucocystophyceae have been compiled from website of culture collections below: http://www.uni-goettingen.de/; http://www.ccap.ac.uk; http://www.ccac.uni-koeln.de/, http://www.utex.org; http://mcc.nies.go.jp

[a]Might correspond to *C. paradoxa* (Kugrens 2001)

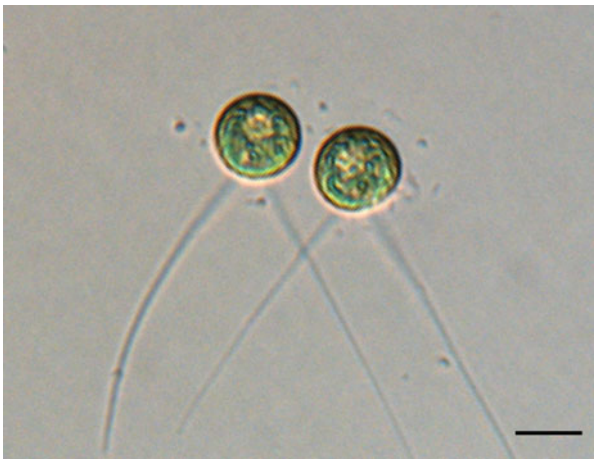

Fig. 2 Two cells of *Gloeochaete wittrockiana*, strain SAG 46.84 (Kies strain IAB 2323). Each cell contains two long pseudocilia. Interference contrast light micrograph. Scale line=10 μm

et al. 2012) provided distinct support for a single plastid primary endosymbiotic event and gave rise to a number of related reviews (Bhattacharya et al. 2014; Löffelhardt 2014; Facchinelli and Weber 2015; Jackson et al. 2015).

The recognition of Glaucophyta is intimately connected to the concept of endosymbiosis between protists and cyanobacteria and the theory of the evolution of eukaryotic cells (Mereschkowsky 1905; Margulis 1981; Margulis and Sagan 2003). After thorough investigations, both Geitler (1959a) and Pascher (1929) concluded that *Cyanophora* species (Fig. 1), *Gloeochaete wittrockiana* (Figs. 2, 3, and 5) and *Glaucocystis nostochinearum* (Figs. 4a, b, 6, and 7), were cases of symbioses between heterotrophic host cells and modified autotrophic cyanobacterial endosymbionts functioning like plastids. Such endosymbionts were named "cyanelles" by Pascher (1929) who created the terms "endocyanome" for the whole consortium and "endocyanosis" for this particular type of endosymbiosis. Nowadays, the kingdom "Archaeplastida," also known as "Plantae," is thought to have resulted from a single successful primary endosymbiotic event between a cyanobacterium and a heterotrophic protist. Once this immensely complicated and lengthy process was successfully completed, the "protoplastid" became the ancestor of all plastids known to date, regardless of differences in traits such as pigmentation and morphology. This does not rule out much more recent instances of endosymbiotic organellogenesis as in *Paulinella* (Nowack et al. 2008) or *Rhopalodia* (Kneip et al. 2008).

In contrast to all other plastids, with the potential exception of the moss *Physcomitrella patens* (Hirano et al. 2016), the muroplasts of *Cyanophora paradoxa* (Fig. 1), *Gloeochaete wittrockiana* (Fig. 5), *Glaucocystis nostochinearum* (Fig. 6), and *Cyanoptyche gloeocystis* (Fig. 4c, d) have thin lysozyme-sensitive cell walls clearly recognizable with electron microscopy (EM) between the two envelope membranes (Kies 1992), which in *C. paradoxa* (Schenk 1970; Aitken and Stanier

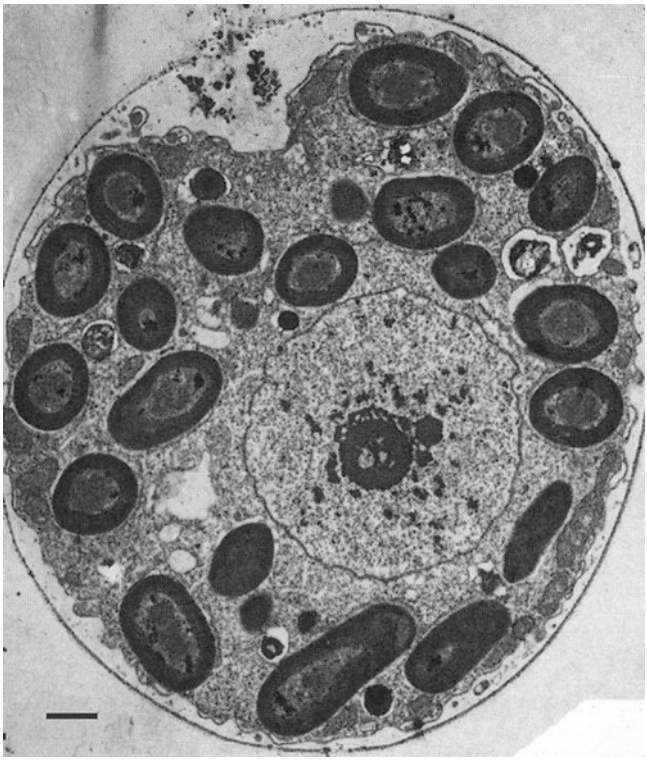

Fig. 3 *Gloeochaete wittrockiana*, strain SAG 46.84 (Kies strain IABH 2323), in longitudinal section, with apical depression, numerous muroplasts, and in the center of the cell a conspicuous nucleus with a nucleolus. Transmission electron micrograph. *Scale line* = 1 μm (Taken from Kies and Kremer (1990))

1979; Pfanzagl et al. 1996a), *G. nostochinearum* (Scott et al. 1984; Pfanzagl et al. 1996b), and *C. gloeocystis* (Pfanzagl et al. 1996b) have been identified as peptidoglycan layers. Skuja's taxonomic treatment of the phylum (Skuja 1954) was adopted: Skuja included in his phylum Glaucophyta *Gloeochaete*, *Glaucocystis*, and all endocyanomes described by Korshikov, Pascher, Geitler, and Skuja. Motile endocyanomes such as *Cyanophora* were not included in this framework. Kies (1979) suggested reviving the class Glaucocystophyceae (Skuja 1954) to accommodate the genera *Cyanophora*, *Gloeochaete*, *Glaucocystis*, and *Glaucosphaera*, which share ultrastructural characters not encountered together in any other algae (see Table 2). An emendation including a typification of several taxa of the Glaucophyta is given by Kies and Kremer (1986). *Cyanoptyche* was confirmed as a new member in 1989 (Kies 1989), whereas *Glaucosphaera* was removed in 1995 (Bhattacharya et al. 1995a).

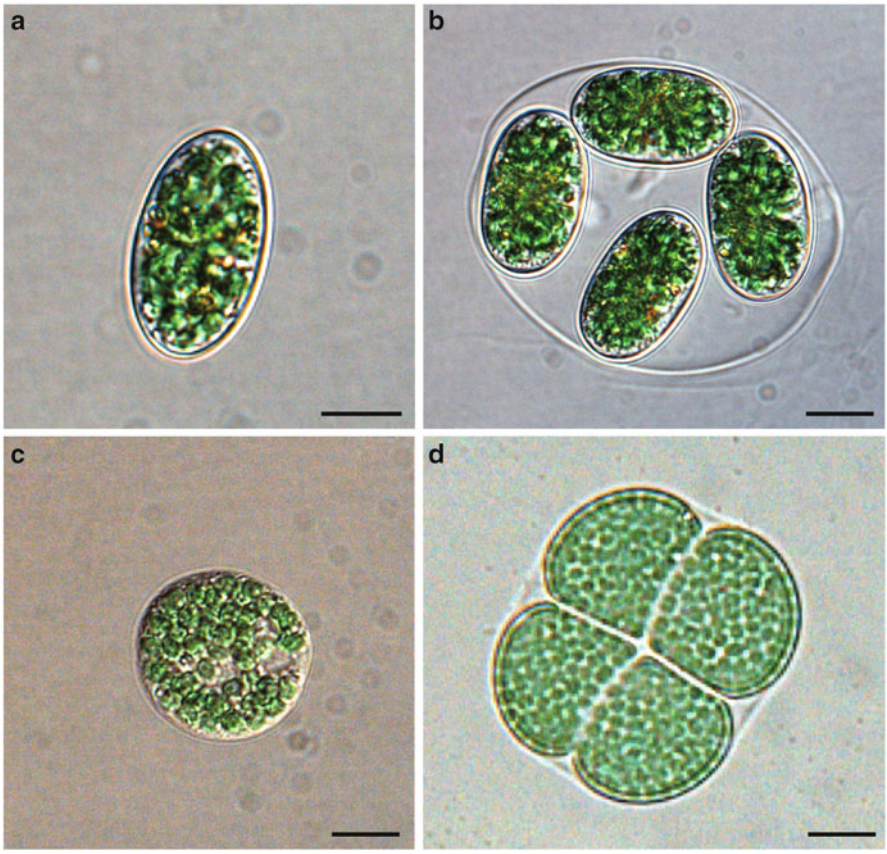

Fig. 4 **a**, **b** *Glaucocystis nostochinearum* SAG 45.88; C-D: *Cyanoptyche gloeocystis* SAG 4.97. A vegetative cell and autospores are shown for each species. *Scale bar* = 10 μm

Practical Importance

The Glaucophyta have not been exploited for economic or medical applications. A potential use of the eukaryotic peptidoglycan is as a model for the impact of beta-lactam antibiotics on eukaryotes, because the doses effective on *C. paradoxa* are similar to those for *E. coli* (Berenguer et al. 1987). In addition, a pigment extract of *C. paradoxa* containing pheophorbide *a*, beta-cryptoxanthin, and zeaxanthin as the main components has been shown to have strong antiproliferative activity against three cancer cell lines (Baudelet et al. 2013).

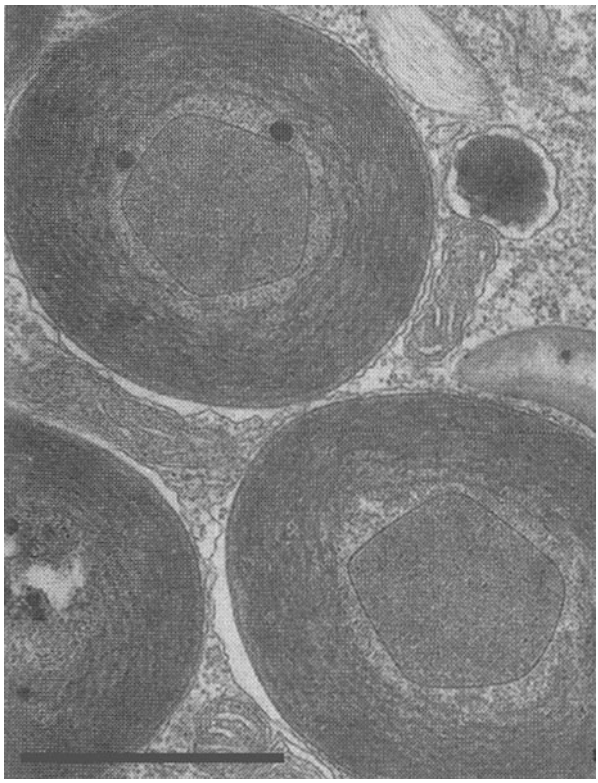

Fig. 5 Muroplasts of *Gloeochaete wittrockiana*, strain SAG 46.84 (Kies strain IABH 2323) with concentric thylakoid membranes. The central part contains a large polyhedral body confined by an electron-dense layer. Transmission electron micrograph. *Scale line* = 1 μm (Taken from Kies and Kremer (1990))

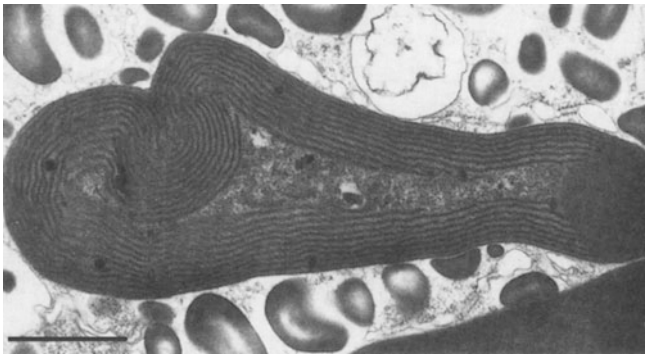

Fig. 6 A muroplast of *Glaucocystis nostochinearum*, strain IABH 2344 (Kies strain), in longitudinal section. Note the irregular, rodlike shape (spherical in all other glaucophytes) and the polar position (central in all other glaucophytes) of the RuBisCo microcompartment (Transmission electron micrograph. Scale line = 1 μm (Taken from Kies and Kremer (1990))

Table 2 Morphological characters of Glaucophyta

Character	*Cyanophora*	*Cyanoptyche*	*Gloeochaete*		*Glaucocystis*
			Zoospore	Vegetative cell	Vegetative cell
Organization	Monadoid	Palmelloid	Monadoid?	Capsalean	Coccoid
Reproduction	Bipartition	Binary fission	*	Successive bipartition	Progressive cleavage autospores
Cell wall	No wall	Mucopoly-saccharidic	No wall	Non-cellulosic	Cellulosic [a, b]
Layer of flat vesicles underneath the plasmalemma	+	+	+	+	+
Apical depression	+	–	+	+	+
pulsating vacuoles	+	+	+	+	+
Golgi bodies	Parabasal	Parabasal perinuclear?	Parabasal	Parabasal	Parabasal
Symmetry of monadoid stages	Dorsoventral	Dorsoventral *	Dorsoventral	*	*
Flagella	2, with mastigoneme	*	2, with mastigonemes	2 pseudocilia	2 reduced flagella
Cross section of flagella	(9 + 9) + 2	(9 + 9) + 2	(9 + 9) + 2	(9 + 9) + 0	(9 + 9) + 0
Kinetid	Cruciate, 2 MLS[c, d]	*	Cruciate 4 MLS	Cruciate 4 MLS	Cruciate[c] 4 MLS

Nuclear membrane fragments during mitosis, open spindle	+	?	*	+	+
Centrioles	–	?	*	–	–
Phycoplast	–	?	*	–	–
Persistent telophase spindle	+	?	*	+	+
Division by infurrowing	+[e]	?	*	+	+
Starch grains free in cytoplasm	+	+	+	+	+
Mitochondria with flattened cristae	+	+	+	+	+
Muroplasts with peptidoglycan wall	+	+	+	+	+

+ = character present, – = character absent, * = not applicable, ? = not investigated; MLS = multilayered structure
References: Kies (1992)
Additional references in Kies (1979) and Trench (1982)
[a]Schnepf (1965)
[b]Robinson and Preston (1971)
[c]Rogers et al. (1981)
[d]Melkonian (1983)
[e]Pickett-Heaps (1972)

Habitats and Ecology

In terms of being reported in the literature, the cosmopolitan *G. nostochinearum* is most frequent, followed by *C. paradoxa* and *G. wittrockiana* (see also Table 1). Because the knowledge of their distribution pattern and ecological niches is incomplete, and there is only a limited and dispersed literature on their ecology, glaucophytes are not easy to collect.

Cyanophora paradoxa, originally found in small eutrophic ditches near Kharkov, Ukraine (Korshikov 1924), was isolated by Pringsheim in England from alkaline water and from a soil sample taken from a fishpond near Erlangen, Germany, by Kies (Kies 1979; Pringsheim 1958). *Cyanophora tetracyanea* has been collected from river plankton in the Gorki district of Belarus and from the littoral zone of Lake Fibysjon, Sweden (Skuja 1956). *Cyanoptyche gloeocystis* and its subspecific taxa have been found in *Sphagnum* bogs (Pascher 1929) and in ponds rich in submerged cormophytes and diatoms (Geitler 1959b). It has been found on the underside of floating leaves of *Potamogeton natans*, a monocotyledonous angiosperm (Pascher 1929). In some instances it occurred together with other glaucophytes such as *Gloeochaete* and *Glaucocystis. Gloeochaete wittrockiana* is epibiotic on filamentous chlorophytes such as *Oedogonium*, *Rhizoclonium*, *Chara*, and *Nitella*, the xanthophyte *Vaucheria*, and the leaves of aquatic mosses and submerged angiosperms. It has been found both in acidic *Sphagnum* bogs, soft water lakes poor in plant nutrients (Skuja 1956), and ditches with medium levels of inorganic nutrients (Kies 1979). Skuja frequently found its zoospores in the plankton of some Swedish lakes. It often occurred together with *Glaucocystis nostochinearum. Glaucocystis nostochinearum* has been found in acid and alkaline waters (Geitler 1959a); it was reported from the plankton of Swedish lakes and ponds where it occurred together with *Gloeochaete wittrockiana* (Skuja 1956) and from swamps and bogs. It was collected from a drainage ditch near Hamburg, Germany, rich in submerged land plants and also containing *Gloeochaete wittrockiana* (Kies 1979). The pH was 6.5–8.2.

Characterization and Recognition

Glaucophytes are distinguished by ultrastructural and biochemical characters. The phylum Glaucophyta can be defined as follows (for references see Tables 1 and 2): Glaucophyta (glaucophytes) are mastigote (Fig. 1) or coccoid algae (Fig. 4a), single or in colonies (Fig. 2). Typical carotenoids of cyanobacteria such as echinenone and myxoxanthophyll are absent. They display characters of oxygenic prokaryotic photosynthesizers (photosystems I and II). The thylakoids are concentrically arranged (Figs. 1, 5, and 6), and the muroplast pigments are chlorophyll *a*, β-carotene, zeaxanthin, β-cryptoxanthin, allophycocyanin, and C-phycocyanin.

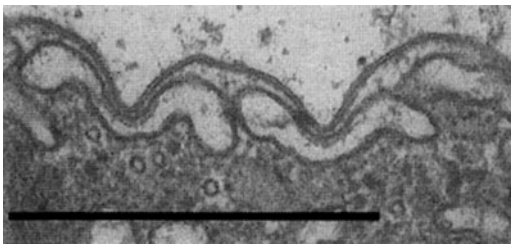

Fig. 7 Pellicle of *Glaucocystis nostochinearum*, strain IABH 2344 (Kies strain). Flat vesicles (lacunae) associated with microtubules form a layer beneath the plasma membrane. Transmission electron micrograph. *Scale line* = 0.5 μm (Taken from Kies and Kremer (1990))

Flagellated vegetative cells and asexual reproductive cells (mastigotes), if present, have a dorsoventral construction. In motile forms, two flagella (Heimann et al. 1989; Fig. 1) both with mastigonemes arise in an apical groove. One is directed toward the direction of swimming, the other laterally. In vegetative cells of *Gloeochaete*, stiff, hairlike extensions called pseudocilia arise (Fig. 2) in an apical depression (Fig. 3). In *Glaucocystis*, reduced flagella are present. The cruciate kinetid contains four multilayered structures (MLS) (Table 2) in *Gloeochaete* and *Glaucocystis* and two in *Cyanophora.*

Glaucophytes contain mitochondria with flattened cristae. An open spindle appears during mitosis, but centrioles and phycoplasts are absent. Cytokinesis occurs by infurrowing of the plasma membrane. Reproduction is by longitudinal binary fission in the mastigotes, by multiple mastigotes or immotile reproductive cells ("autospores," Fig. 4b, d) in capsalean and coccoid members. Sexuality has not yet been reported; a lacuna pellicular system is present (Heimann et al. 1997; Fig. 7). The polysaccharide reserve product (starch) accumulates in the cytoplasm of the host cell in the form of minute granules.

Classification

A classification scheme was first proposed by Skuja (1954). A more recent treatment of the Glaucophyta (Kies and Kremer 1986; Kies 1992) differs from Skuja's scheme in that:

1. The diagnosis of the phylum and class is emended to include ultrastructural and biochemical characters. Typified names instead of descriptive names are used for all taxa.
2. Mastigotes, which comply with the emended diagnosis, are included.
3. Separate orders are established for monadoid (Fig. 1), capsalean (Fig. 3), and coccoid (Fig. 4a) genera.
4. Taxa of uncertain affiliation with the Glaucophyta due to incomplete description and/or lacking ultrastructural and biochemical evidence are treated here as genera and species inquirendae.

Maintenance and Cultivation

Gloeochaete and *Glaucocystis*, the only common glaucophytes, are isolated by use of a capillary pipette (Hoshaw and Rosowski 1973). From fresh natural collections, single cells or colonies are removed and transferred with a sterile capillary pipette in a Petri dish through at least ten drops of sterile culture medium to dilute out undesired organisms. Between each step the capillary pipette is newly pulled through a flame. *Gloeochaete* living epibiotically on filamentous freshwater algae should be isolated together with parts of the filaments. In fresh culture medium multiple mastigotes are formed readily and may be isolated as described.

Axenic cultures have been established from *Cyanophora* and *Glaucocystis* species (see Table 1). Fluorescence-activated cell sorting (FACS) proved to be the method of choice. The criteria adopted were maximum chlorophyll autofluorescence and maximum forward scatter. A total of 20–30% of the sorted single cell cultures grew successfully, and among these more than 20% were axenic (Sensen et al. 1993). Isolates from four genera were deposited in culture collections (Table 1), among them at least five species of *Cyanophora*, whereas the other genera appeared to be monospecific: *Cyanoptyche gloeocystis*, *Glaucocystis nostochinearum*, and *Gloeochaete wittrockiana*. However, recently a more thorough investigation of the genus *Glaucocystis* (Chong et al. 2014; Takahashi et al. 2016) led to a splitting into several species as has also happened for *Cyanophora* (Takahashi et al. 2014). A compilation including the latest results is presented in Fig. 8. The best sources are the SAG (Göttingen, Germany), the NIES (Tsukuba, Japan), and the CCAC (Cologne, Germany) that keep several Kies strains (Table 1).

Culture media recipes can be found in the SAG catalogue and web site (http://www.uni-goettingen.de/). *Cyanophora paradoxa* cultures show a requirement for vitamin B_{12} as an essential cofactor for methionine biosynthesis, which in natural habitats likely is provided by environmental bacteria (Croft et al. 2005).

Biochemistry, Molecular Biology, and Cell Biology

Here, research done during the past 25 years will be reviewed. Almost all data were obtained with *C. paradoxa*, and most of them deal with various aspects of muroplast biology. The clear outcome is that muroplasts are primary plastids sensu stricto.

The Muroplasts of *Cyanophora paradoxa*: Protein Import, Biochemical Pathways, and Plastome Organization

Emphasis is given to processes and structures for which biochemical and cell biological experiments corroborate and extend the information obtained from plastome and genome sequencing.

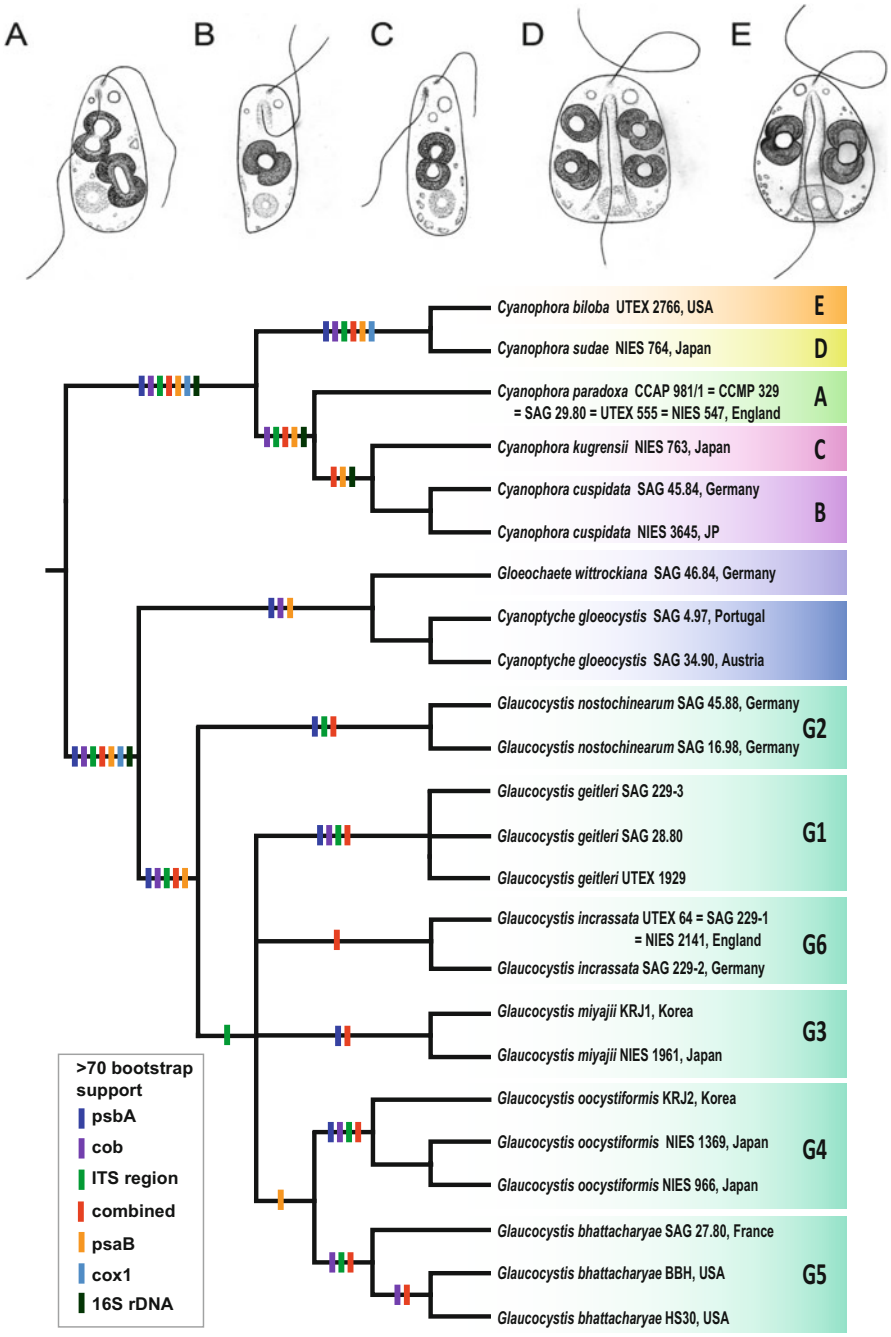

Fig. 8 Phylogenetic relationship of the Glaucophyta based on plastidal psbA, psaB, 16S rRNA, mitochondrial cox1, cob, and nuclear ITS region including ITS 1 and 2, 5.8S, partial SSU, and LSU

Protein Import into Muroplasts

Considerable progress has been made during the past 20 years with respect to components and mechanism of the import apparatus of land plant chloroplasts. It consists of two independent but cooperating translocons, Toc and Tic (Paila et al. 2015), at the outer envelope membrane (OEM) and the inner envelope membrane (IEM), respectively. Important translocon components are Toc75 (channel), Toc34 and Toc159 (receptors), Tic110 (putative channel), Tic20 (putative channel), Tic21, Tic22, and Tic40. GTP is the energy source for OEM translocation, and ATP energizes further translocation across the IEM via chaperone action. There is agreement that the import apparatus constitutes a eukaryotic "invention" which does not preclude the recruitment of suitable cyanobacterial membrane proteins (Reumann et al. 2005; Kalanon and McFadden 2008).

Nucleus-encoded muroplast polypeptides are synthesized in the cytosol as precursors containing cleavable N-terminal transit sequences that are 35–90 aa in length (Steiner and Löffelhardt 2002). These resemble chloroplast stroma-targeting peptides (Bruce 2000) in domain structure, amino acid composition (especially at the processing site; Köhler et al. 2015), and positive net charge. However, the N-terminal motif MA(A)FVxxVP is found with slight variation in nearly all muroplast transit sequences (Steiner and Löffelhardt 2002, 2005) but not in those for land plant or green algal chloroplasts. Pre-FNR and pre-transketolase from *C. paradoxa* were efficiently imported into isolated muroplasts (Ma et al. 2009; Jakowisch et al. 1996). Other precursors as pre-cytochrome c_6 and pre-RuBisCO activase performed even better during in vitro import and were completely internalized after 3–7 min incubation (Burey et al. 2005; Steiner et al. 2000). The energy requirements (ATP, temperature) corresponded to those for chloroplast import. The observed stability of muroplasts due to their peptidoglycan armor is misleading: even a slight osmotic shock causes damage of the OEM and loss of import competence (Steiner and Löffelhardt 2002), CO_2 fixation (Trench 1982), and in organello protein synthesis (Löffelhardt and Bohnert 2001).

In addition to pre-FNR (Jakowitsch et al. 1996), all other *Cyanophora* precursors tested are readily imported into isolated chloroplasts from spinach or pea (Ma et al. 2009; Steiner and Löffelhardt 2002, 2005). However, the inverse heterologous import, i.e., of precursors from land plants into isolated muroplasts, did not occur. Therefore, the N-terminal consensus sequences appear to be the sole recognizable difference between muroplast and chloroplast stroma- targeting peptides. The phenylalanine residue, usually at position three or four, is conserved and might be

Fig. 8 (continued) rDNA (Modified from Chong et al. 2014; Takahashi et al. 2014). Strain number and its origin were indicated beside the species name. Six clades of *Glaucocystis* species complex were marked as G1–G6 (Chong et al. 2014), while three new *Cyanophora* species from Takahashi et al. (2014) have been adopted in this phylogeny. Color bars indicate >70% bootstrap support values for each node from each individual gene. Ink drawings for A–E (*Cyanophora* species) were taken from Takahashi et al. 2014

crucial for successful translocation across the muroplast envelope. Its prevalence was recently confirmed through proteomic studies on isolated muroplasts (Köhler et al. 2015). Indeed, deletion or exchange of this amino acid from *C. paradoxa* pre-FNR led to impeded or even completely abolished import into muroplasts (Steiner et al. 2005a). The obvious next step was to engineer a chloroplast precursor, pre-FNR from *Mesembryanthemum crystallinum*, with the missing phenylalanine in the N-terminal region of the transit sequence. This enabled heterologous import with an efficiency comparable to homologous import (Steiner et al. 2005a).

The high gene content of their plastomes, the PBS light-harvesting antennae, and results of phylogenetic analyses make it possible to categorize muroplasts and rhodoplasts as "plastids with ancestral characteristics." An inspection of putative transit sequences of nucleus-encoded rhodoplast proteins from various red algae revealed N-terminal consensus sequences very similar to those for muroplast stroma-targeting peptides. The crucial phenylalanine residue is always present, even in precursors targeted to secondary plastids derived from endosymbiotic red algae where a phenylalanine residue is created as the first amino acid of the transit sequence after cleavage of the preceding signal sequence (Patron and Waller 2007; Gould et al. 2006; Kilian and Kroth 2005). On the other hand, this is not found in precursors to chloroplasts or secondary plastids from the "green lineage." When this feature is considered as typical for primordial plastids, it might as well have been taken over or adapted from the prokaryotic ancestor: phenylalanine has been reported to occupy a prominent position in the sequence of bacterial proteins targeted to the outer membrane (Struyvé et al. 1991) as porins (C-terminus) or type IV pilins (N-terminus, created by prepilin peptidase cleavage). An outer membrane protein, Omp85, acting as receptor/chaperone for such proteins recognizes their exposed phenylalanine residue and assists in their correct membrane assembly (Voulhoux and Tommassen 2004). When such a preexisting cyanobacterial protein was recruited (after transfer of its gene to the nucleus) for the development of a protein import apparatus in the endosymbiont envelope, it could have been oriented inversely so that precursors with a phenylalanine signature coming from outside, i.e., from the eukaryotic cytosol, would be recognized. Indeed, this reorientation could recently be demonstrated (Sommer and Schleiff 2014). Thus, an Omp85-like protein (due to its sequence similarity, chloroplast Toc75 is included in the Omp85 family) could have been adapted to fulfil dual functions, that of the "Phe-receptor" and that of the protein import channel (Steiner and Löffelhardt 2005; Steiner et al. 2005a). Blue-native gels of isolated muroplasts yielded a distinct signal (αToc75) for the Toc complex at about 550 kDa. (Yusa et al. 2008). The muroplast import apparatus is considered as a prototype that has not undergone many changes relative to that of the ancestral protoplastid which might also apply to rhodoplasts and, likely, to secondary plastids derived from red algal endosymbionts (with respect to the two innermost membranes). Omp85 proteins are suitable for the proposed dual role because: (i) these are the only members of the Omp85 family that can form pores of sufficient diameter to allow protein translocation and (ii) they display a presequence (Phe)-binding domain. Phe in the transit sequence of pre-FNR from *C. paradoxa* was shown to reduce unspecific binding to liposomes but to enhance binding to

proteoliposomes containing Omp85 from *Anabaena variabilis* (Wunder et al. 2007). There is now evidence for the minimal set of components of the Toc/Tic complexes (see section on Genome Analysis of Glaucophytes; Bhattacharya et al. 2014; Löffelhardt 2014). The interaction of both complexes might be more pronounced than in chloroplasts, resulting in fixed positions of import sites coinciding with localized lesions in the organelle wall. The latter are necessary to allow translocation of large proteins that would have problems with the narrow mesh size of the peptidoglycan network and could be generated through the action of lytic transglycosylases bound to the import complex (Steiner and Löffelhardt 2005).

Conservative Sorting

The "conservative sorting" hypothesis posits that organelles (mitochondria, plastids) that are derived from prokaryotic endosymbionts not only had to develop a selective protein import apparatus at their envelope but also retained prokaryotic preprotein translocases at their inner envelope (mitochondria) and thylakoid membranes (chloroplasts). Conservative sorting in land plant chloroplasts is widely accepted (Smeekens et al. 1990). Bipartite presequences, i.e., a transit sequence followed by a signal sequence, are indicative of intraplastidic sorting to the thylakoid lumen or thylakoid integration. According to the translocons involved and the respective energy requirements, the Sec pathway transporting unfolded passenger proteins and the ΔpH-dependent or Tat pathway for folded proteins are defined (Cline and Dabney-Smith 2008), both being paradigms for conservative sorting of (largely) lumenal proteins. Cyanobacteria, the ancestors of plastids, are capable of "exporting" cytosolically synthesized preproteins either to the periplasmic space or into the thylakoid lumen (Mackle and Zilinskas 1994). Muroplasts also possess a periplasmic space between IEM and OEM containing the peptidoglycan wall, seven penicillin-binding proteins, enzymes of peptidoglycan degradation and modification, cytochrome c_6, etc. (Steiner et al. 2000; Löffelhardt and Bohnert 2001). Therefore it is justified to postulate conservative sorting for both the thylakoid and inner envelope membranes (Fig. 9) as was first shown for cyanobacteria with respect to the Sec translocase (Nakai et al. 1993).

Sec pathway: Here, muroplasts have played a leading role for some time because *secY* is a muroplast gene and was shown to complement the thermosensitive *secY24* mutation in *E. coli* (Flachmann et al. 1993). In subsequent work, an expressed sequence tag (EST) for nuclear-encoded SecA was found in *C. paradoxa*. Muroplast SecA appeared to be quite susceptible to inhibition by sodium azide during import experiments with homologous precursors: the amount of mature cytochrome c_6 was reduced and intermediate accumulated in the stroma, whereas thylakoid translocation of the larger intermediate form of PsbO was completely abolished (Steiner et al. 2005b). Cyanobacterial thylakoids do not form tight vesicles upon isolation and thus are not suitable to demonstrate protease protection of internalized, processed lumenal proteins. With improved muroplast fractionation methods, it was possible, at least for PsbO, to show Sec-dependent translocation in organello and, after

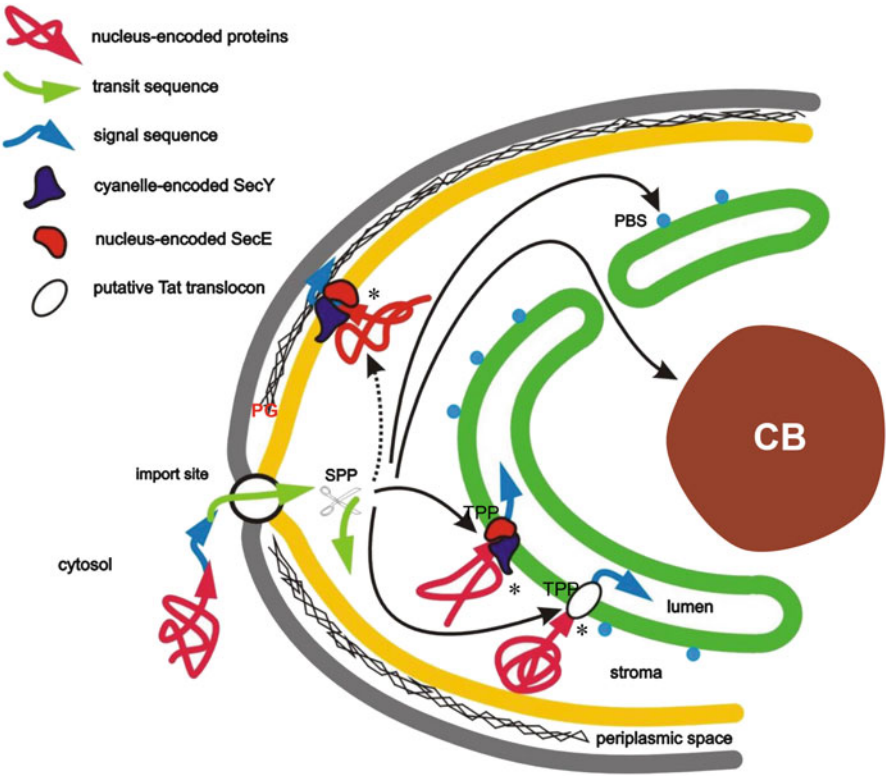

Fig. 9 Conservative sorting within the muroplasts of *Cyanophora paradoxa*. Proteins directed by a specific transit sequence (muroplast stroma-targeting peptide) across the muroplast envelope into the stroma can either stay there, or can be integrated into a microcompartment, or can be sorted by a signal sequence (in case of a bipartite presequence) to the thylakoid lumen or the periplasmic space, respectively. *CB* central body, *PBS* phycobilisome, *PG* peptidoglycan, *Spp* stroma processing peptidase, *TPP* thylakoid processing peptidase, * conservative sorting

muroplast lysis and thylakoid isolation, for the first time protease protection of the mature protein inside of phycobilisome-bearing thylakoids. Nigericin did not interfere; addition of azide to the import assay abolished protease protection of PsbO by inhibiting thylakoid translocation (Steiner et al. 2005b). However, import experiments into isolated thylakoids are only possible in land plant systems. The *Cyanophora* Genome Project revealed contigs for secA and thylakoid processing proteases (TPP) but no additional, nucleus-encoded SecY (Table 3; Steiner et al. 2012): there is but one *secY* gene and one *secA* gene, as in cyanobacteria. The generation of specific antisera directed against muroplast SecY allowed the demonstration of dual localization (Fig. 9) of the Sec translocon in muroplasts (Yusa et al. 2008). SecY-containing bands of distinct size were immuno-decorated on blue-native gels of thylakoid membranes and IEM, respectively (Koike et al. 2007). In land plant chloroplasts, a second Sec translocase was recently shown at the IEM, but

Table 3 Genes for components of protein sorting pathways within the muroplasts of *Cyanophora paradoxa* and for candidate passengers undergoing spontaneous membrane insertion (Steiner et al. 2012)

Protein	Function	Comments
SecY	Sec translocase	One copy on the muroplast genome
SecE (n.d.)	Sec translocase	Should be present. Low sequence conservation
SecA	Sec translocase	N-terminal fragment with STP[a]
TatC	Tat translocase	5 TM domains, negatively charged N-terminus[a]
TatA	Tat translocase	STP, 1 TM domain, highly polar C-terminus[b]
TPP	Signal peptide cleavage	LepB1 homolog[a], 1 TM domain
TPP	Signal peptide cleavage	Fragment, putative LepB2 homolog[a]
mpSRP54	Signal recognition particle	STP, GTP-binding domain[a]
mpFtsY	SRP receptor	GTP-binding domain[b]
Albino3	D1 insertase	STP, 5 TM domains[b]
Vipp1	Thylakoid stabilization	STP, amphipathic α-helix at C-terminus[a]
PsbW	Spontaneous insertion?	STP, SP, 1 TM domain[b]
PsaK	Spontaneous insertion?	2 TM domains[a]

n.d. not detectable, *mp* muroplast, *STP* stroma-targeting peptide, *SP* signal peptide, *TM* transmembrane

[a]Best hits among cyanobacteria

[b]Best hits among green algae and plants

SecY and SecA are derived from nuclear genes different from those giving rise to the thylakoid Sec translocon (Skalitzky et al. 2011).

Tat pathway: In *C. paradoxa* EST databases, nucleus-encoded candidate passengers were found, as pre-PsbU and pre-PsbQ', with bipartite presequences containing the typical "twin-arginine" motif in the signal sequence (Cline and Dabney-Smith 2008) that did not respond to azide. The problem is that the effect of nigericin on muroplasts obviously is weaker than on land plant chloroplasts. In the non-cleavable signal-anchor sequence immediately after the transit sequence, a KR motif is found in both cases (RR only in cyanobacterial pre-PetC). In the presence of azide, but not of nigericin, it was possible to detect low amounts of protease-protected (i.e., internalized) mature protein trimmed by removal of five N-terminal amino acids (preceding the single transmembrane domain) protruding into the stroma. This was interpreted as evidence for operation of the Tat pathway in the muroplast thylakoid membrane (Steiner et al. 2005b). With the availability of the genomic sequence, genes for TatA and TatC could be identified (Table 3; Steiner et al. 2012). There is now also evidence for dual localization of the Tat translocase in cyanobacteria (Aldridge et al. 2008). In the absence of any experimental data, this is also a likely scenario for muroplasts resulting in fully conservative sorting, whereas for chloroplasts an IEM-resident Tat translocase is rather not envisaged (Skalitzky et al. 2011). The muroplast signal recognition particle (SRP) protein and the corresponding receptor (distinct from the cytosolic counterparts) were also identified and included in the compilation of Table 3. Since LHCP is missing from glaucophytes and rhodophytes, a posttranslational SRP pathway should not be operative in the plastids

from both phyla. However, the cotranslational SRP pathway with the important function of thylakoid integration of PSII and PSI reaction centers (Ossenbühl et al. 2006) can be considered as another example of conservative sorting and is expected to be active in muroplasts and rhodoplasts. Consequently, the genes for Albino3/Oxa1/YidC and Vipp1 were identified, whereas the SRP-RNA which is encoded on all rhodoplast genomes could not be found on muroplast DNA (M. Rosenblad, personal communication). Obviously, mpSRP54 alone can fulfill its function without an RNA component. Table 3 is completed by two candidates for spontaneous (i.e., unassisted) thylakoid insertion (Tissier et al. 2002) which seems to be a special feature of galactolipid-rich plastid membranes.

Structure and Biosynthesis of the Unique Eukaryotic Peptidoglycan

In contrast to chloroplasts, isolated muroplasts of *C. paradoxa* are stable in hypotonic medium. This is due to the presence of a lysozyme-sensitive murein sacculus in the muroplast envelope (Schenk 1970). This "organelle wall" with an estimated thickness of 7 nm has hitherto only been found in the eubacterial kingdom. Such a peculiar prokaryotic wall around a eukaryotic organelle, perhaps the most striking biochemical evidence for the cyanobacterial origin of plastids, was assumed to mimic early stages of primary endosymbiosis and justified (for some time) consideration of *C. paradoxa* and glaucophytes in general as "living fossils." The basic components of muroplast peptidoglycan were identified as those known for the $A_1\gamma$-type found in Gram-negative bacteria: *N*-acetylmuramic acid, *N*-acetylglucosamine, L-alanine, D-glutamic acid, *m*-diaminopimelic acid, and D-alanine (Aitken and Stanier 1979). Analogous results were reported for the muroplast wall from *G. nostochinearum* (Scott et al. 1984). Cleavage of purified muroplast peptidoglycan from *C. paradoxa* with *Chalaropsis* muramidase and separation by HPLC yielded a muropeptide pattern different from that of *E. coli*: Only 7 of the 29 major muropeptides investigated by a combination of amino acid analysis and mass spectrometry were identical to bacterial counterparts. The remaining 22 appeared to be derived from known muropeptides of *E. coli* by a substitution leading to an increment in MW of 112 or multiples thereof (Pfanzagl et al. 1996a). The modification was localized to the C-1 carboxylic group of the D-isoglutamoyl moiety, and *N*-acetylputrescine was identified as the substituent (Pittenauer et al. 1993). The structures of all 29 major muropeptides (4 monomers, 8 dimers, 11 trimers, and 6 tetramers) have been elucidated (Pfanzagl et al. 1996a). In fact, the muroplast wall is thicker and more cross-linked than the cell wall of *E. coli*. The substitution (not detected in cyanobacteria) and the reduced thickness (as compared to the cyanobacterial wall) could thus both serve the purpose of increasing the permeability of the peptidoglycan network. This might be especially important for a cell organelle which requires extensive protein import from the cytoplasm. Indeed, *N*-acetylputrescine was also found in the muroplast walls from two other glaucophytes, *G. nostochinearum* and *C. gloeocystis* (Pfanzagl et al. 1996b), indicating that it really constitutes a signature for muroplasts in general, i.e., for the "eukaryotic"

peptidoglycan of an armored organelle. An alternative, less likely function might be in connecting the PG layer to the OM in the absence of murein lipoprotein (Pfanzagl et al. 1996a) as was reported for some rare cases of anaerobic Gram-negative bacteria that show cadaverine or putrescine linked to C-1 of the isoglutamoyl moiety (Kojima et al. 2010).

The biosynthetic pathway of *C. paradoxa* murein appears to be analogous to that of *E. coli* with respect to intermediates, the participating enzymes, and their compartmentation. Penicillin-binding proteins (PBPs) possess transglycosylase and/or transpeptidase activity and perform the last steps of bacterial peptidoglycan biosynthesis by introducing new monomeric building blocks into the growing carbohydrate chain and cross-linking the peptide side chains (Sauvage et al. 2008). Seven PBPs in the size range from 110 to 35 kDa were identified in the muroplast envelope by labelling with a radioactive derivative of ampicillin (Berenguer et al. 1987). Accordingly, β-lactam antibiotics are lethal for *C. paradoxa* in much the same concentrations as for eubacteria. Also, differential sensitivity of individual PBPs toward different penicillin derivatives was demonstrated (Berenguer et al. 1987). Muroplast division is arrested whereas cell division continues, finally leading to colorless, nonviable cells. Dumbbell-shaped muroplasts were also observed upon benzyl penicillin and vancomycin treatment of *C. paradoxa* (Iino and Hashimoto 2003). Indirect evidence was obtained for a periplasmic localization in the muroplasts of *C. paradoxa* of DD- and LD-carboxypeptidases and DD-endopeptidase, enzymes hydrolyzing defined bonds in peptidoglycan (Plaimauer et al. 1991). As in the cytosol of *E. coli* (Barreteau et al. 2008), the biosynthesis of the soluble precursor of peptidoglycan, UDP-*N*-acetylmuramoyl pentapeptide, was shown to occur in the muroplast stroma (Plaimauer et al. 1991). The membrane-bound steps, i.e., the transfer of UDP-*N*-acetylmuramoyl pentapeptide to undecaprenylphosphate (yielding Lipid I) and disaccharide formation with *N*-acetyl glucosamine (yielding lipid II), occur in analogy to *E. coli* (Bouhss et al. 2008) at the inner envelope membrane of muroplasts followed by putrescinylation at C-1 of the D-isoglutamyl moiety and then *N*-acetylation (Pfanzagl and Löffelhardt 1999). Amidation of *Staphylococcus aureus* PG at the same position was recently reported to also occur at the stage of lipid II (Münch et al. 2012). Surprisingly, the muroplast genome encodes only a single protein potentially involved in peptidoglycan biosynthesis during septum formation, FtsW (Löffelhardt et al. 1997). One proven function of *E. coli* FtsW is the recruitment of PBP3 (FtsI) to the divisome. More than 30 eukaryotic genes specifying enzymes responsible for building up the prokaryotic organelle wall must therefore reside in the nuclear genome of *C. paradoxa*. Recently, a homolog to the cyanobacterial division protein SepE, which has a role in assembly and stability of the FtsZ ring (Hamoen et al. 2006), was also identified on the muroplast genome. The expression of *ftsW* and *sepE* appear to be cell cycle independent (Miyagishima et al. 2012).

Muroplast division in *C. paradoxa* shows intermediate features between cyanobacterial and plastid division (Iino and Hashimoto 2003; Sato et al. 2009). This was to be expected since it is strictly dependent upon the formation of a peptidoglycan septum in contrast to all other plastid types which nevertheless rely

on a number of cell division genes of bacterial origin as *ftsZ*, *ftn2* (*arc6*), *minD*, *minE*, etc. (Yang et al. 2008). Interestingly, there is but one gene for (muroplast-targeted) FtsZ on the *C. paradoxa* genome (as in cyanobacteria) and no mitochondrial counterpart, whereas algae and plants possess at least two genes for the chloroplast proteins and, more recently (e.g., in case of stramenopiles), additional genes for the mitochondrial FtsZ were described (Leger et al. 2015). Muroplasts and chloroplasts show in the stroma a distinct inner plastid division (PD) ring, corresponding to the FtsZ ring superimposed by a thicker, electron-dense ring. However, muroplasts lack the outer chloroplast division ring and the adjacent ring formed by the dynamin-related protein ARC5 (DRP5B). These components of the chloroplast division machinery are considered as host cell contributions after the endosymbiotic event. Nuclear genes for MinD and MinE, proteins determining the site of the division septum, and for ARC6 (assumed to tether the FtsZ ring to the IEM) were identified in *C. paradoxa*, and their expression was shown to be regulated by the cell cycle (Miyagishima et al. 2012). In contrast, FtsZ was found to be constitutively expressed. In (cyano)bacteria, various hydrolases function in PG splitting during septum formation. Recently, a homolog of the gene for DipM was detected on the nuclear genome of *C. paradoxa*, and the protein was shown to localize to the intermembrane space of dividing muroplasts at the site of septum formation (Miyagishima et al. 2014a). Again, the expression of DipM followed the cell cycle with a peak in the S phase.

In the *Cyanophora* Genome Project, three different approaches were used for PBP gene identification: (1) domain searches, (2) BLAST searches against the eight PBP genes of *Synechocystis* sp. PCC6803 (Marbouty et al. 2009) and the *Anabaena* sp. PCC7120 homologs, and (3) BLAST searches against *Physcomitrella patens* PBP-like genes. In most cases, the results converged leading to the identification of at least 11 genes or gene fragments (Bhattacharya et al. 2014); examples of which are shown in Table 4. In general, sequence similarity was higher to homologs in cyanobacteria than to those in *P. patens*. In some cases of periplasmic proteins, bipartite presequences consisting of a transit peptide and a signal peptide could be found. This suggests import to the muroplast stroma, followed by export to the periplasmic space. This special variant of "conservative sorting" would necessitate a dual location of Sec (already documented) and Tat (seems possible as another parallel to cyanobacteria) translocases on thylakoid and inner envelope membranes of muroplasts. In a Gram-negative background, the low molecular weight (MW) peptidases VanX and VanY are not linked to vancomycin resistance but rather to D-alanine recycling and to an additional endolysin, respectively. Peptidoglycan biosynthesis requires cleavage of existing glycan chains to allow for insertion of new material. This is performed by soluble and membrane-bound lytic transglycosylases: one gene of this kind could also be identified in *C. paradoxa*. A lysozyme family protein with significant similarity to protist lysozymes displays a signal peptide indicating a vacuolar (lysosomal) location that is likely involved in the autophagosomal digestion of damaged muroplasts. Genes for stromal proteins that are involved in the synthesis of the soluble precursor are also listed in Table 4. The N-terminal transit peptide identifies one such gene in *C. paradoxa* (*glmS*, specifying

Table 4 Nuclear genes involved in biosynthesis and degradation of muroplast peptidoglycan in *Cyanophora paradoxa*

Gene/protein	Function[a]	Localization
PBP1, PBP2	PG transglycosylase/transpeptidase	PS, IEM
PBP1, PBP2	PG transglycosylase/transpeptidase	PS, IEM
ftsI/PBP3	PG transglycosylase/transpeptidase	PS, IEM (septal ring)
PBP4	PG transglycosylase/transpeptidase	PS
dacB/PBP 5	D-Ala-D-Ala-carboxypeptidase,	PS
	D-Ala-D-Ala-endopeptidase	
PBP 8	D-Ala-D-Ala-carboxypeptidase C	PS
vanX	D-Ala-D-Ala-dipeptidase	PS
vanY/endolysin	D-Ala-D-Ala-carboxypeptidase	PS
Lysozyme-like	Muramidase	PS
mlt	Lytic transglycosylase	PS
dipM	PG splitting enzyme	PS (septum site)
glmS	Glucosamine-6-P synthase	Stroma
murA	UDP-*N*-acetylglucosamine-	Stroma
	1-carboxyvinyl transferase	
murB	UDP-*N*-acetylenolpyruvoyl-	Stroma
	glucosamine reductase	
murC	UDP-*N*-acetylmuramate:	Stroma
	L-Ala ligase	
murI	Glutamate racemase	Stroma
murD	D-Glu-adding enzyme	Stroma
murE	DAP-adding enzyme	Stroma
alr	Alanine racemase	Stroma
ddl	D-Ala:D-Ala ligase	Stroma
murF	UDP-*N*-acetylmuramoyl	Stroma
	tripeptide/D-Ala-D-Ala ligase	
mraY	Lipid I synthesis	IEM
murG	Lipid II synthesis	IEM

PS periplasmic space, *IEM* inner envelope membrane
[a]The high MW (1–4) and the medium MW (5–8) PBPs are redundant in *Synechocystis* sp. PCC6803

D-glucosamine-1-phosphate synthase) as a member of the muroplast-resident PG biosynthesis pathway, whereas the cytosolic counterpart would be expected to participate in protein glycosylation. The complete set of enzymes that are involved in UDP-*N*-acetylmuramate biosynthesis as well as the peptide side-chain adding enzymes and the alanine (Alr) and glutamate (MurI) racemases are encoded on the nuclear genome of the alga. The IEM-bound or associated MraY and MurG proteins complete this compilation.

Genes for enzymes of PG biosynthesis were transferred twice into Archaeplastida during the course of evolution – from the more ancient donor of the mitochondrion and from the subsequent cyanobacterial ancestor of plastids. These genes retain a high sequence similarity in *Arabidopsis thaliana* (few genes) and the moss

Physcomitrella patens (almost complete set), but their functions are likely to have changed. As long as chemical and structural proof is lacking, (pleiotropic) effects of antibiotics or gene knockouts on plastid division do not provide sufficient evidence to claim the presence and biosynthesis of PG in the plastids of bryophytes (Takano and Takechi 2010). FtsZ in (cyano)bacteria and muroplasts (derived from a single gene) is assumed to recruit the divisome proteins forming the peptidoglycan septum. In rhodoplasts and chloroplasts, the FtsZ ring is thought to instead recruit the outer PD ring and the dynamin ring to perform the constriction of the OEM. The *C. paradoxa* genome does not encode any of the host cell-derived plastid division proteins, whereas *P. patens* encodes three DRP5B dynamins (Miyagishima et al. 2014b).

With the present state of knowledge, glaucophyte PG – in the sense of a contiguous, stress-bearing layer between the envelope membranes – appears unique among Archaeplastida. In the rhizarian testate amoeba *Paulinella chromatophora*, the situation is different: there is also PG in this eukaryote, but all genes necessary for its biosynthesis are encoded on the endosymbiont (i.e., "chromatophore," photosynthetic organelle) genome which exceeds the size of plastid genomes by a factor of five to ten (Nowack et al. 2008). Unlike their counterparts in *C. paradoxa*, these genes retain their prokaryotic character; i.e., they were not transferred to the nuclear genome, and thus no import of precursor proteins is required for biosynthesis of the sacculus in photosynthetic *Paulinella* species.

The finding of more than one gene to a given function is not uncommon among cyanobacteria. For example, one of two genes with high sequence similarity to *murG* is more closely related to MGDG synthases, the likely function of "MurG" in plants. In an analogous fashion, *murD*-like genes might instead play a role in folate biosynthesis. Thus, one should expect modified functions for "mur-like" genes, e.g., "MurE" of *Arabidopsis* is involved in chloroplast development but not in chloroplast division (Garcia et al. 2008). However, should it become possible to demonstrate PG in bryophyte chloroplasts through novel, highly sensitive detection methods, as in the case of the cell wall-less bacterium *Chlamydia trachomatis* (Liechti et al. 2014), the chloroplast division apparatus of *P. patens* will have to be reevaluated. A first step in that direction was reported very recently (Hirano et al. 2016).

The Photosynthetic Apparatus of *Cyanophora paradoxa* Muroplasts

The first comprehensive investigation of the components of photosynthesis in *C. paradoxa* was performed by Burnap and Trench (1989). These authors purified ferredoxin, cytochrome b_6, and cytochrome c_6 and verified the absence of plastocyanin from muroplasts. They also isolated photochemically active PSI complexes and could resolve five subunits ranging from 66 kDa to 11 kDa. Further data included the preparation of PSII core particles and of phycobilisomes. More than 10 years later another round of research papers on this topic emerged after the muroplast genome sequence was published. This certainly was very helpful since more than 50% of the thylakoid proteins are contained therein. Shibata et al. (2001) prepared

oxygen-evolving thylakoid membranes and solubilized PSII particles. These contained PsbO and PsbV (cytochrome c_{550}; muroplast encoded), but PsbU was lost from the preparation. Enami et al. (2005) described PsbO, PsbV, and PsbU as the extrinsic proteins of the oxygen-evolving complex (OEC) of cyanobacteria and *C. paradoxa* muroplasts. PsbO, PsbV (rhodoplast encoded), PsbU, and PsbQ' were assigned to *C. merolae* rhodoplasts (Enami et al. 2005), whereas the chloroplasts of green algae and land plants were long known to harbor the OEC components PsbO, PsbP, and PsbQ, all of them as the products of nuclear genes. PSI preparations now allowed the identification of ten subunits, whereby N-terminal protein sequencing was adopted. Sequence alignments in some cases yielded higher similarity to cyanobacterial homologs, in other cases to the counterparts from plants and green algae (Koike et al. 2000). In a comparison of supercomplex organization (where unicellular cyanobacteria possess a PSI trimer), the filamentous N_2-fixing *Anabaena* sp. PCC 7120 and *C. paradoxa* had a PSI tetramer and dimer instead, and the lack of LHCI, likely in all glaucophytes (in contrast to all other phototrophic eukaryotes), was corroborated. On the other hand, PSI monomers only were reported for the extremophilic rhodophyte *C. merolae*. Thus, with respect to PSI, glaucophytes are closer to cyanobacteria than to rhodophytes which also are distinct from the former through their LHCI antennae (Watanabe et al. 2011).

The next quantum leap to come was the *Cyanophora* Genome Project that stimulated a number of related investigations, e.g., on the muroplast proteome of *C. paradoxa* (Facchinelli et al. 2013). A total of 510 polypeptides were identified, among them the proteins of the photosynthesis apparatus with few exceptions, e.g., AtpA. Meanwhile, the state of the art with respect to cyanobacterial OEC components has changed to PsbO, PsbV, PsbU, PsbQ', and PsbP' (the latter two with sequence similarity to chloroplast PsbQ and PsbP), PsbP' being present in substoichiometric amounts with a presumed function in assembly/stability of PSII (Bricker et al. 2012). Muroplast proteomics confirmed PsbO and PsbV and identified PsbP' as an additional component (Facchinelli et al. 2013). PsbU is known to be encoded on the *Cyanophora* genome. The precursor contains the twin-arginine motif in the signal sequence and is one of the candidate passengers for the Tat translocase (Steiner et al. 2005a). Muroplast prePsbP' (also equipped with the RR signature) was proven to be imported into the thylakoid lumen via the Tat pathway in heterologous and homologous import experiments (Kleiner 2014), in analogy to PsbP from land plants. Very recently, a contig representing a PsbQ' homolog (J.M. Steiner, unpublished) completed the list for *C. paradoxa* resulting in a very similar OEC subunit structure for cyanobacteria, glaucophytes, and red algae. Thus, the "primitive" muroplasts and rhodoplasts differ from chloroplasts not only with respect to their extrinsic PBS antennae on the stromal side but also with respect to the extrinsic OEC proteins on the lumenal side of the thylakoid membranes. Chloroplasts have lost PsbV and PsbU in the course of evolution, whereas the gene for PsbP expanded to a small multigene family in land plants (Bricker et al. 2012).

Cyanophora RuBisCO belongs to form IB (as in cyanobacteria and chloroplasts) whereas rhodoplasts contain form ID. Common to muroplasts and rhodoplasts is the *rbcL-rbcS* transcription unit on the respective plastomes and the concentration and

compaction of RuBisCO into a microcompartment, the pyrenoid (see below). Calvin cycle enzymes corresponded to major transcripts (frequent in EST collections) and grouped among abundant stromal proteins with respect to spectral counts (Facchinelli et al. 2013). Again, canonical STPs were found throughout. Ferredoxin-NADP+ oxidoreductase (FNR) of *Cyanophora paradoxa* was characterized at the protein and cDNA level (Gebhart et al. 1992; Jakowitsch et al. 1993). The 34 kDa protein showed high amino acid sequence similarity to land plant counterparts and lacked the C-terminal extension of the cyanobacterial homologs responsible for binding to phycobilisomes. The availability of the ^{35}S-labeled precursor was important for the establishment of an efficient muroplast in vitro import system (see section on Protein Import into Muroplasts). A NAD(P)-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purified from a muroplast extract of *C. paradoxa* as a 142 kDa homotetramer with features similar to the cyanobacterial counterpart (Serrano and Löffelhardt 1994). This is in agreement with the postulated duplication of the *GapA* gene early in streptophyte evolution (Petersen et al. 2006).

The gene for the CP12 protein involved in the formation of inactive complexes of Calvin cycle enzymes during night was also characterized (Petersen et al. 2006). A muroplast-localized fructose-1,6-bisphosphate aldolase of class II was fractionated from *C. paradoxa* extracts as a 85 kDa protein and was shown to be bifunctional for fructose-1,6-bisphosphate and sedoheptulose-1,7-bisphosphate cleavage (Flechner et al. 1999). The cDNA of pre-transketolase was sequenced. In a neighbor-net graph, the *Cyanophora* enzyme occupied a position intermediate to the plastid and cyanobacterial homologs (Ma et al. 2009). The single copy gene was downregulated upon shift to low CO_2 conditions, typical for Calvin cycle enzymes (Burey et al. 2007).

Photorespiration: The oxygenase activity of RuBisCO inevitably leads to photorespiration (in different variations) in cyanobacteria and in all oxygenic phototrophs (where peroxisomes and mitochondria are involved in addition to plastids). The *Cyanophora* Genome Project inspired a study about evolution and phylogeny of this pathway in the earliest branching phototrophic eukaryote (Kern et al. 2013). The outcome was that some cyanobacterial genes (originally obtained through endosymbiotic gene transfer [EGT]) were lost, as for glycerate-3-kinase, or later replaced by α-proteobacterial homologs, as for glycine decarboxylase. Only phosphoglycolate phosphatase appears to be derived from Archaea. Glycolate oxidase was described to be of cyanobacterial origin in *Cyanophora* and all other algae/plants. A cyanobacterial origin was also postulated for serine:glyoxylate aminotransferase of *C. paradoxa*, whereas the counterparts from red algae and green algae/plants were found to be derived from proteobacteria through HGT. A similar situation is assumed for hydroxypyruvate reductase. Taken together, *Cyanophora* seems to have retained more cyanobacterial genes of the C_2 pathway than other algae and land plants in accordance with the predicted basal position of glaucophytes among Archaeplastida (Kern et al. 2013). Certainly, more biochemical research in this field is needed, as the lack of glycerate-3-kinase points toward some changes in the C_2 pathway of glaucophytes. Proteomics confirmed the muroplast localization of phosphoglycolate

phosphatase that showed the canonical transit sequence at the gene level (Facchinelli et al. 2013). There is but one experimental paper investigating glycolate metabolism in *C. paradoxa* that revealed glycolate oxidase and glycolate dehydrogenase activity. Furthermore, multiple forms of hydroxypyruvate reductase were shown, whereas serine:glyoxylate aminotransferase could not be detected. This was also taken at that time to indicate some deviations from the glycolate metabolism observed in leaves of land plants (Betsche et al. 1992).

The potential C_4 pathway of CO_2 fixation in algae including *C. paradoxa* was assessed in the light of emerging genome data: most of the respective enzymes, if present at all, appear to be derived from archaea/proteobacteria rather than from cyanobacteria, which are assumed to lack a complete C_4 pathway. Nevertheless, some algae, e.g., diatoms, seem to contain the enzymes necessary for the C_4 pathway, whereas *C. paradoxa*, lacking pyruvate:phosphate dikinase, malic enzyme, and alanine amino transferase, is not likely to perform C_4 photosynthesis (Chi et al. 2014). After all, a pyrenoidal CCM is operative in glaucophytes to cope with low CO_2 conditions (see below).

The Phycobilisomes of *Cyanophora paradoxa*

Phycobilisomes (PBS) are the primary light-harvesting pigment complexes of cyanobacteria, red algae, and glaucophytes and are attached to the stromal surface of the thylakoids (for review see, e.g., Adir 2008). These high molecular weight protein complexes with multiple functions consist of 400–700 subunits originating from more than 20 individual polypeptides with 600–2,000 covalently linked chromophores. Sequential assembly, conformational flexibility, and interaction between the chromophore and protein components are the main features of this complex network. Linker polypeptides play a central role in all of these processes, modulate the spectral characteristics of the phycobiliprotein chromophores, and mediate the attachment of the PBS to the photosynthetic membrane. Two structural domains, the central core complex and the peripheral rods, form this superstructure. For PBS without phycoerythrin, the core is composed of three cylinders, each formed by four allophycocyanin (APC) trimers $(\alpha\beta AP)_3$ with additional minor phycobiliprotein components and core-specific linker proteins. The rods radiate from the core and consist of three to four hexameric phycocyanin (PC)-rod linker (L_R) complexes $(\alpha\beta PC)_6L_R$. The rods are connected to specific domains of the core via rod-core linker polypeptides. In rhodophytes and the model glaucophyte *Cyanophora paradoxa*, the phycobiliprotein genes reside on the plastid genome, while the colorless linker proteins are encoded by the nucleus (Egelhoff and Grossman 1983). In cyanobacteria, the PBS most likely undergo a self-assembly process mediated by the amount of PBS assembly interaction partners and assisted by chaperones and processing enzymes (Anderson and Toole 1998). In muroplasts, where the PBS components are genetically separated, transcription events in the nucleus followed by translation in the cytosol and subsequent protein import must be coordinated with transcription events of muroplast-encoded subunits and in organello biosynthesis. In

Table 5 Components of purified, intact muroplast phycobilisomes

Apparent MW (kDa)	Abundance	Phycobiliprotein	Correlated cyanobacterial gene	Function
98	Medium	Yes	*apcE*	Core-membrane linker
55	Medium	No	n. m. (*cpcK1*)	Rod linker
53	Medium	No	n. m. (*cpcK2*)	Rod linker
38	Low	No	*cpcG2*	Rod-core linker
31	Low	No	*cpcG1*	Rod-core linker
18–20	High	Yes	$cpcA^{a},B^{a}$	Phycocyanin subunits
17–18	High	Yes	$apcA^{a},B^{a},D^{a},F^{a}$	Allophycocyanin subunits
10	Low	No	*apcC2*	Core linker (ApcD associated)
9	Low	No	*cpcD*	Terminal rod linker
8	Low	No	*apcC1*	Core linker

n. m. No orthologous match to rod linkers from phycocyanin-PBS, size comparable to red algal linkers and to an unusual chromophorylated phycoerythrin linker from *Synechococcus* sp. WH 8102, see Six et al. (2005)
[a]Muroplast encoded

vitro PBS assembly could be shown after import of the radiolabeled small core linker precursor protein preApcC1 from *Cyanophora paradoxa* into isolated muroplasts and subsequent isolation of the PBS (Steiner et al. 2003).

Phycobilisome components: *Cyanophora* PBS are of dual genetic origin, as are plastid microcompartments in general. The gene distribution is clear-cut: The seven phycobiliproteins including the "core-membrane linker" ApcE are muroplast encoded, whereas the non-chromophorylated linker polypeptides are nuclear encoded (Table 5; Steiner and Löffelhardt 2011; Watanabe et al. 2012). All these precursors show the canonical transit sequence containing a phenylalanine residue in the N-terminal domain. The functional assignments are based on MS measurements, on 2D gel electrophoresis of purified intact PBS, and on PBS dissociation studies followed by sucrose density gradient fractionation and SDS-PAGE (Steiner et al., manuscript in preparation). The genes encoding all non-chromophorylated PBS subunits in *C. paradoxa* could be identified from abundant ESTs (and later in the Genome Project; Price et al. 2012) and by research conducted in parallel by others (Watanabe et al. 2012).

The two large rod linkers, CpcK1 and CpcK2 (Watanabe et al. 2012), were shown to result from tandem duplications of the *cpcG* (rod-core linker) gene and are – in that respect – not related to the large linker polypeptides from red algae. There is but one similar special case among cyanobacterial PBS: a 59 kDa chromophorylated phycoerythrin linker originating from a fusion of two smaller linkers (Six et al. 2005). Further, two additional truncated *cpcG* genes were found adding up to a third version (*cpcG3*) – up to four genes were reported for filamentous cyanobacteria. CpcG3 might be part of a rudimentary PSI antenna (consisting of a rod only) as was

reported for cyanobacteria (Kondo et al. 2007) and red algae (Busch et al. 2010). The three small linkers are interpreted as follows: The two core linkers, ApcC1 and ApcC2, form complexes with ApcA,B and ApcA,B,D, respectively. In cyanobacteria, just one core linker is common, whereas in red algae also two core linkers are reported. The third small linker is the terminal rod linker CpcD that determines rod length.

A typical cyanobacterial rod linker protein CpcC consists of two domains, an N-terminal pfam00427 (PBS linker domain) and a C-terminal pfam01383 (CpcD/APC linker domain). The two CpcK linker proteins from *Cyanophora* consist of two pfam00427 domains in tandem, while the pfam01383 domain is missing. Two competing models exist for the location of linker proteins in the PBS rod. Novel "skeleton-like" structures have been described in the phycobilisomes of *C. paradoxa* (Watanabe et al. 2012). The authors showed, via native polyacrylamide gel electrophoresis (PAGE), two subcomplexes (ApcE/CpcK1/CpcG2/ApcA/ApcB/CpcD and ApcE/CpcK2/CpcG1/ApcA/ApcB) that may serve as a scaffold for the whole PBS assembly. CpcK1 and CpcK2 correspond to the large pfam00427 (PBS linker domain) tandem-duplicated rod linkers. However, data obtained by different types of native PAGE combined with limited proteolysis (Steiner et al., manuscript in preparation) suggest that these "skeleton-like" structures are most likely protein aggregates originating from phycobilisome degradation. When appropriate protease inhibitors are used, isolated subcomplexes showed a more "classical" pattern in native PAGE where the main APC core particle was complexed to ApcC1, the smaller of the two tandem-duplicated rod linkers (CpcK2) migrated in a complex together with phycocyanin and the terminal rod linker (CpcD), whereas the larger tandem-duplicated rod linker (CpcK1) migrated in a complex with phycocyanin only (Fig. 10; Weisser 2012). Since both complexes show a molecular weight of about 460 kDa, an association of cpcK1 and cpcK2 with three PC trimers (one trimer about 120 kDa without linkers) seems reasonable. Moreover, CpcG2 could be shown to form a separate complex with PC, APC, and ApcC2 (Maluck 2012). Limited proteolysis followed by native and SDS-PAGE allowed to estimate the amount of protected linker protein fragments and therefore the size of the different phycobilisome subparticles. Altogether a model is favored where the two tandem-duplicated rod linkers are part of the same rod with CpcK1 being the core-proximal hexamer rod linker and CpcK2 being the core-distal hexamer rod linker (Fig. 11, right; Steiner et al., manuscript in preparation) as opposed to the model with only one of the large linkers per rod, in more stretched conformation (Fig. 11, left; Watanabe et al. 2012). A schematic view of the *Cyanophora* PBS as a whole is given in Fig. 12.

The Nature of the RuBisCO-Containing Microcompartment of Muroplasts

The conspicuous, electron-dense central body of *C. paradoxa* muroplasts was shown to contain the bulk of RuBisCO (Mangeney and Gibbs 1987) and has been denoted the "carboxysome" in most publications. Despite the fact that eukaryotes contain

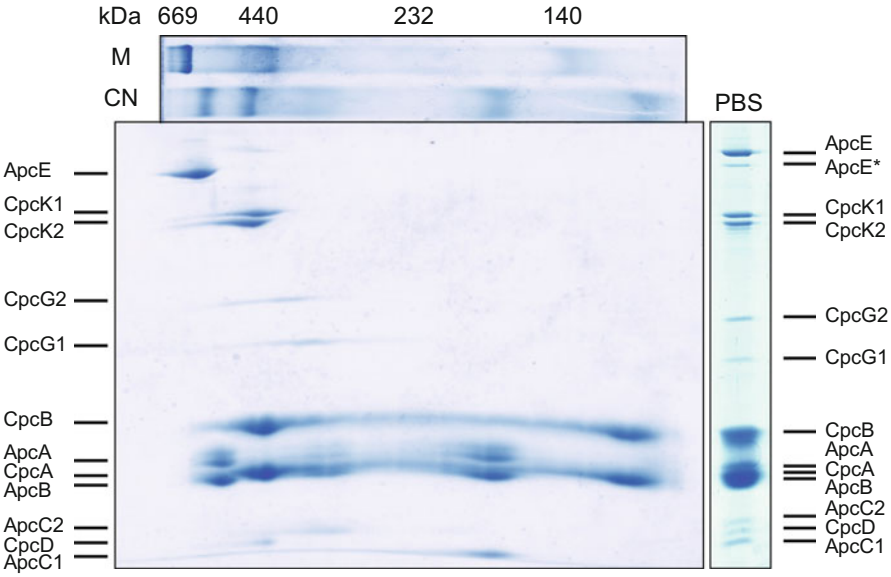

Fig. 10 Colorless native (CN)-PAGE of isolated *Cyanophora* phycobilisomes followed by SDS-PAGE: *upper* horizontal panel (M), high molecular weight marker; *upper* horizontal panel (CN), first dimension (CN-PAGE). *Lower* panel, second dimension (SDS-PAGE). Right lane, SDS-PAGE of intact phycobilisomes; ApcE*, typical degradation product of ApcE

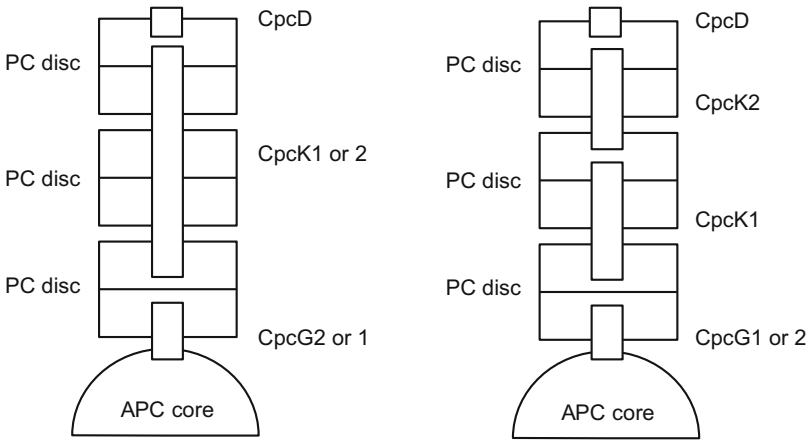

Fig. 11 Comparison of *Cyanophora* phycobilisome substructure models: *left*, skeleton-like structure (Watanabe et al. 2012); *right*, model proposed by Steiner et al., manuscript in preparation

pyrenoids (Meyer and Griffiths 2013) functioning in the carbon-concentrating mechanism (CCM), this coinage emphasized the often-postulated transitional position of glaucophytes between plastids and cyanobacteria. Further, the hypothesis of

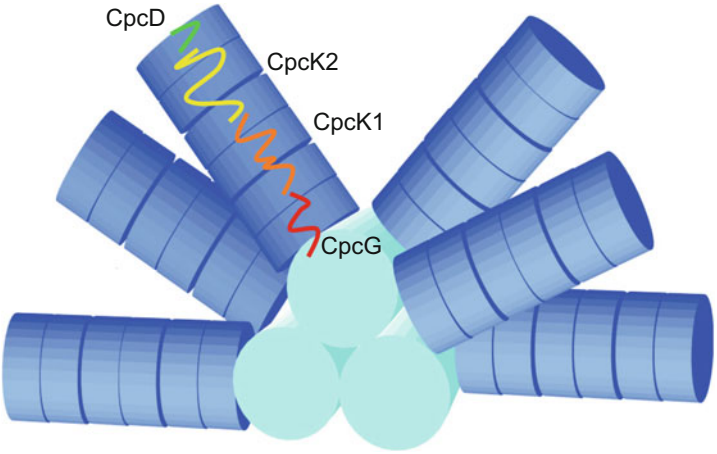

Fig. 12 Complete model of the *Cyanophora* phycobilisome according to Steiner et al. *Blue*, phycocyanin rods; *cyan*, allophycocyanin core; *green*, *yellow*, *orange*, and *red*, linker proteins

Raven (2003) that muroplasts had retained the peptidoglycan wall for osmotic protection since these were the only plastids that had also retained carboxysomes was quite appealing: A carboxysomal CCM (Badger and Price 2003) would lead to a much higher accumulation of bicarbonate in the stroma than a pyrenoidal CCM. However, all attempts to identify genes for carboxysomal shell proteins corresponding to cyanobacterial ccmKLMNO in the *C. paradoxa* genome have failed (Price et al. 2012) as did proteomic studies on isolated muroplast central bodies (Fathinejad et al. 2008). Indeed, it might be problematic to harbor shell protein genes in the nucleus, because they have high affinities to each other and likely self-assemble as carboxysomal prestructures (Kinney et al. 2011), thereby interfering with protein import into muroplasts. On the other hand, evidence was obtained (Table 6; Bhattacharya et al. 2014) for a number of genes (e.g., *LCIB* and *LCIC*) with functions in the pyrenoidal CCM of *Chlamydomonas reinhardtii* (Yamano et al. 2010). LCIB and LCIC were shown to form a hexameric complex (ca. 360 kDa) close to the pyrenoid under light and low [CO_2]. A role for this complex is assumed in trapping of CO_2 that has escaped from the pyrenoid via interaction with the carbonic anhydrase CAH6. Alternatively, Yamano et al. (2010) envisage physical blockage of CO_2 from escaping the pyrenoid (somehow analogous to the function of the carboxysomal shell). Some putative cyanobacterial plastid ancestors – given their filamentous nature (*Lyngbya*) or capability of producing a starch-like reserve carbohydrate (*Cyanothece*) – contain *LCIB* and *LCIC*. These cyanobacteria might use mechanisms of the type discussed above that are superimposed on their carboxysomal CCM. At present, the more recent *Paulinella chromatophora* "plastid" origin (ca. 100 Ma) constitutes the only proven example of "eukaryotic carboxysomes." Here, the necessary genes remain on the genome of the cyanelle (photosynthetic organelle; Nowack et al. 2008), interestingly originating from HGT (Marin et al. 2007). If carboxysomes were transferred to early plastids

Table 6 Genes for proteins potentially involved in the CCM of *Cyanophora paradoxa*

Gene[a]	Function	Comments
LCIA[b]	Bicarbonate transport	TP, complete
LCIA	Bicarbonate transport	TP, complete
LCIB[b]	CCM	TP, complete
LCIB	CCM	TP, complete
LCIB, LCID?	CCM	TP, 3′-truncated
LCIB, LCIC?	CCM	fragment
Rca[b]	RuBisCO activase	TP, complete
CAH8 [b]	Carbonic anhydrase	Beta-CA superfamily, periplasmic
CAH4 [b]	Carbonic anhydrase	Beta -CA superfamily, mitochondrial
CAH5[b]	Carbonic anhydrase	Beta -CA superfamily, mitochondrial
?	Carbonic anhydrase	Gamma-CA family, cytosolic?
?	Carbonic anhydrase	Gamma-CA family, cytosolic

[a]Nomenclature corresponding to the homologs from *C. reinhardtii*
[b]CO2-responsive gene; *TP* muroplast transit peptide, containing phenylalanine in the N-terminal region

via endosymbiosis, the separation between carboxysomal and pyrenoidal CCM could have occurred within the phylum Glaucophyta, i.e., *C. paradoxa* and *Glaucocystis nostochinearum* already progressed toward a pyrenoidal CCM, whereas *Gloeochaete wittrockiana* (Fig. 5; Kies 1976) and *Cyanoptyche gloeocystis* (Kies 1989), with their polyhedral microcompartments confined by an electron-dense, shell-like layer (both features missing in the two former species), might have retained the carboxysomal CCM (Fathinejad et al. 2008). Under such a scenario, the *ccmKLMNO* genes would be expected to reside on the muroplast genomes of *G. wittrockiana* and *C. gloeocystis*. The PG wall, though no longer necessary, was retained for unknown reasons in the plastids of *C. paradoxa* and *G. nostochinearum*. Table 6 includes two genes encoding the putative bicarbonate transporter LCIA (Yamano et al. 2015) and several genes with strong sequence similarity to genes for LCIB, LCIC, and LCID from *C. reinhardtii*. Because these are closely related, an exact assignment is difficult. However, whenever the N-termini are intact, unequivocal muroplast presequences were found for these proteins.

A key enzyme of the CCM is carbonic anhydrase (CA), either co-packaged with RuBisCO in cyanobacterial carboxysomes or located in the lumen of thylakoids traversing the pyrenoid of *C. reinhardtii* (Karlsson et al. 1998). The number of CAs can vary among algae, e.g., from 9 in *C. reinhardtii* to 13 in some diatoms (Tachibana et al. 2011). Five CAs from *C. paradoxa* are shown in Table 6. Two of these belong to the gamma-CA family with high sequence similarity to homologs in plants. The other three contain the conserved Zn-binding site (VCGHSHCGAMKG) of (cyano)bacterial beta-CAs. In the case of the putative mitochondrial CAs, high sequence similarities to *C. reinhardtii* CAH4 and CAH5 are observed. The third beta-CA resembles the periplasmic CAH8. A bona fide muroplast CA (e.g., the stromal CAH6 or the lumenal CAH3 of *C. reinhardtii*) is missing from this compilation. In a recent data mining effort among 15 microalgae, Meyer and Griffiths

(2013) revealed two additional bicarbonate transporters in the *Cyanophora* database via sequence similarity to *Chlamydomonas* homologs: the plasma-membrane-localized ABC transporter HLA3 (Yamano et al. 2015) and CCP1 in the plastid envelope. As a consequence, if we assume a pyrenoidal CCM in *C. paradoxa*, the organism must utilize a mechanism different from that in *C. reinhardtii* (Meyer and Griffiths 2013). There is no evidence in *C. paradoxa* of a muroplast microcompartment traversed by thylakoid membranes. A recent high-resolution ultrastructural study of the *C. reinhardtii* cell (Engel et al. 2015) posits that the thylakoid-derived pyrenoid tubules contain several minitubules thought to transport ATP, RubP, etc. across the starch sheath to the RuBisCO in the pyrenoid interior. Because starch is stored in the cytosol of glaucophytes, such a function may not be necessary here. In the diatom *Phaeodactylum tricornutum*, the carbonic anhydrase CA-1 (CO_2 responsive) is co-packaged with pyrenoidal RuBisCO and does not reside in the lumen of the traversing thylakoid (Tachibana et al. 2011). Mass spectrometric analysis of central body proteins from *C. paradoxa* did not reveal a CA-like protein either. The only outcome of these studies (in addition to RuBisCO LSU and SSU) was RuBisCO activase that was also corroborated by Western blotting and assembly studies after in vitro import into isolated muroplasts (Fathinejad et al. 2008). *C. paradoxa* activase, whereas showing high sequence similarity to both cyanobacterial and plant homologs, lacks the C-terminal extension typical for filamentous cyanobacteria but shows the N-terminal extension present in plant homologs only. Taken together, the domain structure of RuBisCO activase from *C. paradoxa* does not support the carboxysome concept either. Several genes listed in Table 6 were shown to be CO_2 responsive in the closely related *C. cuspitata* SAG 45.84 (Kies strain) underlining their postulated role in the CCM (Burey et al. 2007).

Other Metabolic Pathways in Muroplasts

The *C. paradoxa* genome project, in combination with the muroplast proteome (Facchinelli et al. 2013) and some biochemical investigations, allows interesting insights into the metabolism of a primitive plastid in comparison to the abundant data on chloroplast metabolism.

Glycolysis: With respect to glycolysis, significant deviations from the known chloroplast pathways were found: Phosphoglyceromutase and enolase are present in the muroplast stroma in contrast to the situation in chloroplasts, allowing direct production of PEP from photosynthetically generated 3-phosphoglycerate. On the other hand, hexokinase and phosphofructokinase are missing from muroplasts. Fructose-1,6-bisphosphatase and phosphoglucomutase are sufficient to generate glucose-6-phosphate, the metabolite to be exported to the cytosol (see below).

Glucose-6-phosphate dehydrogenase was purified from a *C. paradoxa* muroplast extract (Fester et al. 1996). The 59 kDa protein forms enzymatically active dimers and tetramers. 6-Phosphogluconate dehydrogenase was identified in the stroma

through proteomics. The corresponding gene showed a canonical muroplast STP (Facchinelli et al. 2013). This points toward a muroplast-localized oxidative pentose phosphate pathway.

Isoprenoid lipid biosynthesis: Proteomics yielded very conclusive results concerning isoprenoid metabolism. With one exception, all enzymes of the 1-deoxy-xylulose-5-phosphate/2-C-methylerythritol-4-phosphate (MEP) pathway of isopentenyl diphosphate synthesis were demonstrated in the muroplast stroma (Facchinelli et al. 2013) but none of the mevalonate pathway. Proteomics also corroborated the muroplast localization of other enzymes of the prenyl lipid pathway (Facchinelli et al. 2013) as geranyl-geranyl diphosphate reductase (phytol biosynthesis), geranyl-geranyl diphosphate synthase (CrtE, muroplast encoded), solanesyl diphosphate synthase (PreA, muroplast encoded), and homogentisate solanesyl transferase (plastoquinone biosynthesis). In most of these cases, nucleus-encoded muroplast proteins possess canonical transit sequences (with F replaced by Y or W in a few instances).

Amino acid biosynthesis: As plastids, muroplasts are the main contributors to amino acid biosynthesis. This became apparent from the genome data where muroplast STPs preceded the respective genes and also from the analysis of the muroplast proteome (Facchinelli et al. 2013).

Photooxidative stress management: *Cyanophora paradoxa* is known to prefer low light intensities for growth (Löffelhardt and Bohnert 2001). A recent survey showed that *C. paradoxa* does not use the ascorbate/ascorbate peroxidase system that plays an important role in coping with reactive oxygen species (ROS), which is unparalleled among phototrophs (Wheeler et al. 2015). Furthermore, *C. paradoxa* is devoid of glutathione reductase (Serrano and Löffelhardt 1994). However, *C. paradoxa* contains catalase, glutathione peroxidase, and peroxiredoxins, and its muroplasts harbor the unusual peroxidase symerythrin (Cooley et al. 2011). In glaucophytes, the low levels of ascorbate synthesized by the unusual enzyme gulonolactone oxidase (land plants and green algae use a gulonolactone dehydrogenase) might have a role as enzyme cofactor but neither in the ascorbate/glutathione antioxidative pathway nor in the xanthophyll cycle (Wheeler et al. 2015).

Miscellaneous: The NADP-dependent malate dehydrogenase of *C. paradoxa* was shown not to be responsive to reductive activation (Ocheretina et al. 2000) as red algal enzymes, in contrast to enzymes of the "green" lineage. Accordingly, attempts to demonstrate thioredoxin *m* in *C. paradoxa* were unsuccessful (Dai et al. 1992). A muroplast pyrophosphatase (sPPase I) was isolated from *C. paradoxa* and characterized by N-terminal sequencing and MW determination via MALDI-TOF mass spectrometry (Gómez-García et al. 2006). The monomeric 30 kDa protein is more related to PPases from heterotrophic eukaryotes than to the smaller cyanobacterial enzymes. This also applies to the plastid enzymes from other algae, e.g., *C. reinhardtii*, and from plants. Taken together, this means that early in plastid evolution, the endosymbiont gene was lost and the product of a host cell gene was relocalized to the organelle.

Genome Analysis of Glaucophytes

Glaucophyte genome-wide analyses are relatively scarce when compared to plants and green algae. This is explained by the limited expressed sequence tag (EST) and complete genome data available from these taxa. For many years, the only sources of EST data were from the Pringsheim (Reyes-Prieto et al. 2006) and Kies strains of *Cyanophora paradoxa* and from *Glaucocystis nostochinearum* (http://tbestdb.bcm.umontreal.ca/searches/login.php). The complete plastid genome sequence of *C. paradoxa* (Pringsheim strain; Stirewalt et al. 1995; Loeffelhardt et al. 1997) was also available (see section on the Muroplast Genome; Table 7). Uses of the EST data from *C. paradoxa* included assessment of the divergence position of glaucophytes within Plantae/Archaeplastida (Reyes-Prieto and Bhattacharya 2007a; Deschamps and Moreira 2009) and estimation of the contribution of cyanobacterial genes to the nuclear genome of glaucophytes via EGT (e.g., Timmis et al. 2004; Reyes-Prieto et al. 2006). Bioinformatic analyses suggested that 6–11% of *C. paradoxa* nuclear genes owed their origin to EGT from the endosymbiont (Reyes-Prieto et al. 2006; Qiu et al. 2013a). The *C. paradoxa* plastid genome has been invaluable to many researchers who have used it to infer the phylogenetic history of this organelle, its gene content, and gene order (e.g., Stirewalt et al. 1995; Rodriguez-Ezpeleta et al. 2005; Sato et al. 2005; Janouškovec et al. 2010; Qiu et al. 2013a). A recent biochemical characterization of the *C. paradoxa* plastid proteome that identified a partial list of 586 non-redundant proteins (Facchinelli et al. 2013) demonstrated their complex evolutionary histories. Maximum likelihood analysis of these proteins by Qiu et al. (2013a) showed that 25% were plastid encoded, 12% were derived from EGT candidates encoded in the nucleus, 7% were of non-cyanobacterial (HGT) origin, and the remaining (56%) were derived from the host or were of ambiguous provenance based on analysis of current data. The phylogenetic origins of non-redundant plastid proteins in *C. paradoxa*, *Chlamydomonas reinhardtii* (1,057 proteins), and *Arabidopsis thaliana* (1,660 proteins) are shown in Fig. 13 (Qiu et al. 2013a). More recently, mitochondrial genomic data have been analyzed from seven different glaucophytes and used to test (and validate) Archaeplastida monophyly (Jackson and Reyes-Prieto 2014). These organelle genomes have a highly conserved gene content but show significant variation in gene order across taxa (Jackson and Reyes-Prieto 2014).

Genome data: A significant step forward for the field of glaucophyte genomics came in 2012 with the publication of the draft genome assembly from the *C. paradoxa* Pringsheim strain CCMP329 (SAG 29.80; Price et al. 2012). This work was supported by the United States National Science Foundation and resulted in the generation of 8.3 billion base pairs (Gbp) of Roche 454 and Illumina GAIIx sequence data that were co-assembled with 279 Mbp of random-shear Sanger sequence from this taxon. The resulting assembly comprised 60,119 contigs, totaling 70.2 Mbp. More recent sequencing of this strain using the long-read PacBio platform suggests that the genome size is closer to 120 Mbp based on the initial assembly output. Pulsed-field gel electrophoresis suggests the existence of at least seven chromosomes in *C. paradoxa* with the smallest being less than 3 Mbp in size

Table 7 Muroplast genes from *Cyanophora paradoxa*. Gene nomenclature follows the guidelines for chloroplast genes (Stoebe et al. 1998). Genes marked with an asterisk are not found on any other plastid genome. Genes underlined are absent from the chloroplast genomes of land plants

Ribosomal RNAs (3): *rrsA, rrlA, rrfA*
Transfer RNAs (36)
Other RNAs (2): *rnpB,* tmRNA
Ribosomal proteins (37): *rpl1, rpl2, rpl3, rpl5, rpl6, rpl7, rpl11, rpl14, rpl16, rpl18, rpl19, rpl20, rpl21, rpl22, rpl28, rpl33, rpl34, rpl35, rpl36, rps2, rps3, rps4, rps5, rps6, rps7, rps8, rps9, rps10, rps11, rps12, rps13, rps14, rps16, rps17, rps18, rps19, rps20*
RNA polymerase subunits (4): *rpoA, rpoB, rpoC1, rpoC2*
Phycobiliproteins (7): *apcA, apcB, apcD, apcE, apcF, cpcA, cpcB*
Photosystem I and II proteins (27): *psaA, psaB, psaC, psaE, psaF, psaI, psaJ, psaM, psbA, psbB, psbC, psbD, psbE, psbF, psbH, psbI, psbJ, psbK, psbL, psbM, psbN, psbT, psbV, psbX, psbY, psbZ, psb30*
ATP synthase subunits (7): *atpA, atpB, atpD, atpE, atpF, atpG, atpH*
Cytochrome b_6/f subunits and ferredoxin (8): *petA, petB, petD, petG, petL, petN, petX, petF*
Anabolic enzymes (13): *rbcL, rbcS, chlB, chlI, chlL, chlN, acpP, nadA*, preA, crtE*, hemA*, hisH, trpG*
Peptidoglycan biosynthesis/muroplast division (2): *ftsW, sepF*
Proteases (2): *clpP1, clpP2*
Chaperones (3): *dnaK, groEL, groES**
Translation factor: *tufA*
Preprotein translocase: *secY*
ORFs with unknown or putative function (37): *ycf3*[a], *ycf4*[a], *ycf5*[b], *ycf16*[c], *ycf17*[d], *ycf21, ycf23, ycf24*[e], *ycf27*[f], *ycf29*[f], *ycf30*[g], *ycf33*[h], *ycf34, ycf35, ycf36, ycf37*[i], *ycf38*[j], *ycf39*[k], *orf27, orf48, orf77, orf91, orf102, orf108, orf163, orf179, orf180**[l], *orf182, orf188, orf206, orf244*, orf299*, orf333*[m]

[a]Role in PS I assembly
[b]Role in PS I function
[c]ABC transporter subunit, ortholog to bacterial sufC, involved in [Fe-S] cluster biogenesis
[d]CAB/ELIP/HLIP superfamily protein
[e]ABC transporter subunit, ortholog to bacterial sufB, involved in [Fe-S] cluster biogenesis
[f]Response regulator of PS I genes (rpaB)
[g]Transcription factor (RuBisCo operon)
[h]Role in cyclic electron transport
[i]PSI stability or assembly
[j]ABC transporter
[k]Photosystem II assembly factor
[l]Symerythrin
[m]Role in assembly/stability of PSII

(Price et al. 2012). Given the initial Sanger/Roche/Illumina and the later PacBio genome data, we posit that the Price et al. (2012) assembly likely captured most of the gene inventory in the gene-rich regions (see below), whereas assembly of the complex (e.g., repeated or with strong nucleotide bias, such as homopolymers) DNA regions was only possible with the PacBio long-read technology. Generation and analysis of a hybrid Illumina/PacBio genome assembly are underway in the Bhattacharya and Andreas P.M. Weber labs. Interestingly, the PacBio results are

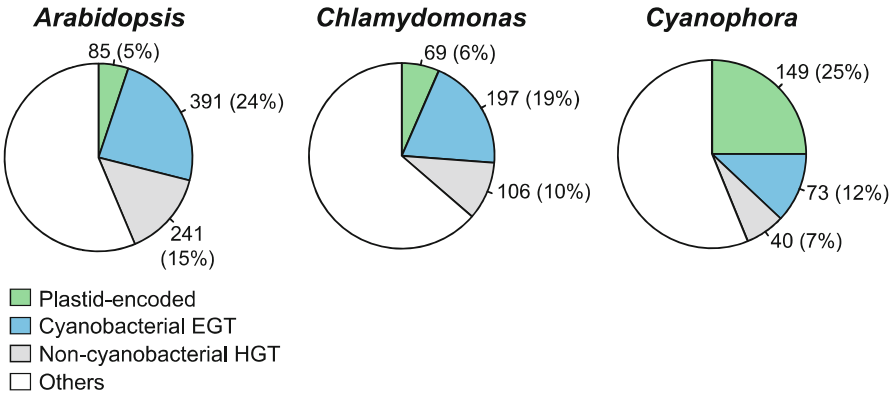

Fig. 13 Results of phylogenetic analysis of single proteins represented as pie charts that show the relative contribution of cyanobacterial and non-cyanobacterial sources to Archaeplastida plastid proteomes (for details, see Qiu et al. 2013a)

more in line with previous fluorescence-activated cell sorting (FACS) work that suggested the haploid genome size in *C. paradoxa* to be ca. 140 Mbp (Löffelhardt et al. 1997).

Consistent with these observations, genome analysis done by Price et al. (2012) demonstrated an unusually high G + C content in *C. paradoxa* (83.8% at third codon positions) that likely explains the highly fragmented, initial assembly. Nonetheless, BLASTN analysis using 3,900 Sanger-derived EST unigenes from the glaucophyte against the draft assembly showed that 99% of the ESTs had hits (at *e*-value ≤1E-10), suggesting that the vast majority of expressed genes were present in these genome data. Given this promising result, 15 Gbp of Illumina mRNA-seq data were used to train ab initio gene predictors to generate 27,921 gene models for downstream analysis (Price et al. 2012). Below we will discuss some of the insights that were gained through analysis of the *C. paradoxa* genome data generated by Price et al. (2012), recognizing that the PacBio results will likely lead to additional novel insights.

Phylogenomic analysis test Archaeplastida monophyly: Given that many multi-gene (i.e., concatenated protein dataset) phylogenies have provided conflicting topologies regarding the monophyly of Archaeplastida in the eukaryote tree of life (e.g., Burki et al. 2007; Baurain et al. 2010; Parfrey et al. 2010; Yabuki et al. 2014; Jackson et al. 2015) and have failed to reach any consensus on this important question, Price et al. (2012) took another approach. Rather than joining proteins, often with uncertain histories into a single dataset, they analyzed each protein separately using maximum likelihood (ML) phylogeny reconstruction and tabulated the overall signal for Archaeplastida monophyly. In their analysis, a total of 4,628 proteins had significant BLASTP hits (*e*-value ≤1E-10) to sequences in a comprehensive local database that were used for comparative analysis (e.g., Moustafa et al. 2009; Chan et al. 2011). Using an automated approach (Chan et al. 2011), they generated 4445 ML trees for *C. paradoxa* proteins that had significant database hits.

Only trees containing ≥3 phyla were considered and a minimum number of terminal taxa (*N*) that ranged progressively from 4 to 40 (Fig. 14a). Using this approach they found that >60% of all trees supported (at bootstrap value ≥90%) a sister-group relationship between glaucophytes and red and/or green algae. The glaucophytes were most often positioned as sister to Viridiplantae in trees that excluded non-Archaeplastida algae, a result that was found even though a large number of trees favored glaucophyte-red-green (Archaeplastida) monophyly (44, 40, 32, 18, and 16 trees at $N = 4$, 10, 20, 30, and 40, respectively), and they had substantial red algal genome data in the database. Most of the trees showed *C. paradoxa* to be monophyletic with other Archaeplastida in a clade ("shared") that also included non-Archaeplastida phyla (GlR/GlVi/GlRVi in Fig. 14). When they sorted the phylogenomic output using the red or green algae as the query to test Archaeplastida monophyly, these results also identified Archaeplastida as the most frequently recovered clade. Expectedly, red and green algae showed far more gene sharing than glaucophytes because they, unlike glaucophytes, are involved in secondary endosymbioses (Harper and Keeling 2003; Moustafa et al. 2009; Baurain et al. 2010; Chan et al. 2011; Bhattacharya et al. 2013). These results demonstrate a highly complex phylogenetic history for glaucophyte and algal genome data in general, showing that EGT and HGT have moved genes between disparate lineages leaving a highly reticulate signal within their genomes. Regardless, the single protein trees overall strongly support a single origin of Archaeplastida and likely a single primary plastid endosymbiosis in their common ancestor (Fig. 14; Price et al. 2012). Future genome projects that add more glaucophytes and other poorly sampled Archaeplastida lineages (e.g., prasinophytes) to the analysis are needed to validate the hypothesis of Archaeplastida monophyly.

Given the extent of gene sharing among algae, Price et al. (2012) investigated the "footprint" of non-cyanobacterial, prokaryotic HGT in the nuclear genomes of Archaeplastida. For this analysis, they constructed a database that included sequences from NCBI Refseq, *C. paradoxa* and the red algae *Calliarthron tuberculosum* and *Porphyridium purpureum* (Bhattacharya et al. 2013). These data were then queried using each *C. paradoxa*, *C. tuberculosum*, and *P. purpureum* protein, as well as those derived from two Viridiplantae (i.e., *Chlamydomonas reinhardtii* and *Arabidopsis thaliana*). The top five bacterial hits (BLASTP *e*-value ≤1E-10) were retained for each Archaeplastida query sequence and used as input for an automated phylogenetic tree-building pipeline (for details of procedure, see Price et al. 2012, supplement). Inspection of the maximum likelihood-generated trees turned up 444 non-cyanobacterial gene families shared by prokaryotes and Archaeplastida. Of these, 15 were present in all three Archaeplastida phyla. One such ancient HGT resulted in the transfer of a thiamine pyrophosphate-dependent pyruvate decarboxylase family protein involved in alcohol fermentation. This analysis turned up 60 other genes that are present in only two of the three phyla (i.e., 24, 10, and 26 genes in Glaucophyta-Viridiplantae, Glaucophyta-Rhodophyta, and Rhodophyta-Viridiplantae, respectively). More recent work has shown that HGT plays a key role in adaptation of algae to their environment and the impacts of this process will likely become more widely appreciated as additional complete algal

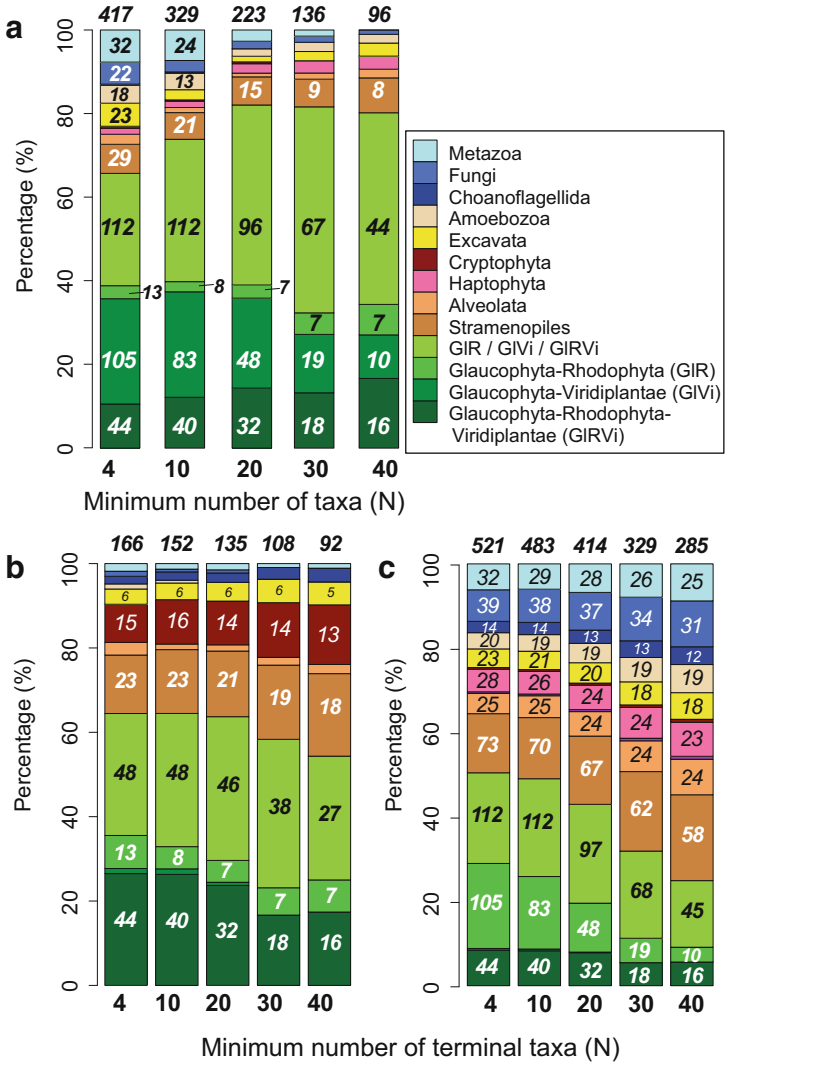

Fig. 14 Maximum likelihood analysis of single proteins derived from the *C. paradoxa* genome assembly (for details, see Price et al. 2012). **a** Percentage of single protein maximum likelihood trees (raw numbers shown in the bars) at bootstrap cutoff >90% that support the monophyly of glaucophytes solely with other Archaeplastida or in combination with non-Archaeplastida taxa that interrupt this clade. These latter groups of trees are explained by *red/green* algal EGT into the nuclear genome of chromalveolates (e.g., diatoms, haptophytes) and euglenids, respectively. For each of these algal lineages, the set of trees with different numbers of taxa (N) ≥4, ≥10, ≥20, ≥30, and ≥40 and distinct phyla ≥3 in a tree are shown. The Archaeplastida-only groups are Glaucophyta-Rhodophyta (GlR), Glaucophyta-Viridiplantae (GlVi), and Glaucophyta-Rhodophyta-Viridiplantae (GlRVi). Trees with evidence of EGT are shown as the single group, GlR/GlVi/GlRVi. **b** The same analysis done with *red* algae as the query to search for support for Archaeplastida monophyly. **c** The same analysis done with *green* algae as the query to search for support for Archaeplastida monophyly

genomes are analyzed (Qiu et al. 2013b; Schönknecht et al. 2013; Foflonker et al. 2015).

Evolution of the plastid translocon and metabolite transport: Important innovations that have been the subject of much study in algae and plants are the evolution of the protein import system for the plastid and the emergence of metabolic connections between the captured cyanobacterial endosymbiont and the host cell. A fundamental outcome of the cyanobacterium-to-plastid evolutionary transition in the Archaeplastida primary endosymbiosis was the establishment of protein translocons for protein targeting into the organelle (e.g., Gross and Bhattacharya 2008, 2009; Reumann et al. 2005; Sommer and Schleiff 2014). Components of the translocons at the outer and inner envelope membranes of chloroplasts (Toc and Tic, respectively) were known in other Archaeplastida and in chromalveolates (McFadden and van Dooren, 2004). The existence of an equivalent protein import system in *C. paradoxa* was suggested by immunological detection of epitopes in this alga using plant Toc75 and Tic110 antibodies and heterologous protein import assays (see section on Protein Import into Muroplasts; Steiner et al. 2005a; Yusa et al. 2008). Analysis of the genome of *C. paradoxa* turned up homologs of Toc75 and Tic110 that are OEM (outer envelope membrane) and IEM (inner envelope membrane) protein conducting channels, respectively, two Toc34-like receptors, as well as homologs of the plastid Hsp70 and Hsp93 chaperones and stromal processing peptidase (Price et al. 2012). These are likely to have formed the primordial protein translocation system in the Archaeplastida ancestor (Gross and Bhattacharya 2008, 2009). In summary, analysis of *C. paradoxa* genome data revealed the presence of the conserved core of translocon subunits derived from the cyanobacterial endosymbiont (i.e., Toc75, Tic20, Tic22), suggesting that the Toc/Tic system was likely to have been in place in the Archaeplastida common ancestor. Toc75 of glaucophytes and likely also of rhodophytes is closer than the homolog of Viridiplantae to the ancestral Omp85 of cyanobacteria in recognizing phenylalanine in the N-terminal part of the transit peptides (see also section on Protein Import into Muroplasts; Wunder et al. 2007). A dual function as receptor and pore is assumed (Steiner and Löffelhardt 2005). This phenylalanine requirement is no longer found in chloroplast import: Toc75 in Chlorophyta and Streptophyta has only retained the pore function, whereas the receptor function is taken over by a small family of proteins, e.g., Toc159. Likely, this went along with the need for import of certain abundant proteins (RuBisCO SSU, LHCPII). In addition, the Tic translocon appears to be more elaborate in land plants: a 1 Mda complex contains Tic20 (pore?), Tic56, Tic100, and Tic214 (Nakai 2015).

Another landmark trait linked to plastid establishment is the coordination of carbon metabolism between the host and plastid that relies on sugar-phosphate transporters. Previous work had shown that plastid-targeted sugar transporters evolved from existing host endomembrane nucleotide sugar transporters (NSTs) through gene duplication, divergence, and retargeting to the photosynthetic organelle (Weber et al. 2006; Colleoni et al. 2010). Analysis of the *C. paradoxa* genome turned up a surprising result in this respect. Price et al. (2012) found that although six endomembrane-type NST genes existed in *C. paradoxa*, there were no genes for

plastid-targeted phosphate translocator (PT) proteins. The search for the missing genes turned up two candidates that encode homologs of bacterial UhpC-type hexose-phosphate transporters. These genes were also found in other algal members of the Archaeplastida, but lost in plants. Both *C. paradoxa* UhpC homologs encode an N-terminal extension that could serve as a plastid targeting sequence. Surprisingly, both of these UhpC genes were derived via HGT in the Archaeplastida ancestor from parasites related to *Chlamydiae* and *Legionella* (Price et al. 2012). Support for the absence of typical NST-derived sugar transporters in the plastid of *C. paradoxa* was found in the analysis of the plastid permeome from this species. Using YFP-fusion constructs in *Nicotiana benthamiana*, Facchinelli et al. (2013) validated the capacity of the UhpC transit peptide to target to the chloroplast inner membrane in *N. benthamiana*, as well as the localization of the complete protein to this site for both *Chlamydiae*-derived transporters in *C. paradoxa*, as predicted by Price et al. (2012). Subsequent work done by Karkar et al. (2015), using the same approach, showed that the UhpC homologs in the red algae *Galdieria sulphuraria* and *C. merolae* are also targeted to the chloroplast inner membrane in *N. benthamiana*. These results demonstrate that the diversification of the PT gene family occurred in the red-green algal ancestor, with the glaucophytes relying on UhpC, a gene that is also retained by algal members of the Rhodophyta and Viridiplantae. Whether these data prove an early divergence of glaucophytes within Archaeplastida is unclear because PT gene loss in this lineage could also explain the current distribution. Regardless, these results bring to a close an intriguing, open question in Archaeplastida evolution and suggest that UhpC could have been the primordial sugar transporter in this supergroup (for details, see Karkar et al. 2015).

Small RNAs in Cyanophora paradoxa: RNAi (RNA interference) is a strategy found among eukaryotes to protect their genomes from the spread of self-replicating genetic entities such as transposable elements and viruses (e.g., Mallory and Vaucheret 2010). This pathway relies on the production of small RNAs (sRNAs) from double-stranded RNA (dsRNA). The initial RNAi signal may be amplified by the generation of multiple secondary sRNAs from a targeted mRNA. This reaction is catalyzed by RNA-dependent RNA polymerases (RdRPs), a phenomenon known as transitivity (Calo et al. 2012) that is particularly important in plants to limit the spread of viruses (Chen et al. 2010). The RNAi process in which sRNAs formed from perfect dsRNAs acting in *cis* by pairing to their cognate producing transcripts is referred to as the small interfering RNA (siRNA) pathway (Obbard et al. 2009), microRNAs (miRNAs) also represent a class of sRNAs widespread in eukaryote genomes that probably evolved from the ancestral siRNA pathway (Piriyapongsa and Jordan 2008). Gross et al. (2013) generated extensive sRNA data from *C. paradoxa* to characterize their genome-wide distribution and to gain insights into their potential functions. Given the monophyly of glaucophytes and Viridiplantae within the Archaeplastida, it was postulated that *C. paradoxa* could represent an ancestral form of the highly developed RNAi system found in plants such as *Arabidopsis thaliana*.

To establish the presence of a putative RNAi pathway in *C. paradoxa*, BLASTP analysis of the glaucophyte genome was done using, as queries, homologs of the

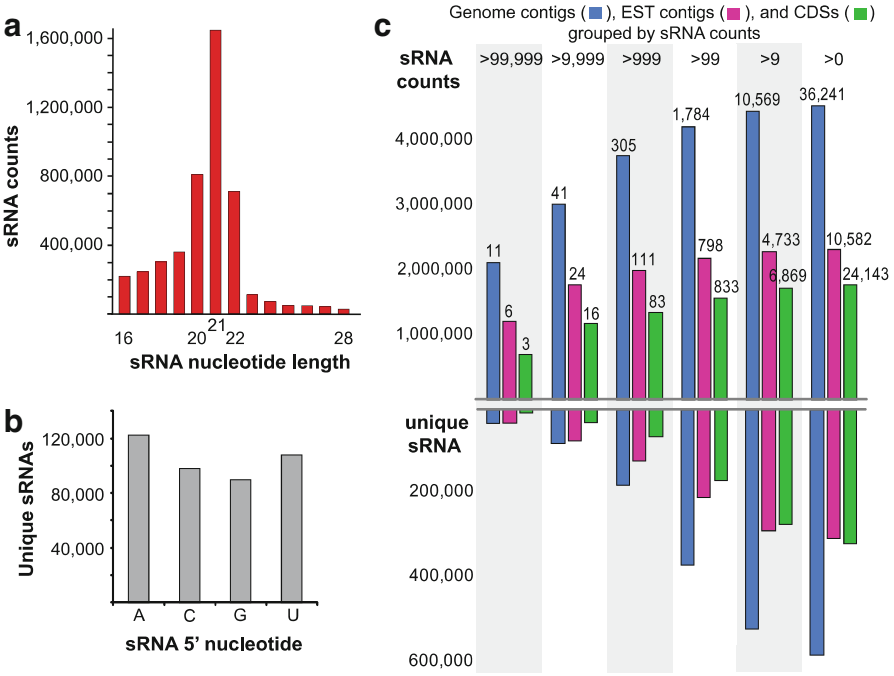

Fig. 15 Analysis of sRNAs from *C. paradoxa*. (**a**) Size distribution of redundant sRNAs in *C. paradoxa* showing the predominance of the 21 nt length class. (**b**) Composition of the 5′ nucleotide of unique sRNAs in *C. paradoxa*. (**c**) The results of mapping redundant (above the x-axis) and unique (below the x-axis) sRNAs to genomic contigs, EST contigs, and CDSs from *C. paradoxa*. The numbers on the top of the colored bars correspond to the number of genomic contigs (*blue*), EST contigs (magenta), and CDSs (*green*) that are associated with the sRNA counts shown at the *top* of the panel

A. thaliana Dicer and Argonaut proteins. These sequences were found as were several putative homologs of *A. thaliana* RdRP. Bioinformatic analysis of 4,739,151 sRNA reads derived from four *C. paradoxa* cDNA libraries showed that sequences had a predominant size of 21 nt (Fig. 15a) with overrepresentation of adenine and uracil in the first nucleotide (Fig. 15b) (Gross et al. 2013). Because *C. paradoxa* sRNAs mapped to over 70% of the EST contigs and to 75% of the predicted CDSs (Fig. 15c), Gross et al. (2013) concluded that sRNA production in this species was primarily associated with mRNA (exonic) sequences. A possible explanation for the significant levels that were found of transcript-derived sRNAs is through the production of secondary siRNA by RdRPs (present in the glaucophyte) during amplificatory cascades of the RNAi signal (for details, see Gross et al. 2013). This intriguing finding has however not been validated due to the lack of genetic tools in *C. paradoxa*. Given the postulated transitivity in *C. paradoxa* and its known presence in the fungus *Mucor circinelloides* (Calo et al. 2012), it is likely that a complex RNAi system was present in the ancestor of all eukaryotes.

In summary, nuclear genome data from glaucophytes have provided a myriad of important insights into the evolution of Archaeplastida. However, much of what has been learned is gleaned from a single draft assembly and several EST databases. As the *C. paradoxa* genome assembly improves, it will provide a valuable reference source for other glaucophyte complete genome projects. These are underway in different labs and become increasingly more tenable as sequencing costs continue to fall and better, long-read technologies are developed. Although we have touched upon some key aspects of glaucophyte genome evolution, we did not address several others that are rapidly advancing. One of these is the work led by the lab of J. Clark Lagarias on phytochrome function and evolution in algae. Analysis of glaucophyte phytochromes demonstrates that *C. paradoxa* (CparGPS1) has an unusual blue/far-red photocycle, whereas *Gloeochaete wittrockiana* (GwitGPS1) has a red/blue photocycle (Rockwell et al. 2014). This is in stark contrast to classical plant phytochromes that are associated with red/far-red photoreception that regulates gene expression for developmental pathways and the shade avoidance response (Rockwell et al. 2006). The surprising diversity of phytochromes in algae (Duanmu et al. 2014; Anders and Essen 2015), and in particular in glaucophytes, indicates that much still needs to be learned about how algae tune their light response to ambient conditions. In this regard, the sequence of a genomic clone of cyanophoropsin, a highly conserved homolog of fungal and bacterial rhodopsins, was described by Frassanito et al. (2010). This trait seems to be unrelated to the known photophobic response of *C. paradoxa* (Häder 1985) because uniform immuno-decoration of the muroplast envelope was achieved using specific antisera directed against an N-terminal recombinant peptide. Therefore, Frassanito et al. (2010) suggest the role of a light-driven proton pump, possibly in conjunction with bicarbonate import into the muroplasts (see section on CCM). Several, but not all, amino acid positions thought to be essential for this function are conserved. Interestingly, corresponding ESTs were overrepresented in low [CO_2] cDNA libraries, indicating that the cyanophoropsin gene is CO_2 responsive (Burey et al. 2007). In addition, a second form of cyanophoropsin was purified as a recombinant protein (Frassanito et al. 2013). Opsins localize to the muroplast envelope; the corresponding genes lack both N-terminal phenylalanine and a canonical stroma-targeting peptide as revealed by terminal amine labeling of substrates (TAILS; Köhler et al. 2015). It is therefore clear that glaucophyte genomes will provide exciting and novel insights into the broader story of algal evolution and help us understand how these taxa thrive in highly variable environments.

The 135.6 kb Muroplast Genome of *Cyanophora paradoxa* SAG 29.80

The list of genes of the completely sequenced muroplast genome of *C. paradoxa* (Stirewalt et al. 1995; Löffelhardt et al. 1997), given in Table 7, contains more than 60 genes that are nuclear encoded or missing in land plants. This gene content is typical for primordial plastids, i.e., those from algae devoid of chlorophyll *b*. The 192 muroplast genes rank between the 174 genes present on the 120 kb plastome

from the diatom, *Odontella sinensis*, and the 251 genes found on the 191 kb plastome from the red alga *Porphyra purpurea* (Reith 1995). With some exceptions, e.g., the *ndh* genes and *infA* (missing from all algal plastomes investigated thus far), *atpI*, or *accD*, the muroplast genome contains the standard set of chloroplast-encoded genes. In addition, the muroplast genome encodes many more ribosomal proteins, several enzymes involved in anabolic pathways other than photosynthesis, chaperones, (putative) transcription factors, and components of ABC transporters and the Sec preprotein translocase (Table 7). The most conspicuous feature of the gross organization of the muroplast genome is the 11.3 kb inverted repeat (IR), which corresponds to about half the size of land plant chloroplast IRs. Another obvious feature is the small intergenic spacer regions between muroplast genes. In a few cases (*orf299/orf244*, *ycf16/ycf24*, *atpD/atpF*, *psbD/psbC*) adjacent genes have been found to overlap by 3–16 bp. Moreover, only few noncoding regions that extend over several hundred bp are observed. Just one single intron has been identified: the 232 bp group I intron in the anticodon loop of $trnL^{UAA}$. These three features explain why muroplasts encodes around 50 genes more than land plant chloroplasts most of which even have slightly larger genomes. A restriction map of muroplast DNA from *C. cuspitata* (Kies-isolate; SAG 46.84) showed significant differences in size (about 10 kbp) and restriction pattern. However, the overall sequence identity to the Pringsheim isolate (SAG 29.80) was above 85% and 18 protein gene loci and the rDNA regions appeared to be conserved (Löffelhardt et al. 1997).

RNA genes: About half of the IR regions are occupied by the two rDNA units. The rDNA spacer is small, as is typical for chlorophyll *b*-less algae, and harbors *trnI* and *trnA* as in most plastids and prokaryotes (Löffelhardt et al. 1997). The *rnpB* gene, also present on the *P. purpurea* plastome, specifies the essential RNA component of RNaseP, a ribonucleoprotein responsible for 5′-processing of plastid tRNAs. This marks another distinction between primitive plastids and chloroplasts. In land plant chloroplasts, the enzyme activity is protein based only, whereas in *C. paradoxa* muroplasts an RNA component with strong similarity to bacterial counterparts is present – the protein component, if any, has not been found yet. In contrast to red algal RnpB, an RNA-only activity (as shown for bacteria) has been demonstrated for the muroplast RNA (Li et al. 2007). Addition of RnpA protein from *E. coli* considerably enhanced the activity, indicating a certain conformational instability of muroplast RnpB. A tmRNA combining properties of tRNAs and mRNAs that ameliorates problems arising from stalled ribosomes was also found to be encoded by a muroplast gene and shown to be processed by RNaseP (Gimple and Schön 2001). This is again typical for primitive organelles whose tmRNAs are examples of reductive evolution compared to their bacterial counterparts (de Novoa and Williams 2004). An RNA component of the algal plastid SRP, encoded on all sequenced rhodoplast genomes, could not be detected on muroplast DNA.

Muroplast gene expression: The codon bias of muroplast genes, likely a selection for translation efficiency, is more pronounced than that of other algae or land plants (Morton 1998). Putative promoter motifs can often be observed that are similar in both sequence and spacing to the canonical sequences from *E. coli* and other

eubacteria. Three muroplast ORFs (*ycf27*, *ycf29*, and *ycf30*), that are conserved among primitive plastid genomes, show significant sequence similarity to prokaryotic transcription regulatory factors of the OmpR and LysP classes. The occurrence of these putative regulators suggests that some transcriptional regulation occurs in muroplasts. *Ycf27* homologous response regulator genes (*rpaB*) appear to be confined to phycobiliprotein-containing organisms. Many genes show short poly-purine stretches complementary to the 3′ end of the cyanelle 16S rRNA (−CCUCCUUU-3′OH) at a distance of 7–12 bases upstream of the initiation codon. Typical ribosome binding sites (Shine-Dalgarno sequences) are AAGG, AGGA, GGAG, and GAGG. The gene arrangements observed suggest a predominance of polycistronic transcripts as reported for chloroplasts (e.g., the large ribosomal protein gene cluster) and cyanobacteria (e.g., phycobiliprotein gene clusters) which could be proven in several cases. Processing of the primary transcripts to smaller mRNAs seems to be rather common (Löffelhardt et al. 1997). The widespread distribution of a specific gene cluster (5′-*rpoB-rpoC1-rpoC2-rps2-atpH-atpG-atpF-atpD-atpA*-3′) strongly supports the hypothesis of a common origin of all plastid types. Three transcription units (*rpoBC1C2*, *rps2-tsf*, and *atpIHFGDAC*) that are widely separated on cyanobacterial genomes seem to have been fused together after the endosymbiotic event. This cluster is found with some variation in gene content, but never in gene order, in muroplasts and rhodoplasts as well as in land plant chloroplasts. The existence of this "diagnostic" cluster in plastids of different evolutionary levels can only be explained when a single primary endosymbiotic event is assumed (Kowallik 1994; Reith 1995; Löffelhardt 2014). In *O. sinensis*, this cluster is bipartite, and it is completely disintegrated in *Chlamydomonas reinhardtii*, which shows that there is no particular selection pressure to maintain or to reach this kind of gene arrangement.

A signature of primitive plastids devoid of chlorophyll *b* is that both subunits of RuBisCO are plastome encoded as first shown for *C. paradoxa* (Heinhorst and Shively 1983) and cotranscribed (Starnes et al. 1985). Interestingly, the *rbcLS* and *atpBE* genes are adjacent and divergently transcribed in muroplasts and land plant chloroplasts.

There are a few cases where the muroplast genome contains cyanobacterial genes and transcription units that are absent from the *P. purpurea* rhodoplast genome in spite of the 30% surplus in size and gene content of the latter. One of them is *groES-groEL*: the chaperonin-10 homolog is nucleus encoded in the red alga. Other examples are *crtE* (specifying geranyl-geranyl pyrophosphate synthase), *hemA* (glutamyl-tRNA reductase), and *orf244-orf299* encoding two components of an ABC transporter, likely for manganese, based on the significant sequence similarity to the cyanobacterial *mntA* and *mntB* genes (Bartsevich and Pakrasi 1995). The *orf333* upstream from muroplast *psbE* is found in this position in cyanobacteria, too, but is absent from all other plastid genomes. ORF333 is the product of a nuclear gene (*hcf136*) in *Arabidopsis thaliana* and is absolutely required for assembly/stability of functional PSII units (Meurer et al. 1998). A special case is *orf180* found only on muroplast DNA (in the *petA-psaM* intergenic region) and on the genome of the peculiar cyanobacterium, *Gloeobacter violaceus*. The gene product, symerythrin,

belongs to the ferritin-like superfamily (FLSF, Cooley et al. 2011). While its in vivo functions are still unknown, the recombinant protein displays oxidase and peroxidase activity. Other members of the FLSF (e.g., the rubrerythrins) have six or seven ligands to the diiron metallocenter, whereas symerythrin has eight ligands. Other unique features comprise the high internal symmetry of the crystal structure and the spontaneously formed carbon-carbon cross-link between a valine and a phenylalanine side chain. This led the authors to assume an ancestral role for this fold in the evolution of FLSF (Cooley et al. 2011). Recently, the muroplast DNA of *G. nostochinearum* was sequenced (B.F. Lang and G. Burger, unpublished) and was found to resemble that of *C. paradoxa* both in size and gene outfit. Interestingly, *orf180* was also detected, almost identical in sequence to the *Cyanophora* counterpart. In summary, such features of the plastome lend support to the often claimed "living fossil" status of glaucophytes, whereas the mosaic structure of the gene-rich nuclear genome of *Cyanophora* rather seems to contradict this view (Price et al. 2012).

Glaucophyte Mitochondrial Genomes

The complete mitochondrial DNAs (mtDNAs) of *C. paradoxa* (51.6 kbp) and *G. nostochinearum* (34.1 kbp) have been sequenced (Price et al. 2012). Glaucophyte mtDNAs do not stand out as particularly large or gene rich. Repetitive regions and larger intergenic distances in the *Cyanophora* metagenome account for the size difference. They encode the basic set of genes typical for animals and fungi, plus those characteristic of many protists and plants (i.e., close to a dozen coding for ribosomal proteins, a few extra subunits of the NDH and SDH complexes, and 5S rRNA). Recently, the mtDNA sequences of *Gloeochaete wittrockiana* (36 kbp) and *Cyanoptyche gloeocystis* (33.2 kbp) were published (Jackson and Reyes-Prieto 2014) with coding capacities strongly resembling those of the other two glaucophytes. Red and green algae share mtDNA-encoded TatC, a protein translocase component (see section on "Conservative Sorting"), and *ccm* genes specifying ABC transporters involved in cytochrome *c* biogenesis (Verissimo and Daldal 2014). Both these gene classes are absent from glaucophyte mtDNAs. In turn, green and glaucophyte algae share *rpl2*, *nad7*, and *nad9*, which are not present in red algal mtDNAs. Finally, glaucophytes possess a mitochondrion-encoded *nad11* that was lost by the two other groups. In conclusion, there is nothing at the level of mitochondrial gene complement that would specifically unite two of the three lineages.

Despite earlier claims likely caused by bacterial contaminants (Kiefel et al. 2004), no genes for mitochondrial division proteins of prokaryotic origin were found on the *C. paradoxa* genome. This is paralleled in green algae and plants, whereas mtMinD, mtMinE, and mtFtsZ were reported for rhodophytes and chromophytes (Leger et al. 2015). There is a single gene specifying (muroplast-targeted) TatC in the genome of *C. paradoxa* indicating the absence of the mitochondrial Tat pathway as, e.g., in land plants, where the AAA-ATPase Bcs1 assists mtRieske Fe-S protein in IM translocation and assembly into the cytochrome bc_1 complex (Wagener et al. 2011).

Interestingly, a contig for a Bcs1 homolog with a predicted mitochondrial localization was detected in the *Cyanophora* genome database (J.M. Steiner, unpublished). This would mean an advanced aspect of *Cyanophora* mitochondria, as primordial mitochondria (e.g., of jakobids) retained the proteobacteria-derived Tat pathway (Wagener et al. 2011).

Metabolic Pathways in the Cytosol of *Cyanophora paradoxa*

Starch metabolism: Early diverging phototrophic eukaryotes seem to play an important role in the conversion of cyanobacterial glycogen into the starch of green algae and land plants during evolution (Deschamps et al. 2008). Reserve carbohydrate granules have long been known to reside in the cytosol of glaucophytes (Kies 1992) and also of rhodophytes and algae derived through red algal secondary endosymbiosis. *C. paradoxa* starch showed a (high) amylose and amylopectin content with chain length distributions and crystalline organization similar to green algae and land plants that use ADP-glucose as the activated monomer for starch synthesis and temporary storage in the chloroplasts (Plancke et al. 2008). However, several starch synthase activities were found in *C. paradoxa* utilizing UDP-glucose, this time in analogy to rhodophytes that also synthesize their (more amylopectin-related) floridean starch in the cytosol. In addition, a multimeric isoamylase complex and multiple starch phosphorylases were demonstrated and of isoamylase: There is a correlation between the presence of starch and the debranching activity of isoamylase; those alpha-1,6-branches that impede the attainment of a crystalline structure are removed (Cenci et al. 2014). These results were obtained at the zymogram level and in some cases also at the gene level (Plancke et al. 2008). Transcription of a granule-bound starch synthase (responsible for amylose formation) was shown to be upregulated upon shift to low [CO_2] (Burey et al. 2007). Furthermore, the cytosolic transglucosidase DPE2 (disproportionating enzyme 2), transferring one glucose moiety from maltose (resulting from starch degradation by beta-amylase) to a cytosolic heteroglucan, could be demonstrated on *C. paradoxa* zymograms (Fettke et al. 2009). The *Cyanophora* Genome Project (http://dblab.rutgers.edu/cyanophora/home.php) (Price et al. 2012) allowed the identification of numerous putative carbohydrate metabolism enzymes using the Carbohydrate-Active enZymes (CAZy) database (Cantarel et al. 2009): about 84 glycoside hydrolases (GHs) and 128 glycosyl transferases (GTs), significantly more than in the green microalga *Ostreococcus lucimarinus* or the extremophilic red alga *Cyanidioschyzon merolae*, but less than in land plants. Many *C. paradoxa* CAZymes are involved in starch metabolism. Synthesis of the polysaccharide within Viridiplantae plastids relies on ADP-glucose-dependent enzymes of the GT5 family associated with glycogen synthesis in bacteria. The major *C. paradoxa* enzyme is phylogenetically related to the UDP-glucose-specific enzyme of heterotrophic eukaryotes (Cantarel et al. 2009) and has been partially purified from this alga (Plancke et al. 2008). This suggests the absence of ADP-glucose pyrophosphorylase in *C. paradoxa*. Surprisingly, another gene was found in the glaucophyte genome whose product is related to the

SSIII-SSIV (GT5) type of starch synthases in Viridiplantae. This gene is phylogenetically related to glucan synthases in chlamydiae, cyanobacteria, and some proteobacteria and is hypothesized to have played a key role in linking the biochemistry of the host and the endosymbiont. The SSIII-SSIV enzyme uses ADP-glucose in bacteria and land plants, suggesting that *C. paradoxa* or, rather, the common ancestor of Viridiplantae and glaucophytes may have used both types of nucleotide sugars for starch synthesis at the onset of the endosymbiosis. Cytosolic ADP-glucose is thought to arise from the cyanobacterial endosymbiont at that time via a sugar nucleotide transporter of host origin (Weber et al. 2006). A third player is thought to have contributed to this merging of the reserve carbohydrate synthesis pathways of host cell and endosymbiont: Chlamydiae, known for their intracellular lifestyle, might have supplied crucial enzymes and transporters to the cytosol and the endosymbiont/phagosome membranes during an earlier long-term, but transitory, infection. This "ménage a trois" could have been instrumental for the transition from glycogen of the heterotrophic host to starch of the eukaryotic phototroph (Ball et al. 2013): SSIII-SSIV (GlgA), isoamylase (presumably after gene duplication and some change in function of the bacterial direct debranching enzyme GlgX), and (at a later stage) the glucose-6-phosphate transporter UhpC likely represent the contributions (via HGT) from chlamydiae. Granule-bound starch synthase is of cyanobacterial origin (EGT), whereas the other enzymes stem from the metabolic repertoire of the host cell. New developments necessitated due to the glycogen-starch transition are glucan, water dikinase (GWD) and phosphoglucan, water dikinase (PWD), genes for which are also found on the *Cyanophora* genome. Degradation of the quasicrystalline starch granules by beta-amylases and phosphorylases is only possible after previous action of GWD and PWD (Cenci et al. 2014). Readers should note that the impact of *Chlamydiales* on Archaeplastida evolution and the validity of the ménage a trois hypothesis are considered controversial by some parties (e.g., Dagan et al. 2013; Deschamps 2014; Domman et al. 2015). More recent biochemical, phylogenetic, and genomic data however provide strong support for this model of Archaeplastida primary plastid establishment (see Ball et al. 2016a, b; Cenci et al. 2017; Gehre et al. 2016).

Biosynthesis of long-chain fatty acids and isoprenoids: De novo biosynthesis of fatty acids is compartmentalized in muroplasts as in plant chloroplasts. Elongation beyond C_{16} occurs in the cytosol with acetyl coenzyme A provided by the action of ATP citrate lyase (ACL). The long-assumed plastid localization of ACL was falsified for *C. paradoxa*, and, for the first time, a heterodimeric structure as in fungi and the prokaryote *Chlorobium tepidum* was proposed (Ma et al. 2001). This now applies for all plants as opposed to the large monomer observed in metazoa. cDNA and genomic sequencing of the gene for the catalytic subunit provided information about intron structure of nuclear genes: introns are numerous, in the size range of 53–65bp, with conserved border and (putative) branch point nucleotides (Ma et al. 2001; Bhattacharya and Weber 1997). The regulatory subunit is also present as evidenced by ESTs (http://tbestdb.bcm.umontreal.ca/searches/login.php).

The mevalonate pathway (missing in green algae), also dependent on acetyl coenzyme A provided by ACL, seems to be restricted to the cytosol of

C. paradoxa as shown by amplification of four selected genes (Grauvogel and Petersen 2007) and was confirmed later through the genome project.

Anaerobic Energy Metabolism

C. paradoxa was long considered an obligatory phototroph, and attempts to grow it on carbon sources as glucose or acetate were unsuccessful (Trench 1982). Therefore, it came as a surprise that the genome project revealed the potential for various fermentative metabolic pathways (Price et al. 2012). The respective gene repertoire is almost as extensive as that of *C. reinhardtii*, the best known model for this trait among green algae, and even exceeds that of picochlorophyta, whereas such genes are rare in red algae (Atteia et al. 2013). It remains to be seen if the corresponding enzyme activities, e.g., acetate:succinate CoA-transferase, hydrogenase (and maturation factors), pyruvate:formate lyase (and activating enzyme), and pyruvate: $NADP^+$ oxidoreductase, can be demonstrated in the appropriate compartments of the *Cyanophora* cell. Cytosol, plastids, and mitochondria are known to be involved in the anaerobic energy metabolism of algae (Atteia et al. 2013). The complex fermentative capabilities conserved between the distant relatives *C. paradoxa* and *C. reinhardtii* likely represent an evolutionarily advantageous combination of anoxic enzymes from the eukaryotic host and the cyanobacterial endosymbiont (Price et al. 2012).

Evolutionary History

No fossil remnants of glaucophytes are known, but their origin among the Archaeplastida is thought to date back to the Mesoproterozoic/Neoproterozoic boundary as that of rhodophytes (Butterfield 2000). Apart from the common possession of multilayered structures in members of the three algal groups mentioned, glaucophytes differ from prasinophycean green algae (flagella with scales, pellicular lacunae absent, intraplastidial starch), green algae (different kinetids and flagella movement, pellicular lacunae absent, intraplastidial starch), and euglenids (different pellicular structure, different type of mitosis, paramylon instead of starch as reserve polyglycan).

Phylogenetic Relationships

The phylum Glaucophyta as one of the three groups containing primary plastids contains all genera described by Kies (1992) based on morphological criteria (Table 2) and the presence of muroplasts. A concatenated phylogenetic analysis of plastid-encoded genes placed *C. paradoxa* and thus the glaucophytes on the first branch after the single primary endosymbiotic event (Martin et al. 1998; Rodríguez-Ezpeleta et al. 2005). This was corroborated by concatenated nuclear genes

(Rodríguez-Ezpeleta et al. 2005; Reyes-Prieto and Bhattacharya 2007a, b). Phylogenomics, made possible through the *Cyanophora* Genome Project, gave additional support (see above). Thus glaucophytes can be considered as direct descendants of the most ancient phototrophic eukaryotes, at least among the species known at present.

Phylogenetic relationships within the Glaucophyta have been investigated thoroughly in two independent studies (Chong et al. 2014; Takahashi et al. 2014). Based on concatenated and single genes of plastid (*psbA*) and mitochondrial (*cob* and *cox1*) origin, and the nuclear internal transcribed spacer (ITS) region, Chong et al. (2014) revealed that strains of *Glaucocystis nostochinearum* (or *Glaucocystis* species complex) were divided into six clades that possibly correspond to individual species (Fig. 8). The monophyletic group of *Glaucocystis* sp. complex was clustered together with the monophyletic *Gloeochaete wittrockiana* and *Cyanoptyche gloeocystis* clade. Five *Cyanophora* species were separated from the rest of the glaucocystophycean clade (see Fig. 8). Within the *Cyanophora* clade, *C. sudae* and *C. biloba* were clustered strongly and separated from the remaining *C. paradoxa* + *C. kugrensii* + *C. cuspidata* clades based on the *psa*B and ITS phylogenies (Takahashi et al. 2014). Although three new *Cyanophora* species were suggested based on morphological and molecular data (Takahashi et al. 2014), it still is a challenge to delimitate species in glaucophytes, because of the lack of authentic (Type) strain(s) and the simple morphology prevailing. However, using a combination of molecular and morphological data, the latter made possible through advanced EM methodology, Takahashi et al. (2016) confirmed the *Glaucocystis* clades proposed by Chong et al. (2014) and delineate six individual species (Fig. 8). Subtle, but significant differences in the peripheral ultrastructure of the cells, i.e., in the vesicle system underlying the plasma membrane (lacunae, cf. Fig. 7), were the key to this problem.

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Rhodophyta

3

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Abstract

Rhodophyta, or red algae, comprises a monophyletic lineage within Archaeplastida that includes glaucophyte algae and green algae plus land plants. Rhodophyta has a long fossil history with evidence of *Bangia*-like species in ca. 1.2 billion-year-old deposits. Red algal morphology varies from unicellular, filamentous, to multicellular thalloid forms, some of which are sources of economically important products such as agar and carrageenan.

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These species live primarily in marine environments from the intertidal zone to deep waters. Freshwater (e.g., *Batrachospermum*) and terrestrial lineages also occur. One of the major innovations in the Rhodophyta is a triphasic life cycle that includes one haploid and two diploid phases with the carposporophyte borne on female gametophytes. Red algae are also well known for their contribution to algal evolution with ecologically important chlorophyll-*c* containing lineages such as diatoms, dinoflagellates, haptophytes, and phaeophytes all containing a red algal-derived plastid of serial endosymbiotic origin. Analysis of red algal nuclear genomes shows that they have relatively small gene inventories of 6,000–10,000 genes when compared to other free-living eukaryotes. This is likely explained by a phase of massive genome reduction that occurred in the red algal ancestor living in a highly specialized environment. Key traits that have been lost in all red algae include flagella and basal body components, light-sensing phytochromes, and the glycosylphosphatidylinositol (GPI)-anchor biosynthesis and macroautophagy pathways. Research into the biology and evolution of red algae is accelerating and will provide exciting insights into the diversification of this unique group of photosynthetic eukaryotes.

Keywords

Red algae • Rhodophyta • Ultrastructure • Evolutionary timeline • Triphasic life history • Genome reduction

Contents

Summary Classification

●**Rhodophyta**
●●**Cyanidiophytina**
●●●**Cyanidiophyceae**
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●●●**Rhodellophyceae**
●●●**Stylonematophyceae**
●●●**Florideophyceae**
●●●●**Hildenbrandiophycidae**
●●●●**Nemaliophycidae**
●●●●**Corallinophycidae**
●●●●**Ahnfeltiophycidae**
●●●●**Rhodymeniophycidae**

Introduction

General Characteristics

The Rhodophyta (red algae) is a well-characterized and morphologically diverse lineage of photosynthetic protists. They range from unicells and uni- or multiseriate (arranged in rows) filaments, to large (up to 3 m) pseudoparenchymatous, branched or unbranched, terete (cylindrical) to foliose (blade-like) thalli, including crustose and erect forms, some of which are calcified (Figs. 1 and 2). More than 7,100 species are currently reported (www.algaebase.org). Diagnostic features of the red algae are: (1) plastids with accessory, water-soluble pigments allophycocyanin, phycocyanin, and phycoerythrin localized in structures termed phycobilisomes located on the outer faces of photosynthetic lamellae (thylakoids, Fig. 3b, c; other pigments include chlorophyll *a,* α- and β-carotene, lutein and zeazanthin); (2) thylakoids present as single lamellae (i.e., not stacked) in plastids (Fig. 3a–c); (3) lack of flagellated structures at any stage of the life history; and (4) food reserves stored as floridean starch [α-(1, 4)-linked glucan] in granules outside the plastid (Fig. 3a, b). Additional traits of some, but not all red algae include: (1) the presence of "pit connections" between cells (a misnomer because these are not connections between cells, rather plugs of proteinaceous material deposited in the pores that result from incomplete centripetal wall formation) (Fig. 3a, d); (2) mitochondria associated with the forming (cis) faces of dictyosomes (Golgi bodies) (Fig. 3e); (3) plastids surrounded by one or more encircling thylakoids (Fig. 3c); and (4) a complex life history composed of an alternation of two free-living and independent generations (gametophyte and tetrasporophyte) and a third generation, the carposporophyte, that occurs on the female gametophyte (terms are defined in the "Life Histories" section). The Rhodophyta currently consists of two subphyla and seven classes (Yoon et al. 2006).

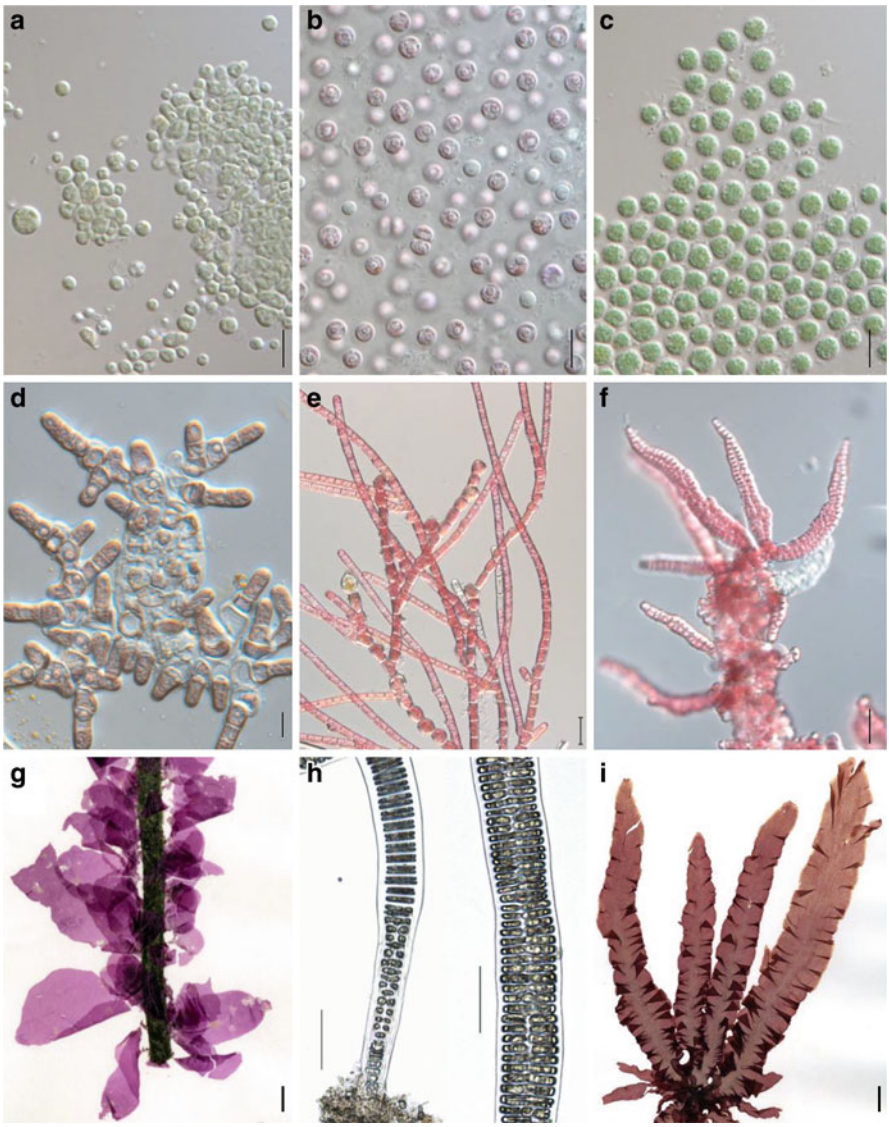

Fig. 1 (**a**–**i**) Representative species of the Rhodophyta. (**a**) *Galdieria phlegrea* (Cyanidiophyceae), (**b**) *Rhodosorus marinus* (Stylonematophyceae), (**c**) *Porphyridium aerugineum* (Porphyridiophyceae), (**d**) *Boldia erythrosiphon* (Compsopogonophyceae), (**e**) *Rhodochaete parvula* (Compsopogonophyceae), (**f**) *Stylonema cornu-cervi* (Stylonematophyceae), (**g**) *Smithora naiadum* (Compsopogonophyceae), (**h**) *Dione arcuata* (Bangiophyceae), (**i**) *Pyropia viridiidentata* (Bangiophyceae). (Scale = 10 μm for **a**–**c**, 20 μm for **d**–**f**, 2 cm for g, 30 μm for h, 10 cm for i)

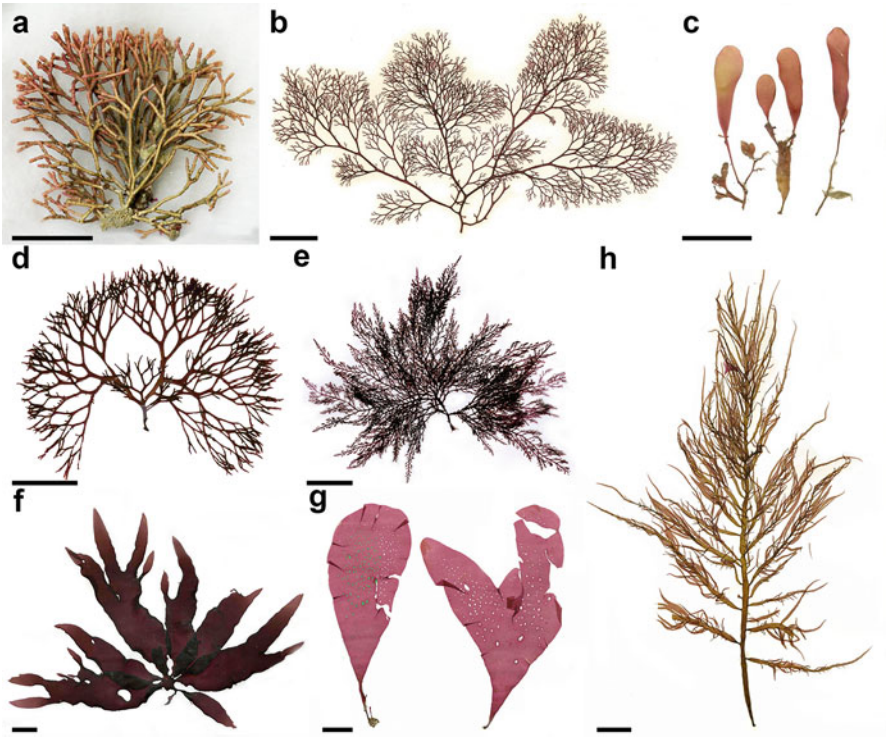

Fig. 2 (**a**–**g**) Habits of Florideophyceae. (**a**) *Actinotrichia fragilis* (Nemaliales, CNU011766) from Jeju, Korea. Scale = 1 cm. (**b**) *Ceramium kondoi* (Ceramiales, CNU013255) from Oeyondo, Korea. Scale = 1 cm. (**c**) *Schottera* sp. (Gigartinales, CNU040701), from Chujado, Korea. Scale = 1 cm. (**d**) *Ahnfeltiopsis flabelliformis* (Gigartinales, CNU033820) from Chujado, Korea. Scale = 2 cm. (**e**). *Gelidium elegans* (Gelidiales, CNU018530) from Jeju, Korea. Scale = 1 cm. (**f**) *Pachymeniopsis lanceolata* (Halymeniales, CNU049476) from Pohang, Korea. Scale = 2 cm. (**g**) *Sparlingia pertusa* (Rhodymeniales, CNU057539), from Uljin, Korea. Scale = 2 cm. (**h**) *Chrysymenia wrightii* (Rhodymeniales, CNU021964). Scale = 2 cm

Florideophyceae, the most species-rich class (6,751 spp.; 95% of all taxa), appears to be a monophyletic group characterized by the presence of tetrasporangia and a filamentous gonimoblast in most species (terms defined in the "Life Histories" section).

History of Knowledge

The process of describing and naming Rhodophyta (along with all plants and eukaryotic photosynthetic organisms) begins with Linnaeus, who placed taxa that currently belong to this phylum in three genera: *Conferva* (filamentous forms), *Ulva* (membranous forms), and *Fucus* (thalloid forms). Lamouroux was the first to use

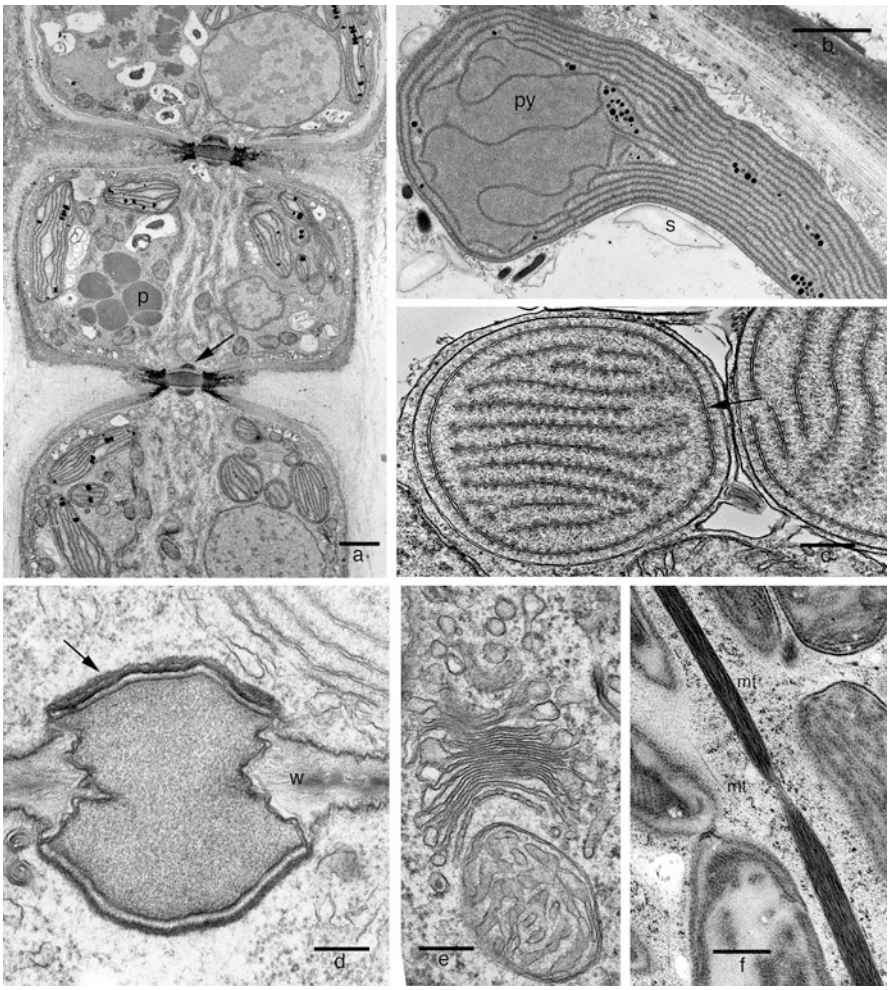

Fig. 3 (**a**–**f**) Thin-section electron microscopy of cellular features of red algae. (**a**) Multicellular epithallial filament of *Neopolyporolithon loculosum*. Cells are joined by pit plugs having dome-shaped outer caps (*arrow*). Cells contain a nucleus, numerous plastids, Golgi bodies associated with mitochondria, endoplasmic reticulum, and protein bodies (*p*). Scale = 1.0 μm. (**b**) Plastid of *Colaconema rhizoideum* containing a large pyrenoid (*py*) penetrated by thylakoids. Starch grains (*s*) are present in the cytoplasm. Scale = 1.0 μm. (**c**) Plastid of *Griffithsia pacifica*. A single peripheral thylakoid (*arrow*) just inward of plastid envelope encircles numerous plate-shaped thylakoids. Phycobilisomes are visible as granules on the surfaces of the thylakoids. Scale = 0.2 μm. (**d**) Pit plug of *Palmaria palmata*. The homogeneous plug core, flanked by cell wall (*w*), is separated from the cytoplasm by thin multilayered plug caps (*arrow*). Scale = 0.2 μm. (**e**) Golgi body-mitochondrion spatial association in *Audouinella saviana* is typical of florideophytes. Scale = 0.2 μm. (**f**) Freeze-substitution preparation of *Antithamnion kylinii* preserves cytoskeleton, including prominent cables of microfilaments (*mf*) and scattered microtubules (*mt*). Scale = 0.5 μm

color to distinguish between groups of thallophytes, and he placed some red algal genera into an order "Floridées."

Red algae (particularly Florideophyceae) were not recognized as a monophyletic assemblage, however, until Harvey (1836) distinguished red, green, and brown algae (Rhodospermeae, Chlorospermeae, and Melanospermeae, respectively) as separate groups based on the spores being the same color as the parent thalli. Although this classification gained immediate acceptance, it was not until the elegant experiments of Haxo and Blinks (1950) that the direct link was established between the colors (presence of various accessory pigments) of algae and their photosynthetic action spectra.

During the nineteenth century, when European nations were sponsoring voyages to discover and explore new lands, plant and animal specimens were sent back to various scientific authorities. Thus, algal specimens were sent to C. A. and J. G. Agardh in Lund, F. T. Kützing in Leiden, P. C. Montagne in Paris, and W. H. Harvey in Dublin, as well as to numerous other algal systematists who published significant (and often magnificent) tomes. Their observations were restricted to morphological and anatomical features of taxa, with no clear understanding of how these features were related to the reproduction or life histories of the organisms.

Convincing documentation of sexual reproduction in red algae was provided by Bornet and Thuret, and further observations made independently by Schmitz and Oltmanns, linked morphological features with stages of sexual reproduction. With these discoveries, the criteria that formed the basis of the classification of the Florideophyceae for many years were established. By early in the twentieth century, a number of orders that are recognized today had been established, and by mid-century the voluminous works of one man, Harald Kylin (summarized in Kylin 1956), had set down an infraordinal classification scheme that was followed for about three decades. Over the past ca. 25 years, many more orders, families, and genera of red algae have been established (Schneider and Wynne 2007, 2013; Wynne and Schneider 2010).

The red algae are classified into the phylum Rhodophyta (Wettstein 1901), as one phylum of the supergroup Archaeplastida with two sister phyla, the Viridiplantae and Glaucophyta (Adl et al. 2005). The Rhodophyta has been traditionally classified into two classes, the Bangiophyceae and Florideophyceae (Gabrielson et al. 1985), or two subclasses, the Bangiophycidae and Florideophycidae (Dixon 1973). Based on cladistics and molecular phylogenetic studies, the Bangiophyceae has been identified as a paraphyletic group (e.g., Gabrielson et al. 1985; Müller et al. 2001; Oliveira and Bhattacharya 2000; Yoon et al. 2006). To reflect phylogenetic relationships, Saunders and Hommersand (2004) developed a revised classification system comprising two phyla (Rhodophyta and Cyanidiophyta), three subphyla (Rhodellophytina, Metarhodophytina, and Eurhodophytina), and five classes (Bangiophyceae, Compsopogonophyceae, Cyanidiophyceae, Florideophyceae, and Rhodellophyceae). This system was updated by Yoon et al. (2006), who inferred seven well-supported phylogenetic lineages in a multigene analysis. They proposed the Rhodophyta contain two subphyla, the Cyanidiophytina with a single class, the

Cyanidiophyceae, and the Rhodophytina with six classes (Bangiophyceae, Compsopogonophyceae, Florideophyceae, Porphyridiophyceae *classis nova,* Rhodellophyceae, and Stylonematophyceae *classis nova*). This seven-class system is now widely accepted for red algal classification. The system presented here and discussed in the "Classification" section represents a slight modification of the system proposed by Yoon et al. (2006, 2010).

Habitats and Ecology

Ecological Importance of Red Algae

Red algae can be found in many different environments – marine, freshwater, and terrestrial. The majority of red algae belong to the Florideophyceae, which are largely multicellular and nearly all inhabit marine habitats. Some species extend into estuarine environments and some are exclusively freshwater, for example, members of the Batrachospermales.

Although red algae rarely form canopies in subtidal communities, they play key roles in nearshore ecosystems. Species of red algae range from the upper reaches of intertidal shores (e.g., members of the Bangiales) to hundreds of meters in depth in clear tropical waters. As understory vegetation in kelp forests as well as turfs on intertidal shores, red algae provide habitat for a wide variety of organisms. This review supplements the earlier review of Gabrielson et al. (1990).

Calcified Red Algae

Calcified red algae are vital components of nearshore ecosystems. They can be found from intertidal shores to the deepest reaches of the euphotic zone and from polar to tropical latitudes (Foster 2001; Nelson 2009). Most calcified red algae belong to the orders Corallinales, Hapalidiales, or Sporolithales. Species in these orders are either geniculate (jointed or articulated) or nongeniculate (typically crustose). In tropical coral reef environments, crustose coralline algae reinforce the skeletal structure of corals, filling cracks and cementing together sand, dead coral, and debris, creating stable substrate, and reducing reef erosion (Adey 1998; Diaz-Pulido et al. 2007). In studying tropical coral reefs, Littler and Littler (2007) concluded that the presence of "massive corals and calcareous coralline algae relative to frondose macroalgae and algal turfs indicates a healthy spatially heterogeneous condition reflecting low nutrients and high herbivory," whereas high coverage of coralline algae suggests high herbivory levels and elevated nutrients, which can inhibit some corals.

Geniculate coralline algae are also widespread on hard substrata. They are sometimes referred to as ecosystem engineers to reflect the way their three-dimensional structure modifies the environment. Their complex, branched axes intermesh and thus resist wave action and disturbance and retain moisture when exposed at low tide, a particular advantage for intertidal species. These turfs provide

habitat and shelter from several of the stresses of intertidal life (e.g., desiccation, wave action, and predation) and, in addition, they provide surfaces for settlement of microphytobenthos and trap sediments for epiphytic filter-feeding taxa. Coralline turfs have been found to harbor high diversity, density, biomass, and productivity of mobile invertebrates (e.g., Cowles et al. 2009; Kelaher et al. 2004). Another ecosystem service provided by coralline algae is the release by some species of compounds that are critical to the settlement and morphogenesis of corals and molluscs (Morse et al. 1996; Roberts 2001; Tebben et al. 2015).

Rhodoliths are free-living coralline algae found in coastal habitats extending to depths of more than 200 m (Foster 2001; Nelson 2009), and they thrive in areas with sufficient water motion to inhibit burial by sediment but not so much as to remove them from their favored habitat (Foster 2001). Rhodolith beds (maërl) are extensive communities found on a wide variety of sediments, from mud to coarse sand. Foster (2001) argued that rhodolith beds may be one of earth's "big four" seaweed-dominated communities together with kelp forests, seagrass meadows, and nongeniculate coralline algae-dominated tropical reefs. Internationally recognized as unique ecosystems, new rhodolith beds continue to be discovered (Foster 2001; Konar et al. 2006; Teichert et al. 2012; Macaya et al. 2015). The three-dimensional structure of rhodolith beds creates microhabitats for diverse invertebrates and algae, including rare and unusual species, as well as serving as nursery grounds for some commercial species of fish (e.g., Hernández-Kantún et al. 2010; Kamenos et al. 2004a, b; Neill et al. 2015; Peña and Bárbara 2008b; Steller et al. 2003; Teichert 2014). Recognition of the ecological importance of these algal-dominated communities and the need for conservation has increased over the past decade (e.g., Barbera et al. 2003; Grall and Hall-Spencer 2003; Peña and Bárbara 2008a). Maërl has a long history of use as a soil additive in Europe, and commercial mining of rhodoliths is carried out in Europe and Brazil, despite concerns about the sustainability and impacts on ecosystem services (Briand 1991; Riul et al. 2008).

Recent studies indicate that rhodoliths and other coralline algae are at risk from the impacts of a range of human activities, such as physical disruption, reduction in water quality, alterations to water movement, and global climate change (e.g., McCoy and Kamenos 2015; Nelson 2009).

Invasive Species

There is increasing recognition of the potential ecological impacts of introduced species – for example, modifying the habitats they invade, displacing native species, altering food webs and community structure, and threatening native biodiversity. Compilations of introduced seaweeds have been published as well as regional surveys (e.g., Davidson et al. 2015; Miller et al. 2011; Nelson 1999; Williams and Smith 2007).

There have been serious consequences accompanying human-assisted introductions of certain red algae, with examples of both filamentous species, such as *Heterosiphonia japonica* (e.g., Newton et al. 2013; Schneider 2010; Sjøtun et al. 2008), and

large foliose species, such as *Grateloupia turuturu* (Araujo et al. 2011; D'Archino et al. 2007; Janiak and Whitlach 2012; Verlaque et al. 2005). Research has examined biological attributes that may determine the invasive nature of these species and their impacts on the receiving communities.

Both the movement of aquaculture species and ballast waters have been implicated in the spread of red algae. Molecular sequencing has been a useful tool in understanding the pathways and the timing of some introductions (Andreakis et al. 2007; Yang et al. 2008). In genera such as *Grateloupia* and *Gracilaria*, where identifying species using morphological characters can be problematic, molecular techniques as well as analyses of proteins and other compounds have proved valuable in distinguishing native from nonnative species (e.g., Kollars et al. 2015; Gavio and Fredericq 2002; Kim et al. 2010; Wilcox et al. 2007).

Biogeography

Studies continue to document the flora of some of the lesser-known areas of the globe (Harper and Garbary 1997; Hommersand et al. 2009; Klochkova and Klochkova 2001; Lindstrom 2006, 2009; Nelson and Dalen 2015; Selivanova and Zhigadlova 1997a, b, c; Wulff et al. 2009). Red algae are significant in these studies because they are generally both more numerous than either green or brown algae and more phylogenetically diverse due to their ancient history and wide environmental tolerances.

During the 1990s, biogeographic studies continued to focus on the role of physiological responses (particularly to temperature) in the distribution of red algae. Much of this work focused on Arctic, Antarctic, and tropical species (e.g., Wiencke et al. 1994 Bischoff-Bäsmann and Wiencke 1996; Bischoff-Bäsmann et al. 1997; Pakker and Breeman 1996). The role of temperature and area, particularly over geological time, was central to the thermogeographic model of Adey and Steneck (2001). This model has been used to explain the predominantly Pacific origin of the Arctic and Atlantic boreal seaweed floras (Adey et al. 2008) and was validated using subtidal seaweed assemblages in the northwestern Atlantic Ocean (Adey and Hayek 2011).

Molecular data are being used to look at the distribution and phylogeography of species (e.g., Gurgel et al. 2004; Montecinos et al. 2012) although phylogeographic patterns are not always evident in these data (e.g., Vis et al. 2012). Studies have also looked at patterns of recolonization in areas affected by Pleistocene glaciations (Hu et al. 2010; Lindstrom et al. 1997; Provan et al. 2005; Yang et al. 2009). Hommersand (2007) analyzed the Australian macroalgal flora in terms of global biogeographic patterns and in relation to vicariance events in the geological history of Australasia. He identified Australasia as "a center of origin and diversity for marine algae, especially the Rhodophyta." Molecular studies provided data to support the hypothesis that many lineages of red algae originated in the southern hemisphere, or at least extant members of lineages, are found there (e.g., Bangiales – Broom et al. 2004; Gelidiales and Gigartinaceae – Hommersand et al. 1994; Nelson

et al. 2011; Gracilariales – Gurgel and Fredericq 2004). Molecular sequencing has indicated that many species actually are species complexes, and the resolution of species boundaries not evident from morphological examination is permitting a clearer understanding of their divergent ecologies (Lindstrom et al. 2011; Boo et al. 2016a, b). The evolution of a domesticated red alga, *Gracilaria chilensis*, has also been studied using a combination of phylogeographic and population genetic tools (Guillemin et al. 2014).

Ocean Acidification, Global Warming, and Red Algae

The long-term ecosystem consequences of human-mediated changes in global climate (e.g., rising temperatures, increased levels of atmospheric carbon dioxide and resulting decreases in seawater pH, changes in UV radiation, and changes in ocean circulation and upwelling patterns) are being investigated. Harley et al. (2012) reviewed how multiple stressors may affect survival, growth, and reproduction of seaweeds in a changing climate: different responses of community members to these stressors may determine persistence or extinction. For turf-forming red algae, which rely on aqueous CO_2, elevated levels should differentially favor their growth, which in turn may enhance their competitive ability (Hepburn et al. 2011). Climate change may also drive shifts in seaweed distributions at both horizontal (geographical) and vertical (elevation) scales (Brodie et al. 2014; Harley et al. 2012). These changes may be stochastic rather than gradual as shown by Harley and Paine (2009).

Roleda and Hurd (2012) summarized the responses of seaweeds to ocean acidification and examined the underlying chemistry, physiological and community-level responses, and interactions with other stressors. The contribution of calcareous algae to global carbonate production was reviewed by Basso (2012) and by McCoy and Kamenos (2015), including the response of coralline red algae to marine acidification and rising temperature. These algae showed decreased net calcification, decreased growth and reproduction, as well as reduced abundance and diversity, leading to death and an ecological shift to dominance by noncalcifying algae. In some regions, the contribution of rhodolith beds to nearshore carbonate production is very significant. Pereira-Filho et al. (2012) calculated that the summits of several seamounts are covered with extensive rhodolith beds within the tropical southwestern Atlantic. These beds are responsible for 0.3% of the world's carbonate production, and Amado-Filho et al. (2012) recorded the production from Brazilian rhodolith beds to be comparable to the world's largest $CaCO_3$ deposits, describing these beds as "major $CaCO_3$ biofactories."

Calcareous organisms can provide insight into geological processes and have the potential to be used as indicators of paleoenvironmental conditions: rhodoliths and crustose coralline algae are particularly useful in this context because of their sensitivity to ecological changes reflecting their depositional setting (e.g., Adey et al. 2015; Frantz et al. 2000, 2005; Fietzke et al. 2015; Halfar et al. 2000, 2007, 2008, 2011; Kamenos et al. 2008).

The effects of ozone depletion and UVB radiation on algae have been summarized by Bischof and Steinhoff (2012). Because there are marked species-specific responses to UVB radiation, there may be significant ecological implications in the responses at a community or ecosystem level with changes in distributional patterns (latitude and depth) as well as succession patterns, trophic interactions, and species diversity. Studies of red algae in polar regions have shown that their distribution on the shore is related to their ability of cope with UVB-mediated damage to DNA. In red algae, mycosporine-like amino acids (MAAs) have been the focus of a number of studies examining their role as UV-screening substances. In general, cellular MAA concentrations in red algae have been shown to be positively correlated with UV dose.

Commercial Importance

Red algae continue to be an important component of seaweed aquaculture, representing about 33% of the harvested weight but nearly 50% of the value, which was about US $6.4 billion in 2012 (FAO 2014). *Eucheuma* spp., including *Kappaphycus*, were responsible for more than 5 million tons of harvested seaweed, and *Gracilaria* 2.7 tons, and *Porphyra* spp., including *Pyropia*, about 1.8 million tons. Production of all species showed significant increases from the 1990s. Major production areas include Korea, Japan, China, Indonesia, and the Philippines, with minor production occurring in Malaysia and Zanzibar. Buchholz et al. (2012) summarize the methods employed in cultivation of farmed red algae including both monoculture methods and integrated multitrophic aquaculture (IMTA–Chopin et al. 2008).

The majority of red seaweeds, either collected from the wild or farmed, are used in the production of human food (Buchholz et al. 2012; Pereira et al. 2012). Direct consumption as sea vegetables is important in the Asia Pacific region, and red algal hydrocolloids are used widely in the food and other industries. New applications are being developed for marine algal products, for example, in functional foods, medicine (as anti-inflammatory, antiviral, anticancer uses), as well as in cosmetics and cosmeceuticals, and as biomaterials in skeletal replacement or regeneration, including dental applications.

Seo et al. (2010) revealed a potential use of rhizoidal filaments in *Gelidium* as raw material for papermaking. The handsheets of *Gelidium* pulp had very high Bekk smoothness and opacity, which are essential properties for high-valued printing paper, when compared to those of wood pulp.

Novel Chemistry

Galloway et al. (2012) showed that different groups (phyla, orders, families) of marine macrophytes, including red algae, have distinct essential fatty acid signatures, and the signatures of red algae were more variable than those of brown,

particularly those in the orders Corallinales, Gigartinales, and Gracilariales. Because animals cannot synthesize these molecules and rely on plant sources, essential fatty acids are useful trophic markers for tracking sources of primary production through food webs.

Some red algae are known to produce secondary metabolites, which appear to play a key defensive role against both herbivory and fouling (e.g., Blunt et al. 2011; Dworjanyn et al. 2006; Oliveira et al. 2013). Amsler et al. (2009) found that chemical defenses against herbivory are very important in structuring Antarctic macroalgal communities but not the single Arctic community examined to date, and they suggested that this may be a consequence of the different evolutionary histories of these regions. Nylund et al. (2013) examined the costs and benefits of chemical defense in *Bonnemaisonia hamifera* and found that although costly in energetic terms, there were significant fitness benefits by protecting against harmful bacterial colonization. Lignin and secondary walls were reported in red algae by Martone et al. (2009), raising questions about the biosynthetic pathways and the convergent or deeply conserved evolutionary history of these traits.

Population Biology

Many of the ecological studies of red algae have focused on aspects of their biology in relation to their life histories and reproductive modes. Although little studied, vegetative reproduction via multicellular propagules is widespread in red algae, increasing local populations, and it may be that this is the way in which some human-mediated introductions are effected (reviewed by Cecere et al. 2011).

Differential responses to environmental factors by isomorphic life history stages have intrigued researchers who have grappled with the implications of the predominance of one phase of an alternating life cycle. A number of studies have modeled the impacts of changes in fertilization success and reproductive output on the abundance of isomorphic generations (e.g., Fierst et al. 2005; Scrosati and DeWreede 1999; Thornber and Gaines 2004). Guillemin et al. (2008) explored genetic diversity in the agarophyte *Gracilaria chilensis*, a species farmed extensively in Chile. Their results suggested that the farming practices favored asexual reproduction and reduced genetic diversity in the farmed stocks. A subsequent study showed that adult tetrasporophytes grew more rapidly than gametophytes under the same conditions. Guillemin et al. (2012) hypothesized that during domestication this difference led to selection of the tetrasporophyte now dominating commercial farms.

Molecular tools are providing new insights into aspects of the ecology and population dynamics of red algae enabling examination of connectivity between populations, as well as the genetic structure of populations at small spatial scales (Andreakis et al. 2009; Donaldson et al. 2000; Engel et al. 1999, 2004; Krueger-Hadfield et al. 2011).

Characterization and Recognition

Ultrastructure

Study of the fine structure of red algae began in earnest in the mid 1960s, and progress was recounted in a series of reviews in the early 1990s. The general features of red algal ultrastructure were reviewed in detail by Pueschel (1990), and knowledge of the fine structure of cell division was summarized by Scott and Broadwater (1990) in the same volume. Broadwater et al. (1992) reviewed the cytoskeleton and spindle. The fine structure of the unicellular red algae was surveyed by Broadwater and Scott (1994).

Although red algae have a typical eukaryotic cell structure (Fig. 3a), they possess a unique combination of cellular features. Their distinctive coloration stems from their water-soluble phycobilin accessory pigments, which are visible ultrastructurally as granules, called phycobilisomes, on the surface of the unstacked photosynthetic membranes of the plastids (Fig. 3b, c). Light energy captured by phycobilisomes is transferred to chlorophyll *a*, which is a constituent of the photosynthetic membranes. The presence of phycobilisomes on single photosynthetic membranes is a feature inherited from the endosymbiotic cyanobacteria that were the progenitors of red algal plastids. Also related to the primary endosymbiotic origin of red algal plastids is the absence of periplastid endoplasmic reticulum (PER) (Fig. 3c). Bounding membranes external to the two membranes of the plastid envelope are typical of many algal lineages and are considered remnants of secondary endosymbiotic acquisition of plastids from another photosynthetic eukaryote. The red algae, like the green algae and glaucophytes, which also became photosynthetic by cyanobacterial primary endosymbiosis, lack PER.

Red algae deposit starch as an insoluble carbohydrate reserve. Floridean starch differs from green-plant starch in being free in the cytoplasm (Fig. 3b), rather than in the plastids, and in consisting solely of amylopectin, without an amylose component. Amylopectin is an α 1–4 linked glucan with abundant α 1–6 linkages, similar to animal glycogen, but in light and electron microscopy the grains of floridean starch appear similar to those of green plants and unlike the fine granules of animal glycogen.

The crucial CO_2-fixing enzyme, ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO), occurs throughout the stroma of plastids, appearing as small granules similar in size to plastid ribosomes. In many lineages of algae, dense aggregations of RuBisCO form visible structures termed pyrenoids (Fig. 3b). Only a small proportion of red algal species possess pyrenoids, but those that do are taxonomically widespread, occurring in some representatives of most of the presently recognized classes. Pyrenoids provide a variety of distinguishing features: number per plastid, location within the plastid, whether thylakoids penetrate the pyrenoid matrix (Fig. 3b), proximity to starch grains, and, in the Rhodellales, the peculiar feature of the pyrenoid is that it is deeply penetrated by an RNA-enriched projection of the nucleus (Waller and McFadden 1995).

One of the most distinctive features of the red algae is the absence of any form of flagellated motility. Centrioles, which have a microtubular substructure similar to

flagellar basal bodies and in some organisms give rise to flagella, are also absent from the red algae. The near universality of flagella or centrioles among eukaryotes and their absence in red algae was reasonably interpreted as evidence that the red algae diverged from the main line of eukaryotic evolution before the advent of eukaryotic flagellation. Molecular evidence provides a different explanation: these structures were lost by an ancestor of all living red algae. Although centrioles are absent, small, ring-shaped, or discoid structures with no structural similarity to centrioles are present at the poles of mitotic and meiotic spindles (Scott and Broadwater 1990).

Another intriguing ultrastructural feature of red algae is the variety of spatial associations that Golgi bodies form with other organelles (Broadwater and Scott 1994). The close association of the cis-face of Golgi bodies with mitochondria is decidedly the most common configuration in red algae (Fig. 3e). This arrangement contrasts strongly with the cis-Golgi being associated with the nuclear envelope, which is found only in some unicellular species. The association of Golgi with endoplasmic reticulum, the typical arrangement in eukaryotes, is also found, and cisternae of ER are often present near the mitochondrion-Golgi pairings, as well.

All but a few genera of multicellular red algae possess persistent intercellular connections, termed pit connections (Fig. 3a, d), which are the product of incomplete cytokinesis (Pueschel 1990). A structure called the pit plug is deposited within the connection, separating the cytoplasm of the two cells, but the cell membranes of the connected cells remain continuous along the sides of the pit plug. Pit connections are present in all members of the Florideophyceae and Bangiophyceae (although in the case of the latter, not in all life history stages) and some members of the Compsopogonophyceae. The proteinaceous plug core is the only universal element of pit plugs. The plug core may be separated from the adjacent cytoplasm by one or two cap layers of differing chemical composition (Pueschel and Cole 1982). In a multilayered plug cap, the cytoplasm-adjacent outer layer may be either a dome (Fig. 3a) or a thin plate (Fig. 3d), but both of these morphological types have similar cytochemical properties. A membrane, termed the cap membrane, may or may not be present, whether cap layers are present or not. The cap membrane and outer cap layer must have originated within the Florideophyceae because neither feature is found in other classes. Evidence for intercellular transport across pit plugs is largely circumstantial (Pueschel 1990), and compelling experimental proof of the function of pit plugs is not yet in hand.

The cytoskeleton is the most poorly known of typical red algal cellular constituents because it is composed mainly of microtubules and microfilaments, both of which are labile in conventional chemical fixation for electron microscopy. Freeze substitution provides a different preparative approach, and using this technique, Babuka and Pueschel (1998) demonstrated thick bundles of microfilaments and numerous cortical microtubules in axial cells of *Antithamnion* (Fig. 3f). Freeze substitution has been used extensively by Kuroiwa and associates (e.g., Miyagishima et al. 2003; Suzuki et al. 1995) to explore the role of ring-shaped structures, some actin – some not, in the division of plastids, mitochondria, and cells of *Cyanidium* and related genera. Light microscopic studies of fluorescently labeled

microfilaments and microtubules, often used in conjunction with specific cytoskeletal inhibitors, have demonstrated a role of one or both of these cytoskeletal elements in cytokinesis (Garbary and McDonald 1996), plastid movement (Russell et al. 1996), fertilization (Kim and Kim 1999; Wilson et al. 2002a, 2003), vesicle transport (Wilson et al. 2006), and the formation of pseudopodia in spores (Ackland et al. 2007). The rotation of plastids in the unicellular alga *Rhodosorus* is another striking example of subcellular movement, but the motive force is unknown (Wilson et al. 2002b). Using time-lapse microscopy, Pickett-Heaps et al. (2001) demonstrated that directional gliding motility is common and widespread in spores and among unicellular species of red algae. Mucilage secretion accompanies this movement, but the mechanism that generates directional motility remains to be elucidated.

Despite the ultrastructural characterization of the many diverse cellular inclusions found in red algal cells, we still have insufficient understanding of their functions. For example, protein bodies (Fig. 3a) are a prominent component of many vegetative cells. It has been proposed that these inclusions might serve as a seasonal nitrogen store (Pueschel 1992), but this idea has not been tested in red algae. Calcium oxalate crystals are common in higher plants and are present in some algal groups, including red algae (Pueschel 1995), but the physiological functions usually assigned to such inclusions in higher plants are unlikely to apply to the algae (Pueschel and West 2007). Progress has been made in the characterization of refractile inclusions that are associated with some kinds of specialized vegetative cells (Paul et al. 2006) and can form distinctive structures, such as the *corps en cerise* in cortical cells of *Laurencia* (Reis et al. 2013). These inclusions consist of halogenated sesquiterpenes, which can be transported to the thallus surface (Salgado et al. 2008) where they have a role in discouraging herbivory and fouling. In cortical cells of *Plocamium,* specialized vacuoles, dubbed mevalonosomes, have been demonstrated by ultrastructural enzyme localization techniques to contain enzymes of the mevalonate pathway (Paradas et al. 2015), whose products also have an antifouling function.

The greatest complexity of cell structure in red algae is found in reproductive cells and specialized vegetative cells. A large portion of the ultrastructural literature addresses the many subcellular changes associated with sporogenesis (Pueschel 1990). Although there is likely a phylogenetic signature in the fine structural details of sporogenesis, the taxonomically diverse survey work needed to explore this potential has not been pursued. The fine structure of the many kinds of specialized vegetative cells, such as rhizoids, gland cells, and hair cells, was studied early in the ultrastructural explorations of red algae (Pueschel 1990). Hair cells have continued to receive attention (Judson and Pueschel 2002; Oates and Cole 1994), as have some kinds of gland cells (Paul et al. 2006). Increased interest in the Corallinales has led to detailed examination of one of the most distinctive types of specialized cells in the red algae, the corallinalean epithallial cell. Although they are apical cells, the epithallial cells undergo terminal differentiation, senescence, and sloughing in a programmatic fashion (e.g., Pueschel et al. 1996). Intercalary meristematic cells divide to produce replacement epithallial cells. This highly unusual process is hypothesized to have an antifouling function or, alternatively, to be an adaptation

to frequent grazing. The fact that the walls of coralline algae are heavily calcified makes these epithallial dynamics all the more complex and interesting.

The discovery, description, and elucidation of phylogenetic affinities of new species of red algae are ongoing and for unicellular red algae, ultrastructural study continues to play a critical role in this endeavor. Given the simplicity of unicellular red algae and the paucity of structural features, one might expect to find molecularly distinct but structurally indistinguishable lineages. Instead, the several rhodophyte orders containing unicellular species possess a variety of distinctive ultrastructural characters. That these simple taxa should differ in their basic cellular features presumably reflects the antiquity of their evolutionary divergences. Scott et al. (2011) summarized the systematics of several of the orders containing unicellular red algae and their ultrastructural features. Compared to the diversity of cellular features of unicellular red algae, the basic features of typical vegetative florideophycean cells are relatively uniform.

Life Histories

The red algal life history is unique in having an additional third phase (i.e., a triphasic life history) in most Florideophyceae (except the Hildenbrandiales, Batrachospermales, and Palmariales). The "basic" biphasic life history is found in the early-diverged red algal lineages as well as in some florideophycean taxa. There are, however, numerous variations in the life histories of red algae.

The triphasic life history is an alternation of generations of three phases, the gametophyte, carposporophyte, and tetrasporophyte. It is generally called a "*Polysiphonia*-type" life history because it was first observed in the genus *Polysiphonia*. The triphasic life history is composed of haploid gametophytes (thalli that produce gametes), diploid carposporophytes, and diploid tetrasporophytes (thalli that typically produce four spores by meiotic division) (Fig. 4a). Gametophytes and tetrasporophytes are generally independent photosynthetic thalli, whereas the carposporophyte is diploid tissue that occurs on or within the haploid female gametophyte as a result of fertilization of the egg cell and subsequent development of the zygote.

Male gametophytic plants produce spermatia (= nonmotile sperm) from spermatangial initial cells. Female gametophytic plants produce carpogonial branches that are composed of a terminal carpogonium (= egg cell) with a trichogyne (a hair-like extension) and differing numbers of subtending cells depending on taxonomic group. Fertilization starts with attachment of spermatia to the trichogyne. Fusion of the gametic nuclei occurs in the carpogonium. The resulting diploid nucleus is either transferred, via an outgrowth from the carpogonium, to another cell (called the auxiliary cell), or remains in the carpogonium. In both cases, mitotic divisions of the diploid nucleus within a filamentous outgrowth (the gonimoblast) eventually result in the production of diploid carposporangia. Carpospores are released from the carposporangia and germinate to give rise to free-living diploid tetrasporophytes. Meiosis then occurs in specialized cells (tetrasporangial initial

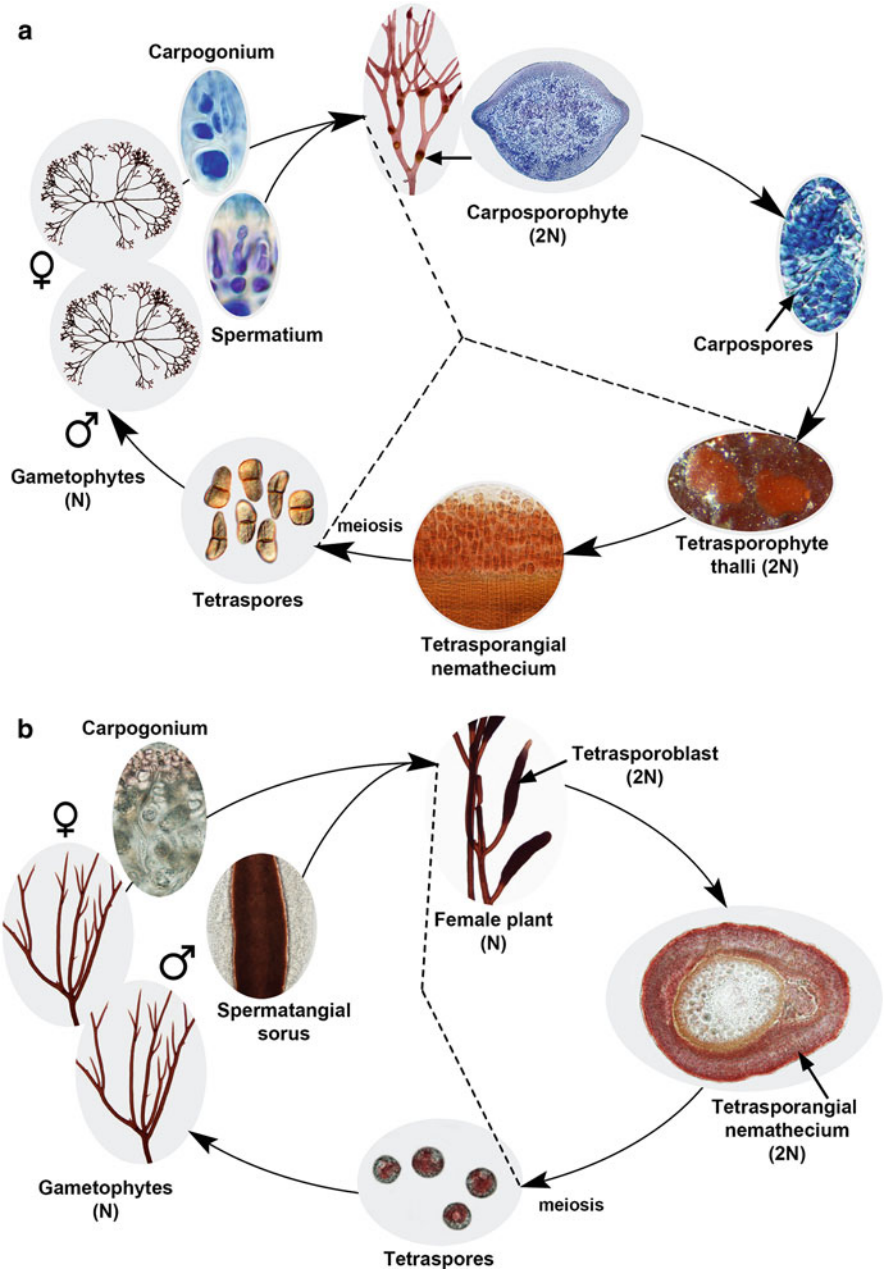

Fig. 4 (**a**, **b**) (**a**) Triphasic life history of *Ahnfeltiopsis catenata.* It shows branched gametophytes and a free-living crust attached to rock. (**b**) Tetrasporoblastic life history of *Pikea yoshizakii*. Tetrasporoblastic life history exhibits a truncated life history in which fertilized females produce tetraspores in nemathecia rather than carpospores in cystocarps

cells) in the tetrasporophyte, and the resulting tetrads of haploid spores are shed from the thallus. Individual spores germinate to give rise to gametophytes, completing the cycle.

The typical *Polysiphonia*-type life history includes isomorphic gametophytes and tetrasporophytes; however, in other red algae heteromorphic generations, in which the tetrasporophyte is morphologically distinct from the gametophyte, also occur. For instance, some species of Gigartinales have a heteromorphic life history in which sporophytes are crustose (see Fig. 4a). Heteromorphic generations also occur in the Nemaliales and Bonnemaisoniales, in which the tetrasporophyte is a minute branched filament. In some species of the Acrochaetiales, the tetrasporophyte is the more conspicuous phase, while the gametophyte is diminutive. The Palmariales are characterized by a life history in which male gametophytes and tetrasporophytes are the conspicuous macrophytes, and female gametophytes are microscopic and after fertilization are overgrown by the tetrasporophytes without benefit of a carposporophyte generation.

Several species of Gigartinales produce tetrasporoblasts and exhibit a truncated life history (Fig. 4b) in which fertilized females produce tetrasporangia in nemathecia rather than carposporangia in cystocarps, bypassing the free-living tetrasporophytic phase, for example, *Pikea yoshizakii* (Boo et al. 2016a). The tetrasporoblastic filaments are homologous to gonimoblast filaments, originating from auxiliary cells following diploid nucleus transfer, and, like the carposporophyte, are also borne on the female gametophyte. Tetrasporangia undergo meiosis, releasing tetraspores that germinate to produce gametophytes.

The biphasic life history is an alternation of generations of two phases: the gametophyte and sporophyte. Among reported sexual species in the Bangiales (Bangiophyceae) (Hawkes 1978), small colorless spermatia (previously referred to as β-spores) are produced (from 16 to 256 per parental cell) which, when released, may fuse with larger pigmented cells. Although formerly referred to as carpogonia, Nelson et al. (1999) concluded that the use of the terms "carpogonium" and "carpospore" is not appropriate for members of the Bangiophyceae, given the significant differences in the ontogeny of the female reproductive structures. The products resulting from this union are termed zygotospores (formerly known as α-spores) and most frequently germinate into the alternate conchocelis phase of the life cycle. The conchocelis phase in the Bangiales regenerates the gametophytic blades or filaments through conchospores (spores produced by the conchocelis phase). Although some species expressing this alternation of generations are reported to be sexual, others apparently are not. In *Pyropia yezoensis*, meiosis has been reported to occur upon germination of the conchospores, resulting in gametophytic thalli that are genetic chimeras (Ma and Miura 1984).

Asexual reproduction occurs in many red algal classes. It can occur through vegetative means (including simple cell division, fragmentation, and production of propagules) and through the production of spores. The term "archeospore" is applied when there is a single-cell product, and "monospore" where single spores are produced by an unequal cell division (Magne 1991). In the Bangiales, archeospores

are produced from conversion of vegetative cells in both the gametophytic and sporophytic phases and are an important means of reproduction. Endosporangia are produced in some members of the Bangiales. Some florideophycean red algae have apomictic (lacking meiosis) and apogamic (no fusion of gametes) life histories.

Evolutionary History

The fossil record of the red algae is meager (except for the Corallinales), due to the delicate or gelatinous nature of the vast majority of taxa. Even when thalli are preserved, it is rare that the minute reproductive structures on which the infraordinal classification is based also remain intact. Despite a growing range of Proterozoic fossils, few can be unambiguously assigned to an extant taxon.

There are, however, two exceptional cases of taxonomically resolved Proterozoic red algae. The first is *Bangiomorpha pubescens* from the Hunting Formation, Somerset Island, Arctic Canada (Butterfield 2000). This well-preserved modern *Bangia*-like fossil is generally considered as the oldest taxonomically known eukaryotic fossil (Fig. 5a–e). Large populations, with material ranging from a single cell to reproductively mature filaments, were embedded in a shallow-water chert/carbonate dated at 1174–1222 million years ago (Ma) (see Knoll 2011 for a review of the age constraints). Within this population, up to 2 mm long, unbranched multicellular filaments of uniseriate, multiseriate, and both uni/multiseriate habits (Fig. 5b, c) were found in clusters of up to 15 individuals (Fig. 5a) (see detail, Butterfield 2000). Two cells were usually paired in a uniseriate filament, suggesting transverse intercalary cell division. In multiseriate filaments, four to eight radially arranged wedge-shaped cells were usually identified in transverse cross-section (Fig. 5d). These transverse and radial intercalary cell division patterns are commonly found in species of modern filamentous Bangiales (e.g., Fig. 1h) and are conspicuously distinct from the apical cell division in other algae and filamentous cyanobacteria. Furthermore, *Bangiomorpha* contains spore-like, spheroidal cells within multiseriate filaments (Fig. 5e), indicating development of sexual reproduction in the ancestral red alga.

The second taxonomically resolved fossil red alga consists of anatomically preserved florideophyte fossils from the phosphorites of the late Neoproterozoic [570 (633–551) Ma] Doushantuo Formation at Weng'an, southern China (Condon et al. 2005; Xiao et al. 1998, 2004). Fossils in Doushantuo phosphorites preserved diverse three-dimensional cellular structures comprising cyanobacteria, acritarchs, animal embryos, and multicellular algae. These fossils provide key paleontological evidence about the early radiation of multicellular eukaryotes (Xiao et al. 2014). In the algal fossils, pseudoparenchymatous thalli exhibit specialized tissues including cell growth patterns (e.g., cortex-medulla differentiation, secondary pit connection between cells) and distinct reproductive structures (e.g., spermatangia, tetraspores and octaspores, and carposporangia, see Fig. 5f–i) that closely resemble key characters of Paleozoic relatives (Brooke and Riding 1998) and modern corallines (Xiao et al. 1998, 2004, 2014). Based on anatomical characters mapped on a molecular

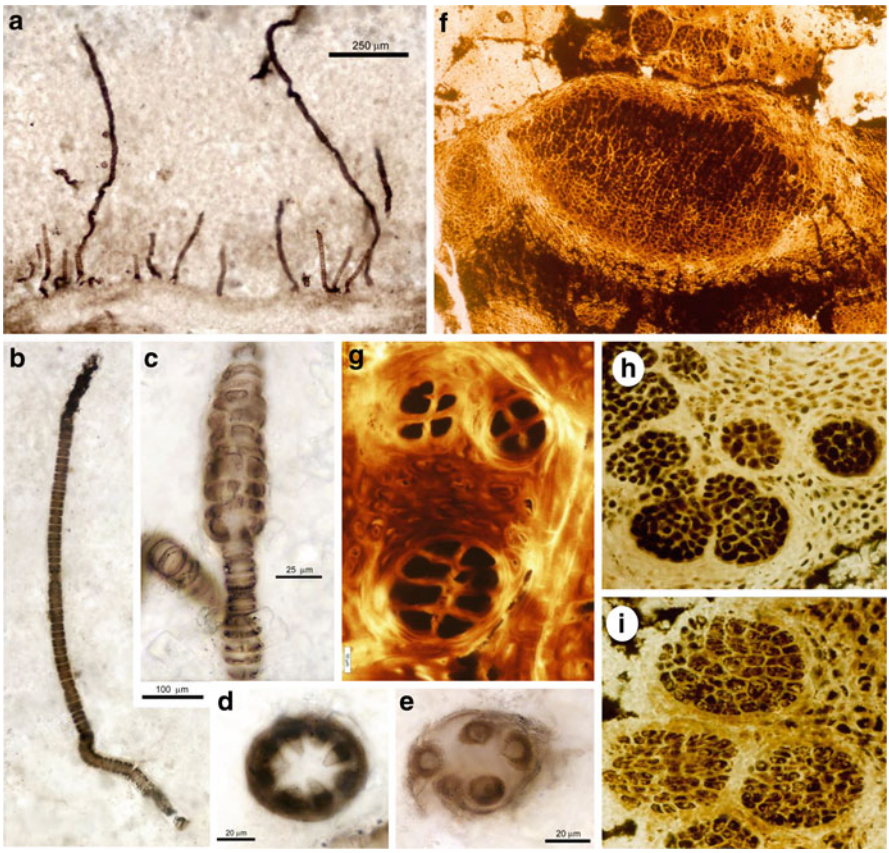

Fig. 5 Proterozoic red algal fossils. (**a**–**e**) *Bangiomorpha pubescens* fossils from the ca. 1200 million-year-old Hunting Formation, Somerset Island, arctic Canada (Courtesy of N. J. Butterfield). (**a**) Population of *Bangiomorpha* that clustered with up to 15 individuals. (**b**) Two paired cells reflecting transverse intercalary cell division. (**c**) Mature thallus showing both uniseriate and multiseriate portions of a filament. (**d**) Transverse cross-section of a multiseriate filament showing eight radially arranged wedge-shaped cells. (**e**) Spore-like spheroidal cells within multiseriate filaments from transverse cross-section. (**f**–**i**) Coralline fossils from the late Neoproterozoic 570 Ma Doushantuo Formation at Weng'an, southern China (Courtesy of S. Xiao). (**f**) A spermatangia-like reproductive structure with filaments. (**g**) Tetraspores and octaspores embedded in algal thallus showing possible tetrasporangium with subtending stalk cells. (**h**, **i**) Carposporangia

phylogeny, Xiao et al. (2004) concluded that these fossils are stem groups that may have diversified into the crown group of Corallinophycidae in the Mesozoic Era. In addition, some Doushantuo algal fossils are related to the zygotosporangia of modern thallose Bangiales (Xiao et al. 1998, 2014), indicating diversification of the Bangiophyceae as well as the Florideophyceae during the Neoproterozoic Era or earlier.

More recently, crown groups of coralline fossils were reported from Mesozoic and Cenozoic sedimentary rocks (Aguirre et al. 2000, 2010). These species have been placed within the Sporolithales (136–130 Ma), Hapalidiales (115–112 Ma), and Lithophylloideae (65.5–61.7 Ma), providing additional time constraints on coralline and florideophyte evolution.

Divergence time estimation using relaxed molecular clocks usually provides an overview of the evolutionary timeline, despite the large degree of uncertainty associated with fossil constraints. To estimate a more reliable timeline, three fundamental requirements are critical: (i) a well-supported accurate phylogeny representing diverse lineages, (ii) reliable fossil calibrations, and (iii) robust molecular clock methods (Soltis et al. 2002). Several divergence time estimations indicated a Mesoproterozoic origin of red algae. For example, based on a phylogeny using six genes from 46 taxa, Yoon et al. (2004) estimated 1,474 Ma for the origin of red algae, after the primary endosymbiosis between a heterotrophic protist and a cyanobacterium sometime before 1,558 Ma. Parfrey et al. (2011) suggested approximately 1,500 Ma for the origin of red algae based on a 15-gene dataset from 88 eukaryotic taxa. Although they used multigene data from diverse eukaryotic phyla, both studies included only limited florideophycean taxa; therefore, they were not able to suggest a detailed timeline for the Florideophyceae, which includes ca. 95% of red algal species.

A comprehensive molecular clock analysis was recently published with special focus on the Florideophyceae (Yang et al. 2016) (see Fig. 6). This analysis was based on a robust seven-gene phylogeny including 91 red algal taxa representing all seven classes and 34 orders (i.e., 27 of 29 florideophycean and seven nonflorideophycean orders). Seven reliable fossils were used as constraint points: *Bangiomorpha,* Doushantuo and Mesozoic coralline fossils, and four land plants (i.e., 471–480 Ma for the liverwort and vascular plant split; 410–422 Ma for the fern and seed plant split; 313–351 Ma for the gymnosperm and angiosperm split, and 138–162 Ma for the monocot-eudicot split, see Magallón et al. 2013). This study suggests that the Florideophyceae diverged approximately 943 Ma, followed by the emergence of the five subclasses: Hildenbrandiophycidae (781 Ma), Nemaliophycidae (661 Ma), Corallinophycidae (579 Ma), and the split of Ahnfeltiophycidae and Rhodymeniophycidae (508 Ma).

This red algal evolutionary timeline was used to interpret the emergence of key morphological innovations (Fig. 6). The triphasic life cycle is the most distinctive feature of red algae, ancestrally present in nonhildenbrandiophycidean Florideophyceae (except the Palmariales and Batrachospermales). Because it is not possible to rule out secondary loss of the carposporophyte phase in the Hildenbrandiophycidae, Yang et al. (2016) suggested that the triphasic life cycle was enabled by the evolution of the carposporophyte sometime between the divergence of ancestral Florideophyceae (943 Ma) and the divergence of Nemaliophycidae (661 Ma). After the development of the carposporophyte (i.e., gonimoblast development on the female gametophyte), two distinct innovations evolved in the postfertilization development in diploid gonimoblast filaments. The first is found in the Corallinophycidae (except Rhodogorgonales), Ahnfeltiophycidae,

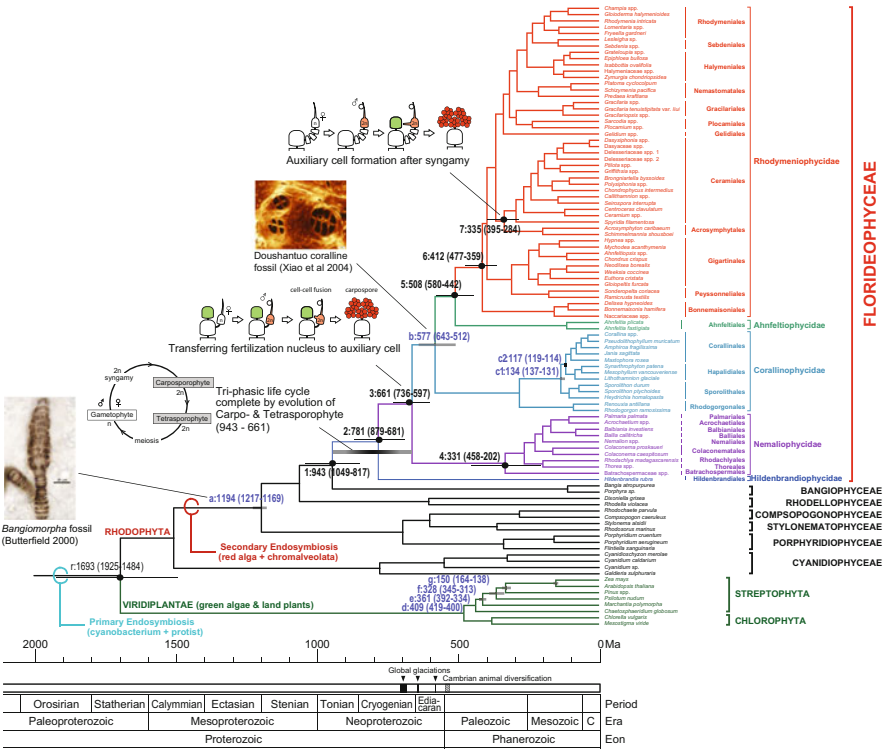

Fig. 6 Divergence time and evolution of the red algae. Primary and secondary endosymbiosis events are indicated with *cyan* and *red lines*, respectively. The three key evolutionary events are indicated with diagrams at the estimated evolutionary timeline. Triphasic life cycle, postfertilization "cell-to-cell fusion" mechanisms between carpogonium (fertilized egg) and an auxiliary cell, and the formation of an auxiliary cell after fertilization (syngamy) are indicated (Modified from Yang et al. 2016)

and Rhodymeniophycidae (661 Ma), where the zygotic nucleus and derivatives in the carpogonium move to an auxiliary cell by "cell-to-cell fusion" mechanisms followed by carposporophyte development, release of carpospores, and eventual sporic meiosis on the tetrasporophyte. The second innovation is only found in the Ceramiales (335 Ma) of the Rhodymeniophycidae, where an auxiliary cell is formed after fertilization (syngamy) followed by movement of the zygotic nucleus to the auxiliary cell. In addition within the Florideophyceae, especially in the Rhodymeniophycidae, there are numerous types of pre- and postfertilization cell-to-cell fusion mechanisms that have been used for ordinal diagnostic characters in florideophyte classification schemes (i.e., Hommersand and Fredericq 1990; Krayesky et al. 2009; Withall and Saunders 2006). The great diversity in pre- and postfertilization strategies in the Rhodymeniophycidae has resulted in the most successful subclass that comprises more than 70% of species richness in the entire Rhodophyta.

Evolutionary Relationships

The monophyly of Rhodophyta, Viridiplantae (green algae and land plants), and Glaucophyta, collectively referred to as the Archaeplastida (Adl et al. 2005), is supported by diverse molecular data (Chan et al. 2011; Hackett et al. 2007; Jackson and Reyes-Prieto 2014; Moreira et al. 2000; Rodriguez-Ezpeleta et al. 2005; Price et al. 2012; Reyes-Prieto and Bhattacharya 2007; Yoon et al. 2002b; Yoon et al. 2004), although a paraphyletic origin of these lineages cannot yet be ruled out (Parfrey et al. 2010; Yabuki et al. 2014; Yoon et al. 2008). However, because of the consistency between plastid and nuclear gene phylogenies, the single primary endosymbiosis hypothesis is widely accepted. This theory posits the origin of the plastid by acquisition of a cyanobacterium in the common ancestor of Archaeplastida >1,500 million years ago (see Fig. 6), followed by divergence of the greens, glaucophytes, and red algal lineages. These three major photosynthetic lineages share two-membrane-bounded plastids. Internal relationships (i.e., red-green monophyly vs. green-glaucophyte monophyly), however, are not fully resolved.

One of the most important evolutionary contributions of the red algae has been as a plastid donor through secondary endosymbiosis to the chlorophyll-*c* containing eukaryotic groups including the SAR group (Stramenopiles; Alveolates – dinoflagellates, apicomplexa, and ciliates; Rhizaria), cryptophytes, and haptophytes (Bhattacharya et al. 2004; Hackett et al. 2007; Yoon et al. 2002a, b) (see, e.g., ▶ Ciliophora ▶ Dinoflagellata ▶ Cryptophyta (Cryptomonads) and ▶ Haptophyta). Although the monophyly of these groups is still debated (Burki et al. 2016; Parfrey et al. 2011), plastid monophyly of the noncyanidiophycean red algal and chlorophyll-*c* containing lineages is strongly supported (Yoon et al. 2002a, b, 2004). Photosynthetic groups from these lineages have plastids bounded by three (i.e., peridinin-containing dinoflagellaes) or four (stramenopiles, cryptophytes, and haptophytes) membranes. Based on molecular clock analysis, Yoon et al. (2004) suggested 1,274 Ma as the date for the red algal secondary endosymbiosis (see Fig. 6).

Phylogenetic relationships between all major groups of Rhodophyta have been studied by Yoon et al. (2006), Le Gall and Saunders (2007), Verbruggen et al. (2010), and Yang et al. (2015). Based on a broadly sampled multigene phylogeny, with a focus on nonflorideophycean red algae, Yoon et al. (2006) identified several well-supported lineages, with the earliest diverged being the Cyanidiophyceae, and a strong monophyly of the Bangiophyceae and Florideophyceae. They proposed the seven-class system, although internal relationships among the four classes Compsopogonophyceae, Porphyridiophyceae, Rhodellophyceae, and Stylonematophyceae remain unresolved. In contrast, Le Gall and Saunders (2007) focused on the internal relationships of the Florideophyceae using combined EF2, SSU, and LSU rDNA sequences. They resolved five subclasses and established the subclass Corallinophycidae. Recently, Yang et al. (2015) largely resolved the internal relationships of the 12 orders of the Rhodymeniophycidae with a strong to moderately supported phylogeny based on

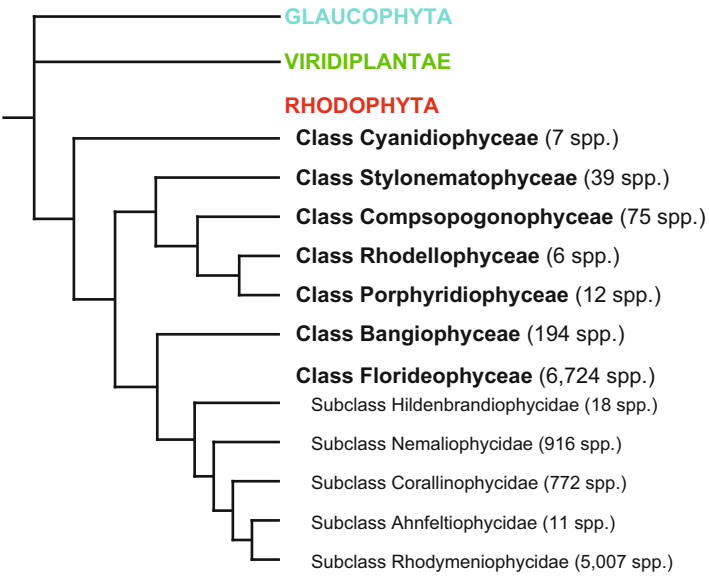

Fig. 7 Schematic phylogenetic relationships of the red algal classes and subclass based on Le Gall and Saunders (2007), Yoon et al. (2006), and Lee et al. (unpublished)

mitochondrial genome data. A more recent analysis using red algal plastid genome data from 45 species in all seven classes, 5 Florideophyceae subclasses, and 12 Rhodymeniophycidae orders resolved the four classes (i.e., Compsopogonophyceae, Porphyridiophyceae, Rhodellophyceae, and Stylonematophyceae) that diverged early (Lee et al., unpublished). After the divergence of the Cyanidiophyceae, the Stylonematophyceae diverged next, followed by the Compsopogonophyceae, and the Rhodellophyceae + Porphyridiophyceae clade (Fig. 7). Results from mitochondrial (e.g., Yang et al. 2016) and plastid genome analyses (Lee et al., unpublished) strongly suggest that organellar genome data can provide sufficient phylogenetic information to resolve most phylogenetic relationships in the Rhodophyta.

Genome Reduction in Rhodophyta

Although the red and green algal lineages putatively share a sister group relationship in the Archaeplastida (as described above), each has followed a vastly different path since their split. Genomes in the green lineage show dramatic expansion of gene families associated with the birth of land plants. In contrast, red algae likely have survived an ancient phase of extremophily (i.e., life in extreme environments such as volcanic hot springs) that resulted in extreme genome reduction (GR). This so-called hot start was followed by diversification into normal habitats and the origin of

multicellularity, without massive gene gains (Bhattacharya et al. 2013; Collén et al. 2013; Collén 2015; Nakamura et al. 2013).

GR is a hallmark of symbionts, intracellular pathogens, and parasites (Keeling and Slamovits 2005; McCutcheon and Moran 2012). The highly simplified gene inventory and reduced functions in these taxa precipitates an obligate association with a host (Keeling and Slamovits 2005; Moran 2002). In free-living organisms, GR is associated with reduced metabolic flexibility and life in specialized niches such as in oligotrophic [e.g., *Prochlorococcus* (Dufresne et al. 2003) and *Ostreococcus* (Derelle et al. 2006)] and extremophilic [e.g., Cyanidiophytina red algae (Qiu et al. 2013), *Galdieria sulphuraria* (Schönknecht et al. 2013) and *Cyanidioschyzon merolae* (Matsuzaki et al. 2004)] environments that are relatively invariant over time. Given the narrowing of genetic potential, GR presumably precludes subsequent taxonomic and ecological diversification. Intriguingly, red algae appear to provide a counter-example to this perspective. The ability of this lineage to diversify and adapt to novel mesophilic habitats, despite a highly reduced gene inventory, ultimately led to the rise of a remarkably successful branch of life that shows immense morphological diversity and complex life cycles (Saunders and Hommersand 2004). The available data suggest that GR in red algae provides a model for deciphering the lower limits of gene diversity in free-living taxa and potentially offers insights into how novel solutions evolved for promoting the diversity of Rhodophyta.

Evidence for Genome Reduction in the Red Algal Common Ancestor

Available complete genome data suggest that red algae encode only a modest gene inventory when compared to Viridiplantae, with extant species typically containing fewer than 10,000 genes, e.g., in the mesophilic unicellular red alga *Porphyridium purpureum* (Bhattacharya et al. 2013) and in the extremophilic unicellular red algae *C. merolae* (Matsuzaki et al. 2004) and *G. sulphuraria* (Schönknecht et al. 2013). Even red seaweeds such as *Chondrus crispus* (Collén et al. 2013) and *Pyropia yezoensis* (Nakamura et al. 2013), which are complex multicellular lineages and have sophisticated life cycles, contain a gene inventory comparable to their unicellular relatives (i.e., 9,606 and 10,327 putative genes, respectively). An analysis of gene family evolution under a phylogenetic framework that incorporated all available genomic data (e.g., novel transcriptomes from the Marine Microbial Eukaryote Transcriptome Sequencing Project; Keeling et al. 2014) is summarized in Fig. 8a. These results correlate the estimated number of core gene families and thallus morphology in each lineage and provide evidence for limited gene expansion in the derived, mesophilic lineages (Qiu et al. 2015). Fig. 8b shows the results of the analysis of orthologous gene families [using OrthoMCL (Li 2003)], based on Dollo parsimony (Farris 1977), and the estimation of gene family gains and losses under the same parameters as described in Qiu et al. (2015).

The results shown in Fig. 8 suggest that the net loss of genes was most severe in the stem lineage of red algae and in the common ancestor of the Cyanidiophytina.

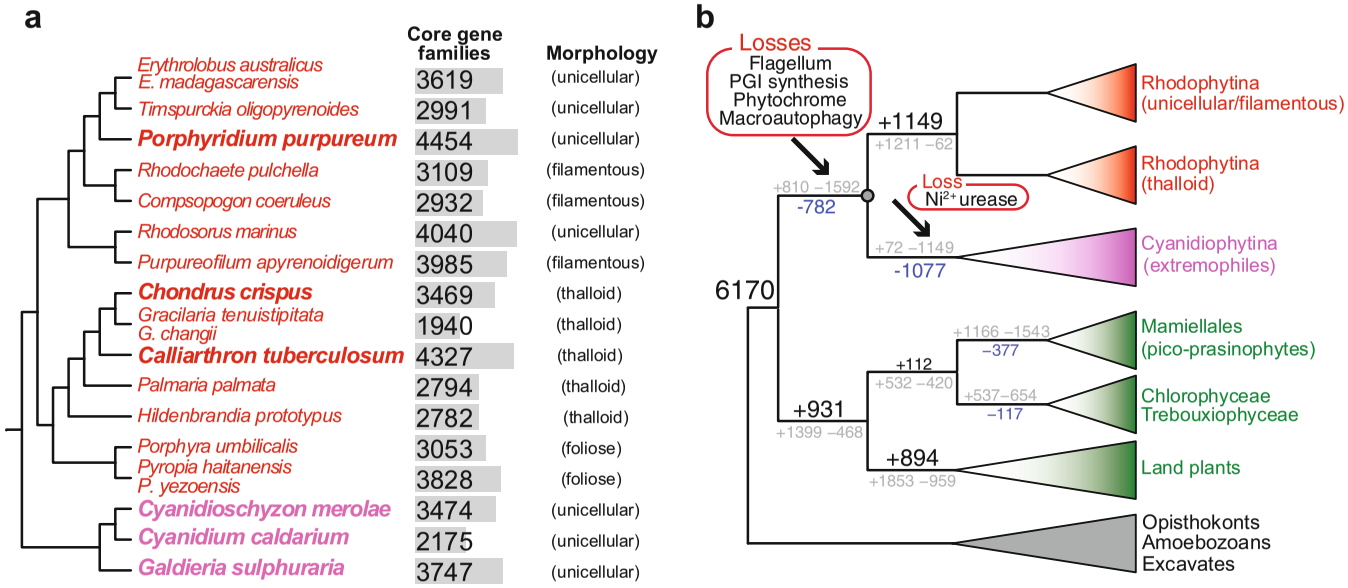

Fig. 8 (**a**, **b**) Genome-wide analysis of red algae. (**a**) The number of core gene families in red algal taxa for which genome or transcriptome data are available. A core gene family is defined as those present in the Cyanidiophytina or Rhodophytina ancestor, or earlier (for details, see Qiu et al. 2015). Using the number of core gene families as a measure, *Rhodosorus marinus* shows a coverage (e.g., 91%) that is comparable to that of *Porphyridium purpureum*, which has a completely sequenced genome. The approximate sequence coverage in other species is expressed likewise. Sequences from *Gracilaria tenuistipitata* and *Gracilaria changii* were pooled. The same was the case for *Pyropia yezoensis* and *Pyropia haitanensis* sequences. Taxa in boldface have complete genome sequences available. The tree topology of the mesophilic lineages is tentative and has no impact on the estimation of core gene families that have more ancestral origins. (**b**) Estimate of the number of gene family gains and losses (*gray* number at each branch) in *red* algae and Viridiplantae. Net gene family gains or losses are shown in *black* and *blue* text, respectively. Net gene family changes, when >600, are shown as proportionately sized numbers. The *arrows* indicate the two major phases of net gene loss in *red* algae. The major functional losses associated with each phase of genome reduction are also shown (for details, see Qiu et al. 2015)

Remarkably, about one-quarter (1,592/6,170, or 26%) of conserved algal "core" genes were lost in the red algal common ancestor. This is in contrast to the pronounced net gene gains in the Viridiplantae stem lineage (+931) and in the lineage leading to land plants (+894; Fig. 8b). Although we expect these numbers to change as more genomes are added to the analysis, the most compelling comparison is between the stem lineages of red and green algae. The Rhodophyta ancestor would have to gain ca. 1,700 genes on this branch to achieve the expansion found in Viridiplantae. The large gene gains at the root of mesophilic red algae (+1,149) needs to be interpreted with caution because some of these genome assemblies are highly fragmented (i.e., leading to over-estimation of gene numbers) and there are contamination issues associated with the EST data included in the analysis (Qiu et al. 2015).

Functions Lost in the Red Algal Ancestor

The impact of GR on red algae is most obviously manifested in the absence of flagella and basal bodies. Other notable losses in the red algal stem lineage include light-sensing phytochromes, glycosylphosphatidylinositol (GPI)-anchor biosynthesis, macroautophagy pathways (Qiu et al. 2015), and then subsequent loss of the nickel-dependent urease pathway in the Cyanidiophytina common ancestor (Qiu et al. 2013) (Fig. 8b). Interestingly, flagella and the GPI-anchoring function are preserved in parasites such as *Trypanosoma* species and *Giardia lamblia* (Das et al. 1994) that also underwent drastic GR. This observation suggests a differential impact of GR in cells adapted to different lifestyles, i.e., intracellular pathogens versus free-living cells. Whereas flagella loss is relatively common in eukaryotes, GPI anchoring is a highly conserved function and plays critical, perhaps indispensible roles in a wide variety of organisms (Kawagoe et al. 1996; Lillico et al. 2003; Takeda and Kinoshita 1995), as is the case for macroautophagy (Mizushima and Levine 2010). It is currently unknown how red algae cope with the loss of these conserved functions.

Classification

Here we follow the seven-class system (Yoon et al. 2006) of the Rhodophyta (see Table 1 and Fig. 7) and describe the basic diagnostic characters and classification status for each class based on the previous review (Yoon et al. 2010).

Cyanidiophyceae is a group of asexual, unicellular red algae that thrive in acidic (pH 0–4) and high-temperature (25–55 °C) conditions around hot springs and/or acidic sulfur fumes (Pinto et al. 2003). This is the first group to diverge, and members contain the ultrastructural character of a Golgi-ER association. The class Cyanidiophyceae contains one order Cyanidiales, two families Cyanidiaceae and Galdieriaceae, and three genera *Cyanidium*, *Cyanidioschyzon*, and *Galdieria*, based on morphological characters. Molecular phylogenetic studies, however, have revealed great hidden diversity in this lineage (Gross et al. 2001; Pinto et al. 2003;

Table 1 Current taxonomic system of the red algae according to Yoon et al. (2006, 2010) and Le Gall and Saunders (2007)

Kingdom Plantae Haeckel
Phylum Rhodophyta Wettstein
Subphylum Cyanidiophytina Yoon, Müller, Sheath, Ott, et Bhattacharya
Class Cyanidiophyceae Merola, Castaldo, De Luca, Gambardella, Musacchio, et Taddei
Order Cyanidiales Christensen
Subphylum Rhodophytina Yoon, Müller, Sheath, Ott, et Bhattacharya
Class Bangiophyceae Wettstein
Order Bangiales Nägeli
Class Compsopogonophyceae Saunders et Hommersand
Order Compsopogonales Schmitz in Engler et Prantl
Order Erythropeltidales Garbary, Hansen, et Scagel
Order Rhodochaetales Bessey
Class Florideophyceae Cronquist
Subclass Hildenbrandiophycidae Saunders et Hommersand
Order Hildenbrandiales Pueschel et Cole
Subclass Nemaliophycidae Christensen
Order Acrochaetiales Feldmann
Order Balbianiales Sheath et Müller
Order Balliales Choi, Kraft, et Saunders
Order Batrachospermales Pueschel et Cole
Order Colaconematales Harper et Saunders
Order Entwisleiales Scott, Saunders, et Kraft
Order Nemaliales Schmitz
Order Palmariales Guiry et Irvine
Order Rhodachlyales Saunders, Clayden, Scott, West, Karsten, et West
Order Thoreales Müller, Sherwood, Pueschel, Gutell, et Sheath
Subclass Corallinophycidae Le Gall et Saunders
Order Corallinales Silva et Johansen
Order Hapalidiales Nelson, Sutherland, Farr, et Yoon
Order Rhodogorgonales Fredericq, Norris, et Pueschel
Order Sporolithales Le Gall, Payri, Bittner, et Saunders
Subclass Ahnfeltiophycidae Saunders et Hommersand
Order Ahnfeltiales Maggs et Pueschel
Order Pihiellales Huisman, Sherwood, et Abbott
Subclass Rhodymeniophycidae Saunders et Hommersand
Order Acrosymphytales Withall et Saunders
Order Bonnemaisoniales Feldmann et Feldm.-Maz.
Order Ceramiales Oltmanns
Order Gelidiales Kylin
Order Gigartinales Schmitz
Order Gracilariales Fredericq et Hommersand
Order Halymeniales Saunders et Kraft

(*continued*)

Table 1 (continued)

Order Nemastomatales Kylin
Order Peyssonneliales Krayesky, Fredericq, et Norris
Order Plocamiales Saunders et Kraft
Order Rhodymeniales Schmitz
Order Sebdeniales Withall et Saunders
Class Porphyridiophyceae Yoon, Müller, Sheath, Ott, et Bhattacharya
Order Porphyridiales Kylin ex Skuja
Class Rhodellophyceae Cavalier-Smith
Order Dixoniellales Yokoyama, Scott, Zuccarello, Kajikawa, Hara, et West
Order Glaucosphaerales Yang, Scott, Yoon, et West
Order Rhodellales Yoon, Müller, Sheath, Ott, et Bhattacharya
Class Stylonematophyceae Yoon, Müller, Sheath, Ott, et Bhattacharya
Order Rufusiales Zuccarello et West
Order Stylonematales Drew

Yoon et al. 2002a, b) from comprehensive sampling in Italy (Ciniglia et al. 2004), Yellowstone National Park, Japan, and New Zealand (Skorupa et al. 2013; Toplin et al. 2008), Iceland (Ciniglia et al. 2014), and Taiwan (Hsieh et al. 2015). As Yoon et al. (2010) suggested, taxonomic revision in the Cyanidiophyceae is required at the order, family, and genus levels.

Compsopogonophyceae is a group of multicellular but simple filamentous, blade, and tubular red algae. It is characterized by having a Golgi-ER association and floridoside as the low molecular weight carbohydrate (LMWC) (Broadwater and Scott 1994; Karsten et al. 2003). *Rhodochaete* and *Compsopogon* contain pit plugs with a simple plug core without a cap or membrane (Scott et al. 1988). The class Compsopogonophyceae is classified into three orders: one freshwater order, Compsopogonales, with two families Boldiaceae and Compsopogonaceae, and two marine orders, Erythropeltidales and Rhodochaetales, with 14 genera. The presence of sex was reported from two sister taxa *Erythrotrichia* and *Rhodochaete* (Hawkes 1988; Magne 1960, 1990), and packets of spores may be indicative of sexual reproduction in *Pyrophyllon* and *Chlidophyllon* (Nelson et al. 2003).

Porphyridiophyceae is a group of unicellular red algae that contain a single branched or stellate plastid without a peripheral thylakoid, a Golgi association with ER/mitochondria (Scott et al. 1992), and floridoside as a LMWC (Karsten et al. 2003). This class has a single order Porphyridiales, one family Porphyridiaceae, and four unicellular genera *Erythrolobus*, *Flintiella*, *Porphyridium*, and *Timspurckia*.

Rhodellophyceae is a class that includes the unicellular red algae *Corynoplastis*, *Dixoniella, Glaucosphaera*, *Neorhodella*, and *Rhodella* and contains three orders Dixoniellales, Glaucosphaerales, and Rhodellales (Scott et al. 2011; Yokoyama et al. 2009; Yoon et al. 2006). Dixoniellales and Rhodellales contain mannitol as the LMWC. The LMWC for the Glaucosphaerales is unknown (Karsten et al. 2003). *Dixoniella*, *Glaucosphaera*, and *Neorhodella* have a Golgi-nuclear association,

differing from *Corynoplastis* and *Rhodella*, which have a Golgi-ER association (Scott et al. 1992, 2011).

Stylonematophyceae comprises diverse morphological forms of unicellular, pseudofilamentous, and filamentous taxa with thick mucilaginous walls and cells lacking pit plugs. A Golgi-ER association and digeneaside and sorbitol as LMWCs are diagnostic characters for this group (Broadwater and Scott 1994; Karsten et al. 2003) although digeneaside is missing in *Chroodactylon*, and dulcitol is present in *Rhodospora*. A single stellate plastid with a pyrenoid is found in most taxa. This class has two orders, Stylonematales and Rufusiales, two families, Stylonemataceae and Rufusiaceae, and 14 genera (*Bangiopsis, Chroodactylon, Chroothece, Colacodictyon, Empselium, Goniotrichopsis, Kylinella, Neevea, Purpureofilum, Rhodaphanes, Rhodosorus, Rhodospora*, *Rufusia,* and *Stylonema*) that are all reported from marine habitats.

Bangiophyceae has either simple unbranched filaments or leaf-shaped foliose thalli, and most species live in the marine environment. The Bangiales includes the most highly valued seaweed aquaculture crops in the world (i.e., *Pyropia*, previously known as *Porphyra*). A biphasic life cycle is common in this group, with a macroscopic gametophyte alternating with a microscopic conchocelis phase. The conchocelis phase in the Bangiales has pit plugs with a single cap layer but no cap membrane (Pueschel and Cole 1982). The class Bangiophyceae includes one order Bangiales, one family Bangiaceae, and 12 currently recognized genera with ca. 130 species. The real diversity, however, is likely underestimated, and further genera need to be formally described (Sutherland et al. 2011).

A sister group relationship of the Bangiophyceae and Florideophyceae has been suggested based on numerous morphological and molecular data including features of the reproductive cells, Golgi association with ER/mitochondria, the presence of pit connections, and the presence of group I introns (Gabrielson et al. 1985; Gabrielson et al. 1990; Freshwater et al. 1994; Ragan et al. 1994; Oliveira and Bhattacharya 2000; Müller et al. 2001; Yoon et al. 2002b; Yoon et al. 2004; Yoon et al. 2006).

Florideophyceae includes around 6,700 species that are mostly macroscopic; they are the most morphologically and genetically diverse of all red algal classes. The triphasic life cycle comprising a carposporophyte, tetrasporophyte, and a gametophyte phase is common in this group. Five subclasses are recognized (Hildenbrandiophycidae, Nemaliophycidae, Corallinophycidae, Ahnfeltiophycidae, and Rhodymeniophycidae) with 29 orders that are distinguished by molecular data, ultrastructural features (i.e., pit plug connection between neighboring cells including number of cap layers and membranes), and reproductive development (i.e., pre- and postfertilization processes) (see review by Saunders and Hommersand 2004).

The subclass **Hildenbrandiophycidae** contains a single order the Hildenbrandiales, with two genera *Hildenbrandia* and *Apophlaea*, characterized by pit plugs with a single cap layer covered by a membrane (Pueschel and Cole 1982). Although zonately and irregularly divided tetrasporangia have been reported, there are no reports of recognizable gametophytic reproductive structures (carpogonia or spermatangia) or a sexual life history. The **Nemaliophycidae** is characterized

by the presence of pit plugs with two cap layers. Ten orders are recognized: Acrochaetiales, Balbianiales, Balliales, Batrachospermales, Colaconematales, Entwisleiales, Nemaliales, Palmariales, Rhodachlyales, and Thoreales. The **Corallinophycidae** is characterized by pit plugs with a domed outer cap layer and calcified thalli. It contains four orders: the Corallinales, Hapalidiales, Rhodogorgonales, and Sporolithales. The **Ahnfeltiophycidae** includes two orders the Ahnfeltiales and Pihiellales that are characterized by having naked pit plugs lacking caps and membranes (Maggs and Pueschel 1989). The **Rhodymeniophycidae** is the most taxon-rich (ca. 5,000 spp.) red algal subclass and is divided into 12 orders: Acrosymphytales, Bonnemaisoniales, Ceramiales, Gelidiales, Gigartinales, Gracilariales, Halymeniales, Nemastomatales, Peyssonneliales, Plocamiales, Rhodymeniales, and Sebdeniales. All have pit plugs covered by a membrane only (Pueschel and Cole 1982).

Summary

Red algae occupy a wide variety of habitats and play important economic and ecological roles on our planet. They remain poorly studied at the genetic level but have a rich history of morphological, biochemical, and life history analyses. Ultimately all of these diverse areas of science will need to unite to provide comprehensive understanding of the features that make red algae unique members of the tree of life. As an example of recent advances, the explosion of genomic data has significantly changed our views of red algal evolution. Rather than being typical photosynthetic members of the Archaeplastida, we now recognize Rhodophyta as a distinct group that does not share the expected large gene inventory with Viridiplantae and Glaucophyta. In fact, they appear to have shed about one-quarter of the ancestral gene set, leading to nuclear genome reduction. This finding may be explained by an ancient adaptation to an extremophilic environment such as in the vicinity of hot springs: this is the so-called hot start hypothesis for Rhodophyta. Despite this surprising revelation about their early evolution, which is expected to result in severely reduced taxonomic diversity [i.e., extant Cyanidiophytina are species depauperate; 6–10 species/lineages (Reeb and Bhattacharya 2010)] and further habitat restriction, the Rhodophytina ancestor managed to re-emerge, diversify into a variety of mesophilic environments, and develop multicellularity and a complex triphasic life cycle. If this hypothesis is correct, then understanding how this feat was achieved remains a major unanswered question to be addressed by future researchers.

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Zygnematophyta

4

John D. Hall and Richard M. McCourt

Abstract

The Zygnematophyta are among the most diverse green algae, with a variety of thallus types (filaments, unicells, colonies), cell wall structure (one to several layers, with varying degrees of ornamentation), and approximately 4,000 described species. The group lacks flagella at all stages of the life cycle. Several types of asexual spores are produced. Sexual reproduction, when present, involves conjugation or the union of two haploid vegetative protoplasts (individual cells of filaments or unicells) to form a zygospore, which undergoes meiosis to produce a new haploid thallus upon germination. Almost exclusively freshwater, these algae are common in ponds, lakes, and streams, in surface mats, or as phytoplankton or benthic growths. Many, but not all, are found in oligotrophic to mesotrophic waters of moderate to low pH, although the diversity of habitats occupied spans a wide range and may be quite specific for individual species. The fossil record extends at least to the Carboniferous. Recent analyses have placed this group as the sister taxon to land plants, despite the dramatic differences in morphology, life cycles, and reproduction. The group includes the well-known *Spirogyra* and numerous beautiful unicellular forms known as desmids, many of which have elaborate external ornamentations (e.g., spines, granules, large lobes). The conjugating green algae are important as ecological indicator species and for the ecological services they provide.

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●●**Desmidiaceae** (e.g., *Cosmarium*, *Micrasterias*, *Staurastrum*)
●●**Peniaceae** (*Penium*)
●●**Closteriaceae** (*Closterium*, *Spinoclosterium*)
●●**Gonatozygaceae** (*Gonatozygon*)

Introduction

The Zygnematophyta, here considered a phylum, is equivalent to the class Zygnematophyceae in the botanical literature. The group comprises those freshwater green algae with two unique characteristics: sexual reproduction by conjugation and absence of flagellate (mastigote) cells in the life cycle. The phylum contains some of the most beautiful microscopic organisms known (Fig. 1). The elegance of their microscopic cellular architecture is obscured by their macroscopic appearance as floating pond scums, green planktonic masses, and slimy films on the surfaces of plants and other substrates.

The Zygnematophyta is usually divided into two groups (considered two orders of the class Zygnematophyceae): Desmidiales and Zygnematales (Table 1). The order Zygnematales traditionally included the families Zygnemataceae and Mesotaeniaceae (Mix 1972). The family Zygnemataceae (14 genera, over 800 species) included filamentous algae with cell walls that lack a median incision or pores. The

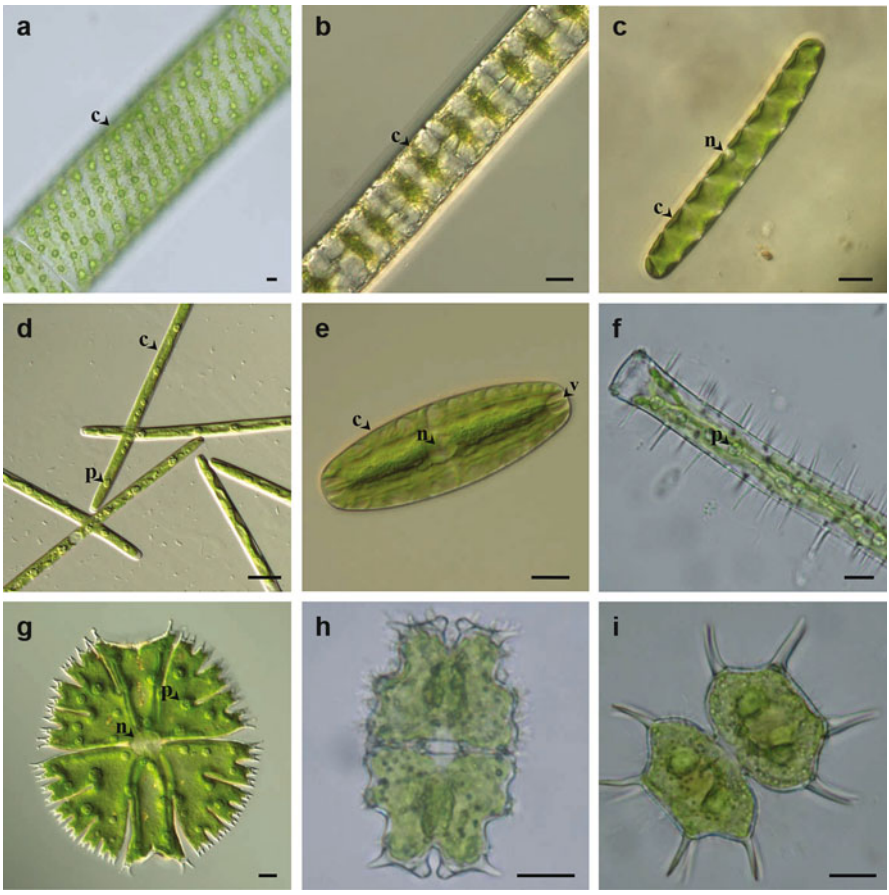

Fig. 1 Structural diversity in the Zygnematophyta. (**a**) *Spirogyra* sp.; (**b**) *Zygnema* sp.; (**c**) *Spirotaenia condensata*; (**d**) *Roya obtusa* var. *montana*; (**e**) *Netrium digitus*; (**f**) *Gonatozygon aculeatum*; (**g**) *Micrasterias rotata*; (**h**) *Euastrum evolutum* var. *glaziovii*; (**i**) *Xanthidium cristatum var. hipparquii.* Structures: *c* chloroplast, *n* nuclear region at site of isthmus between semicells, *p* pyrenoid, *v* apical vacuole. Scale bar = 10 μm in each micrograph

family Mesotaeniaceae contained the saccoderm desmids and was the smallest family (8 genera, approximately 100 species). Molecular phylogenetic studies indicate that the families of the Zygnematales are not monophyletic (McCourt et al. 2000; Gontcharov et al. 2003; Hall et al. 2008a). Zygnematales are generally oblong, rod shaped, or cylindrical, and the smooth cell wall lacks pores; the primary wall is a homogeneous piece, lacking a median constriction. The Desmidiales (41 genera, 3,500+ species) contains the placoderm desmids, which are divided into four families, the Closteriaceae, Gonatozygaceae, Peniaceae, and Desmidiaceae, the latter being the largest of the four families (36 genera, 3,000 species, 12,000 subspecific taxa) (Gerrath 1993; Hall and Delwiche 2007). Most placoderm desmids

Table 1 Classification of Zygnematophyta

Zygnematophyceae	**Desmidiales**	**Closteriaceae**	*Closterium*	*Spinoclosterium*	
		Desmidiaceae	*Actinotaenium*	*Allorgeia*	*Amscottia*
			Bambusina	*Brachytheca*	*Cosmarium*
			Cosmocladium	*Desmidium*	*Docidium*
			Euastridium	*Euastrum*	*Groenbladia*
			Haplotaenium	*Heimansia*	*Hyalotheca*
			Ichthyocercus	*Ichthyodontum*	*Mateola*
			Micrasterias	*Octacanthium*	*Onychonema*
			Oocardium	*Phymatodocis*	*Pleurotaenium*
			Prescottiella	*Sphaerozosma*	*Spinocosmarium*
			Spondylosium	*Staurastrum*[a]	*Staurodesmus*
			Streptonema	*Teilingia*	*Tetmemorus*
			Triplastrum	*Triploceras*	*Vincularia*
			Xanthidium		
		Gonatozygaceae	*Genicularia*	*Gonatozygon*	
		Peniaceae	*Penium*		
	Zygnematales	**Mesotaeniaceae**	*Ancylonema*	*Cylindrocystis*	*Geniculus*
			Mesotaenium	*Netrium*	*Nucleotaenium*
			Planotaenium	*Roya*	*Spirotaenia*
			Tortitaenia		
		Zygnemataceae	*Hallasia*	*Mougeotia*	*Mougeotiopsis*
			Pleurodiscus	*Sangirellum*[c]	*Sirocladium*
			Sirogonium	*Spirogyra*	*Temnogametum*
			Transeauina (Debarya)	*Trigonum*[c]	*Zygnema*
			Zygnemopsis	*Zygogonium*	

[a]*Staurastrum* in the broad sense, including segregate genera recognized by Palamar-Mordvintseva (2003, 2005)
[b]If Yamagishi's (1963) revision were accepted, we would add two genera: *Mougeotiella* and *Neozygnema*
[c]Dubious genera reported only once from India

are unicellular, but filamentous and colonial species are known. Placoderm cell walls have pores and may be intricately ornamented. Each cell consists of two mirror-image parts called semicells that are joined at a narrow midregion or isthmus where the nucleus is located (Fig. 1g). Chloroplasts and other nonnuclear cell contents are divided equally between semicells. The structure of semicells is often complex, with two, three, or more planes of symmetry. The number of lobes on a semicell on end view determines its degree of radiation, e.g., biradiate semicells have two corners, triradiate have three, and so on.

Because of their intriguing structure and reproduction, the zygnematophytes have been extensively studied since the mid-nineteenth century. Research continues in many areas of zygnematophycean biology. In this review, we focus on seminal works and literature published in the last 30 years. Investigators tend to

specialize on one of the three traditional families, a fact reflected in treatments in monographs and books. Reviews and monographs on filamentous Zygnematales (Zygnemataceae) include Transeau (1951), Randhawa (1959), Hoshaw (1968), Kadlubowska (1972, 1984), and Rundina (1998). Major references on unicellular Zygnematales (Mesotaeniaceae) and Desmidiales are *A Synopsis of North American Desmids* (six volumes, Prescott et al. 1972, 1975, 1977, 1981, 1982; Croasdale et al. 1983), Růžička (1977, 1981), Brook (1981), Förster (1982), Croasdale and Flint (1986, 1988), Croasdale et al. (1994), Palamar-Mordvintseva (2003, 2005), Coesel and Meesters (2007), and Brook and Williamson (2010).

Members of the Zygnematophyta have not been exploited for economic use in any major way. A few species have been used in fish aquaculture, and natural populations are abundant enough that one can infer an important role in natural food webs. Some studies suggest that green algae in general and *Spirogyra* in particular may be useful for the detection and recovery of certain metals from contaminated waterways (Gupta et al. 2001; Singh et al. 2007; Rai et al. 2008). Members of the conjugating green algae, including *Spirogyra*, *Mougeotia*, and the Desmidiales, have been used as indicators of trophic status and water quality (Jarnefelt 1952; Rawson 1956; Brook 1965; Coesel 2001).

Biogeography, Habitats, and Ecology

Zygnematophytes are restricted almost entirely to freshwater, although a few species have been collected from brackish waters. Many conjugating green algae are minute, have large geographic ranges, and are able to survive in many marginal habitats; however, biogeographic patterns do exist. Krieger (1937) proposed that many desmids were part of geographic groups and recognized ten continental-scale geographic areas with distinct desmid floras. Coesel (1996) considered this hypothesis in light of modern distributional data and concluded that there are regional floras, although the Arctic/Alpine flora seems to be mostly determined by a minimum average temperature. The apparent existence of regional desmid floras may be the product of insufficient sampling in some regions. However, many conspicuous species characteristics of a particular area are known only from less-studied regions (such as tropical Asia, Africa, and Australia) and, presumably, would have been noticed in areas with a longer history of investigation (such as Europe and North America) if those species existed there (Tyler 1996). Hundreds of local, regional, and national floras documenting the distribution of zygnematophytes have been published. A search of online databases will reveal many of the most recently contributed books and papers. Older floras are referenced in taxonomic treatments and reviews (e.g., Kadlubowska 1984; Prescott 1984).

Zygnematophytes occur in a wide variety of habitats, ephemeral pools, ponds, lakes, streams, rivers, marshes, and bogs, and on every continent. A considerable number of artificial habitats have been colonized by zygnematophycean species. In fact, the widespread occurrence of reservoirs, cattle tanks, roadside ditches,

irrigation canals, and other water impoundments has probably had a significant but unknown effect on the distribution of many species. Within a given habitat, species often show preference for microhabitats. Planktonic species occupy the water column, either permanently as euplankton or temporarily as tychoplankton after being dislodged from the substrate. Relatively few desmid species are truly planktonic. Most conjugating green algae are benthic or periphytic and grow on surfaces or occasionally attached to substrates by means of rhizoids or mucilage. Rhizoids that attach to substrate may be present in all of the filamentous Zygnematales (e.g., *Mougeotia*, *Spirogyra*, and *Zygnema*). Epibiotic species grow on the surfaces of the submerged leaves and stems of plants, especially on plants with highly dissected leaves such as *Utricularia*. Epibionts and species that live in water near plants make up the periphyton.

Some zygnematophytes thrive in habitats subject to extreme physical conditions. For example, some species of saccoderm desmid genera *Ancylonema*, *Cylindrocystis*, and *Mesotaenium* occur on snow and ice. *Cylindrocystis* has also been found in desert crust communities (Lewis and Lewis 2005). Placoderm desmids can be found in Alaskan tundra pools as well as the Antarctic Peninsula and some sub-Antarctic islands, in which freezing temperatures are tolerated for long periods. The filamentous *Zygogonium* is found in very acid pools and rivers (pH < 3) (e.g., Zettler et al. 2002).

Although zygnematophytes occur in a wide variety of habitats, species show distinct preferences for certain habitats characterized by water chemistry and productivity. A generalization often made is that desmids prefer slightly acidic waters (pH 4–7), such as pools in acid peat bogs. Brook (1981) pointed out that, although this generalization is true, a number of species are common in alkaline waters. Acidic habitats support desmid species with the greatest ornamentation and morphological complexity. Generalizations about the distribution of the Zygnemataceae are difficult to make because of the paucity of comparable field measurements. A series of more than 250 collections of *Spirogyra, Zygnema*, and *Mougeotia* from sites across the USA in spring 1982 showed that the temperature and pH of the sites were remarkably uniform at the time of collection (ca. 20 °C and pH 6, in spring season), but this may have been a correlation, rather than a cause (McCourt et al. 1986).

Considerable attention has been devoted to the chemistry and productivity of desmid habitats (Brook 1981). Whereas most placoderm desmids, especially planktonic species, are characterized as oligotrophic (occurring in water of low productivity, low pH, high levels of free CO_2, and low levels of bicarbonate), some species commonly occur in eutrophic waters (high productivity, high pH, low levels of free CO_2, and high levels of bicarbonate). Oligotrophic species requiring high levels of free CO_2 for photosynthesis may be outcompeted in eutrophic lakes by eutrophic species that are able to use bicarbonate directly for photosynthesis (Brook 1981). Desmid assemblages have not been definitively linked to chemical properties of water bodies. Factors controlling local distribution of conjugating green algae remain largely speculative.

Nonetheless, the occurrence of distinct assemblages of desmids in particular water types has led to the effective use of assemblages as general indicators of trophic status and water quality (Brook 1981; Coesel 2001, 2003). Researchers have classified desmid communities into associations, i.e., assemblages, characterized by predictable combinations of species. Heimans (1969) noted that, although it is useful to categorize species as oligotrophic or eutrophic, more than one factor such as pH or eutrophy is involved, and a species' response to these multiple factors may change from one region to another. Coesel (1982) studied desmid assemblages in the Netherlands. He used principal components analysis to categorize over 200 species in quaking fen and lowland marsh habitats into ecologically relevant clusters of species. Cluster composition was affected by pH, successional stage of the *Sphagnum* mat in the fen habitat, oligotrophic or eutrophic nature of water, and rapidity with which these factors changed in water-filled depressions in the *Sphagnum* mat.

Coesel (2001) proposed an index for the use of desmids as biological indicators of conservation value of sites in the Netherlands. This index (scaled from 0 to 10) takes into consideration more information about the species than presence or absence. Species richness, the presence of rare taxa, and the presence of taxa indicative of habitat maturity are included in the calculation of the conservation value of a particular habitat. So sensitive are the desmids to environmental parameters that the desmid flora will sometimes change before there are noticeable differences in the macroflora of a degraded habitat (Coesel 2003).

Little is known about the ecology and habitat preferences of filamentous Zygnematales. For a brief review of published work, see Hoshaw (1968). Filamentous Zygnematales are widely distributed but less frequently reported than other green algae because they often occur in ephemeral pools and because species identification relies on characteristics of the infrequently encountered zygospores. Pessoney (1968), in an unpublished dissertation, described reproduction of *Spirogyra*, *Zygnema*, and *Mougeotia* in temporary, semipermanent, and permanent ponds near Austin, Texas, USA. Conjugation occurred mostly in spring and was more frequent in temporary ponds that were flooded and dried in 2 weeks than in more permanent bodies of water. *Spirogyra* species tended to dominate the pools and showed the highest incidence of conjugation of the three genera.

Species of zygnematophytes are often widely distributed. Individual cells, filaments, or zygospores may be dispersed by currents, wind, insects, water birds, or other agents. Vegetative cells and spores may be carried in the gut of water birds or in mud attached to their feet. Several types of desiccation-resistant spores and cells are produced by zygnematophytes (zygospores, parthenospores, and akinetes). Rewetting of dried mud samples as much as 10 or 20 years old is a common means of starting or recovering cultures, which suggests that spore banks may persist in natural habitats over long periods of time.

Little is known of the role of Zygnematophyta in freshwater ecosystems outside of their role as primary producers. The extent and variety of the animals that graze on them are poorly known. Available data suggest that desmids are an important food source for zooplankton and possibly benthic microinvertebrates (Coesel 1997). It has

been demonstrated that grazing induces physical changes in some species of the genus *Staurastrum* (Wiltshire et al. 2003), which implies a complex relationship between these algae and the herbivores that feed on them.

Conjugating green algae are also subject to parasitism. Chytrids, other fungi, and oomycetes are known to parasitize desmids and filamentous Zygnematales and may have significant effects on algal populations (Kadlubowska 1999; see Kagami et al. 2007 for a review on chytrids). Filamentous Zygnematales are also among the preferred hosts of the parasitic dinoflagellate *Cystodinedria* (Carty 2003).

Zygnematophytes are not immune to the adverse impact of human activities. Hoshaw (1968) remarked on the destruction of habitats of the Zygnemataceae. A number of studies have documented long-term declines in natural desmid populations due to pollution and subsequent eutrophication, development of forests that shade out macrophytes that harbor periphyton, and decreased pH of waters subject to acid rain (e.g., Coesel et al. 1978; Van Dam and Buskens 1993).

Characterization and Recognition

General Features

Unicells and unbranched filaments are the most common forms in the Zygnematophyta, but colonial forms are also known. A single nucleus is usually located in the center of the cell. From one to several axile or sometimes parietal chloroplasts occur in a single cell. Chloroplast shapes range from asteroid (*Cylindrocystis* and *Zygnema*, Fig. 1b) to laminate (*Gonatozygon*, *Mesotaenium*, *Mougeotia*, and *Roya*, Fig. 1d, f) to ribbon-like (*Spirogyra* and *Spirotaenia*, Fig. 1a, c). An axile, ridged chloroplast (stellate) is found in many desmids including *Netrium* (Fig. 1e), *Closterium*, and *Penium*. Species of Desmidiaceae contain some of the largest and most elaborate chloroplasts known among the green algae. Their chloroplasts are often ridged, lobed, and highly dissected. Chloroplasts of most species contain thylakoids stacked as in plants; other species lack grana-like structures or have only rudimentary grana. Pigments include those typical of green algae and embryophytes, i.e., the descendants of a common ancestor that includes all green algae and embryophytic plants: chlorophylls *a* and *b*, β-and γ-carotenes, and several xanthophylls including loroxanthin (Donohue and Fawley 1995). Chloroplasts usually contain one or more pyrenoids (Fig. 1d, g) around which starch is stored.

Cell walls of the Zygnematophyta consist of at least three layers: an outer layer of variable composition depending on species (mucus or an amorphous pectic substance), a primary wall composed of irregularly arranged microfibrils beneath the outer layer, and an innermost layer composed of ribbon-like bands of cellulose microfibrils (Fig. 2). Many placoderm desmids shed the primary wall and retain the secondary wall as the outer covering. Ornamentations such as granules, spines, and verrucae may occur in the outer layer only or in the primary and secondary walls.

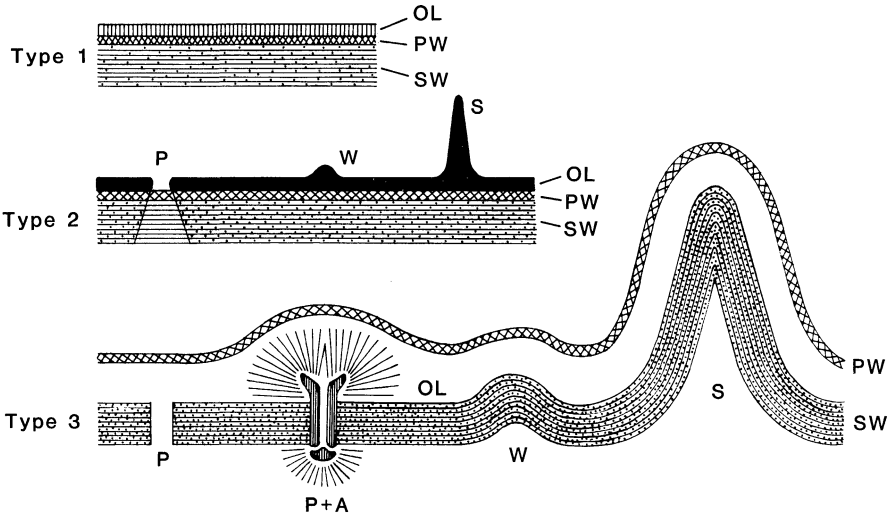

Fig. 2 Cell wall structure of the Conjugaphyta. Wall types *1*, *2*, and *3* are described in Table 2. Structures: *OL* outer layer, *PW* primary wall, *SW* secondary wall, *P* pore, *P+A* pore apparatus, *S* spine, *W* wart (After Mix 1975)

Cell wall pores are of fundamental importance in classification, although their function is not clear. Pores may be absent (Zygnemataceae, Mesotaeniaceae) or present (Desmidiales). If present, pores may penetrate only the outer layer (Closteriaceae, Gonatozygaceae, Peniaceae) or extend completely through the secondary wall (Desmidiaceae). Pores through the secondary wall in some desmids may be differentiated into a complex pore apparatus, with a lined pore channel and a "spider web" of fibrous material at the inner opening (Neuhaus and Kiermayer 1981). Extrusions of fibrous mucilage from the external openings of the pores are visible under the light microscope.

Classification and Taxonomy

Work on mitosis, cytokinesis, and kinetid (flagellar apparatus) ultrastructure in the green algae resulted in major restructuring of systematics of the green algae at the class level and reinterpretation of their presumed relationship to ancestors of land plants. Mattox and Stewart (1984) proposed the establishment of five classes in the Chlorophyta. They placed the Zygnematales (= Zygnematophyceae) in the class Charophyceae (= Charophyta) on the basis of the presence of a primitive phragmoplast in *Spirogyra*, types of enzymes for glycolate and urea metabolism known for a few genera, and the similarities of the group to *Klebsormidium*, an obviously charophycean genus (Pickett-Heaps 1975). We follow the classification of Lewis and McCourt (2004) for the class-level relationships and the classification of Mix (1972), with some modification, at the family level. The Zygnematophyta are one of several

green algal groups that are monophyletic with embryophytes and one of the major lineages of the Chlorobionta, which itself is one of the main lineages of the Archaeplastida (Adl et al. 2012; McCourt 2016). Although recent studies have changed our view of the green algal sister taxon to embryophytes (see discussion below), the working classification proposed by Lewis and McCourt (2004) is still a reasonable one.

The Zygnematophyta are part of a lineage of green algae, known as Charophyta (Karol et al. 2001), equivalent to Streptophyta of other authors (e.g., Wickett et al. 2014), that contains some green algae plus all land plants (McCourt et al. 2004; Leliaert et al. 2012). Early molecular studies with broad taxon sampling and several genes showed the conjugating green algae to be more distantly related to land plants than either the Charophyceae or Coleochaetophyceae (Karol et al. 2001). However, later work with many genes, albeit with fewer taxa, supported zygnematophytes as the sister group (Turmel et al. 2006, 2007). Most recently, the latter hypothesis has garnered strong support from several recent phylogenomic analyses so that the strongly supported consensus is that a filamentous zygnematophyte-like ancestor gave rise to the Zygnematophyta and its sister taxon, all embryophytes (Wickett et al. 2014; Ruhfel et al. 2014). The implications of the latter relationship are profound in regard to the origin of a land flora and the likely morphological and physiological traits of early land-colonizing green algae (Delwiche and Cooper 2015; Davis et al. 2014; deVries et al. 2016). Taxon sampling in larger phylogenomic analyses has been, however, limited (only two zygnematophytes are included in Wickett et al. 2014). Delwiche and Cooper (2015) noted this problem of taxon sampling and suggested that further studies might provide alternative topologies. Davis et al. (2014) also commented that "In some cases, however, high support for relationships should be interpreted cautiously because conflicting topologies are supported by other data. Key examples include the previously mentioned sister groups of land plants . . ." Thus, although the consensus currently is that the zygnematophytes are the sister group to land plants, the issue is not quite a settled question.

Within the zygnematophytes, relationships among the numerous genera are becoming clear, but traditional taxonomy has not been supported by molecular work (Gontcharov et al. 2003, 2004; Hall et al. 2008a). For example, it is not clear if the Zygnematales is a monophyletic group and there is some evidence that the Desmidiales may include *Netrium* and *Roya*, genera not previously recognized as part of this lineage (Gontcharov et al. 2003, 2004; Hall et al. 2008a). There is some evidence that species of the genus *Spirotaenia* may not belong to the conjugating green algae (Gontcharov and Melkonian 2004). Although phylogenetic position of this genus is uncertain, it shares many of the characteristics of other zygnematophytes including reproduction by conjugation and will be treated as a member of the group.

Classification within the conjugating green algae is somewhat unsettled, and structural synapomorphies have not been found for many of the lineages discovered in molecular phylogenetic studies (e.g., Gontcharov and Melkonian 2008). There is some evidence that chloroplast shape may be an important phylogenetic

Table 2 Cell-wall characteristics of the Zygnematophyta

Zygnematales	Type 1. Mesotaeniaceae and Zygnemataceae	1. Cell wall one homogeneous piece
		2. Primary wall not shed
		3. Outer hyaline (mucus) layer smooth
		4. Ornamentations weak or absent
		5. Pores absent
Desmidiales	Type 2. Closteriaceae, Gonatozygaceae and Peniaceae	1. Cell wall may be formed of several segments, which are divided by very slight constrictions
		2. Primary wall not shed
		3. Compact, structured outer layer (warts, spines, and ridges originating from outer layer)
		4. Ornamentations strong and variable
		5. Pores or pore-like gaps only in outer layer
	Type 3. Desmidiaceae	1. Cell wall formed of two segments divided by a marked constriction (isthmus)
		2. Primary wall shed
		3. No continuous outer layer but mucilaginous envelope originating from pore organs from secondary wall
		4. Pores in secondary wall

After Brook (1981)

characteristic (McCourt et al. 1995; Hall et al. 2008a), but groups within the Zygnematales have not been formally circumscribed.

Before differences in cell wall characteristics were revealed, three families of conjugating green algae were recognized: Desmidiaceae, Mesotaeniaceae, and Zygnemataceae. The traditional Desmidiaceae is equivalent to the Desmidiales in this treatment, and species in this group are often referred to in general literature as the "placoderm desmids." Conversely, the Mesotaeniaceae in earlier literature were called the "saccoderm desmids." It is important to recognize that the saccoderm desmids and Zygnemataceae are not natural groups, but rather groupings based on growth habitat (saccoderms are unicellular and zygnemataceans are filamentous). The order Zygnematales as defined here includes those species formerly assigned to the families Zygnemataceae and Mesotaeniaceae. They share the trait of a simple cell wall, but this is likely a primitive trait that does not diagnose a monophyletic group. Nevertheless, the current classification is based primarily on characteristics of the cell walls. These characteristics are outlined in Table 2.

The taxonomy of the Zygnematophyta has involved the descriptions of numerous morphological species. Among the characters distinguishing species in this diverse group of algae are radiation, cell wall ornamentation, cell dimensions, chloroplast number and form, details of cell division and the conjugation process, as well as zygospore color and ornamentation. Intraspecific polymorphisms and aberrant forms are numerous, especially among placoderm desmids (Brook 1981).

The correlation between morphological species and biological species is not clear. Watanabe and Ichimura (1982), working with *Closterium*, have shown the existence

of several ecologically and sexually isolated mating groups within a single morphological species or species complex. This and other studies (Ichimura 1983) suggest that, although a species of *Closterium* may appear to be widely distributed, crossing experiments reveal that reproductively isolated mating groups are restricted to much smaller areas, and several biological species may be involved. In our own studies of the Gonatozygaceae, we have found that some morphological species constitute distinct phylogenetic lineages although reproductive isolation has not been demonstrated (Hall unpub.).

Although the *Closterium* studies imply the existence of more biological species than are presently described, research on polyploidy in desmids and *Spirogyra* has indicated that perhaps too many species have been described because of morphological variation within a polyploid species complex (polyploid derivatives from a single clone). Polyploidy has been reviewed by Nichols (1980). Placoderm desmids display changes in cell volume and degree of radiation associated with ploidy changes (Pickett-Heaps 1983). Clonal cultures of *Spirogyra* have been reported to develop filaments of three or more distinct widths that are the phenotypic expression of euploid increases in chromosome number (Allen 1958; Hoshaw et al. 1985, 1987). Variations in ploidy of *Spirogyra* and the morphological changes associated with them have undoubtedly led to excessive numbers of species descriptions (over 400 species). At the same time, it is likely that cryptic genetic variation may have led to underestimates of species diversity in other genera. The nature of diversity in species of these algae remains a fertile area of research.

Reproduction

A cardinal feature distinguishing the Zygnematophyta from other chlorophytes is sexual reproduction by conjugation involving the fusion of non-flagellate gametes. Asexual reproduction is by fragmentation, cell division, akinetes, and parthenospores. These types of reproduction have been observed either in natural populations or in laboratory cultures.

Conjugation was first observed nearly 200 years ago, but only during the twentieth century were the events adequately interpreted (Fritsch 1935; Randhawa 1959). Investigations of cultures have provided data on the events of entire sexual cycles. Observations have been made of stages from cell or filament pairing to zygospore germination.

Sexual cycles (Figs. 3 and 4) consist of conjugation (the physical joining of cells or filaments and subsequent union of gametes to form a zygote), formation of a thick-walled zygospore, a period of zygospore dormancy, and germination of the zygospore to produce vegetative cells. Sexual cycles of the conjugating algae are haplobiontic. They display zygotic meiosis; growing cells are haploid; and meiosis occurs in the zygote, the only diploid cell in the sexual cycle. Strains of species may be homothallic (conjugation is intraclonal) or heterothallic (conjugation is interclonal between plus and minus mating types). A great deal of work has focused on molecular mechanisms involved in mating-type determination in *Closterium* (Hirano et al. 2015; Sekimoto et al. 2014).

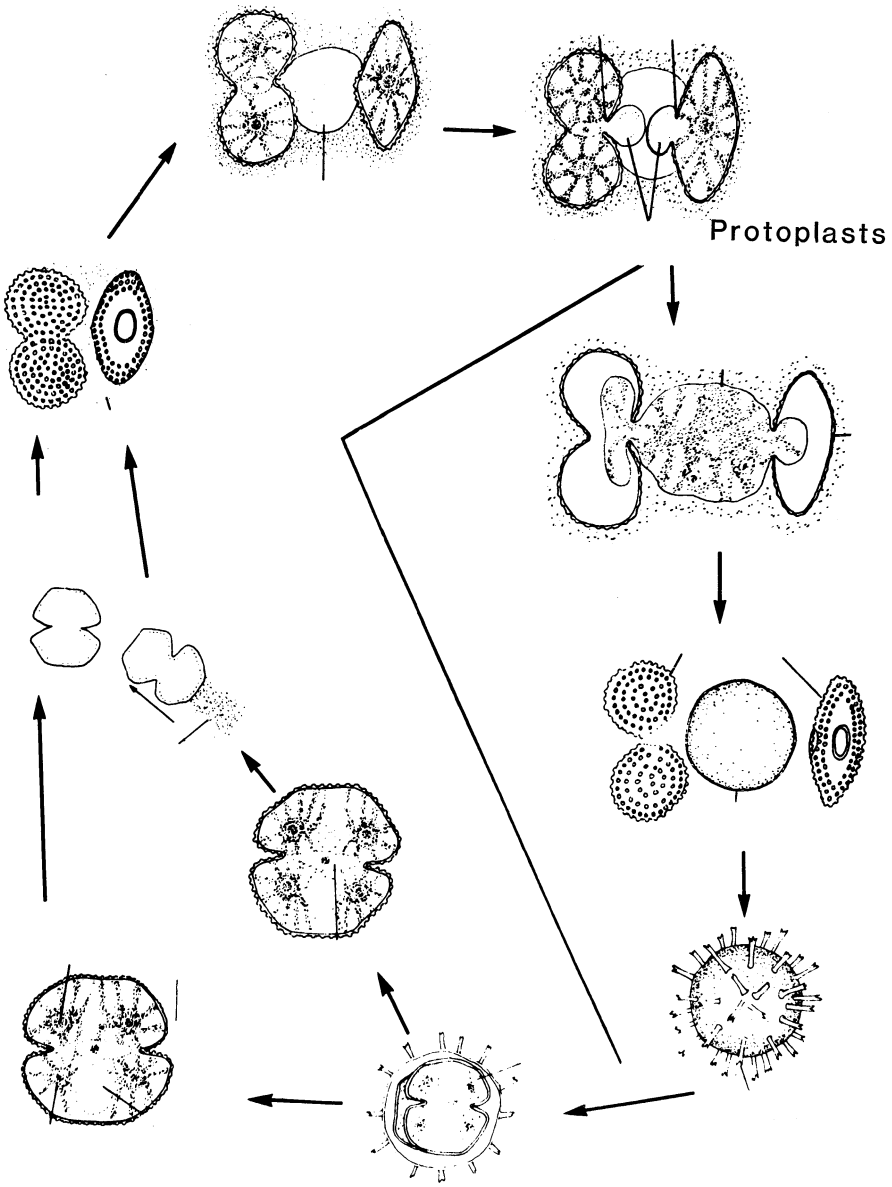

Fig. 3 *Cosmarium* life cycle (After Lee 1980)

Laboratory strains of placoderm and saccoderm desmids have been induced to conjugate by increasing carbon dioxide in the atmosphere surrounding cells (Starr 1964) and by suspending cells in a low-nitrogen medium (Biebel 1973). Brandham (1967) described the movement of *Cosmarium botrytis* cells to form

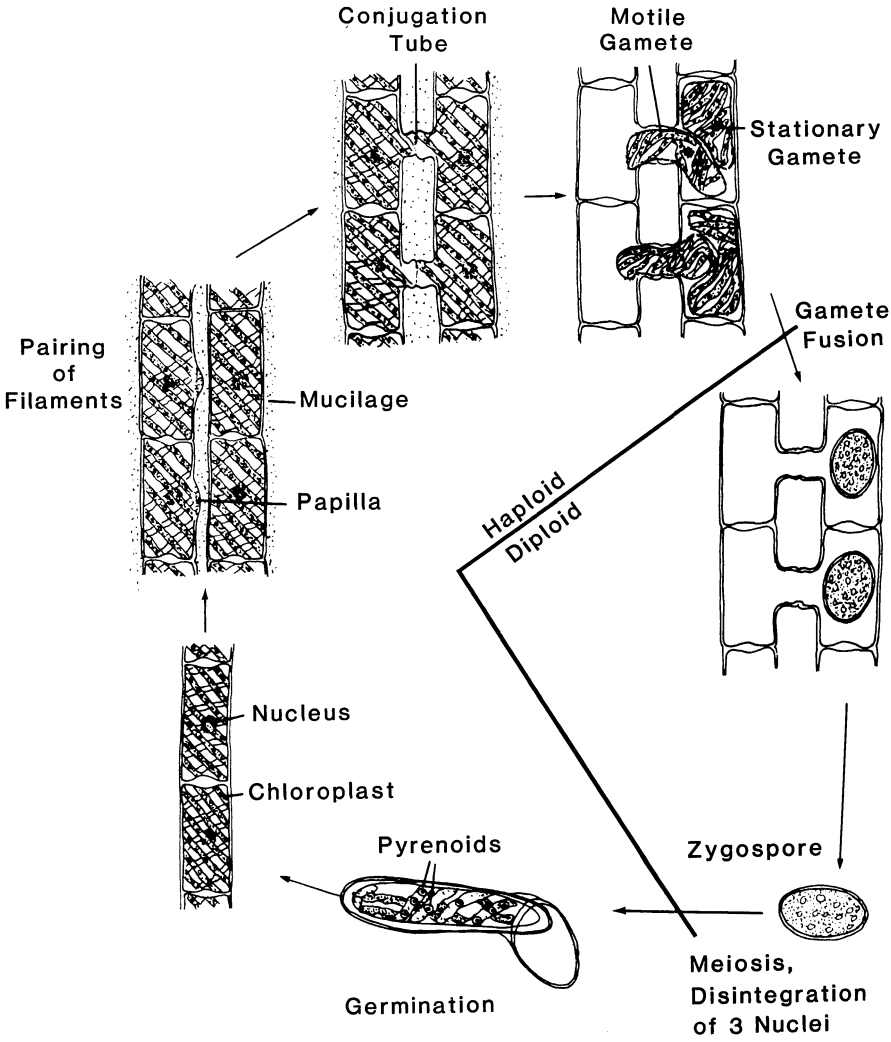

Fig. 4 *Spirogyra* life cycle (After Lee 1980)

pairs; this movement suggests the action of a sexual pheromone. Pheromones were long thought to be involved in initiating sexual reproduction. Hoshaw (1968) reported evidence that a sexual pheromone produced by one mating type of *Zygnema circumcarinatum* facilitated conjugation. A fragment of a gene encoding a putative pheromone thought to be involved in the induction of sexual reproduction has been identified in *Closterium* (Tsuchikane et al. 2003; Fukumoto et al. 2003).

It seems that optimal conditions for conjugation vary from species to species. Filamentous Zygnematales often conjugate when filaments are transferred to nutrient-poor conditions, such as the surface of a 1.2% tap water agar. We have found that freshly collected filaments spread on agar conjugate more readily than filaments from cultures. Scalariform conjugation involves the movement of filaments into a paired ladder-like arrangement after which outgrowths from adjacent cells (papillae) join to form a conjugation tube. In Zygnemataceae with lateral conjugation, a short curved tube joins adjacent cells in a single filament.

Many different mating systems are known among the conjugating green algae. Gametes of saccoderm and placoderm desmids usually leave parent cells and meet in a conjugation vesicle or tube. In conjugation of filamentous Zygnematales, cells of the filament function as gametangia and form isogametes or anisogametes (see glossary in Hoshaw 1968). One gamete may move to the opposite cell, or both gametes may move into and fuse in the conjugation tube. Physiological anisogamy occurs when one isogamete moves and the other isogamete remains stationary, as happens in most species of *Spirogyra* (Hoshaw 1968). Gamete directionality is not fixed. That is, filaments may contain some cells that act as a gamete donor and others that act as a gamete recipient.

The zygospore wall is composed of three major wall layers: exospore (outermost), mesospore (middle), and endospore (innermost). These layers may themselves be multilayered. DeVries et al. (1983) reported the presence of sporopollenin in the two mesospore layers of *Spirogyra hassallii* along with cellulose and/or pectin in exospore and endospore layers. Walls of zygospores are smooth or ornamented and may be black or shades of yellow, brown, or blue.

Meiosis in the placoderm desmid *Pleurotaenium* occurs in the zygospore immediately before germination (Blackburn and Tyler 1981). In contrast, meiosis in *Spirogyra crassa* occurs early in zygospore formation, even before the wall matures (Godward 1966). Zygospore germination involves the rupture of the exospore and mesospore with the emergence of one to four germlings (single cells or the first cell of a filament). The endospore becomes the cell wall of germlings, or cleavage of the single remaining protoplast from meiosis may occur within the endospore during the formation of two, three, or four germlings (Biebel 1973). Chloroplasts from only one gamete survive in germlings, even in homothallic isogamous species where the gametes are genetically identical. In all species of *Spirogyra* studied and some species of *Zygnema*, in which only one gamete moves, chloroplasts persisting in the zygospore and resulting progeny are those of the stationary gamete (Biebel 1976).

Conjugation has infrequently been observed in natural populations of placoderm and saccoderm desmids. In some species, asexual spores are known. Brook (1981) described two types of akinetes formed asexually by placoderm desmids. The protoplast either emerges from the cell to form a spore or secretes a thick wall inside the original cell wall. Akinetes form in many filamentous zygnematalean species by

the second method. A parthenospore is similar to an akinete but forms when gametangial cells form spores without gamete fusion. Less commonly observed are akinetes, thick-walled vegetative cells, which in *Zygnema* form most readily under conditions of high light (6,500 lux) and low nitrogen (Pessoney 1968).

Photomovement of Chloroplasts and Cells

Mougeotia and *Mesotaenium* are useful experimental organisms in the study of phytochrome-mediated movements of chloroplasts (Haupt 1982). The laminate chloroplast moves within the cell to display its maximum surface area or face toward low-intensity white light, whereas in high-intensity white light, the chloroplast aligns itself with the edge profile toward the light. Presumably these motions optimize photosynthetic performance and minimize damage to the photosynthetic apparatus. *Mougeotia* has advantageous features for such research, with its cells each containing a single large chloroplast that can be partially irradiated with microbeam illumination. Unlike chloroplast movements in other organisms, chloroplast orientation in *Mougeotia* proceeds to completion in darkness after exposure to brief light flashes, allowing the processes of light perception and response to be studied separately (Haupt 1982, 1983).

Phytochrome in the peripheral cytoplasm appears to be the photoreceptor pigment in both the low and high light-intensity responses, but the action spectra are very different. Low-intensity white light (or red light) striking the cell from one side transforms red-absorbing phytochrome (P_r) to the far-red-absorbing active form (P_{fr}) more effectively on front and rear cell surfaces than on the flanks. A higher concentration of P_{fr} builds up in the front and rear cortical cytoplasm, and the chloroplast edges move as if repelled by high P_{fr} levels so that the flat surface of the chloroplast turns toward the light. In high-intensity light, the same P_f-P_{fr} gradient is set up, but an additional blue-light photoreceptor in the cortical cytoplasm somehow acts as a switch in the reaction chain; the chloroplast edges, instead of being repelled by high P_{fr} levels, are attracted to them, whereby the edge profile of the chloroplast is displayed to the light. Actin microfilaments are believed to move the chloroplast edge touching the cortical cytoplasm, and calcium apparently plays a key role in the transduction chain. Many details of this process remain unknown, but progress has been made toward a greater understanding of phytochrome signaling in *Mougeotia* and *Mesotaenium* (see Wagner 2001, for details). In particular, full-length phytochrome-coding sequences have been cloned from *Mesotaenium* and *Mougeotia*. In addition, a new photoreceptor, neochrome (similar to the *Adiantum* phy3), has been identified from *Mougeotia scalaris* (Suetsugu et al. 2005). This molecule seems to be the product of the fusion of portions of phytochrome and phototropin genes.

Placoderm and saccoderm desmid cells have long been known to be capable of gliding or somersaulting movements. It has been shown that gliding movement is effected by the localized excretion of mucilage, usually from the apices (Oertel et al.

2004; Rogers-Domozich et al. 1993). Some filamentous Zygnematales, such as *Spirogyra*, are also capable of a gliding motility (Kim et al. 2005).

Cytomorphogenesis

Placoderm desmids, particularly members of the genus *Micrasterias*, have been valuable in research on cytomorphogenesis, the process of growth and differentiation of their elaborately structured cell walls following cell division. Several features make *Micrasterias* especially useful in this research (Kiermayer 1981; Meindl 1993). The cells are relatively large (200–300 μm in diameter), easily centrifuged to produce enucleate semicells, and easy to observe without staining. Experimental production of enucleate, polyploid, or aneuploid cells has shown effects of the nucleus and cytoplasm on differentiation.

Micrasterias cells are typically biradiate, each semicell comprising a polar lobe flanked by two wings (lateral lobes), which are incised and differentiated at the edges in a species-specific pattern. In *M. denticulata*, following mitosis and septum formation at the isthmus, each semicell generates an adjoining mirror image of itself in about 16 h (Kiermayer 1981; Meindl 1993). The septum swells outward under the influence of turgor pressure, the polar lobe and wings begin to form, and the chloroplast flows into the forming semicell and eventually divides into two, but the two halves stay joined as semicells at the isthmus (Fig. 5).

Although all conjugating green algae are thought to use some variation of centripetal cell division, members of the group display a number of interesting and unusual cell division characteristics. In the placoderm desmid *Staurastrum* (*Pleurenterium*), after cytokinesis, the nucleus moves out of the isthmus into the developing semicell. The nucleus is then moved by means of a microtubular system in a circular motion. After most of the semicell has formed, the nucleus then returns to its place in the isthmus of the cell, a process termed nuclear migration (Meindl

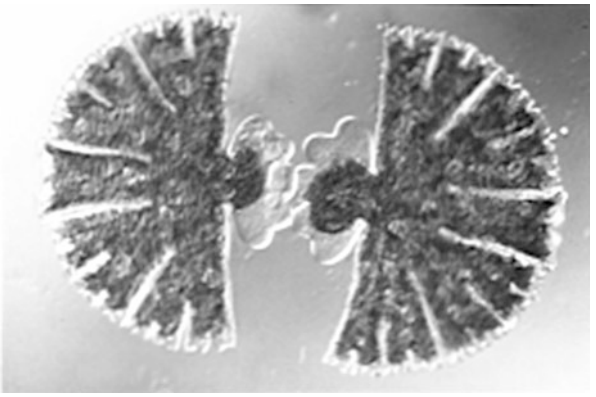

Fig. 5 Early stage in semicell regeneration following cell division in *Micrasterias denticulata*. × (Photomicrograph courtesy of Dr. J. D. Pickett-Heaps, University of Colorado)

1986). Many of the molecular players in this process have been identified (e.g., Holzinger and Lütz-Meindl 2002).

In addition to cytological differences in cell division, some filamentous placoderm desmids display different patterns of cell division. The existence of replicate folds on the cross wall of species of *Desmidium* and *Bambusina* have long been known (Hauptfleich 1888). It was also discovered that *Onychonema* had an unusual mode of cell division that involved the delay of mitosis and the formation of a division vesicle (Krupp and Lang 1985). Additional differences in cell division among filamentous desmids are now known. Hall et al. (2008b) proposed that characteristics of cell division may be a valuable systematic character in filamentous Desmidiaceae.

It is worth noting that among charophytes (=streptophytes), the Zygnematophyta are the only group for which transformation protocols have been developed (Abe et al. 2011 for *Closterium*; Sorensen et al. 2014 for *Penium*; and Vannerum et al. 2010, 2011 *Micrasterias*). These protocols may prove valuable in using zygnematophytes as model systems.

Nuclear Cytology and Genetics

The most significant features of nuclear cytology in Zygnematophyta are (1) the presence during mitosis of stainable material derived from the nucleolus and known as nucleolar substance, (2) the presence of a complex interphase nucleolus, and (3) the absence of a localized centromere on the chromosomes of some species. In *Spirogyra*, large, complex nucleoli have been observed at interphase or prophase (Godward 1966). In *Spirogyra*, *Sirogonium* (Hoshaw and Wells 1982), and *Zygnema* (Harada and Yamagishi 1980), new nucleoli are organized by nucleolar-organizing chromosomes, which possess long secondary constrictions called nucleolar-organizing regions. Placoderm and saccoderm desmids also possess nucleolar-organizing chromosomes and nucleolar substance (Godward 1966; Brook 1981).

According to Godward (1966), *Spirogyra* metaphase chromosomes are of three types: (1) minute dot-like chromosomes in which the centromere position cannot be observed, (2) chromosomes with localized centromeres, and (3) polycentric chromosomes. In some genera such as *Zygnema* and *Mougeotia*, only dot-like chromosomes have been reported. Chromosomes in most saccoderm and placoderm desmids are small (less than 1.5 μm long). The parallel separation of chromatids at anaphase in most species suggests the presence of polycentric chromosomes.

Godward (1966) summarized data on chromosome numbers in the Zygnematophyta. Chromosome numbers in growing cells of saccoderm desmids range from 20 to 592, in placoderm desmids from 9 to 220, and in Zygnemataceae from 4 to 94. Considerable intraspecific variation in chromosome numbers has been reported (Brook 1981).

The genetics of the group was reviewed by Biebel (1976). He noted that their potential for genetic studies has rarely been exploited. Inheritance of genes for mating type and zygospore structure has been demonstrated. Selective abortion of

meiotic products in a zygospore results in incomplete, unordered tetrads. Recessive lethal alleles have been found in *Cosmarium turpinii* that are expressed in the homozygous condition in diploid zygospores but not in haploid vegetative cells.

Maintenance and Cultivation

Laboratory investigations of the conjugating green algae often begin with cultures from one or more of the world's culture collections, thereby bypassing the steps of collection and isolation of experimental organisms. If organisms directly from nature are desired, plankton and periphyton samples frequently contain desmid species, and filaments of the Zygnemataceae occur suspended in submerged masses or as surface scums that permit easy collection by hand. Once collected, cells or filaments can be isolated into unialgal culture by one of the methods described in Stein (1973) and Andersen (2005).

Many growth media are available; formulae and suggestions have been provided by Hoshaw (1968), Stein (1973), Starr (1978), and Andersen (2005). Strains obtained from culture collections often have the growth medium designated. For isolation and early growth, biphasic soil-water medium (Starr 1978) is often preferred; addition of a small quantity of peat is necessary to lower the pH for some species. For organisms requiring soil extract, we have found that we can grow many of these species in axenic conditions by adding 40 mL/L of filter-sterilized soil extract to sterile Bold's basal medium (see Stein 1973, for formula). Most placoderm desmids grow well in general defined medium with the addition of B vitamins. After a suitable growth medium is selected, cultures of Zygnematophyta will often produce luxuriant growth after 2–4 weeks in stationary culture under 40-watt cool-white fluorescent lamps of 50 $\mu Em^{-2}\ s^{-1}$ intensity on a 16:8 h light-dark cycle at 15–20 °C.

Fossil Record

Until the late twentieth century, fossil zygospores of conjugating green algae were largely ignored or identified as form taxa of non-zygnematalean spores (Van Geel 1979). Although fossil zygospores have been used to reconstruct ancient climates and aquatic habitats, a major hindrance to their use in reconstructing past climates is the general lack of knowledge of the ecology of extant species (Ellis and Van Geel 1978). Nevertheless, fossil zygospores provide information independent of pollen records (Van Geel and Van der Hammen 1978). The presence of these algal zygospores (usually *Mougeotia* and *Spirogyra*) at a site implies the past presence of shallow, stagnant, mesotrophic habitats subject to warming temperatures in spring that induce conjugation (Van Geel 1978; Van Geel and Van der Hammen 1978; Jarzen 1979). The presence of fossil desmids in deposits suggests the former presence of slightly acid swamp waters (Tappan 1980). An exception to this is the presence of *Oocardium*, one of the few desmids that prefer limy waters, in which it forms calcareous tubules called tufa (Bradley 1974).

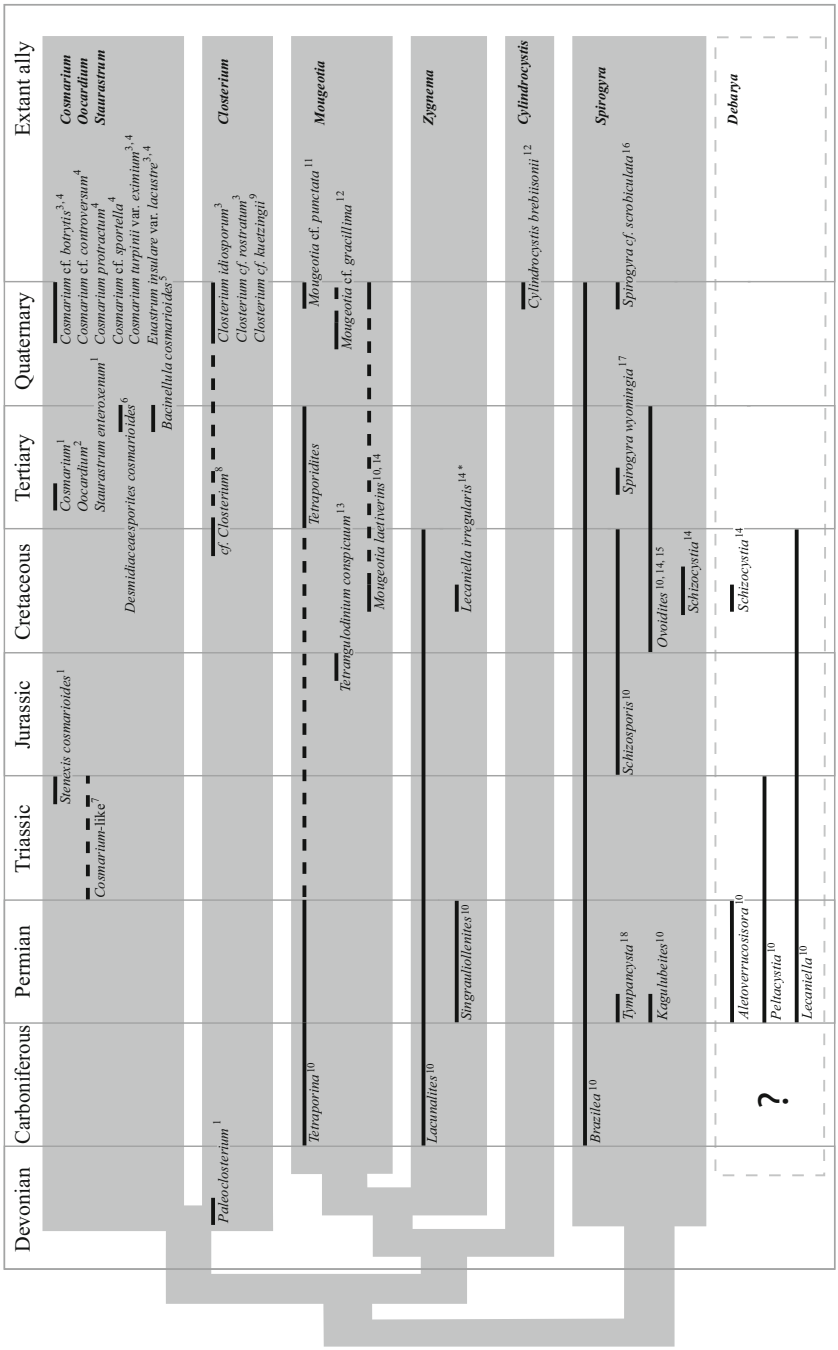

Fig. 6 (continued)

One of the most commonly reported fossil zygospores is that of *Debarya glyptosperma* (Van Geel and Van der Hammen 1978; Ellis and Van Geel 1978), reported originally as the form taxon *Peltacystia* (Van Geel 1979). The commonness of these distinctly keeled zygospores in deposits would not be predicted based on current distribution and abundance of this species, which, although found on every continent, is quite rare. The explanation for this is all the more elusive because, being rare, *Debarya* is not well known ecologically. Based on fossil evidence, *D. glyptosperma* in the Colombian Andes was restricted to cold to cool high-mountain climates (Van Geel and Van der Hammen 1978).

The fossil record of the Zygnematophyta is incomplete but extends to the middle Devonian (Fig. 6; Table 3). Because zygnematalean vegetative cells are fragile, most fossils are those of zygospores, which are usually necessary to identify living as well as fossil Zygnematophyta to species. The oldest zygnematalean fossils are Carboniferous zygospores of *Mougeotia* (reported as the form taxon *Tetraporina*), *Brazilea*, and *Lacunulites*. The phylogenetic placement of some fossil Zygnematophyta is less certain. *Paleozygnema spiralis* has been reported from Cretaceous amber in Germany (Dörfelt and Schäfer 2000). Both vegetative filaments and spores were preserved in the amber. The spore has a superficial spiral pattern similar to early stages of spore formation in *Spirogyra.* Placement of this taxon is uncertain. Based on the supposed phylogenetic affinities of the fossils to modern taxa, it can be deduced that all major lineages of the Zygnematales had diverged before the Carboniferous (Fig. 6; Table 3).

The oldest reported desmid fossils are vegetative cells of *Paleoclosterium leptum* from the middle Devonian. Fossils with obvious affinities to the Desmidiaceae do not appear until the Triassic. Extant genera of the Desmidiaceae (sensu stricto) do not appear until the Tertiary. This late appearance of Desmidiaceae in the fossil record is congruent with the derived placement of the group in molecular phylogenies (McCourt et al. 2000; Gontcharov et al. 2003; Hall et al. 2008a). Similarly, the filamentous nature of the oldest zygnematophycean fossils is congruent with the proposal of Delwiche and Cooper (2015) that the ancestor of charophyte (=streptophyte) algae and embryophytes was a filamentous taxon, most likely a branched one (see their Fig. 3).

Fig. 6 Fossil record superimposed on phylogenetic tree (After Hall et al. 2008a). Branching order based on Hall et al. 2008a. Phylogenetic position of *Debarya* is not known; however, it is thought to be closely related to *Mougeotia*. The placement of the *Debarya* lineage is unknown. *Debarya* is thought to be most closely related to *Mougeotia*. A "?" indicates the point of uncertainty. Sources used for fossil dates are indicated by the *superscript*. *1* Tappan 1980; *2* Bradley 1974; *3* Van Geel et al. 1981; *4* Van Geel et al. 1989; *5* Weyland 1963; *6* Hunger 1953; *7* Schmidt et al. 2006; *8* Waggoner 1994; *9* Van Geel and Van der Hammen 1978; *10* Van Geel 1996; *11* Van Geel 1976; *12* Van Geel 1978; *13* Schrank 2005; *14* Zippi 1998; *15* Hofman and Zetter 2005; *16* Van Geel et al. 1989; *17* Bradley 1970; *18* Afonin et al. 2001. Only new or particularly interesting fossils are referenced; many more fossils including many species within the form genera are known. * There is some disagreement as to the placement of *Lecaniella*, but Zippi (1998) indicates that this fossil belongs to the Zygnemoid lineage rather than the *Debarya* lineage

Table 3 Oldest fossil record of select Zygnematophyta

Taxon	Fossil type	Age	Location	References
Desmidiales				
Baccinellula cosmarioides	Cells	Pliocene	Italy	Weyland (1963)
Closterium sp.	Zygospores	Pleistocene	Colombian Andes	Van Geel and Van der Hammen (1978)
Cosmarium – like	Cells	Triassic	Italian Dolomites, Italy	Schmidt et al. (2006)
Cosmarium sp.	Conjugating cells	Eocene	Wyoming, USA	Tappan (1980)
Oocardium sp.	Layered tufa	Eocene	Wyoming, USA	Bradley (1974)
Desmidiaceaesporites cosmarioformis	Zygospore	Early Miocene	Poland	Hunger (1953)
Paleoclosterium leptum	Cells	Mid-Devonian	New York, USA	Tappan (1980)
Staurastrum enteroxenum	Conjugating cells and zygospores	Eocene	Wyoming, USA	Tappan (1980)
Stenixis cosmarioides	Cells	Late Triassic	Location not given	Tappan (1980)
Zygnematales				
Cylindrocystis brebissonii	Zygospores	Holocene	Germany and The Netherlands	Van Geel (1978)
Debarya glyptosperma (= *Lacunulites*?)	Zygospores	Permian	Western Australia	Van Geel (1979)
Mougeotia sp. (= *Tetraporina*? ?)	Zygospores	Carboniferous	Moscow, USSR	Van Geel (1979)
Spirogyra spp. (= *Brazilea*?)	Zygospores, aplanospores	Carboniferous	Moscow, USSR	Van Geel (1979)
Tympancysta stoschiana	Filaments with chloroplasts	Early Triassic	Russia	Afonin et al. (2001)

The affinities of fossils to extant taxa are rarely known with certainty. Mapping the reported fossils on the phylogeny of extant lineages in light of our incomplete understanding of affinities is somewhat bold, but if taken at face value, it suggests that most of the lineages of extant Zygnematales are very ancient.

Our understanding of evolution in the group has been much affected by molecular phylogenetic studies. It is clear that the traditional families Mesotaeniaceae and Zygnemataceae are not natural groups and that the order Zygnematales may be paraphyletic with respect to the Desmidiales. We now hypothesize that the

Desmidiales are a monophyletic group unto themselves that share a common ancestor with some unicellular and filamentous Zygnematales (as opposed to being derived from one or more unicellular zygnematalean lineages as was often proposed). Among the Desmidiales, most colonial and filamentous forms seem to belong to one or two major lineages, implying few transitions between these two growth habits (McCourt et al. 1995; Gontcharov et al. 2003; Hall et al. 2008a). Among the Zygnematales, however, there have been several transitions between the unicellular and filamentous forms. Although the direction of such transitions is not certain, lineages related to the conjugating green algae (Klebsormidiophyceae and Coleochaetophyceae) comprise filamentous or multicellular organisms, which is the inferred growth habit of the ancestor of conjugating green algae. Continued studies of evolution in the conjugating green algae and related lineages will provide greater insight into the origin and early diversification events in this group.

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Charophyceae (Charales)

5

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Abstract

The charophytes, or stoneworts, are a group of green algae with six extant genera in one family, distributed worldwide in freshwater ponds and lakes. They are among the green algal groups most closely related to land plants and exhibit a complex thallus, with multinucleate internodal cells joined at nodes comprising smaller, uninucleate cells giving rise to whorled branchlets. Two genera (*Chara, Nitella*) contain most of the described species, with a third (*Tolypella*) containing several dozen taxa. The remaining genera have one or a few species. Reproduction is oogamous, with sperm and eggs produced in separate multicellular structures. The thallus is haploid; the zygote is the only diploid cell in the life cycle, and meiosis is followed by the development of a resistant spore. Thalli and spores

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are often encrusted with calcium carbonate. Such spores are abundant in the fossil record of the Charales, which extends to the Upper Silurian, and many genera and families have become extinct. These algae provide important ecosystem services, for example, as colonizing species, as biological agents for producing water clarity, or as the base of the food web. Charophytes are important for the study of evolution of embryophyte development, growth meristems, and cell biophysics. As one of the green algal groups most closely related to land plants, the rich charophyte fossil record may reveal clues regarding the earliest algae that invaded the land.

Keywords
Charales • Charophytes • Stoneworts • Gyrogonites • Plasmodesmata • Cytoplasmic streaming • Bulbils • Globule • Nucule

Contents

Summary Classification

- **Charales**
  - **Characeae** *Chara*, *Lamprothamnium*, *Lycnhothamnus*, *Nitella*, *Nitellopsis*, *Tolypella*

Introduction

Charophytes are large, parenchymatous green algae (Fig. 1) that grow in fresh and occasionally in brackish water. Although less common than aquatic bryophytes or tracheophytes, charophytes are the ecologically dominant benthic macrophyte in some habitats. Charophytes are relatively large for green algae and can grow up to a half meter or more in height, in lush meadows and freshwater ponds, lakes, and flowing waters. The term "charophyte" used here applies to members of the order Charales, which contains extant and fossil taxa (Feist et al. 2005).

Charophytes have a complex thallus with an upright, stemlike main axis punctuated by whorls of branchlets (Fig. 1). Rhizoids anchor the thallus in sandy or muddy substrates. Thalli of some genera (notably *Chara*, *Lamprothamnium*, and *Tolypella*) accumulate calcium carbonate externally and have a musky odor. Worldwide in distribution and occasionally abundant in submerged areas, these macroscopic green

Fig. 1 Charophyte thallus morphology. (**a**) *Chara drummondii*; (**b**) *Nitella haagenii*; (**c**) *Lamprothamnium macropogon*; (**d**) *Tolypella polygyra*. (M. T. Casanova)

algae have been well studied for several centuries by biologists (e.g., Allen 1888, 1889; Braun and Nordstedt 1882; Corillion 1972) .

Six extant genera in the family Characeae and order Charales are recognized; two additional orders and a large number of genera and families are known from the fossil record (Table 1). Two extant genera are common: *Chara* and *Nitella*, each with several hundred species. Of the other four genera, *Tolypella* and *Lamprothamnium* are the most common and diverse, with approximately 16 and 7 species, respectively. A monograph by Wood and Imahori (1965) synonymized numerous species in *Chara* and *Nitella* and recognized only 19 species in *Chara* and 50 in *Nitella*, with numerous subspecific taxa. Wood's taxonomic arrangement has not been widely adopted due to experimental work showing that the earlier taxonomy reflected

Table 1 Classification of green algae commonly known as charophytes (Modified from Feist et al. (2005) and Lewis and McCourt (2004))

Kingdom Chlorobionta
Division Charophyta[a]
Class Charophyceae Smith 1950
[b]Order Moellerinales Lu, Soulié-Märsche and Wang 1996
[b]Order Sycidiales Mädler 1952 em. Feist et al. 2005
Order Charales Lindley 1836
[b]Family Eocharaceae Grambast 1959
[b]Family Palaeocharaceae Pia 1927
[b]Family Porocharaceae Grambast 1962
[b]Family Clavatoraceae Pia 1927
[b]Family Raskyellaceae L. & N. Grambast 1955
Family Characeae Agardh 1824
Chara Linnaeus 1753
Lamprothamnium Groves 1916
Lychnothamnus (Ruprecht 1845) von Leonhardi 1863
Nitellopsis Hy 1889
Nitella Agardh 1824
Tolypella Braun (1849) Braun 1857

[a]This division includes several other lineages of green algae plus embryophytes
[b]Extinct

species-level differences in terms of reproductive isolation (e.g., Grant and Proctor 1972; Proctor 1975; McCracken et al. 1966). Therefore, earlier species names are commonly used in the current literature.

The charophyte thallus is composed of basal rhizoids, with an upright axis consisting of alternating internodes and nodes. Due to their large size and apparent complexity, charophytes may be mistaken for bryophytes or certain aquatic angiosperms (e.g., *Ceratophyllum*) in the field. Charophytes lack the diploid sporophyte generation and multicellular embryos of bryophytes and vascular plants (Graham and Wilcox 2000). However, their plantlike structure, complex asymmetric sperm, and large, protected egg cells (described below) led earlier workers to see them as intermediate in complexity between green algae and embryophytes (Bold and Wynne 1978; Smith 1950). This intermediate position was clarified by molecular studies that show charophytes to be one of several groups of green algae that are more closely related to land plants than they are to the rest of the green algae (Karol et al. 2001; Lewis and McCourt 2004; McCourt et al. 2004; Turmel et al. 2006).

Charophytes fulfill a number of ecological roles in both permanent and temporary water bodies (van den Berg et al. 1998). They are primary producers, sometimes the dominant photosynthesizers in aquatic ecosystems (Schwarz et al. 1999; Porter 2007). Stands of charophytes provide habitat for epiphytic algae, invertebrates (Hawes and Schwarz 1996; James et al. 1998), and structural refuges for zooplankton (Kuczyńska-Kippen 2007) and juvenile vertebrates (fish and frogs). Charophytes

are often early colonizers and water clarifiers (Casanova et al. 2002), and they are directly consumed by a number of arthropods (beetles, amphipods; Proctor 1999) and snails (Elger et al. 2004), fish (Lake et al. 2002), and water birds (Schmieder et al. 2006). In deep lakes they can be the deepest growing plants (Dale 1986; Schwarz et al. 1999). Charophyte communities in temporary wetlands are commonly species rich (Casanova and Brock 1999a) occurring as individual plants (Casanova and Brock 1999b), although monospecific, continuous "beds" or "meadows" are also common (Stross et al. 1988; Pelechaty et al. 2010).

Charophyte life histories are haplobiontic, with one free-living haploid vegetative phase. After meiosis in the zygote, the haploid oospores germinate and produce a protonema, which differentiates into axes, branchlets, and rhizoids at the first node. The rhizoids grow downward (Kiss and Staehelin 1993), anchoring the thallus axis in the sediment, and the axes grow upward (Andrews et al. 1984). Sexual reproduction may be initiated either as soon as possible after germination (e.g., *Chara muelleri,* Casanova and Brock 1999a) or in response to environmental cues (e.g., *Chara australis,* Casanova 1994). Life histories can be annual or perennial (Casanova and Brock 1999a), with annual species most frequently occurring in habitats subject to periodic drying (Blindow 1992a, b) or freezing (Schwarz et al. 1999). However, some species (e.g., *Chara braunii*) with an annual life history occur in areas exposed to long-term flooding (Casanova and Brock 1999b).

Practical applications for charophytes include management of water quality (through encouragement of charophyte colonization) and as an indicator of water regime requirements in riparian and wetland ecosystems (Casanova 2011). Because their large cells are easy to observe and manipulate, charophytes have been useful as model organisms for studies of cell membrane potential and cytoplasmic streaming (Tazawa et al. 1987; Tazawa and Shimmen 2001; Raven and Brownlee 2001; Yamamoto et al. 2006).

Habitats and Ecology

Charophytes are primarily freshwater plants, but they are occasionally abundant in brackish areas, both in contemporary habitats (Shepherd et al. 1999) and in ancient ones, as shown in the fossil record (Soulié-Märsche 1999; 2008). These algae usually occur in quiet or gently flowing waters, from very shallow (several cm) to deep (>10 m (30 m in clear lakes such as Tahoe)), so long as light and oxygen levels are adequate. Some have been found in swiftly flowing rivers (personal obs.), but such occurrences have been rarely noted in the literature. Habitats are typically alkaline (hard water), although some species are known from mildly acidic waters. Rhizoids are usually anchored in sandy substrates mixed with gravel. The upright portions of plants are buoyant and exhibit a characteristic whorled pattern when viewed from above (Casanova 2009).

The family Characeae, which contains all living charophytes, is worldwide in distribution, but individual species range from restricted endemics to broadly

distributed taxa. In general, dioecious taxa are narrowly distributed or endemic, whereas monoecious taxa are usually widely distributed (Proctor 1980).

Autecological studies predominated in the early literature, in which species distributions were characterized by environmental parameters (e.g., temperature, light, depth, water quality characteristics) (Hutchinson 1975). Interspecific interactions have not been investigated as thoroughly, but competitive effects of vascular plants and algae on charophytes have been suggested (Stross 1979; Stross et al. 1988). In fact, Martín-Closas (2003) hypothesized that charophytes dominated freshwater floras after the Permian until angiosperms evolved and came to dominate freshwater habitats from the Lower Cretaceous until the present.

Nutrients are absorbed by charophytes through their rhizoids and photosynthetic thallus (Kufel and Kufel 2002), and charophyte communities can be a significant store of nitrogen in small water bodies (Rodrigo and Alonso-Guillén 2008). Uptake by charophytes removes nutrients from the water column that would otherwise be available for growth of other algae (van den Berg et al. 1998; Siong et al. 2006). In addition, some species have an allelopathic effect on the growth of certain microalgae (Blindow and Hootsmans 1991; Pakdel et al. 2013). Early reports by Forsberg (1964) that even low concentrations of phosphorus were toxic to charophytes have not been supported by subsequent studies (Blindow 1988) and the decline of charophytes following eutrophication can be explained largely by decreases in water clarity and competition with angiosperms (Blindow 1992a).

Susceptibility or resistance to predation has been shown to determine the presence or absence of charophytes in various permanent and ephemeral habitats (Mann et al. 1999; Proctor 1999). A number of studies have investigated the marked zonation of charophytes in lakes, a pattern where charophytes grow in a discrete band with distinct upper and lower depth limits. Studies have invoked light, competition, and herbivory as the controlling factors that set the depth limits (Schwarz et al. 1999, 2000).

Charophytes are well adapted to the submerged aquatic environment. For example, the evolutionary significance of the multinucleate giant cells of charophytes has been explained as a shade-tolerance adaptation (Raven et al. 1979) by which cytoplasmic streaming in giant cells of charophytes optimizes transport of nutrients to various parts of the thallus, analogous to the phloem-like system of transport that evolved in kelps or other large algae.

Characterization and Recognition

The charophyte axis has a distinctive node-internode structure. Internodes consist of so-called giant cells, which are multinucleate. Nodes comprise several, smaller, uninucleate cells that give rise to whorls of leaflike organs of limited growth called "branchlets," and secondary axes (branches of unlimited growth), which also exhibit the node-internode construction. A single apical meristematic cell occurs on each axis tip, the latter exhibiting a pattern of growth and branching similar to the apical meristem of higher plants (Fig. 2) (Cook et al. 1998; Pickett-Heaps 1975; Clabeaux

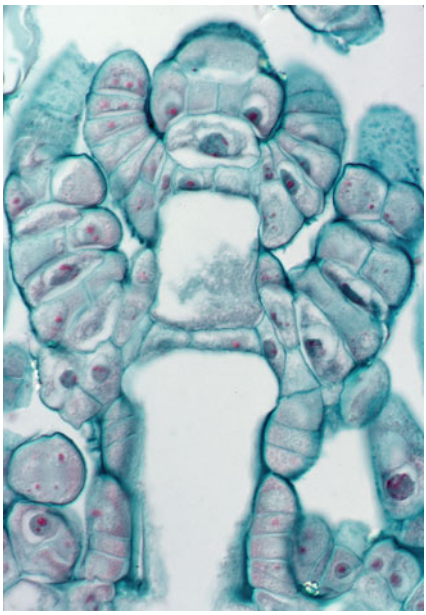

Fig. 2 Apical meristem of *Chara*, longitudinal section. Large intermodal cells show a clear, central vacuolar region; lateral branchlets arise from peripheral cells at nodes. (Photograph courtesy of Dr. Martha Cook, from a specimen from Ward's Natural Science)

and Bisson 2009). Internodes are composed of giant cells, which are multinucleate with numerous ellipsoidal plastids distributed in the cytoplasm surrounding a large central vacuole. The cytoplasm streams actively lengthwise around the cell periphery. Internodal cells may be naked or covered by a single-celled layer of thin cortical cells that grow upward and downward from nodal cells to cover the internodes. Some corticating cells project outwardly as spines. Cortication is common among species of *Chara*, incomplete in the rare genus *Lychnothamnus*, and absent in *Lamprothamnium, Nitella*, *Nitellopsis*, and *Tolypella*. Shape and numbers of ranks of cortical cells are important in delineating species in *Chara* (Wood and Imahori 1965; Casanova 2005).

Nodes consist of several uninucleate cells produced through cytokinesis of 1–3 central cells that give rise to a series of peripheral cells (Cook et al. 1998), with adjacent cells connected by true plasmodesmata. These peripheral cells are initials that give rise to branchlets 3–10 cells in length or to secondary axes that exhibit the node-internode structure of the main axis. Peripheral cells are also the initials for the cortical cells and for stipulodes. Stipulodes are single cells that subtend branchlet whorls at nodes. They may be short and blunt or long and tapering, and they occur in one or two tiers. Stipulodes are present in *Chara*, *Lamprothamnium*, and *Lychnothamnus* in the tribe Chareae and absent in *Nitellopsis* and the Nitelleae (see "Classification" section below).

Growth occurs through division of an apical cell at the tips of the main axes or secondary branches. A single cutting face of the apical cell produces an alternation of internodal cells and nodal initials. The nodal initials develop into the nodes through the cytokinetic pattern described above. While the apical region in charophytes

(Fig. 2) is superficially similar to the apical meristems of higher plants (Pickett-Heaps 1975; Clabeaux and Bisson 2009), the single cutting face is simpler than meristematic development in higher plants (Cook et al. 1998). Cook et al. (1998) interpreted the presence of plasmodesmata and pattern of cytokinesis as a parenchymatous organization of nodal tissue. In this interpretation the internodes and cortical cells are filamentous in construction, whereas the nodes are parenchymatous plates, similar to the earliest histogenetic tissues of plant apical meristems. Homology of these tissues in Charales and higher plants is open to question.

Branchlet morphology differs greatly among genera (Fig. 1). *Chara, Lamprothamnium*, and *Lychnothamnus* produce whorls of branchlets that are essentially monopodial and do not branch dichotomously. Branchlets in *Nitella* are generally not monopodial, and they bifurcate one or several times at the nodes. *Tolypella*, the third common genus, exhibits clusters of branchlets and stalked reproductive structures in clusters at nodes that have the appearance of a bird's nest.

Asexual reproduction occurs through growth of erect axes from nodes on the rhizoids, and through contracted starch-filled branches (Casanova et al. 2007; Casanova 2009), and tubercular, starch-filled outgrowths of the rhizoids called bulbils (Fritsch 1948; Casanova 1994), which may fall away and germinate separate from the thallus.

Sexual reproduction is oogamous. Oogonia and antheridia are the female and male gametangia, respectively, which include gamete-producing cells and associated vegetative cells. Each oogonium contains a single large egg cell, whereas sperm are produced in filaments with numerous antheridial cells, packed inside a spherical antheridium (Pickett-Heaps 1975; Graham and Wilcox 2000). Smith (1950) interpreted the oogonia and antheridia as single-celled structures, each within a larger structure of modified sterile vegetative filaments. He and some authors used the terms "globule" for the male and "nucule" for the female sexual structures, although the more common terms used are antheridia and oogonia, or oosporangia, respectively. Oogonia and antheridia occur on the branchlets at nodes and may be associated with small sterile cells and can be enveloped in mucous. The oogonia are oblong, 200–1000 μm long by 200–600 μm wide. Sexual structures are easily visible with a hand lens or even with the naked eye. Thalli may be dioecious or monoecious. In monoecious species, the two kinds of reproductive structure may occur at the same node (conjoined) or different nodes (sejoined) on the same branch. Sexual structures are relatively easy to remove for experimental crossing studies of monoecious and dioecious species (McCracken et al. 1966; Grant and Proctor 1972).

The egg is surrounded by five jacket cells that spiral in a left-handed (sinistral) twist from the base to the apex, which consists of one or two tiers of cells that form a corona (Fig. 3). The Chareae have one tier of coronal cells, the Nitelleae two. The mature oospore (Fig. 4) displays a basal pentagonal cell and in some genera one or two additional basal cells.

Male antheridia are spherical and range from 200 to 1500 μm in diameter, often bright orange in color. The outside of the antheridium is composed of four or eight shield cells, inside of which is a cluster of modified multicellular filaments, each cell of which produces one sperm. Sperms have two flagella attached slightly below the

Fig. 3 Apex of *Chara* oogonium (female sexual structure) with single tier of five coronal cells. Note spiral jacket cells and transparent sperm swimming around apex. (M. E. Cook)

apex of an asymmetric, helically twisted cell reminiscent of sperm cells in mosses and liverworts (Renzaglia and Garbary 2001).

Sperm cells are liberated when the shield cells separate. Sperm gain access to the egg cells through slits between jacket cells near the apex of the globules (Fig. 3). The zygote and inner jacket cell walls thicken, and the outer parts of the jacket cells fall away leaving an oblong, spiral-embossed spore, which may germinate immediately or go through a period of dormancy (Casanova and Brock 1996). Upon germination, a main axis and a rhizoidal initial are produced, which develop into the mature thallus (Fritsch 1948).

The Characeae possess large chromosomes (Fig. 5) that are relatively easy to stain and count during mitotic cell divisions (Casanova 1997). Young antheridia provide the best material for chromosome observation, but rhizoid squashes can also be successful. Chromosome numbers vary widely in all genera. Counts between 8 and 77 have been published as observed values within *Chara* and *Nitella* (Guerlesquin 1984; Bhatnager 1983). On the basis of this multiplicity of published numbers, both Bhatnager (1983) and Guerlesquin (1984) have attempted to identify the basic or ancestral chromosome numbers for the group. Not surprisingly, the plethora of reported chromosome counts has resulted in basic chromosome number(s) for Characeae of 3, 5, 6, 7, 8, or 11. Grant (1990) hypothesized that a single base, or ancestral, number of $n = 14$ is adequate to explain all extant chromosome numbers in the genus *Chara* and that aneuploidy is either extremely rare or absent. He noted that reported chromosome numbers in *Chara* were invariably multiples of 14, i.e., 14, 28, 42, or 56, in natural populations. Estimates of chromosome numbers in *Nitella* range from 3 to 27, almost invariably multiples of 3 or 9, so the basic

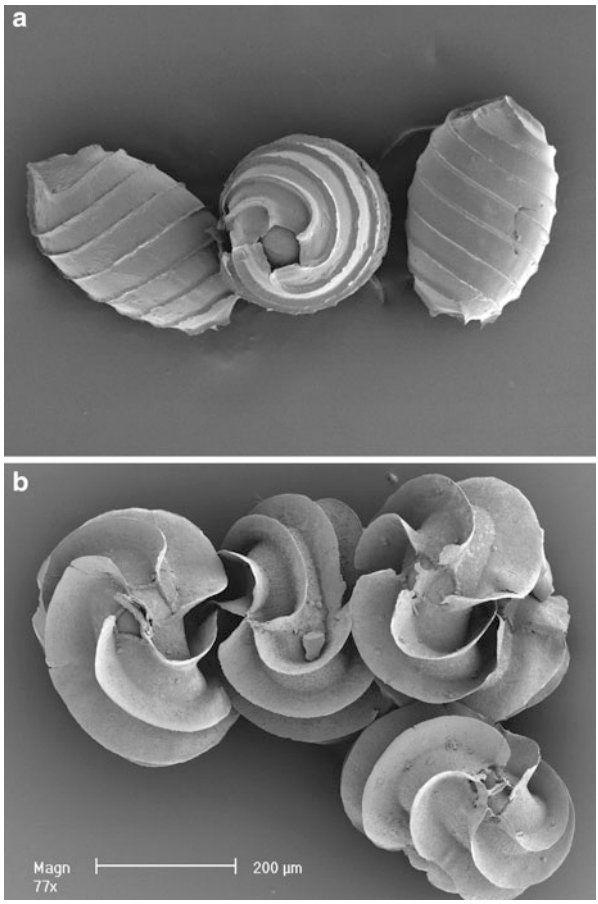

Fig. 4 SEM images of oospores of Characeae showing single-celled (**a**) and two-celled (**b**) basal plate. (**a**) *Chara muelleri*. (**b**) *Nitella sp*. Specimens of both collected from western Victorian swamps, Australia. (M. T. Casanova)

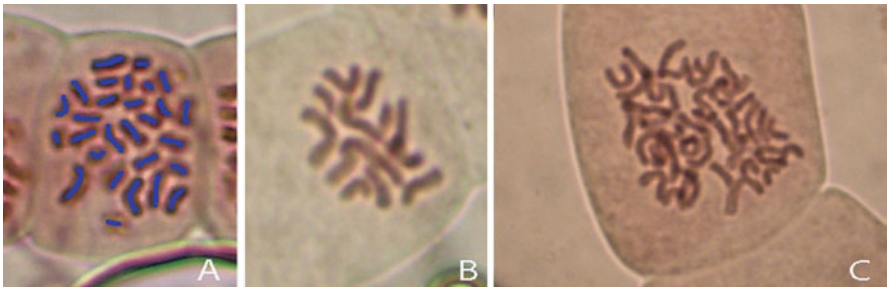

Fig. 5 Metaphase mitotic chromosomes of *Chara, Nitella,* and *Lamprothamnium*. (Photograph courtesy of Michelle Casanova). (**a**) *Nitella leonhardii*, n = 28. (**b**) *Lamprothamnium inflatum*, n = 14. (**c**) *Chara globularis,* n = 42. (M. T. Casanova)

chromosome number is likely to be 3. Grant (1990) also argued that the cytogenetic mechanism and evolutionary history of this group cannot be well understood until chromosome numbers are established and that chromosome counts must be stable and correlated with biological species and not the result of an aberrant cell division product. Further karyotypic work on the Characeae is clearly needed. Grant and Proctor (1972, 1980) postulated that polyploidy is adaptive as a mechanism for producing (and masking harmful) genetic variation in self-fertilizing monoecious species, in contrast to dioecious species, which generally possess half the number of chromosomes. In dioecious species, genetic variation maintained through outbreeding may enhance survival in habitats that vary from one generation to the next (e.g., in temporary wetlands), and in polyploid monoecious species, variation in enzyme activity (through multiple copies of enzymes) is likely to enhance survival during the life of a single plant or population (e.g., in permanent habitats) (Casanova 1997). Grant and Proctor (1972, 1980) suggested that sexual reproduction functions as a mechanism of dispersal and drought avoidance in addition to its role in genetic recombination.

Classification

The genus *Chara* was erected by Vaillant in 1719 for several living species of this genus and formally recognized by Linnaeus (1753) as one of several genera of algae. Understanding of the relationship of the Charales to other green algae and land plants has undergone considerable revision in recent years (reviewed in McCourt et al. 2004; Becker and Marin 2009). The relatively complex morphology and reproduction of charophytes has been long known and led Smith (1950) and others (Margulis et al. 1990, in the first edition of this book) to view the group as a class (Charophyceae) separate from the rest of the green algae (Chlorophyceae). Some workers preferred to elevate the group to division status (e.g., Charophyta of Bold and Wynne 1978). Research on cell ultrastructure and flagellar insertion (Mattox and Stewart 1984), along with molecular phylogenetic studies (McCourt et al. 1996, 1999; Meiers et al. 1999; Karol et al. 2001; Sakayama et al. 2002; 2004a, b, 2005a, b), supported the monophyly of extant members of the group, regardless of rank. In addition, the monophyly of the fossil and extant members of the Charophyceae is well supported (Feist et al. 2005). Figure 6 depicts a consensus molecular phylogeny for the genera of extant Charales (Karol et al. 2001) and also shows the occurrence record of fossils for the major lineages since the origin of the group in the Silurian (dates from Feist et al. 2005). Note that some sister lineages of the extant Charales occur much earlier in the fossil record (Early Devonian) but have since become extinct.

Feist et al. (2005) summarized the history of classification of the charophytes and proposed a classification including both fossil and extant taxa in the phylum (=division) Charophyta, with the single class Charophyceae. Living charophytes are included in the family Characeae in the order Charales, along with five families of extinct taxa known primarily from fossil spores (gyrogonites), with few vegetative

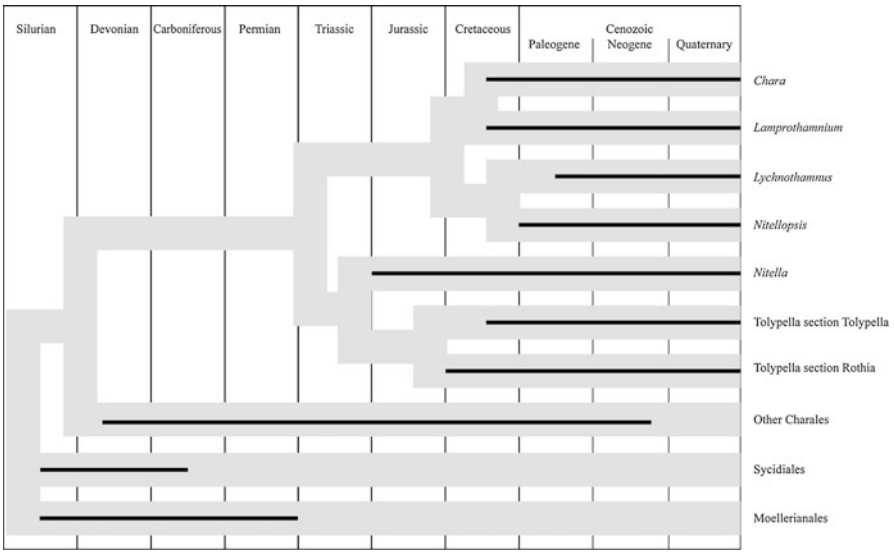

Fig. 6 Phylogenetic relationships of genera in the Charales, and ranges of fossil ages of extant genera and several extinct taxa. The black bars indicate the ages of the earliest known fossils for taxa, as well as fossil ages for extinct taxa in the Charales and the extinct Orders Sycidiales and Moellerinales. Relationships of extant taxa based on molecular phylogenetic studies (McCourt et al. 1999; Meiers et al. 1999; Karol et al. 2001). Fossil ages and phylogenetic relationships of fossil taxa based on Feist et al. (2005). (R. M. McCourt and J. D. Hall)

thalli in the fossil record (but see Kelman et al. 2004). Two additional orders of fossil taxa (Fig. 6) are also included in the Charophyceae (Feist et al. 2005).

Lewis and McCourt (2004) proposed a classification of green algae that assigned extant charophytes to the class Charophyceae in a clade containing several other orders of green algae plus embryophytes or land plants. A separate clade comprises the remaining members of the traditional Chlorophyta. This division of the green algae into two evolutionary lineages, one of which contains several smaller groups (► Chlorokybophyceae, Klebsormidiophyceae, Coleochaetophyceae, by Cook and Graham) and the other larger clade of conjugating green algae (► Zygnematophyta, by Hall and McCourt), was originally based on ultrastructural morphology of flagellar roots and types of mitosis, as well as features of glycolate metabolism (Mattox and Stewart 1984). The hypothesis of two major clades has been strongly supported by molecular data (McCourt et al. 1996; Karol et al. 2001; Becker and Marin 2009). In this scheme, the green algae *sensu lato* do not constitute natural group, and some green algae are clearly more closely related to embryophytes than to other green algae (i.e., Charophyta sensu Karol et al. 2001). A classification of charophytes of this chapter is shown in Table 1.

The evolutionary relationship of Charales to embryophytes remains unresolved (Graham 1993; Lewis and McCourt 2004; Turmel et al. 2006; Becker and Marin 2009). Karol et al. (2001) performed a phylogenetic analysis on a broad sample of

35 green algae and embryophytes using four genes (two plastid, one mitochondrial, one nuclear, ~5000 bp) and found strong support for the hypothesis that the Charales are the sister group (i.e., closest living relatives) of land plants. This hypothesis has been challenged by a study of entire plastid genomes from a smaller number of green algae and land plants (Turmel et al. 2006). The latter study used 76 genes from the complete plastid genomes of nine green algae and embryophytes (~48,000 nt) and found strong support for the hypothesis that conjugating green algae (see ► Zygnematophyta) constitute the sister group to embryophytes. These alternate hypotheses of the embryophyte sister taxon would lead to very different sets of assumptions about the common ancestor of embryophytes and their nearest green algal relative, since the Zygnematophytes are simpler in morphology and reproduction than the charophytes and lack mastigote cells entirely (McCourt et al. 2004). Some of the implications of the sister status of Zygnematophytes and embryophytes are explored in Wodniok et al. (2011).

Additional data with more taxa and more sequence data (including organellar genome data) may resolve this interesting question with significant implications for the evolution of land plants and the origins of their adaptations to a dry habitat.

Maintenance and Cultivation

Charophytes present some unique challenges for cultivation due to their size, life cycle, and, in some cases, dioecy. The erect thallus and rhizoid system often require larger culture vessels (liter sized or more) for the development of adult morphology. The effort needed for culturing charophytes depends on the uses to which they will be put and the length of time the cultures will need to be maintained. Short-term cultures for physiological studies (Beilby and Shepherd 2006), chromosome assessments (Casanova 1997), teaching exercises, or morphological studies (Casanova 2009) can be simply obtained from field-collected material kept in rainwater on a windowsill. Longer term cultures for genetic vouchers or clonal reproductive studies are more difficult to maintain. Because epiphytes are frequent, axenic cultures are difficult to establish from vegetative material. Unialgal cultures (i.e., with a single species of eukaryotic algae and possible bacterial contamination) can be obtained through germination of surface sterilized spores in defined media. However, material for microscopic observation and molecular studies can usually be obtained from branch tips that are relatively free of epiphytes.

Proctor (personal communication) developed a successful means of growing what he termed “clones” (isolates from single vegetative thalli or oospores) in seminatural conditions in a greenhouse. In this method, wide-mouth one-gallon (3.8 L) glass jars are filled to a depth of 3 cm with autoclaved or steam-sterilized alkaline sandy-loam soil. Jars with sandy loam are filled with steamed or filter sterilized water free of chlorine and metal residue from copper pipes. Field-collected sprigs of vegetative branches brushed or manually cleaned of epiphytes are then planted in the sandy loam using clean large forceps or gloved hands. After several weeks, it will be apparent if the sprig has successfully anchored itself in the sediment

with rhizoids. Epiphytes or algal cells associated with the field-collected sprigs may infest some cultures, but the Proctor reported (personal communication) that these often die back without any special treatment. While not always successful, this technique can yield long-lived (>20 years) clonal cultures that require little more than indirect sunlight on a window sill. Such cultures are readily used in the classroom.

The National Institute for Environmental Studies in Japan (NIES; http://mcc.nies.go.jp/) has reported success in growing charophytes in defined media and provided illustrated instructions on culture methods. Watanabe (2005) has also provided methods for *ex situ* cultivation of threatened algal species and included media for Charales.

Evolutionary History

The Charales are exceptional among green algae in having an extensive fossil record, rivaled only by the Dasycladales of the Ulvophyceae (Berger and Kaever 1992; Taylor et al. 2009). Some taxa of both groups deposit calcium carbonate as part of the thallus, which facilitated formation of fossils of vegetative and, in particular, reproductive structures (spores). In Charales, the oogonium is often enveloped by a calcium carbonate "shell" (most *Nitella* and some *Chara* species are exceptions). Fossils resulting from these types of reproductive structures are called gyrogonites, which are often more elaborate in structure than spores of extant Characeae (Fig. 7). Gyrogonites range in size from a few hundred μm to several mm in size. Although some vegetative thalli may be calcified, such as the well-known *Paleonitella* found in the Early Devonian Rhynie chert (Kidston and Lang 1921), gyrogonites are much

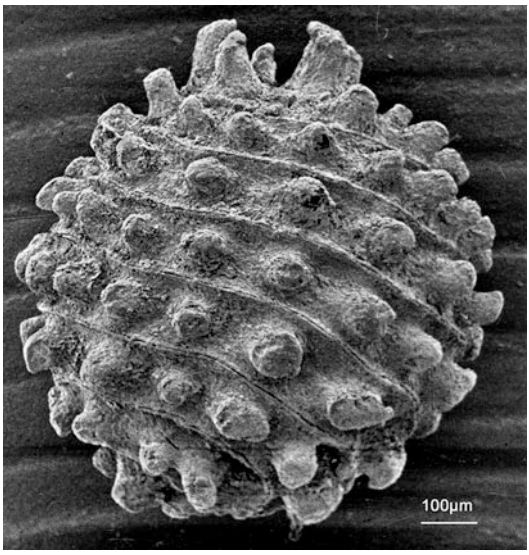

Fig. 7 Gyrogonite of *Maedleriella angusta* Feist-Castel, a species from the Middle Eocene of Southern France (From Feist-Castel 1972)

more common in the fossil record. Therefore, gyrogonite morphology is the basis for most of the taxonomy and stratigraphy of fossil Charales (Feist et al. 2005).

Morphology of gyrogonites provides a rich source of data: shape, dimensions, apical structure, presence and absence of pores, morphology of membranous coverings that occur in some groups, occurrence of a variety of bumps, tubercles, or other ornamentations on the outer surface (Feist et al. 2005). The earliest gyrogonites from the late Silurian and Early Devonian exhibit greater morphological variation than oospores or more recent or extant taxa. The pattern of spiraling of the jacket cells (also called spiral cells) apparently reversed in the Early Devonian (ca. 370 mya) from dextral to sinistral, and the number of jacket cells decreased over time such that all extant taxa now have five sinistral jacket cells, although occasionally spores with six cells are found (M. Casanova, personal observation).

Between the upper Silurian and the present day, charophytes have gone through several periods of diversification and extinction (Grambast 1974). Diversity was greatest during the Devonian, with a secondary peak in diversity in the Late Jurassic and Early Cretaceous (Feist et al. 2005). Since the Miocene, diversity has declined (Grambast 1974) so that only a single family (Characeae) with six genera survives today. Feist et al. (2005) provided a comprehensive overview of the fossil record and evolutionary history of the group.

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Chlorokybophyceae, Klebsormidiophyceae, Coleochaetophyceae

6

Martha E. Cook and Linda E. Graham

Abstract

The freshwater and terrestrial green algal lineages discussed in this chapter include the scaly flagellate *Mesostigma*, the sarcinoid form *Chlorokybus*, the unbranched filamentous members of the Klebsormidiophyceae, and the branched filamentous members of the Coleochaetophyceae. The lineages discussed here, together with two other green algal lineages (Charophyceae and Zygnematophyceae) and the land plants (embryophytes), form a monophyletic group known as Streptophyta or Charophyta. The streptophyte algae share cytological and biochemical characteristics with plants and may shed light on the evolution of plant features. Of special interest is the evolution of mechanisms associated with the transition from freshwater to dry land, a topic currently being energized by whole-genome analyses. Metagenomic studies of these organisms have revealed surprising features that might also have characterized the microbiomes of early streptophytes.

Keywords

Charophycean algae • Charophyte • *Chlorokybus* • *Coleochaete* • *Entransia* • *Klebsormidium* • *Mesostigma* • Plant evolution • Streptophyte • Terrestrial algae

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Summary Classification

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●●**Mesostigmatophyceae** (*Mesostigma*)
●●**Chlorokybophyceae** (*Chlorokybus*)
●●**Klebsormidiophyceae** (e.g., *Klebsormidium*, *Entransia*)
●●**Coleochaetophyceae** (e.g., *Coleochaete*, *Chaetosphaeridium*)

Introduction

The green algal lineages discussed in this chapter include simple freshwater or terrestrial forms distinguished by their close relationship with land plants (embryophytes). The clade Streptophyta (also known as Charophyta) includes embryophytes along with their close green algal relatives, known informally as streptophyte algae or charophyte algae (Bremer 1985; Lewis and McCourt 2004). These algae were originally termed charophycean algae (Mattox and Stewart 1984). The orders Chlorokybales, Klebsormidiales, and Coleochaetales were placed in the class Charophyceae by Stewart and Mattox (1975; Mattox and Stewart 1984) on the basis of a distinctive set of cytological and biochemical characters. These algae form flagellate reproductive cells that are rather similar to spermatozoids of land plants in ultrastructural details. Neither these reproductive cells nor plant spermatozoids have eyespots (which are often present in green algal swarmers). Both have a multilayered structure (MLS) associated with their flagellar apparatus. However, unlike land plant sperm but like motile cells of certain other green algae, charophycean swarmers are typically covered with many small, square scales and

bear similar scales as well as hairs on the flagella. At cytokinesis, members of these charophycean orders have a persistent interzonal spindle, and the genera *Coleochaete* and *Chaetosphaeridium* produce a phragmoplast and cell plate similar to those of land plants (Brown et al. 1994; Cook 2004a; Doty et al. 2014; Marchant and Pickett-Heaps 1973). Streptophyte algae also have peroxisomes and photorespiratory enzymes similar to those of land plants (Frederick et al. 1973). For these and other reasons, streptophyte algae (including the lineages that are the subject of this chapter as well as Zygnematophyceae and Charophyceae (orders Zygnematales and Charales of Mattox and Stewart (1984); see ▶ Charophyceae (Charales) and ▶ Zygnematophyta)) are believed to be closer to the ancestors of land plants (embryophytes) than are other green algae. Cytological (Melkonian 1989; Rogers et al. 1981), biochemical (Iwamoto and Ikawa 2000; Schwender et al. 2001), and molecular (Kim et al. 2006; Nedelcu et al. 2006; Petersen et al. 2006; Rodríguez-Ezpeleta et al. 2007; Simon et al. 2006) evidence indicates that the former prasinophyte *Mesostigma viride* is also a member of the streptophyte algae.

Mesostigma viride Lauterborn is the only member of the Mesostigmatophyceae (Lewis and McCourt 2004; Marin and Melkonian 1999). Likewise, the only member of the Chlorokybophyceae is the single species *Chlorokybus atmophyticus* Geitler (Bremer 1985). Klebsormidiophyceae includes the species *Entransia fimbriata* Hughes (Karol et al. 2001; McCourt et al. 2000; Sluiman et al. 2008; Turmel et al. 2002), the species *Hormidiella attenuata* Lokhorst (Lokhorst et al. 2000; Sluiman et al. 2008), and *Klebsormidium* (Silva et al. 1972) with approximately 20 species currently described (Lokhorst 1996; Novis 2006; Rindi et al. 2008; Škaloud 2006; Sluiman et al. 2008). In addition to these filamentous klebsormidialian forms, some recent studies have indicated that the genus *Interfilum* Chodat, which includes three species composed of unicellular aggregations or cell packets, nests within the genus *Klebsormidium* (Mikhailyuk et al. 2008, 2014; Novis and Visnovsky 2011; Rindi et al. 2011), a condition that will likely require renaming of at least some species of *Klebsormidium* (Novis and Visnovsky 2011; Rindi et al. 2008). Coleochaetophyceae encompasses *Chaetosphaeridium* Klebahn with six species (Thompson 1969) and *Coleochaete* de Brébisson with approximately 20 species currently described (Cimino and Delwiche 2002; Delwiche et al. 2002; Printz 1964). Additional genera are hypothesized to belong to Klebsormidiophyceae and Coleochaetophyceae but have not been sufficiently well studied for accurate systematic placement at this time. The lineages of green algae discussed in this chapter are generally of no particular economic importance. However, in view of their close relationship to land plants, they may well provide excellent simple systems for the study of more complex physiological and reproductive processes of plants, including the transition of aquatic algal ancestors to dry land (Bowman 2013; Delwiche and Cooper 2015; Graham 1984, 1985, 1993, 1996; Graham and Kaneko 1991; Graham et al. 2000).

Habitats and Ecology

The genera included here occupy a variety of freshwater and terrestrial habitats. *Mesostigma viride* is a freshwater flagellate. The sarcinoid form *Chlorokybus atmophyticus*, as its name indicates, has been isolated from terrestrial habitats (Škaloud 2009). *Klebsormidium* travels through the air and occupies diverse freshwater and terrestrial habitats, including biological soil crusts (Mikhailyuk et al. 2015) and urban settings, especially at the base of humid walls and in the shade of tall chimneys (Rindi et al. 2008). Some species of *Klebsormidium* occur in acid mine drainage environments (Brake et al. 2014; Novis 2006) and have been proposed for use in removing metals from these environments (Orandi and Lewis 2013). Surveys of biodiversity within *Klebsormidium* reveal a correlation between phylogeny and particular types of habitat (Mikhailyuk et al. 2015). However, attempts to distinguish different lineages of *Klebsormidium* based on morphology alone have not been successful because some features (e.g., the ease of filament fragmentation) can vary with differing environmental conditions, including light, temperature, humidity, and growth medium (Rindi et al. 2008; Škaloud 2006; Škaloud and Rindi 2013). *Interfilum* occurs on soil, including alpine soil crusts and outcrops (Mikhailyuk et al. 2008; Novis and Visnovsky 2011). *Hormidiella attenuata* has been cultured from the surface of nutrient poor soil in a xeromorphic forest and in a sugar cane field (Lokhorst et al. 2000). *Entransia fimbriata* has been collected near the edge of a small lake formed by damming a river (Hughes 1948) and from a *Sphagnum* bog, in the extension of the lagg into the mat (Cook 2004b). *Coleochaete* and *Chaetosphaeridium* are generally attached to macrophytes or inorganic substrates in nearshore ponds and lakes.

Terrestrial members of the algal lineages discussed here have adapted to the stresses of life on land, including desiccation and higher levels of UV light. The terrestrial form *Chlorokybus* has UV-absorbing amino acids not present in the aquatic *Mesostigma* (Jobson and Qiu 2011). Similarly, aquatic *Entransia fimbriata* lacks UV-absorbing amino acids present in other members of the Klebsormidiophyceae, which may occur in aeroterrestrial habitats (Kitzing and Karsten 2015). Furthermore, the UV-absorbing amino acids of *Klebsormidium* and *Interfilum* have an identical absorption peak that is only slightly different from that of *Hormidiella* (Kitzing and Karsten 2015). These results are consistent with the topology of Mikhailyuk and coauthors (2008), in which *Interfilum* nests within *Klebsormidium*, *Hormidiella* is the sister group to *Klebsormidium*, and *Entransia* is the first to diverge in that lineage. Strains of *Klebsormidium* that occur in alpine soil crust resist desiccation by maintaining a high solute level (Kaplan et al. 2012) and in some cases by maintaining turgor pressure with flexible cells walls that buckle (Holzinger et al. 2011). Even when plasmolyzed, these taxa exhibit little change in cellular structure (Holzinger et al. 2011; Kaplan et al. 2012; Karsten and Holzinger 2014). *Interfilum* is more resistant to desiccation when forming packets of cells than

when in the form of single cells protected by mucilage, presumably due to surface area to volume ratio (Karsten et al. 2014). Likewise, when grown on agar or on quartz sand in a humid environment, some species of *Coleochaete* form packets or clumps of cells similar to *Chlorokybus* or *Interfilum*, rather than the flat discs that occur in a typical aquatic habitat (Graham et al. 2012). Additionally, in such an aeroterrestrial environment, the cell walls produce autofluorescent compounds and are resistant to degradation (Graham et al. 2012).

Characterization and Recognition

Morphology

Mesostigma (Fig. 1a) is a scaly unicellular flagellate with a prominent eyespot (stigma) located in the center of the chloroplast near the flagellar basal bodies (Rogers et al. 1981). The chloroplast includes two prominent pyrenoids surrounded by starch (Manton and Ettl 1965) and several superficial pyrenoids (Rogers et al. 1981). *Chlorokybus* (Fig. 1b) occurs as packets of cells (a sarcinoid thallus). Each cell has a single chloroplast with a prominent pyrenoid surrounded by a starch sheath and a peripheral superficial pyrenoid (Rogers et al. 1980). Members of the Klebsormidiophyceae (Figs. 1c–e) are either packets of cells (*Interfilum*, not shown) or unbranched filaments that may be distinguished from each other by differences in filament or chloroplast structure. *Hormidiella* exhibits a short basal transparent stalk (Lokhorst et al. 2000) (Fig. 1e), while filaments of *Entransia* (Cook 2004b) and *Klebsormidium* (Lokhorst 1996) have no stalk but may be attached with mucilage or some other adhesive. Some filaments of *Entransia* (but not *Hormidiella* or *Klebsormidium*) bear a spine at the end of the apical cell (Cook 2004b) (Fig. 1d). Fimbriate chloroplasts with multiple pyrenoids are characteristic of *Entransia* (Hughes 1948) (Fig. 1d), while chloroplasts of *Hormidiella attenuata* (Lokhorst et al. 2000) (Fig. 1e) and *Klebsormidium* (Lokhorst 1996) (Fig. 1c) have only a single pyrenoid and typically are not lobed, though some species of *Klebsormidium* may exhibit lobed chloroplasts (Lokhorst 1996), at least under certain conditions (Novis 2006; Škaloud 2006).

Coleochaetophyceae (Fig. 1f–i) are branched filaments. Whereas *Chaetosphaeridium* filaments exhibit an unusual sympodial mode of branching, often appearing to be unicellular (Thompson 1969) (Fig. 1f), *Coleochaete* (Pringsheim 1860) may be filamentous (Fig. 1g), pseudoparenchymatous (Fig. 1h), or parenchymatous (Fig. 1i) (Graham 1982). Parenchymatous species form more coherent thalli and more complex gametangia than filamentous species. *Chaetosphaeridium* and *Coleochaete* share distinctive sheathed hair cells (Fig. 1f–i) thought to function as antiherbivore defenses (Marchant and Pickett-Heaps 1977) or to increase absorptive surface area (Cimino and Delwiche 2002; Delwiche et al. 2002).

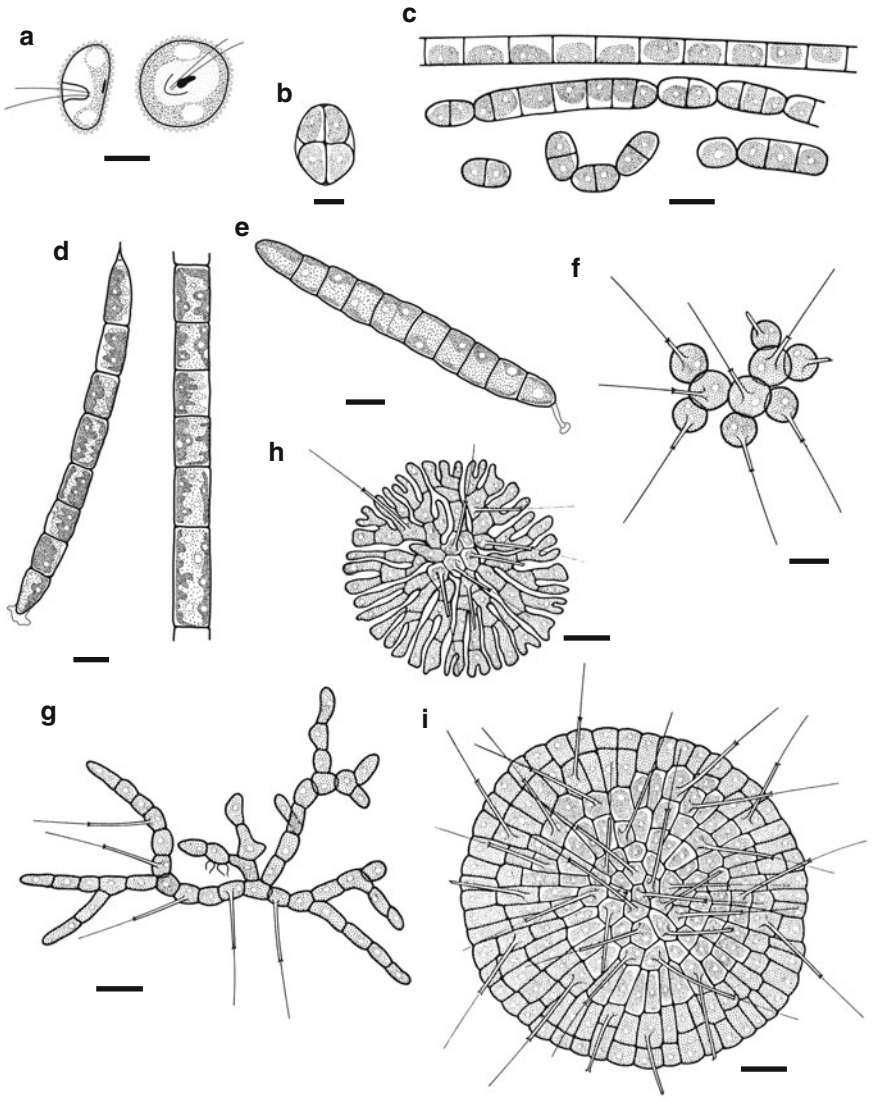

Fig. 1 Morphology of streptophyte algae. (**a**) *Mesostigma viride*, a scaly unicellular flagellate. Scale bar = 5 μm (After unpublished photos of M.E. Cook & L.W. Wilcox). (**b**) *Chlorokybus atmophyticus,* a sarcinoid form. Scale bar = 2.5 μm (After Bourrelly (1966)). (**c**) *Klebsormidium* (*Hormidium*) *subtile*, an unbranched filament. Scale bar = 10 μm (After Smith 1950). (**d**) *Entransia fimbriata,* an unbranched filament that can have a spine at the tip. Scale bar = 16 μm (After Cook (2004b)). (**e**) *Hormidiella attenuata,* an unbranched filament with a stalk at the base. Scale bar = 9 μm (After Lokhorst et al. (2000)). (**f**) *Chaetosphaeridium globosum,* a branched filament with spherical cells. Scale bar = 15 μm (After Thompson (1969)). (**g–i**) Species of the branched filament *Coleochaete.* Scale bars = 40 μm (After Pringsheim (1860)). (**g**) *Coleochaete divergens,* a loosely branched form. h. *Coleochaete soluta,* a loose disc with bifurcated peripheral filament tips. (**i**) *Coleochaete scutata,* a tight discoid form. (**a**), (**d**), (**e**) drawn by L.W. Wilcox. (**b**), (**c**), (**f–i**) drawn by Kandis Elliot

Cell Division

Ultrastructural studies of mitosis and cytokinesis have been conducted on *Chlorokybus atmophyticus* (Lokhorst et al. 1988), on *Hormidiella attenuata* (Lokhorst et al. 2000), and on several species of *Klebsormidium* (Floyd et al. 1972; Lokhorst and Star 1985; Pickett-Heaps 1972). An open spindle (nuclear membrane breaks down) widely separated telophase nuclei, and a cleavage furrow characterizes mitosis and cytokinesis in these organisms. In addition, cytokinesis in *Klebsormidium* includes interzonal vacuoles. The role of microtubules in cytokinesis was not clear. No microtubules were observed with TEM during cytokinesis in *Hormidiella attenuata* (Lokhorst et al. 2000), *K. flaccidum* (Floyd et al. 1972), or *K. subtilissimum* (Pickett-Heaps 1972), while there were microtubules parallel to the furrow in *K. mucosum* (Lokhorst and Star 1985) and in *Chlorokybus* (Lokhorst et al. 1988). Immunofluorescence microscopy demonstrated the presence of interzonal microtubules perpendicular to the division plane between the widely separated telophase nuclei in *Klebsormidium flaccidum* and *K. subtilissimum* (Katsaros et al. 2011). Hooplike cortical microtubules at interphase transitioned gradually to form a spindle during pre-prophase in these taxa. Microtubules and centrioles were closely associated with the single peroxisome during mitosis in *Klebsormidium flaccidum* (Honda and Hashimoto 2007).

Studies of living cells of *Chaetosphaeridium globosum* at the light microscope level and immunofluorescence localization of tubulin on fixed cells of this taxon have revealed the presence of a plantlike phragmoplast during cytokinesis (Doty et al. 2014). Marchant and Pickett-Heaps (1973) investigated the ultrastructure of mitosis and cytokinesis in *Coleochaete scutata.* They observed a phragmoplast similar to that found in *Chara* and land plants. A plantlike phragmoplast was also observed in *Coleochaete orbicularis* (Brown et al. 1994; Cook 2004a; Doty et al. 2014) (Fig. 2) and in *C. soluta* and *C. irregularis* (Doty et al. 2014). In the highly vacuolate cells of *Chaetosphaeridium* and *Coleochaete*, like those of the vascular plant *Arabidopsis* (Cutler and Ehrhardt 2002), the phragmoplast must in some cases negotiate a large vacuole (Cook 2004a; Doty et al. 2014). This process has been termed polar cytokinesis (Cutler and Ehrhardt 2002) because the cell plate forms first on one side of the cell and moves across to the other side of the cell, rather than moving out from the center as it would in cells without a single large vacuole.

Peroxisomes are located between the nucleus and the chloroplast in *Klebsormidium* (Floyd et al. 1972; Lokhorst and Star 1985), *Interfilum* (Mikhailyuk et al. 2008, 2014; Novis and Visnovsky 2011), *Hormidiella* (Lokhorst et al. 2000), *Chaetosphaeridium* (Moestrup 1974), and *Coleochaete* (Marchant and Pickett-Heaps 1973), while in *Chlorokybus* (Lokhorst et al. 1988; Rogers et al. 1980) and *Mesostigma* (Rogers et al. 1981), the peroxisome is associated with centrioles or flagellar basal bodies. Both associations facilitate distribution of the divided peroxisome into two daughter cells during cytokinesis and may represent an evolutionary transition from centriole-associated peroxisomes to the chloroplast-associated peroxisomes that occur in embryophytes (Graham and Kaneko 1991).

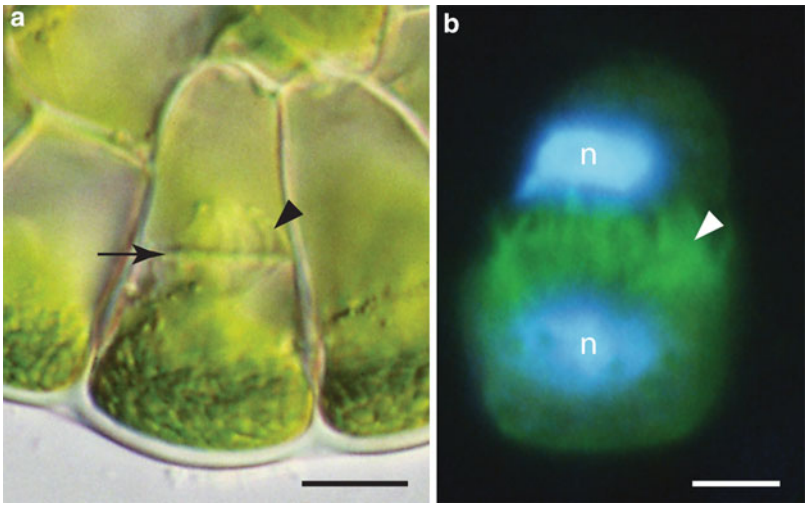

Fig. 2 Cell division involving a plantlike phragmoplast in *Coleochaete orbicularis.* (**a**) Differential interference contrast image with forming cell plate (*arrow*) in center of phragmoplast (*arrowhead*). Scale bar = 10 μm. (**b**) Immunofluorescence localization of tubulin in phragmoplast microtubules (*arrowhead*) between DAPI-stained telophase nuclei (*n*). Scale bar = 5 μm. Micrographs: (**a**) M.E. Cook; (**b**) K.F. Doty (From Graham et al. (2016) *Algae* 3rd edition, used with permission of LJLM Press)

Life Cycle

Chlorokybus and members of the Klebsormidiophyceae and Coleochaetophyceae reproduce asexually by means of biflagellate zoospores. Each zoospore forms from the entire protoplast of one cell and is released by dissolution of the cell wall in *Chlorokybus* (Rogers et al. 1980) or through a pore in the cell wall (Fig. 3a) in members of the Klebsormidiophyceae (Cain et al. 1973; Cook 2004b; Lokhorst 1996; Lokhorst et al. 2000) and Coleochaetophyceae (Thompson 1969; Wesley 1928). Sexuality has not been documented for *Mesostigma*, *Chlorokybus,* or members of the Klebsormidiophyceae. The Coleochaetophyceae are oogamous (a large, nonmotile egg is fertilized by small, biflagellate sperm). So far as is known, sexually reproducing forms have a haplobiontic, haploid life cycle. The only diploid cell is the zygote, and as zygotes typically serve as resting cells, they are often called oospores.

The zoospores of *Chlorokybus atmophyticus* (Rogers et al. 1980), *Hormidiella attenuata* (Lokhorst et al. 2000), and *Klebsormidium flaccidum* (Marchant et al. 1973) have been studied ultrastructurally, as have zoospores of *Chaetosphaeridium* (Moestrup 1974) and zoospores, meiospores, and spermatozoids of *Coleochaete* (Graham and McBride 1979; Graham and Taylor 1986; Graham and Wedemayer 1984; Pickett-Heaps and Marchant 1972; Sluiman 1983). These motile cells are

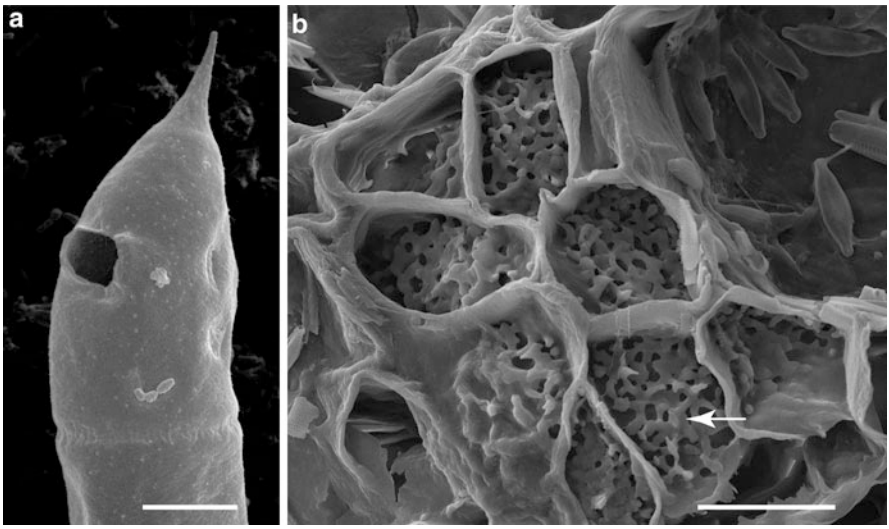

Fig. 3 Scanning electron micrographs of cells with evidence of asexual (**a**) and sexual (**b**) reproduction. (**a**) Empty cell with pore through which a zoospore escaped in filament of *Entransia fimbriata*. Scale bar = 5 μm. (**b**) A layer of elaborate cell wall ingrowths (*arrow*) covers the surface of protective cells adjacent to a zygote in a thallus of *Coleochaete orbicularis.* This thallus has overwintered, and the cell walls on the surface away from the zygote have decomposed, making it possible to see the wall ingrowths. Similar wall ingrowths occur in placental transfer cells at the interface of parent and offspring in land plants. Scale bar = 20 μm. Micrographs: (**a**) M.E. Cook; (**b**) M.E. Cook and L.W. Wilcox (From Graham et al. (2016) *Algae* 3rd edition, used with permission of LJLM Press)

characterized by flagella that emerge subapically and extend to the right when the cell is observed from the side down which the MLS microtubules extend. The MLS of these charophycean swarmers is considered to be homologous to one of the two MLSs of *Mesostigma* (Melkonian 1989; Rogers et al. 1981). The small diamond-shaped scales that occur on the zoospores of *Chlorokybus* and *Chaetosphaeridium* and on the swarmers of *Coleochaete* are similar to the maple-leaf-shaped scales of *Mesostigma* (Marin and Melkonian 1999). Unlike *Mesostigma*, none of the other streptophyte flagellate cells exhibits an eyespot.

Coleochaete is unique among charophycean algae, and similar to embryophytes, in retention of zygotes on parental thalli and in production of more than four meiospores per zygote. In *Coleochaete orbicularis*, there are zygote-associated cells that resemble the placental transfer cells of embryophytes (Graham and Wilcox 1983) (Fig. 3b). Wall ingrowths in these cells provide extensive surface area for efficient transfer of nutrients from the parent thallus to the developing zygotes. Nutritional support of the new diploid generation from the parent gametophyte generation, known as matrotrophy, is thought to be a critical step in the evolution of the plant life cycle (Bower 1908; Graham and Wilcox 2000).

Cell Coverings

Mesostigma is covered with three different layers of scales (Manton and Ettl 1965). The innermost layer is composed of small square scales like those on swarmers of other streptophyte taxa, while the middle layer is formed of larger flat oval scales, and finally the outermost layer consists of elaborate three-dimensional basket-shaped scales. All of these scales are produced in Golgi vesicles (Domozych et al. 1992; Manton and Ettl 1965). The basket scales have been found to include calcium, phosphate, and a sugar keto acid that is part of the pectic polysaccharide rhamnogalacturonan II found in cells walls of higher plants (Domozych et al. 1991). *Mesostigma* has no cell wall, only scales.

Members of the other charophycean lineages have cell walls made of cellulose and other components of plant cell walls. *Coleochaete scutata* exhibits rosette-type cellulose-synthesizing complexes that are similar to those of plants (Okuda and Brown 1992). These complexes are formed in Golgi vesicles and transported to the cell membrane where they produce cellulose microfibrils. Analyses based on rotational symmetry indicate that these complexes have eightfold symmetry in *C. scutata* (Okuda and Brown 1992), in contrast to the rosettes of six particles known from plants and members of the Zygnematophyceae (Tsekos 1999).

Biochemical analysis revealed that cell walls of *Coleochaete* are more like those of plants than are cell walls of *Chlorokybus* or *Klebsormidium.* For example, cell walls of *Chlorokybus* and *Klebsormidium* include less cellulose and more callose than those of *Coleochaete* (Sørensen et al. 2011), and they lack evidence for the pectic polysaccharide rhamnogalacturonan-I found in *Coleochaete* (O'Rourke et al. 2015). On the other hand, the pectic polysaccharide homogalacturonan was present in cell walls of *Chlorokybus* and *Coleochaete,* but not *Klebsormidium* (O'Rourke et al. 2015). In the cell walls of *Coleochaete*, small amounts of lignin-like compounds were identified (Sørensen et al. 2011). Hence, molecules prominent in plant cell walls were inherited from algal ancestors, and analysis of the cell wall components of streptophyte algae may lead to a greater understanding of the complex construction of plant cell walls.

Genomes

The genome of *Klebsormidium flaccidum* has been fully sequenced (Hori et al. 2014). This work revealed that plant genes important for survival on land were already present in *Klebsormidium*, including those associated with protection from high levels of light and with hormone signaling pathways. For example, a gene for transport of the hormone auxin in *Klebsormidium* appears to be intermediate between two forms of the gene present in the vascular plant *Arabidopsis* (Viaene et al. 2013; Hori et al. 2014).

Metagenomes

Long-read shotgun metagenomic analyses for *Coleochaete pulvinata* and *Chaetosphaeridium globosum* indicated that some microorganisms and genes characteristic of land plant microbiomes might have originated from algal ancestors (Knack et al. 2015). In addition to nitrogen-fixing cyanobacteria and rhizobialeans (e.g., *Rhizobium*), the *C. pulvinata* and *Ch. globosum* microbiomes included methanotrophs, as do those of early-diverging bryophytes (e.g., *Sphagnum* peat mosses, whose lineage extends back to at least the middle Ordovician (Laenen et al. 2014)). 16S rDNA amplicon analyses of *Nitella tenuissima* indicated diverse prokaryotic associations and greater similarity of microbiota to that of members of the Coleochaetophyceae than to a chlorophyte of similar size and ecology (Knack et al. 2015).

Sequence evidence for early-diverging fungi and *Nuclearia*, the protist sister to the Kingdom Fungi was also observed in metagenomes of the Coleochaetophyceae, suggesting the remarkable possibility that streptophyte-fungal associations originated prior to the origin of the plant and fungal kingdoms (Knack et al. 2015). Part of the pathway involved in signaling between plants and mycorrhizal fungi had already arisen in *Chlorokybus* (Delaux et al. 2015).

Metagenomic analysis of *C. pulvinata* and *Ch. globosum* (Knack et al. 2015) also allowed inference of presence of protein subunits serving as functional enzyme markers, e.g., diverse types of NifH indicating nitrogen fixation and particulate methane monooxygenases indicating methane oxidation, as well as all enzymes involved in aerobic and anaerobic biosynthetic pathways for vitamin B_{12}.

Maintenance and Cultivation

Mesostigma has been grown in nine parts Woods Hole Medium (Nichols 1973) supplemented with one part soil water extract (Domozych et al. 1991). Cultures of *Chlorokybus* and *Klebsormidium* can be maintained on agarized Bold's Basal Medium (BBM) (Nichols 1973). Liquid or agarized BBM with addition of vitamins and triple nitrate have also been used to grow *Klebsormidium* and *Interfilum* (Mikhailyuk et al. 2014; Rindi et al. 2011). *Hormidiella* can be cultured with Woods Hole Medium or liquid or agarized BBM and has sometimes been supplemented with 2% soil water extract (Lokhorst et al. 2000). *Entransia* prefers BBM with added B vitamins (Cook 2004b).

Coleochaete can also be maintained long term on BBM agar with the plate stored upside down. When actively growing cultures with normal morphology are required, specimens can be transferred to liquid SD11 medium (Hoffman and Graham 1984). Species of *Coleochaete* obtained from culture collections often exhibit abnormal morphology, and better results may be obtained using fresh isolates collected from nature by zoospore isolation (Graham et al. 1986). Cultures of *Chaetosphaeridium*

can also be obtained from nature by zoospore isolation techniques and may be grown in Woods Hole Medium (Delwiche et al. 2002) or in DYIII medium (Doty et al. 2014).

Most of the taxa discussed in this chapter can be maintained with a 16 h light: 8 h dark cycle at 15–20 °C. *Entransia* grows best with shade cloth to provide protection from high levels of light (Kitzing and Karsten 2015). Induction of zoosporogenesis via a dark treatment has been described for *Klebsormidium flaccidum* (Cain et al. 1974).

Evolutionary History

Overall Phylogenetic Position

The green algae are composed of two major clades: the Chlorophyta, including numerous lineages of scaly unicellular prasinophytes, and the Streptophyta (also known as Charophyta), which includes land plants (embryophytes) and their closest green algal relatives (Bremer 1985; Lewis and McCourt 2004; McCourt 1995; McCourt et al. 2004) (Fig. 4). These close algal relatives of plants are not a

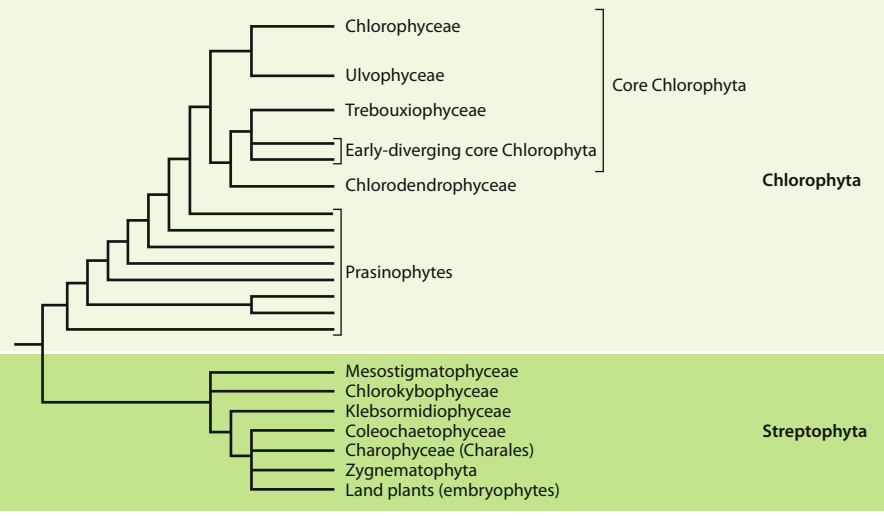

Fig. 4 Cladogram showing relationship of streptophyte algae to other green algae and land plants based on molecular analyses (Civáň et al. 2014; Karol et al. 2001; Leliaert et al. 2012; Timme et al. 2012; Turmel et al. 2013; Wickett et al. 2014). Molecular analyses support previous hypotheses of relationships among major green algal groups based on ultrastructure and biochemistry (Reviewed by Lewis and McCourt 2004)

monophyletic group without plants and have been referred to as charophycean green algae (Mattox and Stewart 1984), charophyte algae (Lewis and McCourt 2004), or streptophyte algae (Becker and Marin 2009).

While *Mesostigma* is a scaly flagellate, this genus is no longer classified with the prasinophytes and is therefore not a member of the Chlorophyta, though one recent molecular analysis found that there was insufficient data to resolve the position of *Mesostigma* (Grievink et al. 2013). Initial studies of organelle genomes (Lemieux et al. 2000; Turmel et al. 2002) placed *Mesostigma* at the base of the green algae, before Chlorophyta and Streptophyta diverged, but most evidence, including duplication of the gene for glyceraldehyde-3-phosphatase dehydrogenase (Petersen et al. 2006), a multilayered structure (MLS) in the flagellar apparatus like that of streptophyte zoospores and spermatozoids (Melkonian 1989; Rogers et al. 1981), the presence of the enzymes glycolate oxidase (Iwamoto and Ikawa 2000) and Cu/Zn superoxide dismutase (DeJesus et al. 1989), as well as data sets with molecules from all three genomes (Karol et al. 2001; Rodríguez-Ezpeleta et al. 2007), indicates that *Mesostigma* is a member of the Streptophyta.

Internal Relationships

While there is abundant evidence uniting *Mesostigma* and the other streptophyte algae with embryophytes, relationships within the streptophyte clade are less clear. Polytomies (Fig. 4) indicate this uncertainty at the base of the Streptophyta and at the top, where the sister group of embryophytes is much debated. At the base of the Streptophyta, some molecular studies identified a clade composed of *Mesostigma* and *Chlorokybus* (Lemieux et al. 2007; Turmel et al. 2013), while others found that *Mesostigma* diverged before *Chlorokybus* (Finet et al. 2010, 2012) or could not resolve the relationship of these two species (Civáň et al. 2014; Leliaert et al. 2011, 2012). Likewise, different molecular studies have identified the sister group of plants as the Charophyceae (Charales) (Karol et al. 2001; Qiu et al. 2007; Turmel et al. 2003), the Zygnematophyceae (Civáň et al. 2014; Timme et al. 2012; Turmel et al. 2006, 2007; Wickett et al. 2014), and a clade composed of the Zygnematophyceae plus Coleochaetophyceae (Finet et al. 2010, 2012; Laurin-Lemay et al. 2012). Other analyses could not resolve this position, finding that either the Zygnematophyceae or a clade composed of the Zygnematophyceae plus Coleochaetophyceae is the sister group of land plants (Leliaert et al. 2011, 2012; Turmel et al. 2013; Wodniok et al. 2011).

Because the extant lineages of charophycean algae have been evolving for hundreds of millions of years since they diverged from a common ancestor with plants, no single lineage is likely to hold the key to plant evolution. Even if the sister group of plants remains elusive, examination of the characters of all extant charophycean algae may lead to greater understanding of the evolutionary transition from simple aquatic algae to terrestrial plants.

Importance for Understanding the Evolution of Land Plants

While none of the recent analyses has identified the Coleochaetophyceae as the sister group to plants, it is likely that a loosely branched filament, perhaps like that of *Coleochaete pulvinata* (Delwiche and Cooper 2015), was the most recent common ancestor of the extant charophycean lineages and plants. The range of morphological form in *Coleochaete* has been used to illustrate possible evolutionary transitions involved in the origins of land plant tissues and complex, multicellular gametangia (Graham 1982, 1984). It has also been proposed that *Coleochaete* illustrates evolutionary steps occurring in the origins of the sporophyte generation and alternation of generations of land plants (Graham 1985, 1990; Graham and Wilcox 2000).

The freshwater habitat of all extant streptophyte algae indicates a freshwater origin for the ancestors of plants (Becker and Marin 2009; Delwiche and Cooper 2015; Graham 1993). Becker (2012) has hypothesized that adaptation to a drier glaciated earth may have lead to the divergence of the photorespiratory pathways that distinguish the Chlorophyta (glycolate dehydrogenase in mitochondria) from the Streptophyta (glycolate oxidase in peroxisomes) (Stabenau and Winkler 2005). Traits of modern streptophyte algae that have the ability to survive in a subaerial habitat, including *Chlorokybus* (Škaloud 2009), *Hormidiella, Interfilum*, and *Klebsormidium* in nature (Karsten and Holzinger 2014; Kitzing and Karsten 2015), as well as *Coleochaete* in laboratory experiments (Graham et al. 2012), may be useful for understanding characteristics of ancient green algae that made the transition to land. These algae may also provide clues to the future adaptation of plants and streptophyte algae in a warmer and drier world.

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Bacillariophyta

7

David G. Mann, Richard M. Crawford, and Frank E. Round

Abstract

The diatoms (Bacillariophyta) are the most species-rich group of autotrophic algae, found in fresh, brackish, and marine waters worldwide, and also in damp terrestrial habitats. They are well represented in marine phytoplankton and may account for 20% of global photosynthetic carbon fixation. However, the vast majority of the estimated 100,000 species are benthic, living attached to surfaces or gliding over sediments using a unique organelle, the raphe system. Flagellate cells are absent, except in the sperm of some lineages. Diatoms possess a similar photosynthetic apparatus to that present in several other stramenopile lineages (with fucoxanthin and chlorophyll c as the principal accessory pigments) but are easily recognized by the unique construction and composition of their cell wall, which is usually strongly silicified and consists of two overlapping halves (thecae); these in turn consist of a larger end piece (valve) and a series of narrow strips (girdle bands). Expansion of the cell occurs by sliding apart of the thecae and addition of new bands to the inner, overlapped theca. At cell division, each daughter cell inherits one of the thecae of the parent and forms a new theca

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internally. Hence, because the silicified wall is inelastic, average cell size usually declines during vegetative growth and has to be restored through expansion of a special cell, the auxospore, usually after sexual reproduction. A few diatoms have lost their plastids and are osmotrophic. Classification has traditionally relied on details of valve structure. There is a rich fossil record.

Keywords
Bacillariophyta • Diatoms • Frustule • Girdle • Silicification • Valves

Contents

Summary Classification

●Bacillariophyta
●●leptocylindrids
●●corethrids
●●melosirids
●●ellerbeckiids
●●arachnoidiscids
●●coscinodiscids

••rhizosolenids
••proboscids
••Bacillariophytina
•••Mediophyceae (polar centrics)
•••Bacillariophyceae (pennate diatoms)
••••Urneidophycidae
••••Fragilariophycidae
••••Bacillariophycidae (raphids)[1]

Introduction

General Characteristics

The Bacillariophyta, commonly known as ***diatoms***, are a group of unicellular (though sometimes colonial), diploid, golden or brown-pigmented algae, most of which occur in freshwater and marine habitats; just a few live on land. The aquatic species can be planktonic or benthic. The vast majority of diatoms are free-living phototrophs but some live as endosymbionts of other protists and a small number have lost photosynthetic capacity and have become obligate heterotrophs. Like related phototrophic stramenopiles (heterokonts), photosynthetic diatoms possess chloroplasts that are bounded by four membranes and contain thylakoids grouped into threes. The principal light-harvesting pigments are fucoxanthin, chlorophyll a, and various forms of chlorophyll c. The most characteristic feature of diatoms is their silicified cell wall, referred to as the ***frustule*** (see section "Cell Wall and Cell Division"), which is unlike anything found in other organisms. It is strong and sometimes massive but, crucially for these photosynthetic cells, transparent. It is composed of several interlocking and overlapping elements, comprising two ***valves***, one at each end of the cell, which are usually large and robust, and a variable number of more delicate ***girdle bands*** covering the space in between (Fig. 1a, b). The vegetative cells are always walled except in the few species that occur as endosymbionts; no free-living flagellated or amoeboid cells exist, except as gametes. In a very few cases (e.g., some stages of the highly unusual, polymorphic diatom *Phaeodactylum*), the cell wall is purely organic.

Diatoms have a simple diplontic life cycle, multiplying profusely by mitotic divisions during the diploid vegetative phase and producing haploid cells only as a result of gametogenesis. A characteristic and remarkable feature of most diatoms is that average cell size decreases during the vegetative phase and has to be restored through formation of a special cell – the ***auxospore*** (see section "Life Cycle"). Auxosporulation is usually preceded by sexual reproduction, the auxospore being a

[1] In the case that the eight informally named groups (leptocylindrids to proboscids) together comprise a monophyletic taxon (see "Taxonomy"), this is called the Coscinodiscophytina, containing a single class, Coscinodiscophyceae.

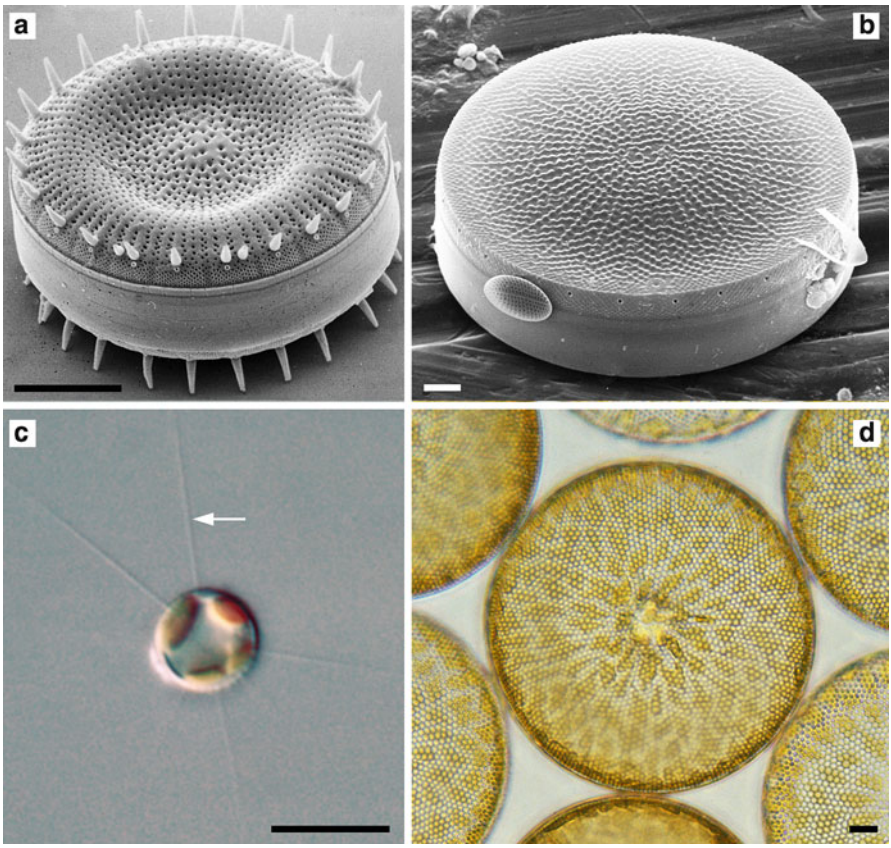

Fig. 1 Cells of planktonic centric diatoms. Scale bars = 10 μm. (**a**) Whole cell of *Stephanodiscus* with concentric undulations of the valve and a crown of spines. SEM. (**b**) Whole cell of *Actinocyclus,* SEM. (**c**) Living cell of *Cyclotella* with radiating fibrils of chitin (e.g., *arrow*) secreted through fultoportulae (Fig. 12c). (**d**) Living cells of *Coscinodiscus*; many small plastids are visible beneath the honeycomb-like pattern of markings on the valve

zygote formed through the fusion of motile or nonmotile gametes, but in some cases the auxospore is formed asexually. During auxosporulation, the cell walls of the old, small vegetative cells are discarded. In the "centric" lineages of diatoms, sexual reproduction is ***oogamous***: here the auxospore is formed by fertilization of a large ***nonmotile egg cell*** by a much smaller, ***anteriorly uniflagellate sperm***. However, in one late-evolving lineage (the pennate diatoms, comprising the majority of extant species), the gametes are relatively large and alike in size and appearance (though not necessarily in activity), and lack flagella. Auxospores (which are not dormant stages, contrary to what might be thought from the use of the word "spore") often possess special wall elements found at no other stage during the life cycle, which allow and control cell expansion and protect the newly enlarged vegetative cell while it forms its new frustule.

Some diatoms are nonmotile, drifting freely in the water column or lying loose on a substratum or growing attached to it. Others are motile, gliding actively over surfaces via a unique type of locomotion associated with a unique organelle, the ***raphe system***, which comprises slits through the cell wall (the ***raphe slits***) and associated elements of the cytoskeleton. Movement is generated by secretion of polysaccharide through the raphe slits, adhesion of the secreted material to the substratum, and active displacement of the secretions relative to the cell by interactions with the cytoskeleton, thus driving the cell forward (Edgar and Pickett-Heaps et al. 1984). Through their raphe secretions, stalks and pads, benthic diatoms often greatly modify their immediate environment, e.g., by gluing sediment particles together or by forming a thick biofilm that is colonized by other algae and microorganisms.

Several hundreds of genera of extant diatoms are recognized, and the number of named species and infraspecific taxa (including fossils) exceeds 60,000 (Kociolek and Williams 2015). Some of these taxa are synonyms, but many species have not yet been discovered or named, and it has been estimated that the final total of extant species will be between 100,000 and 200,000 (Mann and Vanormelingen 2013). Many small-celled diatoms have been poorly researched and some important, highly species-rich habitats have been largely neglected, e.g., the phytobenthos of sublittoral marine habitats. Furthermore, gene sequence data reveal that cryptic and pseudocryptic species are common. Hence the diatoms have a strong claim to be considered one of the most diverse and successful groups of protists. They also have a rich subfossil and fossil record, because their silica shells are resistant to decay. Many extinct fossil genera are known, and many modern genera are represented in the fossil record by extinct species.

Recently, diatoms have become the focus of intense research using genomic and transcriptomic approaches, because of their importance to the functioning of the biosphere and because of their unrivalled ability to metabolize silicon and produce patterned, silicified walls.

Occurrence and Sampling

Diatoms occur in almost all aquatic habitats, both freshwater and marine (Round 1981a), and probably account for about 20% of global net primary production (Mann 1999b). Virtually the whole ocean (70% of the earth's surface), down to depths to which photosynthetically available radiation (of wavelength 400–700 nm) penetrates, is colonized by diatoms, though they are numerically most abundant in regions of upwelling and other productive zones. However, the greatest diversity is probably in marine intertidal communities. For example, in two nearby samples from a North Carolina beach, Friedrich Hustedt (1955) recognized 369 species (of which 89 were new) belonging to 63 different genera. Diatoms occur on land too. Most soils capable of supporting plant growth bear diatoms, and they occur anywhere water drips, collects, or flows – even the moist regions between bryophyte leaves and on the surfaces of angiosperm leaves and lichens in wet tropical forests (Round 1981a).

Diatoms live as motile, attached, or suspended cells. Though the suspended (***planktonic***) species are those most often illustrated, and thus the most familiar to biologists (Figs. 1a–d and 2a–d), the range of form is greater in benthic habitats, and there are far more benthic species than planktonic ones (by a couple of orders of magnitude). Motile species occur in the surface film of soils and on dripping rock faces, and on the sediments of ponds, lakes, streams, rivers, coastal lagoons, and coastal seas. They often coat the surface of estuary muds with a dense brown layer of cells, which play an important ecological role in stabilizing sediments (Underwood and Paterson 2003). These ***epipelic*** diatoms (Fig. 3a) are motile and often migrate vertically upwards through the sediment in the morning and move back into the sediment later in the day, in a rhythm of movement under the control of a biological clock which, in tidal situations, is in synchrony with the tidal cycle (Palmer and Round 1967). Soil diatoms are of similar morphology to those occurring in aquatic epipelon, but they are generally smaller and less motile. Sand in both freshwater and

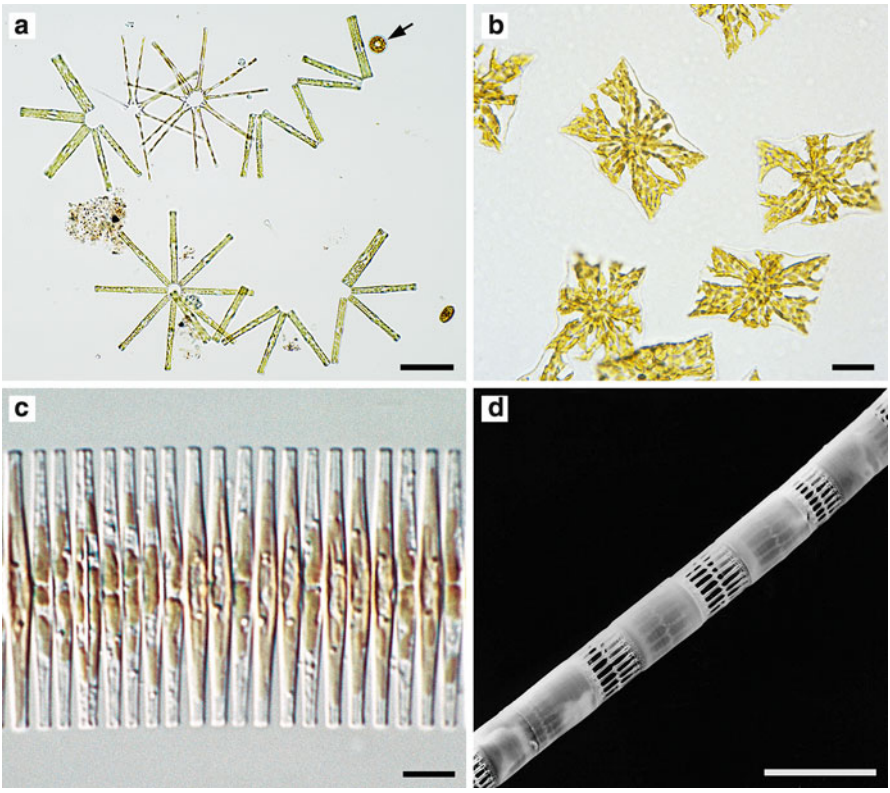

Fig. 2 Planktonic diatoms. (**a**) Freshwater phytoplankton containing a stellate *Asterionella* colony (slender-celled colony at *top*), stellate and zigzag colonies of *Tabellaria*, and a single *Cyclotella* cell (*arrow*). Scale bar = 50 μm. (**b**) The marine *Mediopyxis*: solitary cells. Scale bar = 10 μm. (**c**) A ribbon of *Fragilaria* cells from freshwater Scale bar = 10 μm. (**d**) Filament of *Skeletonema* cells, SEM. Scale bar = 10 μm

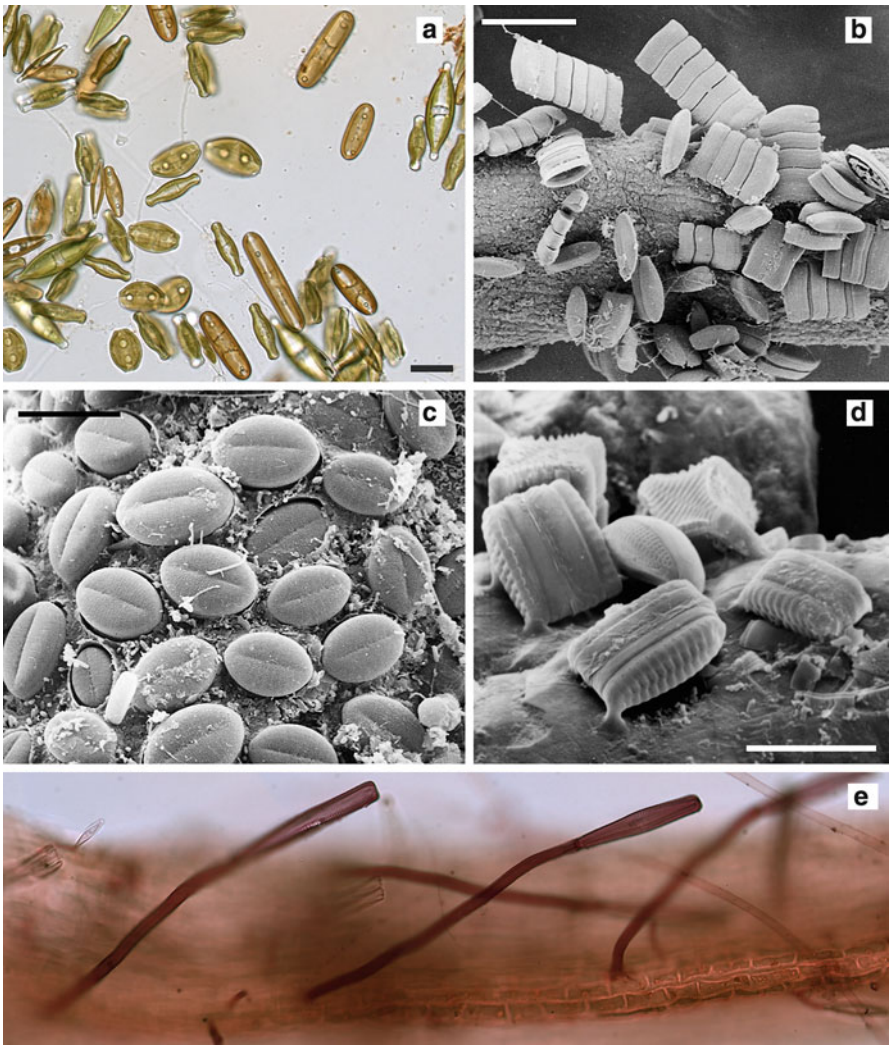

Fig. 3 Diatom communities. (**a**) Freshwater epipelon, containing *Amphora*, *Sellaphora*, *Navicula* and *Hippodonta* cells. Scale bar = 20 μm. (**b**) *Achnanthes* growing epiphytically on a plant surface. SEM. Scale bar = 50 μm. (**c**) *Cocconeis* growing on the green alga *Cladophora*. SEM. Scale bar = 10 μm. (**d**) Epipsammon: *Martyana*, *Amphora*, and *Staurosira* on a sand grain. SEM. Scale bar = 10 μm. (**e**) Carmine-stained cells of *Gomphonema*, attached to a plant surface by long polysaccharide stalks

marine environments may be colonized not only by epipelon but also by extremely small diatoms attached to the surfaces of the sand grains themselves, comprising the ***epipsammon*** (Fig. 3d). Attached (***epilithic***) species coat rock surfaces, the hard surfaces of calcified algae, and the dead fragments of corals and calcareous algae. Filamentous algae in both freshwater and marine habitats are often so densely

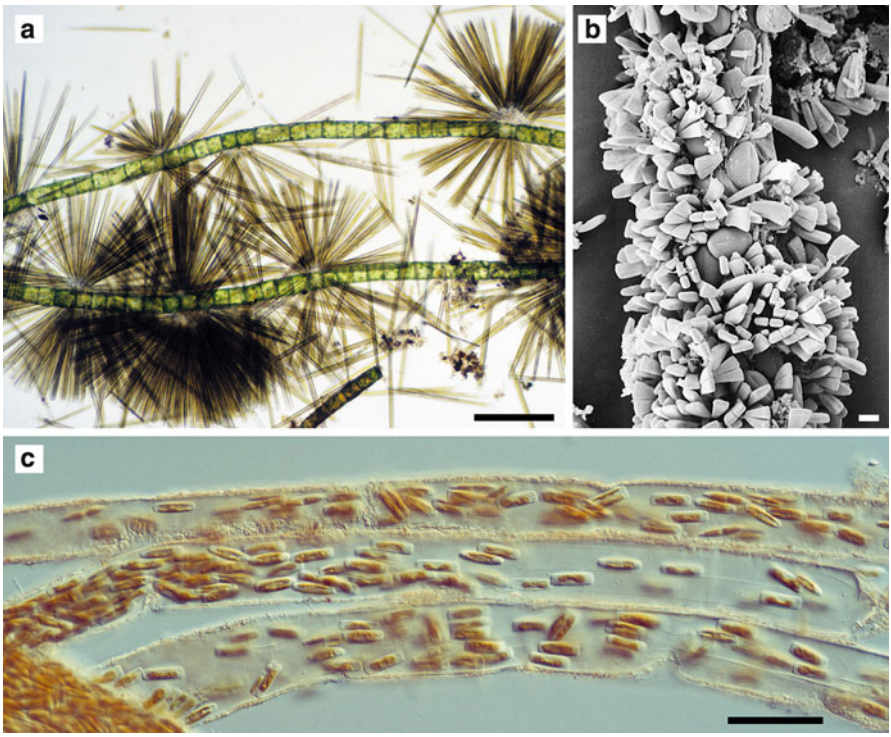

Fig. 4 Attached diatom communities. (**a**) *Ulnaria* epiphytic on filamentous green algae. Scale bar = 50 μm. (**b**) Dense growth of *Rhoicosphenia*, *Gomphonema* and *Cocconeis* on the green alga *Cladophora*. SEM. Scale bar = 10 μm. (**c**) Tube-dwelling *Berkeleya*, scraped from a rock surface. The tubes are made of polysaccharide. Scale bar = 50 μm

covered by ***epiphytic*** diatoms (Figs. 3b, c, e, and 4a) that the host surface may be obscured (Fig. 4b), and animal hosts, from copepod hard parts and limpet shells to sea birds and cetaceans, sometimes have an external diatom microbiota (***epizoon***) involving species-specific forms. Vast areas of ***sea ice*** around Antarctica and the Arctic ice cap are coated on the undersurface with a dense layer of diatoms. Smaller forms are even found in the brine channels of the ice. Man-made objects placed in water soon acquire a covering of diatoms, and glass slides or ceramic tiles have sometimes been deployed for this reason in rivers, so that they can later be removed for assessment of water quality and ecological status (e.g., during biomonitoring under the European Union Water Framework Directive).

Collection of diatoms involves sampling of sediments, plants, or animals, or filtering (or sedimenting) quantities of lake or seawater. Sediments should be sampled by techniques that remove only the top few millimeters or so. If the sediment is then placed in a Petri dish or translucent plastic box, excess water removed, and cover glasses or lens cleaning tissue placed on the surface, motile diatoms will move upwards and attach to the new substratum and can be removed to

a microscope slide for examination or placed in culture media. Sand samples can be washed free of silt and organic matter by repeated agitation and settling and the sand grains then observed directly on a microscope slide. Communities on plant and animal surfaces can be observed directly if the plant or animal is microscopic, or the surface coating of diatoms may be removed from them or stones by scraping with a scalpel. Careful sampling will often show that an upper "canopy" is present. This is more easily detached than the initial colonizers, which grow appressed to the substratum. Planktonic diatoms can be sampled by drawing a net through the water either horizontally behind a drifting boat (or thrown in from the shore) or vertically by lowering a weighted net to a set distance below water level and then drawing it up. More complicated devices can be used if quantitative samples are required, including standard water bottle samplers that can capture known volumes of water from known depths.

Because of the nature of their cell walls, diatoms have left evidence of their evolution in the fossil record since the Cretaceous, often in the form of fairly pure deposits called ***diatomites***, produced by sedimentation of the plankton of fresh and marine waters. Diatomites may be powdery or more rocklike, the latter requiring treatment (grinding, disaggregation using chemicals, freeze–thaw cycles, or sonication) to reduce them to a finer state before examination. The material can often be mounted directly in water or high refractive index mountants but is often better if "cleaned" first (see below).

The fine detail of wall structure is usually critical for identification and has to be revealed by cleaning the cells with strong oxidizing agents (e.g., a mixture of concentrated sulfuric and nitric acids, or hydrogen peroxide; however, though widely used, the latter seems often to lead to erosion of fine detail) to remove organic material, leaving only the silica parts of the cell wall. If the sample contains much carbonate, this may have to be removed first (it can be dissolved with dilute hydrochloric or nitric acids and washing to remove the resulting salts), especially if sulfuric acid is to be used subsequently. After oxidation, samples must be thoroughly washed with deionized water by settling or centrifugation. Then the cleaned frustules (which often separate into their component pieces – valves and girdle bands) can be dried onto cover glasses and mounted in high refractive index media (e.g., Naphrax: Fleming 1954). Final identification of species can then be attempted. Care should always be taken to study the full range of forms present in a population because most diatoms undergo size reduction during the life cycle, and the shape and patterning of the valves can also change. It is not uncommon for the small and large cells in the life cycle to be mistaken for different species.

Because species are generally characterized and identified by the morphology of their silica valves, and because important details of valve structure cannot be seen easily in living cells, it is common for diatom communities to be studied only after cleaning, as described above. This has had the unfortunate side effect that many aspects of the structure and growth of living cells remain unknown, even in common species. Details of plastid form and position are often characteristic of the species or genus in benthic diatoms but must be examined in very fresh material (because gross changes often occur rapidly after sampling) or after fixation (with rapidly penetrating

fixatives such as glutaraldehyde or OsO_4-containing mixtures) and staining. It should also be remembered that the chloroplasts and other organelles often move around the cell in preparation for, or after, cell division (e.g., Mann 1996). Such changes need to be taken into account when interpreting and identifying live diatoms. Living diatoms can be studied for several hours or days in microscope slide preparations in which the coverslip is sealed to the slide using petroleum jelly. Alternatively, they can be examined using water immersion lenses dipped directly into Petri dish cultures, or through the base of the culture vessel using an inverted microscope. With the advent of molecular systematics, it is worth considering whether aliquots of samples should be preserved for subsequent genetic analysis, e.g., by freezing at −80 °C.

The gradual decrease of cell size that occurs in most diatom species during the life cycle has consequences for the maintenance of strains in culture. If conditions for sexual reproduction and auxosporulation are unfavorable in culture, or if the diatom is heterothallic, clonal strains will continue to get smaller and finally die (Chepurnov et al. 2004). Furthermore, even if clones are self-compatible and can complete the life cycle, their progeny may suffer from inbreeding depression and die out after a few sexual generations (Chepurnov et al. 2011). Consequently, most culture collections contain rather few diatom strains, many of which are atypical of the group (e.g., some avoid size reduction, whereas others auxosporulate automictically). Small numbers of diatom species are maintained in the major culture collections, e.g., at the National Center for Marine Algae and Microbiota (NCMA), Bigelow, Maine, USA (https://ncma.bigelow.org/); the Culture Collection of Algae (UTEX), Austin, Texas, USA (https://utex.org/); the Culture Collection of Algae and Protozoa (CCAP), Oban, Scotland, United Kingdom (http://www.ccap.ac.uk/); the Sammlung von Algenkulturen (SAG), Universität Göttingen, Germany (https://www.uni-goettingen.de/en/); the Roscoff Culture Collection, Roscoff, France (http://www.roscoff-culture-collection.org/); and the Microbial Culture Collection, National Institute for Environmental Studies, Tsukuba, Japan (http://mcc.nies.go.jp/). Pedigreed lineages of heterothallic diatom species, as well as homothallic and asexual lineages, are maintained by the specialized diatom culture collection at the Protistology and Aquatic Ecology Research Group, Ghent University, Belgium (http://bccm.belspo.be/about-us/bccm-dcg). Many individual workers also maintain small collections for research. Some progress has been made in cryopreservation of diatoms, but because of the complications caused by the life cycle, cryopreservation is not a permanent solution to culture maintenance, though it can considerably extend the availability of a strain. Not surprisingly, therefore, there is no system for designating “type strains” in diatoms; instead, proposals have been made for using DNA barcodes to help typify taxa (Evans and Mann 2009).

Once cleaned, diatom frustules can be preserved indefinitely either dry or suspended in alcohol; the use of aqueous preservatives (e.g., formalin, Lugol’s iodine) should be avoided because the frustules will slowly dissolve. Large collections of permanent slides of cleaned diatoms, including type specimens, are held by several institutions, notably the Academy of Natural Sciences,

Philadelphia; the Natural History Museum, London; and the Alfred-Wegener-Institut für Polar- und Meeresforschung, Bremerhaven; but many other museums and institutes also hold important collections (Fryxell 1975, lists some and De Wolf and Sterrenburg provide further information at http://home.planet.nl/~wolf0334/). Collections of slides with text catalogues were distributed by several diatomists in the late nineteenth and early twentieth centuries (e.g., Tempère and Peragallo 1915).

Literature

Most of the early literature on the structure, life cycle, and taxonomy of diatoms is in German and includes the following major works: Kützing (1844); Pfitzer (1871); Schmidt (1874–1959); Schütt (1896); Hustedt (1927–1966); Karsten (1928); and Geitler (1932). A widely used, more recent flora for identifying freshwater diatoms is the *Süsswasserflora von Mitteleuropa* by Krammer and Lange-Bertalot (1986–1991; see also the condensed and updated version by Hofmann et al. 2013). An excellent handbook to marine planktonic diatoms was produced in English by Hasle and Syvertsen (1996), though this is not comprehensive, focusing on the more commonly encountered species of temperate and polar regions. Online floras for freshwater diatoms are being assembled in the USA (http://westerndiatoms.colorado.edu) and the UK. There are no up-to-date, comprehensive accounts of marine and brackish benthic diatoms. For these, the French flora of Peragallo and Peragallo (1897–1908) is still indispensable, together with myriad papers scattered through many journals, which are often hard to access (however, digitization of the older literature means that many works can now be accessed at e.g., http://www.biodiversitylibrary.org/, http://gallica.bnf.fr/, https://archive.org/).

Several series of specialist diatom publications are active, including *Bibliotheca Diatomologica*, *Iconographia Diatomologica*, *Diatom Monographs*, and *Diatoms of Europe*. Most of the volumes in these series focus on taxonomy and biodiversity (e.g., Metzeltin and Lange-Bertalot 2007; Levkov 2009). The journal *Diatom Research* (1986–) is published on behalf of the International Society for Diatom Research, which also organizes the biennial International Diatom Symposium, and *Diatom* is published by the Japanese Society of Diatomology. There is an extensive Russian and Japanese literature on diatoms. The earlier Russian papers are catalogued in the Soviet bibliography of algal literature (reprinted in Koeltz 1976 and indexed by Gollerbakh and Krasavina 1971); see also the ongoing *Diatomovye vodorosli* flora of marine and freshwater diatoms (e.g., Glezer et al. 1974).

Of special interest is the collection of electron micrographs edited by Helmcke and Krieger (1953–1977), whereas listings of more recent micrographs have been compiled by Gaul et al. (1993) and Henderson and Reimer (2003). A remarkable catalogue of diatom names was compiled by VanLandingham (1967–1979), which laid the foundation for an online catalogue of diatom names (http://researcharchive.calacademy.org/research/diatoms/names/index.asp currently not updated past September 2011) compiled at the California Academy of Science by E. Fourtanier

and J.P. Kociolek. However, VanLandingham's catalogue contains extra information not present in the online catalogue, viz. key references illustrating the use of taxon names. Another useful resource for nomenclature and taxonomy, collating information from the literature, is the "Diatom New Taxon File" of the Academy of Natural Sciences, Philadelphia, at http://symbiont.ansp.org/dntf.

Ecological, biochemical, physiological, and genetic information on diatoms is widely scattered in a vast and rapidly growing literature. A review of genus-level biodiversity was produced by Round et al. (1990), who also provided an extended, referenced introduction to diatom structure and biology. The multiauthor volume edited by Smol and Stoermer (2010) gives many examples of applications of diatoms in ecological monitoring, paleoecology, and forensics. Eclectic collections of topics are reviewed in *The Diatom World* (edited by Seckbach and Kociolek 2011) and in the much earlier but still useful *Biology of Diatoms* (edited by Werner 1977). Much interesting information about diatoms and the early history of diatom research is summarized in a handbook by Taylor (1929), which also gives information about the derivations of diatom names. The terminology of cell wall structures and morphology is summarized by Ross et al. (1979) and Barber and Haworth (1981). The special terminology applied to sexual stages and auxospores has recently been codified by Kaczmarska et al. (2013).

History of Knowledge

The first diatom taxa were described at the end of the eighteenth century, but the earliest illustrations of a diatom (a *Tabellaria*) appeared much earlier (Anonymous 1703). The name "Diatomeae" was first used by C. A. Agardh in 1824, although the basic two-part nature of the diatom wall had been implicitly recognized by De Candolle in 1805, when he named the genus *Diatoma* (Lamarck and De Candolle 1805). During the first 50 years of the nineteenth century, a large number of species were described. In 1830–1832, Agardh published a *Conspectus Criticus Diatomacearum* containing c. 100 species; by 1844, Kützing could list c. 800 species. The great German scientist Christian Gottfried Ehrenberg studied both living and fossil material from all over the world and produced innumerable illustrations, excellent for their time, many of which appear in the volumes of the *Abhandlungen der Königlichen Akademie der Wissenschaften zu Berlin* (see references in VanLandingham 1967–1979). He noted diatoms attached to the under surface of ice, in soil, on animals, and on sediments, and speculated on many aspects of their biology. Ehrenberg (1854) wrote on the formation of geological strata by the growth and deposition of microorganisms, including diatoms.

The motility and organelles of some diatoms suggested to early workers, including Ehrenberg (1838), that diatoms were animals (the chloroplasts and reserve material being interpreted as organs of digestion), and it was not until the middle of the nineteenth century that they were shown to be autotrophs (Kützing 1844). Around this time there were also numerous arguments about the mechanism of motility and about whether diatoms could live in the dark ocean depths (they can

survive for some time but do not actually photosynthesize and propagate). There was at first little understanding of the diatom life cycle: auxospores were observed but thought to be sporangia (Smith 1856), involved primarily in multiplication and dispersal rather than in regeneration of large cells *per se*.

Throughout the first half of the nineteenth century, the principal focus of diatom research was the description of diatom genera and species. The second half of the century saw classic studies on cell structure by Pfitzer (1871), Lauterborn (1896), Müller (1886, 1889, 1901), and Schütt (1896). Some of their observations were truly remarkable for their detail and accuracy and could be confirmed only when electron microscopy became available (e.g., Pickett-Heaps et al. 1984). Meanwhile, compilations of descriptive data continued, such as in the *Atlas der Diatomaceen-Kunde*, begun by Adolf Schmidt in 1874 and continued by various other authors until 1959. Descriptions of genera and species were augmented from material during nineteenth-century expeditions, including the great oceanographic voyages of H.M.S. Challenger in 1873–1876. Fundamental studies on Arctic (Cleve and Grunow 1880) and Antarctic (Karsten 1905–1907; Heiden and Kolbe 1928) diatoms were also completed at an early date. Of course many more expeditions took place on land and none was more remarkable than that undertaken by Georgi as early as 1772, exploring the waters around Lake Baikal in Siberia. His material was included in the collection of Klaproth in Berlin.

Explanation of one of the unique features of the diatom life cycle – how average cell size decreases with each cell division – was presented formally and independently by MacDonald and Pfitzer (MacDonald 1869; Pfitzer 1869) and analyzed further by Geitler (1932), whose work detailing the shape and pattern changes that accompany size reduction should still be prescribed reading for all undertaking taxonomic studies of diatoms. Discovery of the size restoration stage – auxosporulation – had occurred earlier (Thwaites 1847), but its significance was not then fully understood. Meiosis was shown to be associated with gametogenesis in the pennate diatom *Surirella* by Karsten (1912), thus showing that pennate diatoms are diplonts, but it was not until 1950 that it was finally established that centric diatoms are also diplonts (von Stosch 1950), exhibiting oogamy. Knowledge of chloroplast morphology and division in diatoms, which is still far from complete, was given an excellent foundation by the eccentric Russian biologist C. Mereschkowsky (Sapp et al. 2002), better known for his championship of endosymbiosis, in a series of papers in the early 1900s (e.g., Mereschkowsky 1902–1903, 1904).

The first half of the twentieth century was notable for the massive contribution of Friedrich Hustedt who described nearly 2000 new taxa (most of them small-celled and freshwater) and also published numerous works on the structure, taxonomy, biogeography, and ecology of diatoms, including the seminal *Die Kieselalgen Deutschlands, Österreichs und der Schweiz* (1927–1966). The foundation for our current knowledge of diatom life cycles and sexual reproduction was laid principally by just three workers: L. Geitler (see Schmid 1991), H.A. von Stosch (see Anonymous 1987), and A.M. Roshchin (e.g., 1994, and see Chepurnov et al. 2004).

From the 1960s onwards, the *Deep-Sea Drilling Project* and its successors (currently the *International Ocean Discovery Program*) have provided long cores from all the oceans and stimulated work on the geological record of diatoms. Many new species have been described and evolutionary events documented. Cores have also been made for paleoecological analyses in countless lakes worldwide (though rarely from earlier than the Quaternary) and have documented both natural and anthropogenic environmental changes (e.g., Smol and Stoermer 2010).

The development of transmission electron microscopy (which allowed the study of organelle structure, mitosis, cell division mechanisms, and wall formation) and, since c. 1967, scanning electron microscopy has transformed our knowledge and interpretation of diatom structure and also stimulated a resurgence in systematics. Little physiological or biochemical work on diatoms was undertaken until the 1950s, and there is no comprehensive review of the many recent developments.

The advent of cheap sequencing technologies has provided new insights into diatom systematics and has also allowed the first microsatellite-based investigations of the genetic structure of marine (e.g., Rynearson and Armbrust 2004; Godhe et al. 2013) and freshwater diatom populations (e.g., Evans et al. 2009; Vanormelingen et al. 2015); the only previous studies of population structure were based on isozymes (e.g., Gallagher 1982).

A diatom, *Thalassiosira pseudonana*, was the first eukaryotic microalga to have its genome wholly sequenced (Armbrust et al. 2004), inaugurating a new phase of research into the developmental genetics and metabolism of the group. The genome of another diatom, the highly unusual polymorphic pennate *Phaeodactylum tricornutum*, has also been sequenced (Bowler et al. 2008) and other species have followed (e.g., *Pseudo-nitzschia multiseries*, *Fragilariopsis cylindrus*). Several unexpected features of diatoms have been discovered as a result of genomic studies, such as that they possess a urea cycle, which is thought to help diatoms make particularly effective use of C and N following periods of N limitation (Allen et al. 2011). Diatoms have also been discovered to have unusual actin and microfilament-related components (Aumeier et al. 2015), and many examples of horizontal gene transfer from bacteria have been found (e.g., Bowler et al. 2008; Raymond and Kim 2012). Transcriptome studies are being used to dissect the process of sexual reproduction in raphid diatoms (e.g., Patil et al. 2015; Moeys et al. 2016). The advent of high-throughput sequencing has also provided new insights into the diversity and distribution of marine planktonic diatoms (Nanjappa et al. 2014; Malviya et al. 2016) and the mechanisms that maintain this diversity (Alexander et al. 2015), and has the potential to revolutionize the use of diatoms in biomonitoring (e.g., Kermarrec et al. 2014).

Practical Importance

The importance of diatoms in planktonic communities has long been recognized, and the control of their populations by silica limitation was shown in detail for several freshwater species by Lund (1949 and subsequent publications). The total contribution by diatoms to the algal biomass within many communities is still not clear

because they do not usually grow alone but in assemblages containing other algal groups. Nevertheless, their overall biomass and contribution to carbon fixation are certainly enormous (Mann 1999b estimated that they may account for c. 20% of total global C-fixation), and they are clearly very important in the food chains of aquatic habitats and have been significant players during the evolution of the biosphere (e.g., Falkowski and Knoll 2007; Berger 2007; Renaudie 2016).

Diatoms can be used as indicators of water quality and ecological status, and systems have been devised to utilize diatom populations growing on natural substrata in running waters and in lakes for biomonitoring (e.g., Kelly et al. 2008). Because their frustules are preserved well in many lake and ocean sediments, diatoms are very important for detecting long-term changes (over tens to millions of years) in aquatic environments (Smol and Stoermer 2010). Diatoms are valuable in water supply reservoirs because they oxygenate the water and remove excess nutrients; however, with excessive growth, they can become a nuisance, blocking the filtration devices in water treatment plants. Other undesirable effects include the production of the neurotoxin domoic acid (a noncanonical amino acid) by marine species of the genera *Nitzschia* and *Pseudo-nitzschia* (and apparently by *Amphora coffeaeformis*), causing potentially lethal "amnesic shellfish poisoning" (Trainer et al. 2012).

The sediments left in freshwater and marine basins that have been drained or raised above sea level often yield diatomite because of the fact that, under favorable conditions, planktonic diatoms settle to the bottom and their silica, being relatively insoluble, builds up to form deposits several hundreds of feet thick in places, e.g., Lompoc in California. This material can be processed by relatively simple means to remove organic or calcareous matter and then used in many industries, e.g., as fine abrasives and filtration material (Smol and Stoermer 2010). Fossil diatoms are also important as stratigraphical markers, e.g., for oil exploration (Krebs et al. 2010).

The unique ability of diatoms to fashion intricate cell walls of amorphous silica has stimulated particular interest among cytologists (Pickett-Heaps et al. 1990) and also biochemists and engineers (e.g., Kröger 2007; Wee et al. 2005), because of the potential to develop new methods for synthesizing silica in ambient conditions and new biomimetic materials, and to provide inspiration for architecture (Kooistra and Pohl 2015).

Habitats and Ecology

A division of diatom habitats can be made along freshwater/marine lines and indeed the vast majority of diatom genera (even whole families and orders) occupy either one or the other habitat. However, some genera occur in both and some others, especially among lineages of motile diatoms, are predominantly found in one but "spill" a few species into the other (Mann 1999a; Alverson et al. 2007). It is quite common to find similar life forms in similar habitats, whether marine or freshwater, as a result of convergent evolution (e.g., between *Tabellaria* or *Diatoma* and *Grammatophora*, which all produce zig-zag colonies: Figs. 2a, and 5b, d). Almost

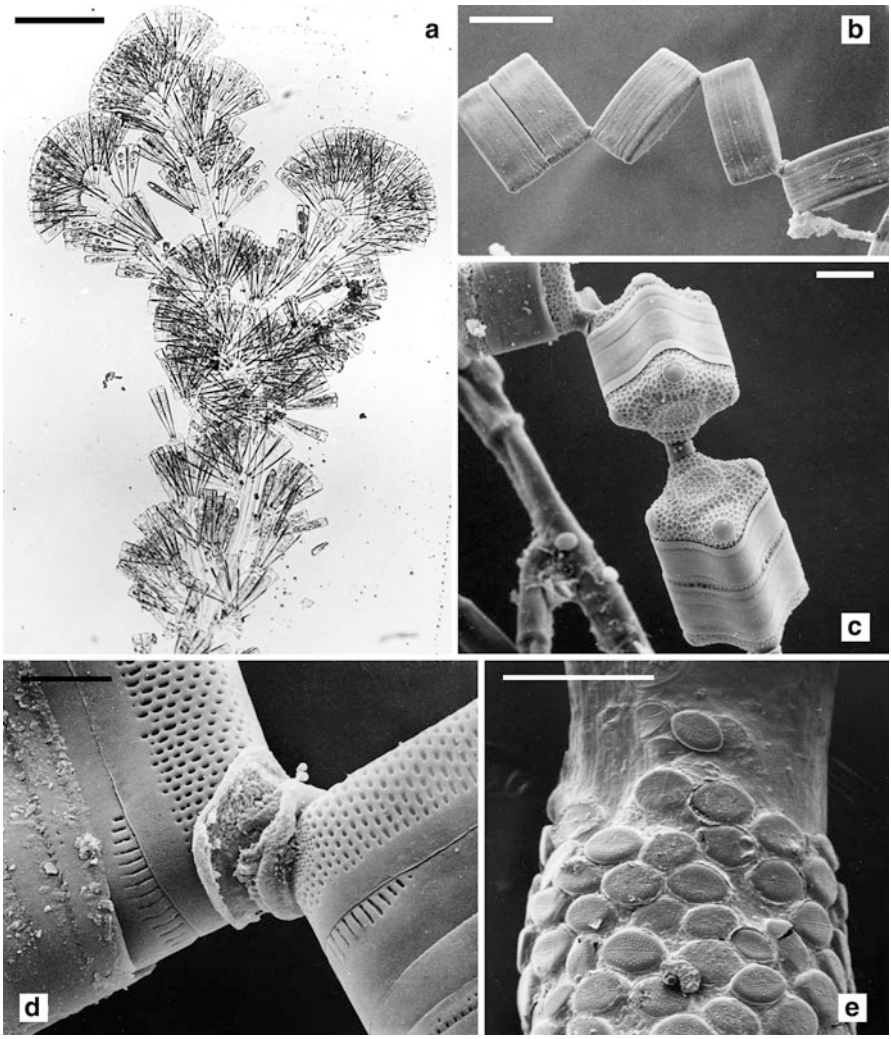

Fig. 5 Colony formation through the formation of mucilage (polysaccharide) pads and stalks. (**a**) *Licmophora* colony. Scale bar = 100 μm. (**b**) Chain of four cells of *Diatoma* linked by pads secreted from the ends of the valves. SEM. Scale bar = 20 μm. (**c**) Chain of *Amphitetras* cells linked by pads secreted through areas of small, unoccluded pores at the corners of the valves. SEM. Scale bar = 20 μm. (**d**) Detail of two *Grammatophora* cells united by a pad of mucilage at their apices. SEM. Scale bar = 3 μm. (**e**) *Cocconeis* on a marine hydroid. SEM. Scale = 100 μm

all diatoms are free-living autotrophs and out of the more than 10,000 described living species, fewer than 10 are colorless heterotrophs (Kamikawa et al. 2015), though this may in part reflect limited sampling of suitable habitat. A similar number of diatoms have been discovered living symbiotically, mainly in Foraminifera (Lee 2011), and a few dinoflagellates (so-called dinotoms) have incorporated diatoms as

permanent endosymbionts, with which they coevolve (Tamura et al. 2005; Pienaar et al. 2007; Saburova et al. 2009). The colorless forms, mostly species of *Nitzschia* (Lewin and Lewin 1967; Li and Volcani 1987; Kamikawa et al. 2015), have secondarily lost the ability to photosynthesize but retain a nonpigmented plastid (leucoplast: Schnepf 1969; Kamikawa et al. 2015).

Free-living diatoms occur in two major types of communities: (i) plankton, occurring in open water masses, and (ii) benthos, which are the communities associated with underwater surfaces and by extension also the subaerial habitats on soil, plants, etc. These gross habitat boundaries disguise a number of sub-habitats and countless niches (Round 1981a).

Plankton

The open waters of oceans and lakes are potentially available for diatom growth, down to the depth to which photosynthetically available light penetrates: populations in tropical oceans have been recorded down to 120–140 meters. However, it is unusual to find living diatoms circulating from the surface to such depths. Instead, the surface waters are extremely barren in parts of the tropical oceans and a deep-lying population occurs in the region of the thermocline in a zone of relatively high nutrient availability derived from the ample store of nutrients in the deep water, although the diatoms may be close to the point of light limitation. In temperate to cold oceans, populations tend to congregate in the surface 30–40 meters.

No diatom cells have a motility mechanism that can move them actively through water, except for the sperm of centric diatoms. Indeed, many planktonic diatoms tend to sink because the specific gravity of silica is significantly greater than that of water; maintenance of such cells in the water column is mainly because of wind- or current-induced turbulence, as can be readily seen when a lake freezes and the water column becomes isolated from wind and wave action – in this case, the diatom population sinks (Lund 1954). A characteristic of many marine planktonic diatoms is the possession of a very large vacuole, accommodated by a wide girdle containing many girdle bands. Some marine diatoms are consequently able to offset the excess weight of the silica wall by incorporating low-density solutes or adjusting ion concentrations in the cell vacuole (Boyd and Gradmann 2002). However, this is feasible only in larger-celled species (Raven and Waite 2004). A further consequence of the large vacuole is that it enables the plastids to spread out over a large surface area in conditions of low light or to clump the plastids round the nucleus (karyostrophy: see Mann 1996), supposedly for protection of the latter against high irradiation in bright sunlight.

There is an enormous range in cell size and form among planktonic genera. Small-celled, pill-box-shaped species of *Stephanodiscus* (Fig. 1a), *Cyclotella*, *Minidiscus*, and *Thalassiosira* may be only 3–5 μm in diameter, whereas the common marine *Coscinodiscus* and *Actinocyclus* (Fig. 1b) species vary between 30 and 600 μm. The largest cells of the centric genus *Ethmodiscus* can reach 2 mm in diameter. Needlelike species are also common among planktonic diatoms, ranging

from small Cymatosirales a few μm long (Hasle et al. 1983), through *Thalassionema* (10–100 μm in length) to *Thalassiothrix*, which can attain lengths of more than 5 mm. Some planktonic diatoms are solitary (e.g., *Stephanodiscus*, *Coscinodiscus*: Fig. 1a, d), but in many others the cells remain attached to each other after division to form colonies, which may be long filaments or stepped chains, e.g., in *Rhizosolenia*, *Chaetoceros*, *Skeletonema* (Fig. 2d), and *Pseudo-nitzschia*; ribbons, e.g., in *Fragilaria* (Fig. 2c) and *Fragilariopsis*; starlike (stellate), e.g., *Tabellaria*, *Asterionella* (Fig. 2a), and *Asterionellopsis*; or zigzags, e.g., *Thalassionema* and *Diatoma* (Fig. 5b). In some diatoms, the ability to form chains is facultative (e.g., *Mediopyxis*: Fig. 2b). In still others, the cells have long extensions or produce long chitin fibrils (Fig. 1c) that slow the rate of sinking, e.g., Walsby and Xypolyta (1977). Colonial morphology, such as in *Asterionella* and *Fragilaria*, can also be argued on physical grounds to be adaptations that slow sedimentation (Reynolds 2006). However, other diatom species growing in the same water may have no apparent mechanism to reduce sinking rate and indeed, sinking is arguably advantageous in some circumstances, e.g., to remove diseased cells from populations or to alleviate diminishing nutrient availability (Raven and Waite 2004). Sinking is enhanced by aggregation in the form of "marine snow" and live cells and empty frustules may be rapidly exported in this way (Smetacek 1985), facilitating deposit of diatom frustules on the ocean floor (rather than dissolution during sedimentation).

Growth in the plankton is dependent upon a supply of silica (generally in good supply in cold temperate oceans and after the winter input in lakes), and the rate of recycling of this element may be critical for the maintenance of populations. Other nutrients (especially N, P, and Fe), light intensity, and temperature are also controlling factors, in combination with the genetically determined physiological capacities and nutrient uptake systems of the cells. Equally important for population dynamics, however, are the "loss processes," which include sinking, outwash (in lakes at certain times of the year), physical or biochemical damage, parasitism, and grazing (Reynolds 2006). Only when the rate of cell growth overcomes these loss factors will the population increase and a diatom "bloom" occur, which can sometimes color the water brown, especially in spring. If a bloom continues for a long time, the available silica may be used up and the majority of cells may die. Small residual populations remain and grow again when conditions are favorable. Some marine planktonic species form thick-walled cells, which seem to help ensure short- or long-term survival of adverse conditions (McQuoid and Hobson 1996). These may be modified vegetative cells or specialized "resting spores" with a morphology quite unlike that of the vegetative cells (Round et al. 1990). A few freshwater diatoms, such as *Aulacoseira italica*, have been shown to sediment to the lake bottom and remain there in a viable vegetative state until the next growing period; in this case, in winter when the turbulence stirs the cells from the lake bottom into the water column (Lund 1954). This is impossible over most of the ocean surface, where the bottom is beyond the action of turbulence sufficient to resuspend the cells, but it may happen in inshore waters. A further ecological attribute of some marine planktonic species is the fixation of atmospheric nitrogen via endobiotic cyanobacteria (e.g., *Richelia intracellularis* in species of several diatom genera: Carpenter et al. 1999).

Benthos

The situation here is much more complex than that of the plankton because of the range of habitats in which either motile or nonmotile attached species occur. Nutrient concentrations are usually higher in benthic habitats than in the water column above. Nevertheless, the growth of benthic diatoms can also be limited by nutrient availability, and it has recently been shown that benthic diatoms perceive gradients of nutrient concentrations, e.g., of silicate (Bondoc et al. 2016), and exhibit directional movements in relation to them.

Epipelon and Soils. The surfaces of sediments of all kinds support a motile microbiota of diatoms. Whereas they can be found at some depth in the deposits and may exist there for some time, they only grow actively in the top few millimeters of the sediment. There are many records of soil diatoms at greater depths, but these are probably species that have been washed down or carried there by animals. In many lakes the epipelic microbiota only colonize sediments down to 5–10 meters below the water surface, depending upon the transmission of light through the water; in the sea, epipelon may extend to much greater depths. The vast majority of diatoms in this habitat are motile biraphid species (having raphe slits on both valves: Fig. 3a) because, after disturbance or burial by inwashed sediment, phototactic movement up to the surface is essential. These species often undergo circadian movements in and out of the surface sediment (Palmer and Round 1967; Round 1981a). A few filamentous species also "float" on the surface sediments in flocs where they seem to maintain themselves and avoid burial. Many epipelic diatoms are grazed by other protists, such as ciliates, and small animals, such as mollusks, and in some marine habitats by fish. Whereas the latter are probably nonselective, grazing by protists (Hamels et al. 2004) and parasitism by chytrids and oomycetes (Canter and Jaworski 1983, Mann 1999b) probably play a major role in controlling the diversity of epipelic and other diatom communities.

Epipsammon. Sand grains are often the site of attachment of small diatoms, and in some habitats every grain is covered by up to a hundred or more diatoms. Some grow adnate (closely appressed) to the surface of the grain, often forming short chains, whereas others perch on small mucilage pads and stand out from the grains, e.g., *Martyana* (Fig. 3d). The subtidal marine sand community (comprising both epipsammic and epipelic species) is probably the least explored in diatom ecology, due to its inaccessibility.

Epiphyton. All photoautotrophic groups, including algae and a few diatoms, are hosts to diatom species. A brown coating of diatoms on angiosperms and on green and red algae along coasts is often obvious to the naked eye. As with the sand-associated microbiota, some species are "glued" onto the plant surfaces (*Epithemia*, *Cocconeis*; Fig. 3c), whereas others are on short pads or stalks, projecting into the water (*Ulnaria*, *Achnanthes*; Figs. 3b and 4a). Yet others occur on long branching stalks (*Gomphonema*, *Licmophora*; Figs. 3e and 5b). Many attach by a corner pad of mucilage and then form zig-zag colonies when the cells remain attached to each other after cell division (*Diatoma*, Fig. 5b; *Amphitetras*, Fig. 5c; *Grammatophora*, Fig. 5d). All these features probably function (here and in other attached

communities) to project cells into positions where they will intercept more nutrients, capture more light, and compete less with adjacent organisms, with the counterbalancing risk of becoming more susceptible to grazers and parasites.

Epilithon. Rock surfaces support a microbiota of attached species. In protected regions, e.g., rock pools, filamentous species may develop upward into the water and some species grow inside mucilage tubes up to several centimeters long, e.g., *Berkeleya* (Fig. 4c) and *Parlibellus*. Recent evidence suggests that the diatoms inside a single tube may not be genetically identical (Hamsher and Saunders 2014): the tube may therefore be a cooperatively assembled structure, produced by several or many pioneer cells.

The relationship between the epilithic and epiphytic floras is not clear. Some genera and even species certainly live in both habitats, but whether any species are actually confined to one or the other requires further study. Both epiphytic and epilithic habitats may be stable for long periods of time (relative to the generation time of individual cells) and allow the establishment of "climax communities."

Metaphyton. Nonattached diatoms occur in the colorless mass of mucilage produced by some algae growing epibiotically (probably also epilithically) and remaining as a gel around the substratum. This community was first studied by Behre (1956) but few have investigated it in detail since then. Medlin (1983) showed that the metaphytic and epiphytic communities were distinct entities and that the epiphyton showed host specificity but the metaphyton did not. The diatoms within the mucilage are weakly motile. This community is very similar to the one developing in some acid streams and bog pools, consisting of masses of mucilage-forming sheets in which diatoms coexist with many other algae. These mucilage-based associations tend to be confined to waters of low pH.

Epizoon. This community is very little studied. Habitats include the feathers of diving sea birds (Holmes and Croll 1984) and the perisarc of hydroids, which often forms a rich substratum for *Cocconeis* (Fig. 5e) and *Grammatophora*. Small crustaceans can have species of *Synedra* (in fresh water) and *Pseudohimantidium* (in the sea) on their appendages; these diatoms seem to be specific to the animals. Shells of mollusks also support attached diatoms and all hard parts of dead animals become coated with diatoms. The skin of cetaceans is the substratum for species of *Bennettella* and *Epipellis* (Holmes 1985; Denys and De Smet 2010), whereas marine turtles bear diverse epizoic communities (Majewska et al. 2015) and may be important natural dispersal vectors for benthic species.

Symbiosis. The first endosymbiotic diatom recorded was *Licmophora* in *Convoluta* (Ax and Apelt 1965) and since then diatoms have been discovered to be endosymbionts of foraminifera (Lee et al. 1979; Lee 2011) and dinoflagellates (e.g., Pienaar et al. 2007; Chesnick et al. 1997; Imanian and Keeling 2014). The *Convoluta* and foraminiferan endosymbionts do not form siliceous wall elements within their hosts but can produce them again when extracted and cultured. Foraminifera also ingest free-living diatoms, and free-living species of diatoms may attach to the outside of the carbonate skeleton. As far as is known, the endosymbionts of dinoflagellates have totally lost the capacity to grow independently. A symbiotic relationship between the Antarctic ice diatom *Amphiprora kufferathii*

and its epiphytic bacteria has been demonstrated by Hünken et al. (2008). The diatom benefits with enhanced antioxidative defenses, and the bacteria utilize hydrogen peroxide produced by the diatom's photosynthesis.

Ice Diatoms. The microbiota of sea ice is a rather mixed one with diatoms being the dominant group (Thomas and Dieckmann 2003). When sea ice forms, the surface plankton is incorporated into the ice where it occupies brine pockets and channels which arise during freezing. The water in the brine pockets can attain salinities up to 4 times that of seawater as temperatures in the sea ice drop to below −10 °C. Some species do not survive, but many can withstand the hypersaline conditions and low temperatures, proliferating to form dense brown layers on the periphery and underside of the ice. Some of the species have narrow temperature requirements with optima around 2 °C and ceasing growth at 5 °C. *Melosira arctica* attaches to the lower surface of multiyear ice in the Arctic and produces long pendant columns.

The Siliceous Wall as Protection

The diatom protoplast of vegetative cells is never exposed, even during cell division, and its robust nature has led to suggestions, reviewed by Hamm et al. (2003), that the silica cell wall functions as a defense against predators. Whatever the truth of this, broken fragments of diatoms are common in fecal pellets and provide ample evidence of grazing in the marine water column, and there are also records of parasitism both in freshwater and the sea (Raven and Waite 2004). Canter showed evidence of infection of diatoms leading to accelerated decline of populations and demonstrated specificity in choice of closely related hosts (Canter and Jaworski 1983; Crawford et al. 1985; Mann 1999b). Penetration by parasites is sometimes achieved between the girdle bands or via apparent "weak points," such as the rimoportulae or raphe, but may also be through the valves (Kühn et al. 1996). The use of silica as a wall material has been suggested by Raven (1983) to reflect its lower energetic cost, relative to carbon.

Tolerance of Ecological Factors

Each individual species has a genetically determined range for existence and for optimal growth, which is then restricted further by competition and grazing. The ranges for very few species have been worked out in detail, but together, the diatoms occupy a remarkably wide span of environments. One important determinant of distribution is salinity. Some diatoms are stenohaline, being restricted to a narrow range of salinity (usually either freshwater or fully marine), but others are less fussy. Some marine diatoms extend down the salinity scale almost to fresh water and many grow optimally at salinities below the average 33–35‰ of seawater. Equally, some tolerate hypersaline conditions but as salinity increases, e.g., in tropical lagoons or salt works, the number of species decreases until at 120‰ only one or two survive

(Ehrlich 1975). However, no species have yet been confirmed as confined to salinities above that of normal seawater.

Extremes of temperature are also tolerated by a few species. For example, some diatoms are able to withstand extremely high temperatures in thermal springs: *Denticula elegans* was found living at 60–62 °C by Cassie and Cooper (1989) at Rotorua, New Zealand, and Cassie (1989) reported *Fragilaria construens* surviving 77 °C. However, most diatoms have much lower tolerance limits, and Hustedt (1959) considered 45 °C to be the upper limit for most species.

Fresh waters are chemically much more diverse than seawater and here there are clear species preferences, e.g., for acid, alkaline, or sulfate-rich waters. Some *Pinnularia* species can tolerate a pH of less than 2 (Sabater et al. 2003). In most cases, the physiological basis of these preferences has not been established. For example, in the case of pH, it is usually unclear whether it is pH itself that is selective or whether it is some other factor, such as the availability of carbon dioxide or bicarbonate, or of silicate or other nutrients, that is causal. The abundance of a few species is clearly correlated with water flow, e.g., *Meridion*. Whatever the physiological mechanisms, however, the combination of adequate taxonomy, identifiable preferences, and the long-term preservation of diatoms in lake and ocean sediments makes diatoms unrivalled for reconstruction of environmental change in aquatic habitats over periods of tens to millions of years (reviewed in Smol and Stoermer 2010).

Just as conditions may become suitable to sustain massive growths of planktonic diatoms, so too may benthic species be favored. This occurs spectacularly and disastrously in rivers in many parts of the world as a consequence of blanket growths of *Didymosphenia geminata* (e.g., Bothwell et al. 2014). This species severely compromises the ecosystem of affected rivers and causes expensive problems for water management.

Characterization and Recognition

Cell

The Bacillariophyta are all unicellular or colonial. Their vegetative cells are diploid and characterized above all by their complex siliceous walls. In many species the ornate pores, thickenings, and spines of the siliceous wall components are clearly visible under high magnifications in the light microscope, but further significant detail is always detectable by electron microscopy. It is possible to identify some species in live material, but traditionally the cells have been treated to separate the wall components and it is above all the morphology of the valves that forms the basis for classification and identification.

Inside diatom cells are the organelles typical of heterokont (stramenopile) algae. The plastids are conspicuous and vary in color from yellowish or greenish hues to a deep brown, and they are therefore sometimes called chromoplasts or chromatophores, rather than chloroplasts. They may be small discoid or lobed structures (Figs. 1d and 2b), or platelike (Fig. 6e), or ribbonlike (Fig. 7d), or highly dissected

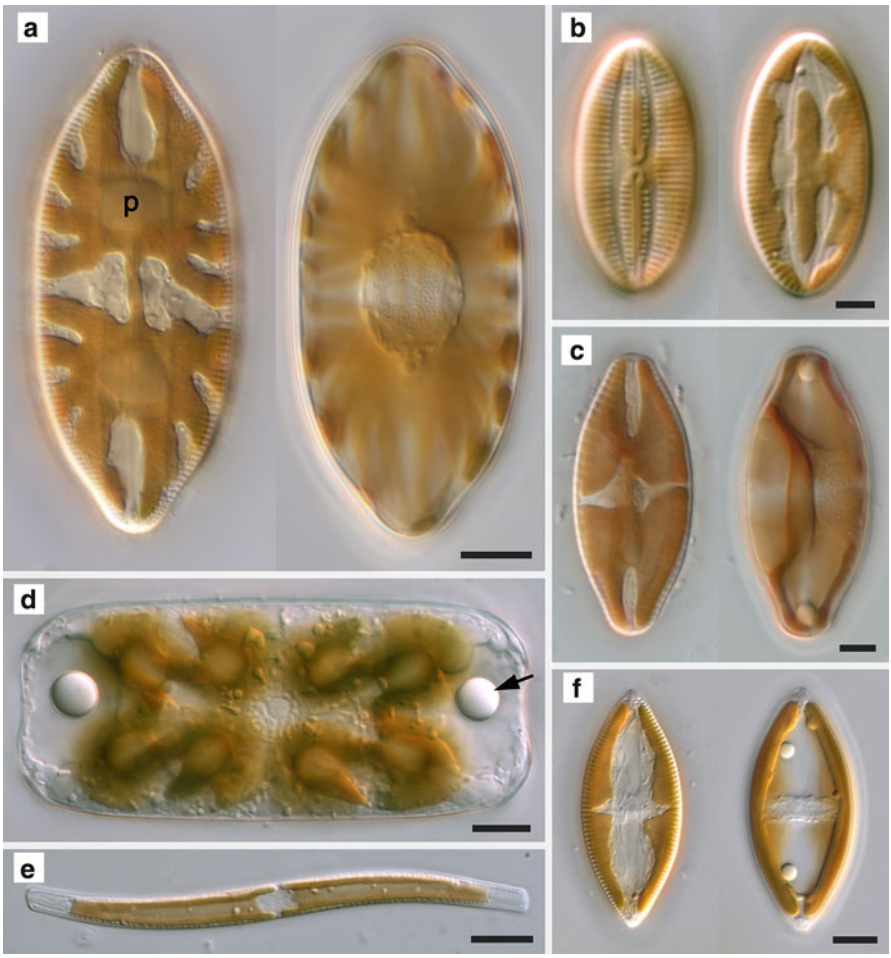

Fig. 6 Living cells of raphid pennate diatoms, all seen in valve view except (**d**, **e**). All scale bars = 10 μm, except (**c**). (**a**) Peripheral and central focuses of *Lyrella* cell. Note the strongly lobed chloroplast, which contains two roundish pyrenoids (e.g., p), and the central nucleus containing a prominent nucleolus and surrounded by a shell of cytoplasm containing Golgi bodies (appearing as short *curved bars*). (**b**) Valve and peripheral focuses of *Fallacia*. The lobes of the chloroplast are clearly related to the pattern of markings on the valves, avoiding the lyre-shaped clear area. (**c**) Peripheral and central focuses of *Placoneis*. Scale bar = 5 μm. (**d**) Amphoroid diatom in girdle view, with a highly convoluted chloroplast and two 'volutin' granules (e.g., *arrow*). (**e**) Sigmoid *Nitzschia* species containing two chloroplasts arranged end to end. (**f**) Peripheral and central focuses of *Navicula* cf. *palpebralis*; there are two chloroplasts, one on each side of the cell. Note also the central, transversely elongate nucleus and two volutin granules

and complex in shape (Figs. 6a–d, f, and 7a). In raphid diatoms, chloroplast morphology and position are usually highly constant within genera and can be used to help identify living diatoms. There is often a clear relationship between the position and shape of the chloroplasts and cell wall structures and other organelles (Fig. 6b).

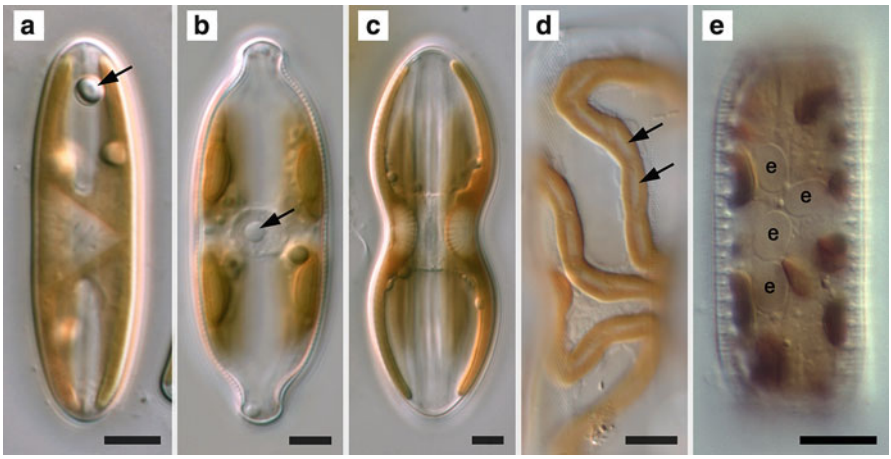

Fig. 7 Detail of chloroplasts and cells of raphid pennate diatoms. (**a**) *Sellaphora bacillum*. Note the H-shaped chloroplasts, the eccentrically placed triangular profile of the pyrenoid (with invaginations) and volutin granule (*arrow*). Scale bar = 5 μm. (**b**) *Neidium* cell with four chloroplasts and a central nucleus surrounded by Golgi bodies (appearing as *curved bars*) and containing a prominent nucleolus (*arrow*). Scale bar = 5 μm. (**c**) *Diploneis* cell with two chloroplasts, one on either side of the cell, each with a prominent invaginated pyrenoid at the center. Scale bar = 5 μm. (**d**) Part of a *Donkinia* cell with ribbon-like chloroplasts. Note the bar-like pyrenoids along the axis of the chloroplast (arrows). Scale bar = 10 μm. (**e**) *Epithemia* cell, containing four endosymbionts (e). Scale bar = 10 μm

The chloroplasts contain chlorophylls a and c, fucoxanthin, and various other carotenoid pigments, e.g., diatoxanthin and diadinoxanthin (Jeffrey et al. 2011; Egeland 2016). One or more pyrenoids are usually present in each chloroplast and are often conspicuous (Figs. 6a and 7a–d). The number of pyrenoids per chloroplast and their structure and positions vary among genera; some have angular shapes (Fig. 7a), probably reflecting a semicrystalline substructure. In a few genera the pyrenoids are penetrated by fingerlike extensions of the cytoplasm (Fig. 7a, c). The chloroplasts are bounded by four membranes, reflecting their ultimate origin through secondary endosymbiosis of a red alga (van den Hoek et al. 1995). Chloroplast (cp-) DNA is usually contained in a peripheral "ring nucleoid," running around the margin of the organelle (Kuroiwa et al. 1981; Coleman 1985), but in large-celled diatoms the arrangement can differ: in *Nitzschia sigmoidea* cp-DNA lines the sides of the linear pyrenoids (Mayama et al. 2004) and in *Pinnularia nobilis* it occurs as scattered granules (Mayama and Shihira-Ishikawa 1994).

The mitochondria have tubular invaginations of their inner membranes (Fig. 12f). Prominent shells of Golgi bodies occur around the nucleus in many pennate and most bipolar centric diatoms (Figs. 6a and 7b), whereas elsewhere among the centrics there are sometimes special associations of a Golgi body, endoplasmic reticulum, and a mitochondrion (e.g., Pickett-Heaps et al. 1990), or of a Golgi body and either a mitochondrion or a chloroplast (Idei et al. 2012). The principal

carbon storage products are oil globules and glistening whitish deposits of chrysolaminarin (a β–1,3 glucose polymer). Polyphosphates are also produced (Kuhl 1962), forming conspicuous "volutin" granules in some species (Figs. 6d, f, and 7a), and it seems likely that diatoms play an important role in transferring phosphorus from the water column to the sediments in the world's oceans (Diaz et al. 2008).

Some diatoms contain endosymbionts. Heterotrophic bacteria have been found in the raphid diatom *Pinnularia* (Schmid 2003a, b), and cyanobacteria are present in the vacuoles of some planktonic diatoms, such as *Hemiaulus* and *Rhizosolenia* (e.g., Janson et al. 1995), and in the cytoplasm of *Epithemia* (Fig. 7e) and *Rhopalodia* (Geitler 1977; Nakayama et al. 2011). These cyanobacteria contribute to the symbiosis principally through nitrogen fixation (e.g., Foster et al. 2011; Kemp and Villareal 2013). The endosymbionts of *Epithemia* and *Rhopalodia* are incapable of independent existence and indeed of photosynthesis (Nakayama et al. 2014). How these cyanobacteria entered diatom cells, despite the presence of the frustule, is a mystery; the only naked cells known in *Epithemia* and *Rhopalodia* are the amoeboid gametes.

Cell Wall and Cell Division

The diatom cell wall (***frustule***) is often likened to a Petri dish (cf. Fig. 1a, b) because it consists of two overlapping halves (***thecae***). However, this is a little misleading, because each theca is itself composite, consisting of a series of hoops (the ***girdle bands***) attached to the edge of a large endpiece (the ***valve***). One theca (the ***hypotheca***) is generally slightly smaller than the other (the ***epitheca***: Fig. 8a–d) and is always younger, being formed after the latest mitosis. During the cell cycle, the hypotheca slides out from beneath the older, overlapping epitheca and new bands are added to its edge; in this way, the cell increases in volume. The volume cannot be increased in any other direction because the siliceous valves and girdle bands, like glass, are essentially inelastic, although they can flex (e.g., in the living cells of the raphid diatom *Craticula*, the valves bow outwards as a result of the turgor of the cell, despite being well-silicified and robust: Mann 1994).

Once the cell has grown sufficiently and the hypotheca has attained more or less the same length and structure (with the same number of girdle bands) as the epitheca, mitosis is initiated. As the division of the nucleus is completed, cytokinesis takes place and two new valves (usually with at least some of their accompanying girdle bands) are formed within the frustule of the parent cell, before the old thecae separate. Then the two daughter cells separate, each inheriting one of the valves of the parent cell and one of the newly formed valves. This highly characteristic, semiconservative mode of cell division, is known only from this phylum and has fundamental consequences for much of diatom biology, e.g., causing average cell size to decrease during the vegetative phase (see below).

In many diatoms, the daughter cells separate fully once the new valves are complete, but in some the valves remain connected by organic material or

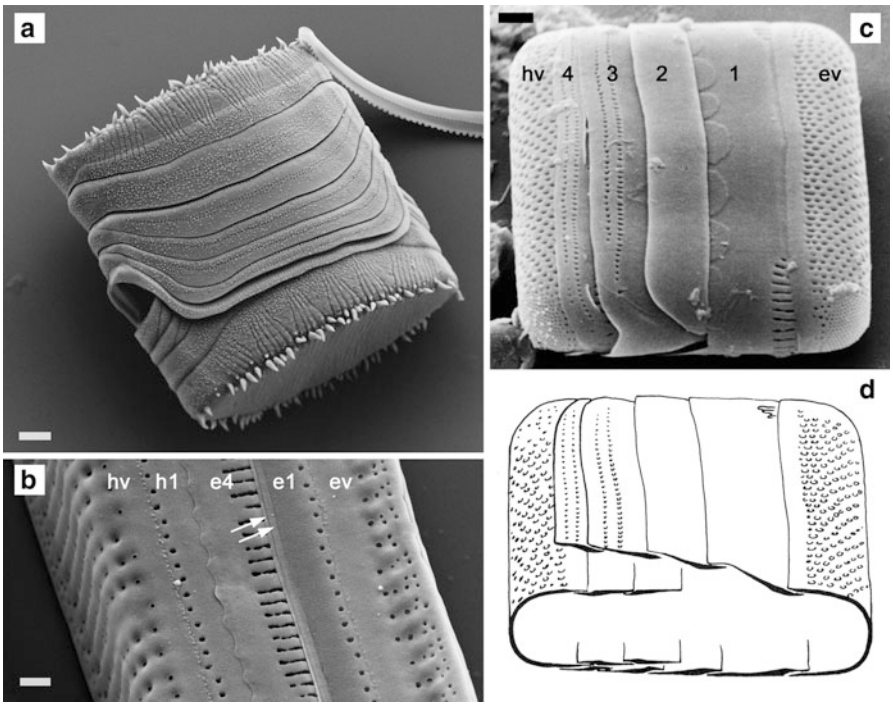

Fig. 8 Frustule and girdle structure. SEM. (**a**) *Diatoma* frustule: the epitheca is at the top, overlapping the hypotheca. Scale bar = 2 μm. (**b**) Detail of a *Nitzschia* frustule. The epitheca comprises the epivalve (*ev*) and four girdle bands, two wide (e1, e4) and two very narrow ones (*arrows*) in between. The epitheca partially overlaps the hypotheca, of which the hypovalve (*hv*) and one band (h1) are visible. Scale bar = 500 nm. (**c**, **d**) Photograph and drawing of a *Grammatophora* frustule. The epivalve to the *right* (ev) is linked to four girdle bands (1–4), which partly obscure the hypovalve to the *left* (hv). Note the variation of markings in the girdle-bands. The schematic cut-away drawing of a *Grammatophora* frustule (**d**) illustrates the spatial relationships of the frustule components in **c**. The two girdle-bands of the incompletely formed hypocingulum (*left*) are assumed. Scale bar = 10 μm

interlocking or fused silica projections (Figs. 2d and 12d, e). In this way, chains can be formed, which, with some important exceptions (these include colonies of cells on branched stalks, thalloid mucilaginous colonies in *Dickieia* and mucilaginous tubes in various raphid diatoms, e.g., *Berkeleya* [Fig. 4c]), are the only means of colony formation. In a few diatoms, the new valves are not smaller than the valves of the parent cell because of an unusual flexibility of the girdle. Consequently, these species can grow in culture indefinitely, without any reduction in the average size of the cells in the population (Chepurnov et al. 2004).

The valves are perforated by numerous small pores, arranged in species-specific patterns. Traditionally, two main types of valve pattern have been recognized (Schütt 1896). In the "***centric***" type of organization, the pores are arranged in radiating rows (striae: Figs. 1a, b, d), subtended at the pattern center (which is not always at the

center of the valve) by a small ring (***annulus***), within which pores are less regularly arranged or absent. Centric diatoms can be circular (Fig. 1a–d), oval, triradiate or triangular, quadrate (Fig. 5c), or many angled; less often they are elongate. Molecular phylogenetic studies have shown that the centric diatoms are not a monophyletic grouping but, depending on the criteria selected for the analysis (see Medlin 2014), fall either into a grade of separate lineages or into two monophyletic classes, comprising the radial and bipolar centrics, respectively (see section "Summary Classification"). In the "***pennate***" type of organization (Fig. 9a–g), the pattern is feather-like (Latin *pinna* or *penna* = feather), the striae lying in two rows either side of a longitudinal bar or rib (the ***sternum***). Pennate diatoms are almost invariably elongate but may be isopolar (Figs. 9b, g) or heteropolar (Figs. 9a, c), bilaterally symmetrical (Figs. 9b) or dorsiventral (Fig. 9d, i). The down-turned side of the valve is known as the ***valve mantle*** and the markings on this may differ from those on the top of the valve (the valve face). Unlike the centrics, the pennate diatoms are always recovered as monophyletic in molecular phylogenies (e.g., Sims et al. 2006; Theriot et al. 2010), but sternum-like structures have evolved independently in some centric lineages, perhaps through elongation of the annulus (e.g., Kooistra et al. 2003a).

The majority of pennate species have two complex slits along or near the midline of the valves – these are known as ***raphe slits***, and it is through them that the organism achieves locomotion (Fig. 9b–i). A model to explain raphe function was proposed by Edgar and Pickett-Heaps (1984) and no major revision of this seems yet to be needed. Mucilage fibrils are secreted into and through the raphe slits, apparently from Golgi-derived vesicles, but remain connected to the protoplast via transmembrane components. In turn, the transmembrane components interact with actin microfilaments lying immediately beneath the raphe and are constrained to stream along the raphe slits (Round et al. 1990). Hence, if the mucilage fibrils become attached distally to a firm substratum, the effect of the streaming will be to generate motion of the whole cell, which occurs at speeds of up to 20 μm or more per second. Mucilage is left behind as a trail when it reaches the ends of the slits, forming part of the "extracellular polymeric substances" released by diatom cells and performing various functions including adhesion and providing structure (Daniel et al. 1987; Underwood and Paterson 2003).

Some genera have raphe slits on both valves (***biraphid***), while others (the ***monoraphid*** diatoms, which are polyphyletic) have slits on one valve only. In the latter, motility is limited and slow and the cells are attached to the substratum for most of the time by mucilage, e.g., *Cocconeis* (Fig. 3c) and *Achnanthes* (Fig. 3b). For accounts of the various diatom polysaccharides, see Hoagland et al. 1993; Underwood and Paterson 2003; Gügi et al. 2015. The raphe slits can run along the midline of the valve (Figs. 9b, c, f) or may be displaced to one side (Fig. 9g, i) or even circumferential (Fig. 9e). The raphe normally consists of a pair of slits running from either side of a clear central area to the apex, where the external fissure often bends and continues as a blind surface groove (Fig. 5c). In several genera, e.g., *Nitzschia* and *Hantzschia* (Fig. 6d), the slits are bridged internally by short bars (fibulae), which appear to function as ties, preventing the valve from splitting along the raphe. In the genus *Eunotia* and its allies, which seem to be an early offshoot of

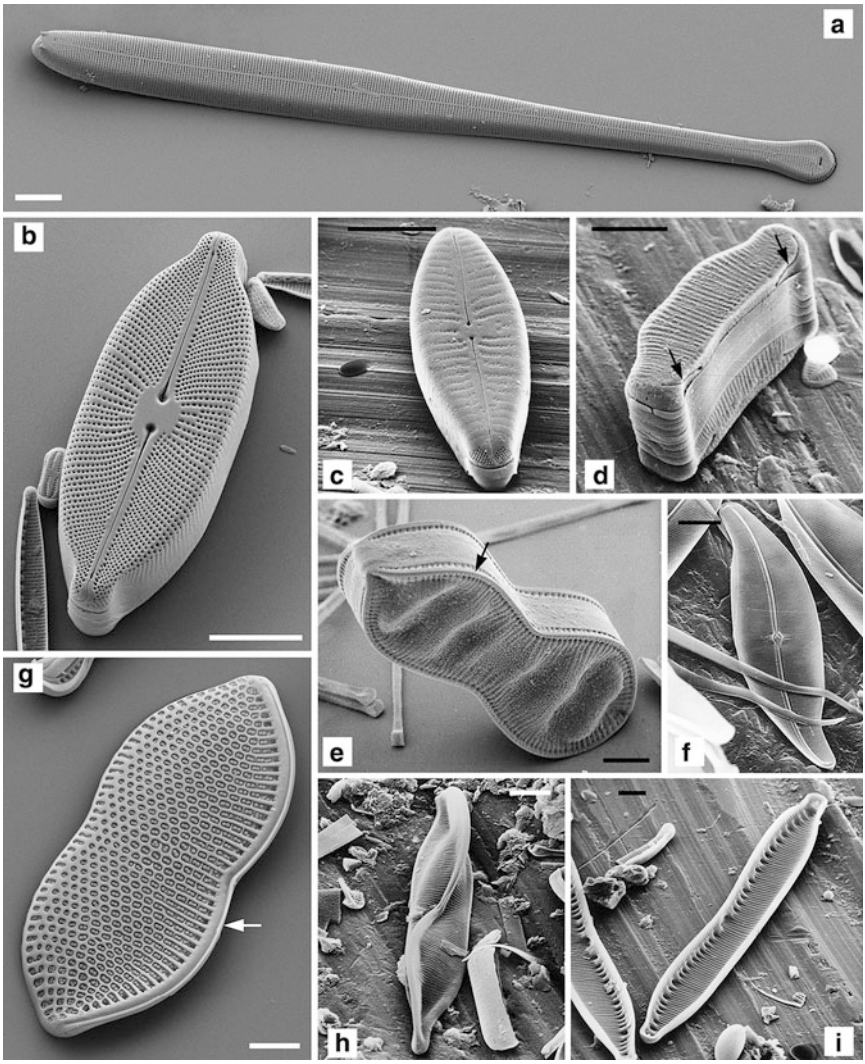

Fig. 9 Pennate diatoms. SEM. All except *Licmophora* (**a**) are raphid diatoms. (**a**) *Licmophora* valve; a stalk (like those shown in Figs. 3e and 5a) is secreted through special pores at the narrower end. Note the sternum running along the center of the valve and bearing transverse ribs on either side. Scale bar = 5 μm. (**b**) *Cosmioneis* frustule. Note the two axial raphe slits and slightly radiating striae. Scale bar = 10 μm. (**c**) *Gomphonema*, with heteropolar symmetry. Scale bar = 10 μm. (**d**) Frustule of *Eunotia*, which has short raphe slits (*arrows*) that run from the valve face over onto the mantle. They are found on the same side in the two valves of each frustule. Scale bar = 10 μm. (**e**) *Cymatopleura* frustule. The valve face is undulate and the raphe (*arrow*) runs round the rim of the valve with a discontinuity at either end. SEM. Scale bar = 10 μm. (**f**) Sigmoid symmetry of *Gyrosigma*. Scale = 10 μm. (**g**) *Psammodictyon* valve. The raphe (*arrow*) is borne on a raised keel at the margin of the valve. Scale bar = 2 μm. (**h**) *Entomoneis* valve: the raphe is elevated on a ridge, which takes a sigmoid curve along the valve. Scale bar = 10 μm. (**i**) The raphe of *Hantzschia* lies to one side of the valve (shown here from the inside) and is subtended on the inside by a number of small bridges (fibulae). Scale bar = 5 μm

the raphid diatom lineage (Theriot et al. 2010), the raphe slits are very short and lateral to the sternum instead of integrated into it (Fig. 9d), but the cells are nevertheless motile. As in the centric series (Figs. 1b and 5c), there is all manner of variation in valve outline and topography in pennate diatoms, including sigmoid (Fig. 9f, h) and keeled (Fig. 9h) forms.

The siliceous girdle bands are frequently split rings, with the splits in adjacent bands lying at 180° to each other. Opposite the split in one band there is a tonguelike extension (ligula) of the adjacent girdle band to fill the gap (Figs. 8a, c, and 10a). In a few genera some of the bands are complete hoops, e.g., in *Grammatophora*, where the bands also bear well-developed septa extending part way into the cell lumen (Fig. 10b). Still other diatoms have a girdle composed of individual segments (Fig. 10c), appearing like diamond-shaped scales.

The pores of the valves and girdle bands, termed ***areolae***, allow transfer of water, nutrients, gases, cellular products, etc. between environment and cell. Only rarely,

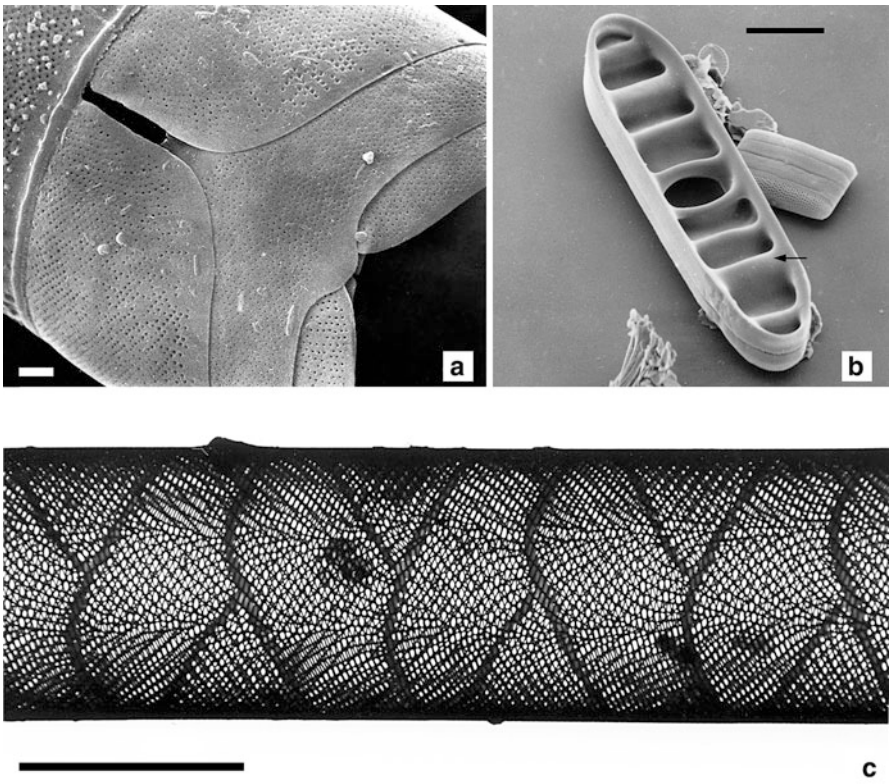

Fig. 10 Girdle bands. (**a**) *Pleurosira:* the gaps left by the split ends of the first and third bands are partially or completely closed by an enlargement of the second band. SEM. Scale bar = 10 μm. (**b**) Girdle band of *Grammatophora* with its characteristic undulate septum (*arrow*). SEM. Scale bar = 10 μm. (**c**) The scale-like girdle bands of *Rhizosolenia*. Transmission electron micrograph (*TEM*). Scale bar = 1 μm

however, are they simple channels through the silica. In most cases, a fine plate of silica, itself perforated by tinier holes, stretches across the pores. These plates are known as ***vela*** or ***pore plates*** and take many different forms, each to some extent characteristic of genera or groups of genera; the position of the velum, towards the inner or outer ends of the areolae, is also of systematic importance. Some of the variation to be found among vela can be seen in Fig. 11a–d. The areolae of the girdle bands are usually similar to those on the valves but much smaller. The last-formed bands (furthest from the valves) are often plain. In some cases, all the girdle bands lack pores.

The valves tend to be more complex than the girdle bands and may have special types of apertures in addition to the areolae. The most common type of special aperture, found in most centric and a few pennate diatoms, is developed internally as a slit between a pair of lips and externally either as a simple opening or a tube and is termed a ***rimoportula*** (Fig. 12a, b) or ***labiate process***. The functions of rimoportulae

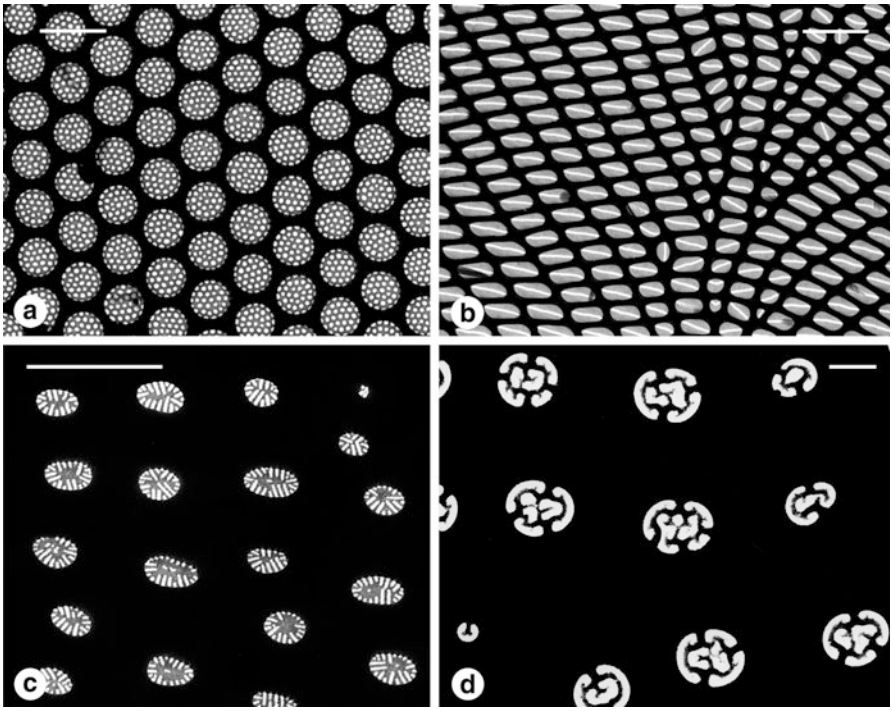

Fig. 11 Details of areola structure. TEM. Scale bars = 1 μm. (**a**) *Roperia*: each areola has many small pores in a thin siliceous velum. (**b**) Each velum of *Rhizosolenia* has just one narrow slit. (**c**) The areolae of *Cocconeis* are variable in size and shape and so is the pattern of slits in the velum**.** (**d**) The vela of *Rhaphoneis* are branching, interconnected projections from the side of the areolae

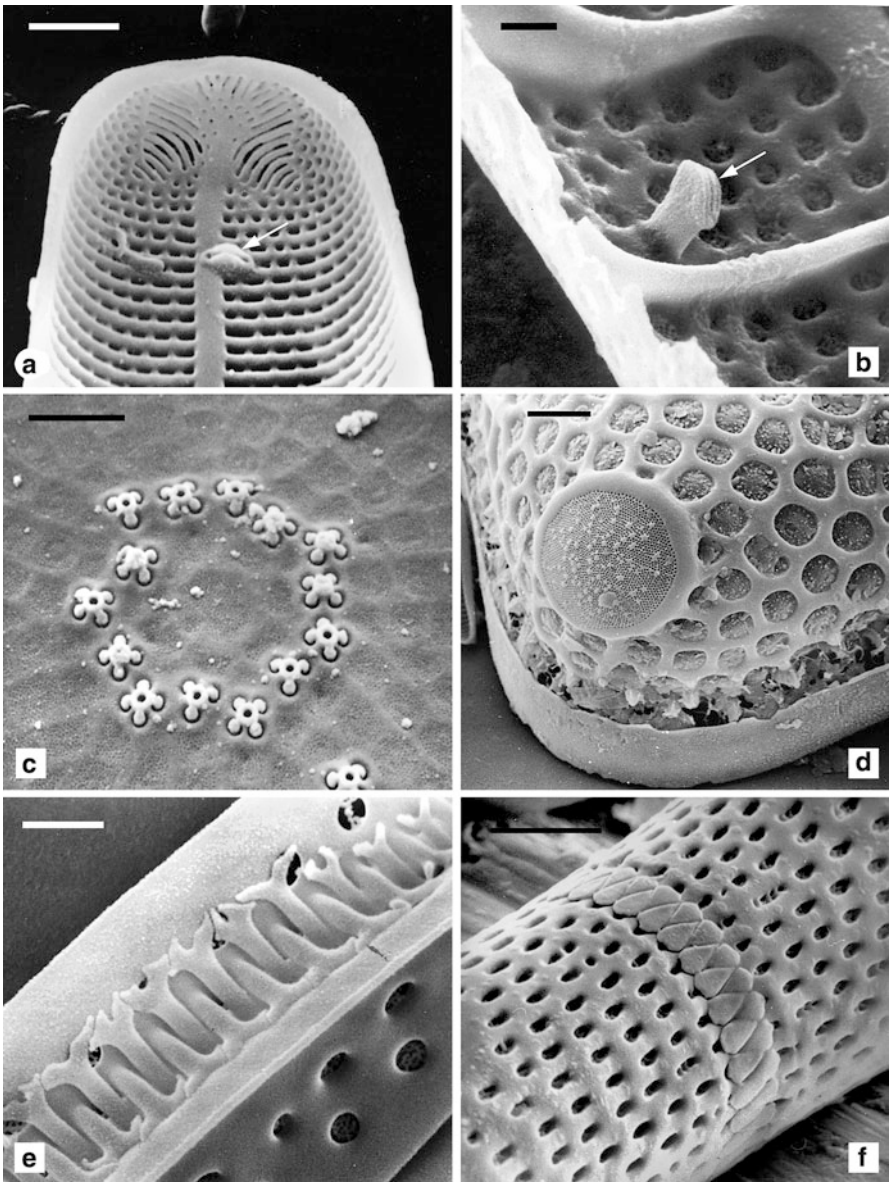

Fig. 12 Special wall structures. SEM. Scale bars = 1 μm. (**a**) Rimoportula of *Cyclophora* (*arrow*). (**b**) Stalked rimoportula of *Triceratium* (*arrow*). (**c**) Internal apertures of the fultoportulae of *Thalassiosira*. (**d**) Ocellus of *Odontella*. (**e**) Linking spines connecting two valves of *Cymatosira*. (**f**) Linking spines connecting two valves of *Aulacoseira*

remain unclear in most cases, although in a few cases they have been shown to be involved in secretion for motility (Medlin et al. 1986; Pickett-Heaps et al. 1990) or endocytosis (Kühn and Brownlee 2005). The other well-known type of process is confined to the centric order Thalassiosirales and is called the ***fultoportula*** or ***strutted process***. This consists externally of a simple tube or opening and internally of a tube surrounded by a number of basal satellite pores separated by buttresses (Fig. 12c), or with the satellite pores developed as tubes. Its function is the secretion of chitin fibers (Fig. 1c) that connect cells together or control sedimentation (Walsby and Xypolyta 1977). Additionally, in many epiphytic, epilithic, and epipsammic diatoms there are areas of simple pores near the periphery or ends of the valves (Figs. 5d, 8c, 9c, and 12a, d), from which stalks or pads of mucilage are secreted to link the cells to the substratum or to one another.

Spines, tubercles, etc. are common on the outer surfaces of the valves but never on the girdle bands, nor on the inner surface of the valves. Some spines (Fig. 12e, f) act to connect cells together in chains and in a few genera the exit tubes of the rimoportulae or fultoportulae act as interlocking devices.

Diatom mitosis and particularly the structure and functioning of spindle and associated structures have been the focus of considerable detailed research, which has contributed significantly to a general understanding of the mechanism of mitosis (Pickett-Heaps 1991). In some species a small dense body of granular material is associated with microtubules and lies near the nucleus during interphase. This ***microtubule organizing center*** (***MTOC*** or ***centrosome***) breaks down at prophase and at the same time a complex and highly ordered spindle develops nearby. Cytokinesis occurs through ***cleavage*** (Round et al. 1990). Mitosis and cytokinesis are followed by the formation of new valves (indeed, this sequence is generally obligatory). The MTOC reforms and migrates to a position between the nucleus and the ***silica deposition vesicle*** (***SDV***), which is a flattened sac beneath the cell membrane in which the new valves are formed. The nucleus and the MTOC usually remain intimately associated with the developing valve, and systems of microtubules (subtended by the MTOC) and microfilaments are present, which may play a role in the expansion of the SDV and the morphogenesis of the valve (Pickett-Heaps et al. 1990). In some diatoms, treatment with microtubule inhibitors results in the formation of distorted valves, although the basic rib–stria system appears to be little affected. A special structure, the ***raphe fiber***, has been found immediately below the forming raphe slits in recently divided cells of raphid diatoms and may be involved in generating the complex shape of the raphe (Pickett-Heaps et al. 1990). A somewhat similar fibrous structure – ***the labiate process apparatus*** – is present while the rimoportulae are formed.

Since 2000, there have been major advances in our understanding of how silicate is acquired by cells and converted into the amorphous hydrated silica of the valves and girdle bands (Hildebrand 2008; Hildebrand & Lerch 2015; Finkel 2016), stimulated by the realization that diatoms achieve feats of chemical engineering in ambient conditions that materials chemists achieve only by using high temperatures

and pressures. Building on earlier studies by Volcani and coworkers (e.g., chapters in Simpson and Volcani 1981) and using modern molecular and genomic approaches, it has been possible to characterize components of the silicon transport system (Hildebrand 2008) and to show that silica deposition in the SDV is catalyzed and mediated by at least two classes of proteins: (1) ***silaffins***, which are peptides rich in serine and lysine that have been extensively modified after translation by methylation, phosphorylation, and covalent linkage with polyamines and silacidins; and (2) ***silacidins***, which contain mostly phosphorylated serine and aspartic and glutamic acids (Sumper and Brunner 2008). It appears that interactions between silaffins, silacidins, the polyamines, and polysaccharides, e.g., chitin, control the detail of silica deposition (e.g., Richthammer et al. 2011). Recently, transcriptomics approaches have added considerably to knowledge of which genes are involved in silicification (reviewed by Finkel 2016).

However, although the biochemical and electrostatic properties of silaffins and silacidins probably take us a long way towards understanding the finer detail of cell wall development, it is not yet clear that they are relevant to larger-scale morphogenesis in diatoms: the creation of the beautifully ordered patterns of ribs and pores of diatom valves still mostly eludes explanation. Pickett-Heaps et al. (1979) proposed that an organic template is formed, onto which silica is deposited from both sides. This may be true for pennate diatoms whose wall is a simple laminate structure but the structure of more complex walls, such as are found in many centric diatoms, suggests the formation of one layer first, onto which a chambered or ***loculate*** system is later superimposed (Crawford 1974a; Schmid and Volcani 1983; Round and Crawford 1984). Lenoci and Camp (2008) have been able to generate patterns very similar to those of many diatoms possessing chambered or folded valves, using a model based on phase separation on a planar surface, and Pickett-Heaps et al. (1990) argue that the cytoskeleton and cell organelles are probably also involved in mesoscale patterning in diatoms; this is supported also by more recent studies using fluorescence labeling (Tesson and Hildebrand 2010).

The initial development of the valve almost always involves sequential formation of a tightly controlled rib–stria pattern, and the way that the pattern varies in relation to disturbances (e.g., Mann 2006) and natural variation in valve size indicates that the rib–stria system and any template controlling its appearance must form as the SDV expands outwards from the initial pattern center (e.g., Schmid and Volcani 1983; Pickett-Heaps et al. 1990), which is usually either the annulus (in centric diatoms) or the sternum (in pennate diatoms). Explanation of the control of rib spacing during the production of the initial layer (which must be very precise, since otherwise the species taxonomy of diatoms would not work as well as it does) is probably the main remaining challenge in understanding diatom morphogenesis.

At the gross level, cell shape in diatoms is created largely during the expansion of the auxospore (see below) and then gradually modified by differential flexing of the girdle during the subsequent phase of slow decline in size during the vegetative phase (Mann 1994), except in species with circular valves where no modification occurs except in teratologies.

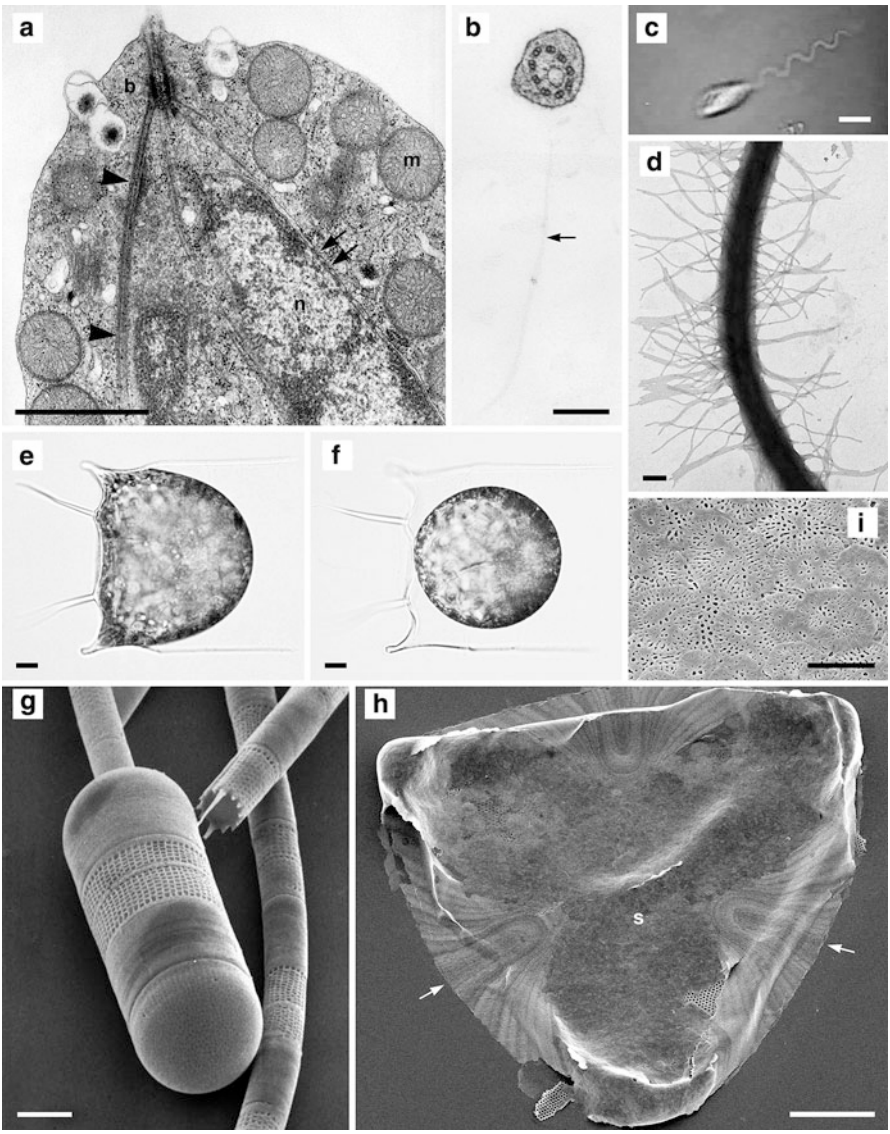

Fig. 13 Sexual reproduction in centric diatoms. Micrographs (**a**–**d**, **h**, **i**) were very kindly provided by Professor Masahiko Idei (Bunkyo University, Japan) and are reproduced here by permission (**a**) Apex of a *Thalassiosira* sperm in longitudinal thin section, showing the flagellar basal body (*b*) subtending a cone of microtubular bundles (e.g., *arrowheads*) that extend over the surface of the nucleus (*n*). Many nuclear pores are evident (*arrows*). Note also abundant rounded mitochondria (*m*) containing tubular invaginations of the inner membrane. TEM. Scale bar = 1 μm. (**b**) Transverse thin section through the flagellum of *Melosira moniliformis* var. *octogona*. The axoneme lacks central microtubules (9+0 configuration) but frequently contains vesicles, as here. Long mastigonemes are attached to either side of the flagellum (e.g., *arrow*). (**c**) Swimming sperm of *Thalassiosira*. High-speed video still, showing quasi-sinusoidal beat. Scale bar = 5 μm.

Life Cycle

As noted above, in some species the girdle bands are sufficiently flexible to allow the new valves to be as large as the old valves, even though they are formed within the parent cell's frustule. However, in most diatoms one of the new valves is smaller than the smaller of the two parent valves by double the thickness of the girdle bands (Crawford 1981). Consequently, a succession of mitotic cell divisions generally results in a diminution of the average valve dimensions. Ultimately, death of the population will result unless the maximum dimensions of the cells are restored. This usually occurs via an auxospore formed following sexual reproduction. Sexual reproduction is morphologically isogamous in most pennate genera, but oogamous, with motile sperm and larger nonmotile egg cells, in the various lineages of centric diatoms (although information is absent for many genera).

Though regarded until recently as almost universally homothallic, diatoms do in fact exhibit a variety of mating systems (Chepurnov et al. 2004; Sato et al. 2011; Davidovich et al. 2012). Some pennate diatoms (probably the majority) are heterothallic, whereas others (and also most centric diatoms) are facultatively or habitually homothallic. Reduced sexuality (via auto- or apomixis) has evolved independently in several lineages (e.g., Mann et al. 2013; Poulíčková et al. 2015).

In oogamous diatoms, ***sperm*** (Fig. 13c) are produced following a series of divisions within a modified cell (***spermatogonium***); they are then released and swim to find the ***egg cell*** (produced within an ***oogonium***: Fig. 13e), presumably guided by chemotaxis. Pennate diatoms lack flagellate stages and here the gametes (Fig. 14b) are usually all alike (morphologically isogamous) and show very limited autonomous movement; in raphid pennate diatoms, it is the sexualized vegetative cells that move, using their raphe systems to find each other and pair actively before meiosis is initiated (Fig. 14a). The cells then often surround themselves with a capsule of mucilage (Fig. 14d), in which gametogenesis and fertilization take place. Araphid pennate diatoms are not generally able to move very effectively and in some genera (e.g., *Tabularia*, *Pseudostaurosira*), the gametes are differentiated into small nonmotile female gametes and ± equally small male gametes that possess curious threadlike appendages that generate spinning and unidirectional movements, which help the gametes find each other (Sato et al. 2011; Davidovich et al. 2012),

Fig. 13 (continued) (**d**) Mastigonemes in two rows on the flagellum of *Hydrosera*. Whole mount, TEM. Scale bar = 200 nm. (**e**) Theca of living *Odontella* oogonium containing partly naked egg cell. Scale bar = 10 μm. (**f**) Egg of *Odontella* with polarized cell contents. Scale bar = 10 μm. (**g**) Pre- and postauxospore cells of *Aulacoseira*. The large hemispherical valves either end of the wider filament are initial valves, i.e. the first valves formed within the spherical auxospore. SEM. Scale bar = 10 μm. (**h**) Ventral side of the auxospore wall (incunabula and perizonium) of *Triceratium*. The center is covered by a mass of small scales (detail in **i**). The triangular shape is created as a result of differential wall hardening through deposition of a complex set of perizonial strips, beginning with a triradiate element with its center on the dorsal side, whose three arms curve back (*arrows*) onto the ventral side. Other bands are then added adjacent to the primary band (cf. Round et al. 1990, fig. 65). (**i**) Incunabular scales of *Triceratium*. SEM. Scale bar = 5 μm

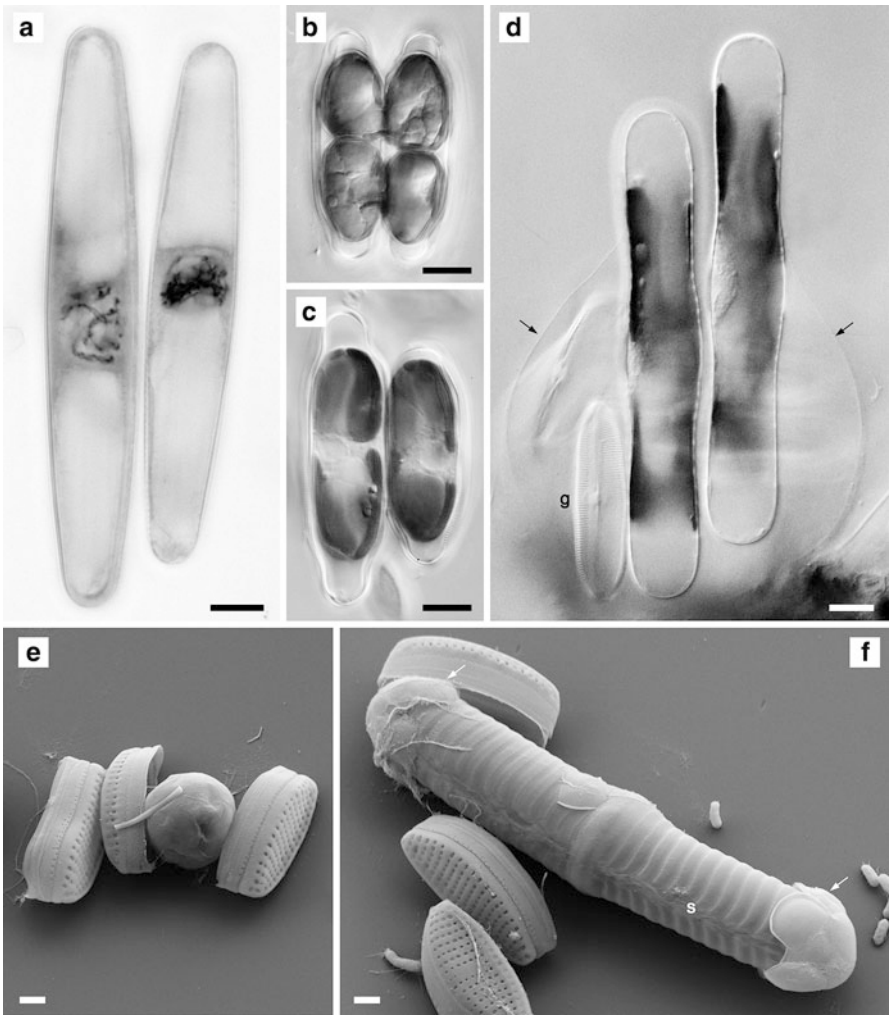

Fig. 14 Sexual reproduction in pennate diatoms**.** The images in (**e**) and (**f**) were very kindly provided by Drs Shinya Sato and Laia Rovira. (**a**) Paired cells of the raphe-bearing pennate diatom *Navicula oblonga* in meiotic prophase (diplotene at *left*, zygotene at *right*). Haematoxylin-stained preparation. Scale bar = 10 μm. (**b**) Paired cells of *Neidium*, each containing two large non-flagellate gametes. The gametes are beginning to move (clockwise) into the adjoining cell. Scale bar = 10 μm. (**c**) Paired cells of *Neidium* following fusion of the gametes and movement of one gamete from each gametangium into the other cell. Each parental frustule therefore now contains a single zygote. Scale bar = 10 μm. (**d**) Expanded auxospores of *Caloneis*, flanked by a valve of one gametangium (*g*). Gametogenesis and fertilization occurs here within a large ellipsoidal mass of mucilage (*arrow*). Scale bar = 10 μm. (**e**) Spherical zygote of *Nitzschia inconspicua* flanked by the thecae of the gametangial frustule. The zygote is covered by incunabula of silica scales. Scale bar = 1 μm. (**f**) Expanded auxospore of *Nitzschia inconspicua*. The scale-case of the zygote has been split into two scaly caps (*arrows*) by the growth of the auxospore, which develops a cylindrical shape through sequential hardening of its wall, outwards from the center, by perizonial strips; these are open on one side, forming a 'suture' (*s*). Scale bar = 1 μm

though only over very short distances. ***Pheromones*** have recently been demonstrated to be involved in the sexualization and chemotaxis of pennate diatoms (Sato et al. 2011; Gillard et al. 2013; Moeys et al. 2016), and the genetic basis of sex determination is now being explored for the first time (Vanstechelman et al. 2013).

Meiosis occurs during gametogenesis (Fig. 14a). The isogametes of pennate diatoms (Fig. 14b) and the egg cells of oogamous diatoms (Fig. 13e, f) all possess plastids, as do some sperm, although it is unclear whether sperm plastids ever survive in the zygote after fertilization of the egg cell. The gametes mostly lack their own siliceous walls, although they are often protected by mucilage capsules or (in centric eggs and many pennate diatoms) by remaining partly enclosed within the frustule of the mother cell (e.g., Idei et al. 2012). Diatom sperm possess two opposite rows of tripartite mastigonemes (Fig. 13b, d) and perform quasi-sinusoidal movements (Fig. 13c) in the plane of the mastigonemes, like the flagellate cells of other heterokont protists, but they differ from them in the structure of the flagellar axoneme, which lacks central microtubules (i.e., the axoneme has a ***9+0 configuration***: Fig. 13b and Idei et al. 2013b). The flagellar apparatus is also unusual in lacking the usual systems of microtubular and fibrous roots, though it sometimes possesses instead a cone of microtubules extending down over the surface of the nucleus (Fig. 13a). No transitional helix is present, and there is no trace of a second flagellum or basal body in the few sperm that have been studied in detail.

Fertilization is followed by development of the zygote into an auxospore, so-called because it is this cell that is able to grow and restore the maximum size characteristic of the species. The zygote produces an organic wall as it differentiates into an auxospore and, as the auxospore expands, silica elements are often inserted into the wall, creating regions that afford some rigidity and probably control expansion. Consequently, an initially spherical (Fig. 14e) or ellipsoidal zygote (Fig. 14c) can be transformed into more complex shapes – elongate cigars with or without a swollen central portion (Fig. 14d, f), bananas, spindles, triradiate forms, or stars (Mann 1994). The special silica elements added to the auxospore wall to stiffen it are sometimes many and intricate (e.g., Poulíčková et al. 2007) and can be divided into (1) the ***incunabula*** – i.e., those elements formed by the zygote before expansion (Trobajo et al. 2006), which can include circular or elliptical scales (Figs. 13i and 14e) or narrow strips of plain silica – and (2) the ***perizonium*** (Figs. 13h and 14f), which comprises several or many bands (often differentiated into transverse and longitudinal series) that are formed sequentially by the auxospore as it expands (Idei et al. 2013a). The development of the auxospore often involves a considerable increase in dimensions (to twice or several times the length of the gametangia in some pennate diatoms: Figs. 14d, e, f). Once expansion is complete, a new cell – the ***initial cell*** – is formed within the auxospore (Fig. 13g). This involves two successive mitoses, each preceding the deposition of a new large valve. The initial valves are usually unlike the valves produced during the vegetative phase because they are formed within and molded by the auxospore, not by another frustule. In addition, the initial valves of chain-forming diatoms differ from those produced subsequently by virtue of the fact that they lack interlocking spines, etc., and come to lie at the ends of the filaments to which they give rise after subsequent cell divisions. Sometimes

modifications of cell shape occur during formation of the initial valves, after auxospore expansion is complete, as a result of contractions of the auxospore away from parts of its wall. The divisions of the initial cell and its immediate descendants are followed by the formation of valves that correspond ever more closely to those typical of the species.

The chloroplasts can be inherited uniparentally or biparentally in diatoms (Mann 1996), and it has recently be shown that recombination can occur between chloroplast genomes located in different plastids within the same cell (D'Alelio and Ruggiero (2015).

The sexual process – and hence restoration of the maximum size – is initiated only when the cells are within a certain size range (Geitler 1932; Chepurnov et al. 2004). Until a ***critical size threshold*** is passed, cells can only reproduce vegetatively. Particular environmental conditions are probably also required for sexualization in many cases, although in culture there seems to be little difference between the conditions required for active vegetative growth and those that permit auxosporulation. Auxospore formation occurs infrequently in nature, because the length of the sexual phase is much shorter than the period of vegetative multiplication during which cell size diminishes (a few days or weeks as opposed to months or years: Mann 1988). Hence it is not surprising that there are rather few records of auxosporulation in natural populations (but see references in Mann 1988 and, e.g., D'Alelio et al. 2010, Jewson & Granin 2015). In temperate planktonic communities, records of auxospores tend to be restricted to the beginning or end of the growing period, i.e., in spring and late summer (e.g., Jewson 1992). Thinning of the population by entering the sexual phase with a large commitment of cells to gamete formation, as in *Corethron pennatum* (Crawford 1995, as *C. criophilum*), could have advantages for survival of the assemblage through periods of low nutrients brought about by a bloom (Crawford et al. 1998) and sidestep the interruption of synthesis that is one costly consequence of the sexual phase (Lewis 1983). In natural populations of single species, small cells generally outnumber large cells; this seems also to reflect the costs of sexual reproduction, in lost synthesis and aborted or unfit gametes and zygotes (Mann 2011).

Taxonomy

Karsten's (1928) system is a convenient starting point for tracing the development of modern diatom classifications. Karsten placed the diatoms in a division (or phylum), the Bacillariophyta, as have many modern workers (see Round 1981b). Within this, he recognized two orders, the Centrales (***centric diatoms***) and the Pennales (***pennate diatoms***), based on the organization of the pattern on the valves – which is radially or concentrically ordered (rarely irregular) in the Centrales, and feather-like in Pennales. This subdivision is also echoed in the features of the sexual reproduction of the two groups – oogamous in centrics but usually isogamous and always lacking flagellate sperm in pennates. Silva (1962) elevated the centrics and pennates to classes (Centrobacillariophyceae and Pennatibacillariophyceae) and created or

amended a number of orders within them, which brought the classification of diatoms into line with that of other major algal groups.

Scanning electron microscopy revealed further groupings beyond those recognized by Karsten and Silva. Round et al. (1990) therefore suggested many changes and new taxa, from classes to genera, in an attempt to summarize likely relationships, based not only on cell wall detail but also on cytological and other information. Analyses of molecular sequence data (especially from 18S rDNA) have subsequently shown that neither the traditional classification nor the revised system by Round et al. can be upheld, although some aspects of each gain support. Unfortunately, there is as yet no agreement about what should replace the older classifications, nor about whether it is sensible to make any changes at all until a clearer picture of diatom evolution emerges.

In the Round et al. (1990) classification, the diatoms (Bacillariophyta) were split into three classes: Coscinodiscophyceae, Fragilariophyceae, and Bacillariophyceae. These three are readily identifiable. The Coscinodiscophyceae equated more or less to the Centrales (Centrobacillariophyceae) and comprised all those diatoms with radial organization of the primary valve pattern, centered upon a small ring (annulus). The Fragilariophyceae and Bacillariophyceae together comprised the Pennales (Pennatibacillariophyceae) of earlier classifications, all having feather-like organization (transverse ribs and rows of pores, subtended by a longitudinal sternum). The two classes were separated by the absence (Fragilariophyceae) or presence (Bacillariophyceae) of a raphe system. The three classes of Round et al. (1990) seem mostly to avoid the charge of polyphyly. However, it is now clear that they do not capture the essential features of diatom evolution, since two of the three classes (Coscinodiscophyceae and Fragilariophyceae), as defined by Round et al. (1990), are almost certainly paraphyletic. Medlin and Kaczmarska (2004) therefore suggested a new system, in which the diatoms are split into two subdivisions, Coscinodiscophytina and Bacillariophytina. The Coscinodiscophytina comprised only centric diatoms (i.e., having a centric organization of the valve pattern); the Bacillariophytina, on the other hand, contained both centric diatoms, classified by Medlin and Kaczmarska into the Mediophyceae, and pennate forms, classified in the Bacillariophyceae. However, in some subsequent analyses the Coscinodiscophytina and the Mediophyceae have both been paraphyletic (e.g., Sorhannus 2007; Theriot et al. 2011), or the Mediophyceae have been monophyletic but not the Coscinodiscophytina (e.g., Ashworth et al. 2012; Nakov et al. 2015). If either of these later reconstructions accurately reflects evolution, the Medlin–Kaczmarska scheme will not satisfy most systematists, who require monophyly of taxa. Medlin (2014) notes, on the other hand, that if certain criteria are met in the molecular analysis, the Coscinodiscophytina and Mediophyceae **are** recovered as monophyletic clades and it has also been suggested (e.g., Medlin 2015, 2016a) that some reproductive and morphological features are consistent with the Medlin–Kaczmarska classification. In summary, there is as yet no consensus on the phylogeny and classification of centric diatoms. However, even if the Coscinodiscophytina and Mediophyceae are not monophyletic, Medlin and Kaczmarska's revision made two significant advances on the previous system

proposed by Round et al. (1990): (1) it recognized that the primary evolutionary radiation took place among diatoms with a centric organization and oogamous reproduction, and (2) it restored unitary status for the pennates, which are monophyletic in most molecular phylogenies and are characterized morphologically by the possession of a single sternum as the pattern center.

At the ordinal to family level, some of the groupings recognized by Round et al. (1990) and earlier authors appear monophyletic in molecular phylogenies and formal analyses of morphological characteristics. Examples are the Cymatosirales, Thalassiosirales, Bacillariales, Sellaphorineae, and Naviculaceae (e.g., Theriot et al. 2010, Ruck and Theriot 2011). However, many do not. Thus, *Proboscia* and *Urosolenia* are not closely related to *Rhizosolenia* (Round et al. placed them together in the same family, Rhizosoleniaceae), and *Achnanthes* and *Achnanthidium* are not related, despite their similarly monoraphid frustules (Round et al. placed them together in the Achnanthales) (e.g., Medlin and Kaczmarska 2004; Sorhannus 2007; Theriot et al. 2010). In contrast, at the genus level, many of the revisions suggested or incorporated by Round et al. (1990) have been supported by later analyses, such as the removal of *Ardissonea* and *Toxarium* from *Synedra* (Medlin et al. 2008), or the separation of *Lyrella*, *Petroneis*, *Fallacia*, *Sellaphora*, and *Placoneis* from *Navicula* (Jones et al. 2005; Bruder and Medlin 2007; Evans et al. 2008). At present, however, there are few or no molecular data for many genera and even where molecular data are available, the phylogenetic trees they yield often contain few nodes that (from bootstrap support values or posterior probabilities, or congruence with morphological or other data sets) can be regarded as reliable. Furthermore, different approaches to alignment and phylogeny reconstruction are adopted by different researchers, with significant effects on the phylogenies obtained (e.g., contrast Medlin & Kaczmarska 2004 with Theriot et al. 2015). Hence it is not surprising that there is no consensus yet about what should replace the Round et al. classification. The completion of current initiatives to develop multigene phylogenies of diatoms (e.g., Ashworth et al. 2013) will hopefully lead to a more satisfactory system. This will probably involve many major changes in how particular groups of species or genera are classified: a good example, showing the difficulties of reconciling existing taxonomy with new understanding, based on molecular and refined morphological analysis, is given by Ruck et al. (2016) in a study of the Rhopalodiales and Surirellales.

Given current uncertainty (except that previous classifications are wrong in many respects), we depart significantly from the previous edition of this book and present a greatly simplified classification (Table 1) modified from Adl et al. (2005). It is based on a comparison of recent published phylogenies and classifications (e.g., Theriot et al. 2010, 2011; Nanjappa et al. 2013; Nakov et al. 2015; Li et al. 2015; Medlin 2016a, b), taking into account the persistent lack of support for many basal nodes in molecular analyses (e.g., Theriot et al. 2015, fig. 1) and the frequent lack of a clear pattern in the distribution of morphological and cytological characters. Decisions about which clades should be recognized among the "radial centrics," ("Coscinodiscophytina") is especially problematic. In order to get an idea of the diversity that Table 1 represents, but ignoring the classification imposed upon it in

Table 1 Major clades and paraphyletic taxa of diatoms. The examples of genera listed include the genera illustrated in this chapter

Division	*Bacillariophyta*	Descriptions and subgroups	Examples of taxa
Subdivision Subdivision Coscinodiscophytina: monophyletic in Medlin and Kaczmarska (200$) (and then comprising the single class Coscinodiscophyceae), paraphyletic in Theriot et al. (2015). Contains several clades of radial centric diatoms whose interrelationships are unclear. Valves generally circular; pattern-center an annulus; sexual reproduction via oogamy; auxospores with scales only	leptocylindrids	Chain-forming, delicate; valves circular, striae radiating from a central circular annulus; unique simple process present near the annulus; girdle bands segmental; auxospore forming a dormant resting stage (not present in other centric clades)	*Leptocylindrus*, *Tenuicylindrus*
	corethrids	Solitary; valves circular; radially symmetrical; articulating spines secreted from around the valve margin; rimoportulae absent; girdle bands segmental	*Corethron*
	melosirids	Usually chain-forming, sometimes forming special "separation valves"; valves circular, radially symmetrical; rimoportulae small, scattered on the valve face or marginal; girdle bands hooplike or segmental	*Aulacoseira*, *Melosira*, *Podosira*, *Stephanopyxis*
	ellerbeckiids	= "paralids" of Mann in Adl et al. (2005): Chain-forming, heavily silicified; valves circular, radially symmetrical; small tube processes present, restricted to the mantle; girdle bands hooplike	*Ellerbeckia*
	arachnoidiscids	Solitary, heterovalvar; valves circular, radially symmetrical; one valve with its center surrounded by radial slits (apparently modified rimoportulae); girdle bands hooplike	*Arachnoidiscus*
	coscinodiscids	Solitary, isovalvar; valves usually circular, striae radiating from a central, subcentral, or submarginal circular annulus; rimoportulae central, scattered on the valve face or marginal; girdle bands hooplike	*Actinocyclus*, *Actinoptychus*, *Coscinodiscus*, *Stellarima*, and many others
	rhizosolenids	Chain-forming, with a long perivalvar axis, rarely solitary; valves circular, almost radially symmetrical or with the pattern-center displaced towards one side; rimoportula single, associated closely with the annulus, sometimes developed into a spine; girdle bands segmental	*Guinardia*, *Rhizosolenia*
	proboscids	Usually solitary, with a long pervalvar axis; valves circular, extended into an eccentric beak (proboscis); rimoportulae and other processes absent; girdle bands segmental	*Proboscia*

(*continued*)

Table 1 (continued)

Division	*Bacillariophyta*	Descriptions and subgroups			Examples of taxa
	Subdivision Bacillariophytina	Valves usually elongate or structurally bipolar or multipolar, as a result of anisometric expansion of the auxospore, constrained by a perizonium (not present and likely secondarily lost in Thalassiosirales)			
		Class Mediophyceae (possibly paraphyletic with respect to the Bacillariophyceae)	Pattern-center an annulus (which is sometimes elongate rather than circular); valve outline and structure highly varied, mostly bi- or multipolar; sexual reproduction via oogamy		Thalassiosirales, Cymatosirales, Lithodesmiales, Chaetocerotales, Biddulphiales, *Attheya* and others; includes *Amphitetras*, *Cyclotella*, *Cymatosira*, *Hydrosera*, *Mediopyxis*, *Odontella*, *Pleurosira*, *Skeletonema*, *Stephanodiscus*, *Thalassiosira*, *Triceratium*
		Class Bacillariophyceae (pennate diatoms)	Pattern-center a sternum; sexual reproduction via morphological isogamy, rarely anisogamy		
			Subclass Urneidophycidae	Nonmolecular characters as for Bacillariophyceae	*Asterionellopsis*, *Delphineis*, *Plagiogramma*, *Rhaphoneis*, *Talaroneis*
			Subclass Fragilariophycidae (core araphids)	Nonmolecular characters as for Bacillariophyceae	*Asterionella*, *Diatoma*, *Fragilaria*, *Grammatophora*, *Martyana*, *Rhabdonema*, *Tabellaria*
			Subclass Bacillariophycidae (raphids)	Possession of a raphe system	*Achnanthes*, *Berkeleya*, *Caloneis*, *Cocconeis*, *Cymatopleura*, *Diploneis*, *Donkinia*, *Entomoneis*, *Epithemia*, *Eunotia*, *Fallacia*, *Gomphonema*, *Gyrosigma* *Hantzschia*, *Hippodonta*, *Lyrella*, *Navicula*, *Neidium*, *Nitzschia*, *Placoneis*, *Psammodictyon*, *Sellaphora*, and many others

1990, readers should refer to the atlas of genera by Round et al. (1990), although many further genera have been described since that book was written.

A complementary approach, in which molecular phylogenies are used to test explicit hypotheses concerning the evolution of specified traits, has recently been applied and has provided insights into variation and changes in salinity preference (Alverson et al. 2007), cell size (Nakov et al. 2014), growth form (Nakov et al. 2015), and reproductive behavior (Mann et al. 2013; Poulíčková et al. 2015).

At the species level, studies of reproductive isolation and fast-evolving genes indicate that the diatoms are even more speciose than was already known. Common freshwater and marine diatoms have proved to be complexes of several or many species that are difficult or impossible to identify reliably using the light microscope (e.g., Sarno et al. 2005; Amato et al. 2007; Evans et al. 2008; Souffreau et al. 2013; Vanormelingen et al. 2013). It is likely that many other "species" are likewise composite, with potentially adverse consequences for ecological studies, biomonitoring, biogeography, and other sciences dependent on consistent and accurate identification. To help obviate difficulties, DNA barcoding is being developed (e.g., Mann et al. 2010; Zimmermann et al. 2011).

Maintenance and Cultivation

Enrichment and Isolation from Nature

Diatoms are relatively easy to culture in mixed populations simply by enriching natural water with nutrient solutions or transferring subsamples to artificial media (see below). It is convenient to do this in Petri dishes, which can then be observed directly at low magnification with a stereo-microscope or inverted microscope to check for growth. It should always be remembered that diatoms require dissolved silicate for growth and this is usually added to media, although it is sometimes assumed that supply will be adequate if soft-glass dishes are used. Light may be natural or artificial and alternating light/dark cycles or silicon-starvation can be used to achieve a degree of synchrony (e.g., Darley and Volcani 1971). Temperature should be adjusted by experimentation; most diatoms grow over a wide range but some, e.g., ice diatoms, can have a very narrow range.

To isolate clones, individual cells or colonies can be picked out from mixed cultures or natural samples with a micropipette, washed in sterile medium, and transferred to new sterile media. Alternatively, natural samples can be spread on agar plates, using normal microbiological technique. If the plates have been previously dried for a short time in an oven at 30 °C or in a flow hood, the liquid of the sample will quickly be absorbed and the individual cells will be trapped on the agar surface, where they can either be picked off immediately using a micropipette or allowed to grow into colonies. If the latter approach is taken, discrete colonies can be removed after a few days or weeks by cutting out agar blocks, each with a colony originating from a single cell, or subsampled using a micropipette and transferred to clean agar or liquid media. Clones may survive for months or years (especially if the growth rate is reduced by use of low

light and temperature), but as previously noted, many cannot be kept indefinitely because of size reduction and a mating system that enforces outbreeding. Thus clonal cultures may not always be ideal for maintaining diatoms in culture and unialgal cultures may be more suitable for long-term survival. It should be remembered in any case that meiosis and recombination are likely to occur in clones of homothallic and automictic diatoms maintained for months or years in culture and that consequently cells should be reisolated before critical experimental work is undertaken.

Axenic Cultures

The usual mixtures of antibiotics (e.g., of streptomycin, ampicillin, or penicillin) can be added to cultures to suppress bacteria and, through repeated transfer, produce axenic cultures (Andersen 2005).

Culture Media

Growth media suitable for freshwater and marine diatoms and other algae are given in the handbook edited by Andersen (2005). Relatively high quantities of silicate are of importance for culturing diatoms, but otherwise no special requirements are necessary for routine culture. Apart from vitamins, no organic additives to media are generally needed, except of course for the few obligate heterotrophs. However, some diatoms have so far remained recalcitrant ("unculturable"), particularly large-celled species from marine intertidal sandflats (e.g., Droop et al., 2000).

For freshwater diatoms, we frequently use WC medium, which was developed originally by Guillard and Lorenzen (1972). This contains:

36.76 mg	$CaCl_2.2H_2O$
8.71 mg	K_2HPO_4
36.9 mg	$MgSO_4.\ 7H_2O$
28.42 mg	$Na_2SiO_3.\ 9H_2O$
12.6 mg	$NaHCO_3$
85.01 mg	$NaNO_3$

Micronutrients:

3.15 mg	$FeCl_3.\ 6H_2O$
0.18 mg	$MnCl_2.4H_2O$
0.01 mg	$CuSO_4.5H_2O$
0.022 mg	$ZnSO_4.7H_2O$
0.01 mg	$CoCl_2.6H_2O$
0.006 mg	$Na_2MoO4.2H_2O$
1.0 mg	HBO_3
4.36 mg	Na EDTA

Vitamins:

0.1 mg	Thiamine.HCl
0.5 µg	Biotin
0.5 µg	Vitamin B12

Make up to 1 l with deionized water. Alternatively, stock solutions can be made at 1000× concentration and added at 1 mL L^{-1}. The vitamins should be added after autoclaving. WC is a fairly nutrient-rich medium, usually adjusted to around pH 7 (with drops of HCl). Diatoms from acid oligotrophic waters may be better grown in a modified GG medium (von Stosch and Fecher 1979).

For marine diatoms, we have found Roshchin medium (Roshchin 1994) to be effective: dissolve 202 mg KNO_3, 17.9 mg $Na_2HPO_4.12H_2O$, 1.2 mg $Na_2S_2O_3.5H_2O$, and 10 mg Na_2SiO_3. $9H_2O$ in 1 L filtered seawater; trace elements and vitamins can be added as for WC medium. Again, stock solutions can be made at 1000× strength. The medium is sterilized by pasteurization or filtration, since autoclaving leads to precipitation of some components. Alternatively the well-known series of "f" media can be used, in particular f/2 medium (Andersen 2005). If fully defined marine media are required, an artificial seawater mix can be used instead of natural seawater.

Evolutionary History and Biogeography

Fossil Record

The fossil record of diatoms has been briefly summarized by Sims et al. (2006) and Harwood et al. (2007). The earliest generally accepted records of diatoms are of "*Pyxidicula*" species, from the late Early Jurassic of Germany (Toarcian; c. 190 Mya) (Rothpletz 1900). However, the original source of the material is unknown. Rothpletz boiled a fossil sponge in HCl (hence the original specimen was destroyed), and the resultant siliceous residue (diatoms) was mounted and sectioned (Medlin 2015, 2016a). The earliest diverse, well-preserved diatom assemblages studied in modern times are from the Early Cretaceous, especially a deposit from the Weddell Sea (Gersonde and Harwood 1990; Harwood and Gersonde 1990). Paleozoic records have been reported but are now discounted as contamination. The absence of diatoms from Paleozoic or PreCambrian deposits has sometimes been ascribed (e.g., Round 1981b) to conversion of the diatomaceous silica to porcelanite and later to chert (a process described by Calvert 1977). However, although many diatom deposits have undoubtedly been lost through diagenesis, the order of appearance of major diatom groups in the fossil record agrees reasonably well with molecular phylogenies (Sims et al. 2006; Kooistra et al. 2007) and tentative dating of molecular trees suggests that the fossil record, though imperfect, does not hugely underestimate the origin of the diatoms: a Mesozoic or latest Paleozoic (late Permian) origin is the most likely (Kooistra

and Medlin 1996; Sorhannus 2007; Medlin 2011, 2015, 2016a). The date of origin of the pennates, however, is particularly controversial (Medlin and Desdevises 2016). In the Tertiary, an extensive fossil record has been preserved and is used for stratigraphic correlation and for calibrating the molecular clock in phylogenetic studies.

Nevertheless, although the fossil record is more reliable than some have thought, dissolution and fragmentation of the more delicate species certainly does occur and results in a modified picture of the natural assemblages that originally existed. For example, biochemical markers indicate that the microfossil record of *Rhizosolenia* and related genera (whose frustules are composed largely of girdle bands) underestimates their age (Sinninghe Damsté et al. 2004), and the blanket bogs of boreal regions often contain a rich diatom flora of strongly silicified acidophilic species whereas a few centimeters down in the peat there are often no diatom remains, presumably because of dissolution. Further sources of serious bias for evolutionary studies is the greater likelihood that planktonic species will become fossilized, because of their much greater initial abundance and distribution, relative to benthic species, and the lack of suitable depositional environments for marine littoral species. The Eocene diatomites at Oamaru in New Zealand are an important exception, preserving a highly diverse assemblage of well-preserved near-shore marine diatoms (Edwards 1991). On land, the short life of most lakes and destruction of deposits by glacial and other erosion lead to a surprisingly poor fossil record for freshwater diatoms, though there are some remarkable exceptions (e.g., the Eocene Giraffe Pipe deposits in NW Canada: Siver et al. 2010). Preservation of internal structure is extremely rare, but diatoms with cell content have been discovered in late Cretaceous cherts in Mexico (Beraldi et al. 2015).

Freshwater and terrestrial diatoms are usually considered to occur somewhat later in the geological sequence than marine ones, but some recently discovered Early Cretaceous deposits in Korea may be of terrestrial origin (Harwood et al. 2007). Multiple invasions into freshwaters have been documented using molecular phylogenies (Sims et al. 2006), and some have been demonstrated to occur in the reverse direction (Alverson et al. 2007). Molecular clock methods have been used to date invasion times in the Thalassiosirales (Alverson 2014). Recently, diatoms have been found preserved in amber (Girard et al. 2009).

The fossil record is still underused as a source of information for phylogenetic reconstruction and systematics at the generic and species level. Increasingly, however, the fossil record is being used in conjunction with neontological analysis and molecular phylogenies to estimate the tempo of evolution in particular diatom genera or families (e.g., Souffreau et al. 2011). In a few cases, it has been possible to use fossil material to detect anagenetic changes within what appears to be a single lineage, such as the evolution of *Stephanodiscus yellowstonensis* from *S. niagarae*-like ancestors in Yellowstone Lake, Wyoming (Theriot et al. 2006). For some marine planktonic groups impressive fossil records are available,

documenting morphological evolution over many millions of years (e.g., Yanagisawa and Akiba 1990).

Biogeography

During most of the twentieth-century species, species concepts and delimitation in diatoms – and consequently data on species distributions – were based almost entirely on the morphology of the valve as seen with the light microscope. Latterly, details observable with EM have gained importance and this, coupled with insights from mating experiments and (still more recently) molecular sequence data, has been accompanied by an explosion in the descriptions of new species. There has certainly also been a trend towards narrower species definitions – a coarse-grained taxonomy has been replaced by a much finer one (Mann 1999b). Furthermore, whereas it was always accepted (e.g., Hustedt 1942) that some diatoms appeared to be restricted to particular regions because of dispersal constraints, as opposed to ecological restrictions, a much greater proportion of new species are now being claimed to be endemic to particular small regions or lakes.

It is doubtful whether many of the claims of endemism are justified, given the difficulties and inconsistencies in identifying diatoms (partly because there are so few critical revisions of any diatom genera [Kociolek and Williams 2015] and partly because of problems in accessing all the relevant literature), the rather limited sampling of many parts of the world (especially in Africa, S America, and SE Asia, and more generally in the tropical zone both in the sea and on land), and the very real problem of how to detect microeukaryote species when they are rare (i.e., occurring at frequencies of less than, say, 1 in 10^6). Likewise, claims that particular species have been introduced (e.g., Coste and Ector 2000) also need to be treated with caution (e.g., Gómez and Souissi 2010). Some diatoms do seem to be restricted to particular regions by geographical barriers, rather than the availability of suitable habitats: examples are discussed by Vanormelingen et al. (2008) and include the unmistakable genus *Eunophora*, apparently restricted now to temperate Australasia. There is also clear evidence for isolation by distance between populations of some heterothallic species, even on scales of a few tens or hundreds of kilometers (Vanormelingen et al. 2015). On the other hand, there are also examples, confirmed by barcode and/or mating data, of species and haplotypes with extremely wide distributions (e.g., Evans et al. 2009; Rimet et al. 2014), and geographical pattern disappears very quickly as one ascends the taxonomic hierarchy from species to genera, implying rather rapid spread of diatom lineages, relative to higher plants and vertebrates. On the other hand, there is also evidence of range contractions. For example, the genus *Arachnoidiscus* was formerly present in Europe (e.g., in the Miocene: Hajós 1986), but is now extinct there, the nearest populations being in the Indian Ocean.

Origin of the Diatoms

There is still a huge gap in our understanding of how and when diatoms acquired their unique morphology and life-cycle characteristics. Originally, the diatoms were kept as a quite separate group, allied to various algal/animal groups. Pascher (1914, 1921) seems to have been the first to suggest that the diatoms have features in common with the Chrysophyceae and Xanthophyceae. To reflect this, he placed all three groups together in the phylum Chrysophyta. Ultrastructural and molecular sequence data have confirmed the general thrust of Pascher's idea, placing the diatoms unambiguously among the heterokont protists ("stramenopiles") within the chromalveolates (e.g., Andersen 2004; this position is recognized in the overall classification of protists by Adl et al. 2005). However, a close relationship to silica scale-producing algae, such as the Chrysophyceae, is not likely according to molecular and ultrastructural evidence (e.g., Derelle et al. 2016). At present, molecular phylogenies indicate that the closest known relatives of the diatoms are the Bolidophyceae and Parmales, which are small groups of marine autotrophic picoplankton with the same kind of four-membrane-bound plastids as diatoms and other autotrophic heterokonts (Guillou et al. 1999; Ichinomiya et al. 2011). The relationship with the Parmales was earlier predicted by Mann and Marchant (1989), because Parmales produce silica scales that, in their pattern and apparently space-filling ontogeny, resemble diatom valves and girdle bands. In particular, the round plates produced by Parmales often possess ring structures (annuli) at their centers (Booth and Marchant 1987), like centric diatom valves (Round and Crawford 1981). However, although Parmales scales seem to develop centrifugally from an annulus, as in diatoms, the two groups differ significantly in their morphogenesis, because Parmales plates develop within the cell (Yamada et al. 2016), whereas diatom valves and girdle bands are always formed peripherally in association with the cell membrane; and also in the control of silicification, because cell growth and division are not prevented by silicon depletion in Parmales (Yamada et al. 2014), whereas in diatoms they are.

There is therefore some support for the suggestions of Round and Crawford (1981, 1984) and Mann and Marchant (1989) that the diatom frustule originated as a ***scale-case***. Both sets of authors postulate that diatoms evolved from cells bearing uniform scales, via an early stage where scales were differentiated into larger valve-like scales and narrower ones resembling the segmental girdle bands of modern rhizosolenids (cf. the differentiation of round shield plates and triradiate girdle and dorsal plates in Parmales), and a later stage when the proto-girdle bands became thinner and stretched to form hoops encircling the cell. This assumes that valves and girdle bands have a common origin and indeed their fine structure is often so similar that this is a reasonable assumption, and it seems that girdle bands are also formed centrifugally, like valves (e.g., Sato 2010). Furthermore, cells covered evenly with scales are known in diatoms, in the auxospores of some centric species, e.g., of *Melosira* and *Ellerbeckia* (Crawford 1974b; Schmid and Crawford 2001) and in several pennates (e.g., Mann et al. 2013). The Round–Crawford and Mann–Marchant schemes differ principally in the assumptions made about the nature of the scales and scaly cell in the early ("Ur") diatoms. In the Mann–Marchant scheme, the scales

of the ancestral diatom are abutting space-filling components of a cyst wall, whereas Round and Crawford envisaged the scales as discrete imbricating elements covering growing vegetative cells, as in modern synurophytes. In a series of opinion papers, Medlin (e.g., 2007) has suggested that that silica may originally have had the property in diatoms of inducing a temporary resting state, which is consistent with the "Ur" diatom being a cyst.

No precursors of diatoms are known from the fossil record. Though it now seems clear that the Bolidophyceae–Parmales are their nearest relatives, the diatoms are an extremely well-characterized, distinctive, and monophyletic group, and it is nomenclaturally convenient to regard them as a separate phylum, which allows maintenance and gradual refinement of the lower-level classification of diatoms developed during the twentieth century.

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Phaeophyta

8

Hiroshi Kawai and Eric C. Henry

Abstract

Brown algae (Phaeophyceae) are benthic macroalgae constituting a primary element of coastal ecosystems in temperate and cold water seas and are also economically important. Currently ca. 2,000 species in ca. 300 genera are recognized. They are mostly marine and only a few genera are known in freshwater habitats. They are photosynthetic organisms, sharing chloroplasts originated from secondary endosymbiotic events with photosynthetic heterokonts (chromists, stramenopiles) and surrounded by four layers of membranes. Major photosynthetic pigments are chlorophylls *a* and *c* and fucoxanthin and produce laminaran as the storage polysaccharide. All known species are multicellular, with cell walls composed of alginates, fucoidan (fucan), and cellulose, and traversed by plasmodesmata. Basal taxa generally show isomorphic life history and apical growth. Derived taxa have evolved heteromorphic life histories and modified life history patterns, some with only a diploid generation (thallus), as well as diverse growth patterns such as diffuse and intercalary growth. They commonly reproduce by asexual heterokont zoospores, or zygotes formed by fusion of motile or nonmotile female gametes with heterokont male gametes.

Keywords

Algal bed • Alginate • Brown algae • *Ectocarpus* • Fucoidan • Fucoxanthin • Heterokont • Heterokonta • Kelp • Life history • Multicellular alga • Ochrophyta • Phaeophyceae • Secondary endosymbiosis • Stramenopile • Tripartite tubular mastigoneme

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Contents

Summary Classification

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●●**Dictyotales** (*Dictyopteris, Dictyota, Lobophora, Padina*)
●●**Sphacelariales** (*Cladostephus, Halopteris, Phaeostrophion, Sphacelaria*)
●●**Onslowiales** (*Onslowia, Verosphacela*)
●●**Syringodermatales** (*Microzonia, Syringoderma*)
●●**Desmarestiales** (*Arthrocladia, Desmarestia, Himanthothallus*)
●●**Ascoseirales** (*Acroseira*)
●●**Sporochnales** (*Carpomitra, Nereia, Sporochnus*)
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●●**Ectocarpales s.l.** (*Acinetospora, Chordaria, Dictyosiphon, Ectocarpus, Scytosiphon*)
●●**Laminariales** (*Aureophycus, Agarum, Alaria, Chorda, Laminaria*)
●●**Asterocladales** (*Asterocladon*)
●●**Fucales** (*Durvillaea, Fucus, Himanthalia, Sargassum, Xiphophora*)
●●**Nemodermatales** (*Nemoderma, Zeacarpa*)
●●**Tilopteridales** (*Cutleria, Phyllariopsis, Haplospora, Tilopteris*)
●●**Stschapoviales** (*Halosiphon, Platysiphon, Stschapovia*)
●●**Ralfsiales** (*Analipus, Mesospora, Neoralfsia, Ralfsia*)

Introduction

General Characteristics

Brown algae (Phaeophyceae, Heterokonta/Ochrophyta) are multicellular organisms that vary in size from microscopic branched filaments less than a millimeter in diameter to large macroscopic fleshy thalli that may attain lengths in excess of 50 m (Bold and Wynne 1985; van Den Hoek et al. 1995; Graham and Wilcox 2000). They are essential elements of coastal ecosystems, often forming dense algal beds comparable to the forests in terrestrial ecosystems (Dayton 1985). They commonly reproduce by asexual heterokont zoospores, or zygotes formed by fusion of motile or nonmotile female gametes with heterokont male gametes. They are photosynthetic organisms, with chloroplasts originated from a secondary endosymbiotic event in a common ancestor shared with photosynthetic heterokonts and surrounded by four membranes (Fig. 1). All known species are multicellular, with cell walls composed of alginates, fucoidan (fucan) and cellulose, and traversed by plasmodesmata (cytoplasmic continuity retained through cell division) (Figs. 2 and 3) (Terauchi et al. 2012, 2015).

Brown algae comprise approximately 2,000 species in around 300 genera (AlgaeBASE: http://www.algaebase.org/). Genera such as *Cystoseira, Dictyota, Ectocarpus, Sargassum*, and *Sphacelaria* include large numbers of species, which present considerable taxonomic difficulty and are in need of critical review (Stache-Crain et al. 1997; Peters et al. 2010; Ni-Ni-Win et al. 2010; 2011a, b;

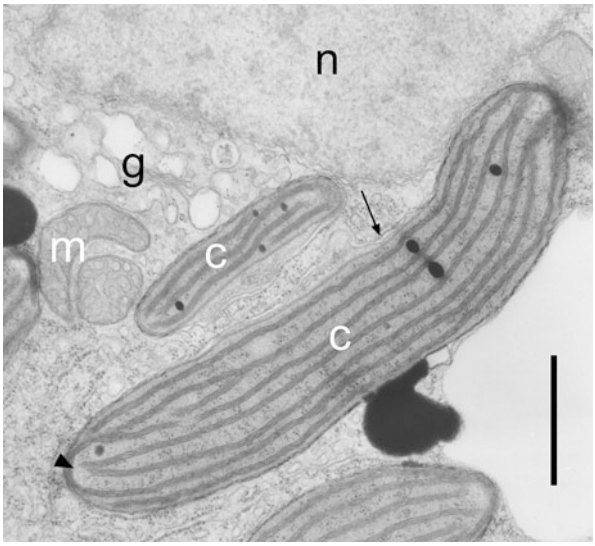

Fig. 1 Brown algal chloroplasts (*c*) of *Saccharina* sp. with four surrounding membranes. The outermost chloroplast membrane with attached ribosomes is shown by an *arrow*. The chloroplast has girdle lamella, and ring-shaped DNA within the girdle lamella (*arrowhead*). *g* Golgi body, *m* mitochondrion, *n* nucleus. Scale bar, 1 μm (TEM micrograph courtesy of Taizo Motomura)

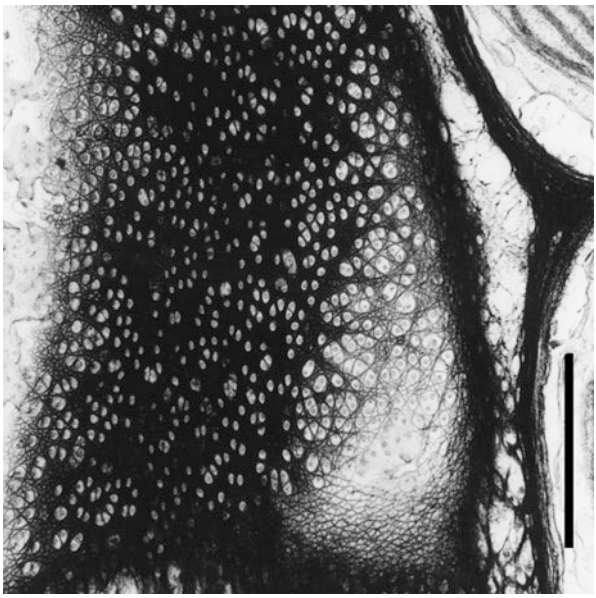

Fig. 2 Tangential section of a cell wall showing plasmodesmata (cytoplasmic continuity between cells) in *Chorda asiatica*. TEM micrograph. Scale bar, 1 μm

Tronholm et al. 2010; Silberfeld et al. 2014a). Furthermore, a number of cryptic species have been discovered in the course of taxonomic re-examinations employing molecular phylogenetic analyses. The life histories of brown algae are varied. Most involve meiosis and fertilization, with an alternation of haploid and diploid phases. The haploid gametophyte generation often exhibits varying degrees of morphological reduction. In some taxa, the reduced generation has become cryptic so that they superficially lack alternation of generations.

Morphology varies greatly among brown algae. Some of the smaller species form inconspicuous filamentous tufts and cushions or grow as crusts in close contact with the substrate. Larger forms include a variety of simple and branched thalli with differentiation into foliose blade, stem-like stipe, and basal holdfast. Some thalli are exceedingly mucilaginous. The larger fleshy species include the intertidal and upper subtidal seaweeds known as wracks and kelps.

Occurrence

Brown algae are almost exclusively marine organisms that inhabit the intertidal and subtidal zones of coastal regions throughout the world. They grow to varying depths depending on the availability of light and substrates. Only a few species grow in estuarine and freshwater habitats. Most of the freshwater taxa are considered to be relatively recent descendants of marine taxa and are scattered among diverse phylogenetic groups, although the taxonomic positions of some taxa need reexamination (McCauley and Wehr 2007).

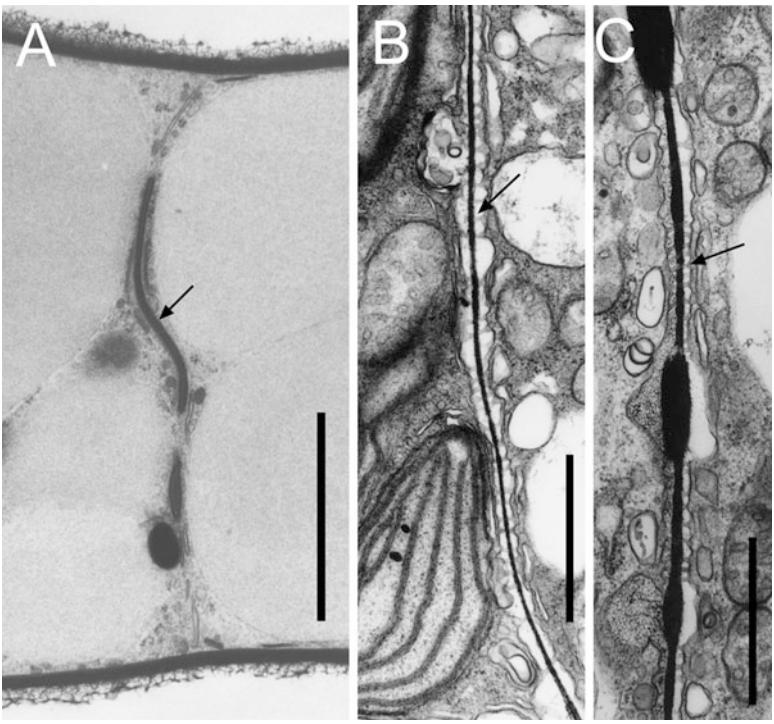

Fig. 3 Formation processes of cell walls and plasmodesmata. (**a**) Cross section of *Scytosiphon* gamete germling showing the initial stage of the deposition of wall substances (arrow). Freeze substitution TEM micrograph (Courtesy of Chikako Nagasato). (**b**, **c**) TEM micrograph by chemical fixation showing the early stages of cell wall formation and plasmodesmata (arrows) in *Chorda* meristematic cells

Most brown algae grow attached to a great variety of surfaces including rock, concrete, metal, and wood structures, as well as the surfaces of other organisms such as other macroalgae or shells. A few species that grow on macroalgae and sea-grasses penetrate the tissues of their living hosts (Kawai and Tokuyama 1995; Burkhardt and Peters 1998; Garbary et al. 1999), sometimes exhibiting a semiparasitic habit (Peters 1989). Some brown algae grow free-floating in sheltered habitats such as bays and estuaries. In the Sargasso Sea in the middle North Atlantic Ocean, a large biomass of floating *Sargassum* species propagates only vegetatively.

Many brown algae are easily collected from their natural habitats and can be grown and maintained in culture. Some taxa (e.g., *Cladosiphon, Laminaria, Saccharina, Sargassum, Undaria*) are grown on a large scale in the field (mariculture) and used for food and industrial materials. Numerous culture strains available for research purposes are deposited in several culture collections: KU-MACC (Kobe University Research Center for Inland Seas, Japan), NCMA (National Center for Marine Algae and Microbiota, USA), SAG (Sammlung von Algenkulturen der Universiät Göttingen, Germany), and UTEX (The Culture Collection of Algae,

University of Texas at Austin, USA). Most of the available strains are unialgal, and some are axenic. Some strains are cryopreserved in the culture collections (Heesch et al. 2012).

Literature and History of Knowledge

Papers concerning brown algae appear in a range of botanical and marine biological journals, including the specialist algal journals *Phycologia, Journal of Phycology*, *European Journal of Phycology*, *Phycological Research*, *Algae, Cryptogamie Algologie*, *Journal of Applied Phycology*, and *Botanica Marina.* Pioneering studies on the morphology and development of a wide range of brown algae were described in the publications of Kylin (1933), Kuckuck (1929, 1964), and Sauvageau (1929, 1931, 1933); Fritsch (1945) comprehensively reviewed the literature before 1945. Brown algae are also discussed in comprehensive phycology textbooks in English (Wynne 1981; Bold and Wynne 1985; South and Whittick 1987; van den Hoek et al. 1995; Graham and Wilcox 2000; Lee 2008; Reviers et al. 2015). Life histories of the brown algae have been reviewed by Wynne and Loiseaux (1976), Pedersen (1981), Peters (1987), and Hori (1993). Ecological and physiological studies of brown algae were reviewed by Lobban and Harrison (1994). Entire genome sequences of brown algae were first reported in the model species *Ectocarpus siliculosus* (Cock et al. 2010), followed by the kelp species *Saccharina japonica* (Ye et al. 2015), and diverse information on their genomes has become available.

Traditionally the classification schemes devised by Kylin (1933), Papenfuss (1955), and Wynne and Loiseaux (1976) have been widely accepted, but the basic concept of the fundamental importance of life history patterns and thallus constructions has been challenged by recent biological studies (e.g., life histories, sexual pheromones), and have been considerably revised based on molecular analyses, mostly using ribosomal DNA and RuBisCO gene sequences (Tan and Druehl 1996; Siemer et al. 1998; Reviers and Rousseau 1999; Draisma et al. 2001; Sasaki et al. 2001; Cho et al. 2004; Kawai et al. 2007; Lim et al. 2007; Draisma et al. 2010). More recently, molecular phylogenetic studies using multiple gene data sets have elucidated the general phylogenetic relationships among orders (Phillips et al. 2008; Silberfeld et al. 2010, 2011, 2014b; Kawai et al. 2015a). Information on taxonomic names is available at AlgaeBASE (http://www.algaebase.org/) and Index Nominum Algarum (http://ucjeps.berkeley.edu/INA.html).

Brown marine algae were recognized by European phycologists as a distinct group of organisms in the early nineteenth century. Detailed studies of their structure and reproduction, along with the first attempt at cultivation, began to appear in the latter half of the century. Some of the more notable works include that of Thuret, who made the first microscopical observations of fertilization in algae using the eggs and flagellated sperm of *Fucus* (Thuret 1854). Improved microscopical and histochemical techniques have made possible critical investigations of brown algal anatomy (Gantt 1980). Alternation between macroscopic sporophyte and microscopic gametophytes in the life histories of kelps was first reported by Sauvageau (1915), who

also made the first culture studies of many other brown algae. The culturing of brown algae was greatly advanced by development of growth media by Shreiber (1927) and Provasoli (Tatewaki 1966; Provasoli 1968; Starr 1978). Culturing techniques of brown algae are reviewed in Kawai et al. (2005b; for details see below).

Practical Importance

Species of Laminariales with foliose thalli (3–4 families) and Fucales (*Ascophyllum* and *Durvillaea*) are utilized commercially as sources of alginates, the salts of alginic acid, a major component of brown algal cell walls. Alginates, polysaccharide compounds composed of mannuronic and guluronic acid subunits, can form highly viscous solutions and readily form gels (Percival and McDowell 1967). Alginates are used widely in the manufacture of products including foods, cattle and poultry feeds, adhesives, dyes, and explosives (Chapman and Chapman 1980). Species harvested for alginate extraction include *Ascophyllum nodosum, Durvillaea potatorum, Eisenia bicyclis, Ecklonia cava, Macrocystis pyrifera, Laminaria* spp., and *Saccharina* spp.

Brown algae are a particularly valuable natural source of vitamins and minerals, notably iodine (Critchley and Ohno 1998). The larger species of brown algae are utilized as fertilizers and growth promoters by coastal agricultural communities. Extracts of some, for example, *Ascophyllum nodosum,* are manufactured and sold widely for use in agriculture and horticulture (Chapman and Chapman 1980). Fucoidan (one of the sulfated polysaccharides in brown algae) extracted from some taxa (*Cladosiphon, Laminaria*, *Undaria*) are used as a functional food. A number of species, primarily belonging to Laminariales (*Ecklonia*, *Laminaria, Saccarhina, Undaria,* etc.), are widely cultivated and used as food in Japan, Korea, and China.

Habitats and Ecology

Geographical Distribution

Studies of the biogeography of benthic marine algae (e.g., van den Hoek 1975; Lawson 1978; South 1975; Santelices 1980; Womersley 1981; Lüning 1990) include information on the distribution of brown algal species. Brown algae dominate many benthic marine biotas. The characteristics of the marine biotas of four types of region – polar, cold temperate, warm temperate, and subtropical/tropical – have been outlined by Womersley (1981). The polar regions, which possess more limited algal biotas, include some distinctive taxa, for example, the Antarctic *Himantothallus* in the Demarestiales, *Ascoseira,* and the Arctic species of *Laminaria*, *Saccharina*, *Saccorhiza* and *Chorda*. A high species diversity is found among the marine algal biotas of cold temperate regions. Fucales (e.g., *Ascophyllum*, *Fucus* spp., *Hormosira banksii*) dominate the intertidal zone of rocky shores and the kelps

form dense communities in the subtidal and lower intertidal zones. The coastal regions of the world with the greatest numbers of species and genera of brown algae include Japan, Pacific North America, Southern Australia, and Britain, all regions of temperate climate with a significant cold temperate component. Large kelps are absent on tropical coasts and are not conspicuous in warm temperate regions. The Dictyotales and *Sargassum* spp. (Fucales) are the common brown algae of tropical and subtropical regions (Womersley 1981; Silva et al. 1996; Silberfeld et al. 2014a).

Temperature and salinity strongly affect horizontal distributions of species of benthic marine algae (Druehl 1981). Experimentally determined lethal or growth- and reproduction-limiting temperatures correlate with phytogeographic boundaries of several species of marine benthic algae, including some phaeophycean taxa (van den Hoek 1982).

Measuring Seaweed Vegetation

Methods have been developed for describing and analyzing the composition, distribution, and structure of local communities of benthic marine algae (Russell and Fielding 1981). Protocols for long-term monitoring of seaweed communities are described by Kautsky and Maarel (1990), Kautsky (1993), Shirayama et al. (2002), and Kawai and Henry (2007).

Environmental gradients and the physiological tolerance limits of species have been studied with the goal of understanding zonation, the vertical banding of seaweed communities. Biotic interactions between different algal species and intertidal animals are significant in determining the vertical limits of seaweed distribution (Chapman 1974; Russell and Fielding 1981).

Kelp Ecosystems

Large brown algae called kelps are either Ascoseirales (*Ascoseira*), Desmarestiales (*Himantothallus*), Fucales (*Durvillaea*), Laminariales (*Alaria*, *Chorda*, *Ecklonia, Eualaria*, *Macrocystis, Laminaria, Nereocystis, Saccharina*), or Tilopteridales (*Sacchorhiza, Phyllariopsis*) and dominate the kelp communities of the world. *Chorda* is dominant on sheltered coasts in Arctic regions. Kelp communities dominated by *Alaria*, *Laminaria,* and *Saccharina* occur in the northern Atlantic and on the coasts of China and Japan, whereas *Macrocystis*-dominated communities are found along the Pacific coast of North America and the temperate Atlantic and Pacific coasts of South America and New Zealand (Lüning and tom Dieck 1990; Santelices et al. 1980). *Ecklonia*-dominated kelp communities are found in Australasia and South Africa (Mann 1982). *Durvillaea* and *Lessonia* kelp beds occur in the circumpolar cold temperate regions of Australasia, South America, and around the

subantarctic islands (Hay 1979). *Ascoseira* and *Himantothallus* are primary elements of Antarctic regions.

Kelp species have very high levels of productivity, on the order of 1,000 g of carbon per square meter annually (Mann 1982). Growth in many species is seasonal (*Macrocystis pyrifera* is an exception) with the highest rates in winter and early spring (Chapman 1974; Kain 1979). Several methods have been used to describe and measure the growth and productivity of kelp species (Kain 1979, 1982), including measurements of standing crop, morphological parameters (including the use of punched holes), chemical components such as alginic acid (cell walls), fucoidan, laminaran, and mannitol (food reserves) (Hellebust and Craigie 1978), and in situ measurements of the rate of photosynthesis (Kremer 1978). In *Laminaria* spp., reserves are built up during the summer when levels of radiant energy are high and are used up during the winter growth phase (Chapman 1974). Many so-called kelps growing seasonally from holdfasts are perennials. Some, like *Laminaria saccharina,* have life spans of less than 3 years (Kain 1979), whereas others (*Durvillaea*) may live for 7 or more years. Thalli of *Laminaria hyperborea* up to 15 years old have been recorded (Kain 1979). Sea urchins are often the most important herbivores in kelp communities. In many localities, they feed on detritus, drifting kelp, and ephemeral algae and cause little disturbance to the attached kelps. Along the northern Pacific coasts, the increase in sea urchin (*Strongylocentrotus* spp.) populations related to the decrease in populations of their major predator, the sea otter (Mann 1982), has led to considerable damage to the kelp beds.

Fucoid Communities

Fucales species are dominant in the rocky intertidal zone in many temperate regions of the world, whereas in the tropics they more commonly form subtidal communities. *Fucus* spp. and *Ascophyllum* abound on temperate shores in the northern hemisphere. In the southern hemisphere, subtidal fucoid communities are often better developed than the intertidal ones, e.g., in Australasia extensive dense stands of *Sargassum* and species in the Cystoseiraceae and Seirococcaceae. *Durvillaea* belongs to Fucales, but is treated as kelp in this chapter (see above).

Fucoids are highly productive, but stresses in the intertidal environment may cause them to exude a considerable amount of soluble organic matter that is rapidly taken up by bacteria (Mann 1982) so that in contrast to kelps, a significant proportion of fucoid productivity is not realized as algal biomass. Intertidal northern hemisphere fucoids grow predominantly during the summer and to a limited extent accumulate storage carbohydrates that permit survival during the winter, when levels of radiant energy and the rate of photosynthesis are very low (Chapman 1974). Sea urchins, starfish, limpets, chitons, littorinid snails, and fish influence the structure of intertidal fucoid communities by grazing, affecting the species composition and biomass (Mann 1982).

Effects of Environmental Factors on Growth and Reproduction

Brown algae are notorious for their morphological plasticity in response to the environment. Thallus form changes in response to variation in such factors as irradiance, water movement, temperature, and salinity. The more dramatic effects occur in the larger species. Kelp species growing on high wave-energy coasts tend to have highly digitate fronds, whereas in calmer water the laminae are more likely to be entire. Unattached fucoids (e.g., *Ascophyllum, Fucus,* and *Hormosira*) in extremely sheltered localities tend to develop a number of distinctive characteristics. Holdfasts are lacking, branching is more profuse, reproduction is often by means of fragmentation, and sexual reproduction is inhibited (Norton et al. 1981).

Changes from the asexual to sexual modes of reproduction are environmentally elicited. Seasonal changes in light quality, irradiance, and temperature have been shown to regulate the fertility of kelp gametophytes. In many species, low temperatures (e.g., 5 °C) and a certain quantum dose of blue light are required to induce gametogenesis, which in nature occurs in winter and spring (Lüning 1981). In *Scytosiphon,* a member of the Scytosiphonaceae, a photoperiodic response controls the transition between the two phases (blades and crusts) of the heteromorphic life history. Blades in laboratory culture develop in response to short days; in nature, blades develop in winter and spring, crusts in summer. Temperature affects reproduction in many species of brown algae. In *Ectocarpus siliculosus*, the development of unilocular (meiotic) sporangia on the sporophyte occurs only below 13 °C so that the gametophyte generation is initiated at lower temperatures. At higher temperatures, only asexual plurilocular sporangia are formed (Müller 1964). Species of brown algae from different orders (Ectocarpales, Sphacelariales, and Fucales) produce their gametes in winter or spring in temperate regions when seawater temperatures are low. Relatively little is known of the factors initiating the developmental sequence leading to gametogenesis in the gametophytes of most brown algae.

Trans-ocean Introductions: Some brown algal species have been introduced across the oceans by human activities intentionally (e.g., fisheries) or accidentally (e.g., associated with ship transportation) and have spread widely and become a considerable threat to local ecosystems. *Sargassum muticum* and *Undaria pinnatifida* were introduced from Asia to North America and Europe attached to young oysters, or as ship hull communities (Critchley and Dijkema 1984; Uwai et al. 2006; Russell et al. 2008). Another Asian-origin *Sargassum* species, *S. filicinum,* has recently spread on the North American Pacific coast (Miller et al. 2007).

Collection of Brown Algae: Brown algae are best collected in buckets and bags from the intertidal zone of the seashore when the tide is low, preferably during spring tides. They may be collected from subtidal habitats by snorkeling and SCUBA diving. Specimens from deep water may occasionally be found in storm drift, caught in fishing nets, or by dredging. Brown algae in the temperate and cold water regions should be kept moist and cool and processed as soon as possible after collection. Only the more tolerant intertidal species survive more than 2 or 3 days of temporary storage in a cold room or refrigerator (see below*).* Tropical and subtropical species are generally better kept at room temperature.

Specimens for herbaria must be prepared from healthy and, if possible, entire thalli. Most species can be preserved as dried herbarium specimens. Individual specimens are spread in a shallow dish of seawater, cleaned of sand, any epibionts, and, if very bushy, carefully pruned of some fronds so that the form can be seen clearly. The specimen is then floated and arranged over a sheet of mounting paper in a tray of clean water, and the paper is slowly tilted and removed from the tray, allowing the water to drain off and the seaweed to remain spread over the paper. Covered with a piece of cheesecloth or plastic nonwoven fabric and pressed between several sheets of newspaper, specimens are stacked and placed in a press. Newspaper must be changed frequently during the first day or two and then daily until the specimens are quite dry. Instead of changing newspapers for removing moisture of the specimens, the pressed specimens on mounting paper and covered with cheese cloth may be put between corrugated cardboard sheets, and the stack placed in a continuous air flow from a fan, so that the moisture is removed through the channels of the corrugated cardboard. Steady firm pressure is essential to obtain flat, unwarped sheets. Delicate soft algae adhere very firmly to the mounting paper and less firmly to the cheesecloth, which can be peeled off when drying is complete. Coarse, thick, or wiry algae do not adhere satisfactorily but can be attached to paper with glue or strips of gummed paper after they are dry. The collector's name, date, and site of collection, code number, and other data are recorded in pencil on each herbarium specimen before mounting. A permanent label is affixed later. Dried specimens, stored flat and away from light, will keep indefinitely and not lose their color.

To preserve specimens, the larger brown algae may be soaked in 50% glycerol, to which some phenol has been added to discourage the growth of microorganisms. The thalli will remain soft and flexible. Brown algae are commonly preserved in seawater-formalin at a concentration of 2–5%. For critical light and electron microscope studies, great care should be taken to select a suitable schedule for the fixation and preparation of the tissue. For this purpose, recent studies on related species should be consulted (see also references in Gantt 1980). For DNA extraction for molecular analyses, fresh specimens should be quickly dried in silica gel and kept desiccated, or frozen.

Some species (e.g., *Desmarestia* spp., *Dictyopteris* spp., *Spatoglossum* spp.) are remarkably delicate and apt to die and turn green when exposed to air or fresh water, revealing the strong acidity within their cell vacuoles when so damaged (Sasaki et al. 1999, 2004).

Characterization and Recognition

Definition of the Class

Brown algae are heterokont, multicellular algae with varying life cycles involving an alternation of haploid and diploid nuclear phases. In a large portion of the taxon, there is a corresponding alternation of independent haploid gametophyte and diploid sporophyte generations. Some orders tend toward an increase in size and morphological complexity of the sporophyte generation and reduction of the gametophyte

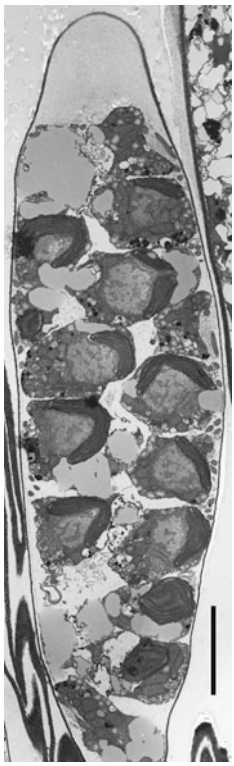

Fig. 4 Unilocular sporangia of *Saccharina japonica* in longitudinal section (TEM micrograph courtesy of Taizo Motomura). Scale bar, 5 μm

generation. Meiosis occurs in cells known as unilocular zoidangia (meiosporangia, Fig. 4) borne on sporophytic thalli, resulting in the formation of haploid spores (meiospores). These are, in most taxa, motile cells (zoospores) having two unequal flagella; they settle, germinate, and give rise to the gametophyte generation. The gametes are generally produced in plurilocular gametangia (Fig. 5) borne on gametophyte thalli. Fertilization varies from isogamous (gametes of different sex are the same size) to oogamous (gametes are nonmotile eggs and motile spermatozoids).

Sexual attractants (pheromones) are known to be involved in the sexual reproduction in brown algae. Attractants, mostly volatile hydrocarbons of low molecular weight (Müller 1981a; Maier and Müller 1986), which are secreted by female gametes to attract male gametes, have been isolated from several species and characterized chemically. In Laminariales, the sexual attractant also induces the release of sperm from the spermatangia (antheridia) (Maier 1995). The diploid zygote develops into the sporophyte generation. In many species, asexual reproduction involving the production of zoospores (motile cells) in multichambered plurilocular sporangia borne on either the gametophyte or sporophyte generation is very common. Brown algae produce heterokont reproductive zoids and sperm with characteristic ultrastructure: two unequal flagella inserted laterally, the anterior bearing mastigonemes and the posterior one lacking them (Figs. 6 and 7). The

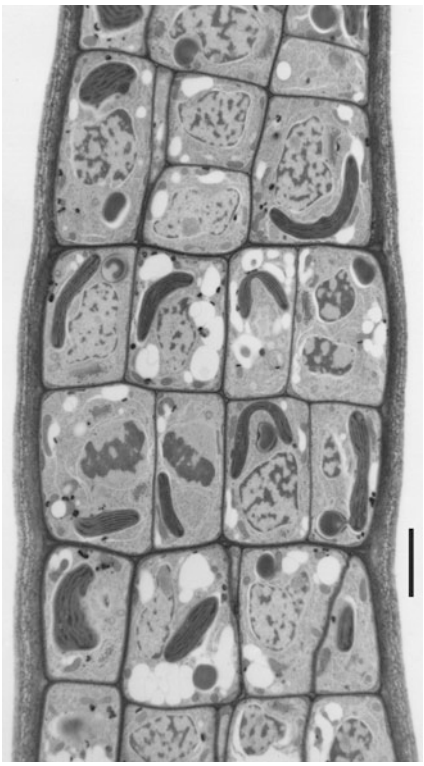

Fig. 5 Immature plurilocular sporangia of *Ectocarpus* sp. Freeze substitution (TEM micrograph courtesy of Taizo Motomora). Scale bar, 2 μm

genes of the proteins constituting mastigonemes have been identified in a unicellular heterokont (*Ochromonas*, Yamagishi et al. 2007) and shown to be comparable to the corresponding genes of the proteins in brown algae.

Most brown algal zoids show photo-orientation responses (phototaxis). In the posterior flagellum, a green autofluorescent substance and a basal swelling, appressed to an eyespot of carotenoid globules in the adjacent chloroplast (Fig. 8), is involved in the photoreception of blue light (Kawai et al. 1990; Kawai 1992a; Kawai and Kreimer 2000). In contrast, zoids of Laminariales and sperm that lack phototaxis in some other orders lack these structures.

Structural characteristics of brown algal chloroplasts (plastids) include the arrangement of thylakoids in groups of three, the presence of a lamella lying just inside the plastid membrane (called the girdle lamella), and the plastid endoplasmic reticulum, which is continuous with the nuclear envelope. The chloroplast DNA is arranged in a peripheral ring (Coleman 1985). The characteristic brown accessory pigment fucoxanthin is active in photosystem II of photosynthesis (Braun and Braun 1974); carotenes, violaxanthin, and chlorophylls *a, c* and c_1 are also present. Pyrenoids, appendages of chloroplasts, are observed in species of the orders Ectocarpales *s.l.* and Scytothamnales (Kawai 1992b) (Fig. 8). Pyrenoids are lacking or not obvious in other orders including basal taxa, and those in Ectocarpales (Nagasato et al. 2003) and

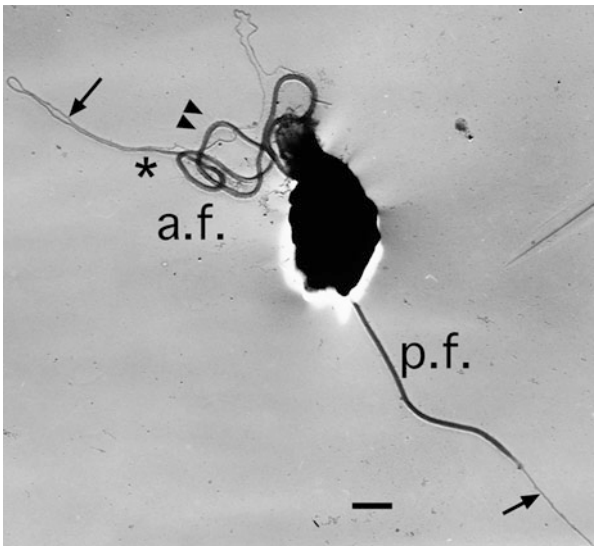

Fig. 6 Sperm of *Stschapovia flagellaris* with hairy anterior flagellum having very long acronema at the tip, and posterior flagellum with shorter acronema. Whole mount TEM micrograph. Scale bar, 2 μm

Scytothamnales (Tanaka et al. 2007) have distinctive morphological features; therefore, pyrenoids are considered to have evolved (or became elaborated) multiple times in brown algae (Silberfeld et al. 2011). The carbon storage material commonly found in the phaeophytes is laminaran, a β-(1, 3) glucan containing mannitol.

Brown algal cell walls are composed chiefly of three kinds of polymer: cellulose, alginic acid, and the heterogeneous fucose-containing sulfated polysaccharides (fucan, fucoidan). The microfibrillar organization of cellulose contributes to the skeletal role of the walls (Kloareg et al. 1986). The cellulose-synthesizing terminal complexes (TCs) associated with the tips of microfibril impressions in the plasmatic fracture face of the plasma membrane (observed by freeze-fracture techniques) consist of subunits arranged in a single linear row (Peng and Jaffe 1976; Tamura et al. 1996). Alginic acid, a polymer of mannuronic and guluronic acids, and other sulfated polysaccharides such as fucoidan (McCandless and Craigie 1979), even though they are chemically more complex than cellulose, do not form structural wall components. Alginic acid was long considered to be a defining characteristic in the Phaeophyta (Phaeophyceae) among the Chromista, but has now also been shown to be present in members of Schizocladiophyceae, Xanthophyceae, and Chrysomeridales (Chi et al. 1999; Kawai et al. 2003). Whatever skeletal functions alginates possess are thought to derive from their property of forming gels and viscous solutions. Fucans apparently have a role in the desiccation resistance of intertidal brown algae by virtue of their strong affinity for highly hydrated magnesium ions (Percival 1979).

Physodes, vacuole-like refractive bodies containing osmiophilic material of phenolic nature, are common in brown algae cells. Chemical analysis (Ragan 1976) has shown physodes to contain primarily phloroglucinol derivatives along with tannins and terpenes. The phenolic compounds in brown algal cells are thought to act as herbivore deterrents in a similar way as the tannins in higher plants (Targett and

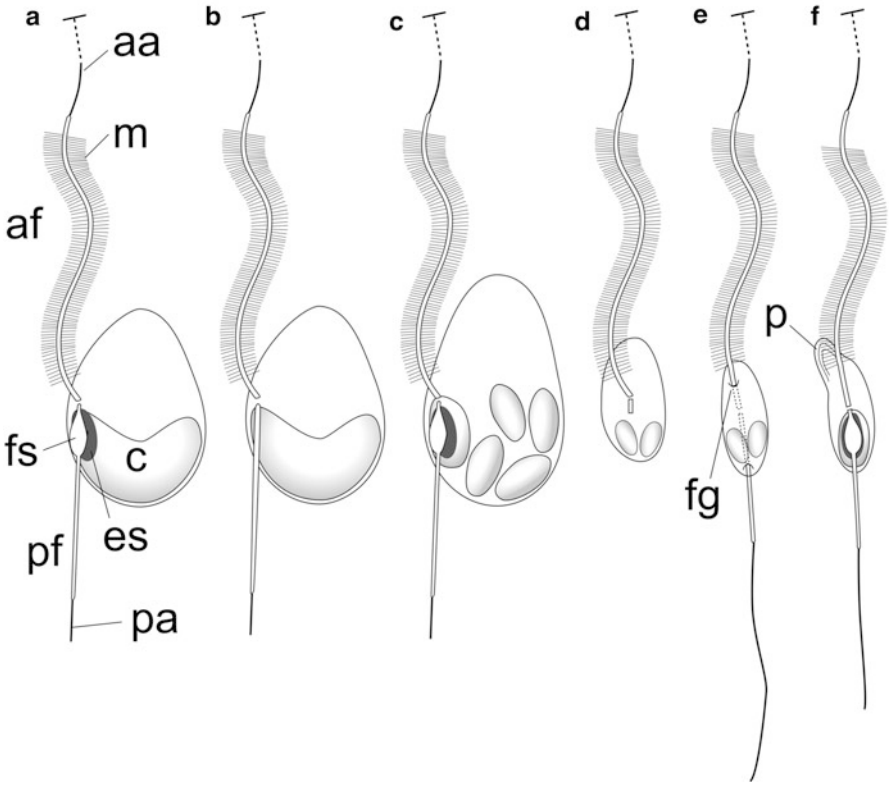

Fig. 7 Schematic representation of brown algal flagellated cells. (**a**) Typical (perhaps plesiomorphic) type with long anterior flagellum (*af*) and short posterior flagellum (*pf*). Chloroplast (*c*) has eyespot (*es*) spatially associated with flagellar swelling (*fs*) on the basal part of the posterior flagellum. Anterior flagellum has long acronema (*aa*), which is easily detached by fixation. Posterior flagellum has relatively short, but more persistent acronema (*pa*). (**b**) Laminarialean type without eyespot and flagellar swelling and lacking phototaxis. (**c**) Female anisogamous gamete with multiple chloroplasts. (**d**) Dictyotalean sperm with remnant posterior flagellum and reduced chloroplast. (**e**) Laminarialean sperm with long posterior flagellum, reduced chloroplasts, and deep flagellar gullet (*fg*). (**f**) Fucalean sperm with proboscis (anterior protuberance) and eyespot

Arnold 1998), but they are also suggested to play a role in polyspermy block and protection against UV radiation (Schoenwaelder 2002).

Classification of the Phaeophyceae

In the ordinal classification of the Phaeophyceae since Kylin (1933), basic construction of the thallus, growth mode, and life history pattern have been regarded as characters reflecting higher-rank phylogeny. Based on the general concept using phenotypic characters, 13–17 orders have been recognized in the Phaeophyceae: Ascoseirales, Chordariales, Cutleriales, Desmarestiales, Dictyosiphonales, Dictyotales,

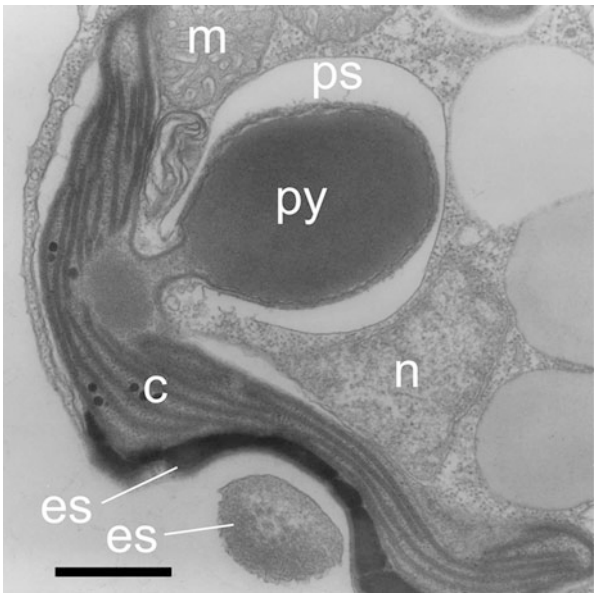

Fig. 8 Chloroplast with protruded type of pyrenoid with pyrenoid sac in *Ectocarpus* gamete. TEM micrograph. Scale bar, 0.5 μm

Discosporanigiales, Durvillaeales, Ectocarpales, Fucales, Laminariales, Ralfsiales, Scytosiphonales, Sphacelariales, Sporochnales, Syringodermatales, and Tilopteridales. However, molecular phylogenetic data showed some incongruence with some of the conventional ordinal assignments. Major updates are as follows: de Reviers and Rousseau (1999) proposed to reduce Durvillaeales to family status within the Fucales, as well as the unification of the Chordariales, Dictyosiphonales, Ectocarpales, and Scytosiphonales into Ectocarpales *sensu lato* (de Reviers and Rousseau 1999). Cutleriales was shown to be sister group of Tilopteridales, and so was merged with Tilopteridales (Silberfeld et al. 2010). Silberfeld et al. (2014b) proposed new subclasses Discosporangiophycidae, Ishigeophycidae, and Dictyophycidae in addition to the subclass Fucophycidae proposed by Cavalier Smith (1986), as well as a new order Phaeosiphoniellales. Kawai et al. (2015a) proposed Stschapoviales and moved Halosiphonaceae and Stschapoviaceae from Tilopteridales to this order together with newly proposed Platysiphonaceae.

The orders and families are listed, as is customary, in sequences of increasing structural complexity. The list of genera, not exhaustive because it is based on only the more accessible recent taxonomic literature, is in alphabetical order. However, the ordinal and familial assignment of ectocarpalean taxa are currently considerably confused in Ectocarpales excluding Scytosiphonaceae, because of the insufficient resolution and the coverage of taxa in relevant molecular phylogenetic studies, although several new families have been described based on molecular data. Therefore, familial assignment is suspended in those taxa in the present chapter.

Representative families and genera in the orders are listed in Table 1. Diagnostic characters of the above-mentioned 17 orders currently recognized within the Phaeophyceae are presented in Table 2.

Table 1 Orders, families, and genera of Phaeophyceae

I. Order Discosporangiales
1. Choristocarpaceae: *Choristocarpus*
2. Discosporangiaceae: *Discosporangium*
II. Order Ishigeales
1. Ishigeaceae: *Ishige*
III. Order Dictyotales
1. Dictyotaceae: *Canistrocarpus*, *Chlanidophora, Dictyopteris, Dictyota, Dictyotopsis, Distromium, Exallosorus, Herringtonia, Homoeostrichus, Lobophora, Lobospira, Newhousia, Padina, Rugulopteryx, Scoresbyella, Spatoglossum, Stoechospermum, Stypopodium, Taonia, Zonaria*
IV. Order Sphacelariales
1. Cladostephaceae: *Cladostephus*
2. Phaeostrophiaceae: *Phaeostrophion*
3. Sphacelariaceae: *Battersia, Herpodiscus, Sphacelaria, Sphacella, Sphacelorbus*
4. Sphacelodermaceae: *Sphaceloderma*
5. Stypocaulaceae: *Halopteris, Phloiocaulon, Protohalopteris, Ptilopogon*
V. Order Onslowiales
1. Onslowiaceae: *Onslowia, Verosphacela*
VI. Order Syringodermatales
1. Syringodermataceae: *Microzonia, Syringoderma*
VII. Order Desmarestiales
1. Arthrocladiaceae: *Arthrocladia*
2. Desmarestiaceae: *Desmarestia, Himanthothallus, Phaeurus*
VIII. Order Ascoseirales
1. Acroseiraceae: *Acroseira*
IX. Order Sporochnales
1. Sporochnaceae: *Austronereia, Bellotia, Carpomitra, Encyothalia, Lucasia, Nereia, Perisporochnus, Perithalia, Sporochnema, Sporochnus, Tomaculopsis*
X. Order Scytothamnales
1. Asteronemataceae: *Asteronema*
2. Bachelotiaceae: *Bachelotia*
3. Splachnidiaceae: *Scytothamnus, Splachnidium, Stereocladon*
XI. Order Ectocarpales sensu *lato*
1. Adenocystaceae: *Adenocystis, Caepidium, Chordariopsis, Utriculidium*
2. Ectocarpaceae (Ectocarpales *sensu stricto* + Chordariales + Dictyosiphonales)
Acinetospora, Acrothrix, Acrotrichium, Actinema, Adenocystis, Ascoseirophila, Asperococcus, Australofilum, Botrytella, Buffhamia, Chilionema, Chordaria, Chuckchia, Cladochroa, Cladosiphon, Cladothele, Clathrodiscus, Climacosorus, Coelocladia, Coilodesme, Corycus, Corynophlaea, Cylindrocarpus, Delamarea, Dermatocelis, Dictyosiphon, Ectocarpidium, Ectocarpus, Elachista, Elachistiella, Entonema, Epinema, Eudesme, Feldmannia, Flabellonema, Fosliea, Geminocarpus, Giraudia, Gononema, Halonema, Halorhipis, Halorhiza, Halothrix, Hamelella, Haplogloia, Hecatonema, Herponema, Heterosaundersella, Hincksia, Hummia, Internoretia, Isthmoplea, Kuckuckia, Kuetzingiella, Kurogiella, Laminariocolax, Laminarionema, Leathesia, Leblondiella, Leptonematella, Levringia, Leblondiella, Liebmannia, Litosiphon, Melastictis, Mesogloia, Mesogloiopsis, Microcoryne, Microspongium, Mikrosyphar, Myriactula, Myriocladia, Myriogloea, Myrionema, Myriotrichia, Nemacystus, Neoleptonema,

(*continued*)

Table 1 (continued)

Omphalophyllum, Papenfussiella, Petrospongium, Phaeophysema, Phaeostroma, Phaeostromatella, Pilinia, Pilocladus, Pleurocladia, Pogotrichum, Polycerea, Polytretus, Proselachista, Protectocarpus, Punctaria, Pylaiella, Rhadinocladia, Saundersella, Sauvageaugloia, Soranthera, Spermatochnus, Sphaerotrichia, Spongonema, Stegastrum, Stictyosiphon, Stilophora, Stilopsis, Streblonema, Streblonemopsis, Strepsithalia, Striaria, Suringariella, Tinocladia, Trachynema, Ulonema, Vimineoleathesia, Xanthosiphonia, Zeacarpa, Zosterocarpus
3. Scytosiphonaceae (Scytosiphonales)
Chnoospora, Colpomenia, Compsonema, Diplura, Endarachne, Endopleura, Hapterophycus, Hydroclathrus, Iyengaria, Jolyna, Myelophycus, Melanosiphon, Petalonia, Rosenvingea, Scytosiphon, Sorapion, Stragularia, Symphyocarpus
XII. Order Laminariales
1. Akkesiphycaceae: *Akkesiphycus*
2. Pseudochordaceae: *Pseudochorda*
3. Chordaceae: *Chorda*
4. Aureophycaceae: *Aureophycus*
5. Agaraceae: *Agarum, Costaria, Dictyoneurum, Thalassiophyllum*
6. Laminariaceae: *Arthrothamnus, Costulariella, Cymathere, Feditia, Laminaria, Macrocystis, Nereocystis, Pelagophycus, Phyllariella, Postelsia, Pseudolessonia, Saccharina, Tauya*
7. Alariaceae: *Alaria, Ecklonia, Eckloniopsis, Egregia, Eisenia, Eualaria, Pleurophycus, Pterygophora, Undaria, Undariella*
8. Lessoniaceae: *Lessonia, Lessoniopsis*
9. *Phaeosiphoniellaceae: *Phaeosiphoniella*
XIII. Asterocladales
1. Asterocladaceae: *Asterocladon*
XIV. Order Fucales
1. Bifurcariopsidaceae: *Bifurcariopsis*
2. Durvillaeaceae: *Durvillaea*
3. Fucaceae: *Ascophyllum, Fucus, Hesperophycus, Pelvetia, Pelvetiopsis, Silvetia*
4. Himanthaliaceae: *Himanthalia*
5. Hormosiraceae: *Hormosira.*
6. Notheiacaea: *Notheia*
7. Sargassaceae*: Acrocarpia, Anthophycus, Axillariella, Bifurcaria, Brassicophycus, Carpoglossum, Carpophyllum, Caulocystis, Cladophyllum, Coccophora, Cystophora, Cystoseira, Halidrys, Hormophysa, Landsburgia, Myagropsis, Myriodesma, Nizamuddinia, Oerstedtia, Phyllotricha, Platythalia, Polycladia, Sargassopsis, Sargassum, Scaberia, Sirophysalis, Stephanocystis, Stolonophora, Turbinaria*
8. Seirococcaceae: *Axillariella, Cystosphaera, Marginariella, Phyllospora, Scytothalia, Seirococcus*
9. Xiphophoraceae: *Xiphophora*
XV. Order Nemodermatales
1. Nemodermataceae: *Nemoderma*
2. Zeacarpaceae: *Zeacarpa*
XVI. Order Tilopteridales
1. Culteriaceae: *Cutleria, Mutimo, Zanardinia*
2. Phyllariaceae: *Phyllariopsis, Saccorhiza*

(*continued*)

Table 1 (continued)

3. Tilopteridaceae: *Haplospora, Tilopteris*
XVII. Order Stschapoviales
1. Halosiphonaceae*: Halosiphon*
2. Platysiphonaceae: *Platysiphon*
3. Stschapoviaceae: *Stschapovia*
XVIII. Order Ralfsiales
1. Heterochordariaceae: *Analipus*
2. Mesosporaceae: *Acrospongium, Hapalospongidion, Mesospora*
3. Neoralfsiaceae: *Neoralfsia*
4. Ralfsiaceae: *Heteroralfsia, Jonssonia, Lithoderma, Myrionemopsis, Petroderma, Porterinema, Pseudolithoderma, Ralfsia*

*Classified in a separate order Phaeosiphoniellales in Silberfeld et al. (2014b)

Order Discosporangiales

The genera *Choristocarpus* and *Discosporangium*, with uniseriate filamentous thalli with apical growth cells, have been classified in Sphacelariales (Prud'homme van Reine 1982). However, recent molecular studies (Draisma et al. 2001; Burrowes et al. 2003; Kawai et al. 2007) revealed that they form a monophyletic clade that first branches off from all other brown algae, sharing the following characters which are considered to be plesiomorphic characters in the brown algae: (1) apical (and diffuse) growth; (2) uniseriate, subdichotomously branched filaments; (3) multiple chloroplasts per cell without pyrenoids; and (4) lack of heterotrichy and phaeophycean hairs. Regarding their higher-rank systematic positions, reinstatement of Discosporangiaceae and Discosporangiales (Schmidt 1937) was proposed, and the inclusion of Choristocarpaceae in the order was also suggested (Kawai et al. 2007). *D. mesarthrocarpum* has unique disk-shaped plurilocular reproductive organs.

Order Ishigeales

The Ishigeales have branched, terete or flattened parenchymatous thalli of up to 10–20 cm high. The genus *Ishige*, including two species distributed in the warm temperate Pacific Ocean, has been classified in Chordariales. However, the lack of prominent pyrenoids in the chloroplast (Hori 1971) and the indication of isomorphic life history (Tanaka in Hori 1993), as well as preliminary molecular phylogenetic data (Tan and Druehl 1994; Peters and Ramírez 2001), made this systematic position doubtful. Later Cho et al. (2004) proposed a new order Ishigeales to accommodate the genus.

Order Dictyotales

The isomorphic sporophyte and gametophyte thalli are foliose and parenchymatous. Dictyotales are unique in brown algae including taxa with calcified thalli (i.e., *Padina* and *Newhousia*). *Dictyota* has a single apical cell, whereas other genera have several to many localized apical cells forming dichotomously branching thalli

Table 2 Characteristic features of brown algal orders

Order	Thallus structure or prominent generation	Chloroplast	Life history pattern	Reproduction (sexual/asexual)	Remarks
Discosporangiales	Uniseriate, filamentous	Multiple, discoid, without pyrenoids	Isomorphic	Isogamy?/unizoids	Most basal taxon in Phaeophyta, only a few species recognized
Ishigeales	Parenchymatous, foliose or terete	Multiple, discoid, without pyrenoids	Isomorphic	Isogamy/unizoids	Only a few species recognized
Dictyotales	Parenchymatous, foliose or membranous	Multiple, discoid, without pyrenoids	Isomorphic	Oogamy/tetraspores in most taxa	Large order including many tropical/subtropical taxa
Sphacelariales	Parenchymatous, filamentous, terete or foliose	Multiple, discoid, without pyrenoids	Isomorphic	Isogamy, anisogamy, oogamy/unizoids, propagules	
Onslowiales	Uniseriate, filamentous, partly with longitudinal walls	Multiple, discoid, without pyrenoids	Isomorphic	anisogamy/unizoids, propagules	
Syringodermatales	Foliose thalli formed by adhesion of filaments; some with longitudinal walls	Multiple, discoid, without pyrenoids	Isomorphic or heteromorphic[a]	Isogamy/unizoids	Only known from relatively deep habitats, life histories remarkably diverse
Desmarestiales	Pseudoparenchymatous, terete or foliose	Multiple, discoid, without pyrenoids	Heteromorphic	Oogamy/unizoids	Some spp. forming large thalli exceeding 5 m, important ecological element in Antarctic region

Ascoseirales	Parenchymatous, terete	Multiple, discoid, without pyrenoids	Only diploid generation is evident	Isogamy	Distributed only in cold water regions of the southern hemisphere
Sporochnales	Pseudoparenchymatous, terete	Multiple, discoid, without pyrenoids	Heteromorphic	Oogamy/uni-zoids	
Scytothamnales	Parenchymatous, terete	Single or multiple, embedded pyrenoid with tubular invaginations	Heteromorphic	Isogamy/uni-zoids	Characterized by distinctive pyrenoids
Ectocarpales *s.l.*	Uniseriate, pseudoparenchymatous or parenchymatous; filamentous, terete, foliose or saccate	Single to multiple, discoid or ribbon-shaped, with projected pyrenoid	Isomorphic[b] or heteromorphic	Isogamy, anisogamy/uni-zoids	Including taxa formerly classified in Chordariales, Dictyosiphonales and Scytosiphonales
Laminariales	Parenchymatous, foliose or terete	Multiple, discoid, without pyrenoids	Heteromorphic	Oogamy/uni-zoids	Largest thalli attaining to 50 m and major component of kelp forests
Asterocladales	Uniseriate, filamentous, partly with longitudinal walls	Multiple, separate pyrenoids grouped in center of cell	Isomorphic	Anisogamy/uni-zoids	
Fucales	Parenchymatous, foliose or terete	Multiple, discoid, without pyrenoids	Only diploid generation is evident	Oogamy	Some spp. forming large thalli exceeding 10 m and major component of algal beds
Nemodermatales	Pseudoparenchymatous, crustose	Multiple, discoid, without pyrenoids	Isomorphic	Anisogamy	Characterized by intercalary or tufted lateral unilocular zoidangia in upright filaments

(*continued*)

Table 2 (continued)

Order	Thallus structure or prominent generation	Chloroplast	Life history pattern	Reproduction (sexual/asexual)	Remarks
Tilopteridales	Parenchymatous, terete or filamentous	Multiple, discoid, without pyrenoids	Heteromorphic, nearly isomorphic or only haploid generation is evident	Oogamy or asexual/uni-zoids	Distributed only in cold or cool water regions of the northern hemisphere
Stschapoviales	Parenchymatous, terete	Multiple, discoid, without pyrenoids	Heteromorphic or regenerating a single type of thallus	Oogamy or asexual/uni-zoids	Distributed only in cold water regions of the northern hemisphere
Ralfsiales	Parenchymatous, crustose, or terete	Single or multiple without pyrenoid	Isomorphic or heteromorphic	Isogamy/uni-zoids	Predominantly crustose

[a]Gametophyte may be considerably reduced and not free-living
[b]In some taxa, sporophyte and gametophyte thallus sizes are not remarkably different but are distinguishable by their gross morphology

or arranged along the entire margin and hence forming fan-shaped thalli. Cells that are cut off from the apical cell undergo further longitudinal and transverse divisions (Katsaros and Galatis 1988; Gaillard and L'Hardy-Halos 1990). Sexual reproduction is anisogamous or oogamous. The sperm have only an anterior flagellum, but have a second flagellar basal body (Manton 1959), except for *Zonaria angustata*, which is reported to have two flagella (Phillips and Clayton 1991).

Order Sphacelariales

The Sphacelariales is a well-defined order (Prud'homme van Reine 1982, 1993) in which branched filaments grow from a conspicuous apical cell. During growth, segments cut off from the apical cell by transverse division subsequently enlarge and undergo further transverse and longitudinal segmentation. Sphacelariales show isomorphic life histories. Isogamy occurs in *Cladostephus,* isogamy or anisogamy in *Sphacelaria,* and anisogamy or oogamy in the Stypocaulaceae. Kawai et al. (2005a; 2015a) suggested classifying *Phaeostrophion* in Phaeostrophiaceae in Sphacelariales, and thereby to emend the order to also include foliose taxa.

Order Onslowiales

Onslowiales comprises two genera *Onslowia* and *Verosphacela*. The isomorphic sporophyte and gametophyte thalli of apical growth are filamentous and irregularly branched, and slightly polystichous with transverse walls (Searles and Leister 1980; Henry 1987; Draisma et al. 2010).

Order Syringodermatales

Syringodermatales include the genera *Syringoderma* and *Microzonia*, formerly included in Dictyotales (Henry 1984; Burrowes et al. 2003). The fan-shaped macrothalli develop by the cohesion of filaments arising from a marginal meristem. The gametophyte morphologies are remarkably divergent among *Syringoderma* species, being either filamentous or reduced to only 4 or 2 cells (Henry and Müller 1983; Henry 1984; Kawai and Yamada 1990).

Order Desmarestiales

In most species, the pseudoparenchymatous thallus is derived from apical meristems situated at the base of a hair (trichothallic). A cortex and a meristoderm (a meristematic surface layer of tissue) are formed from the lateral outgrowths of axial filaments. A parenchyma-like anatomy is formed from this filamentous growth in the Antarctic *Himantothallus* (Moe and Silva 1981). The taxonomy of the species of *Desmarestia* is confused, and a worldwide revision of the 40 or so species is needed (Moe and Silva 1977). Some members show strong acidity by accumulating SO_4^{2-} ion within cells, and the evolution of this character is considered to have occurred once in the order (Peters et al. 1997).

Order Ascoseirales

The Ascoseirales comprise the single Antarctic genus *Ascoseira*, which has a large parenchymatous thallus consisting of a dissected lamina, holdfast, and stipe. Growth

is intercalary with reproductive structures borne in conceptacles that produce large cells containing eight large eggs capable of developing directly into the sporophytic thallus (Moe and Henry 1982) and eight vegetative vestigial cells (Clayton 1987).

Order Sporochnales
The sporophytic thalli are filamentous and growth occurs in meristems located at the base of dense apical tufts of hairs. The pseudoparenchymatous (= formed of filamentous aggregates) fronds are formed from the downgrowth of corticating filaments below the apex. The minute, filamentous gametophytes (microthalli) produce eggs and sperm (Caram 1965).

Order Scytothamnales
The Scytothamnales have branched, terete, parenchymatous thalli, up to 30 cm high, growing from a small holdfast or crustose base. The cells include one or more stellate chloroplasts with a central pyrenoid with tubular invaginations and lacking a pyrenoid sac (Peters and Clayton 1998; Tanaka et al. 2007).

Order Ectocarpales *sensu lato* (including Chordariales, Dictyosiphonales, Ectocarpales *sensu stricto*, and Scytosiphonales)
The taxa formerly classified in Chordariales are mucilaginous, simple, or branched thalli of closely compacted filaments (pseudoparenchymatous). A few genera (in the Elachistaceae, and *Papenfussiella*) also have free filaments. Growth is intercalary in the Elachistaceae, Leathesiaceae, and Chordariaceae and apical in the Acrotrichaceae, Spermatocthnaceae, Splachnidiaceae, and Notheiaceae. Macrothalli are sporophytes with unilocular and, in some species, plurilocular sporangia. Sexual life histories have been described for a number of species (Müller 1981b; Peters 1987).

The taxa formerly classified in Dictyosiphonales were defined by their polystichous character. That is, their cells divide longitudinally as well as transversely to form parenchyma. Longitudinal cell divisions produce a parenchymatous thallus. This is least developed in the Myriotrichiaceae, which possess finely divided, almost filamentous thalli. The macrothallus sporophytes possess both unilocular and plurilocular sporangia in most species. Gametophytes, where known, are filamentous and produce anisogametes (Fiore 1977). Adenocystaceae (Rousseau et al. 2000) is considered to have its closest phylogenetic relationship with this order.

Ectocarpales *sensu stricto* have simple thalli consisting of branched filaments in which growth occurs by intercalary cell division. Reproduction is by zoids produced in plurilocular and unilocular structures. Sexual reproduction is either isogamous or anisogamous, but the life histories of only a few species have been studied in detail (Wynne and Loiseaux 1976). *Ectocarpus siliculosus* and species in the Myrionemataceae (Loiseaux 1967), for example, have more or less isomorphic sporophyte and gametophyte generations (Müller 1967). The Sorocarpaceae, classified previously in the Ectocarpaceae, was erected (Pedersen 1977) for three genera with terminal hairs, sympodial branching, and distinctive aggregates of plurilocular sporangia (sori). Some authors have placed them in the Chordariales. Some authors

have merged Ectocarpales with Chordariales, Scytosiphonales, Tilopteridales, and Dictyosiphonales because the boundaries separating these taxa are indistinct (Russell and Fletcher 1975).

In the taxa formerly classified in Scytosiphonales, the thalli are parenchymatous with variously shaped, simple, or branched forms. Growth is intercalary. The cells contain one plastid. The macrothalli are gametophytes, bearing only plurilocular reproductive structures, some of which are gametangia. Mating is isogamous or anisogamous (Nakamura and Tatewaki 1975; Clayton 1979; 1980). The sporophytes are filamentous or crustose microthalli that produce unilocular sporangia. Taxonomy of the order was revised by Kogame et al. (1999).

Order Laminariales

The Laminariales (see Bold and Wynne 1985) have large parenchymatous thalli differentiated into lamina, stipe, and holdfast. The thallus consists of an outer meristoderm, a cortex, and a central medulla. The trumpet-shaped filaments (hyphae) constituting the medulla are distinctive to the order, comparable to sieve tubes in higher plants. Growth occurs at intercalary meristematic regions. The macrothallus sporophytes reproduce by means of spores formed in unilocular sporangia. Microscopic gametophytes produce sperm or eggs. Egg formation (Lüning 1981) and fertilization (Lüning and Müller 1978) are similar to that in the Desmarestiales, but the laminarialean egg has vestigial flagella (Motomura and Sakai 1988).

Members of Laminariales are major components of lower intertidal to subtidal vegetations in cold water regions, except the Antarctic. Higher rank taxonomy of the order has been considerably revised based on life history and molecular studies (Kawai 1986; Kawai and Kurogi 1985; Kawai and Sasaki 2000; Kawai et al. 2008, 2013; Kawai 2014). Kawai et al. (2008, 2013) reported a novel laminarialean species with distinctively simple sporophyte structure and forming sori on the discoid holdfast. Phylogeography of Laminariales was recently reviewed by Bolton (2010) and Kawai (2014).

Order Asterocladales

Asterocladales comprise two filamentous genera with characteristic chloroplast configurations. Vegetative cells include several elongate plastids per cell, which are linked in a stellate configuration via their stalked and protruding pyrenoids without invaginations, as seen in Scytothamnales (Müller and Parodi 1994; Müller et al. 1998; Uwai et al. 2005; Tanaka et al. 2007).

Order Fucales

The Fucales have fairly large parenchymatous thalli. Branching is either dichotomous, monopodial and radial, or bilateral. The thallus is differentiated into a meristoderm, cortex, and medulla; growth results from the division of apical cells and cell division in associated meristematic regions. Ooogonia and spermatangia are borne on specialized branches known as receptacles. Meiosis occurs in the first division of the sexual parental cells. The haploid gametophyte generation is so greatly reduced that it is not recognizable as such.

Members of Fucales are major components of coastal vegetations of cold water regions of the northern hemisphere (*Fucus, Ascophyllum, Pelvetia*, etc.) and southern hemisphere (*Durvillaea*), and warm temperate to tropical coastal ecosystems (*Sargassum, Cystoseira*, etc.).

Order Nemodermatales

Nemodermatales comprise Nemodermataceae and newly described Zeacarpaceae (Kawai et al. 2016). Both families are monotypic including *Nemoderma tingitanum* and *Zeacarpa leiomorpha,* respectively. It has crustose thalli characterized by the formation of reproductive sori with intercalary or tufted lateral unilocular zoidangia in upright filaments. Each cell includes multiple chloroplasts without pyrenoids. By molecular phylogeny, *N. tingitanum* was shown to be distinctive from other crustose algae, and treatment in a separate order was suggested (Phillips et al. 2008). Later, *Zeacarpa* was shown to phylogenetically most related to *Nemoderma* and also transferred from Ralfsiales to Nemodermatales.

Order Tilopteridales

Tilopteridales comprise Tilopteridaceae, Phyllariaceae, and Cutleriaceae. The family Tilopteridaceae includes three polystichous, filamentous genera (South 1975; Hooper et al. 1988). *Haplospora* sporophytes resemble the gametophytes, and sexual reproduction involves eggs and sperm. The sporophyte of *Haplospora* bears meiotic sporangia that produce a multinucleate nonmotile spore. In *Haplospora* a life history alternating between nearly isomorphic filamentous sporophytes forming monospores and asexual gametophytes forming two types of monospore-like reproductive cells (i.e., eggs and neutral spores) and sperm are reported, whereas the sporophyte is considered to be reduced in *Tilopteris* (Kuhlenkamp and Müller 1985). In either case, sexual reproduction is considered to be reduced. In contrast, some members of Cutleriaceae (e.g., *Cutleria*, *Mutimo*), which used to be classified in its own order Cutleriales, have a life history alternating between heteromorphic terete or membranous gametophytes and crustose sporophytes, whereas *Zanardinia* is isomorphic (Fritsch 1945). Phyllariaceae comprise kelp-like genera such as *Sacchorhiza* and *Phyllariopsis* and have characteristic anatomy with multinucleate conducting filaments (i.e., solenocysts) comparable in function to the trumpet-shaped hyphae in laminarialean kelp.

Order Stshapoviales

Kawai et al. (2015a) proposed to classify Halosiphonaceae, Stschapoviaceae, and Platysiphonaceae in Stschapoviales, mainly based on molecular phylogeny. These are cold water taxa having polystichous, terete thalli with assimilatory filaments in whorls or on the distal end. *Halosiphon* shows a typical heteromorphic life history with large sporophyte and monoecious gametophytes, but *Stschapovia* and *Platysiphon* are suggested to have a modified life history without alternation between two apparent generations, as in Fucales (Kawai and Sasaki 2004; Kawai et al. 2015a, b).

Order Ralfsiales
The validity of Ralfsiales has been challenged, but it was shown to be a monophyletic group after emendation (Lim et al. 2007). The Ralfsiales primarily have crustose thalli, but some have terete erect thalli (e.g., *Analipus, Heteroralfsia*) (Kawai 1989), characterized by discoidal early development of the thallus, intercalary plurilocular gametangia with terminal cells, terminal unilocular zoidangia, and a crustose phase in the life history (Nakamura 1972; Lim et al. 2007). Many members have a single parietal chloroplast without pyrenoids. The new families Mesosporaceae (Tanaka and Chihara 1982) and Neoralfsiaceae (Lim et al. 2007) have been added to the order.

Maintenance and Cultivation

Isolation from Nature

Collecting and Establishing Unialgal and Axenic Cultures: Collected specimens are transported in plastic bags, bottles, or containers suitable to their size, avoiding excess solar irradiation and temperature shocks relative to the prevailing habitat conditions. An insulated cool box or refrigerator is convenient for the temporary storage and transport of brown algae under most climatic conditions, although tropical species may be adversely affected by such low temperatures. Most intertidal taxa are more tolerant of stresses such as desiccation and rapid temperature changes, compared to subtidal taxa. Fertile specimens collected under desiccating conditions tend to release zoids and eggs as soon as they are reimmersed in seawater, such as in the containers used for transportation. Therefore, they may preferably be transported moist in plastic bags or plastic containers, instead of immersed in seawater.

A number of techniques are available for initiating cultures, some of which are more suited to particular orders of the Phaeophyceae (Kawai et al. 2005b). Either vegetative tissue or reproductive cells may be used for establishing unialgal cultures. In both cases, clean specimens without epiphytes and epizoa are preferred, and fertile specimens should be selected in the field. When fertile portions of the specimens are recognizable, only those portions need to be excised and transported to the laboratory, to avoid damage and contamination from the other specimens. Acid-containing taxa (e.g., some *Desmarestia* spp., *Dictyopteris* spp. and *Spatoglossum* spp.) and some other delicate subtidal taxa (e.g., *Dictyota*, *Sporochnus*, etc.) should be transported in containers with a relatively large volume of sea water and separated from other algae, or the thalli will deteriorate very rapidly, which damages the other algae.

For unialgal cultures, sterilized grass vessels or plastic Petri dishes are commonly used. Suitable sizes depend on the targeted algal sizes, but 55–90 mm (diameter) dishes and 200–300 mL vessels are commonly used. Aeration is usually not required for the culture of this size, but is often used for larger cultures.

Unialgal cultures of marine brown algae grow readily in culture media of sea water enriched with basic autotroph nutrients. The most widely used formula is

Provasoli's ES (PES) medium and modified Provasoli's ES medium (PESI), omitting vitamins but adding iodine (Tatewaki 1966). Related literature and recipes of the culture media are listed in Harrison and Berges (2005).

Isolation from vegetative thalli: Most brown algal species have high potential for regeneration and totipotency, so that unialgal cultures can be established for many species from vegetative tissues. However, in practice epiphytic algae and cyanobacteria tend to grow faster than the desired species and overwhelm it. Therefore, isolation by this technique is effective only for minute taxa and some larger ones with apical meristematic growth (e.g., Dictyotales, Sphacelariales, Discosporangiales). For those species, a razor blade may be used to first cut out a small fragment including the apical cell(s) into a Petri dish filled with sterilized seawater. Then, under a dissecting microscope, smaller pieces of tissue including intact apical cell(s) are cut out and transferred into individual wells of a multiwell plate or individual Petri dishes (or test tubes) filled with culture medium, using a clean fine forceps (sterilized by wiping with 70% ethanol) or sterilized capillary pipettes. GeO_2 and antibiotics can be used to eliminate diatom and cyanobacteria contamination, respectively. After 1–2 weeks of culture in an illuminated chamber of suitable temperature, unialgal isolates can be selected using an inverted microscope to carefully inspect each isolate for contaminants.

Isolation from swimming zoids: Many species release zoids (zoospores and gametes) vigorously and synchronously 1 or 2 days after collection, rather than immediately after, if specimens are properly stored in cool and dark conditions. They tend to release zoids soon after reimmersion in seawater, stimulated by temperature rise and illumination. For zoid isolation, place a small fragment of fertile tissue in a small Petri dish, depression slide, or watch glass filled with sterilized seawater and examine the zoid-release under dissecting microscope. When release starts, observe any phototactic behavior of the zoids under a dissecting microscope to determine the orientation of the taxis and isolate clean zoids using a fine pipette into new Petri dish filled with sterile seawater culture medium. Wynne's hanging drop method (Chapman 1973) is useful when mass release of zoids fails and the isolation of individual reproductive structures is precluded. Alternatively, mature reproductive organs (unilocular zoidangia, plurilocular gametangia, etc.) may be cut out from the squashed or fragmented tissues including them and individually isolated and precultured until new offspring (germlings) from them become available. Then the germlings may be reisolated into individual Petri dishes to establish unialgal cultures.

Axenic culture: For axenic cultures, thalli should be cleaned thoroughly using sterile seawater. Guillard's antibiotic mixture, which consists of penicillin G 124.5 mg per liter, streptomycin sulfate 50 mg per liter, and chloramphenicol 20 mg per liter made up in sterile sea water, has been used to purify isolates of laminarialean (Druehl and Hsiao 1969) and *Dictyosiphon* (Saga and Sakai 1982) gametophytes.

Routine sterility tests should be carried out. For this purpose, Fries (1977) recommended transferring pieces of algae into test tubes containing culture medium to which is added 2 g glucose, 2 g galactose, 1 g mannose, 1 g asparagine, 0.5 g yeast extract (Oxoid), and 0.5 g bacteriologic peptone per liter.

Evolutionary History

Fossil Record

The fossil evidence of the evolution of the Phaeophyta is very scanty. This is perhaps because of their generally soft-bodied habit, very limited occurrence of calcified taxa (e.g., *Padina* spp.), and relatively recent evolution compared with red and green algae. The oldest undoubted brown algal fossils are kelps from the Tertiary. Parker and Dawson (1965) described from the upper Miocene fossil kelp, *Julescranea grandicornis,* which is intermediate in appearance between *Pelagophycus* and *Nereocystis. Paleohalidrys, Cystoseirites, Cystoseira,* and *Paleocystophora* have been assigned to the Cystoseiraceae in the Fucales, described from Miocene deposits in California.

Sister Taxa and Divergence Time of Phaeophyceae

It is rather difficult to infer the origin of the brown algae or to clarify the sister relationships with other Phyla, because of the sparse fossil record and lack of known unicellular taxa. The available evidence consists of molecular phylogenetic analyses, and the compositions of accessory photosynthetic pigments (i.e., carotenoids) and cell walls (e.g., presence/absence of cellulose and alginates), fine structure of flagellar apparatus (e.g., presence/absence of transitional helix and rhizoplast), and presence and absence of periplasmic opaque substances. Based on this information, Schizocladiophyceae is considered to be the closest sister group of Phaeophyceae, and both group with Phaeothamniophyceae and Xanthophyceae (Bailey and Andersen 1998; Bailey et al. 1998; Kawai et al. 2003).

The divergence time of Phaeophyceae from Schizocladiphyceae is estimated to be ca. 260 Ma in the Permian Period, based on molecular phylogeny calibrated based on the fossil records of diatoms and brown algae (i.e., *Padina* and *Julescranea*).

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Raphidophyceae (Raphidophyta)

9

Takeo Horiguchi

Abstract

The Raphidophyceae are flagellated unicellular algae that live in diverse marine, brackish, and freshwater habitats. Ten genera are currently recognized: *Gonyostomum*, *Merotricha*, *Vacuolaria*, *Chattonella*, *Chlorinimonas*, *Fibrocapsa*, *Haramonas*, *Heterosigma, Psammamonas*, and *Viridilobus* (the first three are freshwater representatives). They are wall-less heterokonts, i.e., the forward flagellum possesses tubular mastigonemes, and both flagella arise from a shallow pit at or near the apex of the cell. All known raphidophytes are photosynthetic and bear multiple plastids containing chlorophylls *a* and c_1 and/or c_2. With the exception of *Chlorinimonas sublosa*, marine species possess fucoxanthin as a major carotenoid, while freshwater representatives lack this pigment. Marine raphidophytes are widely recognized as ichthyotoxic organisms; species such as *Chattonella* spp., *Fibrocapsa japonica*, and *Heterosigma akashiwo* have been associated with finfish kills. Knowledge of the raphidophyte life cycle, cyst formation, and vertical migratory behavior is important for understanding mechanisms of bloom formation. Molecular phylogenetic analyses suggest that (1) the greenish colored freshwater species diverged from brownish colored marine raphidophytes, (2) all three species of the genus *Haramonas* and a species of *Psammamonas* are sand-dwelling and evolved from a marine planktonic ancestor by acquiring characters of benefit to benthic habitats, (3) *Chlorinimonas* is also sand-dwelling, a characteristic that must have been acquired independently from *Haramonas* and *Psammamonas*, and (4) basal lineages of the Raphidophyceae, *Fibrocapsa, Haramonas*, and *Psammamonas*, possess unique carotenoids such as fucoxanthinol (*F. japonica* and *P. australis*)

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and 19′-butanoyloxyfucoxanthin (*H. dimorpha*), but the significance of the presence of these pigments is currently unknown.

Keywords
Chattonella • *Fibrocapsa* • Flagellate • *Gonyostomum* • HAB • Heterokontophyta • *Heterosigma* • Ichthyotoxic • Raphidophyceae • Stramenopiles

Contents

Summary Classification

●**Raphidophyceae**
●●**Chattonellales**
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Introduction

General Characteristics

Members of the Raphidophyceae are flagellate unicellular algae. They are wall-less heterokonts: the forward flagellum (approximately the same length as the cell) bears tubular mastigonemes, and both flagella arise from a shallow pit at or near the apex of the cell (Heywood 1978b; Mignot 1976) (Fig. 1). They live as either motile or palmelloid individuals with a usual length of 10–80 μm. They bear multiple plastids containing chlorophylls *a* and c_1 and/or c_2. Marine species possess a xanthophyll, fucoxanthin, as a major carotenoid, although freshwater representatives lack this

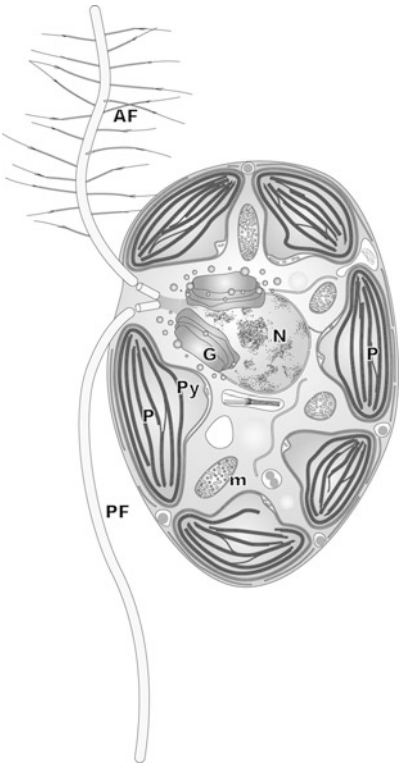

Fig. 1 Schematic illustration of a longitudinal section through a typical marine raphydophyte (*Heterosigma*). A nucleus is surrounded by Golgi body (*G*) and mitochondria (*m*). Plastids (*P*) are located in the periphery of the cell and each chloroplast possesses a projected pyrenoid (*Py*), which is traversed by several thylakoids. The cell possesses an anterior flagellum (*AF*) with tubular mastigonemes and a smooth posterior flagellum (*PF*) (Illustration by Dr. Takeshi Nakayama)

pigment. Sexual reproduction has been documented for a freshwater species (Cronberg 2005; Figueroa and Rengefors 2006). Cyst formation involving alternation of haploid and diploid phases without apparent gamete conjugation in marine raphidophytes has been reported (Yamaguchi and Imai 1994), although another type of cyst formation involving sexual fusion has also been suggested (Demura et al. 2012). The Raphidophyceae is a small group of organisms, with only ten genera (three freshwater and seven marine representatives) currently recognized, all of which are photosynthetic.

Occurrence

Freshwater raphidophyte species usually occur in acidic or neutral pH habitats where the vegetation is abundant. They occur as plankton, among aquatic plants, or adjacent to the mud. Marine species are found in coastal waters, embayments, or in the brackish waters of estuaries. Members of the marine genera *Chlorinimonas*, *Haramonas*, and *Psammamonas* are sand-dwelling (Horiguchi 1996; Yamaguchi et al. 2010; Grant et al. 2013). Although some raphidophyte species are rather rare, e.g., *Haramonas* spp., members of the genera *Gonyostomum*, *Vacuolaria*,

Chattonella, *Heterosigma*, and *Fibrocapsa* are often locally abundant and widely distributed.

Raphidophyte cultures can be obtained from the following sources: Commonwealth Scientific and Industrial Research Organization (CSIRO), The Australian National Algae Culture Collection (ANACC) (Australia), The National Institute for Environmental Studies (NIES) (Japan), The Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA) (USA), and the Culture Collection of Algae at the University of Texas at Austin (UTEX) (USA). For further information regarding algal collections of the world, see Kasai et al. (2005).

History of Knowledge

Ten genera are currently recognized in the class Raphidophyceae. *Gonyostomum* (Diesing 1865), *Vacuolaria* (Cienkowsky 1870), and *Merotricha* (Mereschkowsky 1879), the three freshwater genera, were first described over a century ago. These genera were grouped into the Chloromonadida (Klebs 1892). Later, Biecheler (1936) recognized that the marine alga *Chattonella* also belongs to this group. Subsequently, the genus *Heterosigma* was established in 1968 by Hada (invalid, no designation of type species) and was later validated by Hara and Chihara (1987). The genus *Fibrocapsa* was established based on material from Japan analyzed by Toriumi and Takano (1973) and the genus *Haramonas* was proposed later (Horiguchi 1996). More recently, the genera *Chlorinimonas* (Yamaguchi et al. 2010), *Viridilobus* (Demir-Hilton et al. 2012), and *Psammamonas* (Grant et al. 2013) have been established.

In the recent phycological literature, these protists are frequently treated as a class of algae, the Raphidophyceae (Heywood 1983; Silva 1980). They have been termed "Chloromonadophyceae" by phycologists and "Chloromonadida" by protozoologists, but these terms are inappropriate since the genus *Chloromonas* does not belong to the Raphidophyceae. Loeblich and Loeblich (1978) include within the family Vacuolariaceae the following genera regarded as valid by most phycologists: *Chattonella*, *Gonyostomum*, *Merotricha*, and *Vacuolaria.* Also included are *Trentonia* and *Swirenkoimonas.* Too little is known to include *Swirenkoimonas* with the raphidophytes. *Trentonia* is probably synonymous with *Vacuolaria* (Fott 1968; Heywood 1983). However, the most controversial aspect of this scheme is treating *Fibrocapsa*, *Heterosigma*, *Olisthodiscus*, and *Hornellia* as synonymous with *Chattonella.* The genus *Hornellia* is probably synonymous with *Chattonella*, since the description of *Hornellia marina* (Subrahmanyan 1954) resembles that of *Chattonella subsalsa* (Biecheler 1936). As noted by Heywood (1990) in the original volume of this handbook, Loeblich and Fine (1977) argued that *Fibrocapsa japonica* (Toriumi and Takano 1973) should be named *Chattonella japonica*, that *Heterosigma inlandica* (Hada 1968) should be named *Chattonella inlandica*, and that *Olisthodiscus luteus* (Carter 1937) should be named *Chattonella luteus.* Heywood (1990) discussed the taxonomic confusion concerning marine raphidophytes at both generic and species ranks. Although Loeblich and Fine

(1977) argued that *Chattonella*, *Heterosigma*, *Fibrocapsa*, and *Olisthodiscus* are congeneric, most researchers now consider these genera to be autonomous (*Olisthodiscus* may in fact not be a raphidophyte at all; see below). In the recent literature, based on molecular phylogenetic study, Yamaguchi et al. (2010) proposed that the class Raphidophyceae should consist of a single order, Chattonellales, which contains a single family, Vacuolariaceae.

Hara and coworkers (Hara and Chihara 1982; Hara et al. 1994) recognized seven species of *Chattonella*, i.e., *C. subsalsa*, *C. antiqua*, *C. marina*, *C. ovata*, *C. minima*, *C. globosa*, and *C. verruculosa*. One original member of the genus *Chattonella*, *C. verruculosa*, is now regarded to be a member of the class Dictyochophyceae and has been transferred to the new genus *Pseudochattonella* (Hosoi-Tanabe et al. 2007). Another new genus, *Verrucophora* was established for a species, formally referred to as *Chattonela* cf. *verruculosa* from the North Sea and the Skagerrak (Edvardsen et al. 2007). The type of species of *Verrucophora*, *V. farcimen*, is closely related to *P. verruculosa*, but not identical. Although Edvardsen et al. (2007) transferred *C. verruculsa* to a new genus, a new nomenclatural combination proposed by Hosoi-Tanabe et al. (2007) seems to have priority. Similarly, another member of the genus *Chattonella*, *C. globosa*, was found to be a member of the Dictyochophyceae and was transferred to a new genus, *Vicicitus* (Chang et al. 2012).

Among true *Chattonella* species, there have also been taxonomic problems. *Chattonella antiqua*, *C. marina*, and *C. ovata* have been distinguished from each other solely based on their morphological characters. Recent genetic analyses, however, revealed that these three species are almost identical (e.g., Bowers et al. 2006; Kamikawa et al. 2007). After careful examination of both morphology and genetic diversity, Demura et al. (2009) concluded that these three species should not be treated as independent species. However, they also found that there were distinct tendencies toward specific differentiation with regard to genetic divergence, morphology, and ecophysiological differences. Therefore, they concluded that these three taxa occupy an intermediate stage between a single, unified species and three distinct and independent species; they proposed to treat them as varieties within a species, i.e., *C. marina* var. *marina*, *C. marina* var. *antiqua*, and *C. marina* var. *obata*. Klöpper et al. (2013) demonstrated that the strains identified as *C. subsalsa* in fact consist of two different species, and the strains from the western Adriatic coast (Mediterranean Sea) more closely match the original species description. Using microsatellite markers, Demura et al. (2014) attempted to reveal putative sources of populations of *C. marina* var. *antiqua* and *C. marina* var. *marina* along Japanese coasts.

A toxic marine species, *Heterosigma akashiwo*, has been the focus of extensive ecological, biochemical, physiological, and molecular studies. Readers are advised to note that in the 1970s and 1980s, this alga was erroneously identified as *Olisthodiscus luteus*, until Hara and Chihara (1987) sorted out the taxonomic confusion. Not like *Heterosigma*, true *Olisthodiscus luteus* (Carter 1937) is benthic and swims without rotating movement. Although often assigned to the class Raphidophyceae, true *Olisthodiscus luteus* is different from members of the class

in many ultrastructural features (Hara et al. 1985; Inouye et al. 1992). Furthermore, preliminary molecular phylogenetic study indicates that *O. luteus* is not a member of the Raphidophyceae (unpublished data by H. Yamaguchi, Yamaguchi et al. 2008). In addition to confusion regarding *O. luteus*/*H. akashiwo*, there has been debate as to which specific epithet should be used. It is now generally accepted that there is only one species in the genus *Heterosigma* and the species name *H. akashiwo* is appropriate and valid (for details, see Hara and Chihara 1987).

Practical Importance

Freshwater raphidophytes are generally rare and, when present, often occur in low densities. However, *Gonyostomum semen* forms dense blooms and affects lakes used for recreation. The alga discharges mucilaginous strands upon contact, thereby covering bathers with a slimy layer causing itching and other allergic reactions (Cronberg et al. 1988; Figueroa and Rengefors 2006). Members of the marine genera *Chattonella*, *Fibrocapsa*, and *Heterosigma* are often locally abundant (Hollande and Enjumet 1956; Subrahmanyan 1954; Hallegraeff and Hara 1995) and are regarded as nuisance algae worldwide.

Marine raphidophytes often cause extensive negative impact on fisheries all over the world. One of the worst cases reported was the killing of >14 million yellowtail fish (*Seriola quinqueradiata*) by *Chattonella antiqua* in Harmina-nada, Seto Inland Sea, Japan, in 1972. This resulted in the loss of 71 billion yen and a loss of 6.3 billion yen was recorded in subsequent years (1977–1979) in the same area (Okaichi 1997). *C. marina* killed 1700 t of bluefin tuna (*Tunnus maccoyii*) (US $40 million loss) in South Australia (Hallegraeff et al. 1998). In New Zealand, significant mortality of Chinook salmon (NZ $17 million loss) caused by *Heterosigma* was documented (Chang et al. 1990).

Habitats and Ecology

Freshwater raphidophytes have been reported from North America (Drouet and Cohen 1935), South America (Skvortzov et al. 1969; Menezes and Bicudo 2010), Australia (Ling and Tyler 2000), Asia (Jao 1978), and Europe (Fott 1968; Kusber 2003; Cronberg 2005). Marine raphidophytes are known from the coasts of all continents except for the Antarctic.

Species of *Gonyostomum* have frequently been reported from the planktonic fraction or from the vicinity of aquatic plants in water of pH 3.2–7.0. *Gonyostomum latum* was found in water of pH 6.7–7.0 (Fott 1968). The most frequently occurring *Gonyostomum*, *G. semen*, has been reported in water of pH 4.4–6.2 (Drouet and Cohen 1935; Heywood 1980); most reports of its occurrence were from the warmer months of the year (e.g., April to October in the northern hemisphere). Since *G. semen* frequently lives in the immediate vicinity of *Sphagnum*, water squeezed from *Sphagnum* moss may provide a good source of this raphidophyte. *G. semen*

seems to have expanded its habitats to more nutrient-rich waters. Blooms of this species appear every summer in many lakes in southern Sweden, in large parts of Finland, Norway, France, and Czech Republic (Cronberg 2005). Recent studies, including genetic analyses, also show expansion of *G. semen* in Northern Europe (Lebret et al. 2013; Hagman et al. 2015). *Vacuolaria* species also occur with aquatic plants in fresh waters of acidic or neutral pH (Heywood 1983). *V. virescens*, the most frequently occurring species, has been reported from water of pH 4.0–8.3 (Graffius 1966), but it is usually found in neutral or slightly acidic conditions. *V. virescens*, reported from bogs, ponds, lakes, and mountain streams (Cienkowsky 1870; Graffius 1966; Poisson and Hollande 1943; Spencer 1971), occurs in the plankton near aquatic plants or in the layer of water adjacent to the bottom mud. *V. virescens*, tolerant of low temperatures, was found to be present in large numbers in a pond with patches of surface ice (Spencer 1971). *V. viridis* has been collected on only a few occasions from swamps and small ponds containing rich aquatic vegetation (Fott 1968). *Merotricha* (only a single species described, *M. bacillata*) has also been found in the plankton or in the vicinity of aquatic plants from bogs, reservoirs, ponds, and the mouth of a river (Graffius 1966; Mereschkowsky 1879; Palmer 1942; Skvortzov et al. 1969).

Chattonella subsalsa, first collected in southern France in organic-rich brackish water (Biecheler 1936), was also present in the port of Algiers, France (Hollande and Enjumet 1956) and in Delaware's Inland Bays, USA (Portune et al. 2009). *C. subsalsa* occurs during the late summer or early autumn in water rich in organic material, frequently at high densities (Biecheler 1936; Hollande and Enjumet 1956; Mignot 1976). *C. antiqua* has been observed from various parts of Japan and has also been found along the Dutch coast (Vrieling et al. 1995). *C. antiqua* was found to grow well at 25 °C, at salinities between 25 ‰ and 41 ‰ under light intensity above 0.04 ly min^{-1}. The pH (7.6–8.3) did not affect growth rate (Nakamura and Watanabe 1983). *C. antiqua* is known to exhibit characteristic diurnal vertical migration, i.e., the cells are concentrated near the surface during day time and near the bottom at night. The species can form red tides during summer, when the thermal stratification is striking and this vertical migration is thought to be advantageous over diatoms. The migratory ascent at daytime keeps them in the euphotic zone and the descent at night provides access to the nutrient-rich bottom waters (Watanabe et al. 1983; Imai and Yamaguchi 2012). Shikata et al. (2013) demonstrated that the blue light regulates diurnal vertical migration behavior in *C. antiqua*. *C. marina* has a wide distribution and has been found in India (Subrahmanyan 1954), Japan (Imai 1989), Hong Kong (Kai et al. 2006), Russia (Morozova and Orlova 2005), a Swedish fjord (Waite and Lindahl 2006), North America (Bowers et al. 2006), Mexico (Band-Schmidt et al. 2004), Australia (Hallegraeff et al. 1998), and New Zealand (Rhodes et al. 2001). *C. ovata* has been reported in Japan (Hara et al. 1994) and Hong Kong (Kai et al. 2006). *C. minima* was originally reported from Seto Inland Sea, Japan, and seems to have very limited distribution. Because of its ability to produce dormant resting cysts, *Chattonella* species seem to adapt well to the temperature regime in temperate seas such as the Seto Inland Sea of Japan where extensive blooms occur (Imai and Itoh 1987). For various aspects of the biology of *Chattonella* spp.,

including biological control of their blooms, see the comprehensive review by Imai and Yamaguchi (2012).

Heterosigma akashiwo also has a global distribution and occurs in subtropical or temperate, marine or brackish waters. Species occurrence includes Canada, Japan, New Zealand, North America, England, Norway, Peru, Portugal, Chile, Singapore, Korea, Ireland, Denmark, China, Spain, Thailand, Namibia, Australia, and Mexico (Ki and Han 2007 and references therein). The optimum pH for growth of *H. akashiwo* was described as 8.5–9.0 (Iwasaki and Sasada 1969). *H. akashiwo* grows well at a salinity range from 20 ‰ to 30 ‰ with maximum growth at 25 ‰ (Haque and Onoue 2002), but the organism can also tolerate low salinity such as <6 (Strom et al. 2013). During the summer, *H. akashiwo* is the dominant species in the phytoplankton of Narragansett Bay, Rhode Island. It grows to maximum densities from May to August when nitrogen concentration is low and phosphate concentration is close to its yearly maximum (Tomas 1979). Laboratory experiments indicate that at saturating and subsaturating nitrogen (N) concentrations, N uptake preference is as follows: $NH_4^+ > NO_3^- >$ urea (Herndon and Cochlan 2007). The organism is known to exhibit characteristic diurnal vertical migration as described for *C. antiqua* (Watanabe et al. 1983; Yamochi and Abe 1984). The occurrence of cysts has been reported (Imai et al. 1993; Kim et al. 2015).

Fibrocapsa japonica, the only species in the genus, was originally isolated in seawater of pH 8.4 and at a temperature of 18.6 °C (Toriumi and Takano 1973). *F. japonica* has a worldwide distribution mainly in coastal warm and cold temperate regions and has been found in North America (Pacific and Atlantic sides), South America (Brazil), Europe (Atlantic and Mediterranean Sea), East Asia, Australia, and New Zealand (De Boer et al. 2005). A culture study using three *F. japonica* strains from different climate regions revealed the species is viable between 4 °C and 32 °C, thus indicating that the species is eurythermal. The species living in cold temperate regions, e.g., the German Wadden Sea, must experience temperatures below 4 °C, and the presence of a resting stage is expected to survive in this region (De Boer et al. 2005).

Three species in the genus *Haramonas* have been described. *H. dimorpha* was found in the bottom sand (mud) of a tropical mangrove river in northeast Australia (Horiguchi 1996) and later it was found in sand samples from Okinawa, subtropical Japan (Horiguchi, unpublished observation). A relatively localized bloom of *H. dimorpha* on the sand surface below the low tide mark on an Australian beach was noted (Chiovitti et al. 2006). *H. viridis* is a cold temperate species described from island of Sylt (eastern North Sea), Germany (Horiguchi and Hoppenrath 2003). The third species, *H. pauciplastida* was found in the beach sand of Vancouver Island, Canada (Yamaguchi et al. 2008). This genus is thus distributed from tropical to cold temperate regions. Another characteristic of *Haramonas* is having dimorphic phases in a life cycle, i.e., spherical nonmotile cells alternate with elongated motile cells (Fig. 2a, b).

The genus *Chlorinimonas* presently contains only one species, *C. sublosa*, which was discovered in sand samples of temperate regions in Japan. In culture, *C. sublosa* stays at the bottom of the culture vessel and does not behave like “typical” plankton.

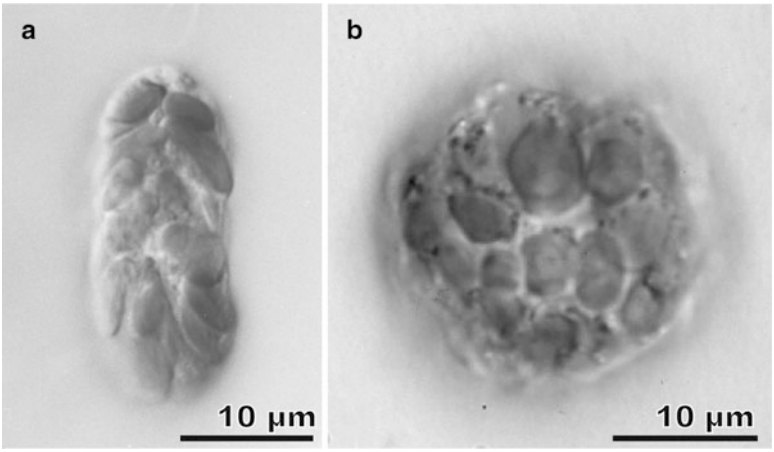

Fig. 2 LM photographs of the marine raphidophyte *Haramonas dimorpha*. (**a**) Typical motile cell. (**b**) Nonmotile spherical cell

The genus *Viridilobus* contains a single species, *V. marinus*, which can form dense blooms in Delaware's Inland Bays in the United States and can even grow when the salinity is almost zero (Demir-Hilton et al. 2012). The genus *Psasmmosa* also consists of a single species, *P. australis*, which is sand-dwelling and possesses two different morphological phases in a cell cycle. It can also produce unique "rafts," formed from 2 to 30 or more cells. The amoeboid movement of cells was also noted (Grant et al. 2013).

Although members of the Raphidophyceae are photosynthetic, mixotrophy, ingestion of bacteria in particular, was observed in *Heterosigma akashiwo* and in *Chattonella ovate*, *C. subsalsa*, and *Fibrocapsa japonica* (see Jeong 2011 and references therein).

Characterization and Recognition

Cell Structure

Raphidophyte cells vary from ovoid or pyriform to approximately spherical in shape; some species are flattened dorsiventrally and bear a furrow on the ventral surface. Biochemical, ultrastructural, and molecular information suggests that raphidophytes belong to the Heterokontophyta (photosynthetic stramenopiles) (e.g., Ali et al. 2002; Horn et al. 2007). The anterior flagellum beats rapidly and is responsible for the forward movement of the cell. The other flagellum moves infrequently and lacks tubular mastigonemes; it trails posteriorly over the ventral surface of the cell.

Plastids of freshwater species are usually bright green in color, while marine representatives are yellowish brown, although there are a few exceptions.

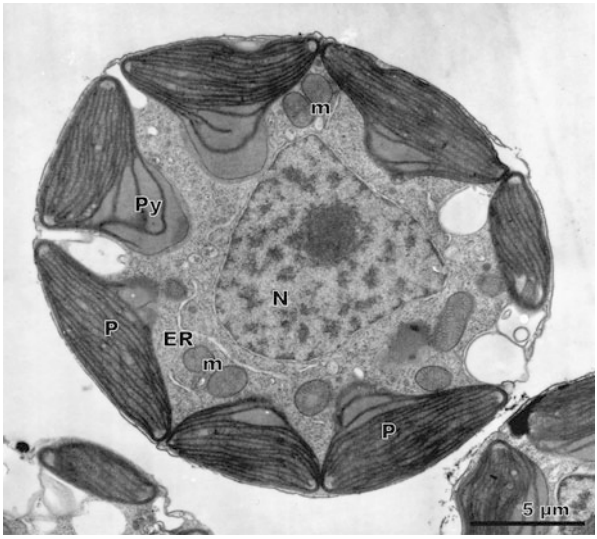

Fig. 3 TEM cross section through the cell of a marine raphidophyte (*Heterosigma akashiwo*) showing the general arrangement of organelles. *ER* endoplasmic reticulum, *N* nucleus, *m* mitochondria, *P* plastid, *Py* pyrenoid (Photograph courtesy of Dr. Yoshiaki Hara)

Chlorophylls *a* and c_1 and/or c_2 are present. The carotenoid pigments of freshwater raphidophytes are β, β-carotene, diadinooxanthin, heteroxanthin, and vaucheriaxanthin (Bjørnland and Liaaen-Jensen 1989). Fucoxanthin has been identified in all marine genera as a major carotenoid (Bjørnland and Liaaen-Jensen 1989), except for one species, *Chlorinimonas sublosa*. In the latter species, like freshwater representatives, no fucoxanthin was detected and diadinoxanthin was identified as a major xanthophyll (Yamaguchi et al. 2010). Distribution of minor carotenoids among marine raphidophytes is variable (Mostaert et al. 1998). Multiple plastids are present in the outer region of the cell between the plasmalemma and the layer of cytoplasm surrounding the nucleus (=exoplasm) (Figs. 1, 2a, b, and 3). Plastids are usually planoconvex or discoid in shape and may attain sizes up to 3 µm wide by 5 µm long. Lamellae, consisting of three thylakoids, extend approximately parallel to the longitudinal axis of the plastid (Figs. 1, 3, and 4a). A girdle band is present in *Gonyostomum*, *Vacuolaria*, and *Heterosigma* (Fig. 1) (Heywood 1980; Hara and Chihara 1987) but typical girdle lamellae appear to be absent in *Chattonella*, *Fibrocapsa*, *Haramonas*, and *Chlorinimonas* (Mignot 1967, 1976; Hara and Chihara 1985; Yamaguchi et al. 2008, 2010). Pyrenoids, present in the plastids of most marine species (Figs. 1, 3, and 4a), have not yet been reported in freshwater species (Heywood 1980; Loeblich and Fine 1977; Mignot 1967, 1976; Hara and Chihara 1982, 1985, 1987; Horiguchi 1996; Horiguchi and Hoppenrath 2003; Yamaguchi et al. 2008, 2010; Demir-Hilton et al. 2012). However, some species of freshwater representatives, e.g., *G. depressum*, may in fact possess a pyrenoid (Fig. 4a, Yoshiaki Hara and Hanae Takahira, personal communication 2013). The reserve food material is suggested to be 1, 3-β-D-glucan, which is comparable to chrysolaminarin of diatoms (Chiovitti et al. 2006). No eyespots have been reported.

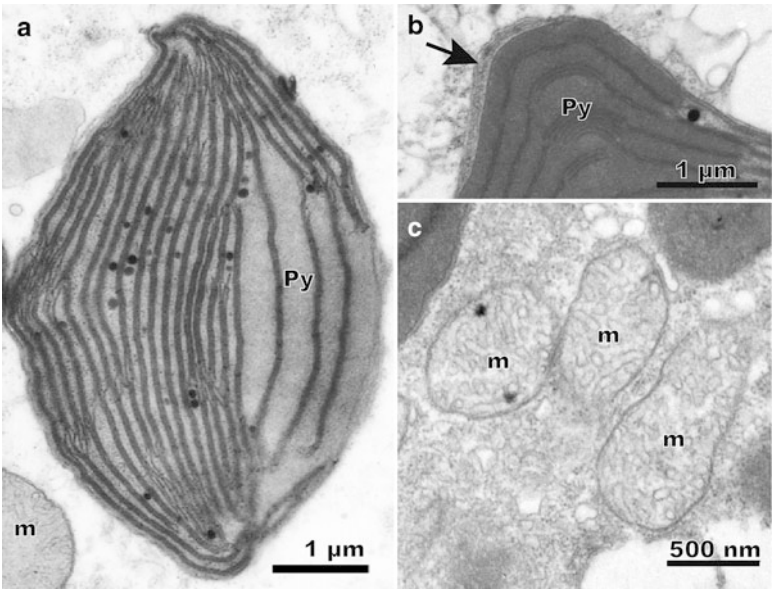

Fig. 4 TEM photographs of selected raphidophytes. (**a**) Close-up of plastid of a freshwater raphidophyte, *Gonyostomum depressum*, showing the presence of a pyrenoid (*Py*). *M* mitochondria (Photograph courtesy of Dr. Yoshiaki Hara and Ms. Hanae Takahira) (**b**) Close-up of the pyrenoid region of *Haramonas viridis*, showing the periplastidal network (*arrow*). (**c**) Close-up of raphidophyte mitochondria (*m*) (*Haramonas dimorpha*), which contains tubular cristae

The plastids of raphidophytes are of secondary endosymbiotic origin as in other heterokont algae. The plastid is surrounded by four membranes: the inner and outer envelope membranes (IEM and OEM), the periplastid membrane (PPM), and the outermost membrane, referred to as the chloroplast endoplasmic reticulum (CER) (Ishida et al. 2000). Small vesicles, termed the periplastidal network (Hibberd 1976), are present between the OEM and PPM at the surface of the projected pyrenoid (Figs. 1 and 4b). The process of plastid division in *Heterosigma akashiwo* was investigated, and it was revealed that an electron-opaque annular structure (plastid-dividing ring or PD ring) girdles the constricting isthmus of the dividing plastids. The inner membranes (IEM and OEM) constrict in advance of the outer two membranes, and the PD ring was observed at the outer surface of the inner pair (Hashimoto 1997). The membrane topology and plastid protein targeting system of *H. akashiwo* was investigated as a model system of organisms with multiple plastids of secondary origin (Ishida et al. 2000). The CER membrane is connected to the endoplasmic reticulum (ER) and in turn, the ER membrane is continuous with outer nuclear envelope. Therefore, the chloroplasts (plastids) of raphidophytes are located within the ER lumen, as in single-plastid containing heterokonts (Ishida et al. 2000). Using an in vitro system, Ishida et al. (2000) hypothesized that nuclear-encoded plastid protein precursors that have been cotranslationally transported into the ER lumen are sorted in the ER and transported to the plastid through the ER lumen

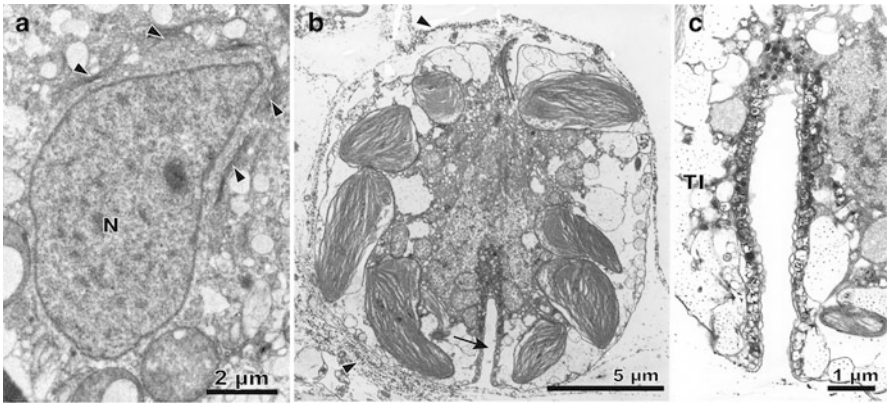

Fig. 5 TEM photographs of the marine raphidophyte *Haramonas* spp. (**a**) Close-up showing a tear-drop shaped nucleus (*N*) and surrounding Golgi bodies (*arrowhead*) in *H. viridis*. (**b**) TEM longitudinal section through a nonmotile cell of *H. dimorpha*, showing the "tubular invagination" (*arrow*). Note that the cell is surrounded by mucilaginous material (*arrowheads*). (**c**) Close-up of the tubular invagination (*TI*) of *H. dimorpha* (Images shown with permission from Phycological Research, Wiley and Sons)

(Ishida et al. 2000; Ishida 2005). *H. akashiwo* has been also used to study various aspects of plastid molecular biology (e.g., Duplessis et al. 2007).

Raphidophyte mitochondria, which possess tubular cristae, are especially numerous in the layer of cytoplasm surrounding the nucleus (Fig. 4c), although some occur in the more peripheral regions of the cell. A distinctive feature is the presence of a large Golgi network over the anterior surface of the nucleus (Figs. 1 and 5a) (Heywood 1980, 1990; Mignot 1967, 1976). A contractile vacuole, which may reach up to 10 µm in diameter, occurs between the Golgi and the kinetosomes in freshwater genera but not marine genera (Heywood 1983; Mignot 1967, 1976; Toriumi and Takano 1973; Hara and Chihara 1982, 1985, 1987; Horiguchi 1996; Horiguchi and Hoppenrath 2003; Yamaguchi et al. 2008, 2010; Demir-Hilton et al. 2012).

Neither scales nor cell walls are present in the raphidophytes, but extracellular material may be produced by extrusome organelles, mucocysts, and trichocysts, which occur in many species. Trichocysts can expel their mucilaginous contents considerable distances (Drouet and Cohen 1935; Toriumi and Takano 1973). Oboe-shaped mucocysts are a characteristic feature of *Chattonella subsalsa* (Biecheler 1936; Klöpper et al. 2013). Material produced by the mucocysts may surround a motile individual with mucilage so that it becomes palmelloid. Members of the genus *Haramonas* produce copious amounts of mucilage (Fig. 5b) (Horiguchi 1996; Horiguchi and Hoppenrath 2003; Yamaguchi et al. 2008).

An unusual structure, the tubular invagination, has been found in all three species of the genus *Haramonas*. The structure can be observed throughout the cell cycle. It opens directly to the outside of the cell (Fig. 5b, c) and appears hollow and devoid of any kind of material. The plasmalemma of the tubular invagination is supported by a

single layer of many underlying small, flattened vesicles, resembling the amphiesma of dinoflagellates (see ▶ Dinoflagellata). These vesicles are, in turn, surrounded by one or two layers of small spherical vesicles, which contain fibrous materials. The function of this structure is currently unknown (Horiguchi 1996; Horiguchi and Hoppenrath 2003; Yamaguchi et al. 2008).

The large nucleus (up to 20 μm in length) and chromosomes (1–12 μm in length at metaphase) have prompted several investigations of nuclear cytology (Heywood 1978a, 1980; Mignot 1967; Poisson and Hollande 1943). Interphase chromatin is often recognizable as fine threads. Chromosomes condense during mitosis and their chromatids become attached to opposite poles by kinetochore microtubules. Spindle microtubules, formed around the kinetosomes, enter the nucleus through gaps at the poles of the nuclear envelope at prophase. By metaphase the chromosomes have become aligned across the equator of the nucleus, and the one or more nucleoli have begun to disperse. Well-spread chromosome preparations from metaphase cells indicate that there are 97 ± 2 chromosomes in *V. virescens* and 65–75 chromosomes in *G. semen* (Heywood 1980). In V. *virescens*, a Golgi and contractile vacuole occur at each pole of the mitotic nucleus; this arrangement ensures their segregation to progeny cells. The original nuclear envelope remains intact over most of its surface until telophase; at this stage new nuclear envelope has begun to be assembled over much of the surface of the chromosome groups (Heywood 1978a). Light microscopic investigation suggests that nuclear envelope behavior is similar in other raphidophytes (Heywood 1978a). Little is known about the biochemistry of raphidophyte nuclei, but their nuclear DNA has been analyzed and found to have a guanine plus cytosine content of 35% in *G. semen* and 34% in *V. virescens* (Rae 1976). Nemoto et al. (1987) reported that light irradiation is necessary for nuclear DNA replication in *Chattonella antiqua* and that the timing of the replication is dependent upon only the timing of the onset of the last irradiation.

Flagella and Flagellar Apparatus

The raphidophytes possess two heterodynamic flagella. The anterior flagellum possesses tripartite tubular mastigonemes, while the posterior flagellum is smooth in surface (Fig. 1) (Karpov 2000). There is no transitional helix in the transition zone of the flagella (Hibberd 1979). Neither flagellar swelling nor flagellar autofluorescence has been detected in the raphidophyte algae (Kawai and Inouye 1989). Only a limited amount of information concerning flagellar apparatuses is available for the Raphidophyceae (Mignot 1967, 1976; Heywood 1980; Vesk and Moestrup 1987; Horiguchi and Hoppenrath 2003; Yamaguchi et al. 2008, 2010). The flagellar root system of *H. akashiwo* was described as comprising three roots, i.e., (1) the rhizoplast, a massive crossbanded fibrous root, which extends from near the proximal ends of both basal bodies to the anterior surface of the nucleus (Fig. 6a), (2) a compound microtubular root with a layered structure, associated with the anterior flagellum and extending the anterior surface, and (3) the rhizostyle, which passes between the two basal bodies leading anteriorly to a vesicle in the flagellar

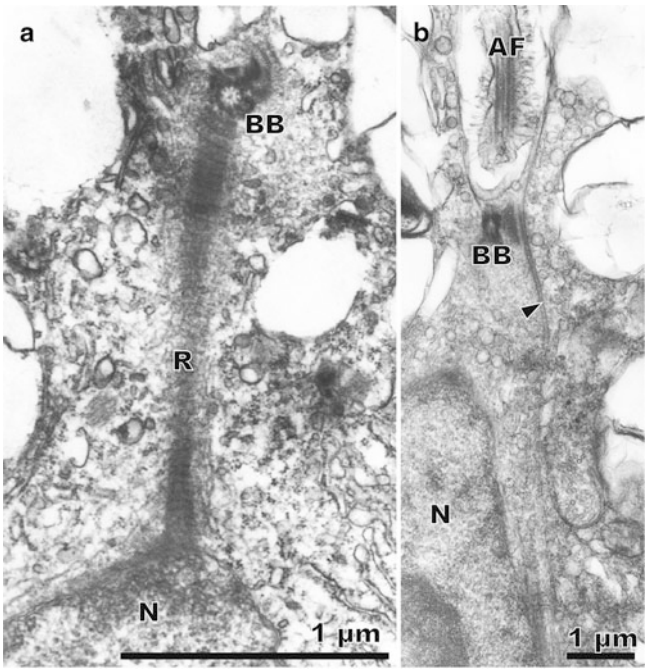

Fig. 6 TEM photographs of the marine raphidophyte *Haramonas viridis*. (**a**) Close-up showing part of the flagellar apparatus, with one of basal bodies (*BB*) and rhyzoplast (*R*) visible, the latter connecting the nucleus (*N*) and basal body. (**b**) Image shows the rhyzostyle (*arrowhead*) running toward the anterior and posterior parts of the cell. *AF* anterior flagellum, *BB* basal body (Images shown with permission from Phycological Research, Wiley and Sons)

groove region and following the nucleus posteriorly, terminating deep in the cytoplasm (Vesk and Moestrup 1987). All raphidophyte species so far examined possess a rhizoplast (Fig. 6a). The presence of a rhizostyle (Fig. 6b) also seems to be a common feature of the class (Vesk and Moestrup 1987; Horiguchi 1996; Horiguchi and Hoppenrath 2003). The presence of a layered structure associated with basal body of the anterior hairy flagellum was reported in *H. akashiwo* (superficially resembling the MLS (multilayered structure) of green plants but with a different structure) (Vesk and Moestrup 1987). This structure has been found in *Chattonella subsalsa* and *Vacuolaria virescens* and *Gonyostomum semen. Haramonas* spp. seem to have similar structures, but details have yet to be confirmed.

Toxicity

Members of marine raphidophytes are widely recognized as ichthyotoxic organisms. The following species have been associated with finfish kills: *Chattonella antiqua*, *C. marina*, *C. subsalsa*, *C. ovata*, *Fibrocapsa japonica*, and *Heterosigma akashiwo*.

The mechanism(s) of toxicity by these raphidophycean flagellates are not fully understood. Production of brevetoxin or brevetoxin-like compounds was reported for *C. antiqua*, *C. marina*, *F. japonica*, and *H. akashiwo* (Khan et al. 1997; Keppler et al. 2006). *C. antiqua*, *C. marina*, and *C. ovata* are known to produce reactive oxygen species (ROS) such as superoxide, hydroxyl radicals, and hydrogen peroxide, and the ROS generated by *Chattonella* spp. was thought to involve gill tissue injury (Ishimatsu et al. 1996; Hiroishi et al. 2005). It was demonstrated that *F. japonica* and *H. akashiwo* also generate superoxide and hydrogen peroxide (Oda et al. 1997). Marshall et al. (2002) demonstrated that *C. marina* cells contain high levels of potentially toxic polyunsaturated fatty acids such as eicosapentaenoic acid (EPA). Later they found that the presence of superoxide together with a low concentration of EPA accelerated fish mortality rates threefold and thus hypothesized that a synergistic effect between ROS and FFA accounts for the ichthyotoxicity of *C. marina* (Marshall et al. 2003). In a study using *C. marina*, *F. japonica*, and *H. akashiwo* (and a few toxic dinoflagellates), Dorantes-Aranda et al. (2015) demonstrated that ROS plays an important role only with *C. marina* and that ROS may also cause a synergistic effect with the lipids in the alga, producing other toxic compounds through lipid peroxidation. They also suggested that other unknown compounds are involved in ichthyotoxicity by *H. akashiwo*, *F. japonica*, and *C. marina*, some of which clearly have a lipid component (Dorantes-Aranda et al. 2015). *H. akashiwo* was known to have allelopathic interactions with a diatom species, *Skeletonema costatum* (Yamasaki et al. 2007).

Life Cycle and Cyst Formation

Members of the Raphidophyceae reproduce asexually by binary fission. Sexual reproduction was demonstrated in a freshwater species, *Gonyostomum semen* (Cronberg 2005; Figueroa and Rengefors 2006). The fusion of gametes was observed under stressed conditions, such as in old cultures or in medium with N or P depletion (Figueroa and Rengefors 2006). The gametes seem smaller and lighter in color than the vegetative cells (Cronberg 2005; Figueroa and Rengefors 2006). There are discrepancies between two reports concerning the sexual process. According to Cronberg (2005), meiosis, i.e., gamete formation, takes place within the cyst (resting cyst) and fused gametes become diploid vegetative motile cells, while Figueroa and Rengefors (2006) reported that the resting cyst is formed by fusion of gametes and a motile diploid vegetative motile cell is released from the resting cyst. The resting cyst is reported to be spherical, 27–39 μm in diameter, and with a few red droplets (Cronberg 2005; Figueroa and Rengefors 2006).

To understand the seasonal occurrence of noxious red tide raphidophytes such as *Chattonella* spp., *F. japonica*, and *H. akashiwo*, information on life cycles and cyst formation is extremely important. Subrahmanyan (1954) documented sexual reproduction and zygote formation in *C. marina* (as *Hornellia marina*), but the fate of the zygote was not observed. As for *Chattonella*, the cysts of this genus were first identified in the Seto Inland Sea, Japan (Imai and Itoh 1986), and it was subsequently

found that the cysts overwinter in the sediments and play an important role in initiating red tides the following summer (Imai and Itoh 1987). The cysts of *Chattonella* are hemispherical in shape with a diameter of 25–35 μm and usually attaching to a solid surface (Imai 1989). Cyst formation was induced by N depletion in the culture medium, and for germination, the cysts required a dormancy period (>4 months) at low temperature (11 °C) (Imai 1989).

By using microfluorometric analysis, Yamaguchi and Imai (1994) reported the life cycle of *Chattonella antiqua* and *C. marina*. The vegetative motile cells are thought to be diploid. The cyst was formed after meiosis, thus the cyst stage is haploid. The germinated small cell becomes a diploid vegetative motile cell, thus suggesting the occurrence of DNA diploidization without cell fusion (asexual diploidization) sometime after excystment (within 2 days) (Yamaguchi and Imai 1994; Imai and Yamaguchi 2012). On the other hand, Nakamura et al. (1990) observed fusion of "small cells (gametes)" and subsequent formation of the cyst (diploid), suggesting the presence of sexual reproduction. Using a microsatellite marker genotyping technique, Demura et al. (2012) confirmed that vegetative cells of 286 strains analyzed were heterozygous for at least some loci and thus diploid. The result suggests that most *Chattonella* strains undergo sexual reproduction. If asexual diplodization were the case, vegetative cells would be expected to be homozygous, even though diploid. The cysts of *F. japonica* were found to be similar in morphology to those of *Chattonella* but smaller (15–20 μm in diameter) and attaching to the solid substrata (Yoshimatsu 1987). Cyst formation in *H. akashiwo* was also reported (Itakura et al. 1996). The cysts, which are covered with sediment particles and can form a cyst cluster, are mostly spherical, about 10 μm in diameter, possessing a distinct wall and a diagnostic feature called the "structure underneath the lid of germination pore" or SLUG (Kim et al. 2015).

Cell Fixation and Molecular Identification of Species

Because of their delicate nature, it can be difficult to fix raphidophyte flagellates without their cell envelope collapsing by commonly used chemical fixatives. Katano et al. (2009) demonstrated that Hepes-buffered paraformaldehyde and glutaraldehyde works well for fixation of *Chattonella* species (and possibly other raphidophytes, too) and that these fixed cells are amenable to flow cytometry.

Members of the Raphidophyceae can easily change morphology, and it is sometimes difficult to identify species with certainty. For example, Imai (2000) reported that in *Chattonella antiqua* cultures, *C. marina*-like cells were occasionally produced. Precise identification of these harmful species is extremely important to fisheries management. Because some of these harmful species seem to have expanded their distribution rather recently, and toxicity can differ between strains, it is important to know the genetic relationships between strains and species located in geographically separated regions. Molecular methods for species identification

have been developed (Connell 2000, 2002; Tyrrell et al. 2001; Akase et al. 2004; Kai et al. 2006; Bowers et al. 2006; Hosoi-Tanabe et al. 2006; Ki and Han 2007; Kamikawa et al. 2007), and microsatellite markers for identification of *Chattonella* spp. (Demura et al. 2007) and *Heterosigma akashiwo* (Nagai et al. 2006) have been developed.

Maintenance and Cultivation

Enrichment of raphidophytes from mixed natural samples has been achieved by phototaxis (Chapman and Haxo 1966; Mignot 1976; Spencer 1971). Clonal cultures have been obtained for most genera (e.g., Heywood 1973; Loeblich and Fine 1977), and in some instances axenic cultures have been established (Cattolico et al. 1976; Iwasaki and Sasada 1969). Raphidophyte cells are usually sufficiently large and distinctive to be distinguished from other protists under a dissecting or inverted microscope and picked out by a micropipette to establish clonal cultures.

Media and conditions for culturing the freshwater species *Gonyostomum semen* and *Vacuolaria virescens* have been described (Chapman and Haxo 1966; Guillard and Lorenzen 1972; Heywood 1973; Spencer 1971). A series of culturing experiments on raphidophytes was reported by Heywood (1973). The medium used in these studies (Table 1) gave satisfactory growth at 22 ± 1 °C when the cultures were aerated with 4% CO_2 in air and were illuminated by Ecko brand 30 W daylight fluorescent tubes at a light intensity of 210 fc. Cultures were maintained in alternating light and dark regimes or in continuous light; under a continuous light regime, a doubling time of 70.5 h was recorded (Heywood 1973). Subsequently, a completely synthetic medium that promoted more rapid growth was developed (Table 2) which allowed a doubling time of 46.0 h at 24 ± 1 °C under continuous light.

For the culturing of marine species, various types of media have been utilized; most such species are easy to maintain in culture. The widely used media include Provasoli's enriched seawater (Provasoli 1968), f/2 culture medium (Guillard 1975), and modified SWM3 medium (Chen et al. 1969; Yamasaki et al. 2007).

Table 1 Composition of GSP medium containing soil and peat extract

KNO_3	100 mg
K_2HPO_4	10 mg
$MgSO_4$-$7H_2O$	10 mg
Ferric citrate	1 mg
Citric acid	1 mg
Soil extract	100 ml
Peat extract	100 ml
Distilled water	800 ml

From Heywood (1990)
pH adjusted between 5.2 and 6.5

Table 2 Composition of raphidophyte medium (in milligrams per liter)

KNO_3	90
K_2HPO_4	29
$MgSO_4{\cdot}7H_2O$	89
NH_4CL	20
$ZnSO_4{\cdot}7H_2O$	20
$CaCO_3$	8
H_3BO_3	1
$MnSO_4{\cdot}4H_2O$	6
$FeSO_4{\cdot}7H_2O$	4
$Na_2MO_42H_2O$	2
$CoSO_45H_2O$	2
$CuSO_45H_2O$	0.1
EDTA	50
Biotin	1
Thiamine	1
Vitamin B_{12}	0.01

From Heywood (1990)
For *Vacuolaria virescens* the pH was adjusted between 6.3 and 6.5
For *Gonyostomum semen* the pH was adjusted between 5.5 and 5.8

Evolutionary History

There is presently no raphidophyte fossil record. Molecular phylogenetic analyses clearly indicate that members of the Raphidophyceae belong to the division Heterokontophyta (autotrophic stramenopiles) (Potter et al. 1997; Ali et al. 2002; Horn et al. 2007). This phylogenetic placement is justified particularly well by the ultrastructure of their flagella, i.e., an anterior flagellum with tubular mastigonemes. However, the exact phylogenetic affinities of the Raphidophyceae to other members of the Heterokontophyta have not been elucidated.

Yamaguchi et al. (2010), Demir-Hilton et al. (2012), and Grant et al. (2013) published phylogenetic trees of the Raphidophyceae based on the SSU rRNA gene, which has been sequenced from representatives of most raphidophyte genera. Figure 7 summarizes the phylogenetic relationships between genera within the class. The genus *Fibrocapsa* appears to have diverged first within the lineage. The three species of *Haramonas* together with *Psammamonas australis* formed a robust clade as the next deepest diverging lineage, followed by a clade containing the three freshwater genera. Sister to the freshwater raphidophyte clade, a clade containing the marine genera *Chlorinimonas*, *Heterosigma*, and *Chattonella* is resolved. The tree allows some insights into the evolution of raphidophycean algae to be inferred. First, the greenish colored freshwater species diverged from brownish colored marine raphidophytes (Figueroa and Rengefors 2006; Yamaguchi et al. 2010). It is highly likely that the freshwater species are derived from a marine raphidophyte, and loss of fucoxanthin and gain of diadinooxanthin, heteroxanthin, and vaucheriaxanthin

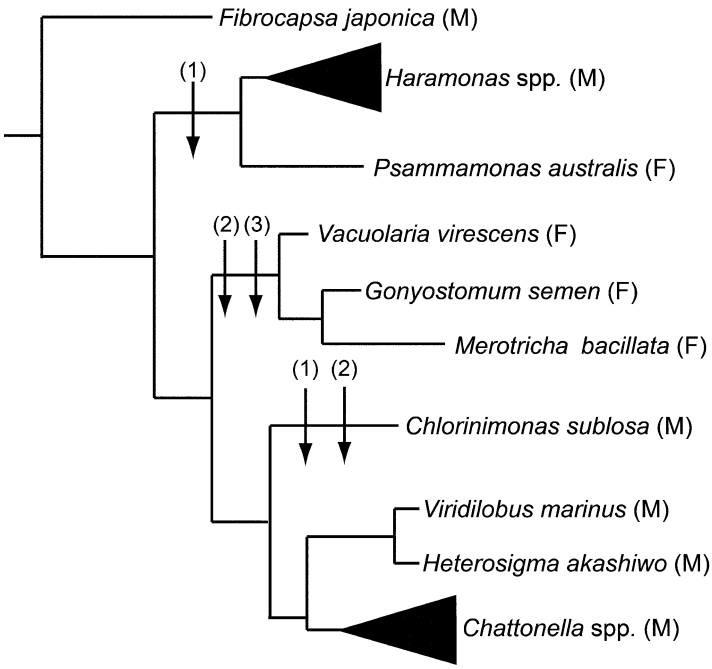

Fig. 7 Schematic diagram depicting the evolutionary relationships between raphidophyte genera based on SSU rDNA phylogenies (see text). (*M*) marine species, (*F*) freshwater species. (*1*) Indicates gain of sand-dwelling habit. (*2*) Indicates loss of fucoxanthin and gain of diadinoxanthin. (*3*) Indicates gain of freshwater-dwelling habit

(Bjørnland and Liaaen-Jensen 1989) took place only once in the lineage leading to freshwater raphidophytes. Second, although being a marine species, *Chlorinimonas sublosa* lacks fucoxanthin and possesses diadinoxanthin like in freshwater representatives. If this SSU-based tree topology is correct, replacement of photosynthetic pigments must have occurred independently in this lineage. Third, all three species of the genus *Haramonas* and a species of *Psammamonas* are sand-dwelling in habit. Since all other marine raphidophytes are planktonic, these three species appear to be derived from a single marine planktonic ancestor, and to have acquired characters that helped them adapted to a benthic habitat. Fourth, *Chlorinimonas* is also sand-dwelling a characteristic it presumably acquired independently from the *Haramonas*/*Psammamonas* lineage. Finally, basal lineages of the Raphidophyceae, viz., *Fibrocapsa*, *Haramonas*, and *Psammamonas*, possess unique carotenoids such as Fucoxanthinol (*F. japonica* and *P. australis*) and 19′-butanoyloxyfucoxanthin (*H. dimorpha*); the significance of the presence of these pigments is currently unknown (Mostaert et al. 1998; Grant et al. 2013). Molecular data from more raphidophyte taxa and additional genes will hopefully provide a more complete framework for understanding the evolutionary history of this fascinating and important algal group.

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Chrysophyta

10

Jørgen Kristiansen and Pavel Škaloud

Abstract

The *chrysophytes* (more than 1,200 described species) are unicellular or colonial algae characterized by *heterokont* flagella and chloroplasts with *chlorophyll a* and *c,* and by their endogenous silicified *stomatocysts*. They occur mainly as phytoplankton in temperate freshwaters, and their distribution is ecologically determined, mainly by temperature and pH.

Cells are naked or in many cases surrounded by an envelope, e.g., of species-specific silica scales manufactured from the chloroplast ER and *Golgi* vesicles and transported to the cell membrane and extruded. Photoreceptor systems include a swelling on the short flagellum and a corresponding stigma in one of the chloroplasts. Photosynthesis results in *chrysolaminaran*. But in many species, e.g., in colorless species, organic compounds can be taken up from the water or by phagocytosis. Life history includes mitotic divisions and *encystment*. In many species, *sexuality* – cell fusion followed by *encystment* of the zygote – has been observed. Classification was traditionally based on morphological criteria, including ultrastructure, but in recent years molecular methods have resulted in profound changes in our concepts of relationships and evolution.

Keywords

Occurrence • Ecology • Cell construction • Life history • Cultivation • Classification • Phylogeny

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●●●Ochromonadales
●●●Paraphysomonadida
●●●Chrysosaccales
●●●Segregatales
●●●Apoikiida

Introduction

General Characteristics

The Phylum Chrysophyta is a group of golden-brown microscopic algae and related colorless forms, most of them flagellates (Fig. 1). About 1,200 species in about 112 genera (Kristiansen and Preisig 2001) have been estimated, but many more species will certainly be described. The classification of the phylum with the classes Chrysophyceae and Synurophyceae is shown in Table 1, but the latter class, erected in 1987, should now again be included in the Chrysophyceae due to several recently published molecular investigations (Takishita et al. 2009; Del Campo and Massana 2011; Yang et al. 2012). On the other hand, several taxa previously associated with

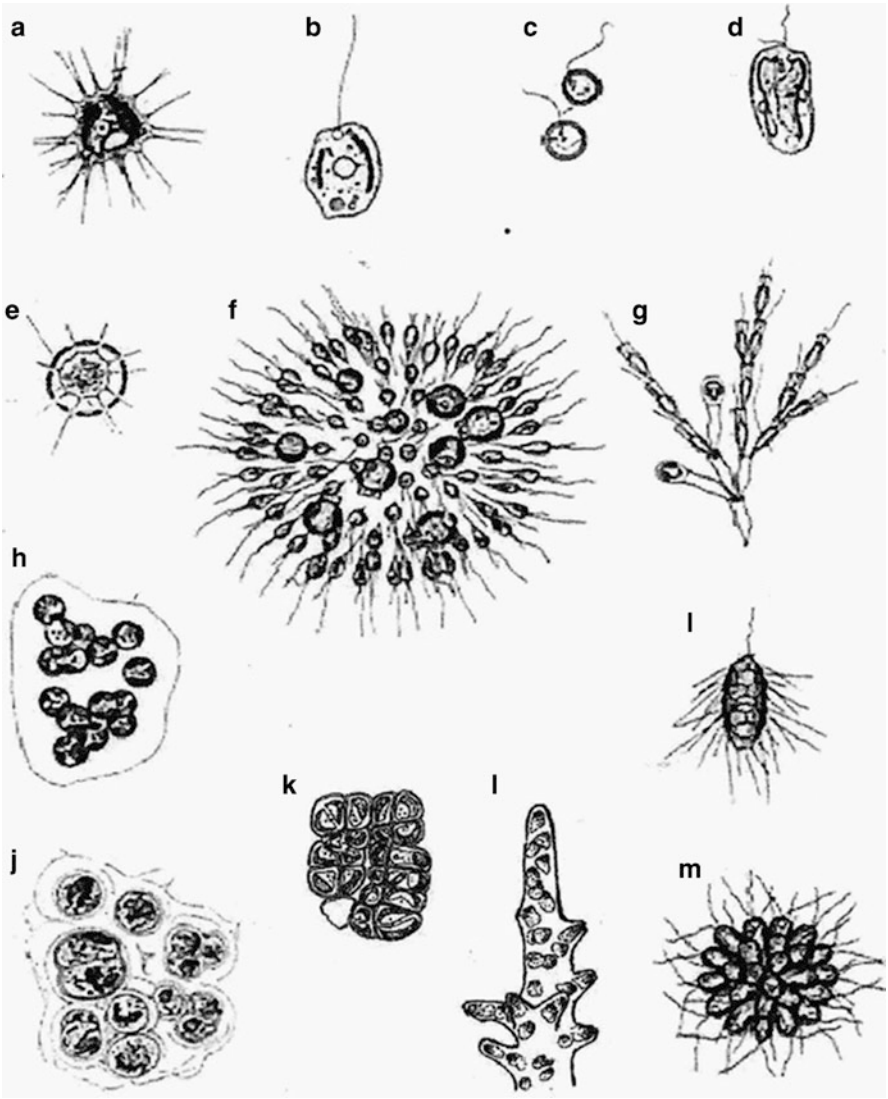

Fig. 1 Morphological diversity within the Chrysophyta. *A. Chrysamoeba. B. Chromulina, C. Chrysococcus, D. Ochromonas, E. Chrysothecopsis*, *F. Uroglena, G. Dinobryon, H. Chrysocapsa, I. Mallomonas, J. Gloeochrysis, K. Phaeoplaca, L.*, *Hydrurus, M. Synura* (After Kristiansen 2005, with alterations)

chrysophytes have been shown to belong to other evolutionary lineages and separated as independent classes: Phaeothamniophyceae, Dictyochophyceae, Pelagophyceae, and Bicosoecophyceae. Accordingly, they are not included here.

A survey of all the genera has been given in the "Encyclopedia of Chrysophyte Genera" (Kristiansen and Preisig 2001).

Table 1 Classification of the Chrysophytes

Traditional classification based on morphological data (after Preisig in Kristiansen and Preisig 2001, altered)	Updated classification based on molecular data
Class CHRYSOPHYCEAE Pascher 1914	**Class CHRYSOPHYCEAE** Pascher 1914
Order **CHROMULINALES** Pascher 1910	Order **OCHROMONADALES** Pascher 1910
Family **Chromulinaceae** Engl. 1897	Order **CHROMULINALES** Pascher 1910
Family **Dinobryaceae** Ehrenb. 1834	
Family **Paraphysomonadaceae** Preisig and Hibberd 1983	Order **PARAPHYSOMONADIDA** Scoble et Cavalier-Smith 2014
Family **Chrysolepidomonadaceae** M. C. Peters & R. A. Andersen 1993	
Family **Chrysamoebaceae** Poche 1913	
Family **Chrysocapsaceae** Pascher 1912	Order **CHRYSOSACCALES** Bourrelly 1957
Family **Chrysosphaeraceae** Pascher 1914	
Family **Chrysothallaceae** Hub.-Pest. 1941	
Order **HIBBERDIALES** R. A. Andersen 1989	Order **HIBBERDIALES** R. A. Andersen 1989
Family **Hibberdiaceae** R. A. Andersen 1989	
Family **Stylococcaceae** Lemmerm. 1899	
Order **HYDRURALES** Pascher 1931	Order **HYDRURALES** Pascher 1931
Family **Hydruraceae** Rostaf. 1881	
Class SYNUROPHYCEAE R. A. Andersen 1987	
Order **SYNURALES** R. A. Andersen 1987	Order **SYNURALES** R. A. Andersen 1987
Family **Mallomonadaceae** Diesing 1866	
Family **Synuraceae** Lemmerm. 1899	Order **SEGREGATALES** Boenigk et Grossmann 2016
	Order **APOIKIIDA** Boenigk et Grossmann 2016

Occurrence

The great majority of described species are found in plankton of fresh water. Some others are epibiotic or neustonic (i.e., attached to the water surface). A few species are benthic, e.g., found attached to the bottom in streaming mountain rivers. Relatively few known species occur as marine plankton. For example, species of the colorless genus *Paraphysomonas* may play an important role during the formation of sea ice (Ikävalko 2001). However, a recent culture-independent analysis of chrysophyte diversity revealed the existence of several unknown, marine clades (del Campo and Massana 2011) raising the question of the major predominance of chrysophytes in freshwater habitats.

Some chrysophyte species are very common and cosmopolitan, others are rare with peculiar disjunct distributions; however, our knowledge is still very fragmentary but rapidly increasing. Due to the rising number of investigations undertaken almost all over the world, the knowledge of the global distribution of the chrysophytes has increased considerably, especially for the silica-scaled forms, because of their reliable EM identification and documentation based on the silica scales. Thus a number of distribution types have been established (Kristiansen 2001). Of the ~250 species of silica-scaled chrysophytes, about 50 species are widely distributed or cosmopolitan. They have dispersed to suitable localities almost all over the world (Kristiansen 2000). The other species have more or less restricted distributions determined by climatic, historic, ecological, and dispersal factors. The following distribution types have been recognized: Northern temperate-subarctic-arctic species, species with bipolar distribution, and tropical species. A large group of species are endemic, having only been found within a restricted area. In fact, almost all new species start as endemic for the type locality, but most of them sooner or later will also be found in other localities and thus loose endemic status. In 2004, of the 172 described *Mallomonas* species, 69 were considered endemic (Kristiansen and Lind 2005).

The distribution of a species is due to dispersal, mainly of stomatocysts, by birds and by air. Thus the distribution pattern at a given time depends on several factors: dispersal capacity of the species, available vectors, suitable available habitats, and, perhaps most important, sufficient time (Kristiansen 2008). This is in contrast to the ubiquity hypothesis advocated mainly by Finlay and Clarke (1999) that all species are everywhere, only the environment determines the occurrence. This problem is still under discussion, and a sort of compromise has been offered by Řezáčová and Neustupa (2007). However, the ubiquity hypothesis has been contradicted, e.g., by studies in North America where quite similar neighboring water bodies had different floras of silica-scaled chrysophytes (Siver and Lott 2012b).

In addition, the rapidly increasing amount of molecular investigations has revealed the existence of cryptic lineages within the presumably cosmopolitan species, showing restricted distribution patterns. For example, the cosmopolitan *Synura petersenii* s.l. (Fig. 2) has been shown to consist of at least 10 well-defined species, some of them occurring in geographically separated areas (Boo et al. 2010; Kynčlová et al. 2010; Škaloud et al. 2012, 2014). Probably the most striking example is the distribution pattern of *S. hibernica* restricted to an extremely small biogeographic area of western Ireland (Škaloud et al. 2014).

Literature

Important identification works: The most comprehensive identification work today on freshwater chrysophytes is in the "Süsswasserflora von Mitteleuropa" by Starmach (1985) and Kristiansen and Preisig (2007), the latter based on electron microscopy of silica scales. In addition, there are regional floras from, e.g., British Isles and North America, where the chrysophytes have been treated by Kristiansen and Preisig (2011), Siver (2003), and Nicholls and Wujek (2003), respectively.

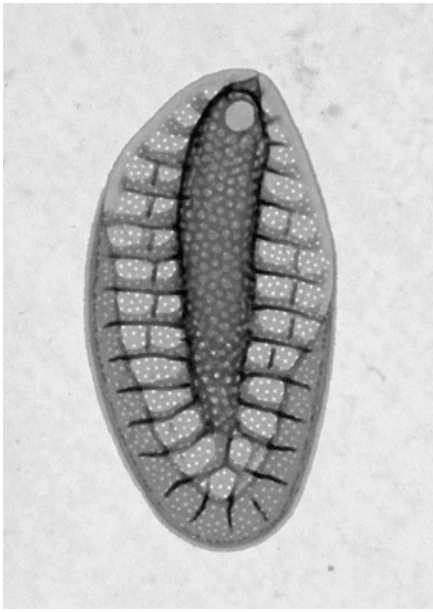

Fig. 2 Silica scale of *Synura petersenii*. × 20,000

A survey of all chrysophyte genera has been compiled by Kristiansen and Preisig (2001). A general account of chrysophytes and their biology has been given by Kristiansen (2005). Further useful references are Pienaar (1980), Kristiansen and Takahashi (1982), and Round (1986).

The scale-bearing species, as seen in the electron microscope, were first surveyed by Takahashi (1978) and, more recently, the Synurales by Kristiansen and Preisig (2007). This was supplemented by the work on *Paraphysomonas* and related genera by Preisig and Hibberd (1982, 1983) and by Scoble and Cavalier-Smith (2014). A recent review on the taxonomy of silica-scaled chrysophytes has been published by Škaloud et al. (2013).

History of Knowledge

Knowledge of the chrysophytes was initiated by the Danish naturalist O. F. Müller who, in his famous work *Animalcula Infusoria* (1786), depicted and named the colorless *Volvox vegetans,* which is now known as *Anthophysa vegetans.* A systematic survey of microorganisms, among them many chrysophytes, culminated in Ehrenberg's (1838) magnificent work, in which species of *Synura, Dinobryon,* and *Uroglena* were depicted and described.

The first precise descriptions of chrysophytes are found in the authoritative work of Stein (1878); many of his illustrations are still used in modern textbooks.

Many species were subsequently described and placed with other flagellates in the animal kingdom. Pascher (1913, 1914, and in a long series of papers) established the botanical position of these algae. He defined the class Chrysophyceae, showing also how chrysophytes resemble the diatoms, the brown algae, and others. He demonstrated parallel evolution in the major algal groups: like other algal taxa, the Chrysophyceae evolved from flagellates to multicellular organization levels, which retained swarmers of the ancestral flagellated types. Based on these principles, Bourrelly (1957) published his *Recherches sur les Chrysophycées,* including all available light microscopy information on these protists. Later, Bourrelly (1965) considered flagellar number as the main taxonomic criterion.

Knowledge of the chrysophytes has advanced considerably since then by the introduction of electron microscopic techniques that reveal cell structure, flagellar systems, and cell envelopes. Understanding of their taxonomy has greatly progressed, so that life cycles and sexuality can be recognized, and studies on the ecology and distribution of the individual species can be carried out. Chlorophylls and the accessory pigments have been identified and their functions elucidated by improved biochemical methods (Kristiansen 2005). However, our knowledge is still fragmentary and based on investigations of rather few species. Introduction of molecular methods has greatly enhanced our understanding of taxonomic relationships, as will be discussed in the final chapter.

Practical Importance

The practical use of chrysophytes is restricted to the laboratory: *Ochromonas* species have served as experimental organisms for many investigations of general biological importance, viz., the freshwater species *Ochromonas danica* for secretion of organic compounds such as vitamins into the environment (Aaronson et al. 1971). *Poteriochromonas malhamensis* has been used for determining the toxicity of lead compounds (tetraethyl lead) as antiknock additives to gasoline (Röderer 1980).

Because of their narrow ecological spectra, silica-scaled chrysophytes can serve as indicators for changes in trophic conditions, in particular of pH in lakes (Smol et al. 1984; Siver and Hamer 1990). Silica structures, such as stomatocysts and scales (Figs. 3 and 4), are used in sediment studies in geology and limnology, often together with pollen analysis, to study the history of lakes (e.g., Nygaard 1956; Munch 1980; Smol 1980; Adam and Mahood 1981; Carney and Sandgren 1983; Cronberg 1986; Siver and Smol 1993; Siver and Marsicano 1996). Changes in pH (acidification) and anthropogenic influence can readily be followed.

Some chrysophytes, e.g., the genera *Synura* and *Uroglena*, may become a nuisance when they occur in great quantities, because they excrete fishy-smelling ketones and aldehydes (Collins and Kalnins 1972). They may foul drinking water reservoirs (Watson et al. 2001; Watson and Satchwill 2003).

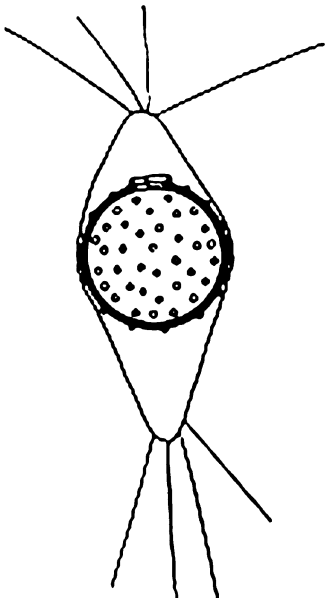

Fig. 3 Stomatocyst of *Mallomonas teilingii* within the scaly envelope

Fig. 4 Silica scale of *Mallomonas acaroides*

Habitats and Ecology

Chrysophytes occur mainly as phytoplankton, and standard phytoplankton methods are used in their collection. Although planktonic species are obtained in plankton nets of suitable mesh, e.g., 20 μm, a great many nanoplankton species pass through. These must be obtained directly from water samples brought to the laboratory. Most chrysophytes are very fragile; thus, transport to the laboratory should take place in a thermos or on ice and living material should be examined as soon as possible. Immediate preservation of field samples for light microscopy and counting is made by Lugol's solution modified with the addition of acetic acid; glutaraldehyde is used for electron microscopy.

Material from water samples should be concentrated (by filtration or centrifugation) for examination in the laboratory. To determine species diversity and abundance, an inverted microscope is indispensable. Lugol-fixed material is inspected in sedimentation chambers of defined volume viewed from below in an inverted microscope for quantification; this is also a way to detect many very small forms.

To detect and identify many of the scale-covered species, electron microscopic examination is required. Material is dried on formvar-coated grids and often shadow casting with a heavy metal (e.g., gold-palladium or chromium) is necessary to enhance contrast and to show three-dimensional structures in TEM. SEM is increasingly used for identification (e.g., Siver 1991).

Most chrysophytes occur as plankton in lakes and ponds. Only few, such as *Hydrurus*, are found attached to stones in running waters (Parker et al. 1973). Some few species occur as neuston attached to the surface layer; *Chromophyton* may cover small forest ponds with a golden layer, in the quantity of two million cells per cm^2 (Molisch 1901; Frølund 1977).

Typical freshwater chrysophyte habitats are humic, neutral, or slightly acidic lakes and ponds with a moderate supply of nutrients. Here the chrysophytes may constitute the main phytoplankton biomass. In more acidic, low nutrient, or alkaline waters, few species occur but sometimes at high cell numbers. Ponds surrounded by agricultural land, unless polluted by cattle, are often very rich in chrysophytes. Species of scaled chrysophytes can be arranged along a trophic gradient in relation to their trophic demands, their trophic scores (Siver and Marsicano 1996).

Many species have well-defined occurrence ranges regarding pH; they can thus be arranged as acidobiontic, acidophilic, indifferent, alkaliphilic, and acidobiontic species (compare Kristiansen 1975 and 2005). The ecological tolerances of species differ greatly even between species of which many are distinguishable only by electron microscopy (Fig. 5). *Synura sphagnicola* (Fig. 6), for example, occurs only in acidic water, while other *Synura* species occur only in alkaline water or are more broadly adapted, e.g., the nearly ubiquitous *S. petersenii*. However, as already mentioned, this species has been shown to include a number of cryptic species with presumably different ecological preferences.

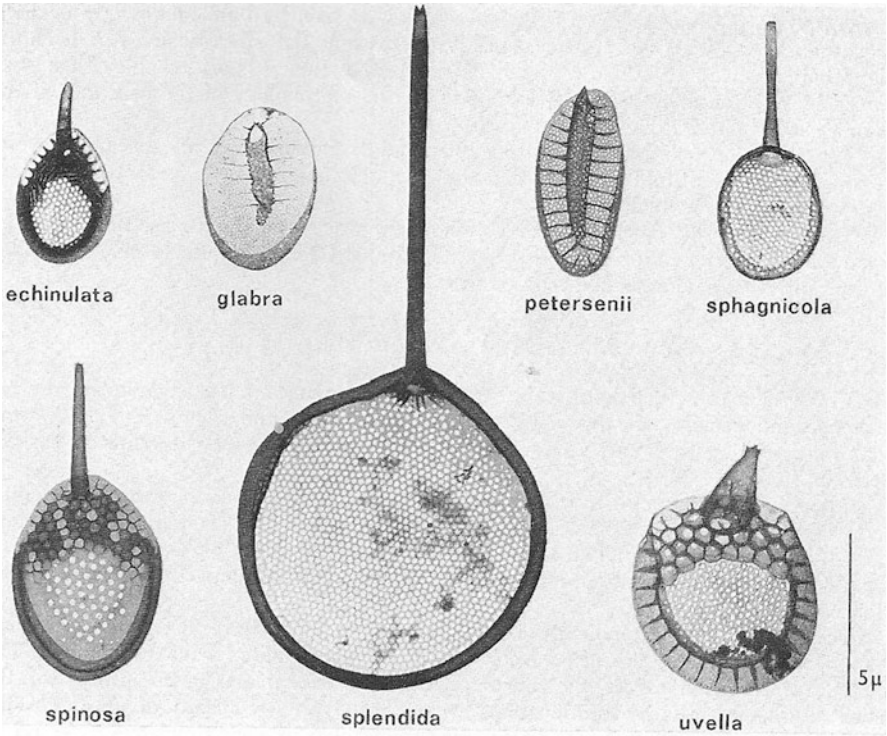

Fig. 5 Scales of species of *Synura*, originally defined on structural characters, but now additional molecular information is necessary

Most species have their main occurrence in spring, often just after ice break. Many species are restricted to cold or cool water, thus in temperate regions occurring in spring and autumn, others prefer warmer water in summer.

There are only few true marine species described (Scoble and Cavalier-Smith 2014). Until recently, the sea was considered to be crowded with chrysophytes, but as several "splinter groups", e.g., Dictyochophyceae (Ostroff et al. 1980; Moestrup and Thomsen 1990), Phaeothamniophyceae (McLachlan et al. 1971), and Pelagophyceae (Lewin et al. 1977) have been shown to have other affinities (Moestrup 1995), the number of marine species has been reduced considerably. Among the most abundant former marine chrysophytes are the silicoflagellates (Dictyochophyceae). However, as already mentioned, the marine diversity of true chrysophytes is probably much greater than previously realized (del Campo and Massana 2011).

Species of *Paraphysomonas* (Preisig and Hibberd 1982) are found both in fresh and sea water, and they may occur in quantities during sea ice formation (Ikävalko 2001). As colorless phagotrophic organisms attached to "marine snow," they may play an important role in the marine food web (Lim et al. 1999).

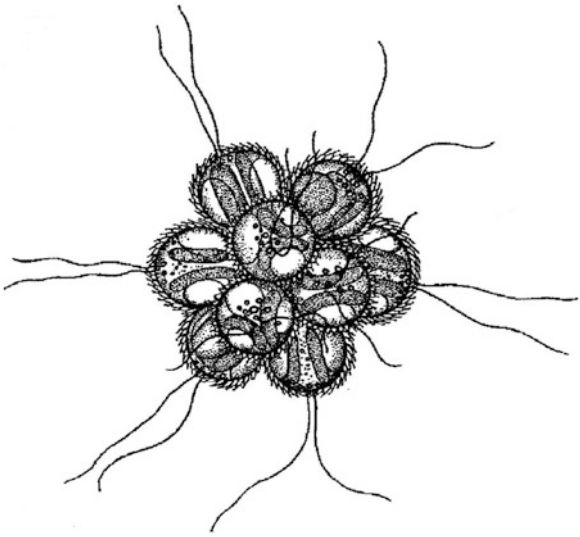

Fig. 6 *Synura sphagnicola* a motile, colonial scale-bearing chrysophyte. × 400 (From Korshikov 1927)

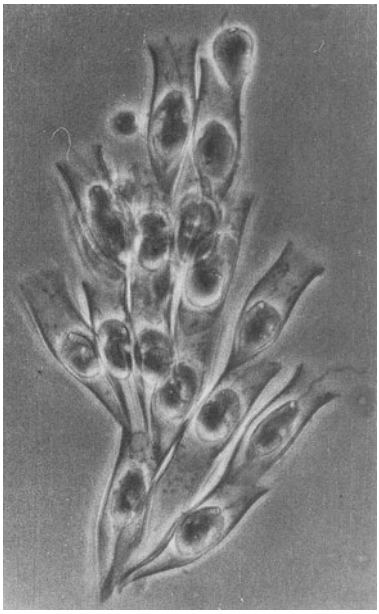

Fig. 7 *Dinobryon cylindricum* var. *alpinum,* a colonial, loricate chrysophyte. × 600

All chrysophytes form endogenous cysts (statospores, stomatocysts) during their life history. In *Dinobryon cylindricum* (Fig. 7), encystment occurs either in the exponential phase of population growth (intrinsic, mainly sexual resting cysts) or in the stationary phase (extrinsic, induced by nutrient depletion). Two clones must be

present in order to produce sexual cysts in *Dinobryon cylindricum,* whereas asexual cysts are produced by individuals, pioneers in a new habitat. These *Dinobryon* produce asexual cysts at a low rate, which gradually slows down during the end of the growth period. They produce sexual cysts rapidly during rapid growth. These two strategies result in almost the same number of cysts. The cysts sink into the sediment; the germination rate during the next spring is unknown (Sandgren 1983a, b). *Dinobryon* stomatocysts in surface waters of an arctic lake germinated during the same summer, whereas those in the sediment only germinated the next spring, when turnover exposed them to light (Sheath et al. 1975).

Chrysophytes excrete a great variety of organic compounds (Aaronson et al. 1971), corresponding to 20% of the carbon they fix by photosynthesis. These compounds include carbohydrates, enzymes, and vitamins and are utilized by bacteria and heterotrophic protists. Small chrysophytes, together with cryptomonads and prymnesiophytes, make up an important part of the nanoplankton of many lakes where they are the main food for zooplankton.

Because *Dinobryon* has an effective phosphate-uptake mechanism, it is especially abundant in waters with low phosphate concentrations (Lehmann 1976). However, most species (excluding Synurales) are mixotrophic, partly covering carbon and phosphorus demand by ingestion of bacteria (Sanders and Porter 1988).

Silica is required for scale-bearing species. *Synura* and *Paraphysomonas* require silica in the water at a concentration of at least 1 μM in order to grow well; they are able to deplete a medium almost completely of silica. Very low silica content results in unstable colony structure and failure to form cysts and scales. The silica requirement is further demonstrated by the inhibitory effect of germanium dioxide on growth (Klaveness and Guillard 1975; Lee 1978).

Most of the chrysophytes have chlorophyll *a*- and *c*-containing chloroplasts and can photosynthetically utilize inorganic carbon from CO_2 in the synthesis of organic compounds. An exogenous supply of organic carbon compounds, e.g., vitamins of the B group, mainly B_{12}, is also necessary. This will normally be present in the water, either excreted by bacteria, released by the decomposition of algal cells, or brought by sewage. Organic compounds are also obtained by phagocytosis of particulate food by many species. Colorless forms are exclusively dependent on phagocytosis and/or uptake of dissolved organic compounds (Pringsheim 1952).

Characterization and Recognition

Cell Structure

The Chrysophyta, a group of protists containing single-celled individuals as well as quite complex colonial forms, can briefly be defined by the following biochemical and structural criteria: chloroplasts with chlorophylls *a* and *c* (Andersen and Mulkey 1983) but lacking *b,* fucoxanthin as the most important accessory pigment, β-1, 3-glucan (chrysolaminaran) as storage product, swarmers with heterokont flagella (i.e., one long hairy and one shorter smooth, the latter in many cases only to be

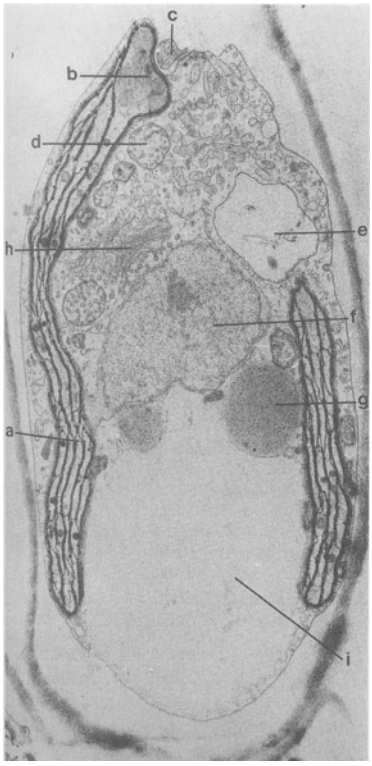

Fig. 8 A longitudinal thin section of a *Dinobryon* cell showing the position of most organelles. The chloroplasts (**a**), one of them with a stigma (**b**) in juxtaposition to the flagellar swelling (**c**). Several mitochondrial sections (**d**) with tubular cristae. A vacuole (**e**) is above the nucleus (**f**) and two lipid droplets (**g**). The golgi body (**h**) can be seen. The chrysolaminaran storage vacuole (**i**) occupies a major portion of the posterior part of the cell. × 8,700

detected by EM). Endogenous silicified cysts (stomatocysts) are present throughout the class.

The basic morphological type in the Chrysophyceae is the motile cell or swarmer (flagellate), from which other structural types or organization levels presumably evolved (Pascher 1914). The swarmer cell is naked or surrounded by an envelope; it occurs either solitary or in colonies. It is provided with one or two visible flagella; contractile vacuoles, most often located anteriorly, are present, and in most cases a chloroplast with a stigma (eyespot) is also present (Kristiansen 1986, 2005).

Many species have a tendency to form lobed or branched cytoplasmic extensions. In some species, the cell is amoeboid during the greater part of its life history (rhizopodial organization level), and either motile or sessile. The palmelloid level of organization is characterized by immotile cells located within mucilage as the dominant stage of the life cycle. Many motile species have such a stage during their life cycle as well. The coccoid level of organization, in which the cell is immotile and surrounded by a distinct wall, is displayed by a few genera.

Chrysophyte cells exhibit a number of structural characteristics by which they can be distinguished from other protists (Figs. 8 and 11), including distinctive flagellar basal bodies and subsurface microtubules, golgi appressed to the nucleus,

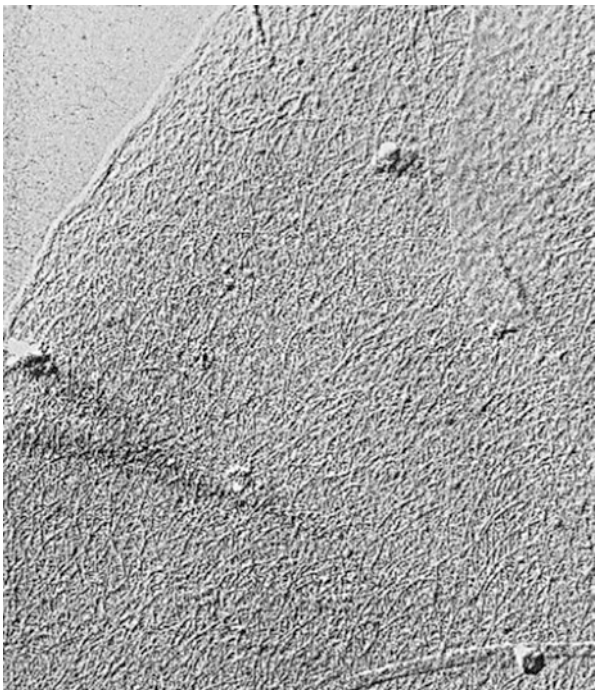

Fig. 9 Detail of the lorica of *Dinobryon pediforme,* showing the fibrillar construction. ×40,000

chloroplast endoplasmic reticulum, and a flagellar swelling opposite the distal face of the plastid with the stigma (Bold and Wynne 1978).

Most chrysophytes occur as naked cells. The cell membrane is in direct contact with the water; in *Ochromonas,* it is covered with a fuzzy layer and with surface blebs and vesicles. These may serve to trap bacteria and other particles that are subsequently engulfed as food (Kahan et al. 1978).

In many chrysophytes, the cells are surrounded by a wall or lorica of several different shapes. For example, it is vase- or beaker-shaped in *Dinobryon,* flask-shaped in *Lagynion,* or globular in *Chrysococcus.* The lorica consists of imbricate scales in *Epipyxis;* in *Bitrichia,* it has a peculiar double construction. In *Lagynion,* the lorica is fixed to a substratum. A ring-shaped part of the lorica fastens *Chrysopyxis* around an algal filament (Kristiansen 1972). The lorica is an interwoven system of fine fibrils consisting of cellulose; or in some cases it consists of chitin (Herth et al. 1977). In *Dinobryon,* the cellulosic fibrils are secreted during rotation of the protoplast and thus show a more or less helical arrangement (Franke and Herth 1973; Herth 1979; Fig. 9). In *Chrysococcus,* the dark and opaque lorica is impregnated with manganese and iron compounds. In *Ochromonas,* simple lorica forerunners have been observed (Schnepf et al. 1968).

Cells of several genera, mainly in the order Synurales, are covered by an armor of silica scales, spines, and bristles. By means of X-ray microanalysis, they have been proved to be composed of silica, which is consistent with the inhibition of scale formation by germanium dioxide (Klaveness and Guillard 1975; Lee 1978). An

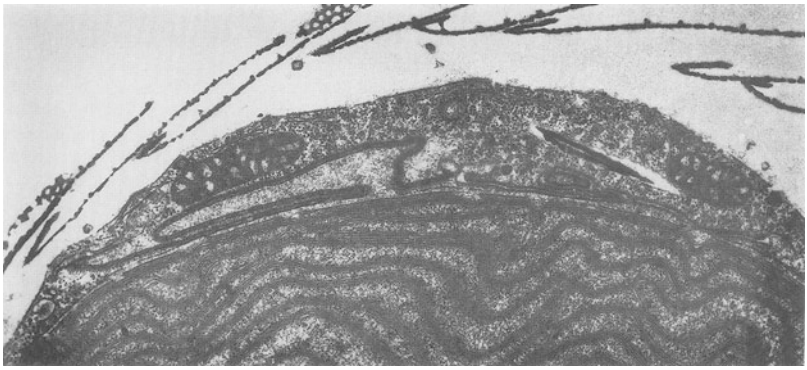

Fig. 10 *Synura petersenii,* formation of silica scales from the chloroplast. *Above*, part of the scaly armour is seen. × 17,500

additional organic component has been demonstrated in *Synura* scales (McGrory and Leadbeater 1981).

Silica scales and associated structures are produced internally; two different but related mechanisms are involved. In the Synurales, scale deposition vesicles are produced from the chloroplast endoplasmic reticulum (CER) on the outer side of the chloroplast. In *Synura* (Schnepf and Deichgräber 1969), the adjacent part of the CER bulges into such vesicles (of golgi body origin), functioning as molds for the scales (Fig. 10). "Hairy" golgi body vesicles that transport material fuse with the scale-producing vesicle. The mature scale is extruded from the cell and brought into correct position in relation to the other scales and the cell surface. *Mallomonas* bristles are formed in a similar way. They are initiated as flat sheets and then rolled into hollow tubes, which are then hinged to the scales (Wujek and Kristiansen 1978; Mignot and Brugerolle 1982). Beech et al. (1990) have shown the mechanism in *Mallomonas splendens*, how the bristles are extruded and brought in correct position and then with their foot glued to the scale.

In the Paraphysomonadida, scale production takes place somewhat differently. One vesicle produces scales while another vesicle from the endoplasmic reticulum functions as a mold (Preisig and Hibberd 1983).

Scale structure is species specific and very complicated, and it was understood only after electron microscopy came into common use. A scale generally consists of a perforated basal plate provided with ribs, spines, and other ornamentation (Fig. 5). In *Mallomonas,* some scales bear long, often complicated, bristles (Asmund and Kristiansen 1986; Kristiansen 2002). Scanning EM shows the three-dimensional structure of the scales (e.g., Siver 1991). Scales are deposited on the cell surface in an imbricate, often screwlike pattern. Several scale types are produced in the same cell and deposited on the surface in a definite sequence, as apical, body, and caudal scales (e.g., Belcher 1969b). Organic surface scales of a complicated flowerpot-like shape that cover both cell and flagella have been reported in *Sphaleromantis* (Manton and Harris 1966). A species of *Chromulina* is covered with simple oval scales (Pienaar 1977).

The flagellar system shows a complicated structure and an interesting evolution. The primitive heterokont condition is the presence of two dissimilar flagella: one flimmer (mastigonemate, hairy) flagellum and one shorter, smooth flagellum, both inserted apically in the cell. In more advanced forms, the short flagellum may be somewhat or almost completely reduced and/or transformed into a photoreceptor (Hibberd 1976).

Basal bodies are located anteriorly in the cell, in most cases at an angle to each other. Only in *Mallomonas* and *Synura* are they parallel. These basal bodies are interconnected by a system of fibers and connected by a fibrous band to the stigma region of the chloroplast (Kristiansen and Walne 1976). Systems of microtubules spread as microtubular roots below the cell membrane, and a rhizoplast proceeds into the cell and connects with the nuclear envelope (Figs. 11 and 12). In the transitional region above the basal body, the transitional helix (Hibberd 1979) is a general feature.

The longer, hairy flagellum is most often forwardly directed, beating with uniplanar sine waves starting from the base (Jarosch 1970). It has two rows of mastigonemes (flagellar hairs*)* causing the pulling effect of its movement. In *Ochromonas,* the mastigonemes are single in one row, in tufts in the other. Each mastigoneme consists of a base and a stiff shaft and bears two terminal and several lateral filaments (Bouck 1971). These mastigonemes are produced in the perinuclear space between the nucleus and chloroplast (Leedale et al. 1970). They are transported via golgi vesicles to the base of the flagellum. These vesicles fuse with the plasmalemma, thus the mastigonemes become extracellular and are transferred to the plasmalemma of the flagellum (Hill and Outka 1974). The short flagellum bears fine lateral filaments. The short flagellum, generally directed laterally, beats in helical waves. It may bear a swelling or be completely transformed into a photoreceptor. In some genera it is reduced so that it is only visible by electron microscopy (Belcher 1969a; Belcher and Swale 1967), accordingly these have originally been considered uniflagellate.

Small and simple flagellar scales occur in *Synura* and *Mallomonas* (Hibberd 1973; Bradley 1966). In *Sphaleromantis*, they are similar to the rather complicated body scales, making the flagella appear coarse and stiff (Manton and Harris 1966).

Photoreceptor systems are present in almost all motile chrysophytes; they consist of a swelling on the short flagellum with the photoreceptor and a stigma (often called the "eyespot") functioning as a screen. The stigma, located anteriorly in a chloroplast lobe in juxtaposition to the photoreceptor (Fig. 12), consists of a number of red (carotene) lipid droplets densely arranged just within the chloroplast membranes. A stigma is present in most motile chrysophytes; it does not occur in *Chrysamoeba,* some species of *Chrysococcus* (Belcher and Swale 1972a), in Synurales, and in most colorless forms (Hibberd 1976).

The swelling is placed proximally on the smooth flagellum and often has a complicated internal structure. In *Sphaleromantis* (Manton and Harris 1966), *Chromulina* (Belcher and Swale 1967), and *Chrysococcus* (Belcher 1969a), this flagellum is very short, so that it almost exclusively consists of a photoreceptor and is placed in a pocket in direct juxtaposition to the stigma. In *Mallomonas*, it is reduced

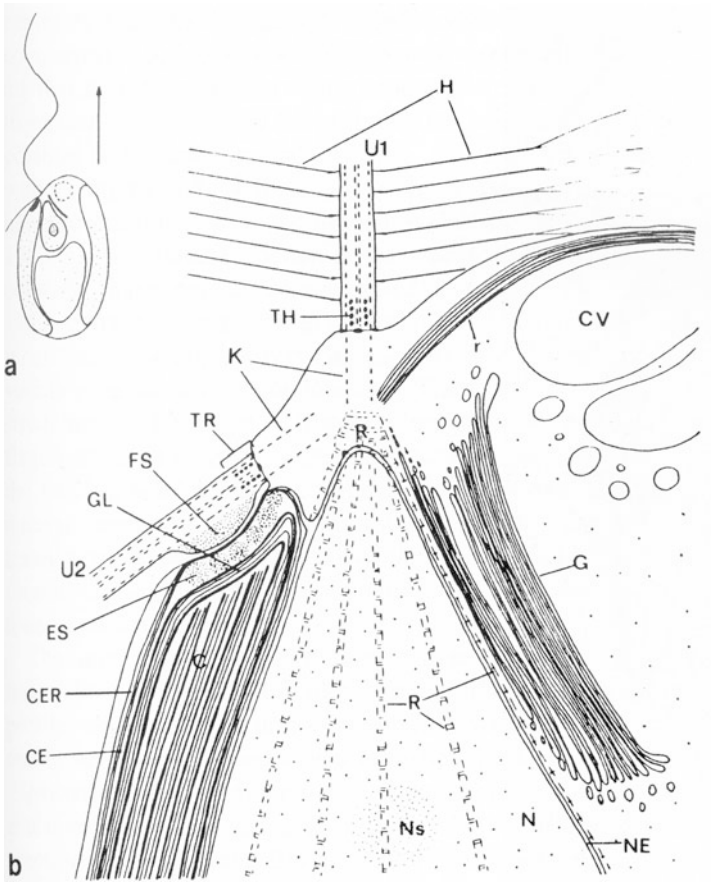

Fig. 11 Basic organization of a chrysophycean cell. (**a**) Diagram showing the flagella and other important organelles as seen with the light microscope (chloroplasts, eyespot, nucleus, golgi body, chrysolaminaran vacuole). (**b**) Diagram of anterior part of cell as seen in thin section with the transmission electron microscope: *C* chloroplast, *CE* chloroplast envelope, *CER* chloroplast endoplasmic reticulum, *CV* contractile vacuole, *ES* eyespot, *G* golgi body, *GL* girdle lamella, *H* flagellar hairs, *K* flagellar basal bodies, *N* nucleus, *Ns* nucleolus, *NE* nuclear envelope, *R* rhizoplast, *r* microtubular flagellar root, *TH* transitional helix, *TR* transitional region, *U1* anteriorly directed flimmer flagellum, *U2* laterally directed smooth flagellum (With permission from: D. J. Hibberd 1976, *Bot. Journ. Linn. Soc.* 72: 55–80, Copyright 1976, The Linnean Society of London)

to a peduncle, hardly protruding beyond the scale cover, and bearing the photoreceptor (Bourrelly 1957). Since no stigma is present in this genus, the shading effect may be due to the chloroplast itself.

In colorless forms, where the chloroplast has been lost or reduced to a leucoplast, there is most often also a reduction of the photoreceptor system. In the genus *Paraphysomonas,* a colorless counterpart to *Spiniferomonas*, there are all transitions from stigma-bearing species with complete photoreceptor system to species without

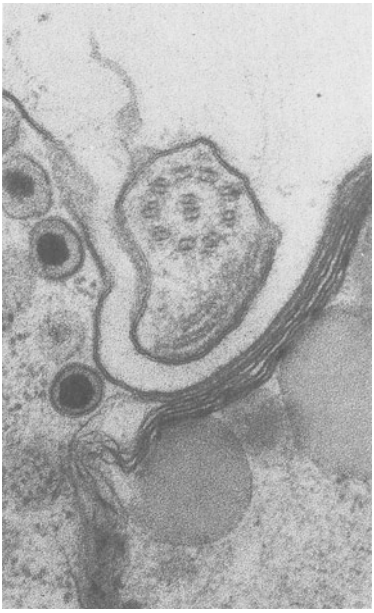

Fig. 12 Flagellar swelling with photoreceptor in juxtaposition to stigma-bearing part of the chloroplast (*Dinobryon*), × 64,000 (EM: J. Kristiansen, from Kristiansen and Walne (1977), with permission from British Phycological Society)

stigma but still with the leucoplast in juxtaposition to the flagellar swelling, and finally to species without stigma and swelling, and with no spatial relationship between leucoplast and flagellum (Preisig and Hibberd 1982, 1983). A similar reduction series is present in *Spumella,* a colorless counterpart to *Ochromonas* (Mignot 1977).

The nucleus surrounded by a double nuclear membrane is normally located in the center of the cell. In most cases, the outer nuclear membrane is continuous with the chloroplast ER, and the nucleus is thus intimately associated with the chloroplast (Fig. 13).

Close to the nucleus is the golgi body (Fig. 14). In most cases it consists of a single but very conspicuous set of vesicles often visible even in the light microscope. There are several golgi structures in *Hydrurus*. A close association exists between the nucleus and the forming face of the golgi: vesicles cut off from the outer nuclear membrane fuse to form golgi cisternae. Vesicles released from the edges of these cisternae are associated with the formation of scales, transport of flagellar hairs, and exocytosis of various substances.

The mitochondria have tubular cristae. The number of mitochondria per cell is difficult to discern. Many mitochondria profiles may be seen in thin sections, but they usually represent one or very few long and coiled mitochondria.

Microtubules occur mainly as peripheral systems below the cell membranes, emanating as microtubular bundles from the basal bodies as flagellar roots. They serve as a cytoskeleton to maintain cell shape. *Ochromonas* cells treated with colchicine, which prevents the assembly of microtubules, lose their specific shape

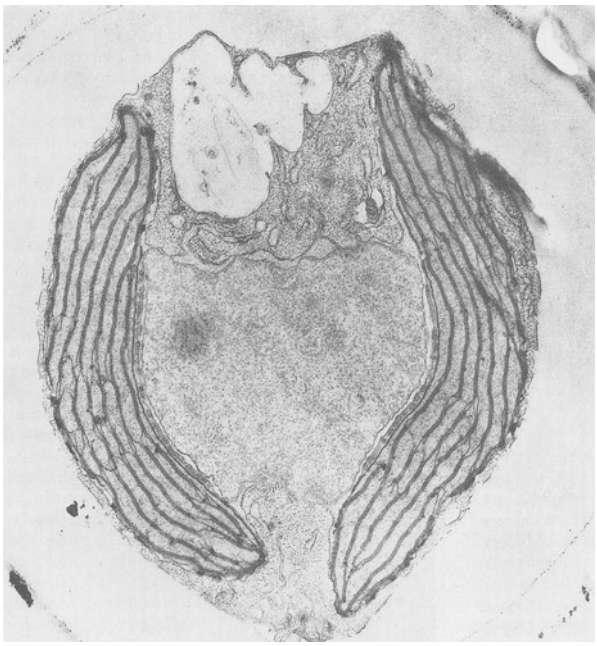

Fig. 13 Transverse section of a *Dinobryon* cell, showing the nucleus and the chloroplast wihin the chloroplast ER. On the inner face of the chloroplasts the periplastidial reticulum is seen. × 16,100. EM

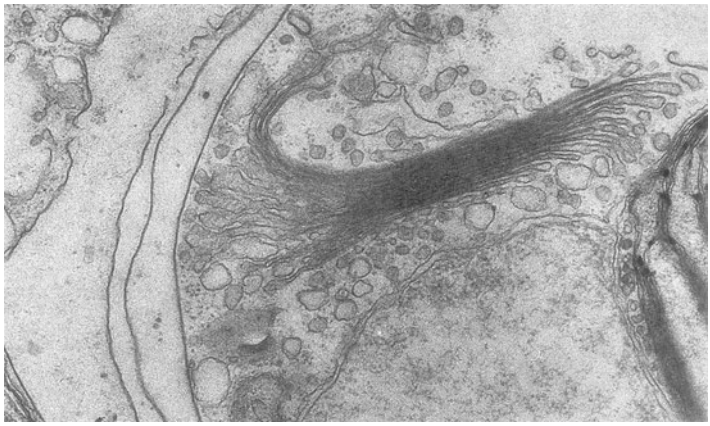

Fig. 14 The relation between the golgi body (*above*), nucleus (*below*), and chloroplast (*right*) in *Dinobryon.* × 30,200. EM

and become spherical (Bouck and Brown 1973). Massively developed microtubular systems occur in the tetrahedral swarmers of *Hydrurus* and *Chrysonebula* (Hoffman et al. 1986; Hibberd 1977a). A bundle of microtubules is situated in the stalk of *Poteriochromonas* (Péterfi 1969).

Most species possess one or two plastids (Fig. 12). The plastids are often lobed and located in close connection with the nucleus. They are surrounded by four membranes (Gibbs 1962), the outermost of which, called the chloroplast endoplasmic reticulum, is continuous with the outer nuclear membrane. The compartment between the next membrane and the inner chloroplast membranes contains the periplastidial reticulum, which functions in the transport of proteins into the plastid (Gibbs 1979).

The chloroplast contains photosynthetic lamellae, each consisting of three thylakoids. A girdle lamella is present, except in *Mallomonas* and *Synura.* The chloroplast DNA is ring-shaped and located just within the girdle lamella. Pyrenoids are either immersed or semi-immersed in the plastid; they are sometimes traversed by thylakoids. Only in *Hydrurus* are they stalked. Colorless chrysophytes have leucoplasts, e.g., *Spumella, Heterochromulina,* and *Paraphysomonas.* In *Anthophysa* and some species of *Paraphysomonas,* the small leucoplast even possesses a stigma (Belcher and Swale 1972b; Preisig and Hibberd 1983).

The chloroplasts of the chrysophytes contain chlorophyll *a* as the main photosynthetic pigment. In addition, chlorophylls of the *c*-group occur, normally both *c*1 and c2, but in *Mallomonas* and *Synura* only *c*1. The golden-brown color of the plastid is due to the occurrence of accessory pigments, mainly xanthophylls; the most important is fucoxanthin, comprising up to 75% of the total pigment in *Ochromonas danica.* Diatoxanthin has been demonstrated in *Sphaleromantis* and *Ochromonas,* diadinoxanthin in *Sphaleromantis* (Aaronson and Baker 1959). β-carotene is present in all chrysophytes. Carotene is concentrated in the part of the plastid differentiated as the stigma.

The product of photosynthesis is chrysolaminaran (chrysose or leucosin). It is a β-1,3-glucan and is deposited as a peculiarly refringent storage product in a posterior vacuole. Lipids are deposited in small vesicles in the cytoplasm. The chrysophytes are known to produce a great variety of fatty acids.

It is doubtful if any entirely photoautotrophic chrysophytes exist. In darkness, *Ochromonas* can grow osmotrophically on dissolved organic compounds, in which case the plastids will eventually be reduced (Pringsheim 1952). Many photosynthetic naked chrysophytes are capable of phagocytosis. Cell membrane flow transports trapped particles to the apex where phagocytosis occurs. Chrysophytes take up any particles, even inorganic ones. Bacteria, small algae, and quite large diatoms that completely distort the cell may be ingested. The phagocytic vacuole is then transported to a special digestion vacuole at the posterior end of the cell (Cole and Wynne 1974). Rhizopodial species are especially adapted for this feeding method. The food uptake mechanism has been studied in detail first in *Ochromonas* (Doflein 1922), showing how bacteria were trapped in a cytoplasmic basket and then engulfed. In *Epipyxis* (Wetherbee and Andersen 1992; Andersen and Wetherbee 1992), food particles, e.g., bacteria, are captured by the flagella and brought into the cytoplasmic feeding basket supported by one of the flagellar roots and from there transported into a digestion vacuole.

Life History

The swarmer is the predominant stage in the life cycle at the monadoid level of organization. However, swarmers also occur as a regular phase in the life cycles of most species at other levels. Swarmers fall into two main types: *Ochromonas*-like swarmers have two flagella, while in *Chromulina* swarmers only one is visible. A special swarmer of tetrahedral shape occurs in *Hydrurus* (Joyon 1963) and *Chrysonebula.* In some coccoid and filamentous forms, the cell divides into several immotile offspring cells (called autospores) liberated by rupture of the parent cell wall.

Sexuality was believed to be rare among the chrysophytes; although it mostly escapes attention and requires much patience to demonstrate, it is likely to be more prevalent. Sex is most often observed in small loricate monads (i.e., surrounded by a special envelope) such as *Kephyrion, Stenocalyx, Chrysolykos,* and the solitary *Dinobryon* species. Undifferentiated cells act as gametes, fuse apically, and produce a globular zygote. The empty loricae of the gametes, which remain attached to the zygote, make it easily recognizable (Fott 1959).

In colonial species of *Dinobryon,* sexuality has also proved to be of great importance. Cyst formation involves autogamic processes (fusion of nuclei formed by a prior mitosis) or gametic fusion of cells liberated from male colonies with loricate cells in female colonies to form zygotic cysts (Sandgren 1981).

In *Synura* and *Mallomonas*, normal scale-bearing cells act as gametes, with posterior fusion (Wawrik 1972). *Synura* is heterothallic; sexuality is induced at high cell density. Single cells liberated from male colonies act as gametes and copulate with cells in female colonies; subsequently the zygotes encyst and remain in the colony (Sandgren and Flanagin 1986).

The endogenous cyst, the stomatocyst (often also called the "statospore"), the characteristic resting stage of the Chrysophyceae, has a very special morphology: a globular, silicified wall with an opening called a porus, closed by a pectic plug. In many species the porus is surrounded by a collar. The stomatocyst wall may be smooth or bear ornamentation, including protuberances such as spines in various arrays depending on species (Fig. 3). In *Hydrurus* and a few other genera, a distinctive stomatocyst occurs that is ellipsoidal with an equatorial ring.

The stomatocysts are usually classified following an artificial taxonomy based upon size and shape, the outer wall ornamentation, as well as pore and collar morphology (Kamenik 2010). Guidelines for the description and nomenclature of stomatocysts have been worked out by Cronberg and Sandgren (1986), and the stomatocyst atlas by Duff et al. (1995) contained 240 taxa. But already in 2001 an enlarged edition was necessary (Wilkinson et al. 2001).

Stomatocyst formation has been studied in detail in *Ochromonas*, *Mallomonas*, and *Dinobryon* by electron microscopy (Hibberd 1977b; Andersen 1982; Sandgren 1980a, b). Two basic types of cyst formation are known, but they have in common the internal formation of the silica wall in the silicalemma, a silica-depositing vesicle

derived from the Golgi body. At maturity, the porus is closed by a plug of fibrillar pectic material.

In *Ochromonas tuberculata* and *Mallomonas caudata*, the uninucleate cell transforms directly into a cyst. The internal silica wall is formed by deposition on a basal lamella, and the porus is formed by resorption of part of the already deposited wall. The cyst also contains one nucleus. The external cytoplasm disintegrates after having deposited the external wall structures. In *Ochromonas sphaerocystis*, the external cytoplasm does not disintegrate but is retracted through the porus. In *Dinobryon,* the process is more complicated. The cell moves to the lorica mouth and secretes a surrounding encystment chamber. After formation of the silica-depositing vesicle, the cyst wall is produced with the porus preformed. After the wall ornamentation has been deposited, the remaining external cytoplasm is retracted through the porus and the plug is formed.

Cyst germination has been examined in only a few species. The plug dissolves and a motile naked cell escapes. In *Ochromonas,* a single normal swarmer separates; in *Leukochrysis* and *Kybotion,* small amoeboid cells emerge. In *Mallomonas,* the germination products are small scaleless monads. In *Dinobryon,* a special germination chamber is formed from the porus of the stomatocyst. The cell divides twice to form four cells that wander into this germination chamber, from where they are eventually released as naked, free-swimming monads (Sheath et al. 1975).

Cell division is longitudinal, and in motile cells it starts from the anterior end of the cell. In scale-bearing forms, the scaly armor appears to be reestablished as division proceeds. Mitosis, studied in detail only in *Ochromonas* and a few others, is of a special type: the rhizoplasts from the two basal bodies act as poles for the organization of the spindle microtubules (Slankis and Gibbs 1972).

Maintenance and Cultivation

General algal culture methods, including those for chrysophytes, are described in Andersen (2005) and references given there. Table 2 presents culture media used with success for chrysophytes.

For ultrastructural and many taxonomical investigations, pure cultures are not always necessary. Enough material may be obtained in other ways: by collecting naturally occurring high concentrations (blooms) or by concentration of motile cells (e.g., *Synura*) using their phototactic behavior.

Crude cultures to enrich for rare chrysophytes may consist only of the natural sample placed in a cool north-facing window, and successively several chrysophyte species will appear, e.g., attached to the water surface. In many cases, enrichment cultures with nutrients added are more adequate. For freshwater nanoplanktonic species of *Spiniferomonas,* Preisig and Hibberd (1982, 1983) added modified Chu 10 medium to their natural water samples, after larger organisms had been filtered off.

Table 2 Examples of culture media

Chromulina placentula	Chu 10 modif.	Belcher and Swale 1967
Chrysococcus cordifieris	Pringsheim biphasic	Belcher and Swale 1972a
Chrysococcus rufescens	Pringsheim biphasic	Belcher 1969a
Dinobryon divergens	Dy-III-medium	Lehmann 1976
Mallomonas papillosa	Pringsheim biphasic	Belcher 1969b
Ochromonas danica	Aaronson and Baker	Aaronson and Baker 1959
Ochromonas minuta	Pringsheim org. Medium	Hill and Outka 1974
Ochromonas sphaerocystis	Chu 10 modif.	Andersen 1982
Ochromonas tuberculata	Bold's Basal + leaf extract	Hibberd 1977b
Paraphysomonas spp.	Lake water + Chu 10 modif.	Preisig and Hibberd 1982
	Sea water +1iver extract	Caron et al. 1999
Phaeaster pascheri	Pringsheim biphasic	Belcher 1969c
Poteriochromonas spp.	Pringsheim org. Medium	Schnepf et al. 1968
Synura petersenii	WC modif.	Klaveness and Guillard 1975
	Waris modif. Enriched	Schnepf and Deichgräber 1969
Standard chrysophyte medium	Dy-V-medium	Andersen et a1. 2005

Naked marine chrysophytes can be cultured by the addition of modified Erdschreiber medium to original water samples.

Many chrysophytes, because they are extremely fragile and delicate, do not tolerate the procedures necessary to get them into unialgal or axenic culture. For unialgal cultures, vitamins, and other organic growth factors must be included in the media. In chemically defined media necessary for most physiological investigations, these organic compounds must be added as specific vitamins, amino acids, etc. Media based on soil or liver extract, although they contribute a wide and undefined spectrum of vitamins and other organic and inorganic nutrients, unpredictably support growth of some organisms and not of others.

Erdschreiber solution, rich in phosphate and nitrate with added soil extract, is one of the media frequently used. Pringsheim's biphasic soil water medium is one of the most successful for growing freshwater chrysophytes, e.g., *Mallomonas papillosa, Chrysococcus cordiformis,* and *Phaeaster pascheri.* The soil in the bottom of the culture vessel slowly releases small amounts of nutrients.

Bold's Basal Medium, an inorganic synthetic medium, has been adapted for chrysophytes such as *Synura petersenii* and *Ochromonas tuberculata*; organics such as vitamin mixtures, leaf-, soil or peat-extract are added.

Chu 10, an inorganic medium containing silica, with addition of organic compounds is useful for silica-scale-bearing algae.

A standard medium for all chrysophytes is Dy V (Andersen 2005), based on Lehman's original Dy III medium for *Dinobryon* (Lehman 1976).

Highly enriched media, such as Pringsheim's organic medium (Pringsheim 1952) containing glucose, liver extract, yeast extract, and tryptone have been used for the cultivation of mixotrophic forms such as *Ochromonas minuta* and

Poteriochromonas malhamensis. In nonaxenic cultures, *Ochromomas* feeds on the bacteria that grow in the medium.

Colorless chrysophytes (e.g., *Anthophysa vegetans* and *Spumella elongata*) can grow in soil-water medium if the necessary extra organic nutrition such as starch or a barley seed are added (Belcher 1976). The phagotrophic *Paraphysomonas* species feed on bacteria naturally growing in nonaxenic culture (Lee 1978) or in sea or lake water enriched with liver extract or Chu 10 (Caron et al. 1999; Preisig and Hibberd 1982).

Chrysophyte cultures are maintained by numerous Culture Collections. Among these may be mentioned the following: UTEX, Austin, Texas, USA; NIVA, Oslo, Norway; CCAP, Oban, Scotland, UK; CAUP, Prague, Czech Republic; NCMA, Bigelow, Maine, USA; EPSAG, Göttingen, Germany.

Evolutionary History

Fossil Records

The siliceous structures of Chrysophyceae are very resistant and therefore common in many geological deposits, providing a better understanding of the evolutionary origin and stratigrafic distribution of these algae. Chrysophycean cysts (stomatocysts) are more heavily silicified than the other siliceous structures (scales and bristles), and so they are more likely to be present in the older sediments. On the other hand, natural classification is almost impossible as the stomatocyst descriptions are rarely accompanied by descriptions of their vegetative cells (Duff et al. 1995).

Cysts from freshwater deposits are grouped into an artificial family Chrysostomataceae, whereas those from marine sediments are grouped into the equally artificial Archaeomonadaceae. Since different genera could produce similar or even identical stomatocysts (Findenig et al. 2010), their fossil records are primarily important for the timing of the evolutionary origin of chrysophytes. Siliceous scales and bristles are generally preserved for a shorter geologic period (Siver et al. 2009; Siver and Wolfe 2005). However, in contrast to the stomatocysts, they could also be used to trace the evolutionary history and diversification of particular chrysophyte genera, or even species.

Although the oldest known chrysophyte-like structures have been reported from the Cambrian sediments (Allison and Hilgert 1986), their affinity to the Chrysophyceae is doubtful as they do not resemble any siliceous structures of modern taxa. Therefore, they may belong to any unrelated or even extinct lineage. The oldest certain fossils of chrysophytes are represented by siliceous stomatocysts of Archaemonadaceae, recovered from Tertiary or Upper Cretaceous marine deposits (Riaux-Gobin and Stumm 2006). At present, the oldest stomatocysts are from Southern Ocean sediments of Lower Cretaceous (Aptian-Albian, $\sim$ 112 Ma),

which may indicate the initiation of silicification within chrysophyte algae (Harwood and Gersonde 1990). In addition, since the stomatocysts are commonly found in fossil marine sediments, chrysophytes are presumed to have a marine origin (Tappan 1980).

The oldest records of fossilized chrysophyte scales and bristles have been reported from the Paleogene age. Recently, the oldest known microfossils of scales have been recovered from a Paleocene kimberlite deposit (∼ 60 Ma) by Siver et al. (2013a). The scales could be assigned to the genus *Synura*, though two of four taxa discovered represent presumably extinct species. Scales and bristles of other genera of silica-scaled chrysophytes (*Mallomonas*, *Spiniferomonas*) are known from younger, Middle Eocene freshwater deposits (∼ 47 Ma; Siver and Wolfe 2005, Siver et al. 2009; Siver and Lott 2012a). Other chrysophyte fossils are very rare. Identifiable remnants of *Dinobryon*, *Lagynion*, and *Cyrtophora* have been found in coprolites from Wyoming, dating from the Upper Eocene (Tappan 1980).

In general, the fossil record of chrysophytes is still very incomplete and poorly understood; there is much work to be done before it can be utilized to infer the timing of their evolutionary origin and to trace the diversification of particular lineages. Therefore, the origin and divergence times of extant genera are primarily estimated based on molecular clock calculations. According to the reconstruction of stramenopile diversification times, chrysophytes most likely originated in the Permian (∼ 279 Ma; Brown and Sorhannus 2010). This estimation is in accordance with the study of Jo et al. (2013), who estimated the origin of the chrysophytes as ∼ 250 Ma. Interestingly, the diversification of *Mallomonas* species was dated to -133-119 Ma (Jo et al. 2013; Siver et al. 2013b, 2015), implying that this genus evolved much earlier than the paleontological record indicates.

Classification

The first systematics of chrysophyte algae was introduced by Pascher (1913), who stressed the organization levels as foundations for taxonomy, with the flagellar number being of major importance. Uniflagellate organisms were placed in the Chromulinales, whereas those organisms possessing two flagella of reportedly equal length were classified in the Isochrysidales. Organisms having two unequal flagella were grouped in the Ochromonadales. A year later, Pascher (1914) established the class Chrysophyceae, encompassing those protists with golden brown pigmentation. However, he took a different approach to their classification, placing emphasis upon vegetative life forms (flagellate, capsoid, or amoeboid) rather than the number and shape of flagella. In his newly proposed system, all flagellates were classified in the order Chrysomonadales.

Pascher's classification was widely accepted in the years that followed. Bourrelly (1957, 1965) divided the Chrysophyceae into three subclasses: the

Acontochrysophycidae (no flagella), the Heterochrysophycidae (one flagellum or two unequal flagella), and the Isochrysophycidae (two equal flagella). Within the Heterochrysophycidae, he recognized two orders: the Chromulinales (one flagellum) and the Ochromonadales (two flagella). All chrysophycean genera forming siliceous scales and spines were united in the family Synuraceae, within the Ochromonadales. Later on, Silva (1980) has pointed out that the name Mallomonadaceae has priority over Synuraceae.

The subsequent ultrastructural studies have shown that the number of flagella is a quantitative character based on reduction of the short flagellum, having no taxonomic value (Kristiansen 1986). Preisig and Hibberd (1983) used the ultrastructural features to split the silica-scaled chrysophytes into the families Mallomonadaceae (the parallel insertion of flagellar basal bodies, presence of girdle lamella and flagellar scales, and lack of stigma) and Paraphysomonadaceae. In his review, Kristiansen (1986) followed this classification scheme, raising the families to an order status, the Mallomonadales and Ochromonadales. Increasing evidence of morphological and chemical (unique chlorophyll composition) differentiation of Mallomonadales culminated in their establishing as an independent class, the Synurophyceae (Andersen 1987). (Table 1).

However, several recently published phylogenies of Stramenopiles or chrysophyte algae (e.g., Ben Ali et al. 2002; Takishita et al. 2009; Del Campo and Massana 2011; Yang et al. 2012; Škaloud et al. 2013; Scoble and Cavalier-Smith 2014) show the close affinity of Synurophyceae with Chrysophyceae, with the former class often nested within the paraphyletic Chrysophyceae. Therefore, the two classes should be combined again, with the synurophyte algae being members of the order within Chrysophyceae, the Synurales.

Phylogeny

Phylogenetic relationships among the chrysophyte taxa were first inferred by Andersen et al. (1999), who investigated SSU rDNA sequences. Both the NJ (neighbour-joining) and MP (maximum parsimony) analyses resolved the seven distinct clades. Later, Andersen (2007) improved the dataset considerably by including several new chrysophyte taxa and conducted Bayesian phylogenetic analyses of the nuclear SSU rDNA and rbcL genes. The newly published sequences of the genus *Chrysosphaerella* were inferred as distantly related to *Paraphysomonas*, indicating the artificial concept of the Paraphysomonadaceae.

A detailed SSU rDNA phylogeny of chrysophyte algae, based on the data set including nearly all available sequences from cultured species and environmental DNA, was published by Klaveness et al. (2011). More recently, Scoble and Cavalier-Smith (2014) published a detailed phylogenetic reconstruction of Chrysophyta based on 239 SSU rDNA sequences, showing the existence of diverse chrysophyte-related environmental clades EC1 and EC2. In their investigations of heterotrophic *Spumella*-like flagellates, Findenig et al. (2010) and Grossmann et al. (2016)

demonstrated a significant cryptic diversity of these organisms forming a number of distinct lineages across the Chrysophyta. Accordingly, 7 new genera (*Acrispumella*, *Apoikiospumella*, *Chromulinospumella*, *Cornospumella*, *Pedospumella*, *Posteriospumella*, and *Segregatospumella*) and 2 new orders (Apoikiida and Segregatales) have been described.

According to the phylogenetic reconstruction based on recently available SSU rDNA and *rbc*L sequences of morphologically well-characterized taxa, nine orders can be presently recognized within the Chrysophyta (Fig. 15):

Ochromonadales Pascher 1910

This order represents the most diverse lineage, comprising a number of flagellate genera, including the solitary flagellates (e.g., *Ochromonas*, *Spumella*), colonial forms (e.g., *Uroglena*, *Chrysonephele*), or loricate monads (e.g., *Dinobryon*, *Poterioochromonas*). The morphologically similar loricate genera *Dinobryon* and *Epipyxis* occupy separate, phylogenetically distant positions within the order. Similarly, the phylogenetic reconstruction indicates several independent losses of plastid during the evolution of the lineage.

Chromulinales Pascher 1910

The order comprises the solitary (e.g., *Chromulina*, *Oikomonas*) and colonial (*Chrysosphaerella*, *Cyclonexis*) flagellates, as well as the amoeboid organisms (*Chrysamoeba*). The colonial genus *Chrysosphaerella* produces siliceous spines and scales.

Apoikiida Boenigk et Grossmann 2016

The order includes two heterotrophic, bacterivorous biflagellated genera: a colonial genus *Apoikia* forming swimming colonies of cells held together by mucilage and a solitary genus *Apoikiospumella*.

Chrysosaccales Bourrelly 1954

This order includes the morphologically diverse assemblage of taxa, including the *Ochromonas*- or *Chromulina*-like flagellates, coccoid chrysophytes (e.g., *Chrysosphaera*), cells embedded in mucilage (*Chrysosaccus*) or amoeboid loricate organisms (*Lagynion*).

Hydrurales Pascher 1931

The order presently comprises three morphologically distinct genera – colonial, freshwater *Hydrurus* forming macroscopic thalli usually growing in cold water, pseudoparenchymatous marine chrysophyte *Phaeoplaca*, and the *Ochromonas*-like flagellate isolated from Antarctic sea ice.

Hibberdiales R. A. Andersen 1989

The order groups the colonial organisms having the palmelloid level of organization. Cells either secrete a buoyant mucilaginous material to which the cells adhere (*Kremastochrysis*), are closed in a spherical capsoid colonies (e.g., *Hibberdia*,

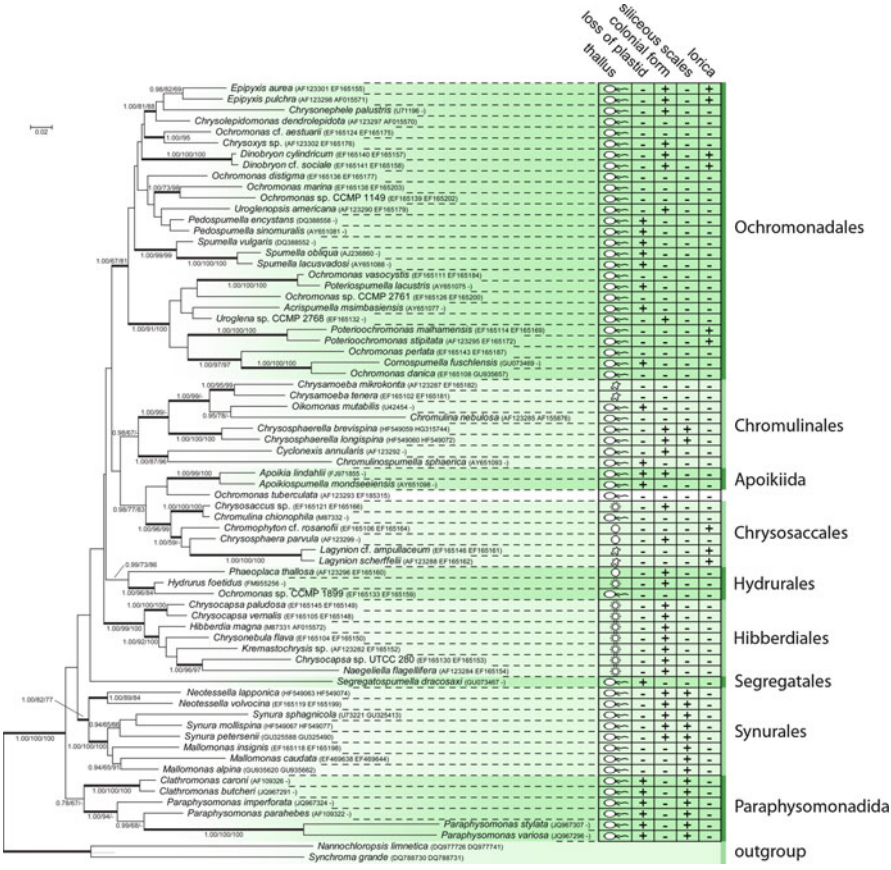

Fig. 15 Phylogeny of Chrysophyta, obtained by a Bayesian analysis of the combined and partitioned SSU rDNA + *rbc*L dataset using a GTR+G+I model for all partitions. Values at the nodes indicate statistical support estimated by three methods – MrBayes posterior-node probability (*left*), maximum-likelihood bootstrap (*middle*), and maximum parsimony bootstrap (*right*). Thick branches represent nodes receiving the highest PP support (1.00). GenBank accession numbers for the concatenated sequences (SSU rDNA and rbcL, respectively) accompany each species name. Scale bar shows the estimated number of substitutions per site. Basic characteristics of depicted chrysophyte taxa are provided in a graphical form. Thallus: the vegetative form is graphically indicated, including the monadoid, amoeboid, coccoid, and palmelloid organization. Other symbols indicate the presence/absence of plastid loss, colonial form, siliceous scales, and loricate structures

Chrysonebula), or they are grouped in a center of mucilaginous matrix extending a number of gelatinous tubes (*Naegeliella*).

Segregatales **Boenigk et Grossmann 2016**

The order currently comprises a single organism *Segregatospumella dracosaxi*, a bacteriovorous heterotrophic flagellate living in fresh water.

Synurales R. A. Andersen 1987

The order comprise three genera of autotrophic scale-bearing flagellates, namely, the solitary *Mallomonas* and the colonial *Synura* and *Neotessella*. The two former genera comprise the most common chrysophycean members of the freshwater planktonic communities worldwide.

Paraphysomonadida Scoble et Cavalier-Smith 2014

The order includes solitary, heterotrophic, flagellated genera *Paraphysomonas* and *Clathromonas*. Cells are covered with siliceous spine, basket, or plate scales. In addition, the order probably comprises a diverse, morphologically uncharacterized, environmental clade EC1.

Even though DNA sequence data are still lacking for several morphologically distinct genera (e.g., *Eusphaerella*, *Spiniferomonas*), the reconstructed phylogeny enables some inferences about evolutionary trends in the chrysophytes. First, the Synurales has the position as a nested group within the Chrysophyceae. Therefore, its recognition as a separate class, the Synurophyceae, is obviously not correct (see the "Classification" section) and should not be followed.

Second, the basal position of the Paraphysomonadida, a group with a significant portion of marine organisms, corroborates the hypothesis that the chrysophytes are of a marine origin (Tappan 1980).

Third, the silica-scaled chrysophytes do not form a monophyletic group, indicating either at least two independent origins of the ability to produce the siliceous structures (at the base of the Chrysophyta and within the Chromulinales) or multiple independent losses of this ability during the chrysophyte evolution.

Fourth, the phylogenetic reconstruction also indicates at least 10 independent reductions and losses of plastids, after which many distinct genera evolved.

Fifth, the morphologically simplest chrysophyte genera, *Ochromonas* and *Chromulina*, are both polyphyletic. The genus *Ochromonas* forms at least nine independent lineages, within the orders Ochromonadales, Hydrurales, and in a sister position to the order Apoikiida. The *Ochromonas* swarmer type might therefore be considered as the most primitive chrysophycean form from which the other morphological types evolved.

In general, the evolutionary history of the chrysophytes seems to be very complex, with several independent origins of morphologically similar taxa. Sequencing of additional taxa, together with extending the fossil data, will undoubtedly yield deeper insight into the evolution of this remarkable group of protist organisms.

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Eustigmatophyceae

11

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Abstract

Eustigmatophyceae (eustigmatophytes) are a distinct lineage of ochrophyte (stramenopile) algae with a relatively small number (~30) of described species, but with evidence for a substantial taxonomic diversity yet to be explored. Eustigmatophytes are all unicellular coccoid algae, usually spherical or ovoid, but sometimes with a more distinctive shape (e.g., stipitate, tetrahedral, or with branched projections). Most eustigmatophytes live in freshwater, but some are common in terrestrial habitats and one subgroup is mostly marine. Reproduction

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occurs primarily via autosporogenesis, but many members of this class form zoospores with an anterior mastigoneme-bearing flagellum and a (sometimes missing) posterior bare flagellum. Sexual reproduction has not been directly observed, but genomic evidence suggests its presence in some species. Eustigmatophytes are distinguished from other ochrophytes by a suite of cytological features (not all are necessarily present in all taxa): a pigmented lipidic body (reddish globule), a swelling at the base of the anterior flagellum associated with an extraplastidial stigma (eyespot), lamellate vesicles (with a putative reserve product), and plastids without a girdle lamella and lacking continuity with the nuclear envelope. Also characteristic is the lack of chlorophyll *c* and violaxanthin as the dominant xanthophyll. Because of their tendency to accumulate large amounts of lipids, including polyunsaturated fatty acids, eustigmatophytes are extensively used for biotechnology applications. The potential for commercial use has sparked a renewed interest in the basic biology of Eustigmatophyceae, including initiation of genome sequencing projects, although attention remains highly biased toward a single lineage comprising the genera *Nannochloropsis* and *Microchloropsis*.

Keywords

Algae • Biofuels • Genomics • PUFAs • Eustigmatophyceae • Molecular phylogenetics • *Nannochloropsis* • Ochrophyta • Stramenopiles • Taxonomy

Contents

Summary Classification

●**Eustigmatophyceae**
●●**Eustigmatales**
●●●**Eustigmataceae group** (*Eustigmatos*, *Vischeria*, *Chlorobotrys*, *Pseudocharaciopsis*)
●●●**Monodopsidaceae** (*Monodopsis*, *Pseudotetraëdriella*, *Nannochloropsis*, *Microchloropsis*)
●●●***Pseudellipsoidion* group** (*Pseudellipsoidion*, *"Pseudocharaciopsis" ovalis*)
●●***Goniochloridales*** (*Goniochloris*, *Pseudostaurastrum*, *Trachydiscus minutus*, *Vacuoliviride*, *Tetraëdriella subglobosa*)
●●**Eustigmatophyceae *incertae sedis*** *(Botryochloropsis)*

Introduction

General Characteristics

Eustigmatophytes are a distinct group of ochrophyte (heterokont or stramenopile) algae. In the vegetative state, these organisms appear as solitary green or yellow-green coccoid cells or less frequently in loose colonies, with primarily autosporic reproduction. They may resemble some xanthophyte or chlorophyte algae but differ by a unique combination of ultrastructural and biochemical characteristics. A conspicuous characteristic of most eustigmatophyte vegetative cells is a cytoplasmic reddish globule consisting of unknown lipidic substances. Eustigmatophyte plastids lack a girdle lamella. The outer plastid membrane, a cisterna of the plastid endoplasmic reticulum, is typically not continuous with the nuclear envelope; however, this connection has been preserved in some species. Vegetative cells as well as zoospores possess vesicles containing a probable reserve material deposited in a lamellar pattern. Zoospores occasionally form in many species and bear one or two subapical flagella. One longer flagellum always has mastigonemes and a characteristic basal swelling. Typically, a red extraplastidial eyespot (stigma) is present at the extreme anterior, although some taxa recently assigned to the class do not possess this feature. Eustigmatophyceae lack chlorophylls *b* and *c*. Violaxanthin is the dominant xanthophyll and is involved in both light harvesting and in a photoprotective xanthophyll cycle. The Eustigmatophyceae is usually considered a small class, as only around 30 species in 15 genera have been described. However, recent studies have shown that many additional eustigmatophytes await recognition or description.

Occurrence

Eustigmatophytes thrive worldwide primarily in freshwater and terrestrial habitats, with the exception of the marine and brackish species of the genera *Nannochloropsis*

and *Microchloropsis*. These organisms are generally inconspicuous because of their small size and are rarely a dominant component of the microbial community.

Literature

There is no recent monograph on the group. The works by Hibberd (1980, 1982, 1990) are still useful as summaries of the first phase of modern research on eustigmatophytes. Hibberd (1981) also published a thorough taxonomic revision and a formal classification of eustigmatophytes that is the starting point for current taxonomic and systematic work on the group. Santos (1996) published the most current general review of eustigmatophytes. Useful information on the morphology and ecology of eustigmatophyte species, including identification keys, can be found in algal floras and compendia (Ettl and Gärtner 1995; John 2011; Ott et al. 2015). Many eustigmatophyte species are included in older floristic works as members of the "Heterokonten" or the Xanthophyceae (Pascher 1939; Ettl 1978).

History of Knowledge

The class Eustigmatophyceae was established by Hibberd and Leedale (1971) after they investigated 12 genera of coccoid algae from the Xanthophyceae and found unique ultrastructural features (Hibberd and Leedale 1970, 1972). The pigment composition of eustigmatophytes was also important in the definition of the new class, because their signature pigments differ significantly from those in xanthophytes (Whittle and Casselton 1969, 1975a, b). The taxonomic revision of eustigmatophyte diversity by Hibberd (1981) led to the recognition of 12 species in six genera. Subsequently, the advent of molecular phylogenetic methods confirmed that the Eustigmatophyceae is a monophyletic lineage distinct from the Xanthophyceae and all other classes of ochrophytes (Bhattacharya et al. 1992; Karlson et al. 1996; Andersen et al. 1998).

Since the seminal work of Hibberd (1981), knowledge of eustigmatophyte diversity has expanded with the transfer of additional species from the Xanthophyceae (Schnepf et al. 1996; Přibyl et al. 2012; Fawley and Fawley 2017) and the descriptions of new taxa (Lubián 1982; Karlson et al. 1996; Krienitz et al. 2000; Suda et al. 2002; Trzcińska et al. 2014; Fawley et al. 2015), including four new monotypic genera (Preisig and Wilhelm 1989; Neustupa and Němcová 2001; Hegewald et al. 2007; Nakayama et al. 2015). Recent culture-based and environmental DNA cloning studies have shown that the diversity of the Eustigmatophyceae is much greater than previously expected (Fawley et al. 2014). After 2010, research on eustigmatophytes entered a new phase with the determination of the first complete genome sequences and development of tools for targeted genetic manipulation in the representative genus, *Nannochloropsis* (Kilian et al. 2011; Pan et al. 2011; Radakovits et al. 2012; Vieler et al. 2012a; Corteggiani Carpinelli et al. 2014; Wang et al. 2014, 2016).

Practical Importance

Eustigmatophytes became the focus of attention and intensive research in recent years due to the potential commercial production of biofuels and bioproducts by some species. Most biotechnology-oriented studies have been performed on the minute marine species of the genus *Nannochloropsis* (including species belonging to the recently segregated genus *Microchloropsis*; Fawley et al. 2015). There has been exponential growth in the number of research papers published on this topic in recent years (e.g., Zou et al. 2000; Krienitz and Wirth 2006; Li et al. 2011; Simionato et al. 2011; Doan and Obbard 2012; Bartley et al. 2014; Xiao et al. 2015); most of the relevant literature concerning the lipid-related biotechnological research has been reviewed by Ma et al. (2016). Briefly, *Nannochloropsis* spp. are valued primarily for the ability to produce two types of lipidic substances – neutral lipids, i.e., various forms of triacylglycerol (TAG) and long-chain polyunsaturated fatty acids (LC-PUFAs), especially eicosapentaenoic acid (EPA). Depending on the cultivation conditions, the intracellular lipid levels in *Nannochloropsis* spp. may reach up to 55–60% of dry weight biomass and may exhibit elevated content of higher saturated fatty acids, being thus suitable for transesterification to biodiesel. *Nannochloropsis* spp. have also been extensively investigated for the production of commercially valuable carotenoid pigments (Lubián et al. 2000), sterols (Suen et al. 1987; Volkman et al. 1993; Patterson et al. 1994), and vitamin E (α-tocopherol; Durmaz 2007). *Nannochloropsis* spp. may also be used as cell reactors for the production of heterologous proteins (Chen et al. 2008) and have long been used as a food source in aquaculture (Duerr et al. 1998; Pfeiffer and Ludwig 2007; Patil et al. 2007; Ferreira et al. 2009).

Industrial use of other genera of the Eustigmatophyceae has been investigated to a lesser extent. *Monodopsis subterranea* (= *Monodus subterraneus*) and *Trachydiscus minutus* both produce large quantities of EPA (Cohen 1994; Hu et al. 1997; Qiang et al. 1997; Lu et al. 2001; Iliev et al. 2010; Řezanka et al. 2010; Cepák et al. 2014; Jo and Hur 2015). Members of the closely related genera *Vischeria* and *Eustigmatos* have also been noted for producing EPA, but also high amounts of β-carotene, and their lipid metabolism also make them promising biofuels producers (Volkman et al. 1999; Li et al. 2012a, b; Aburai et al. 2013; Zhang et al. 2013; Gao et al. 2016). Eustigmatophytes in general may also be regarded as promising antioxidant sources, for example, as documented by a recent survey of various strains from the Coimbra Collection of Algae (ACOI; Assunção et al. 2016).

Environmental bioremediation uses may also be envisaged for eustigmatophytes. For example, cells of *Microchloropsis gaditana* (as *Nannochloropsis gaditana*) were reported to accumulate practically 100% of the copper or zinc present in the medium (Moreno-Garrido et al. 2002). The eustigmatophyte strain nak-9, subsequently described as *Vacuoliviride crystalliferum* (Nakayama et al. 2015), was reported to exhibit a high efficiency in eliminating radioactive caesium from the medium by cellular accumulation (Fukuda et al. 2014). Inoculation of rice grown in hydroponic conditions with *Nannochloropsis* sp. ameliorated the impact of arsenic toxicity on plant growth (Upadhyay et al. 2016). Using municipal wastewater as a source of

nutrients for cultivation of *Nannochloropsis* sp. for biotechnological applications was also tested (e.g., Jiang et al. 2011).

Habitats and Ecology

Habitat preferences may differ substantially even within eustigmatophyte lineages and numerous transitions between freshwater and terrestrial habitats seem to have occurred during eustigmatophyte evolution. A transition to the marine environment probably occurred only once, in the lineage leading to an ancestor of the genera *Nannochloropsis* and *Microchloropsis*. An ancestor of the freshwater species *Nannochloropsis limnetica* must then have secondarily transitioned from marine to freshwater habitats (Fawley and Fawley 2007). Little is currently known about possible biogeographical patterns exhibited by eustigmatophyte species.

Members of the closely related genera *Vischeria* and *Eustigmatos* have been frequently isolated from soils (Petersen 1932; Vischer 1945; Neustupa and Němcová 2001) and from various subaerial habitats, such as tree bark (Nakano et al. 1991; Neustupa and Škaloud 2010), rocks (Czerwik-Marcinkowska and Mrozinska 2009), or desert crusts (Flechtner et al. 1998; Büdel et al. 2009), but they are also found in freshwater (Ott et al. 2015). The genus *Monodopsis* also occurs in soils worldwide as well as in freshwater (Ettl and Gärtner 1995; Ott et al. 2015). *Pseudellipsoidion edaphicum* was isolated from soil in Central Europe, whereas the related species *Pseudocharaciopsis ovalis* was reported from both soil and freshwater habitats (Neustupa and Němcová 2001).

Most members of the clade *Goniochloridales* (Fig. 1) and some other eustigmatophytes (e.g., *Pseudotetraëdriella kamillae*) are freshwater phytoplankton or associated with vegetation in freshwater (Ettl 1978; Schnepf et al. 1996; Přibyl et al. 2012; Fawley et al. 2014). The predominantly marine genus *Nannochloropsis* also comprises a freshwater species, *N. limnetica*, a member of picoplankton communities in lakes and ponds (Krienitz et al. 2000; Fietz et al. 2005; Fawley and Fawley 2007). An extremely abundant population of *N. limnetica* dominating the phytoplankton community and reaching up to 5.7×10^9 cells l^{-1} was reported in a hypertrophic lake in Germany (Krienitz et al. 2000), and it seems to be abundant primarily in periods of cold water (Fawley and Fawley 2007).

Mesotrophic and eutrophic lakes and ponds with neutral or slightly basic pH have proved to be a rich source of phylogenetically diverse eustigmatophyte strains (Fawley et al. 2014). On the other hand, many members of the Eustigmatophyceae are found associated with vegetation in acidic conditions and *Sphagnum* bogs (Ott et al. 2015; Karen and Marvin Fawley, pers. observation). *Chlorobotrys regularis* is frequently found in low-pH habitats such as *Sphagnum* bogs (Hibberd 1974), but some strains of *Chlorobotrys* isolated and held in the ACOI collection were isolated from lakes and ponds. Two unidentified eustigmatophytes were recorded by sequencing environmental 18S rDNA libraries from a peat bog in Switzerland (Lara et al. 2011; Fig. 1). Most recently, *Tetraëdriella subglobosa*, originally found by Pascher (1930) in acidic pools in Czechoslovakia, proved to be a

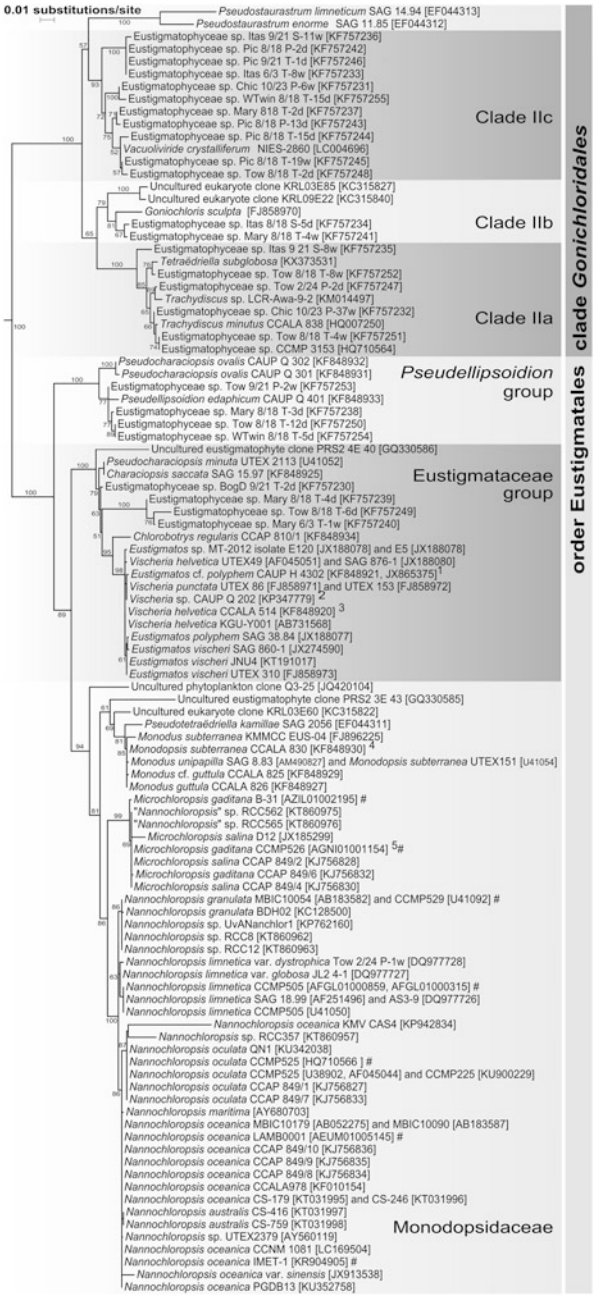

Fig. 1 The phylogenetic diversity of Eustigmatophyceae deduced from 18S rRNA gene sequences. The tree was inferred using the maximum likelihood method (RAxML) from an alignment of nearly all available eustigmatophyte 18S rRNA gene sequences plus sequences from selected non-eustigmatophyte ochrophytes used as an outgroup (not shown). The main eustigmatophyte

eustigmatophyte upon its re-isolation from an acidic pool near the shore of Lake Mácha, Czech Republic (Fawley and Fawley 2017).

The only known eustigmatophytes inhabiting marine or brackish habitats belong to the picoplanktonic genera *Nannochloropsis* (including also the freshwater species *N. limnetica*) and *Microchloropsis*. These species can form blooms in rock pools and enriched or polluted waters (Ryther 1954; Bourrelly 1958). An extensive bloom of *Microchloropsis* (=*Nannochloropsis*) *gaditana* was reported from brackish Comacchio lagoons in Italy (Andreoli et al. 1999a), whereas *Nannochloropsis granulata* was encountered as a large-scale bloom-causing species in China (in Bohai Sea and Yellow Sea; Zhang et al. 2015).

Eustigmatophytes may also be found in various less usual habitats. Frost et al. (1997) reported an unidentified eustigmatophyte endosymbiont living inside the freshwater sponge *Corvomeyenia everetti*. An unknown eustigmatophyte was detected in a wastewater treatment pond by sequencing an environmental library of the *rbc*L gene (Ghosh and Love 2011). A *Nannochloropsis* species related to *N. limnetica* was reported from a permanently ice-covered lake in Antarctica (Bielewicz et al. 2011). *Trachydiscus minutus* was found to constitute a dominant planktonic alga in a eutrophic cooling pond of a nuclear power plant (Přibyl et al. (2012). A new eustigmatophyte, *Eustigmatos calaminaris*, was recently described from Zn- and Pb-loaded calamine mine spoils (Trzcińska et al. 2014). Finally, *Vacuoliviride crystalliferum* was isolated from green-colored sediment in a bottle of glue (Nakayama et al. 2015).

Characterization and Recognition

General Appearance

Eustigmatophytes are spherical, polyhedral, stellate, ovoid, fusiform, or discoid in shape and vary in size generally between 2 and 25 μm in the longest dimension, although some species may have much larger cells (for example, some *Characiopsis*-like organisms, personal observation). The morphological diversity

Fig. 1 (continued) lineages are annotated following the scheme proposed by Fawley et al. (2014). The number sign (#) marks strains for which nuclear genome sequence has been reported (see Table 1). Number in superscript at some taxa indicate that taxa (strains) with identical 18S rRNA gene sequences exist that were not included in the figure: [1]*Eustigmatos vischeri* CCAP 860/7 [KJ713283]; [2]*Chloridella neglecta* SAG 48.84 [KF848924] and *Eustigmatos magna* CCMP387 [U41051]; [3]*Eustigmatos polyphem* CAUP Q 102 [KF848922], *Vischeria stellata* SAG 33.83 [KF848919], *Chloridella simplex* CCALA 279 [KF848923], and "*Ophiocytium majus*" CCAP 855/1 [AM490835]; [4]*Monodus guttula* CCALA 828 [KF848928], *Monodus* sp. CAUP D 901 [KF848926], and *Monodopsis* sp. MarTras21 [KP347780]; [5]*Microchloropsis gaditana* MBIC10418 [AB052271], MBIC10063 [AB183586], CCMP527 [AFGN01000274] #, *Microchloropsis salina* CCMP537 [AF045049] #, and CCMP1776 [AFGQ01000729, AFGQ01000649] #

of eustigmatophytes is documented in Fig. 2. Most vegetative cells are free floating, but *Pseudocharaciopsis* spp. and other *Characiopsis*-like algae may normally or occasionally be attached. Eustigmatophytes form green or yellow-green cultures, so some may be confused with coccoid members of the Xanthophyceae or Chlorophyta (green algae). However, eustigmatophytes can be distinguished by the presence of a red-orange pigmented body in the cytoplasm that is especially prominent in older vegetative cells. This lipidic body also has a characteristic yellow fluorescence when excited with ultraviolet light (Přibyl et al. 2012). Some eustigmatophytes also have highly sculpted cell walls (Santos and Santos 2001; Přibyl et al. 2012; Fawley et al. 2014; Fawley and Fawley 2017), although careful examination is required to detect the sculpting on small cells. Because many xanthophytes were described before the recognition of the distinctions between the two classes, some taxa currently classified as Xanthophyceae will likely be reassigned to the Eustigmatophyceae upon more detailed study.

Vegetative Cell Structure

Careful light and, for most species, electron microscopical observations of eustigmatophyte vegetative cells (Hibberd and Leedale 1972; Lee and Bold 1973; Hibberd 1974; Preisig and Wilhelm 1989; Santos and Leedale 1995; Schnepf et al. 1996; Neustupa and Němcová 2001; Hegewald et al. 2007; Přibyl et al. 2012; Nakayama et al. 2015) have revealed key morphological and ultrastructural characteristics for the whole group, as well as features specific to particular taxa. Most eustigmatophytes contain a single-lobed parietal plastid (Fig. 2); however, multiple plastids have been observed in *Pseudellipsoidion* (Neustupa and Němcová 2001), *Pseudocharaciopsis ovalis* (Hibberd 1981), and *Pseudotetraëdriella* (Hegewald et al. 2007). The plastid lamellae are evenly spaced (Fig. 3a–c) and are composed of three unappressed thylakoids. Additional thylakoids commonly appear between some of the lamellae, particularly at the edge of the plastid, forming granum-like stacks. The plastids do not contain a girdle lamella, and the longitudinal lamellae terminate close to the plastid envelope (Fig. 3a–c). The plastid envelope consists of four membranes with the outermost representing the plastid endoplasmic reticulum (PER). Connection of the PER with the nuclear envelope, otherwise a general characteristic of ochrophyte algae, has been reported only from the genera *Nannochloropsis* (incl. *Microchloropsis*) and *Monodopsis* (Antia et al. 1975; Santos and Leedale 1995; Karlson et al. 1996; Krienitz et al. 2000; Suda et al. 2002).

Pyrenoids are present in vegetative cells of several species, but never in the zoospores. In some eustigmatophytes, the pyrenoid is polyhedral (*Vischeria* spp., *Eustigmatos* spp., *Chlorobotrys regularis*) or globular (*Pseudocharaciopsis minuta*) and separated from the plastid itself by a narrow stalk (Fig. 3b), whereas the genera *Monodopsis* and *Vacuoliviride* form a bulging pyrenoid without a stalk (Santos and Leedale 1995; Nakayama et al. 2015). A pyrenoid has been reported for two species of the genus *Nannochloropsis* (Antia et al. 1975), but its presence could not be confirmed by subsequent studies (Santos 1996). The organism studied by

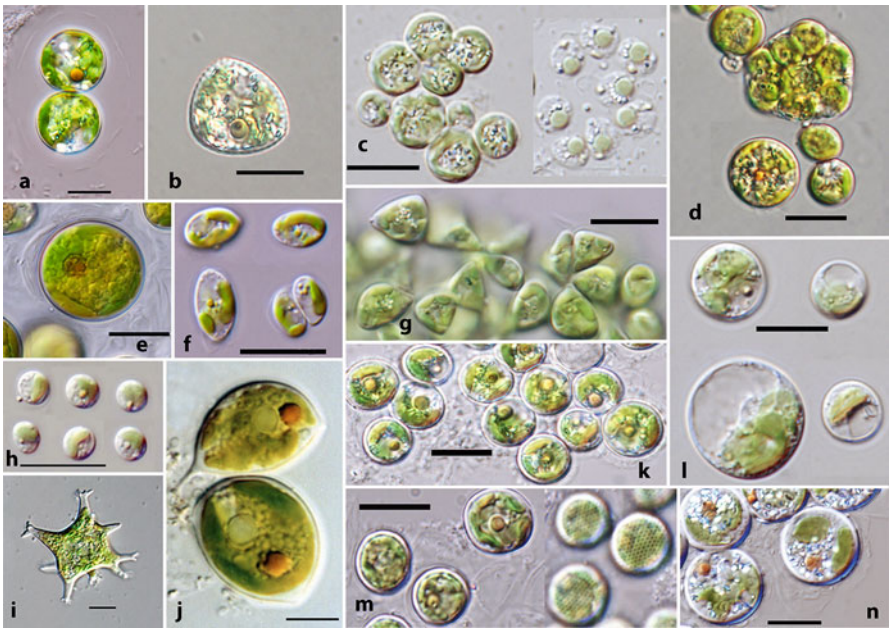

Fig. 2 Light micrographs of representative Eustigmatophyceae. (**a**) *Chlorybotrys* sp. UP3 5/31-7m (Eustigmataceae). (**b**) *Goniochloris sculpta* SAG 29.96 (Clade IIb). (**c**) Unidentified strain Mary 8/18 T-4d (Clade Ia); vegetative cells (*left*) and zoospores (*right*). (**d**) *Pseudellipsoidion edaphicum* CAUP Q 401 (*Pseudellipsoidion* group). (**e**) *Eustigmatos polyphem* (Eustigmataceae group). (**f**) *Monodus unipapilla* Skal1 4/27-2w (Monodopsidaceae). (**g**) Unidentified strain Itas 8/18 S-5d (Clade IIb). (**h**) *Nannochloropsis limnetica* CCMP 2271 (Monodopsidaceae). (**i**) *Pseudostaurastrum* sp. strain 10174 (*Goniochloridales*). (**j**) *Characiopsis acuta* ACOI 456 (Eustigmataceae group). (**k**) Unidentified strain Pic 8/18 T-15d (Clade IIc). (**l**) Unidentified strain Pic 9/21 T-1d (Clade IIc). (**m**) Unidentified strain Chic 10/23 P-37 (Clade IIa), illustrating wall sculpting (*right*). (**n**) Unidentified strain WTwin 8/18 T-15d (Clade IIc). *Bars* = 10 μm

Mohammady et al. (2004) under the name *Nannochloropsis salina* and exhibiting a prominent pyrenoid with a starch envelope is undoubtedly a green alga. The pyrenoid matrix of eustigmatophytes appears finely granular or homogeneous and is not penetrated by either plastid lamellae or individual thylakoids (Fig. 3b). The plates of refractive material that can be seen lying against the faces of the pyrenoids in the light microscope are represented in sections by flattened vesicles containing material that appears very finely lamellate after fixation. Smaller vesicles with apparently identical contents, called lamellate vesicles or refractile granules, also lie freely in the cytoplasm of both the vegetative cells and zoospores (Fig. 3g). These highly characteristic structures of all eustigmatophytes (Santos 1996) probably represent a storage material in the form of a β-1-3-linked polysaccharide (Schnepf et al. 1996).

Mitochondria contain tubular cristae as in other stramenopiles (Fig. 3c, f). A mitochondrion-dividing ring reminiscent of those known from the red alga

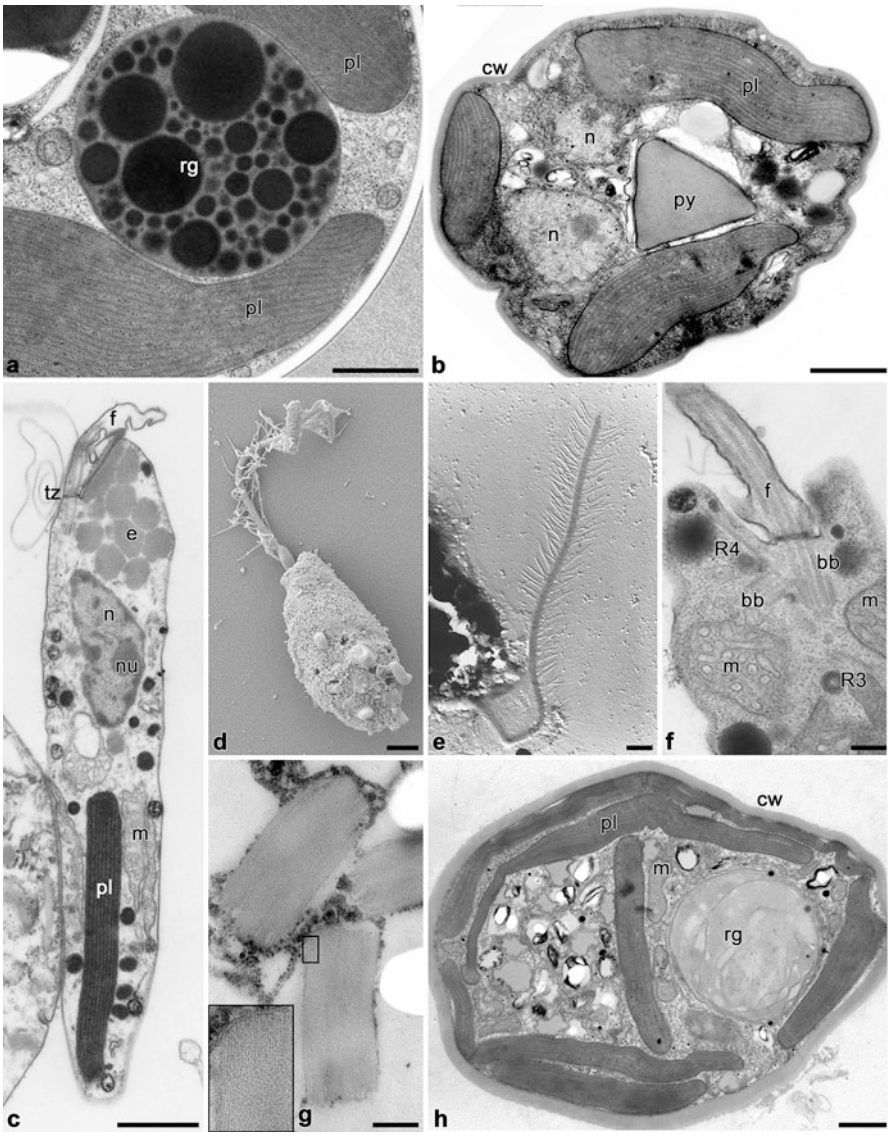

Fig. 3 Cell ultrastructure in the Eustigmatophyceae. (**a**) Detail of a plastid (without the girdle lamella) and a reddish globule of *Vischeria helvetica* (*pl* plastid, *rg* reddish globule). (**b**) Section of a vegetative cell of *Eustigmatos magna*, presumably on a way to cytokinesis (note the two nuclei; *cw* cell wall, *n* nucleus, *p* pyrenoid, *pl* plastid). (**c**) Zoospore of *Vischeria helvetica* (*e* eyespot, *f* flagellum, *m* mitochondrion, *n* nucleus, *nu* nucleolus, *pl* plastid, *tz* transitional zone of the flagellum). (**d**) Zoospore of *Trachydiscus minutus* in a scanning electron microscope. (**e**) Detail of the flagellum bearing mastigonemes, *Vischeria stellata*. (**f**) Longitudinal section of the flagellar apparatus of *Vischeria stellata* uniflagellate zoospore showing basal bodies with R3 and R4 flagellar roots; R1 and R2 are not visible on the section (*f* flagellum, *m* mitochondrion, *R3* flagellar root 3, *R4* flagellar root 4). (**g**) Lamellate vesicles containing material that appears very finely lamellate after

Cyanidioschyzon merolae has been described in the eustigmatophyte *Nannochloropsis oculata* (Hashimoto 2004). The nucleus is more or less spherical (Fig. 3b) but is relatively inconspicuous in the light microscope and can usually be clearly identified only in younger cells. Much more conspicuous is a vacuole with granular contents exhibiting Brownian movement. A highly characteristic structure of most eustigmatophytes, especially of older cells, is the so-called reddish globule (e.g., Fig. 2a, b, j, n). Its color varies from pale yellow-brown to dark red-brown and becomes larger and darker with age. In some species, the reddish globule is composed of a number of smaller droplets (Fig. 3a), but it is homogeneous in *Chlorobotrys regularis* (Hibberd 1974) or with a core less electron dense than the rest of the globule in *Vacuoliviride crystalliferum* (Nakayama et al. 2015). Prior to reproduction, the reddish globule does not divide but is completely inherited by one of the daughter autospores (Neustupa and Němcová 2001). A unique, rod- or V-shaped crystalline structure associated with the reddish globule was observed in *Vacuoliviride crystalliferum* (Nakayama et al. 2015).

The cell wall of eustigmatophytes is in one piece (Fig. 3b, h), but more than one layer can be seen, especially in older cells. The cell wall is generally smooth, but ornamentation with sculpting is seen in some members of the *Goniochloridales* (Fig. 2b, m; Přibyl et al. 2012; Fawley et al. 2014; Fawley and Fawley 2017). The composition of the eustigmatophyte cell wall has not been thoroughly investigated for most taxa, but it seems to be composed primarily of cellulose (Okuda et al. 2004; Vieler et al. 2012a). Recently, the cell wall of *M. gaditana* was studied in great detail and found to exhibit a bilayer structure consisting of a cellulosic inner wall protected by an outer hydrophobic alganean layer comprising long, straight-chain, saturated aliphatic hydrocarbons with ether cross-links (Scholz et al. 2014). The genus *Chlorobotrys* is unique in having a refractile wall exhibiting a high degree of flexibility and in being surrounded by concentric mucilaginous layers separated by tripartite membrane-like structures (Fig. 2a), probably composed of pectic materials with very little cellulose (Hibberd 1974). Biomineralization of manganese on the stalk surface was observed in *Pseudocharaciopsis minuta* (Wujek 2012).

Zoospore Structure

Eustigmatophyte genera, with the exception of *Nannochloropsis*, *Microchloropsis*, *Monodopsis*, *Chlorobotrys*, *Vacuoliviride*, and *Tetraëdriella*, are known to produce naked, somewhat amoeboid, oval, or lageniform (flask-shaped) zoospores, with one or two subapically inserted flagella (Figs. 2c and 3c–f). The zoospores generally harbor a single anteriorly positioned nucleus, a single plastid always without a pyrenoid, one or more mitochondria, a number of vesicles with lamellate or spiral

Fig. 3 (continued) fixation (enlarged lower-left figure), *Pseudocharaciopsis ovalis*. (**h**) Section of a vegetative cell of *Pseudocharaciopsis ovalis* (*cw* cell wall, *pl* plastid, *m* mitochondrion, *rg* reddish globule). (**a**–**e**): bar = 1 μm; (**f**–**g**): bar = 0.2 μm; (**h**): bar = 1 μm

content, and a Golgi body (Hibberd and Leedale 1972; Lee and Bold 1973; Preisig and Wilhelm 1989; Santos and Leedale 1992; Schnepf et al. 1996).

The zoospores of some eustigmatophytes bear just one emergent flagellum (Fig. 3c–f), but at least in those species studied by electron microscopy a second bare kinetosome (basal body) lies closely associated with the one subtending the flagellum. A pair of unequal flagella was found in the zoospores of *Pseudocharaciopsis* spp. (Lee and Bold 1973; Hibberd 1981; Neustupa and Němcová 2001) and *Botryochloropsis similis* (Preisig and Wilhelm 1989), where the posterior flagellum is shorter, very narrow, and not readily apparent in the light microscope. Except for a short proximal part, the posterior flagellum exhibits a simplified structure of the axoneme consisting of only two central microtubules. The single flagellum of the unimastigote zoospores and the anterior flagellum in bimastigote zoospores bear tripartite tubular hairs (mastigonemes) of the same type as found in other ochrophytes (Fig. 3d, e) and exhibit a characteristic basal swelling appressed against the anterior region of the cell body containing the eyespot, if present (Hibberd and Leedale 1972; Santos and Leedale 1991; Schnepf et al. 1996).

The eyespot (stigma) is by far the most conspicuous feature of the eustigmatophyte zoospores and is often considered to be characteristic for the group. However, it is reportedly absent from zoospores of all members of the clade *Goniochloridales* studied so far (*Pseudostaurastrum limneticum*, *Goniochloris sculpta*, and *Trachydiscus minutus*; Schnepf et al. 1996; Přibyl et al. 2012) and also from one member of the Eustigmatales (*Pseudotetraëdriella kamillae*; Hegewald et al. 2007). The eyespot is red-orange in color and its size differs depending on the species (being very large and filling almost the whole of the extreme anterior end of the zoospore in some eustigmatophytes). It lies outside and quite separate from the plastid (Fig. 3c). It is composed of a number of osmiophilic globules of variable size, and neither the eyespot as a whole nor the globules are membrane bound. One large D-shaped droplet lies closely against the cell membrane opposite to the flagellar swelling. The basal swelling/eyespot complex is a probable photoperceptive system with the eyespot serving to enhance contrast. The identity of the actual photoreceptor substance is unknown, but it is thought to emit a green autofluorescence observed in the basal swelling/eyespot region upon excitation with a blue-violet light (Santos et al. 1996).

The transitional region between the basal body and the axoneme consists of a transverse partition and a transitional helix with three to five gyres surrounding the proximal few nanometres of the central pair of the axoneme (Fig. 3c). Flagellar roots have been reconstructed for zoospores of *Vischeria stellata* (Santos and Leedale 1991). Roots R1 and R2 originate on the opposite sides of the flagellum-bearing basal body and run anteriorly supporting the anterior part of the cell associated with the flagellar swelling. Root R3 is attached with dense material between the basal bodies and runs close to the plasma membrane down to the posterior end of the cell, whereas root R4 extends from the bare basal body. The fibrous rhizoplast connects the basal bodies and extends deep into the cell, where it splits into several branches spreading over the nuclear surface (Santos and Leedale 1991).

Reproduction and Life Cycle

Eustigmatophytes reproduce primarily by the formation of autospores (Fig. 2d, f). There may be two D-shaped or four or eight polyhedral autospores per autosporangium; in the more elongate species, the autospores are arranged more or less longitudinally within the parent cell wall before release. Details of cytokinesis in eustigmatophytes have yet to be worked out, but nuclear and plastid division were followed at the ultrastructural level in *Nannochloropsis oculata* (Murakami and Hashimoto 2009). The nucleus divides by closed mitosis, and the inner nuclear envelope constricts and pinches off before the outer nuclear envelope.

An alternative reproduction mode in eustigmatophytes is zoosporogenesis. Some species produce zoospores in relatively small quantities, and the genera *Nannochloropsis*, *Microchloropsis*, *Monodopsis*, *Vacuoliviride*, and *Chlorobotrys* (Hibberd 1974; Antia et al. 1975; Santos 1996; Nakayama et al. 2015) even appear to lack this ability completely. On the other hand, reproduction solely by zoospores was described in *Pseudostaurastrum limneticum* (Schnepf et al. 1996) and *Pseudotetraëdriella kamillae* (Hegewald et al. 2007). Factors inducing zoosporogenesis have not been systematically investigated, but zoospore production in *Trachydiscus minutus* is induced by darkness, suppressed by light, and depends on the temperature (Přibyl et al. 2012). In *Characiopsis*-like eustigmatophytes, zoospores are formed briefly after subculturing old cultures (personal observation). Profound morphological variability in vegetative cell shape, including formation of thick-walled resting cells, was reported as part of the life cycle of *Pseudocharaciopsis ovalis* and *Vischeria* sp. strains (Neustupa and Němcová 2001). Similarly, Fietz et al. (2005) observed thick-walled resting stages in *Nannochloropsis limnetica* germinating by releasing a single cell.

Sexual reproduction is unknown in eustigmatophytes and was suggested to be absent in *Nannochloropsis oceanica* based on evidence from genome sequencing (Pan et al. 2011). However, analyses of the genome sequence of two strains of *Microchloropsis* (=*Nannochloropsis*) *gaditana* unveiled a suite of genes encoding homologs of proteins involved in meiosis, including the meiosis-specific proteins Spo11, Hop1, Hop2, Mnd1, Dmc1, and Msh5 (Radakovits et al. 2012; Corteggiani Carpinelli et al. 2014). This suggests the possible existence of a cryptic sexual cycle in this species.

Genomics, Molecular Biology, and Biochemistry

Knowledge of eustigmatophyte biology at the biochemical and molecular level is heavily biased toward the genus *Nannochloropsis* (sensu *lato*), with very limited information available for other eustigmatophytes. A major change in this field came with the advent of eustigmatophyte genome sequencing, which yielded data now being explored by in silico analyses and inspiring direct experimental research. The latter has been boosted by development of a suite of methods of genome

manipulations for different species of *Nannochloropsis* and *Microchloropsis*. Exogenous DNA can now be readily introduced into the cells by electroporation and integrated with high efficiency and specificity into the nuclear genome by homologous recombination with the target region (Chen et al. 2008; Kilian et al. 2011; Radakovits et al. 2012; Vieler et al. 2012a; Li et al. 2014a; Kaye et al. 2015; Iwai et al. 2015). These techniques enable gene knockout, overexpression, or expression of modified or foreign genes in *Nannochloropsis* or *Microchloropsis* genomes. For example, overexpression of an endogenous Δ12 desaturase driven by a stress-inducible promoter led to enhanced deposition of LC-PUFAs in TAG, demonstrating the power of genetic manipulations for improving the biotechnological utility of eustigmatophytes (Kaye et al. 2015). Moog et al. (2015) used predicted localization signals of several authentic proteins of *N. oceanica* to drive tagged variants of green fluorescent protein (GFP) into different compartments of transformed *N. oceanica* cells. This study thus opens up new possibilities for cell biological research of eustigmatophytes. Most recently, CRISPR/Cas9 technology has been successfully applied to *N. oceanica* for targeted genome editing (Wang et al. 2016).

Below, general characteristics of eustigmatophyte nuclear and organellar genomes are briefly reviewed, and examples of interesting insights into the molecular fabric of eustigmatophyte cells enabled by in silico analyses of genome data are discussed. An overview of the most important aspects of the eustigmatophyte metabolism as unveiled in recent years by a combination of in silico and direct experimental approaches is then provided.

Nuclear Genomes

Thanks to the efforts of several research groups utilizing the increasingly available next-generation sequencing technologies, more or less complete nuclear genome sequences have become available for most *Nannochloropsis* species (the only exception being the recently described *Nannochloropsis australis*) and for both *Microchloropsis* species (Table 1). Genome sequences for more multiple strains have even been reported for *Nannochloropsis oceanica* and *Microchloropsis gaditana*. The reported genome size varies from ~25 to ~35 Mbp, and the number of predicted genes ranges from ~6,600 to ~12,000. The differences in these values within the two genera and even between strains of the same species are striking and may partly reflect technical issues stemming from different strategies employed for sequencing, assembly, and annotation of the genomes. These uncertainties notwithstanding, *Nannochloropsis* and *Microchloropsis* species apparently belong to the category of algae with small genomes (for comparison, see, e.g., Kim et al. 2014), with relatively high gene density and low intron density (Radakovits et al. 2012; Vieler et al. 2012a; Corteggiani Carpinelli et al. 2014; Wang et al. 2014). Whether this can be generalized to eustigmatophytes as a whole is presently unknown.

The most complete information about genome organization is available for *M. gaditana* B-31, which was estimated to have 30 chromosomes based on the number of putative telomeric ends identified in the assembly (Corteggiani Carpinelli et al. 2014) and for *N. oceanica* IMET1, which was suggested to have 22 chromosomes using pulsed-field gel electrophoresis (Wang et al. 2014). The telomeric

Table 1 Sequenced nuclear genomes of eustigmatophytes. Note that the species here assigned to the genus *Microchloropsis* are treated as species of the genus *Nannochloropsis* in the respective references and database records

Species	Genome assembly size (Mbp)	Number of predicted genes	References
Nannochloropsis granulata CCMP529	30.1	8,060	Wang et al. (2014)
Nannochloropsis limnetica CCMP505	33.5	?	Xu et al. unpublished (GenBank accession number AFGL00000000.1)
Nannochloropsis oceanica CCMP1779	28.7	11,973	Vieler et al. (2012a)
Nannochloropsis oceanica CCMP531	35.5	7,268	Wang et al. (2014)
Nannochloropsis oceanica IMET1	30.1	9,915	Wang et al. (2014)
Nannochloropsis oceanica LAMB0001	27.6	6,639	Pan et al. (2011)
Nannochloropsis oceanica OZ-1	28.0	?	Xu et al. unpublished (GenBank accession number AFGK01000000.1)
Nannochloropsis oculata CCMP525	34.5	7,254	Wang et al. (2014)
Microchloropsis gaditana B-31	26.3	10,486	Corteggiani Carpinelli et al. (2014)
Microchloropsis gaditana CCMP526	29.0	8,892	Radakovits et al. (2012)
Microchloropsis gaditana CCMP527	25.6	?	Xu et al. unpublished (GenBank accession number AFGN00000000.1)
Microchloropsis salina CCMP537	26.9	6,562	Wang et al. (2014)

repeat of *M. gaditana* B-31 corresponds to the "human-type" motif TTAGGG (Corteggiani Carpinelli et al. 2014), but whether it is common in eustigmatophytes in general is uncertain, because direct testing of the presence of this telomeric sequence in *Eustigmatos polyphem* and *Vischeria punctata* by Southern hybridization failed to confirm this (Fulnečková et al. 2013). Based on the analysis of Pan et al. (2011), *Nannochloropsis oceanica* LAMB0001 is monoploid (haploid), while the ploidy of other eustigmatophytes with sequenced genomes was not investigated closer. An analysis of gene orientation along the *M. gaditana* genome revealed a pattern suggesting frequent deployment of a single bidirectional promoter to control

the expression of two neighboring genes with head-to-head orientation (Jinkerson et al. 2013)

Comparative analyses of the gene complements of *Nannochloropsis* and *Microchloropsis* spp. revealed a surprising level of differences. Corteggiani Carpinelli et al. (2014) clustered genes annotated in genomes of two strains of *Microchloropsis (=Nannochloropsis) gaditana* and two strains of *Nannochloropsis oceanica* and found that only ~4600 clusters comprise homologs present in all four strains, although the total number of annotated genes were between ~9000 and ~11,000 in each strain. This was not only because of differences between the two species, as the strains of the same species also differed in the presence/absence of hundreds of genes. An analysis by Wang et al. (2014) including six strains and five *Nannochloropsis-Microchloropsis* species found an even smaller set of ~2700 core genes shared by all the taxa, whereas the pan-genome, i.e., the totality of all clusters of homologous genes and gene singletons in the six strains was a surprising ~38,000 genes. While these numbers are certainly impacted by genome annotation artifacts, it seems well established that there is considerable diversity within the *Nannochloropsis-Microchloropsis* group at the level of gene repertoire (Wang et al. 2014), which is in stark contrast to the low differences between the strains and species in their 18S rRNA gene sequences (Fig. 1). The evolutionary origin and functional significance of this diversity is yet to be worked out.

In addition to these general aspects of the gene content of eustigmatophyte genomes, many crucial insights into the molecular underpinnings of various structures and processes in eustigmatophyte cells have been obtained by in silico analyses of the sequenced genomes. Analyses of different functional gene categories generally show standard sets of genes expected for a unicellular alga, although relative enrichment of some gene categories, including genes related to lipid metabolism, organic acid metabolism, and stress response, was noted (Radakovits et al. 2012; Vieler et al. 2012a; Corteggiani Carpinelli et al. 2014; Wang et al. 2014). Most attention has been paid to investigating genes related to metabolism, which is discussed in a separate section below. Here, some of the more interesting findings concerning other aspects are highlighted.

Above all, genome analyses provided some insights into regulatory and signaling processes in eustigmatophyte cells. For example, several studies addressed the repertoire of transcription factors and found that the MYB family is the dominant group in eustigmatophytes, whereas some families common in many other eukaryotes, e.g., homeobox and MADS-box genes, are missing (Vieler et al. 2012a; Hu et al. 2014; Thiriet-Rupert et al. 2016). Eustigmatophytes proved to possess the core components of the machinery for RNA-mediated silencing, including Argonaute, Dicer, and RNA-dependent RNA polymerase (Corteggiani Carpinelli et al. 2014). This suggests the ability to employ RNA interference as a defense mechanism against parasitic genetic elements (transposons or viruses), but the machinery may also be involved in processing of miRNAs and deploying them for regulation of endogenous gene expression, as putative miRNA genes were identified in the *N. oceanica* genome (Vieler et al. 2012a). Eustigmatophyte genomes also harbor homologs of blue light receptors common in eukaryotes in general

(cryptochromes) or specific for ochrophytes (aureochromes), suggesting the ability to sense blue light in the environment (Vieler et al. 2012a; Thiriet-Rupert et al. 2016).

Analyses of the *N. oceanica* genome by Vieler et al. (2012a) also led to a discovery that subsequently proved to be important concerning the evolution and function of mitochondria in eukaryotes. These authors pointed to the fact that the nuclear genome encodes homologs of bacterial MinC and MinD proteins with predicted mitochondrial targeting signals and speculated that these might be novel components of mitochondrial division machinery, given the known function of Min proteins in bacterial cell division. Leger et al. (2015) subsequently showed that not only eustigmatophytes but also a number of other eukaryotic lineages possess a previously unnoticed mitochondrial Min system (comprised of MinC, MinD, and MinE proteins) apparently inherited from the bacterial ancestor of mitochondria and presumably involved in regulating mitochondrial division mediated by the mitochondrial FtsZ protein. This example suggests that eustigmatophytes may prove useful as model organisms for investigating general questions of eukaryotic molecular and cell biology.

Organellar Genomes

In contrast to the nuclear genome sequences so far restricted only to the genera *Nannochloropsis* and *Microchloropsis*, organellar genomes have been surveyed more broadly in eustigmatophytes. Specifically, sequences of both organellar genomes have been published not only for most *Nannochloropsis* species (except *N. australis*) and for both *Microchloropsis* species (Radakovits et al. 2012; Wei et al. 2013; Corteggiani Carpinelli et al. 2014; Starkenburg et al. 2014) but also for three species from different branches of the eustigmatophyte phylogeny: *Monodopsis* sp. MarTras21 (representing a sister lineage of the *Nannochloropsis-Microchloropsis* group), *Vischeria* sp. CAUP Q 202 (representing the more distantly related Eustigmataceae group), and *Trachydiscus minutus* (a representative of the clade *Goniochloridales*) (Ševčíková et al. 2015, 2016; Yurchenko et al. 2016). This sampling allows for inferring not only the general characteristics of eustigmatophyte organellar genomes but also their evolutionary plasticity within the group.

Eustigmatophyte mitochondrial genomes (mitogenomes) are conventional in their architecture (circular-mapping molecules), size (from ~38 to ~46 kbp), and gene content (26–29 tRNA genes, 3 rRNA genes, and 37–40 protein coding genes plus nonconserved ORFs specific for particular eustigmatophyte subgroups) (Starkenburg et al. 2014; Ševčíková et al. 2016). An early study proposed that eustigmatophyte mitochondria use the standard genetic code, in contrast to the mitochondria of superficially similar xanthophytes employing a deviant genetic code with the codon AUA coding for methionine rather than isoleucine (Ehara et al. 1997). This has been corroborated by full genome sequencing, which also revealed the presence of a gene for the Ile-tRNA cognate to the AUA codon in eustigmatophyte mitogenomes (Ševčíková et al. 2016). *Trachydiscus minutus* and members of the *Nannochloropsis-Microchloropsis* lineages independently lost the nucleus-encoded mitochondrion-targeted translation termination factor mRF2 and consequently do not use UGA as a termination codon, but no indication of UGA

being reassigned in these taxa as a sense codon (as in mitochondria of many other eukaryotes) was found. An unusual feature of eustigmatophyte mitogenomes is the presence of the *atp1* gene. This is an ancestral condition retained also by non-ochrophyte stramenopiles such as oomycetes, whereas all other ochrophyte classes have lost the mitochondrial *atp1* gene and the Atp1 protein is encoded by a nuclear copy. Eustigmatophyte mitogenomes also uniquely share a truncated *nad11* gene encoding only the C-terminal part of the Nad11 protein, while the N-terminal part is encoded by a separate gene in the nuclear genome. Whereas the gene order of most eustigmatophyte mitogenomes is highly similar, the *Vischeria* sp. CAUP Q 202 genome has been extensively reshuffled, coinciding with the loss of several mitochondrial genes and accelerated evolution of mitochondrial gene sequences in the *Vischeria* lineage (Ševčíková et al. 2016).

All sequenced eustigmatophyte plastid genomes (plastomes) are typical circular-mapping molecules (from ~115 to ~126 kbp in size) with short and long single-copy regions separated by inverted repeats, with the number of genes subsumed to the inverted repeat somewhat differing between the species (Starkenburg et al. 2014; Yurchenko et al. 2016). Their gene content is highly similar (25–28 tRNA genes, 3 rRNA genes, the *ssrA* gene for tmRNA, 124–128 genes coding for typical conserved plastid proteins, and a varying number of nontypical or nonconserved genes) and generally resembles that of other ochrophytes, with several notable exceptions. Firstly, eustigmatophytes plastomes are interesting in that they possess the gene *ycf49*, so far additionally found only in plastomes of cyanidiophyte red algae and the glaucophyte *Cyanophora paradoxa* (Ševčíková et al. 2015). Secondly, the gene for the ClpC protein has been split into three separate genes, encoding the N-terminal domain and the two AAA+ domains as separate polypeptides that presumably assemble into a functional protein (Starkenburg et al. 2014; Ševčíková et al. 2015; Yurchenko et al. 2016). The split of the N-terminal domain is shared with the sole-sequenced plastome of a chrysophyte, *Ochromonas* sp. CCMP1393, supporting the notion that Eustigmatophyceae and Chrysophyceae are related ochrophyte lineages (Ševčíková et al. 2015). Thirdly, the plastomes of *Vischeria* sp. CAUP Q 202 and *Monodopsis* sp. MarTras21 were surprisingly found to harbor a six-gene cluster (inserted between the *ycf54* and *rpl21* genes) acquired from a bacterial donor via horizontal gene transfer (Yurchenko et al. 2016). In silico analyses of these genes revealed that they constitute a novel putative operon, denoted *ebo*, which is quite widespread in bacteria and encodes enzymes of an uncharacterized pathway of secondary metabolism. The significance of the *ebo* operon for eustigmatophyte biology is not yet clear, but its presence in members of two main subgroups of the Eustigmatales indicates it must have been acquired early in eustigmatophyte evolution and secondarily lost in the *Nannochloropsis-Microchloropsis* lineage (Yurchenko et al. 2016).

Metabolism

For most eustigmatophyte species, biochemical analyses have been generally restricted to the composition of plastid pigments (Whittle and Casselton 1975a; Preisig and Wilhelm 1989; Santos 1996; Schnepf et al. 1996; Karlson et al. 1996;

Krienitz et al. 2000; Lubián et al. 2000; Suda et al. 2002). The group is unique among ochrophyte algae in that no form of chlorophyll *c* is detectable by HPLC. All species contain β-carotene. Violaxanthin is the major xanthophyll along with vaucheriaxanthin (-ester) and sometimes other minor forms (zeaxanthin, canthaxanthin, astaxanthin), but fucoxanthin, diadinoxanthin/diatoxanthin, or heteroxanthin are not detected. Violaxanthin is both a light-harvesting pigment (Owens et al. 1987; Keşan et al. 2016) and a component of the xanthophyll cycle protecting the photosynthetic apparatus against an excess of light via non-photochemical fluorescence quenching (Lubián and Montero 1998; Gentile and Blanch 2001; Bína et al. 2017). The major light-harvesting antenna of eustigmatophytes, homologous to the better-known diatom FCP (Fucoxanthin Chlorophyll Protein), is accordingly called VCP (Viola−/Vaucheriaxanthin Chlorophyll Protein) (Sukenik et al. 2000; Carbonera et al. 2014; Litvín et al. 2016). Very recently, the molecular architecture and subunit composition of the photosystem I (PSI) supercomplex were reported for two species of the *Nannochloropsis*-*Microchloropsis* group, revealing unprecedented features of the PSI antenna complexes (Basso et al. 2014; Alboresi et al. 2017; Bína et al. 2016).

Carbon metabolism in eustigmatophytes appears fairly standard and includes glycolysis and gluconeogenesis, the Krebs cycle, oxidative and reductive pentose phosphate pathway, as well as the glyoxylate cycle (Radakovits et al. 2012; Vieler et al. 2012a). Analyses of the predicted gene complements in *M. gaditana* and *N. oceanica* suggested the operation of several carbon-concentration mechanisms in these algae that would enable both C_3- and C_4-type carbon assimilation (Radakovits et al. 2012; Vieler et al. 2012a). Genes for enzymes of polysaccharide metabolism were also annotated in eustigmatophyte genomes and include those for biosynthesis and degradation of both the main cell wall component, i.e., cellulose, and the main storage polysaccharide, i.e., a β-1,3-glucan (chrysolaminarin) (Vieler et al. 2012a; Corteggiani Carpinelli et al. 2014; Scholz et al. 2014). Genes predicted to encode enzymes responsible for the synthesis of sulfated fucans were also identified, suggesting that like in some other ochrophytes, these polysaccharides may also be present in the eustigmatophyte cell wall (Corteggiani Carpinelli et al. 2014). Precursors for isoprenoid biosynthesis are formed solely by the plastid-located non-mevalonate (DOXP) pathway, as no homologs of enzymes of the cytosolic mevalonate pathway were found in the *M. gaditana* genome (Radakovits et al. 2012). Several vitamin B12-dependent enzymes were found to be encoded by the *M. gaditana* genome, suggesting that vitamin B12 may be beneficial or even essential for eustigmatophyte growth under some conditions (Jinkerson et al. 2013).

A new frontier in eustigmatophyte research was defined by the recent identification of phytohormones, specifically abscisic acid (ABA), cytokinins (CKs), and gibberellin (GA), in *Nannochloropsis oceanica* (Lu et al. 2014; Lu and Xu 2015). The *N. oceanica* genomes encode homologs of enzymes mediating the synthesis of ABA and CKs in plants, and the pathways of ABA and CK synthesis are transcriptionally up- and downregulated, respectively, upon nitrogen depletion.

CKs stimulate cell cycle progression in *N. oceanica* whereas ABA acts as a growth repressor, indicating an antagonistic role of the two regulators in response to nitrogen deprivation. Like some other algae and many anaerobic non-photosynthetic protists, *Nannochloropsis* and *Microchloropsis* have genes encoding the enzyme [FeFe]-hydrogenase (*hydA*) as well as factors involved in hydrogenase maturation (*hydE*, *hydF*, and *hydG*; Radakovits et al. 2012; Vieler et al. 2012a). In agreement with these *in silico* insights, *N. oceanica* was found to produce H_2 when grown at anaerobic conditions and supplied with an abiotic electron donor (Vieler et al. 2012a), but the actual physiological role of hydrogenase in eustigmatophytes remains unknown.

Of all metabolic pathways in eustigmatophytes, the most attractive for researchers have been those concerning the synthesis and degradation of fatty acids and lipids. The significance of these metabolic processes in eustigmatophytes is immediately apparent from the fact that the complement of genes encoding enzymes of lipid metabolism is markedly expanded in *Nannochloropsis* and *Microchloropsis* genomes compared to other algae (Radakovits et al. 2012; Wang et al. 2014). Multiple paralogs are found for many of the enzymes, and phylogenetic analyses suggested that the expansion could have partly originated from acquisition of new genes by horizontal gene transfer (Wang et al. 2014).

This genetic constitution underpins the long-known ability of eustigmatophytes to accumulate large amounts of neutral lipids, i.e., forms of TAG with varying profiles of esterified fatty acids (Ma et al. 2016). TAG accumulates in lipid droplets in eustigmatophyte cells. In *Nannochloropsis* sp., an abundant hydrophobic lipid droplet surface protein (LDSP) was characterized (Vieler et al. 2012b). It is unique in its primary sequence but is structurally similar to other lipid-droplet-associated proteins (oleosins) from other organisms. Physiological experiments established stress factors as the main trigger for TAG accumulation, with TAG accumulation serving as a carbon sink under conditions limiting cell growth. Nitrogen limitation stands out as the most effective factor. Recent studies provided a detailed view of changes in gene expression and the activity of different biochemical pathways leading to an increased TAG production upon nitrogen depletion (Li et al. 2014b; Meng et al. 2015). High light intensity also stimulates TAG production, and the molecular mechanism of this phenomenon was recently studied in fine detail using a combination of transcriptomic, lipidomic, and metabolomic approaches (Alboresi et al. 2016).

Not only the production of TAG as such but also certain features of the metabolism of fatty acids make eustigmatophytes highly attractive for biotechnological exploitation. This concerns primarily the ability to synthesize substantial amounts of nutritionally valuable LC-PUFAs. Of these, the most important is EPA (C20:5 n-3), which is found not only in the highly studied *Nannochloropsis*-*Microchloropsis* group but seems to be abundant in eustigmatophytes in general (Cohen 1994; Volkman et al. 1999; Řezanka et al. 2010; Ma et al. 2016). The actual EPA content in eustigmatophyte cells varies considerably depending on environmental conditions such as nutrient status, salinity, light intensity, or temperature (Sukenik 1991, Cohen

1994, Lu et al. 2001, Hoshida et al. 2005, Khozin-Goldberg and Cohen 2006, Pal et al. 2011). EPA is mostly present in the membrane lipids (glyco- and phospholipids) (Cohen 1994; Khozin-Goldberg and Cohen 2006; Vieler et al. 2012a; Ma et al. 2016), but in *T. minutus*, its considerable amounts were identified in TAG as well (Řezanka et al. 2011). An analysis of the *N. oceanica* genome revealed a complete set of genes encoding membrane-bound ER-localized desaturases, namely, putative Δ9-, Δ12-, Δ6-, Δ5-, and ω3-desaturases, which implies the synthesis of EPA outside of the plastid and its import into the plastid for glycolipid synthesis (Vieler et al. 2012a). Arachidonic acid (C20:4 n-6) is also present in smaller quantities in at least some eustigmatophytes (Řezanka et al. 2014). The fatty acid profile of members of the *Vischeria/Eustigmatos* group proved to be unusual due to a high concentration of long-chain hydroxy fatty acids (Volkman et al. 1999).

Systematics

Although eustigmatophytes were sometimes considered as a separate division or phylum Eustigmatophyta (Hibberd 1981, 1990; Ettl and Gärtner 1995; John 2011) or as a taxon (named Eustigmatales) with no explicitly assigned taxonomic rank (Adl et al. 2012), most often they have been treated as the class Eustigmatophyceae within the broadly defined phylum (division) Ochrophyta (Heterokontophyta) (Santos and Leedale 1991; Andersen 2004; Cavalier-Smith and Chao 2006; Graham et al. 2008; Yang et al. 2012; Ševčíková et al. 2015). The formal taxonomic scheme for eustigmatophytes established by Hibberd (1981) recognized a single order, Eustigmatales, divided into four families (Eustigmataceae, Chlorobotrydaceae, Pseudocharaciopsidaceae, Monodopsidaceae). Each family was characterized by a unique combination of character states concerning the presence/absence of zoospores, number of flagella, presence/absence of mucilage, and cell shape. One additional family, the Loboceae, was established to accommodate the newly described alga *Pseudotetraëdriella kamillae* (Hegewald et al. 2007).

However, the "one order/five families" system is incongruent with the phylogenetic relationships within the Eustigmatophyceae and cannot accommodate the diversity as revealed by recent sampling that has yielded a large number of uncharacterized or unidentified isolates (Fig. 1). Above all, the traditional classification does not capture the division of eustigmatophytes into two phylogenetically deeply diverged lineages (Fig. 1). The first lineage includes all eustigmatophyte taxa known to Hibberd (1981) and hence can be equated to his order Eustigmatales. The second lineage comprises taxa that were recognized as eustigmatophytes or described only in the past 25 years, so it naturally constitutes a new candidate eustigmatophyte order. However, in order to formally erect the order based on the International Code of Nomenclature for algae, fungi, and plants (Melbourne Code; http://www.iapt-taxon.org/nomen/main.php), the family-level classification of this group needs to be resolved. Hence, some of us employed the International Code of

Phylogenetic Nomenclature (PhyloCode; https://www.ohio.edu/phylocode/index.html) as an alternative to describe the second principal eustigmatophyte group as the clade *Goniochloridales* (Fawley et al. 2014).

A comprehensive classification of the two main eustigmatophyte groups that would be consistent with the phylogenetic relationships as revealed by molecular phylogenetic analyses is yet to be worked out. Some of the existing families and genera have proven to be para- or polyphyletic, and taxa need to be established to accommodate some newly recognized or described lineages. However, extensive revisions of many taxa are hampered by the lack of cultures corresponding to type species. As mentioned above, further work is also required to resolve the concept of families in the *Goniochloridales.* One to several separate families are conceivable based on the current picture of the phylogenetic diversity of the group (Fig. 1). In the Eustigmatales, three main lineages are apparent that can perhaps be conveniently recognized as three families. One of them fits the delimitation of the family Monodopsidaceae, provided that the family Loboceae, proposed to accommodate *P. kamillae* (Hegewald et al. 2007), is abandoned to avoid paraphyly of Monodopsidaceae. In fact, the formal description of Loboceae is invalid according to the International Code of Nomenclature for algae, fungi, and plants, as the name is not typified by a genus name. The current circumscription of the families Eustigmataceae, Chlorobotrydaceae, and Pseudocharaciopsidaceae appears to be too narrow from the phylogenetic point of view (Fig. 1). They may possibly be merged into a single monophyletic family characterized by the presence of a bulging pyrenoid connected to the plastid with a narrow stipe. On the other hand, a new family needs to be established for a clade comprising *Pseudellipsoidion edaphicum* and *Pseudocharaciopsis ovalis* together constituting a strongly supported third monophyletic clade of Eustigmatales (Fig. 1). To facilitate communication about the eustigmatophyte phylogenetic diversity before formal taxonomic revisions of the eustigmatophyte families are completed, informal names were proposed for different (presumably) monophyletic subgroups of both Eustigmatales and *Goniochloridales* (Fawley et al. 2014). For simplicity, these informal groups are only indicated in Fig. 1 and are not discussed further.

The following overview of eustigmatophyte classification includes all genera and species previously assigned to this group on the basis of ultrastructural, biochemical, and/or molecular evidence.

Order Eustigmatales

Genus *Eustigmatos* D.J. Hibberd 1981

Type species: *Eustigmatos vischeri* D.J. Hibberd

Very similar to *Vischeria* but the cell wall is always smooth and featureless. Four species (*E. vischeri*, *E. magnus*, *E. polyphem*, *E. calaminaris*) very closely related to or intermixed with *Vischeria* spp. in molecular trees (Fig. 1),

suggesting that the genus *Eustigmatos* should be merged with the genus *Vischeria*.

Genus *Vischeria* Pascher 1938

Type species: *Vischeria stellata* (Chodat) Pascher (basionym: *Chlorobotrys stellata* Chodat).

The cells isodiametric, the zoospores elongate lageniform, with a single emergent flagellum, the cell wall typically raised into projections or ridges. Three species (*V. stellata*, *V. punctata*, *V. helvetica*) studied in detail and confirmed as eustigmatophytes (Hibberd 1981), nine other species described by Pascher (1939) are yet to be reinvestigated.

Genus *Chlorobotrys* Bohlin 1901

Type species: *Chlorobotrys regularis* (West) Bohlin (basionym: *Chlorococcum regulare* West).

The cells occur in pairs or colonies, surrounded by lamellate mucilage. Zoospores not observed. One species, *C. regularis*, was confirmed as a eustigmatophyte (Hibberd 1974); several other described species (Ettl 1978) are yet to be studied in detail.

Genus *Pseudocharaciopsis* K.W. Lee and H.C. Bold 1974

Type species: *Pseudocharaciopsis texensis* K.W. Lee & Bold, considered a junior synonym of *Pseudocharaciopsis minuta* (A.Braun) Hibberd (basionym: *Characium minutum* A.Braun ex Kützing)

The cells ovoid/ellipsoidal and capable of producing a stipe. The zoospores with two emergent flagella. 18S rRNA gene sequences determined for the two *Pseudocharaciopsis* species (*P. minuta* and *P. ovalis*) indicate that this genus is polyphyletic (Fig. 1). In addition, a strain identified as *Characiopsis saccata*, hence representing the genus *Characiopsis* currently classified in the class Xanthophyceae, is closely related to *P. minuta* (Fig. 1). The taxonomy of the genera *Pseudocharaciopsis* and *Characiopsis* is thus in an urgent need of revision (see also below).

Genus *Monodopsis* D.J. Hibberd 1981

Type species: *Monodopsis subterranea* (J.B. Petersen) D.J. Hibberd (basionym: *Monodus subterranea* J.B. Petersen)

Unicellular forms with spherical, ovoid, ellipsoid, or cylindrical cells, 5–10 μm in diameter. Zoospores not observed. One validly described species (*M. subterranea*). The combination *Monodopsis unipapilla* was mentioned in the literature (Santos and Leedale 1995; Santos 1996) but not yet validly published, for a species originally known as *Monodus unipapilla* and closely related to *M. subterranea* (Fig. 1). Other species of the traditionally xanthophyte genus *Monodus* may need reclassification to *Monodopsis*. One such candidate is *Monodus guttula*, nominally represented by two strains with sequenced 18S rRNA gene (Fig. 1) whose identification yet need to be critically examined.

Genus *Pseudotetraëdriella* E. Hegewald 2007

Type species: *Pseudotetraëdriella kamillae* E. Hegewald & J. Padisák

The vegetative cells with four lobes, zoospores elongate-ovate with one emergent flagellum and without an eyespot, globular resting spores. So far monotypic.

Genus *Nannochloropsis* D.J. Hibberd 1981

Type species: *Nannochloropsis oculata* (Droop) D.J. Hibberd (basionym: *Nannochloris oculata* Droop)

The cells spherical, ovoid, ellipsoid, or cylindrical, <5 μm in maximum dimension. Zoospores not observed. Previously included two species now classified in the separate genus *Microchloropsis* (see below). The five formally described species occur in marine habitats (*N. oculata*, *N. granulata*, *N. oceanica*, *N. australis*) or in freshwater (*N. limnetica* with several varieties described; Fawley and Fawley 2007). One more species, "*Nannochloropsis maritima*", appeared recently in the literature (Hu et al. 2013) and is represented by an 18S rDNA sequence in GenBank (accession number AY680703), but it has not been formally described.

Genus *Microchloropsis* M.W. Fawley, I. Jameson & K.P. Fawley 2015

Type species: *Microchloropsis salina* (D.J. Hibbard) M.W. Fawley, I. Jameson & K.P. Fawley (basionym: *Nannochloropsis salina* D.J. Hibberd)

Cells small (2–8 μm), cylindrical or rarely spherical with a single parietal chloroplast. Pyrenoids absent. Some cells with an orange or red lipid body free in the cytoplasm. Refractive granules or short rods usually present in the cytoplasm. Reproduction by autospore production. Young autospores usually hemispherical but become cylindrical or spherical as they mature. Two described species (*M. salina* and *M. gaditana*).

Genus *Pseudellipsoidion* J. Neustupa and Y. Němcová 2001

Type species: *Pseudellipsoidion edaphicum* J. Neustupa & Y. Němcová

The vegetative cells with ellipsoidal and globular morphology, zoospores of irregular or oval shape with one emergent flagellum. Pyrenoid absent. So far monotypic.

Clade *Goniochloridales*

Genus *Goniochloris* Geitler 1928

Type species: *Goniochloris sculpta* Geitler

Vegetative cells dorsoventrally flattened and irregularly triangular in frontal view, with the cell surface sculptured. Reclassification of this genus from the Xanthophyceae to the Eustigmatophyceae is based on the 18S rRNA gene sequence from the type species *G. sculpta* (Fig. 1; Přibyl et al. 2012). The taxonomic assignment of the number of remaining known *Goniochloris* species (Ettl 1978) awaits further studies.

Genus *Pseudostaurastrum* Chodat 1921

Type species: *Pseudostaurastrum enorme* (Ralfs) Chodat (basionym: *Staurastrum enorme* Ralfs)

Tetrahedral or plate-like cells with typically four branched projections. Two species (*P. enorme*, *P. limneticum*) have been studied by modern methods confirming their classification within the Eustigmatophyceae; a few other described species (Ettl 1978) are very likely related given the highly characteristic morphology.

Genus *Trachydiscus* H. Ettl 1964

Type species: *Trachydiscus lenticularis* H. Ettl

Disc-shaped cells with the cell surface sculptured by numerous cell wall protuberances (warts, bulges, or papillae). Reclassification of this genus from the Xanthophyceae to the Eustigmatophyceae is based on a cytological, biochemical, and molecular genetic evidence for the species *Trachydiscus minutus* (Fig. 1; Přibyl et al. 2012). However, the additional six known *Trachydiscus* species, including the type species, are morphologically rather different from *T. minutus*, so it must yet to be confirmed whether they (and hence formally the genus *Trachydiscus* as such) belong to the Eustigmatophyceae and specifically to the *Goniochloridales*.

Genus *Tetraëdriella* Pascher 1930

Type species: *Tetraëdriella acuta* Pascher

Cells pyramidal or tetragonal with walls ornamented by regularly arranged rows of depressions. The genus *Tetraëdriella* is traditionally classified in Xanthophyceae (Ettl 1978) but is here included in eustigmatophytes, specifically as a member of Goniochloridales, based on the recent reinvestigation of the species *Tetraëdriella subglobosa* including evidence from its 18S rRNA gene sequence (Fawley and Fawley 2017; see also Fig. 1). The morphological features of other *Tetraëdriella* species, including the types species, suggest that they are related to *T. subglobosa.* Ultrastructural and molecular evidence for eustigmatophyte affinity of several other *Tetraëdriella* species was presented at a conference (Santos and Santos 2001) or mentioned in the literature (Ott et al. 2015), but the actual data are yet to be published.

Genus *Vacuoliviride* T. Nakayama, T. Nakamura, A. Yokoyama, T. Shiratori, I. Inouye & K.-I. Ishida 2015

Type species: *Vacuoliviride crystalliferum* T. Nakayama, T. Nakamura, A. Yokoyama, T. Shiratori, I. Inouye, and K.-I. Ishida

Vegetative cells solitary, nonmotile, spherical to ellipsoidal, 6–30 μm in diameter, and covered by smooth cell wall. Cells include refractile granules, a large vacuole, and a reddish globule, frequently with a rod- to V-shaped crystalline structure, one to several greenish chloroplasts possessing bulging pyrenoid with longitudinal slit. Cells reproduce by 2–8 autospores. So far monotypic.

Eustigmatophyceae *Incertae sedis*

Genus *Botryochloropsis* H.R. Preisig and C. Wilhelm 1989

Type species: *Botryochloropsis similis* H.R. Preisig and C. Wilhelm

Spherical cells in mucilage aggregated in irregular colonies, zoospores with two emergent flagella. Pyrenoid absent. No molecular data (and no authentic culture) are available for this genus, so its exact position within eustigmatophytes is unknown. So far monotypic.

Additional Eustigmatophytes

The actual diversity of eustigmatophytes is not restricted to the taxa listed above. The many unidentified strains that have been assigned to the Eustigmatophyceae based on their 18S rDNA (Fawley et al. 2014; Fig. 1) and *rbc*L (Prior et al. 2009) sequences illustrate the extent of the hitherto unnoticed diversity of this class. Many of these strains will probably prove to represent new taxa (species and even genera), but it is possible that others can eventually be identified as previously described species after a detailed scrutiny is carried out. Indeed, several algal taxa, traditionally classified in Xanthophyceae, have been occasionally considered as candidate members of Eustigmatophytes. For example, Hibberd (1981) admitted a possibility that *Pleurochloris commutata*, the type species of the genus *Pleurochloris*, may be a eustigmatophyte based on certain morphological features of vegetative cells and zoospores. Interestingly, most of the currently known eustigmatophytes were previously classified as members of the xanthophyte family Pleurochloridaceae comprising a large number of genera and species that have not been studied by modern methods. It is very likely that many additional members of the Pleurochloridaceae will eventually be reclassified to Eustigmatophyceae.

One of the taxa of Pleurochloridaceae with possible eustigmatophyte affinities is the genus *Chloridella* Pascher. 18S rRNA gene sequences were obtained from two strains nominally representing two species, *Chloridella neglecta* (the type species of the genus) and *Chloridella simplex*, showing that both algae belong to the tight *Vischeria/Eustigmatos* cluster (Fawley et al. 2014; Fig. 1). This led Ott et al. (2015) to classify *Chloridella* as a eustigmatophyte genus. However, neither of the two strains is authentic, and their morphology has not yet been properly studied to check the identification as provided in the respective culture collections. Whereas *C. simplex* indeed resembles *Eustigmatos* species (reportedly differing by the lack of zoospores), *C. neglecta* is more reminiscent of *Pleurochloris meiringensis*, a confirmed member of Xanthophyceae (Andreoli et al. 1999b). Thus, classifying *Chloridella* as a eustigmatophyte is premature, and critical revision of this genus is needed to clarify its circumscription and phylogenetic position. A few more genera (e.g., *Gloeobotrys*, *Gloeoskene*, or *Merismogloea*) were listed as candidate

eustigmatophytes by Ott et al. (2015) based on the fact that they had been proposed to be synonymous with established eustigmatophytes or that some of their members (but not type species themselves) had been demonstrated to be eustigmatophytes.

Evidence for additional eustigmatophytes was informally presented at conferences, but bona fide publication of the data is still missing. Specifically, ultrastructural and molecular data were obtained from several strains from the ACOI collection identified as different species of the genus *Characiopsis*, indicating their eustigmatophyte nature (Santos and Santos 2001; Amaral et al. 2011, 2015). Indeed, *Characiopsis* was listed as a genus of eustigmatophytes by Ott et al. (2015), but this was based on an assumption that the alga known as *Pseudocharaciopsis minuta* (and belonging to Eustigmatophyceae; Fig. 1) is in fact the type species of *Characiopsis* Borzì. However, as discussed in detail by Hibberd (1981), the type species of the genus may actually be *Characiopsis borziana* Lemmermann, whose phylogenetic position remains unknown. Dashiell and Bailey (2009) announced a new eustigmatophyte genus ("*Microtalis*") with two new species and strains labeled "*Microtalis aquatica* Bailey,JC" and "*Microtalis reticulata* Bailey,JC" that are available from the National Center for Marine Algae and Microbiota (CCMP3153 and CCMP31547, respectively). Sequences of several genes from the former strain were published by Yang et al. (2012) and indicate that "*Microtalis aquatica*" is closely related to *Trachydiscus minutus* and several unidentified strains (Fig. 1). As mentioned above, *T. minutus* morphologically differs significantly from the type species of the genus *Trachydiscus*, so treating the clade including *T. minutus* and *M. aquatica* as a new genus may prove substantiated when characterization of the strains CCMP3153 and CCMP31547 is eventually published.

A few taxa have been assigned to the Eustigmatophyceae in error. *Ophiocytium maius* (strain CCAP 855/1) was suggested to be a eustigmatophyte based on its *coxI* sequence (Ehara et al. 1997). However, it has proven to be a misidentified member of the *Vischeria/Eustigmatos* cluster (Fig. 1); the genuine *O. maius* (strain SAG 855-1) is undoubtedly a xanthophyte (Maistro et al. 2009). Ott and Oldham-Ott (2003) included the genus *Ellipsoidion* in the Eustigmatophyceae, apparently because early reports on eustigmatophytes featured an alga (strain CCAP 822/1) then identified as *Ellipsoidion acuminatum* (Hibberd and Leedale 1970, 1971, 1972). However, Hibberd (1981) reexamined the strain and eventually identified it as *Characiopsis ovalis*, basing his taxonomic revision of the species name to *Pseudocharaciopsis ovalis* (Chodat) Hibberd. Thus, there is at present no evidence that *E. acuminatum* or the type species of the genus *Ellipsoidion*, *E. anulatum* Pascher, are eustigmatophytes; both species and the whole genus thus formally remain in the Xanthophyceae. Molecular data that were published for a single confidently identified *Ellipsoidion* species, the authentic strain of *Ellipsoidion parvum*, showed that this strain is a green alga conspecific with *Neocystis brevis* (Eliáš et al. 2013). Hence, the actual identity and phylogenetic position of the genus *Ellipsoidion* remain highly uncertain.

Maintenance and Cultivation

Eustigmatophytes presently maintained in culture collections were originally isolated using a wide variety of techniques. Single-cell isolation is possible for all the larger forms. Terrestrial and some freshwater species grow well on nutrient agar, and plating may also be used (Prior et al. 2010; Fawley et al. 2014). Agar plate techniques have been used to isolate the many new stains of freshwater Eustigmatophyceae that are presented in Fig. 1. For the minute forms, particularly when occurring in blooms, dilution techniques work well.

Cultures of freshwater and terrestrial species generally grow well in a wide variety of mineral media or in biphasic soil/water cultures where they may survive for many months, even years. Clonal cultures of most strains are available and stocks are most conveniently maintained on nutrient agar slants containing dilute (e.g., Chu No. 10) or rich (e.g., Bold's Basal Medium) media (Nichols 1973). Two of the authors have had success isolating and maintaining Eustigmatophyceae on the high-nutrient medium, WH+ (Fawley et al. 1990; Fawley et al. 2014), and the low-nutrient medium originally designed for chrysophytes, DYIV (Andersen et al. 1997). The ACOI Collection of Algae holds ca. 80 strains of eustigmatophytes (Santos and Santos 2004) that have been kept for 15 years in liquid Desmidiacean medium M7 (for chemical composition, see Schlösser 1994), with a controlled pH of 6.4–6.6. This is a suitable medium for all strains, but relatively slow growth is observed for sensitive genera, namely, *Pseudostaurastrum*, *Tetraëdriella*, *Chlorobotrys*, and *Goniochloris*. A new medium composed of a one tenth dilution of WH+ with 0.1 g^{-1} MES buffer at pH 5.5 (Karen and Marvin Fawley, unpublished) is proving effective for isolating Eustigmatophyceae from acid environments. Marine forms grow easily in standard seawater media such as Erdschreiber or ASP2 (Provasoli et al. 1957), either as standing liquid batch cultures or on agar slants for stock cultures. These forms tolerate a wide range of salinity and a half-normal salinity or even a freshwater medium is usually more convenient for maintenance of stocks. Cryopreservation has been successfully tested for several eustigmatophyte species (Osório et al. 2004; Gwo et al. 2005).

Evolutionary History

There is no fossil record known for eustigmatophytes; hence, reconstruction of their evolutionary origin and diversification has relied solely on comparative analyses of morphological, biochemical, and molecular characters. All these characters firmly place eustigmatophytes into a broader group of ochrophyte (or heterokontophyte) algae, which in turn form a prominent clade within stramenopiles (or heterokonts) (Santos and Leedale 1991; Andersen et al. 1998; Andersen 2004). Phylogenetic analyses of multigene matrices generally indicate that Eustigmatophyceae are a sister group of a clade comprising Chrysophyceae (incl. Synurophyceae) and

Synchromophyceae, altogether forming the group Limnista (Yang et al. 2012; Ševčíková et al. 2015). However, the recent analysis of stramenopile phylogeny based on a 245-protein dataset and including sequences from *Nannochloropsis gaditana* as a representative eustigmatophyte placed this organism closer to Raphidophyceae and the PX clade (Phaeophyceae plus Xanthophyceae), although with unconvincing statistical support (Noguchi et al. 2016). The phylogenetic position of eustigmatophytes among ochrophytes thus needs to be further tested.

Phylogenetic relationships within eustigmatophytes have been investigated primarily with the aid of 18S rRNA gene sequences, which confirmed eustigmatophyte monophyly (Andersen et al. 1998) and revealed the existence of two principal deeply separated subgroups, *Goniochloridales* and Eustigmatales (Fig. 1; Přibyl et al. 2012; Fawley et al. 2014). Relationships within the *Goniochloridales* are not yet clear; however, four clades within this lineage are supported by analysis of the 18S rRNA gene sequence data (Fig. 1). Within the latter subgroup, three major lineages can be recognized (their names here follow the nomenclature introduced by Fawley et al. 2014): Eustigmataceae group, Monodopsidaceae, and *Pseudoellipsoidion* group. Each group is well supported by analyses of 18S rRNA gene sequences, but their relative branching order needs to be established using a higher number of molecular markers. The phylogenetic position of *Botryochloropsis similis* is unknown given the absence of molecular data. However, a characteristic combination of morphological features documented for this species by Preisig and Wilhelm (1989), specifically the presence of an eyespot, zoospores with two flagella, and plastids with no pyrenoid, suggests that *B. similis* possibly belongs to the *Pseudellipsoidion* group.

Even though incomplete, the phylogenetic tree reconstructed for eustigmatophytes enables some inferences about evolutionary trends in this group. First, eustigmatophytes apparently emerged in a freshwater or terrestrial habitat and frequent transitions between these two habitat types seem to have occurred during their evolution, whereas the *Nannochloropsis* lineage secondarily moved to the sea, from which the species *N. limnetica* came back to the freshwater. Second, since an eyespot is altogether absent in all species of the *Goniochloridales* clade investigated so far (Schnepf et al. 1996; Přibyl et al. 2012) as well as in *Pseudotetraëdriella kamillae* (Hegewald et al. 2007), it is actually possible that the characteristic extraplastidial eyespot, regarded as one of the defining features of the whole eustigmatophyte class, arose only after some eustigmatophyte lineages had diverged. Third, zoospores were presumably lost independently in the lineages leading to *Nannochloropsis* and to *Monodopsis*, since they have been retained by *Pseudotetraëdriella kamillae* (Hegewald et al. 2007). Fourth, up to three independent losses of the continuity between the plastid ER and the nuclear envelope can be inferred to have occurred (in the *Goniochloridales*, in the Eustigmatophyceae group, and in the *Pseudellipsoidion* group). Fifth, species with zoospores with a single emergent flagellum (*Vischeria/Eustigmatos* cluster, *Pseudotetraëdriella kamillae*, members of the *Goniochloridales* with zoospore morphology investigated in detail, and potentially also *Pseudellipsoidion edaphicum*) do not form a monophyletic grouping, which indicates multiple independent losses of the posterior flagellum. Altogether, the evolutionary history of eustigmatophytes appears complex and heavily

influenced by homoplasy. A more detailed reconstruction remains a task for the future, with the very real possibility of a greatly expanded number of taxa included in the class.

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Xanthophyceae

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Silvia Maistro, Paul Broady, Carlo Andreoli, and Enrico Negrisolo

Abstract

The Xanthophyceae is a clade of stramenopilan photoautotrophs containing about 118 genera and 600 species. Morphology ranges from free-living or attached unicells to colonies and unbranched or branched filaments and siphons. A large majority are found in freshwater and soil, while some occur in brackish and marine habitats. Although abundant growth of a few species can occur in nature, none are known to be of practical importance. They are characterized by possession of chlorophylls *a*, c_1, and c_2 and a range of xanthophylls, but not fucoxanthin, in generally yellowish-green, discoidal, parietal chloroplasts. Thylakoids are in groups of three, and most species investigated have a single thylakoid forming a girdle band around the periphery of the chloroplast. Chloroplasts are surrounded by chloroplast endoplasmic reticulum. Pyrenoids, when present, are typically semi-immersed and are not associated with granules of storage products. A cell wall consisting of two overlapping parts occurs in some coccoid and filamentous species. Reproduction is generally asexual but some, e.g., *Vaucheria*, exhibit sexual reproduction. The taxonomic status of a significant number of species is uncertain, especially those that are rarely observed, e.g., species of Chloramoebales, Heterogloeales, and Rhizochloridales. Transfer of species to the Eustigmatophyceae and other groups is likely. There are molecular phylogenetic data for fewer than 20% of species. Four major clades are recognized. Two

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of these contain both coccoid and filamentous species. Many traditional orders, families, and genera are paraphyletic or polyphyletic. It is presently convenient to retain the traditional classification of seven orders based on morphology until these difficulties are resolved following the inclusion of more species in phylogenetic analyses.

Keywords

Coccoid • Filamentous • Freshwater • Heterokont • Phototroph • Siphonous • Soil algae • Stramenopile • Tribophyceae • Yellow-green algae

Contents

Summary Classification

●**Xanthophyceae**
●●**Chloramoebales** (e.g., *Chloramoeba*)
●●**Rhizochloridales** (e.g., *Rhizochloris*, *Stipitococcus*, *Myxochloris*)
●●**Heterogloeales** (e.g., *Heterogloea*, *Mallodendron*, *Pleurochloridella*, *Characidiopsis*)
●●**Mischococcales** (e.g., *Goniochloris*, *Botrydiopsis*, *Chlorellidium*, *Gloeobotrys*, *Gloeopodium*, *Mischococcus*, *Characiopsis*, *Chloropedia*, *Trypanochloris*, *Centritractus*, *Ophiocytium*)
●●**Tribonematales** (e.g., *Neonema*, *Xanthonema*, *Tribonema*, *Heterodendron*, *Heterococcus*)
●●**Botrydiales** (*Botrydium*)
●●**Vaucheriales** (*Vaucheria*, *Asterosiphon*)

Introduction

General Characteristics

Xanthophyceae are photoautotrophic, stramenopilan protoctists possessing green parietal chloroplasts. They are distinguished from members of the Chlorophyta by the absence of chlorophyll *b*, the presence of small amounts of chlorophylls c_1 and c_2, and the lack of starch as a storage carbohydrate. Their color is usually of a more yellow shade than is typical for the Chlorophyta due to the presence of β-carotene and xanthophylls (e.g., violaxanthin, heteroxanthin, and vaucherioxanthin) and the absence of fucoxanthin, a brown pigment present in most other photosynthetic stramenopiles. This has led to their common name of yellow-green algae. Motile cells are typically of the heterokont type, with unequal-length flagella. The longer bears hairs and is directed anteriorly during swimming, while the shorter is smooth and held posterolaterally. About 600 species have so far been described, belonging to over 90 genera.

Occurrence

Xanthophyceans occur from the tropics to the polar regions. The great majority live in freshwater as phytoplankton and periphyton in lakes, ponds, streams, and rivers. Most taxa are seen only rarely but some commonly form visible growths, especially filaments of *Vaucheria* and *Tribonema* in flowing waters. Xanthophyceans often comprise a significant part of the soil microflora (Ettl and Gärtner 1995). Species of *Vaucheria* can form thick velvety mats that help bind sediment in salt marshes, damp soil, and rarely in the coastal marine environment. Members of this clade are represented in all the major algal culture collections.

Literature and History of Knowledge

The taxon has been reviewed by Hibberd (1980, 1990) and Ott (1982). The group was monographed by Pascher (1937–1939), whose account was revised by Ettl (1978). Silva (1979) dealt with many problems of nomenclature. Descriptions of xanthophyceans in the terrestrial ecosystem are provided by Ettl and Gartner (1995). Phylogenetic relationships and taxonomic status of many taxa are under continuing scrutiny using molecular techniques that are deeply changing the perception of diversity and classification of xanthophyceans (see below).

The distinguishing characters of the Xanthophyceae remained unrecognized until the latter part of the nineteenth century, and prior to 1899, the few known species were included in the Chlorophyta. The studies of Braun (1855) and Borzi (1889, 1895) led to the recognition within the green algae of a group, the Confervales, which included species previously scattered among the various families of green

algae. The Confervales were characterized by the possession of numerous discoid yellow-green chloroplasts apparently lacking pyrenoids; fat or oil and not starch as the assimilatory storage product; and zoospores typically containing two lateral discoid chloroplasts and bearing, as it was then believed, only a single flagellum. Unicellular, filamentous, and siphonous forms possessing these characters were all known at this time. An important cytological study of the group was published by Bohlin (1897a), who extended its classification by dividing the Confervales into three families. He also described the amoeboid mastigote *Chloramoeba* (Bohlin 1897a, b) which, although placed in the mastigote group Chloromonadina, was regarded as a possible progenitor of the Confervales.

A palmelloid form (*Chlorosaccus*) belonging to this complex was described by Luther (1899) which in several features was intermediate between "flagellate" and "algal" organization. *Chlorosaccus* would have been included with *Chloramoeba* in the mastigote group Chloromonadina had its palmelloid state not been predominant. The zoospores of *Chlorosaccus* were similar to those of the Confervales except that they had a second short flagellum, and Luther, attempting to confirm the unimastigote condition of the motile cells in the Confervales, made the important discovery that the zoospores of both *Tribonema* and *Botrydiopsis* possessed a second flagellum much shorter than the first. *Chlorosaccus* could thus be regarded as a form intermediate between the Chloromonadina and Confervales.

In the absence of any significant features distinguishing these two groups, and because of the important differences between the Confervales and the remainder of the Chlorophyceae, an entirely new class, the Heterokontae, was erected to include both the Chloromonadina and the Confervales. Luther's view of the Heterokontae as an isolated and well-defined evolutionary series of algae was quickly adopted by most phycologists. The alternative name for the class, Xanthophyceae, was proposed by Allorge (1930) and first validly published by Fritsch (1935). However, Fritsch (1935) still placed *Vaucheria* and three other siphonous, filamentous genera within the Siphonales, Chlorophyceae but expressed uncertainty in this.

A very large part of the increase in knowledge of the systematics and cytology of the Xanthophyceae following these early studies is due to the work of a single investigator, Adolf Pascher. Several decades of study led to his final monumental treatment of the group (Pascher 1937–1939), *Heterokontae*, Volume II in the second edition of Rabenhorst's *Kryptogamen Flora*, in which Pascher himself authored approximately two-thirds of the included genera.

Other than those already mentioned, there were few cytological studies of xanthophyceans prior to the advent of electron microscopy The classic work of Vlk (1931, 1938) demonstrated that the long flagellum of zoospores is a "flimmergeissel," bearing lateral "flimmer" (now known to be tubular, tripartite stiff hairs), and that the short flagellum is a "peitschengeissel" (whiplash flagellum) not bearing hairs but with a thick basal part and a thinner distal part. Koch (1951) and Manton et al. (1952) provided further information of the external morphology of the flagella, the latter authors using electron microscopy for the first time on a Xanthophycean.

The next use of electron microscopy was with observations by Greenwood et al. (1957) and Greenwood (1959) on the compound zoospore of *Vaucheria.* These were followed by several investigations of chloroplast structure in the early 1960s.

Modern electron microscopy studies dealt mainly with the siphonous genera *Vaucheria* and *Botrydium* and with *Tribonema* (Lokhorst 2003; Lokhorst and Star 2003a, b; Ott 1982). Only a handful of more than 400 coccoid species have been investigated (Begum and Broady 2001; Andreoli et al. 1999).

Finally, modern phylogenetic approaches, based on analyses of genes, are dramatically changing concepts of the boundaries of this group as well as the understanding of the relationships among various xanthophycean clades (see below).

Practical Importance

The Xanthophyceae have no known practical importance except for *Vaucheria* having a role in stabilization of sediments. They have yet to be thoroughly investigated for production of biochemicals of potential biotechnological use.

Habitats and Ecology

Xanthophyceae occur predominantly in freshwater habitats. Most taxa are seen only rarely and many have never been seen again since they were originally described, probably because they mostly occur only in small numbers. Also, the inclusion of several taxa within Xanthophyceae requires confirmation by molecular analyses (Maistro et al. 2009). Most unattached forms occur in still waters among other algae or submerged vegetation, particularly in low pH habitats that are also often rich in iron. Coccoid xanthophyceans are relatively well represented in the algal biota of soils (Ettl and Gärtner 1995; Vischer 1945), although several soil algae originally attributed to the Xanthophyceae have now been shown to be Eustigmatophyceans. Species of filamentous *Tribonema* are common among floating mats in still water, particularly in late winter. Coccoid and filamentous species have been isolated from terrestrial habitats in Antarctica (Andreoli et al. 1999; Broady 1976; Maistro et al. 2007; Negrisolo et al. 2004; Rybalka et al. 2009 – and other citations therein). The most conspicuous forms in nature are species of the siphonous genera *Botrydium* and *Vaucheria.* The former occur as large green vesicles up to several mm in diameter, often in very large numbers on the surface of drying mud, while *Vaucheria* is widespread in freshwater, brackish, and marine habitats in still or running water and on soil. Many species are among the dominant autotrophs in salt marshes. Functional chloroplasts of *Vaucheria litorea* are sequestered within cells of the gut by the marine sacoglossan mollusk *Elysia chlorotica* (Pierce et al. 2009). *Heterococcus* occurs as a photobiont in species of the crustose lichen *Verrucaria* (Tschermak-Woess 1988).

Characterization and Recognition

Most xanthophycean species exist vegetatively as green to yellow-green coccoid unicells, though the relatively small number of filamentous and siphonous species are usually more abundant and more commonly found. A few monadoid, rhizopodial, and palmelloid forms have also been described, but doubt must be cast on whether many of these truly belong to this taxon. It is important that these are reinvestigated using modern methods. Their heterokont motile cells most easily distinguish xanthophyceans from chlorophytes. In the absence of these, a negative reaction with dilute iodine in potassium iodide is still a useful means of distinguishing them from chlorophytes as xanthophyceans never store starch; however, the test is often inconclusive on very small cells. In these cases data on pigment composition is necessary combined with a multigene molecular phylogenetic analysis, the only way to unambiguously assign a heterokont species to the Xanthophyceae (Maistro et al. 2009).

Pigment Composition

Chlorophylls *a* and *c* (both c_1 and c_2) are found in xanthophyceans. The *c* chlorophylls are present in extremely low concentrations and are likely to be missed unless specifically sought. Chlorophyll *e* has also been reported once but it has never been found again and so its existence seems unlikely. The presence of chlorophyll *b* in any presumed xanthophycean is an *a priori* reason for its transference to one of the classes of green algae. Presence or absence of chlorophyll *b* is the most reliable taxonomic criterion when the gross assignment of very small planktonic species is in question; when chlorophyll *b* is lacking, xanthophyceans may be distinguished from Eustigmatophyceans by their distinctive xanthophyll composition (Whittle 1976; Whittle and Casselton 1975).

The yellow-green color of xanthophyceans is partly due to the masking of the green color of chlorophyll *a* by carotenoid pigments, but is probably due primarily to the absence of fucoxanthin, the pigment responsible for the overall brown color of Chrysophyceans, Haptophyta (Prymnesiophyceans), diatoms, and the brown seaweeds. Carotenoids typically present in the Xanthophyceae include β-carotene and the xanthophylls diatoxanthin, which is dominant, diadinoxanthin, heteroxanthin, and vaucheriaxanthin ester (Rowan 1989; Stransky and Hager 1970; Whittle 1976; Whittle and Casselton 1975). Minor xanthophylls include neoxanthin and cryptoxanthin monoepoxide and several others have been identified less commonly. A blue-green color, produced by some species when treated with concentrated hydrochloric acid, thought to be caused by a reaction with xanthophyll pigments, is sometimes said to be a test for the group, but it is unreliable as some species produce no color change, some turn a shade other than blue green, and others give a variety of colors.

Vegetative Cell Structure

Xanthophyceans usually contain several discoid parietal chloroplasts (Fig. 1a, b) which show little variation in form. Absence of pyrenoids from xanthophycean chloroplasts was once considered to be characteristic but detailed light microscope investigation and electron microscopy has shown that many species do, in fact, possess pyrenoids (Broady et al. 1997; Hibberd and Leedale 1971; Massalski and Leedale 1969).

The chloroplast lamellae usually comprise three thylakoids which show varying degrees of coherence depending on species. In the majority of investigated forms a single lamella, the girdle band, encircles the others around the edge of the chloroplast, enclosing between itself and the ends of the parallel lamellae a region of less dense matrix (Falk and Kleinig 1968; Hibberd and Leedale 1971; Massalski and Leedale 1969; Fig. 1e, f), almost certainly the location of the chloroplast DNA. The absence of a girdle band in *Bumilleria sicula* (Massalski and Leedale 1969), *Bumilleriopsis filiformis* (Böger and Kiermayer 1974; Hibberd and Leedale 1971), and *Pseudobumilleriopsis pyrenoidosa* (Deason 1971a) is probably due to secondary loss, since these three species are relatively advanced filamentous forms and seem closely related.

The chloroplasts in both vegetative cells and zoospores are bounded by the usual chloroplast envelope of two membranes and also by a two-membrane layer of chloroplast endoplasmic reticulum (PER), the outermost membrane of which is continuous with the outermost membrane of the nuclear envelope (Fig. 2). The region between the chloroplast envelope and the PER, the periplastidial compartment, contains a membranous reticulum, the periplastidial network. This network is best developed where the nuclear envelope and PER are confluent and appears either tubular or vesicular in transverse section (Hibberd and Leedale 1971; Massalski and Leedale 1969; Fig. 2). It shows occasional connection with the inner membrane of the PER (Falk and Kleinig 1968), and clusters of 3–6 spherules with dense walls can commonly be seen in tangential sections (Hibberd and Leedale 1971; Massalski and Leedale 1969). The composition of the spherules and their exact relationship with the periplastidial network has not yet been established.

Pyrenoids are typically of a semi-immersed type, forming a bulge on the inner face of the chloroplast (Deason 1971a; Falk 1967; Hibberd and Leedale 1971; Massalski and Leedale 1969). They are traversed by normal three-thylakoid lamellae but these are more widely spaced than in the remainder of the chloroplast and may also show discontinuities (Massalski and Leedale 1969). Thylakoid-free outpushings of the chloroplast in *Tribonema viride* (Falk and Kleinig 1968), the germlings of *Vaucheria woroniniana* (Marchant 1972), and three strains of *Xanthonema* (Broady et al. 1997) have been described as projecting pyrenoids, but these attributions must remain in doubt pending more thorough investigation. No storage material or capping vesicle has been found to be associated with pyrenoids in the Xanthophyceae but lipid droplets (plastoglobuli) (Fig. 1c), which are normally

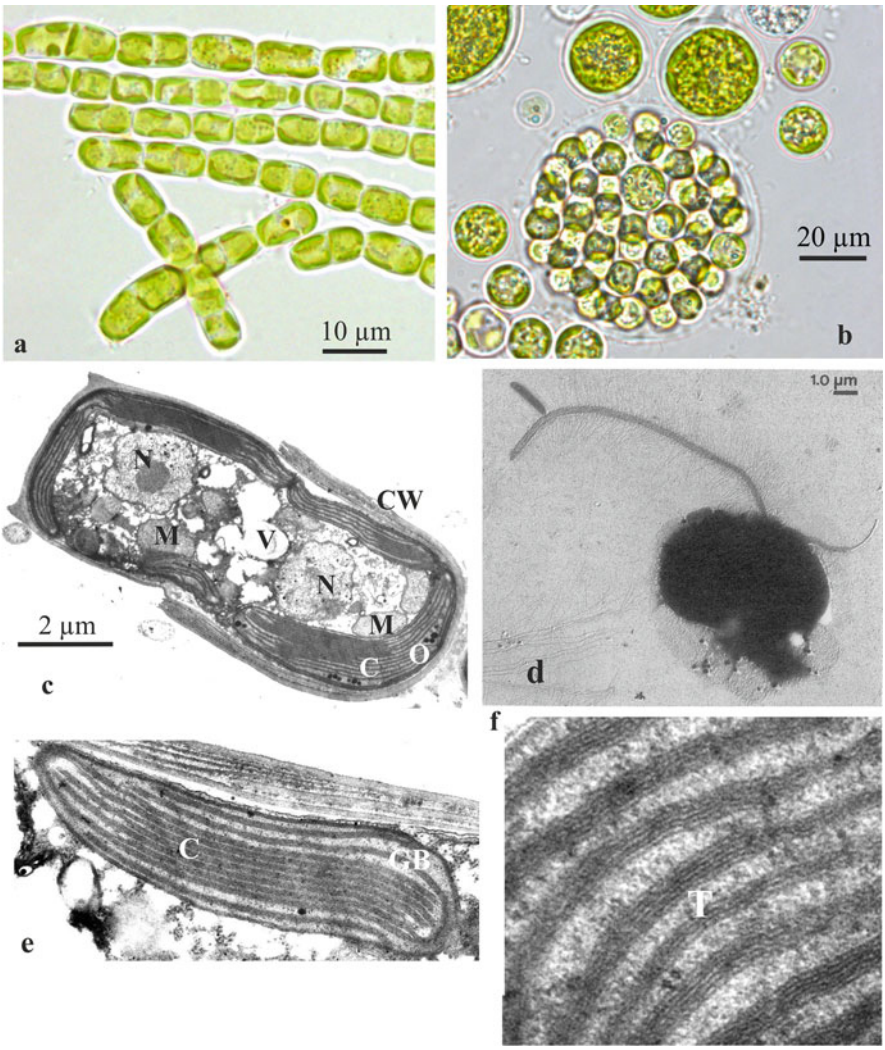

Fig. 1 Cell structure in the Xanthophyceae. (**a**) Vegetative cells of the filamentous form *Xanthonema* sp., light microscopy; (**b**) Vegetative cells of the coccoid form *Botrydiopsis*, light microscopy; (**c**) Section of a vegetative cell of *Xanthonema* sp. (C = chloroplast, CW = cell wall, M = mitochondrion, M = nucleus, O = lipoidal globule, V = empty vacuole); (**d**) Zoospore of *Ophiocytium majus*, light microscopy, phase-contrast, fixed and embedded cell; (**e**) Portion of the vegetative cell of *Xanthonema* sp. (C = chloroplast, GB = girdle band); (**f**) High magnification of the chloroplast of *Xanthonema* sp. to show distribution of thylakoids (T)

distributed randomly in the chloroplast matrix, are often seen to be concentrated at the periphery of the pyrenoid (Deason 1971a; Falk 1967; Hibberd and Leedale 1971; Massalski and Leedale 1969).

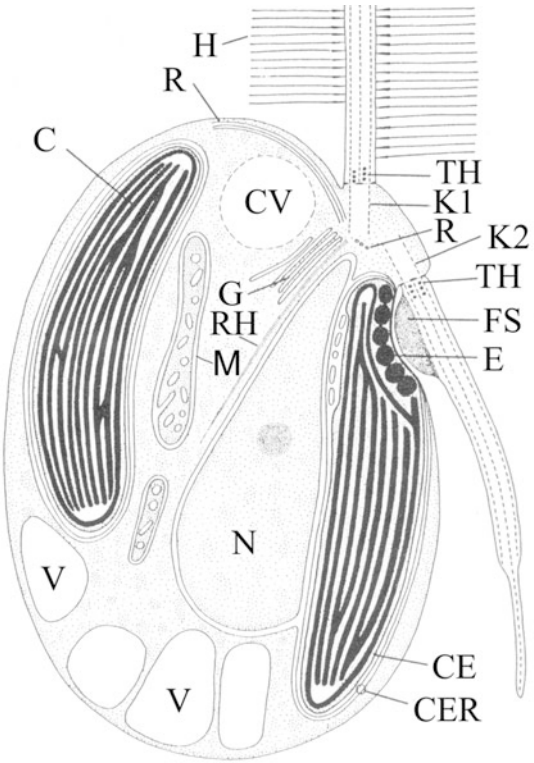

Fig. 2 Schematic representation of a xanthophyte zoospore (C = chloroplast, CE = chloroplast envelope, CER = chloroplast ER, CV = contractile vacuole, E = eyespot, FS = flagellar swelling, G = Golgi body, H = flagellar hairs, K1 = kinetosome long flagellum, K2 = kinetosome short flagellum, M = mitochondrion, N = nucleus, R = microtubular flagellal roots, RH = microfibrillar flagellal root (rhizoplast), TH = transitional helix, V = empty vacuole)

The nucleus in vegetative cells is more or less regular in shape (Fig. 1c), although part of the envelope has been seen to extend toward a pair of centrioles (Deason 1971a; Hibberd and Leedale 1971); this may be the general rule. The outer membrane of the nuclear envelope is confluent with the outer membrane of the PER although the area of contact is not usually extensive. Mitosis has been studied at the ultrastructural level in only one species of *Vaucheria* (Ott and Brown 1972) and *Tribonema regulare* (Lokhorst and Star 2003b). The most noteworthy feature of nuclear division is that the nuclear envelope remains completely intact at all stages, and it is not penetrated at the poles by gaps or fenestrae as commonly found in other examples of "closed" division. Following migration to opposite poles of each pair of centrioles normally associated with each nucleus, a spindle consisting of both continuous and noncontinuous microtubules forms within the nuclear envelope. The nucleolus fragments by metaphase and the centrioles duplicate by early anaphase. Anaphase is characterized by the formation of an extremely long interzonal spindle, probably by extension of the continuous spindle microtubules. By early telophase the spindle is very narrow and consists of only about 24 microtubules. Each nucleus is then cut off from the spindle by invagination of the nuclear envelope and the nucleolus reforms. Even if this type of closed division is found to be typical of the Xanthophyceae as a whole, it is likely that the very long interzonal spindle is confined to *Vaucheria* and perhaps the other siphonous forms.

Conventional chromosome cytology of the Xanthophyceae is extremely difficult owing to the generally small cell and nuclear size, but counts of 17 chromosomes have been published for four species of *Tribonema* (Iorya 1974).

Golgi bodies in the Xanthophyceae are typically small, consisting of 3–7 cisternae (Falk 1967; Falk and Kleinig 1968; Hibberd and Leedale 1971; Massalski and Leedale 1969). Each Golgi body lies against a flattened or concave face in the nuclear envelope, with one edge close to the centriole pair; the same positional relationship exists in the zoospores between the nucleus, Golgi body, and kinetosomes (Fig. 2). Species of *Vaucheria* are unusual in that each of the numerous Golgi bodies in the siphonous filaments is associated with an ER cisterna and a mitochondrion (Greenwood 1959; Ott and Brown 1974).

Mitochondria in the Xanthophyceae have tubular invaginations of the inner mitochondrial membrane into the matrix.

Cell Wall Structure and Composition

The cell wall of many xanthophyceans is delicately sculptured and in others, particularly the filamentous and large coccoid species, the wall consists of two overlapping halves (Bohlin 1897a; Ettl 1978; Pascher 1937–1939). In most cases the bipartite nature is not obvious, becoming apparent only after staining or swelling with alkalis or when the two halves separate for some reason such as zoospore release. It must be stressed that a bipartite wall is present in by no means all, perhaps not even a majority of, species. In species with a bipartite wall, the parts may be more or less equal in size or very unequal, but in all cases studied in detail (Deason 1971a; Hibberd and Leedale 1971; Falk and Kleinig 1968), the gradually tapering edges overlap widely. In the case of filamentous species, the walls are formed of interlocking H-shaped pieces (Bohlin 1897a; Ettl 1978; Pascher 1937–1939; Fig. 1c). Treatment with dilute KOH or NaOH characteristically swells the wall and reveals a complex lamellate construction (Bohlin 1897a), the walls appearing to be composed of closely stacked, deeply U-shaped elements. Electron microscopy of sectioned material shows that the cell walls of the species investigated have a distinctly layered structure and that the tapering edges of the overlapping walls are held together by a separate layer of cementing material (Falk and Kleinig 1968; Hibberd and Leedale 1971). Bipartite cell walls also stain strongly with dilute basic dyes, such as methylene blue and Congo red, and are very resistant to strong acids; for all these reasons the walls have often been described as being composed predominantly of an acid pectic compound. However, cellulose has been identified by a variety of techniques as a major constituent of the continuous tubular wall of *Vaucheria* (Mizuta et al. 1989; Tomaselli 2004). Although *Vaucheria* may not typify the whole group in this respect, its cell wall resembles that of most xanthophycean species in giving a strong positive reaction with Schiff's reagent *without* prior acid hydrolysis. This neglected and poorly understood reaction is largely confined to the Xanthophyceae (Prat 1947) and forms a further useful diagnostic character. However, since all species do not react, a negative reaction is inconclusive.

Motile Cells

While the vegetative cells of many coccoid xanthophyceans may be difficult to distinguish from green algae or Eustigmatophyceans, their motile stages (mainly zoospores) are characteristic (Ettl 1978; Hibberd and Leedale 1971; Lokhorst and Star 2003a; Massalski and Leedale 1969; Pascher 1937–1939; Fig. 2). Xanthophycean motile cells, with the exception of the spermatozoids (motile gametes that fertilize larger nonmotile gametes) and extremely large compound zoospores (synzoospores) of *Vaucheria*, are remarkably constant in structure. They are mostly ovoid or pyriform in shape, vary from about 5–20 μm in length, and are completely naked, bounded only by a plasmalemma. A degree of plasticity of shape and ameboid movement are therefore also common characteristics of these cells. The zoospores bear two flagella of unequal length and are bilaterally symmetrical with an obliquely truncate anterior end into which the flagella are inserted in the median plane of symmetry. The long flagellum is usually about the same length as the body of the zoospore while the second is from half to one-quarter this length. The long flagellum beats in an approximate sine wave, propagated at the tip of the flagellum, "pulling" the cells through the water. The cells rotate about their axis and describe a somewhat helical path. This may be due to the action of the short flagellum, which is difficult to see during life but appears to beat laterally. Electron microscopy shows that the long flagellum bears lateral hairs (Fig. 1d) as does the shorter anterior flagellum of the two relatively long flagella of *Vaucheria* spermatozoids (Koch 1951; Moestrup 1970). The hairs are straight and stiff, vary in length between 1 and 2 μm depending on species, and are about 20 nm thick with a short tapering basal part attached to the flagellar membrane at the narrow end, a tubular shaft, and two long fine terminal filaments of equal or unequal length. Appendages of this kind are referred to either as tubular mastigonemes (Bouck 1972) or tripartite tubular hairs (Moestrup 1982). The short flagellum of xanthophycean zoospores and the long posterior flagellum of *Vaucheria* spermatozoids are always free of appendages but may terminate in a hair point (Fig. 1d). The very large synzoospores of *Vaucheria* are exceptional in that both members of the numerous pairs of subequal flagella are smooth (Greenwood et al. 1957; Koch 1951; Ott and Brown 1974).

The two flagella of a typical zoospore are inserted at an obtuse angle to each other into a raised dome of cytoplasm on the anterior truncate face of the cell (Fig. 2). The long flagellum emerges freely but the proximal 1 μm of the short flagellum bears an asymmetrically wedge-shaped swelling with electron-dense contents at its proximal end. The swelling lies in a shallow depression in the cell immediately over the eyespot (Fig. 2).

The kinetosomes are constructed of the usual 9 triplet microtubules, but the transition region between the kinetosome and the axoneme has a characteristic structure shared with the Chrysophyceae and Eustigmatophyceae (Hibberd 1980). Thus, the lumen of the axoneme is traversed by a partition with a central axosomal thickening that always occurs exactly at the level at which the flagellum enters the cell body. Close to this and surrounding the proximal few nm of the central pair of axonemal tubules is a dense helix usually with 3–4 gyres, called the transitional helix

(Hibberd 1979, 1980; Fig. 2). This helix has also been called a "coiled fiber" (Bouck 1971) and a "Spiralkorper" (Casper 1972) in members of the Chrysophyceae.

A detailed three-dimensional description of the flagellar apparatus including the kinetosomes has been done solely for the zoospore of *Tribonema* by Lokhorst and Star (2003a). Conversely for other xanthophycean motile cells, the analysis of the kinetid is not complete, but it appears that two types of root originate near the kinetosomes: a single narrow cross-banded fibrous root (rhizoplast) that runs closely against the inner surface of the anterior prolongation of the nucleus and at least three microtubular roots extending in various directions through the cytoplasm (Hibberd 1980; Fig. 2). There are three or four component microtubules in each of these roots near the kinetosomes. The spermatozoids of *Vaucheria* are unusual in having an anterior "proboscis," similar to that in the spermatozoids of the brown alga *Fucus*, which contains a band of eight or nine microtubules originating near the kinetosomes (Moestrup 1970).

There are one to several but most commonly two chloroplasts in each zoospore, the number being species specific. One, usually designated as occupying a ventral position, always terminates immediately below the truncate face of the cell, while the dorsal chloroplast extends into the anterior end (Fig. 2). In species where pyrenoids occur in the chloroplasts of the vegetative cells, they are always present in the chloroplasts of the zoospore. When there is only a single chloroplast in each zoospore, this occupies the ventral position. Zoospores of most species possess a reddish refractile eyespot (stigma) at the anterior end of the ventral chloroplast. This consists of a single layer of densely osmiophilic globules immediately within the chloroplast envelope and directly beneath the swelling on the short flagellum (Figs. 1d and 2). The presence of eyespots has been reported by Van den Hoek et al. (1995) and observed in *Tribonema regulare* during the germination stage (Lokhorst and Star 2003a). In *Vaucheria*, however, their absence is genuine; the spermatozoids lack chloroplasts (Moestrup 1970) and the synzoospores have chloroplasts without eyespots (Ott and Brown 1974).

The nucleus in xanthophycean zoospores is pyriform and occupies a characteristic position, lying against the inner face of the ventral chloroplast with its tip close to the kinetosomes; the outer membrane of the nuclear envelope is confluent with the outer membrane of the chloroplast ER over a wide area (Fig. 2). In the compound zoospores of *Vaucheria*, one nucleus is attached to each pair of kinetosomes, but there is no association with the chloroplasts (Greenwood et al. 1957; Ott and Brown 1974). *Bumilleria sicula* is unusual in having patches of a delicate network with 15 nm openings occurring in the perinuclear space (Massalski and Leedale 1969). A further unusual feature in this species is that endoplasmic reticulum has been found organized parallel to areas of the nuclear envelope with circular fenestrations exactly opposed to the nuclear pores.

Zoospores generally contain only a single Golgi body, which lies with its forming face close against the nuclear envelope and with one edge near the kinetosomes (Fig. 2). When more than one Golgi body is present, all of them lie against the anterior prolongation of the nucleus (Massalski and Leedale 1969). The contractile vacuoles occur in the anterior region of the cell adjacent to the Golgi body and

kinetids (Fig. 2). They show no particularly noteworthy structural features, but coated vesicles may often be found in close proximity. Zoospores of some species contain several characteristic types of vesicles and inclusions. Most commonly seen are small peripheral vesicles containing a single spherical densely osmiophilic body (Deason 1971a; Hibberd and Leedale 1971; Massalski and Leedale 1969), possibly produced in the Golgi body (Deason 1971b). Others, with less dense, spirally wound fibrous contents, occur at the posterior end of the zoospores of *Ophiocytium majus* (Hibberd and Leedale 1971) and appear to contain the adhesive material by which the zoospore attaches itself during settling (Hibberd 1980). Peculiar fibrous discoid vesicles apparently produced in the perinuclear space have been found in the zoospore of more than one species (Deason 1971a; Massalski and Leedale 1969) and possibly give rise to the cell wall material as the cells settle. Irregularly shaped droplets, probably of lipoidal material, are generally distributed in the cell, and large, apparently empty vacuoles regularly occur in the posterior ends of zoospores (Fig. 2).

Reproduction and Life Cycles

The majority of xanthophyceans reproduce asexually in a variety of ways, the method being dependent on the basic cell form. The few monadoid or rhizopodial forms uncertainly attributed to this clade divide bilaterally and also appear to produce endogenous cysts similar but not identical to those characteristic of the Chrysophyceae. The palmelloid forms can reproduce by division but also produce zoospores or their non-mastigote equivalent, "hemiautospores" (Ettl 1978) or "aplanospores." The majority of species, the coccoid forms, reproduce primarily by the production of autospores, small replicas of the parent cells (Fig. 1b). Zoospore formation is also common among the coccoid species, although in culture large numbers are normally produced only after a change to fresh medium or some other stimulation. The most usual number of both aplanospores and zoospores produced by each parent cell is two to four, but many more may be found in multinucleate species showing a marked increase in vegetative cell size. Synzoospores probably result from incomplete cleavage. They occur in siphonous, filamentous, and some multinucleate coccoid forms; species producing them usually also have normal bimastigote zoospores. Filamentous forms reproduce either by cell division, leading to filament growth and subsequent fragmentation, or by zoospores. As in the coccoid forms, zoospores are common but are produced in large numbers in culture only following some environmental shock.

Little information is yet available on the fine structure of zoosporogenesis, but a similar pattern of events apparently occurs (Deason 1971b; Falk 1967; Hibberd 1980). The chloroplasts move away from the walls of the cell to take up a more internal position, one or two coming to lie against each nucleus. An eyespot appears in the chloroplast or in one of the chloroplast pair relatively late in the formation process. The pair of centrioles normally associated with each nucleus comes to lie at one end of the chloroplasts and each pair produces two flagella in a vesicle. The

Golgi body maintains its position relative to the centrioles and nuclear envelope. Flagellar hairs are formed in the perinuclear compartment and are then transported to the cell surface in vesicles of the endoplasmic reticulum. Zoospore cleavage occurs by the formation and coalescence of vesicles which separate the nuclei and associated chloroplasts. Zoospore release occurs by rupture or gelatinization of part of the mother cell wall or, in species with bipartite walls, by separation of the two sections of the wall.

Asexual reproduction in *Vaucheria* is by means of very large compound zoospores (synzoospores) bearing numerous pairs of slightly unequal flagella or by means of multinucleate aplanospores (Van den Hoek et al. 1995). A sporangium is formed by septation of the tip of a filament. Organelles accumulate before separation of the zoosporangium by a septum (Ott and Brown 1974). The normal association between the mitochondria and Golgi bodies disappears and flagella form in internal vesicles from the pair of centrioles accompanying each nucleus. These vesicles eventually coalesce to produce large internal flagellar pools that then migrate to the surface of the maturing zoospore and evaginate so that the flagella become situated on the zoospore surface. Septation of the zoospore from the vegetative filament occurs during this stage. The compound nature of the zoospore is revealed by the production of an incipient cleavage furrow which "attempts" to cut off the chloroplasts from the remainder of the zoospore.

Published information on zoospore settling is so far available only for *Vaucheria* (Ott and Brown 1975) and *Ophiocytium* (Hibberd 1980). In the latter, the body of the zoospore gradually rounds up and the flagella are withdrawn. The posterior end of the cell then rapidly elongates and narrows forming the stipe of the new vegetative cell. The distal end of this stipe becomes surrounded by finely fibrillar adhesive material, apparently released from dense vesicles with fibrillar contents present in the swimming zoospores. Later in settling, the axonemes disintegrate and the eyespot disappears. The bipartite nature of the cell wall is established from the earliest stage of wall formation. The first-formed wall material is very dense and composed of two overlapping parts; this is also apparently formed from vesicles normally present in the zoospore. As this wall forms, the chloroplasts come to lie closely against the wall, the nucleus regains its more or less spherical shape, and a normal lamellate and less dense wall is laid down.

In *Vaucheria*, the flagella are retracted into the body of the zoospore, and the peripheral layer of cytoplasm, which contains only nuclei and vesicles, alters in appearance. The nuclei sink into the cytoplasm and the vesicles disappear, probably giving rise to the cell wall, and are replaced by chloroplasts and other organelles. The withdrawn axonemes disintegrate and a bulge forms at one end of the settled cell. The various organelles and vesicles then become arranged as in the vegetative cells and a large central vacuole forms.

Sexuality in the Xanthophyceae has rarely been observed except in the siphonous genera *Botrydium* and *Vaucheria.* In *Botrydium* sexual reproduction is by means of bimastigote isogametes. Sexual reproduction in *Vaucheria* is oogamous. Antheridia and oogonia are formed either on special lateral branches of the main filament or are

sessile. They are separated from the main filament by a cross-wall. The antheridium produces a large number of colorless spermatozoids that are structurally different in a number of ways from xanthophycean zoospores, as already described. The mature oogonium is uninucleate, containing a single oosphere, and fertilization through a special pore produces a thick-walled resting zygote (oospore). Meiosis occurs at germination, yielding a new siphonous filament.

With the exception of the oospores of *Vaucheria*, resting stages are seen only rarely. Endogenously produced cysts with a bipartite wall appear to be confined to the few monadoid or rhizopodial forms. The most common resting stage in coccoid and filamentous forms is the akinete, a single-celled spore in which the wall is formed by a thickening of the parent cell wall. A particular type of aplanospore with a bipartite cell wall of quite different shape from that of the parent cell has also been described for some coccoid and filamentous forms, including *Tribonema.* Nagao et al. (1999) studied the process of akinete formation in relation to the acquirement of freezing tolerance in the freshwater *Tribonema bombycinum.* Both akinetes and this type of aplanospore produce zoospores or hemiautospores on germination.

Traditional Classification

The clade is named Xanthophyceae. An alternative and nomenclaturally typified name for this class, Tribophyceae, based on the generic name *Tribonema*, has been published (Hibberd 1981). This proposal resulted from changes in the International Code of Botanical Nomenclature, which allows names of taxa above the rank of family (to which the principles of typification and priority do not necessarily apply) to be considered as automatically typified when they are ultimately based on generic names. A class name based on *Tribonema* has not yet been validly published. The name Xanthophyceae is based on the generic name *Xanthonema* established by Silva (1979).

Current formal classification of Xanthophyceae does not reflect results obtained by modern molecular phylogenetic studies. Indeed, the latter approach has revealed that many orders, families, and genera currently defined by morphological characters (Ettl 1978) are paraphyletic or even polyphyletic and do not form clades (Andersen et al. 1998; Bailey and Andersen 1998; Maistro et al. 2007, 2009; Negrisolo et al. 2004; Potter et al. 1997; Rybalka et al. 2009; Zuccarello and Lokhorst 2005). A modern biological classification must be based on a rigorous phylogenetic approach and every taxonomic unit should be a monophyletic group. The provision here of new formal names would be premature as several key taxa have not yet been analyzed within a phylogenetic framework (Maistro et al. 2009). Below, we follow a pragmatic approach and retain the formal classification of seven orders provided by Ettl (1978) based largely on morphology of vegetative stages with, in some cases, additional information from reproductive characteristics. Taxa marked with an asterisk (*) are those found to be para-/polyphyletic in molecular phylogenetic analyses.

The unicellular flagellates are placed in the order Chloramoebales, while the ameboid forms belong to Rhizochloridales. The palmelloid and coccoid species are contained respectively in Heterogloeales and Mischococcales. Filamentous taxa form the Tribonematales, while siphonous taxa are assigned to Botrydiales or Vaucheriales. In the previous version of this chapter (Hibberd 1990), all siphonous forms were placed in Vaucheriales, but there is now overwhelming phylogenetic evidence that siphonous forms do not constitute a monophyletic group. Adl et al. (2005) revised the classification of Xanthophyceae and assigned all taxa to Tribonematales except for *Vaucheria* that was assigned to Vaucheriales. This classification is also strongly contradicted by phylogenetic analyses (Maistro et al. 2007, 2009; Negrisolo et al. 2004).

Chloramoebales, Rhizochloridales, and Heterogloeales contain few species that in many cases are known only from original descriptions and have yet to be rediscovered (Ettl 1978; Hibberd 1990; Pascher 1937–1939). Their inclusion within Xanthophyceae requires corroboration through rigorous phylogenetic analyses (Maistro et al. 2009). The remaining four orders encompass the majority of species with Mischococcales containing the most.

In the traditional classification, features defining each family include: habit (free-living or attached; solitary, colonial, or filamentous), whether cells are coenocytic or uninucleate, and presence or absence of surrounding mucilage and of branching. Classification into genera and species is based mainly on cell shape and size, cell envelope characters (lorica or cell wall, wall smooth or thickened in various ways), and to a lesser extent on cell contents (chloroplast number, presence or absence of a pyrenoid). However, several of these features result from convergent evolution and are poor indicators of phylogenetic relatedness (Maistro et al. 2007, 2009; Negrisolo et al. 2004).

Order Chloramoebales

The cells are solitary, free-swimming, naked, and more or less constant in shape or ameboid, with two unequal flagella or rarely only one flagellum, one to several chloroplasts, one or two anterior contractile vacuoles, and sometimes an eyespot. Reproduction is by longitudinal fission. Palmelloid stages and bipartite cysts are known. Species are usually freshwater, but are also found in marine and brackish waters. The order includes one family, Chloramoebaceae, with 11 genera and 14 species.

Family Chloramoebaceae

A poorly-known, certainly unnatural family; several species may belong in other phyla. Ettl (1978) recognizes the separate family Bothrochloridaceae for forms in which the flagella are inserted into a gullet-like depression.

Order Rhizochloridales

The cells are naked and ameboid, with pseudopodia or filopodia, but lack flagella. They are solitary or colonial, free-living or attached, and sometimes loricate. They

contain one to several chloroplasts, sometimes with an eyespot. Reproduction is by fission or by the production of zoospores that bear two unequal flagella. Endogenously produced resting stages with a bipartite wall are found in some forms. Species are freshwater, marine, or brackish.

Family Rhizochloridaceae
The cells are always free-living solitary amebas without a lorica. Relatively few (2–16) chloroplasts are present. The family comprises one genus, *Rhizochloris*, found in freshwater.

Family Stipitococcaceae
The cells produce a delicate hyaline vase-shaped lorica borne on a mostly filiform stipe. The lorica has one or more pores through which the filopodia extend. One to several chloroplasts are present, and some species possess an eyespot and contractile vacuole. Reproduction is by division into two zoospores. Freshwater species are epiphytic on filamentous algae. The family includes three genera, *Stipitococcus*, *Stipitoporos*, and *Rhizolekane*, with 10 species.

Family Myxochloridaceae (Chlamydomyxaceae)
The cells are large ameboid plasmodia containing numerous nuclei and chloroplasts and several contractile vacuoles. Reproduction, where known, is by division into smaller plasmodia, into zoospores bearing two flagella of unequal length, into small uninucleate amebas, or into endogenous uninucleate cysts with a bipartite silicified wall. Freshwater forms usually live in the hyaline cells of the leaves of the moss *Sphagnum*. There are two monospecific genera, *Myxochloris* and *Chlamydomyxa*.

Order Heterogloeales
The cells are palmelloid in organization, i.e., nonmotile, possessing neither flagella nor pseudopodia, but, in some species, containing permanent contractile vacuoles and an eyespot. They are solitary or colonial, free-living or attached, and surrounded by mucilage or not. Species are freshwater, marine, or brackish.

Family Heterogloeaceae
The cells are spherical or ellipsoidal, embedded in structureless mucilage forming free-floating or attached gelatinous masses. Reproduction is by simple division or production of zoospores with one or two unequal-length flagella. The family includes three genera, *Heterogloea*, *Gloeochloris*, and *Helminthogloea*, with seven species.

Family Mallodendraceae
The cells are naked, attached by a thick gelatinous stipe that can branch at division giving rise to arbusculate colonies. Reproduction is by longitudinal fission and by zoospores. Species are freshwater or brackish. There is only one genus, *Mallodendron*, with two species.

Family Pleurochloridellaceae
The cells are solitary, spherical, and unattached with a cell wall but no surrounding mucilage. Reproduction is by zoospores or autospores. There is one genus, *Pleurochloridella*, with two freshwater species. The placement of this family within Xanthophyceae is very doubtful and requires molecular corroboration. Indeed *Pleurochloridella botrydiopsis*, the only species so far analyzed using a molecular approach, was not included within Xanthophyceae (Maistro et al. 2009).

Family Characidiopsidaceae
The cells are solitary with a distinct cell wall, growing attached by means of a stipe and gelatinous disc. There is no surrounding mucilage. Reproduction is by zoospores. There is one genus, *Characidiopsis*, with four freshwater species.

Order Mischococcales*
The cells are coccoid in organization, i.e., having a distinct cell wall and lacking flagella, contractile vacuoles, and eyespot in the vegetative state. They contain one to several chloroplasts and nuclei (usually one nucleus and two chloroplasts). The cell wall is in one or two pieces and is either smooth or sculptured. Reproduction is by autospores, zoospores, or hemiautospores. Bipartite cysts are known for some species. The majority of species of Xanthophyceae are classified in this order.

Family Pleurochloridaceae*
The cells are solitary, free-living, and mostly uninucleate. Classification is based mainly on cell shape and cell wall features. Their shape varies from spherical through ellipsoidal to fusiform or polygonal. The cell wall is smooth and featureless or ornamented in a variety of ways and is in one or two pieces. Reproduction is by means of autospores or zoospores. The family comprises 38 genera with 190 species, freshwater or marine.

Several species formerly classified here have been transferred to the Eustigmatophyceae and many more probably belong there.

Family Botrydiopsidaceae*
The cells are characterized by their capacity for prolonged growth without division, producing large multinucleate cells with numerous chloroplasts. They can be solitary, free-living, or attached. They are spherical to irregular in shape, with the cell wall in one piece and unsculptured. Reproduction is by zoospores, aplanospores or autospores. There are four genera with seven freshwater species. The genus *Botrydiopsis** is polyphyletic.

Family Botryochloridaceae*
The cells remain attached after autosporulation, forming regular to irregular colonies which may or may not be embedded in mucilage. Cell shape varies from spherical to

fusiform. Reproduction is by zoospores or autospores. There are eight genera with 48 freshwater species. *Chlorellidium** is polyphyletic.

Family Gloeobotrydaceae
Species in this family are colonial forms in which the cells are not mutually attached but embedded in a common mucilage. Colonies are attached or free-floating. The cells are mostly spherical or ellipsoidal. Reproduction is by zoospores or autospores. There are eight genera with 27 freshwater species (14 in *Gloeobotrys*).

Family Gloeopodiaceae
The cells secrete a mostly stratified mucilaginous stipe by which they are attached to the substratum. They are unicellular or form small arbusculate colonies. There is one genus, *Gloeopodium*, with six freshwater or brackish species.

Family Mischococcaceae
The cells are united in arbusculate, di-, or tetrachotomous colonies. These colonies are borne on mucilaginous stipes, the cells in twos or fours at the ends of the ultimate branches. Stipes are produced by swelling of the inner layers of the mother cell wall during autospore formation. There is one genus, *Mischococcus*, with six freshwater species.

Family Characiopsidaceae
The cells are solitary or in groups of two or four, always attached to the substratum either directly, by means of a mucilaginous pad, or by a stipitate extension of the cell wall. The cell wall is in one or two parts, smooth or sculptured. Reproduction is mostly by zoospores, rarely by autospores. There are eight genera with 98 freshwater, brackish, and marine species, including 65 species of *Characiopsis*.

Family Chloropediaceae
The cells are arranged in flat tabular colonies, attached directly to the substratum. Reproduction is by zoospores or autospores. There is one genus, *Chloropedia*, with two freshwater species.

Family Trypanochloridaceae
The cells are irregularly rounded to stellate with a single parietal chloroplast and a central nucleus. Reproduction is by large numbers of autospores produced from the central cytoplasm only. There is only a single species, *Trypanochloris clausiliae*, occurring in the outermost layers of the shells of *Clausilia*, a genus of small terrestrial gastropods.

Family Centritractaceae*
The cells are solitary, uninucleate, free-living, prominently elongate, and with a cell wall in two pieces. Reproduction is by zoospores and aplanospores. There are three

genera, *Bumilleriopsis**, *Pseudobumilleriopsis* (Maistro et al. 2009), and *Centritractus*, with 19 species.

Family Ophiocytaceae (Sciadiaceae)
The cells are elongate-cylindrical with a cell wall in two unequal parts, always with two nuclei or multinucleate with two to many chloroplasts. They are solitary or colonial, free-living, or stipitate. Reproduction is by zoospores. There is one genus, *Ophiocytium*, with 14 freshwater species.

Order Tribonematales*
The order includes all Xanthophyceae with filamentous organization in which cells are mainly uninucleate with one to several chloroplasts. Classification into families is based mainly on the degree of differentiation of the filaments. Reproduction is by cell division, zoospores, and aplanospores. Species are freshwater and marine.

Family Neonemataceae
Uniseriate to mostly multiseriate filaments with exterior layers of mucilage; the individual cells are separated from each other. Reproduction is by cell division, zoospores, and aplanospores. There are two genera, *Neonema* and *Chadefaudiothrix*, with four freshwater species.

Family Tribonemataceae*
Species in this family are unbranched uniseriate filaments without exterior layers of mucilage and can be either attached or free-floating. The cell wall is usually clearly differentiated into two parts, the half-walls from adjacent cells forming H-shaped pieces. Reproduction is by zoospores and aplanospores. Thick-walled resting stages (akinetes) are also produced. There are six genera: *Brachynema*, *Heterotrichella*, *Xanthonema**, *Heterothrix*, *Bumilleria*, and *Tribonema**, with 49 freshwater and marine species (Maistro et al. 2009). Some possible polyphyletic species have been identified within *Tribonema* using the *rbcL* gene (Zuccarello and Lokhorst 2005).

Family Heterodendraceae
Species in this family are branched, uniseriate filaments, forming small arbusculate growths attached by an enlarged basal cell. Reproduction is by zoospores. There is only one genus, *Heterodendron*, with two freshwater species.

Family Heteropediaceae
Species in this family form branched uni- to multiseriate filaments, differentiated into pseudoparenchymatous basal and erect filamentous parts. The cell wall is not differentiated into H-shaped pieces. Reproduction is by cell division and zoospores. Zoospores are formed either in all cells or in separate zoosporangia. Palmelloid stages are known. The family comprises six genera with 50 freshwater species (45 in *Heterococcus*).

Order Botrydiales
There is just a single family and genus in this order with the characteristics as described below.

Family Botrydiaceae
These consist of a macroscopic aerial globular part up to several mm in diameter, containing a large number of nuclei and chloroplasts, and subterranean colorless branched rhizoids. Asexual reproduction is mainly by means of zoospores and autospores. Sexual reproduction is by fusion of iso- or anisogametes giving rise to a zygote. Gametes are formed in undifferentiated vegetative cells. Members are terrestrial, often found on drying mud in freshwater environments. There is one genus, *Botrydium*, with eight species.

Order Vaucheriales*
The order Vaucheriales comprises xanthophyceans with siphonous organization (nuclear division taking place without cell wall formation during vegetative growth).

Family Vaucheriaceae*
Species in this family consist of branched siphonous filaments with unlimited apical growth, forming a thallus usually several cm in extent, the filaments containing large numbers of chloroplasts and nuclei in a peripheral cytoplasmic layer surrounding a central vacuole. Cross-walls are formed only during reproduction. Sexual and asexual reproduction in *Vaucheria* is described above. Reproduction in *Asterosiphon* is asexual only, by aplanospores. In *Asterosiphon*, the filaments are regularly branched, forming rosettes up to 1 cm diameter on moist soil. Species of *Vaucheria* commonly form extensive growths on moist soil or grow submerged; many species amphibious. Species occur in freshwater, marine, and brackish habitats. *Asterosiphon* is monospecific; *Vaucheria* includes over 70 species.

Molecular Phylogenetics

Molecular phylogenetic data exist for approximately one-sixth of known xanthophycean species (Andersen and Bailey 2002; Bailey and Andersen 1998; Maistro et al. 2007; Negrisolo et al. 2004; Rybalka et al. 2009, 2013; Zuccarello and Lokorst 2005). Each of these can be firmly placed in one of the four major clades into which the class can be divided. These clades have been designated as the Botrydiopsalean, the Chlorellidialean, the Tribonematalean, and the Vaucherialean (Maistro et al. 2009). The Botrydiopsalean clade contains only coccoid forms and may contain most of the species currently included in Mischococcales but this requires phylogenetic confirmation. The Chlorellidialean clade includes strains with a wide range of morphological expression ranging from coccoid unicells to branched filaments of *Heterococcus* (currently in Tribonematales). There are no

unique features of morphology visible by light microscopy that define this group, although the clade receives very strong molecular support (Maistro et al. 2009). The Tribonematalean clade contains filamentous unbranched species of *Bumilleria*, *Tribonema* and *Xanthonema*, *Heterothrix*, as well as new genera awaiting formal description. Also included are coccoid species of *Bumilleriopsis*, *Pseudobumilleriopsis* and *Ophiocytium*, and siphonous *Botrydium*.

All species except those of *Botrydium* have a bipartite cell wall (Hibberd 1990) that could constitute a synapomorphic feature for this clade. The siphonous thallus of *Botrydium* would then have been acquired by secondary loss of this feature (Maistro et al. 2007). However, the position of *Botrydium* is not fully resolved although tests for alternative phylogenies did not reject the phylogenetic position of *Botrydium* as sister group of other taxa present in the Tribonematalean clade. If further studies show this to be so, then the *Botrydium* clade could be regarded as the traditional order Botrydiales as defined by Ettl (1978). The Vaucherialean clade contains siphonous species of *Vaucheria* and conforms with earlier concepts of order Vaucheriales (Ettl and Gärtner 1995; Adl et al. 2005).

Siphonous *Asterosiphon dichotomus* has been placed as sister species of the Tribonematalean clade, but alternative topology tests did not reject its position as sister species of *Vaucheria* (Maistro et al. 2009). If this latter phylogenetic hypothesis is preferred, then *Vaucheria* + *Asterosiphon* would represent the Vaucheriales sensu Ettl (1978) and Rieth (1980). However, current evidence suggests that *A. dichotomus* should be placed outside the Vaucherialean clade and its unusual morphology of a rosette-shaped, dichotomously branched thallus (Ettl and Gärtner 1995) supports this contention.

Some coccoid species remain outside major clades in poorly resolved positions. Molecular data are unavailable for other coccoid and filamentous taxa as well as for all Chloramoebales, Heterogloeales, and Rhizochloridales.

Maintenance and Cultivation

For the great majority of species that occur in nature as walled unicells, single cell isolation is probably the best isolation technique, especially as most species are usually found only in small numbers. Plating or dilution may be tried when numbers are sufficient. Biphasic soil/water media usually offer the best chance of growth, although since most species multiply relatively slowly it may take several weeks for any growth to become apparent. Biphasic cultures may also be used for long-term maintenance. Agnotoxenic cultures also generally grow well in a wide variety of defined mineral media (listed by Nichols 1973). Axenic cultures may be obtained by repeated washing of single cells or by plating where the species grow well on agar. They are usually maintained on slopes containing proteose peptone, 0.1%; KNO_3, 0.02%; K_2HPO_4, 0.002%; and $MgSO_4$ $7H_2O$, 0.002%.

Species of *Vaucheria* mostly grow well in biphasic soil/water media. They are isolated from thalli that are reproducing sexually by inoculating media with washed oospores, which then germinate to produce a clean vegetative thallus. Zoospores

may also be used as an inoculum in the few species producing them. A simple but effective method for producing axenic cultures of *Vaucheria* has been devised by Åberg and Fries (1976).

Evolutionary History

The Xanthophyceae constitutes one of the major phyletic lines that emerged within the photosynthetic Stramenopiles (e.g., Cavalier-Smith and Chao 2006). Fossil xanthophyceans are extremely rare (e.g., Butterfield 2004) and totally insufficient to trace the evolution of the clade and to establish the evolutionary relationships with other stramenopilans. Before the advent of molecular techniques, the placement of Xanthophyceae and other stramenopileans algae was assessed using biochemical and structural data. These showed evolutionary relationships with other stramenopilean taxa: e.g., Xanthophyceae, Phaeophyceae, and Chrysophyceae share cell wall structure and growth, the form of the resting spore, part of pigments, storage products, and the flagella of motile cells (Pascher 1937–1939). Ultrastructural characteristics, particularly of motile cells, of the Xanthophyceae, Chrysophyceae, Phaeophyceae, and Bacillariophyceae, added support for a common ancestry of these four groups (Hibberd 1976, 1979; Hibberd and Leedale 1971, 1972; Massalski and Leedale 1969).

The main features of motile cell structure common to the Chrysophyceae, Xanthophyceae, and Phaeophyceae include: (1) a long anterior flagellum bearing two opposite rows of tubular hairs and a smooth posteriorly directed flagellum, which, except in the spermatozoids of the xanthophycean *Vaucheria* and some brown algae, is shorter than the anterior flagellum; (2) a swelling at the proximal end of the short flagellum closely associated with the cell membrane it overlies; and (3) an eyespot consisting of a single layer of pigment droplets within one of the chloroplasts. Other features of cell structure common to these three phyla but not confined to them include: (1) bilateral symmetry; (2) chloroplast ER; (3) a chloroplast girdle band; (4) a transitional helix; (5) a pyriform nucleus positioned close to the kinetosomes, which in the Chrysophyceae and Xanthophyceae and probably Phaeophyceae is attached to them by means of (6) a fibrillar root (rhizoplast); and (7) a constant positional relationship between the Golgi body, nucleus, and kinetosomes. The Bacillariophyceae share several features of ultrastructure with these three phyla, including possession of tubular hairs by the single flagellum of the male gametes of some centric forms, and a girdle band.

In addition to these four lineages, the Raphidophyceae also possess heterokont flagella and chloroplasts with a girdle band, and on this basis have been considered to be allied with them.

Biochemical characters, mainly pigment composition, when considered in isolation, are less helpful than structural characters in indicating the possible relationships of the Xanthophyceae. Indeed different distributions of various pigments suggest different relationships with various protist lineages belonging to the Chromalveolata (Adl et al. 2005). Thus, the absence of chlorophyll *b* and the synthesis of

c chlorophylls in the Prymnesiophyceae, Dinophyceae, and Cryptophyceae as well as in the Xanthophyceae, Chrysophyceae, Phaeophyceae, and Bacillariophyceae, provides reasonably strong evidence for the common ancestry of the chlorophyll *c*-related genes in all these groups (Ragan and Chapman 1978). However, distribution of a number of major pathways of carotenoid biosynthesis, particularly that leading to fucoxanthin, suggests that Chrysophyceae, Bacillariophyceae, Phaeophyceae, and Prymnesiophyceae have a common phylogenic heritage. Other pathways indicated that the Xanthophyceae and Raphidophyceae appear to be phylogenetically close to this line and to each other, but the absence of the fucoxanthin pathway and its replacement in these two phyla and the Eustigmatophyceae by the vaucheriaxanthin pathway indicates affinity, although the Eustigmatophyceae also shows some significant differences from the Xanthophyceae, supporting its separation as a distinct clade.

Molecular phylogeny has revolutionized the perception of Xanthophyceae placement in the tree of life. There is robust molecular evidence that Xanthophyceae are one of the phyla of photosynthetic protists included in the Stramenopiles (e.g., Cavalier-Smith and Chao 2006; Riisberg et al. 2009; Yang et al. 2012). Furthermore all photosynthetic heterokonts form a monophyletic group that is supported by various types of molecular evidence (e.g., Cavalier-Smith and Chao 2006; Riisberg et al. 2009; Yang et al. 2012). Relationships among the 16 photosynthetic heterokont algal classes are not yet fully understood but molecular analyses are rapidly filling the gap (e.g., Cavalier-Smith and Chao 2006; Kai et al. 2008; Riisberg et al. 2009; Yang et al. 2012). Multiple gene phylogenies show that the Xanthophyceae together with Chrysomerophyceae, Aurearenophyceae, Phaeothamniophyceae, Phaeophyceae, and Schizocladiophyceae form a well-defined group named clade PX (Cavalier-Smith and Chao 2006; Kai et al. 2008; Riisberg et al. 2009; Yang et al. 2012). This clade derived its name from the two most species-rich classes, Xanthophyceae (X) and Phaeophyceae (P) (Kai et al. 2008). Within clade PX the phylogenetic relationships are not yet fully resolved and further analyses are necessary to properly identify the position of Xanthophyceae. The clade PX together with Raphidophyceae form a clade named SI that constitutes one of the three major phyletic lines that emerged during the evolution of photosynthetic Heterokonta (Yang et al. 2012).

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Hyphochytriomycota and Oomycota

13

Gordon W. Beakes and Marco Thines

Abstract

The anteriorally uniflagellate Hyphochytriomycota and biflagellate Oomycota are in the Kingdom Straminipila (commonly referred to as stramenopiles) which are part of the SAR superkingdom. Both appear to be basal to the large assemblage of golden-brown algae, the Ochrophyta. Both feature osmotrophic nutrition and have traditionally been considered as zoosporic "fungi," but are unrelated to organisms in the monophyletic kingdom Mycota. The Hyphochytriomycota is a small group encompassing around half a dozen genera, which have simple nonmycelial, holocarpic thalli, traditionally encompassing three families: the endobiotic Anisolpidiaceae, the polycentric Hyphochytriaceae, and the monocentric Rhizidiomycetaceae. Recently the former have been shown to be placed among the early diverging Oomycota, leaving just the latter two families in the monophyletic Hyphochytriomycota clade. Hyphochytriomycota are widespread in occurrence, and most are saprotrophs or parasites, infecting the resting spores of Oomycota and Glomeromycota. In contrast, the Oomycota are a large and diverse assemblage, consisting of two major (class level) clades, the Saprolegniomycetes and Peronosporomycetes, and several early diverging classes most of which are simple holocarpic organisms that lack mycelial organisation. Many of these early-diverging clades are as yet poorly resolved because of sparse taxon

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sampling. The early-diverging orders include the Eurychasmales and Olpidiopsidales, both of which are marine seaweed parasites, the nematode infecting Haptoglossales and crustacean infecting Haliphthorales. The Saprolegniomycetes mostly have fungal-like mycelial thalli and include the orders Atkinsiellales s.lat., Leptomitales, and Saprolegniales, which are mostly saprophytes or parasites of invertebrates and, occasionally, vertebrates such as fish and amphibians. A few species in the Saprolegniales are root infecting parasites of plants. The Peronosporomycetes are the second major fungal-like class, and include the largely saprotrophic Rhipidiales, the facultively parasitic Pythiales s.lat., which can infect both animals and plants and the predominantly plant pathogenic Albuginales and Peronosporales sensu lato. Indeed, the Oomycota are significant parasites of both animals and plants, impacting both natural ecosystems and causing significant economic losses in both aquacultural and agricultural systems. The molecular systematics of the Oomycota is still in a state of flux, and in this account a relatively conservative approach has been taken. It is apparent that most of the early-diverging genera are almost exclusively marine and that the Peronosporales represents the main terrestrial and plant pathogenic lineage. Most early-diverging genera lack the oogamous sexual reproduction that characterizes this group and suggests that the oogenesis evolved around the time of emmergence from the sea to the land and freshwater ecosystems. It is also clear that obligate biotrophy in the white blister rusts (Albuginales) and downy mildews (Peronosporales s.str.) has evolved independently.

Keywords

Albugo; *Aphanomyces* • Biflagellate zoospore • Oogamy • Biotrophy • Ecology • Evolution • *Hyphochytrium* • Oomycetes • *Phytophthora* • *Pythium* • Plant pathogen; *Rhizidiomyces* • RxLR-effectors • *Saprolegnia* • Stramenopile • Systematics • Zoosporogenesis • Zoospore ultrastructure

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Where s. lat. is used after a name, there are significant subclades which suggests this taxon will require splitting, although at present the low statistical support, or incomplete taxon sampling means it cannot be done with confidence.
Where names are placed between " " means names have not been formally published.
~Before the name means this Order, Family or genus appears to be para- or polyphyletic and will require taxonomic revision.
[a]Indicates Family or species has not been sequenced, so taxonomic position not confirmed by molecular data.
[b]Recent multigene trees, suggest this clade may merit a new order and family rank (Paralagenidiales, Paralagenidiaceae) (Spies et al. 2016).
[c]This family's Order placement still not fully resolved.
[d]Recent multigene trees, suggest this clade may merit order rank (Pythiales), but may also require further splitting (Spies et al. 2016).
[e]Recent multigene trees, suggest this clade may merit order rank as Peronosporales s. str. (Spies et al. 2016).

Introduction

General Characteristics

Historically, the zoosporic fungi studied by mycologists encompassed chytrids, hyphochytrids, labyrinthulids, thraustochytrids, oomycetes, and plasmodiophorids. All generally had walled thalli that fed by osmotrophic absorption, although many had small holocarpic thalli rather than a typical mycelium. These organisms are a polyphyletic assemblage with only the Chytridiomycota now included in the kingdom Fungi (Fig. 1a; Adl et al. 2012). The Plasmodiophorids are now placed in the Cercozoa, a sister clade to Rhizaria (Heuhauser et al. 2010), which together with all other biflagellate fungal-like groups fall within the recently defined "SAR" (Straminipila, Alveolata, Rhizaria) superkingdom (Fig. 1a; Burki et al. 2007, 2008; Burki and Keeling 2014). Molecular studies confirm that both the anteriorly uniflagellate Hyphochytriomycota and the biflagellate Oomycota (Fig. 1b; Tsui et al. 2009; Van der Auwera et al. 1995) are part of the same lineage as the chlorophyll c containing Ochrophyta (Cavalier-Smith and Chao 2006), which together form the sister clade to the Labyrinthulomycota and Opalinids (Tsui et al. 2009). Dick (2001)

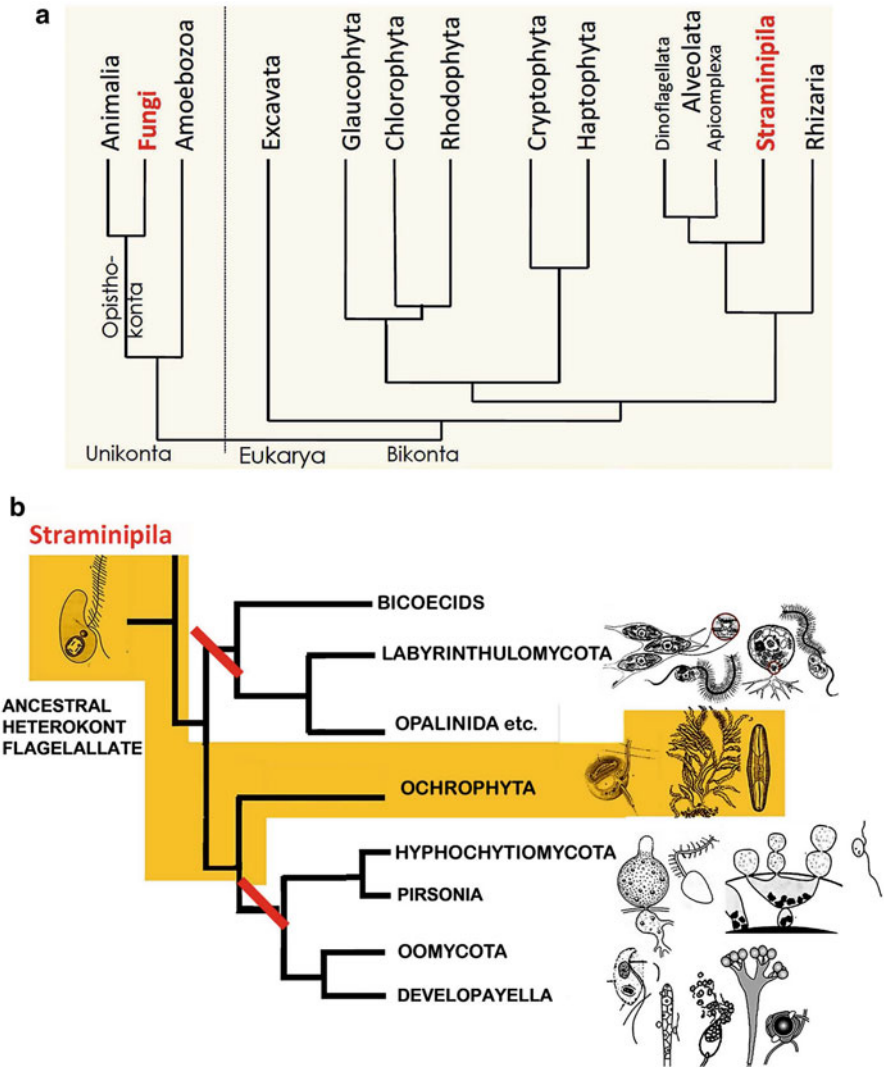

Fig. 1 General phylogeny. (**a**) Overview summary tree of main Eukaryote supergroup clades, showing relative phylogenetic postions of the Fungi and Straminipila, which is part of the Straminipila, Alveolata, Rhizaria (SAR) superclade (Based on Burki et al. 2008). (**b**) Schematic tree of the Straminipila clade, showing probable relationships between the Hyphochytriomycota and Oomycota, with respect to other members of the clade. The *red bars* represent possible plastid loss events as proposed by Tsui et al. (2009) on which Figure is based. However, not all data supports multiple plastid losses, others have proposed multiple plastid acquisitions rather than losses (see Beakes et al. 2014a) (Adapted from Beakes et al. (2011) from *Protoplasma* with permission)

placed all of these mastigonate fungal-like groups in his revised Kingdom Straminipila, while Cavalier-Smith and Chao (2006) placed the Hyphochytriomycota and Oomycota in the phylum Pseudofungi, together with a number of protists, including the bacteriotrophic flagellate *Developayella* (Leipe et al. 1994) and the parasitoid *Pirsonia* (Kühn et al. 2004).

Members of the Hyphochytriomycota are characterized by their small holocarpic, chytrid-like thalli and zoospores with a single, anteriorly-directed flagellum (Figs. 2a and 7a). At the end of a motile period, the zoospores encyst, germinate, and grow to form simple epi- or endobiontic chytrid-like thalli (Fig. 2a–e). Hyphochytriomycota is a small phylum/subphylum consisting of a single order (Hyphochytriales) containing only four or five described genera encompassing around two dozen species. These were grouped by Karling (1942, 1977) into three families: the Anisolpidiaceae, Rhizidiomycetaceae, and Hyphochytriaceae. Molecular sequencing studies of *Hyphochytrium catenoides* (Van der Auwera et al. 1995) and *Rhizidomyces inflatus* (Hausner et al. 2000) confirmed that the Hyphochytriomycota form a well-defined clade sister to the Oomycota (Fig. 1b). However, recent sequence data for the marine phaeophyte parasite *Anisolpidium ectocarpi* have revealed that the Anisolpidiaceae fall within the basal Oomycota, close to *Olpidiopsis spp.* (Gachon et al. 2015), and thus, are excluded from the Hyphochytriomycota (Table 1).

In contrast, the Oomycota is a large and diverse phylum/subphylum containing mostly fungal-like organisms (Fig. 2q, r–u; Money 1998; Richards et al. 2006). There are around 1500 or more species grouped into about a 100 genera, the majority of which, however, contain fewer than five species (Table 1; Dick 2001). They typically produce biflagellate zoospores (Fig. 7b–e) and many saprolegniomycete genera produce two generations of zoospores (diplanetic; Fig. 7b, d) or aplanospores and zoospores. The anterior flagellum is mastigonate (Fig. 7e), while the posterior flagellum is smooth with a terminal acroneme (Fig. 7c; Vlk 1939; Manton et al. 1951; Fig. 2). Characteristics that separate Oomycota from true Fungi include having a diploid rather than haploid vegetative thallus (Win-Tin and Dick 1975), cell wall microfibrils composed of cellulose and glucans rather than chitin (Bartnick-Garcia 1970), and a different biochemical pathway for lysine biosynthesis (Vogel 1960). In addition, they store β1–3 mycolaminarins rather than glycogen as their main carbohydrate reserve (Wang and Bartnicki-Garcia 1974). Molecular phylogeny based on ribosomal subunit genes confirmed that the Oomycota share the same common ancestor as the Ochrophyte algae (Fig. 1b: Adl et al. 2012; Cavalier-Smith and Chao 2006; Förster et al. 1990; Gunderson et al. 1987; Leipe et al. 1994; Rilsberg et al. 2009).

Occurrence

The Hyphochytriomycota are found in both soil and water in freshwater, marine, and terrestrial environments and are cosmopolitan in distribution (Fuller 1990, 2001; Gleason et al. 2009). There are both saprotrophs and low-impact parasites, particularly of other chromistans (Oomycota and Phaeophyta) and possibly of crustacea. Although relatively small numbers of species have been described, environmental

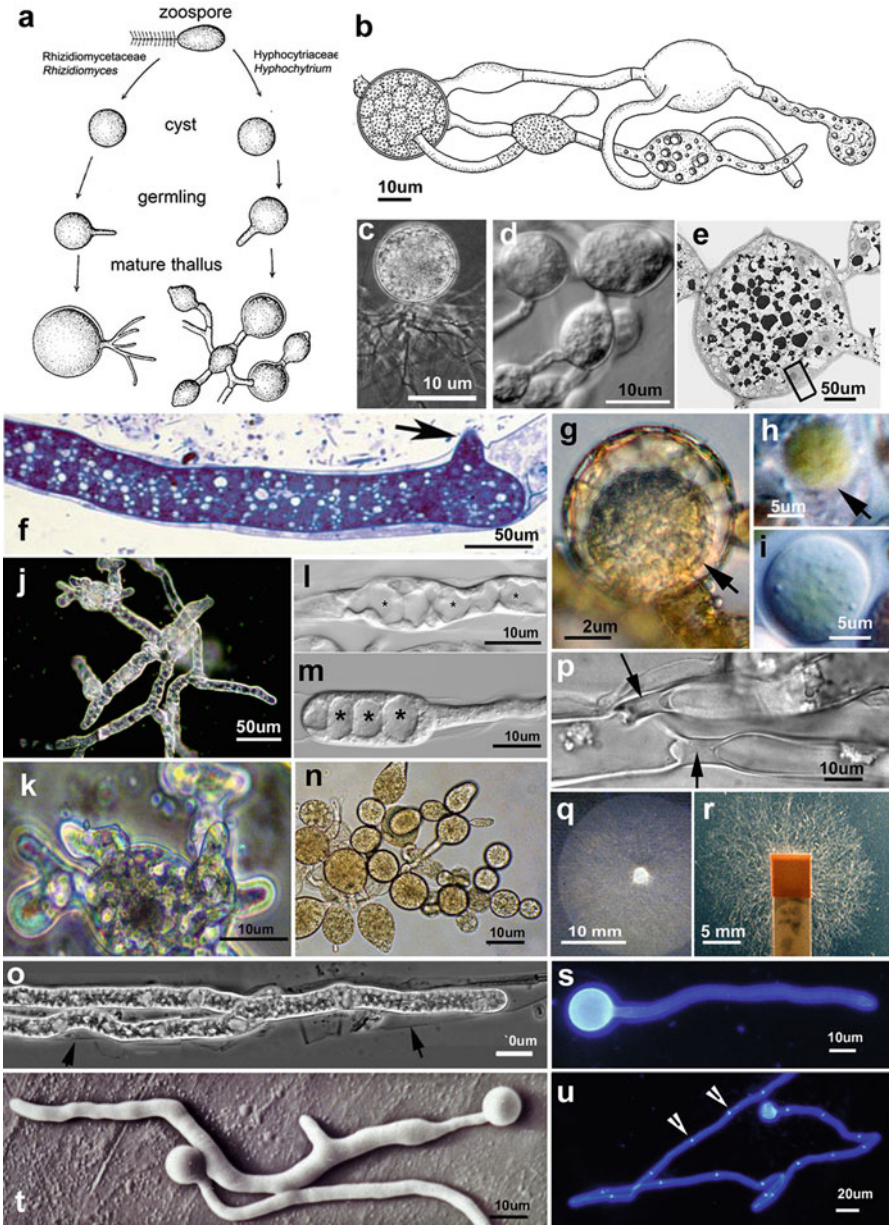

Fig. 2 The vegetative thallus. (**a**) Schematic hyphochytridiomycete life style diagram showing monocentric thallus development in *Rhizidiomyces* and polycentric development in *Hyphochytrium* (Adapted from Fuller 1990, with permission*) (**b**) Diagram of the polycentric thallus of *Hyphochytrium catenoides* showing swollen thalli, interconnectd by short hyphal-like segments (From Karling (1977), with permission of Charles Lubrecht) **(c)** Light micrograph of *Rhizidiomyces apophysatus*, showing spherical thallus, with basal rhizoids (From Fuller and Jaworkski (1987) with

sequencing from both marine (Diéz et al. 2001; Massana and Pedró-Alió 2008; Massana et al. 2002, 2004, 2006) and freshwater (Richards et al. 2012) environments has revealed many unknowns that fall within the hyphochytrid clade, suggesting that as a group they are both more diverse and widespread than generally appreciated. Hyphochytrid cultures are not widely available although both *H. catenoides* and *R. inflatus* are listed in the American Type Culture Collection (ATCC).

Oomycetes are also ubiquitous in marine, freshwater, and terrestrial ecoystems, where they occur as widespread saprotrophs infesting decaying plant and animal detritus (Dick 1990, 2001; Hulvey et al. 2010; Newell and Fell 1995; Riethmüller and Langer 2004) or as necrotrophic and biotrophic pathogens of a wide range of animals (Fig. 3a–k: Karling 1981; Phillips et al. 2008) and plants (Fig. 4a–v: Constantinescu 1991; Dick 2001; Thines 2014; Voglmayr 2008). Plant pathogenic species (Fig. 4a–v) show the greatest diversity, and recent molecular studies have explored the phylogenetic relationships between these pathogens and their hosts (Choi and Thines 2015; Göker et al. 2007; Thines et al. 2008, 2009a; Voglmayr 2003, 2008). Many oomycete plant pathogens, notably *Albugo* and *Hyaloperonospora* infecting

Fig. 2 (continued) permission) (**d**) Light micrographs of mature polycentric thallus of *H. catenoides* in culture (From Gleason et al. (2009) with permission *J. Euk. Microbiol.*) (**e**) TEM of developing thallus showing central vacuole system with electron dense inclusions (From Clay et al. (1991) with permission *Mycol. Res.*) (**f**) A toluidineblue-stained thallus of *Haptoglossa polymorpha*, infecting a rhabditid nematode, showing dense cytoplasm and short discharge tubes (From Beakes et al. (2011) with permission *Protoplasma.*) (**g**) Light micrograph of a naked thallus of *Eurychasma dicksonii*, strain Euo5within a hyperplasic infected cell of the host *Ectocarpus*. (**h**–**i**) Light micrographs of young developing thalli of holocarpic oomycete *Olpidiopsis porphyrae*, in thallus of *Porphyra* (All from Sekimoto (2008) with permission *Protist*) (**j**–**k**) Darkfield and phase contrast light microgrphs of the irregularly lobed thallus of the early diverging, crustacean parasite *Halocrusticida* (syn. *Halodaphnea*) *okinawensis*, Beakes, unpublished micrographs. (**l**–**m**) Differential interference contrast (DIC) light micrographs of crustacean parasite *Haliphthoros* sp. showing irregularly swollen, vacuolate (*) thallus, with peripheral spore initials developing. Photo courtesy Satoshi Sekimoto. (**n**) Irregularly beaded thallus of an in vitro culture of the early diverging saprolegniomycete parasite of nematodes, *Chlamydomyzium oviparasiticum* (From Glockling and Beakes (2006b) with permission *Mycol. Res.*) (**o**) Phase contrast light micrograph showing the elongate holocarpic (sparsely branched) thallus of the related species *Ch. dictyuchoides in vivo*. The digested remnants of nematode cuticle are arrowed (From Beakes et al. (2014b) with permission *Fung. Biol.*) (**p**) Part of a branched sporulating mycelium of *Sapromyces elongatus* (Rhipidiales) showing constricted thalli, sealed with thick cell wall plugs. Beakes, unpublished. (**q**) A colony of *Saprolegnia parasitica*, growing on agar media, showing typical fungal-like colony of advanced Oomycote. Beakes unpublished. (**r**) A colony of *Phytophthora cinnamomi*, growing over surface of agar from a soil incubated dipstick bait. Courtesy of Adrienne Hardham. (**s**) Calcofluor stained, UV-fluorescence light micrograph of a germinating cyst of *Saprolegnia diclina*, showing typical narrow hyphal-like germ tube. (**t**) Low temperature SEM of germinating cysts of *S. diclina* showing beginning of branched mycelial-thallus. (**u**) Calcofluor stained, UV-fluorescence light micrograph of a germinating cyst of *S. parasitica,* showing septate (plugged – *arrows*) hyphae characteristic of this species. **s**–**u**. Beakes unpublished

Table 1 A provisional taxonomic framework for the Hyphochytiomycota and Oomycota based on molecular data

Kingdom: Straminipila	Superphylum: Pseudofungi
Phylum: Hyphochytriomycota	
Class: Hyphochytriomycetes	
Order: Hyphochytriales	
Family Hyphochytriaceae	*Canteriomyces, Cystochytrium*, ***Hyphochytrium***
Family Rhizidiomycetaceae	*Latrostium, Reesia,* ***Rhizidiomyces***
Phylum: Oomycota	
Basal orders – Class(es) incertae sedis	
Order Eurychasmales	
Family Eurychasmaceae	***Eurychasma***[a]
Order Haptoglossales	
Family Haptoglossaceae	***~Haptoglossa***
Order Olpidiopsidales s.lat.	
Family Anisolpidiaceae	***Anisolpidium***
Family Olpidiopsidacae s.lat.	***~Olpidiopsis***
?Family Pontismataceae	*Petersenia, Pontisma*
?Family Sirolpidiaceae	*Sirolpidium*
Order "Haliphthorales"	
Family Haliphthoraceae	***~Haliphthoros, Halocrusticida (****syn. Halodaphnea****),***
Order and Family incertae sedis	***Halioticida***
Rozellopsidaceae	*Rozellopsis*
Ectrogellaceae	*Ectrogella*
Class: Saprolegniomycetes	
Order Atkinsiellales s.lat.	
Family "Atkinisellaceae"	***Atkinsiella***
Family Crypticolaceae	***Crypticola***
Family Lagenismatacae	***Lagenisma***
Order and Family incertae sedis	***~Chlamydomyzium, ~Cornumyces,***
Order Leptomitales	
Family Leptomitaceae	***Apodachlya,*** *Apodachyella*, ***Blastulidium, Leptomitus***
Family incertae sedis	
Leptolegniellaceae	*Aphanomycopsis, Brevilegniella, Ducellieria, Eurychasmopsis, Leptolegniella, Nematophthora, Pythiella*
Order Saprolegniales	
Family Verrucalvaceae	***~Aphanomyces, Aquastella,*** *Pachymetra,* ***Plectospira, Sommerstorffia, Verrucalvus***
Family Saprolegniaceae s.lat. Clade spp. with eccentric oospores	***~Achlya, Brevilegnia, Dictyuchus, Thraustotheca***

(*continued*)

Table 1 (continued)

Kingdom: Straminipila	Superphylum: Pseudofungi
Clade spp. centric oospores	***Aplanes, Aplanopsis, Calyptralegnia,*** *Couchia*, ***Isoachlya, Newbya, Protoachlya, Pythiopsis, ~Saprolegnia,*** *Scoliolegnia*
Clade - uni-oosporiate, centric oospores	***Geolegnia, ~Leptolegnia***
Class: Peronosporomycetes	
Order and Family incertae sedis	***Salispina***
Order Rhipidiales	
Family Rhipidiaceae	*Araiospora, Aqualinderella, Mindeniella, Nellymyces, Rhipidium*, ***Sapromyces***
Order "Paralagenidales"	
?"Paralagenidiaceae"	***Paralagenidium***
Order Albuginales	
Family Albuginaceae	***Albugo****,* ***Pustula, Wilsoniana***
Order Peronosporales s.lat.	
Salisapiliaceae[a]	***Salisapilia***
Family Pythiaceae s. lat. subclades ?Myzocytiopsidaceae subclade	Holocarpic or eucarpic with narrow filamentous sporangia, many with vesiculate zoospore differentiation *Gominocheate,* ***~Myzocytiopsis (part)***
?Salilagenidiaceae subclade	***Salilagenidium (marine Lagenidium spp.)***
?Lagenidiaceae subclade	***~Lagenidium, Myzocytiopsis (part)***
?Lagenaceae s.lat. subclade	*~****Lagena****,* ***Lagenidium*** *(part),* ***Pythiogeton, Pythium*** *(part),* ***Myzocytiopsis*** *(part),*
?Pythiaceae s.str. subclade	Subclades with spp. with filamentous sporangia
	Lagenidium (part), Pythium **s.str,**
Family Peronosporaceae s.lat. subclades	Subclades with more or less globose to ovoid sporangia, zoospore differentiation often intra-sporangial with transient vesicle or without (downy mildews)
Section 1 subclade Globose to elongate sporangia formerly in *Pythium*.	***Globisporangium, Elongisporangium***
Halophytophthora sp. clade marine saprotrophs	***~Halophytophthora*** s.lat.
Section 2a,b subclades: Saprotrophs,	***Phytopythium*** *(syn. Ovatsporangium), Calycofera*
facultative stem and leaf pathogens, many of which produce elicitors	***Pilasporangium*** ***~Phytophthora***
Section 3 subclades Downy Mildews: 3a Graminicolus downy mildews (GDM)	Obligate biotrophs of Angiosperms ***Baobabopsis, Eraphthora, Graminivora, Peronosclerospora, Poakatesthia Sclerospora, Scleropthora, Viennotia***
3b: Brassicolous downy mildews (BDM)	***Hyaloperonospora, Perofascia***

(*continued*)

Table 1 (continued)

Kingdom: Straminipila	Superphylum: Pseudofungi
3c: Downy mildews with coloured conidia (DMCC)	***Pseudoperonospora, Peronospora***
3d: Downy mildews with pyriform haustoria (DMPH)	***Basidiophora, Benua, Bremia, Novotelnova Paraperonospora, Plasmopara, Plasmoverna, Protobremia,***

Those genera that are not emboldened have not been included in molecular phylogenies until the end of 2016
Those prefixed by a ~ appear to be paraphyletic or polyphyletic and are in need of revision
Families prefixed with a ? are in Dick (2001) and although reflected by clades, but it is uncertain whether all will eventually be given family level designation. These subclades are mostly based on a recent unpublished study of Spies et al. (2014, 2016)
Those Orders and Families in quotation marks " ", have not been formally published
[a]The phylogenetic position of this family/genus still not fully resolved. Some analyses have it as sister clade to *Halophytophthora* in the Peronsporaceae s. lat. clade

Arabidopsis, have provided model systems for exploring the molecular interactions between biotrophic pathogens and their hosts (Jiang and Tyler 2012; Kemen and Jones 2012; Thines and Kamoun 2010; Thines et al. 2009c). The occurrence and diversity of marine oomycetes have been greatly underestimated (Hulvey et al. 2010; Nigrelli and Thines 2013), and many recent studies on marine picoplankton samples have revealed many unknown stramenopiles within the Oomycota clade (Diéz et al. 2001; Massana and Pedró-Alió 2008; Massana et al. 2002, 2004, 2006; Richards et al. 2012).

Culture collection holdings of oomycetes are largely confined to the saprophytic and facultatively parasitic species with the largest collections held in the major culture collections such as the American Type Culture Collection (ATCC), Maryland; the Commonweath Agricultural Bureau International fungal collection (CABI), Egham; the Centraalbureau voor Schimmelcultures (CBS), Baarn, and the National Biological Resource Centre (NBRC), Chiba. Some academic institutions hold specialist collections, mainly of *Phytophthora* and *Pythium* isolates, such as the World Oomycete Genetic Resource Collection at the University of California, Riverside; the Department of Agriculture Mycology Culture Collection Ottawa; and a collection of *Aphanomyces* and fish-pathogenic *Saprolegnia* isolates in the Oomycete Culture Collection, Real Jardin Botanico (CSIC), Madrid. The Culture Collection for Algae and Protozoa (CCAP) in Oban has recently established a small collection of dual clonal cultures of marine oomycetes on their seaweed hosts (Strittmatter et al. 2013).

Literature and History of Knowledge

While there have been no dedicated monographs on the Hyphochytriomycota, they were included by Karling (1977) in his richly illustrated monograph of the chytrids sensu *lato*. In this he illustrates over half of the 23 accepted species and gives the

Fig. 3 **Animal pathogenic Oomycota**. (**a**–**d**) Achelminth pathogens. (**a**–**b**) DIC micrograph of a zoosporic *Haptoglossa* sp., infecting rhabditid nematodes showing both zoospore initials and in situ encysted spores that have formed infective gun cells (**b**). Courtesy S. Glockling (**c**) Encysted zoospores of *Ch. dictyuchoides* germinating around the mouth orifice of a rhabditid nematode host (From Beakes et al. 2014b with permission *Fungal Biol.*) (**d**) Tapered thallus lobes of a glutaraldehyde preserved thallus of *Aquastella acicularis*, infecting the rotifer *Polyarthra vulgaris*, an example of holocarpic relative of *Aphanomyces.* Beakes unpublished. (**e**) European white-clawed crayfish (*Austropotomobius pallipes*) that have been challenged and killed by crayfish plague, *Aphanomyces astaci.* (**f**) Sporulating mycelium of *Ap. astaci*, showing undifferentiated hyphae and discharged cluster of primary cysts. (**g**) detail of the underside of the body segments of an infected animal, showing white discolouration. (**e**–**g**) Beakes unpublished. (**h**) Atlantic salmon (*Salmo salar*) eggs, infected with *Saprolegnia diclina*, showing typical white fungal-like vegetative mycelia. Beakes unpublished. (**i**) A wild brown trout (*Salmo trutta*) infected with *Saprolegnia parasitica*, showing extensive white mycelial lesions on the skin. (**j**) Secondary cyst of *S. parasitica*, showing hooped bundles of boathook spines that characterize fish-lesion isolates. (**i**–**j**) Bruno et al. (2011) with permission. (**k**) Winter saprolegniasis, gizzard shad (*Dorosoma cepedianum*) from Murray River showing small irregular lesions typical of *S. parasitica* infections of coarse fish. Courtesy of James Puckridge

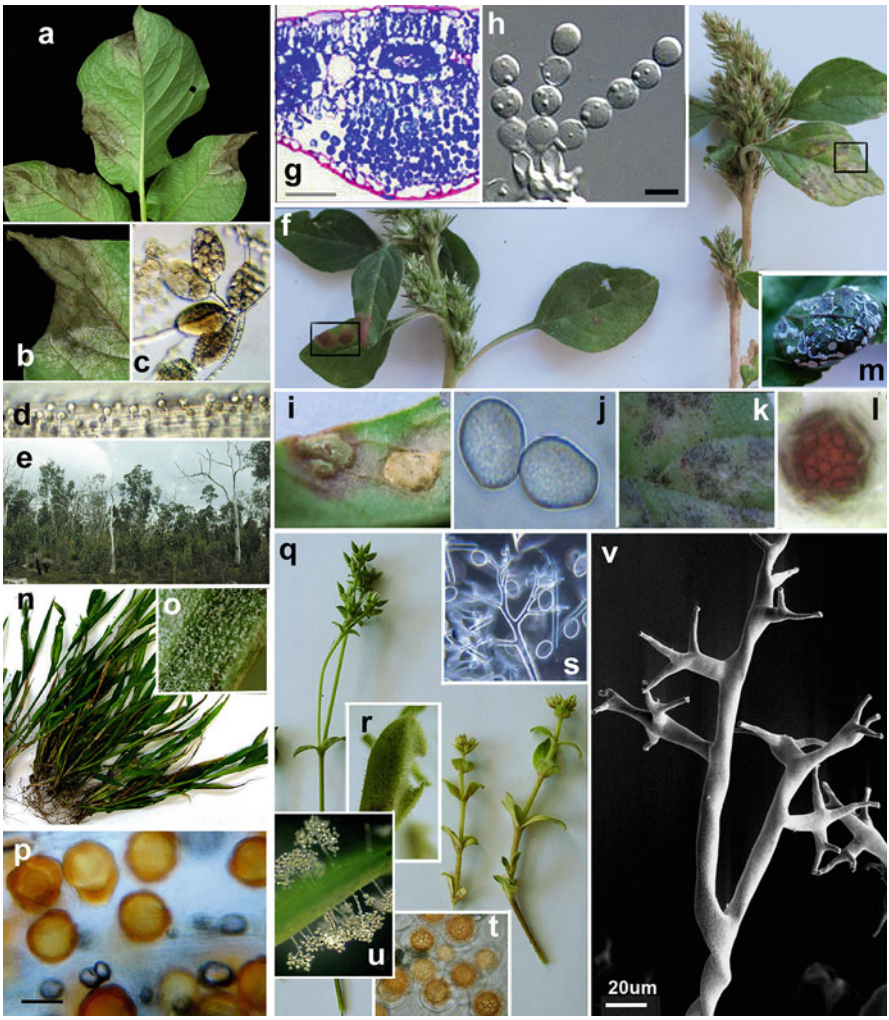

Fig. 4 Plant pathogenic Oomycota(all images unless otherwise stated Thines, unpublished): (**a**–**b**) Leaf lesions caused by the hemibiotrophic late blight pathogen, *Phytophthora infestans* on *Solanum tuberosum.* Courtesy of David Cooke. (**c**–**e**) The root infecting, *Ph. cinnamomi*. e. Mature sporangia showing zoospore release (**c**) and encysted zoospores, germinating on a eucalyptus root surface. (**d**) Native jarrah Forest dieback caused by *Ph. cinnamomi.* (**d**–**e**) courtesy of Adrienne Hardham. (**f**–**m**) The obligate biotrophic white blister rusts (Albuginales). (**g**–**l**) *Amaranthus* infected with *Wilsonia bliti* (f) General view of infected plants showing general symptoms of infections, with lesions shown by boxes. (**g**) Cross section through infected leaf showing pustule with parallel chains of condiosporangia (courtesy Annerose Heller), which are shown in detail in the DIC micrograph (h) of a chains of conidia (courtesy Young-Joon Choi). (**i**) Detail of blister like pustules on the underside of leaves. (**j**) Pear-shaped dispersive (secondary) conidia. (**k**) Detail of

important references for their study. Other reviews of this phylum include those by Fuller (1990, 2001), Dick (2001), and Beakes et al. (2014a). The first observations of zoosporic fungi possessing a single anterior flagellum were first made in the late nineteenth century (Zopf 1884), although the hyphochytrids were not formally separated from the posteriorly flagellate chytrids until later (Karling 1939, 1943).

The most recent comprehensive monograph of the Oomycota is the scholarly overview by Dick (2001) which lists much of the extensive taxonomic and general biological literature up to end of the millennium. Two important plant pathogenic genera, *Pythium* and *Phytophthora*, have been previously monographed, respectively, by Plaats-Niterink (1981) and Waterhouse (1970). The former is currently being comprehensively updated (previewed by de Cock et al. 2012). Recent literature sources for the plant pathogenic downy mildews can be found in Thines and Choi (2016), Lebeda and Spencer-Phillips (2007), Thines (2014), Thines et al. (2009a, b), and Voglmayr (2008). A review of the updated taxonomy of the Albuginaceae is given in Choi et al. (2006, 2008) and Thines and Voglmayr (2009). The three principal genera in the Saprolegniales (*Achlya*, *Aphanomyces* and *Saprolegnia*) have been monographed, respectively, by Johnson (1956), Scott (1961), and Johnson et al. (2002 updated from Seymour 1970). For other taxa, particularly the holocarpic species, reference should be made to Dick (2001), Karling (1981), and Sparrow (1960).

Detailed accounts of the extensive historical studies on the Oomycota have been given by both Dick (2001) and Johnson et al. (2002) and only highlights will be covered here. During the late eighteenth century, there were a number of reports of what we now recognize to be *Saprolegnia* infections of fish (reviewed by Hughes 1994). Similarly the first documented plant pathogenic oomycete was by Persoon who described the white blister rust *Aecidium candidum*, which was subsequently transferred by de Roussel in 1806 to the genus *Albugo* (Choi et al. 2007), although at that time it was still not recognized as an oomycete (Dick 2001). Much of the early documentation of the Oomycota stems from the pioneering researches of Pringsheim, de Bary, Regel, and Tulasne among others (Dick 2001). The higher oomycete taxa were recognized almost as soon as sufficient species had been described to put them into groups. De Bary separated the "Peronsporei" from the mucoraceous phycomycetes and shortly after proposed the "saprolegnieen" and "peronosporeen" family

Fig. 4 (continued) upper leaf showing dark fleck-like oospores within tissue. (**l**) Mature oospore of *W. bliti* showing reticulate oospore ornamentation. (**m**) Blister-like leaf lesion of *Albugo "armoraciae"*. (**n**–**p**) Graminicolousdowny mildews – *Sclerospora graminicola*. (**n**–**o**) Infected plants of *Setaria viridis* and details of leaf surface showing white conidiophores. Detail of orange-pigmented thick-walled angular oospores, typical of these mildews. (**q**–**v**) Downy mildews of herbaceous angiospermae. (**q**–**u**) *Cerastium* sp. infected with *Peronospora* sp. (**q**) plants (unifected *left*, infected *right*) showing stunting and chlorosis. (**r**) Detail of lower surface of a leaf, showing darker regions in areas where oospores have formed. (**s**) Branched sporangiophores (darkfield), with terminal disarticulating conidiosporangia. **t**. Mature reticulate oospores within infected leaf tissue. (**u**) Cotyledons of *Microthlaspi erraticum*, showing abundant condiophores of *Hyaloperonospora thlaspeos-perfoliat*ion both upper and lower surfaces. (**v**) SEM micrographof conidiophore of *Plasmopara nivea*, showing branchlets that bore the now-detached conidiosporangia

concepts (de Bary 1881). *Albugo* was eventually recognized as an oomycete when its sexual stages were described by Léveillé (1847) and subsequently placed in the "peronosporeen group" by de Bary (1881). The first attributable oomycete parasites of aquatic plants, protozoa and invertebrate animals were described by Schenk (1858), Cornu (1872), and Zopf (1884). The first plant pathogenic member of the oomycetes to be described was *Albugo candida* (Persoon, in Gmelin 1792). By the mid nineteenth century, there had also been descriptions of the first three downy mildew genera: *Peronospora* (Corda 1837), *Bremia* (Regel 1843), and *Basidiophora* (Roze and Cornu 1869). However, it was not until the end of the century that Schröter (1893) placed these plant pathogenic species into their own separate family, the Peronosporaceae.

In the latter half of the twentieth century, the taxonomic synthesis of the Oomycota was forged by three outstanding scholars of zoosporic fungi: Dick (1973a, b; 2001), Karling (1981), and Sparrow (1960, 1976). In the second edition of "*Aquatic Phycomycetes,*" Sparrow (1960) listed four major oomycete orders, the Saprolegniales, Leptomitales, Lagenidiales, and Peronosporales. In his final synthesis, Sparrow (1976) proposed splitting of the oomycetes into two 'galaxies', which Dick (2001) later formalized into the subclasses Saprolegniomycetidae and Peronosporomycetidae and also introduced a new order the Eurychasmales, in which he placed a number of little known marine taxa. He considered this new order to be part of the "saprolegnian galaxy" together with the Leptomitales and Saprolegniales. His "peronosporalean galaxy" included the orders Peronosporales, which encompassed most important plant pathogens, and Lagenidiales, which encompassed most of the holocarpic parasites of invertebrates and algae. Dick had continued to refine the classification of the Oomycota (Dick 1976a, b, 1997, 1998; Dick et al. 1984) culminating in his final synthesis which he outlined in his encyclopaedic treatise, "*Straminipilous Fungi*" in which he expanded the number of orders to around a dozen (Dick 2001). However, as a result of subsequent molecular studies, a substantial revision of Dick's (2001) scheme has recently been proposed (Beakes et al. 2014a).

Economic and Practical Importance

No hyphochytrid (i.e., excluding *Anisolpidium*) is known to cause any economically significant disease of plants or animals. Artemchuk and Zelezinskaya (1969) described a species (*Hyphochytrium peniliae*) that caused a severe mycosis of a freshwater crawfish, but there have been no subsequent reports of this disease, and Dick (2001) questioned whether this organism was even a hyphochytrid. Both *Rhizidiomyces* spp. and *Hyphochytrium catenoides* are known to parasitize oospores of plant pathogenic oomycetes (Ayers and Lumsden 1977; Sneh et al. 1977; Wynn and Epton 1979), and *Rhizidiomycopsis stomatosa* (Sparrow 1977) infects the resting spores of the endomycorrhizal fungus *Gigaspora margarita* (Schenck and Nicolson 1977; Sparrow 1977). Hyphochytrids may, therefore, adversely affect populations of both potentially harmful oomycetes and beneficial mycorrhizal fungi in soil ecosystems (Sneh et al. 1977). The closely related parasitoids belonging to the genus *Pirsonia* infect centric diatoms and bring about a decline in planktonic

blooms (Kühn 1997; Schnepf et al. 1990). Members of the genus *Anisolpidium* infect both freshwater algae (Canter 1950) and filamentous phaeophyte seaweeds (Karling 1943; Küpper and Müller 1999); however, this genus is now thought to be an oomycete (Gachon et al. 2015).

In contrast, the economic importance of the Oomycota is well known. Many are devastating and economically important plant pathogens (Fig. 4a–v), with even threatening natural ecoystems such as the Jarrah forest in Australia (Fig. 4e). In the mid-nineteenth century, de Bary and Berkeley established that the species we now know as *Phytophthora infestans* (Fig. 4a, b) was the causal agent of the devastating potato blight epidemic responsible for the great Irish famine (Berkeley 1846; de Bary 1876; Yoshida et al. 2013). Other species cause serious losses to wild and farmed fish (Fig. 3i, k) and crustaceans (Fig. 3e, g), and there are a few species that can opportunistically infect mammals, including humans (Bruno et al. 2011; de Grooters et al. 2013; Mendoza 2005; Phillips et al. 2008; Schurko et al. 2004; Van West 2006). Economically important genera include the obligate biotrophic white blister rusts (*Albugo*; Fig. 4f–l) and the downy mildews (e.g., *Bremia*, *Peronospora*, *Sclerospora* etc.; Fig. 4n–v) and facultatively parasitic genera such as *Aphanomyces, Phytophthora* (Fig. 4a–e)*, Pythium* and *Saprolegnia*. White blister rusts (Fig. 4f–l) and downy mildews (Fig. 4n–v) infect plants, the latter often causing stunting (Fig. 4f, q) and may result in significant yield losses to many economically important crop plants (Constantinescu 1991; Thines and Choi 2016; Van Wyk et al. 1995). The graminicolous downy mildews (GDM; Fig. 4n, o) in particular pose a serious threat to agriculture in the semi-arid tropics (Bock et al. 2000; Kenneth 1981; Telle et al. 2012; Vilgoen et al. 1997). Many *Phytophthora* species cause economically and ecologically devastating dieback diseases of trees and scrubs, such as sudden oak death caused by *Phytophthora ramorum* (Davidson et al. 2003) and jarrah forest dieback (Fig. 4e) caused by *Phytophthora cinnamomii* (Newhook and Podger 1972; Podger 1972). *Aphanomyces euteiches* causes serious economic losses by infecting the roots of leguminous crops such as peas and beans (Gaulin et al. 2007). Comprehensive catalogues of oomycete diseases of crop plants have been given by, among others, Dick (2001) and Constantinescu (1991).

Both *Nematophthora,* which infects nematode eggs (Dick 2001), and *Lagenidium giganteum,* which infects mosquito larvae (Kerwin 2007), have been explored as potential biocontrol agents. Marine species such as *Atkinsiella*, *Haliphthoros* (Fig. 2l, m)*, Halodaphnea* (Fig. 2j, k), and *Salilagenidium* spp. can cause serious economic losses to cultured crustaceans (crabs and prawns etc.) in coastal aquaculture systems (Hatai 2012; Hatai et al. 1980). *Aphanomyces astaci* (Fig. 3e–g), which was introduced to Europe around a century ago on imported signal crayfish (*Pacifastacus leniusculus*) from north America, now threatens to wipe out the native European white-clawed crayfish (*Astacus astacus*) which has no innate resistance to this pathogen (Cerenius et al. 1988; Edgerton et al. 2004).

Saprolegnia infections of fish and their eggs have been extensively documented and are responsible for significant losses to salmonids worldwide (Bruno et al. 2011; Van West 2006). Epizotoic ulcerative syndrome (EUS) caused by *Aphanomyces invadans* (syn *A. piscida*) is an emerging disease of farmed fish in warmer countries,

from the Indian subcontinent eastwards (Johnson et al. 2004; Lilley et al. 1998). Equine phycomycosis is an opportunistic pathogen of mammals caused by *Pythium insidiosum* (Krajaejum et al. 2011; Schurko et al. 2004), which, although largely affecting domesticated livestock in tropical countries, can cause potentially fatal infections to humans (Mendoza 2005). A newly recognized holocarpic lagenidiaceous species (*Paralagenidium karlingii*) has recently been shown to be the cause of fatal mycoses in dogs (de Grooters et al. 2013). In contrast to their importance as pathogens, no hyphochytrid or oomycete is known as a source for any economically important product, although, as with other heterokonts, they are able to synthesize valuable fatty acids (Domergue et al. 2005), but have not so far been commercially exploited.

Habitats And Ecology

Hyphochytriomycota, in common with the Chytridiomycota and Oomycota, are likely to be encountered in soil and water samples from any area of the world (Gleason et al. 2009; Thines 2014). Soil samples baited with pollen and boiled grasses commonly yield isolates of *Rhizidiomyces* (Figs. 2c, 6a–b) and *Hyphochytrium* (Fig. 2b, d). Gleason et al. (2009) demonstrated that *H. catenoides* is capable of surviving extreme environmental conditions. Viable colonies were recovered after subjecting dried material to extremes of pH (2.8–11.2), hypersalinity and freezing temperatures. Species belonging to the genera *Hyphochytrium, Latrostium,* and *Rhizidiomyces* have all been reported to infect algal thalli (Canter 1950). *Hyphochytrium infestans* was isolated from the decaying ascocarps of ascomycetous fungi, while both *Hyphochytrium* and *Rhizidiomyces* spp. infect oogonia of *Saprolegnia* and *Pythium* spp. and the resting spores of endomycorrhizal fungi (Fuller 2001; Schenck and Nicolson 1977; Sparrow 1977).

The Oomycota are likewise ubiquitous in marine, terrestrial, and aquatic ecosystems worldwide. Water moulds in the Saprolegniales have been recovered from almost every freshwater ecosystem but appear most abundant at the margins of lakes and ponds (Dick 1976; Johnson et al. 2002; Willoughby 1962; Wood and Willoughby 1986). In general population levels of saprolegniaceous water molds appear higher in cooler and wetter seasons, often showing peaks in spring and autumn (Ali-Shtayeh et al. 1986; Dick and Ali-Shtayeh 1986). Stagnant water and anaerobic environments also have their own distinctive communities of oomycetes, in which members of the Leptomitales and Rhipidiales (such as *Sapromyces*, Fig. 2p) predominate, and these fungi are often referred to as sewage fungi (Emerson and Natvig 1981; Riethmüller and Langer 2004). In coastal ecosystems, genera such as *Halophytophthora* and *Salisapilia* are now known to play a major role in the initial colonization, degradation and recycling of organic substrates, such as cord grass and mangrove leaves (Hulvey et al. 2010; Nakagiri et al. 1994; Newell and Fell 1995; Nigrelli and Thines 2013). Oomycota also infect a wide range of invertebrate animals such as crustaceans (Fig. 3e–g; Duffey et al. 2015; Hatai et al. 1980, 1992), insects (Frances et al. 1989; Kerwin 2007; Martin 1977), nematodes

(Figs. 2f, o; 3a–c; Dick 2001; Glockling and Beakes 2000a; Karling 1981), and rotifers (Fig. 3d; Molloy et al. 2014).

Oomycota play significant roles in terrestrial ecosystems. In soils, saprotrophic or facultatively pathogenic genera such as *Aphanomyces, Phytophthora*, and *Pythium* spp. predominate (Ali-Shtayeh et al. 1986; Arcate et al. 2006; Duncan 1990; Gaulin et al. 2007). Many oomycetes are obligate plant pathogens infecting annual or perennial herbs (Fig. 4f, q) and grasses (Fig. 4n). The white blister rusts (Fig. 4f–l) are found almost exclusively on herbaceous angiosperm hosts (Dick 2001; Choi et al. 2008; Constantinescu and Fetehi 2002; Spencer 1981; Spencer and Dick 2001; Thines 2009; Thines and Voglmayr 2009; Volgmayr and Riethmüller 2006; Van Wyk et al. 1995) with genera that appear to be restricted to specific host lineages (e.g., *Albugo* s.str. to the Rosidae, *Albugo* s.lat. to the Solanales, *Pustula* to the Asteridae, and *Wilsoniana* to the Caryphyllidae; Thines and Voglmayr 2009). Recent molecular phylogenetic studies have revealed downy mildew and white blister rust species that are restricted to a single host species (Choi and Thines 2015; Choi et al. 2007, 2008; García-Blázquez et al. 2008; Göker et al. 2004; Ploch et al. 2010; Thines et al. 2009b; Voglmayr 2003; Voglmayr et al. 2004). In contrast, some downy mildew genera such as *Peronospora* and *Plasmopara* have a very wide host ranges (Voglmayr and Constantinescu 2008; Voglmayr et al. 2004). While a few species of downy mildews are known to be parasitic to trees (e.g., *Plasmopara cercidis*, *Pseudoperonospora celtidis*), shrubs, and lianae (e.g., *Plasmopara viburni*, *Plasmopara viticola*, *Plasmopara australis*, *Pseudoperonospora humuli*, *Peronospora sparsa*), it is the hemibiotrophic genus *Phytophthora* that is more commonly encountered as pathogens of woody plants (Fig. 4e; Davidson et al. 2003; Newhook and Podger 1972).

Characterization and Recognition

Thallus Organization

Spore germination in monocenric hyphochytrids such as *Rhizidiomyces* results in the formation of a primary rhizoid from which the basal rhizoidal system develops, while the spore body expands to form the main vegetative thallus (Fig. 2a, c; Karling 1971; Sparrow 1960). In the polycentric *Hyphochytrium*, a much broader germ tube emerges, into which a nucleus moves and divides, and develops into a rhizomycelium of interconnected thalli (Fig. 2a, b, d; Wells 1982; Karling 1977). Young thalli of *Hyphochytrium* contain many small vacuoles with electron-dense inclusion bodies (Fig. 2e; Clay et al. 1991). Hyphochytriomyota are characterized by the presence of both chitin and cellulose in their cell walls (Bartnick-Garcia 1970; Clay et al. 1991; Fuller 1960). Immunogold labeling reveals the vegetative thallus walls are predominantly composed of cellulose, while chitin is principally located in the septa and the sporangial discharge tubes and restraining vesicle (Clay et al. 1991).

Many early-diverging Oomycota also have simple endobiotic holocarpic thalli, which directly differentiate into sporangia on maturity (Figs. 5b, c, f; 6a, b). Many

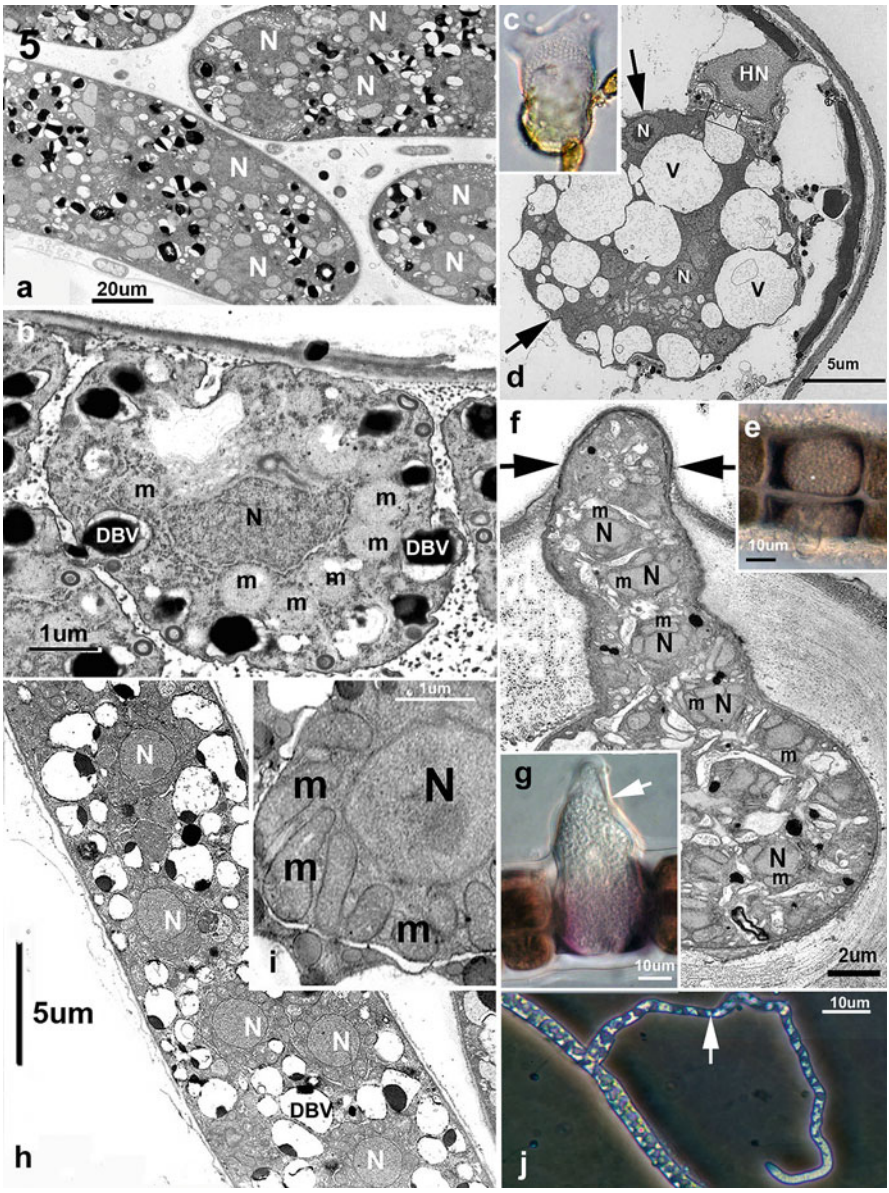

Fig. 5 Thalli of early-diverging Oomycota. (**a**) TEM of a series of young thalli of *Ha. heterospora* showing densely packed non vacuolated cytoplasm scattered with dense body vesicles, mitochondria and nuclei. (**b**) TEM detail of a developing zoospore of a zoosporic *Haptoglossa* sp. showing characteristic zonation of mitochondria (m) and peripheral DBV around the central nucleus (N). Courtesy Sally Glockling. (**c**) Mature thallus of *Eurychasma dicksonii* infecting *Ectocarpus* filament, showing characteristic peripheral net of primary cysts from which zoospores have been released and escaped. (**d**) TEM section of a young thallus of *E. dicksonii* in an expanded vacuolated host cell. Note close proximity of host nucleus (HN) and unwalled nature of the

holocarpic oomycetes, such as the parasities of algae *Ectrogella* (Ragukumar 1980), *Eurychasma* (Sekimoto et al. 2008a), and *Olpidiopsis* (Sekimoto et al. 2008b), have naked plasmodia stages during the earliest stages of infection (Fig. 5d). Taxa that have more extensive lobed, branched or segmented thalli (Fig. 2j, k, n) include the marine crustacean parasites, such as *Atkinsiella* (Karling 1981), *Haliphthoros, Halodaphnea* (Sekimoto et al. 2007), and algal parasites, such as *Lagenisma* (Schnepf et al. 1977, 1978a, b, c; Thines et al. 2015a) and *Petersenia* (Molina 1981; Pueschel and van der Meer 1985) as well as terrestrial genera such as *Chlamydomyzium* (Beakes et al. 2014b; Glockling and Beakes 2006b) and *Cornumyces* (Inaba and Hariyama 2006). Although none of these species produce typical hyphal-like thalli, most can be cultured on solid artificial media, where they form slow-growing irregular colonies (Glockling and Beakes 2006b; Sekimoto et al. 2007). Most thalli in the Rhipidiales (Fig. 2p) and Leptomitales form more typical fungal-like colonies on solid media, although they still have segmented thalli with regular constrictions. It appears that as in the Hyphochytriomycota, the Oomycota also have the capacity to synthesize chitin or chitin-like analogues, as evidenced by the widespread presence of chitin synthase genes within the phylum (Badreddine et al. 2008). In the Leptomitales, the pores in the constricted regions are plugged with refractile chitin-containing cellulin granules (Huizar and Aronson 1986).

The majority saprolegniomycete and peronosporomycete species have branched filamentous mycelial thalli that grow as fungus-like colonies on agar media (Fig. 2q, r). The hyphal tips contain accumulations of vesicles although they lack a well-defined Spitzenkörper analog found in most Fungi (Bartnicki-Garcia 1996). Hyphae vary in diameter from around 2 μm in genera such as *Pythiogeton* and *Verrucalvus* to nearly 150 μm in many genera in the Saprolegniaceae (Dick 2001). Hyphal vacuoles contain soluble β1–3 glucans (mycolaminarins), which are a major storage reserve in the Oomycota (Bartnicki-Garcia and Wang 1983; Wang and Bartnicki-Garcia 1974) as in the Ochrophyta. Most obligate biotrophic plant pathogens produce extensive intercellular hyphae in the infected leaf tissues from which haustoria intrude into the surrounding host cells (Hickey and Coffey 1977, 1978). The hyphae of the relatively

Fig. 5 (continued) pathogen thallus (*arrowed*). From Sekimoto et al. 2008a, *Protist* with permission. (**e**) Mature sporangium of *E. dicksonii*, showing peripheral network of primary cystospores, which is a characteristic feature of this genus. From Sekimoto et al. 2008a, *Protist*with permission. (**e**–**g**) LM showing a young and mature holocarpic thallus of *Olpidiopsis. bostrychiae*, infecting cells of the red seaweed *Bostrychia moritziana*. Note in mature thalli, the distended cell with elongate discharge tube terminated by a cap of wall material (*arrowed*, **g**). Beakes, unpublished. (**g**) Near-median TEM micrograph of *Olpidiopsis porphyrae* infecting ared seaweed of the genus *Porphyra*. The cytoplasm is fully differentiated in zoospore initials typical of holocarpic species. From Sekimoto et al. (2008b), *Mycol. Res*. with permission. (**h**) None-median TEM through thallus of *H. milfordensis* showing peripheral uninucleate (N) spore initials separated by vacuoles. (**i**) Detail of a zoospore initial showing regular array of mitochondria (m) around the central nucleus. Both Beakes unpublished. (**j**) Phase contrast micrograph of sporulating thallus of *H. milfordensis*, showing well formed refractile spore initials and elongate, hyphal-like, discharge tube (*arrowed*). Beakes and Sekimoto unpublished.

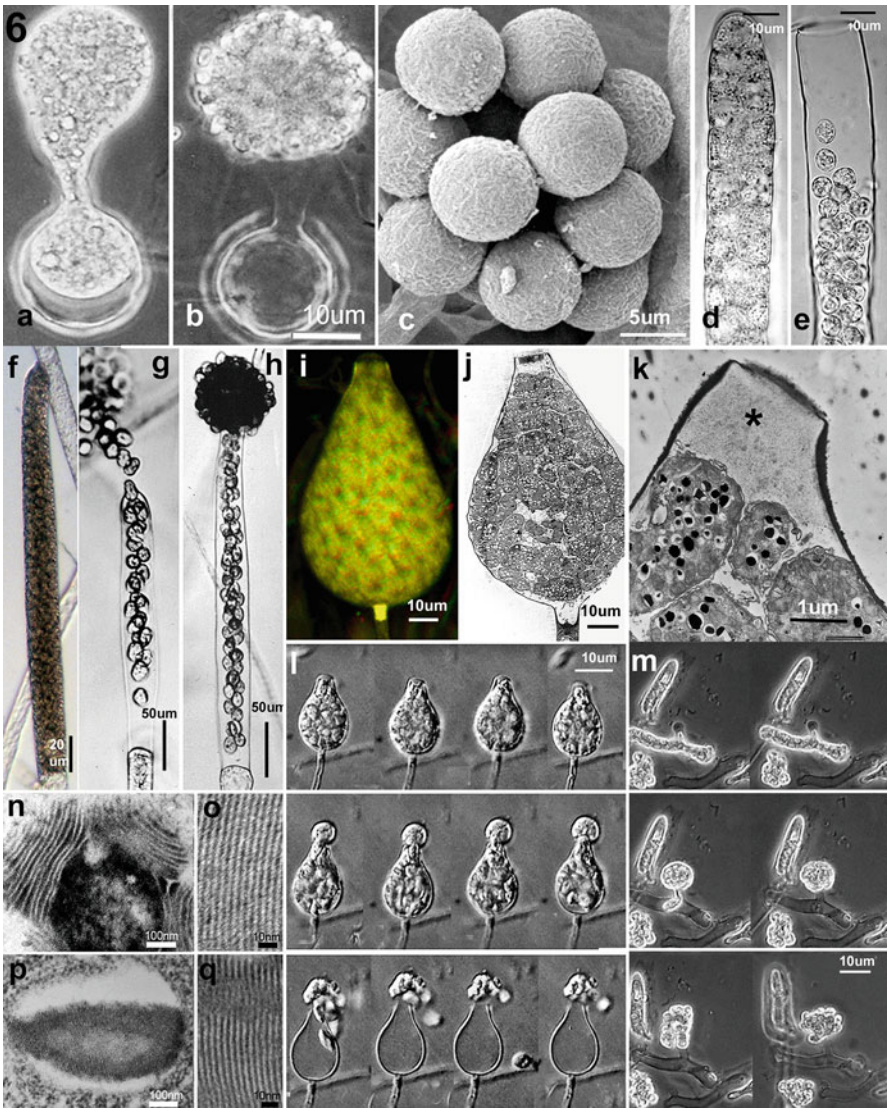

Fig. 6 **Asexual zoosporogenesis**. (**a**–**b**) Sporogenesis in the Hyphochytriomycota. Light micrograph of *Rhizidiomyces apophysatus*, showing cytoplasmic discharge from a mature thallus and formation of a zoospore-filled vesicle. From Fuller and Jaworkski (1987) with permission. (**c**–**h**) Sporogenesis in the Saprolegniales (**c**) SEM of a discharged ball of primary cysts in *Aphanomyces leavis.* Beakes and Lilley unpublished. (**d**) A mature sporangium of *Thraustotheca*, in which the encysted spores are released by gradual dissolution of the entire original sporangium wall. (**e**) A partially discharged sporangium of *Calyptralegnia*, in which the apex of the sporangium dissolves allowing the encysted spores to gradually escape (Beakes unpublished). (**f**) A mature zoosporangium of *Saprolegnia ferax* packed with zoospores. (**g**) A discharging zoosporangia of *S. diclina* and (**h**) *Achlya flagellata*, showing acumulating primary spore ball at mouth of sporangium. Courtesy Dr. N.P. Money. (**i**–**m**) Sporogenesis in the Peronosporales (all Beakes unpublished).

early diverging white-blister rusts, such as *Albugo*, form small stalked globose haustoria (Coffey 1975; Mims and Richardson 2002; Soylu et al. 2003), whereas *Phytophthora* (Coffey and Wilson 1983) and the downy mildews form generally larger digit-like to globose saccoid haustoria (Göker et al. 2003; Voglmayr et al. 2004).

Sporogenesis

In the Hyphochytriomycota, the expanded thallus is directly converted into simple zoosporangia. The cytoplasm then differentiates into uninucleate and uniflagellate zoospores during the zoosporogenesis phase of development (Karling 1977). In *Rhizidiomyces*, the sporangial cytoplasm flows into an external vesicle (Fig. 6a) where the completion of zoospore differentiation takes place (Fig. 6b; Clay et al. 1991; Fuller and Jaworksi 1987). In *Hyphochytrium*, the zoospores form within the sporangium and are released via open discharge tubes (Karling 1977).

Asexual reproductive structures provide many of the morphological characters which have been traditionally used to define genera (Dick 2001; Coker 1923; Johnson et al. 2002; Sparrow 1960), although recent molecular studies have revealed the inherent unreliability of many of these traditional morphological characters (Thines 2006). In early diverging Oomycota genera with plasmodial thalli, the thallus becomes walled prior to spore differentiation (Fig. 5f; Molina 1981; Raghukumar 1980; Schnepf et al. 1978b; Sekimoto et al. 2008a, b). Holocarpic endobiotic species usually form one or more elongate exit tubes (Figs. 2f, 3a, 5g, f, j) to facilitate the release of their spores from their hosts (Glockling and Beakes 2000a; Karling 1981). In species such as *Haliphthoros milfordensis*, these discharge tubes may be very long and hyphal-like in appearance (Fig. 5j). In *Aphanomyces*, primary aplanospores differentiate within what appear to be undifferentiated hyphae (Hoch and Mitchell 1972, Johnson et al. 2002). However, most species with eucarpic thalli form septum-delimited sporangia with a characteristic morphology (Fig. 6f–j) that are typically formed terminally (Johnson et al. 2002; Sparrow 1960). In the

Fig. 6 (continued) (**i**) Stereo z-series projection of mature zoosporangium of *Halophytophthora vesicula* containing fully differentiated zoospores and highly refractile basal plug. **j**. Median LS TEM of a mature zoosporangium of *Hp. vesicula* tightly packed zoospores and callose-like plug delimiting the sporangium. (**k**) Detail of sporangium apex of *Hp. vesiscula*, showing loosely fibriallar material, which forms the apical papillum (asterisked). (**l**) Video sequence showing vesiculate discharge of zoospores in *Hp. vesicula*. (**m**) Video sequqence showing discharge of spore mass into vesicle in a lagenidiaceous *Myzocytiopsis* sp. It takes about 10 min before the zoospores become fully motile and the vesicle ruptures. (**n**–**q**) Electron micrographs of the dense-body (DB)/finger-print (FP) vesicles associated with sporogenesis (all Beakes unpublished). (**n**) Densebody vesicle from primary cyst of *Achlya* (Saprolegniaceae), showing cap of lamellate material associated with the dense vesicle inclusion body. (**o**) High magnification detail of regular lamellate arrays from a oosphere DBV in Saprolegniaceaeshowing a periodicity of ca. 15 nm. (**p**) A finger-print vesicle from zoospore of *Pythium* Pythiaceae) showing reticulate array of lamellate material. (**q**) High magnification detail of regular lamellate arrays from a cyst of *Phytophthora* (Peronosporaceae) showing a periodicity of ca. 15 nm

Saprolegniales, sporangia are delimited by a double-walled septum (Gay and Greenwood 1966), whereas in the Peronosporales they are separated by a callose plug (Fig. 6i, j; Hohl and Hammamoto 1967). There are a significant number of taxa that produce nonmotile primary aplanospores (e.g., *Achlya*: Fig. 6h; *Aphanomyces*: Figs. 3f, 6c; *Calyptralegnia*: Fig. 6d, *Eurychasma*: Fig. 5c, *Protascus*: Fig. 7n, and *Thraustotheca* Fig. 6e). Many downy mildew genera in the Peronosporaceae such as *Bremia*, *Hyaloperonospora* (Fig. 4u) and *Peronospora* (Fig. 4s) form condiosporangia that germinate directly by means of germ tubes.

Zoospores or aplanospores are released (Fig. 6g, h, l) following the dissolution of the apical papillum wall (Beakes 1987; Gay and Greenwood 1966). In the Peronosporales, the papillum usually contains a plug (Fig. 6k, m), which often gives rise to extra-sporangial vesicles into which partially differentiated cytoplasm (Fig. 6m) or fully differentiated zoospores (Fig. 6l) are released (Beakes 1987; Glockling and Beakes 2006b; Lunney and Bland 1976). In the hyphochytrid *Rhizidiomyces* (Fig. 6a, b; Fuller and Reichle 1965) and some Peronosporomycete genera, such as ~*Lagenidium* (Gotelli 1974), *Myzocytiopsis* (Fig. 6m; Glockling and Beakes 2006a), and *Pythium* (Lunney and Bland 1976), the final stages of zoospore differentiation take place within the extra-sporangial vesicle, outside of the thallus. In other Peronosporomycete genera, such as *Phytophthora* and *Halophytophthora* (Fig. 6l), fully motile zoospores form within the sporangium and are also released into a transient restraining vesicle (Hyde et al. 1991a), while in downy mildew species such as *Plasmopara* spp. and *Pseudoperonospora* spp., zoospores are directly released from the sporangium (Thines 2006). In most downy mildews, the sporangia are formed on determinate sporangiophores of distinctive branched morphology (Fig. 4s, v). In most leaf-borne plant pathogens, the mature reproductive structures are disseminated by disarticulation (Fig. 4s) and dispersed by wind and rain splash (Dick 2001, Thines 2006). In the white blister rusts, basipetally maturing chains of conidia/sporangia are produced subepidermally by sporogenous hyphae (Fig. 4g, h), remiscent to true rusts, and again disseminated by disarticulation after lysis of the plant epiermis (Fig. 4j; Heller and Thines 2009; Kemen and Jones 2012; Mims and Richardson 2002).

In the genus *Saprolegnia*, two morphologically distinct types of zoospore are produced, traditionally referred to as primary and secondary zoospores (Beakes 1987; Coker 1923; Holloway and Heath 1977a; Sparrow 1960). Primary zoospores simply serve to disperse the spores from the immediate vicinity of the parent sporangium and are generally weak swimmers. They are usually pip or pear shaped and have apically inserted flagella (Fig. 7b)) which are retracted upon encystment (Holloway and Heath 1977a). The resulting primary cysts (Fig. 7o) typically release the stronger swimming dispersive secondary zoospores (Fig. 7d) which are typically reniform in shape and have laterally inserted flagella (Fig. 7d, g, i) that are shed upon encystment (Holloway and Heath 1977a). This ability to produce two generations of zoospore appears to have been lost in many Saprolegniomycete genera (Beakes et al. 2014a; Johnson et al. 2002), such as where the primary spore initials encyst at the mouth of the exit tube as in *Aphanomyces* (Fig. 3f, 6c) and *Achlya* (Fig. 6h) or within the sporangium as in *Dictyuchus* and *Thraustotheca* (Fig. 6d).

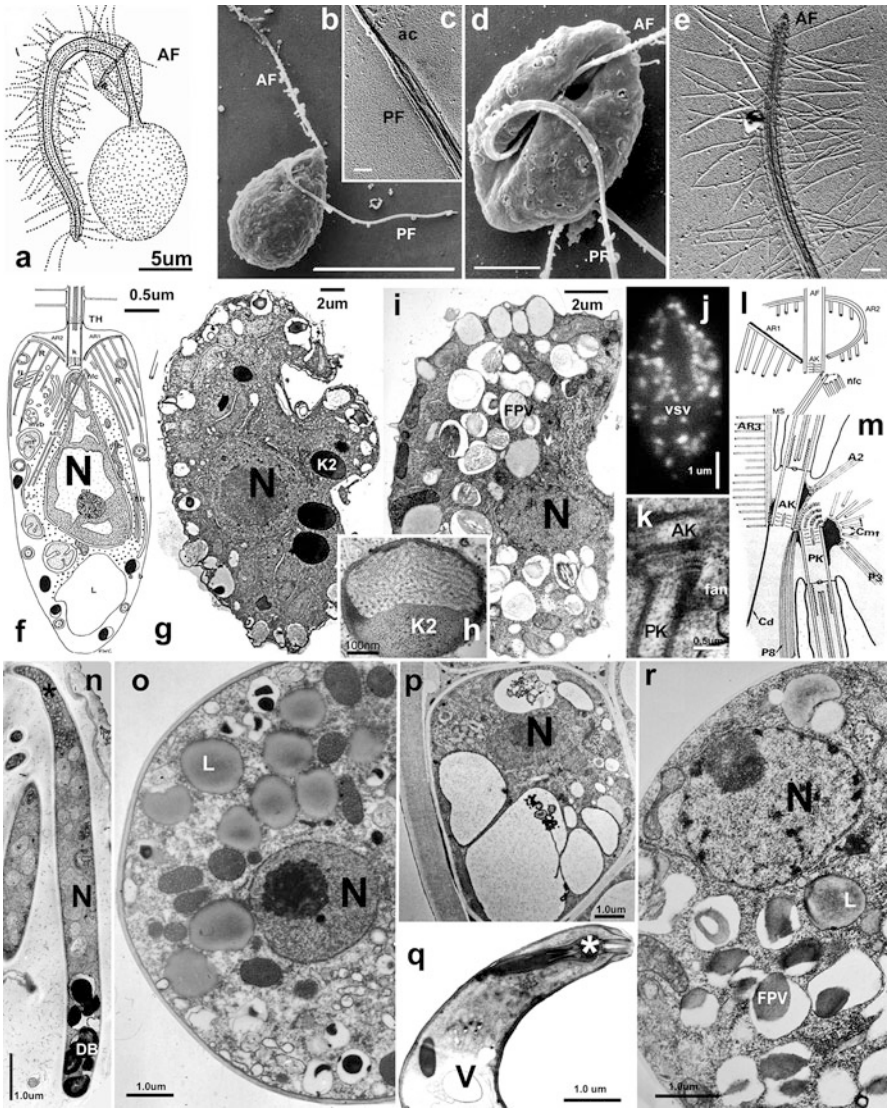

Fig. 7 Zoospore and cyst structure. (**a**) Drawing based on a whole-mount preparation of a Hyphochytriomycota zoospore (*Rhizdiomyces apophysatus*). From Karling (1977) with permission of Charles Lubrecht. (**b**) SEM of a primary zoospore of *Saprolegnia parasitica*, showing anterior (AF) and posterior (PF) flagella attached at apex of pip shaped spore. (**c**) Shadowed TEM whole mount of terminal acronme of posterior flagellum (PF) of *S. ferax*. (**d**) SEM of secondary zoospore of *S. parasitica*, showing ventral grove from which anterior (AF) and posterior (PF) flagella emmergee. Shadowed TEM whole mount of anterior flagellum (AF) of *S. ferax* showing tripartite mastigonene hairs, that give the straminipiles their name **b**–**e**; **g**–**h**: From Beakes (1989), Oxford Clarendon Press with permission. (**f**) Schematic diagram of TEM longitudinal profile of a zoospore of *Hyphochytrium catenoides*. From Fuller 1990. From Cooney et al. (1985) *Can. J. Bot*. with permission. (**g**) Longitudinal LS section of a secondary zoospore of *S. parasitica*, showing central

The ability to produce both primary zoospores (Fig. 7b) and cysts (Fig. 7o) has been entirely lost in Peronosporomycetes, which only form secondary type zoospores and cysts (Fig. 7i, n–r; Beakes 1987; Dick 2001; Hohl and Hammamoto 1967; Lunney and Bland 1976; Sparrow 1960). In the downy mildews, the complete loss of zoospore production has taken place independently in several genera (e.g., *Bremia*, *Hyaloperonospora*, *Paraperonospora*, *Peronosclerpospora*, and *Peronospora*; Göker et al. 2007; Thines 2006; Thines et al. 2009a; Voglmayr et al. 2004). The recent finding of a complete absence of genes associated with flagellum formation and function in the genome of *Hyaloperonospora* indicates that, in some downy mildews at least, this is an irreversible loss (Baxter et al. 2010).

In order to maximize zoospore production, sporangium proliferation frequently occurs throughout the Oomycota following zoospore discharge. Regrowth may take place through the basal septum (internal renewal), or from a lateral branch (cymose renewal) or by outgrowth of the sporangiophore from sites where sporangia were discharged as in some *Phytophthora* species and the grass parasite *Viennotia* (Thines et al. 2007; Thines 2009). In the Albuginales, sporangia are produced in basipetal sequence by the sporogenous hyphae as occurs in true rust fungi (Fig. 4g, h; Heller and Thines 2009; Kemen and Jones 2012; Mims and Richardson 2002).

Encysted zoospores in Oomycota (cysts; Fig. 7o–r) are typically spherical, although in some nematode infecting species, such as *Protoascus* (Fig. 7n), may be elongate and spindle-shaped. They are typically uninucleate and thin walled and contain abundant lipid and vacuolar dense-body/fingerprint vesicles (Fig. 7n–r). Most secondary cysts germinate directly by means of a vegetative germ tube, thus completing the asexual life cycle (Fig. 2s–u). However, the encysted zoospores of the nematode-infecting genus *Haptoglossa* (Beakes and Glockling 1998, 2000, 2002) germinate to produce specialized infection structures known as gun cells

Fig. 7 (continued) nucleus (N) and electron-dense kinetosome-associated bodies (K2) adjacent to the ventral groove. (**h**) TEM detail of a kinetosome associated K-body from *Achlya flagellata* (Saprolegniaceae). (**i**) Near median LS through zoospore of *Phytophthora palmivora*, showing disposition of fingerprint vesicles and lipid around the nucleus. (**g**–**i**) From Beakes (1989), Oxford Clarendon Press, with permission. (**j**) Fixed zoospore of *Phytophthora cinnamomi*, stained with FITC labeled monoclonal antibody (vsv-1) which labels the ventral vesicle fraction. (**k**) Kinetosomes associated with spore of *Haptoglossa erumpens*, showing both anterior (AK) and posterior (PK) kinetosomes and intervening striate fan structure. (**j**, **k**) Beakes unpublished. (**l**–**m**) Schematic diagrams showing basal bodies and rootlet system associated with secondary zoospores of *Hyphochytrium catenoides* (**l**) and *Phytophthora* (**m**). From Barr and Allen (1985) *Can. J. Bot.* with permission). (**n**) Elongate cyst of nematode parasite *Protascus subuliforme*, showing apical vesicles (asterisk), basal cluster of dense body vesicles (DB) and central nucleus (N). Courtesy Sally Glockling. (**o**) Section of a primary cyst of *Achlya flagellata*, showing nucleus and dispersed lipid bodies and mitochondria. (**p**) Cyst of Sapromyces elongatus that had encysted with sporangium. Note single nucleus and basal vacuoles (V) derived from coalesced dense body vesicles (Beakes unpublished). (**q**) Infection gun cell of *Haptoglossa erumpens*, showing basal vacuole (V) and inverted injection tube (asterisked). From Beakes and Glockling (2002), *Fung. Genet and Biol.* with permission). (**r**) Cyst of *Phytophthora palmivora*, showing nucleus (N) and array of finger-print vesicles (FPV). Beakes unpublished.

(Fig. 7q; Robb and Barron 1982). These cells contain a needle-like structure within an inverted tube (Beakes and Glocklng 1998, 2000, 2002). Upon contact with a suitable host, the tube everts and the needle ruptures the host cuticle, resulting in the injection of a minute infective sporidium into the body cavity of the nematode (Glockling and Beakes 2000b).

Most Oomycota also produce vegetative resting structures, variously referred to as chlamydospores in the Peronosporomycetes (Hemmes 1983) and gemmae in Saprolegniomycetes (Dick 2001; Johnson et al. 2002). These structures are delimited by similar septa to sporangia but are thicker-walled. They typically contain abundant storage reserves, particularly lipid (Beakes 1994; Hemmes 1983). When environmental conditions become favorable, they either germinate by producing germ tubes or convert into zoosporangia.

Sexual Reproduction

Sexuality has never been documented in the Hyphochytriomycota sensu stricto (i.e., excluding Anisolpidiaceae; Karling 1977), although structures that have been described as resistant sporangia have been reported in *Rhizidiomyces* spp. and *H. catenoides* (Karling 1977) which might explain why these species appears to be able to survive extreme environmental conditions (Gleason et al. 2009).

Most early diverging Oomycota (Table 1) are usually stated to lack a sexual stage (Sparrow 1976; Karling 1981). However, as Sparrow (1976) points out it seems improbable that all such species are genuinely asexual and suggested that they must have some form of cryptic (i.e., non oogamous) sexual reproduction. The best documented evidence supporting this comes from *Lagenisma coscinodisci*, which has recently been established to be an early diverging member of the Saprolegniomycetes closely related to *Atkinsiella* (Thines et al. 2015a). This species produces zoomeiospores which form cysts that conjugate to form the diploid resting zygote (Schnepf et al. 1977). Recent unpublished observations suggest that this might also be the form of sexual reproduction in *Eurychasma,* although this has only been observed on certain host seaweeds (Gachon, personal communication). Further support that conjugative, nonoogamous, sexual reproduction is prevalent in early diverging Oomycota also comes from *Anisolpidium ectocarpii* (Johnson 1957; Karling 1943, 1981). This species has recently been shown to be an early diverging member of the Oomycota closely related to marine *Olpidiopsis* spp. (Gachon et al. 2015) and reproduces by the fusion of adjacent protoplasts, derived from different cysts (Johnson 1957). Plasmogamy is immediately followed by nuclear fusion (karyogamy). The resulting zygote nucleus divides repeatedly as the cell enlarges and the wall thickens (Johnson 1957). All of these recent observations suggest that oogamy might have evolved at around the time of the Peronosporomycete divergence (Fig. 9a, b) and may even have arisen independently in saprolegniomycete and peronosporomycete lines (Thines et al. 2015a). However, the paraphyletic/polyphyletic genus *Olpidiopsis* needs further investigation in this respect, as

oogenesis has been reported in freshwater species such as *Olpidiopsis varians* (Martin and Miller 1986c) but not in any of the marine species (Sekimoto et al. 2008b, 2009).

In holocarpic Peronosporomycete species, such as *Lagenidium* and *Myzocytiopsis* adjacent thallus segments differentiate into male and female gametangia which have been interpreted as antheridial and oogonial segments, and give rise to a typical oosporic zygote (Dick 1995; Glockling and Beakes 2006a; Karling 1981; Martin and Miller 1986c). In the holocarpic, basal saprolegniomycete genus *Chlam ydomyzium* thick-walled oospore-like structures are formed, but without the apparent involvement of antheridial segments (Beakes et al. 2014b; Glockling and Beakes 2006b). Unfortunately, no information is available regarding nuclear changes that take place during resting spore formation in this genus to confirm whether this is a genuine sexual process, such as described in *Saprolegnia* species that lack antheridia (Beakes 1980b).

Oomycota as a group were named after their distinctive oogamous sexual reproduction present in the vast majority of species, involving the production of spherical to ovoid female oogonia, containing one (Fig. 8i) to several (Fig. 8a, f) large eggs (oospheres), and the associated male antheridia (Fig. 8f, i). In the diploid Oomycota gametangial meiosis preceeds gamete formation (Beakes and Gay 1977; Dick and Win-Tin 1973; Howard and Moore 1970). The female oosphere nuclei and male antheridial nuclei are the only haploid stages in the life cycle (Howard and Moore 1970; Beakes 1980b). The diploid state is restored by the fusion of the gamete nuclei, which normally takes place before the oospores (zygotes) have reached maturity (Beakes 1980b; Beakes and Gay 1977; Howard and Moore 1970). The morphology of gametangia (Fig. 8a, f, i) and oospores (Fig. 8g, h, k–p) have been widely used as key taxonomic characters (Dick 1969, 1990, 2001; Sparrow 1960). In the genus *Saprolegnia*, but also in the Albuginales, species identification is almost entirely dependent upon sexual characters (Choi et al. 2007, 2008; Coker 1923; Johnson et al. 2002; Ploch et al. 2010; Thines et al. 2009c; Voglmayr and Riethmüller 2006). It seems likely that in the Oomycota gametangium differentiation is regulated and coordinated by diffusible steroid hormones (antheridiols and oogoniols), whose functions have been well documented particularly in *Achlya bisexualis* (McMorris and Barksdale 1967; Raper 1939) and certain *Phytophthora* spp. (Ko 1988). In contrast to Saprolegniomycetes, several Peronosporomycetes were reported not to be able to synthesise their own sterols and require these as supplements in order to reproduce sexually (Jee and Ko 1997; Kerwin and Washino 1983). The male antheridia typically are formed on either subtending branches (monoclinous; Fig. 8a) or hyphal compartments (hypogynous) or from separate hyphae produced either on the same thallus (diclinous; Fig. 8f) or in the case of heterothallic species from separate thalli (Dick 1972, 1995, 2001). Many *Phytophthora* species show a unique type of amphigynous antheridium-oogonium association, where the oogonium penetrates the young antheridium which then forms a collar around the base of the oogonium (Fig. 8i; Hemmes and Bartnick-García 1975; Beakes et al. 1998).

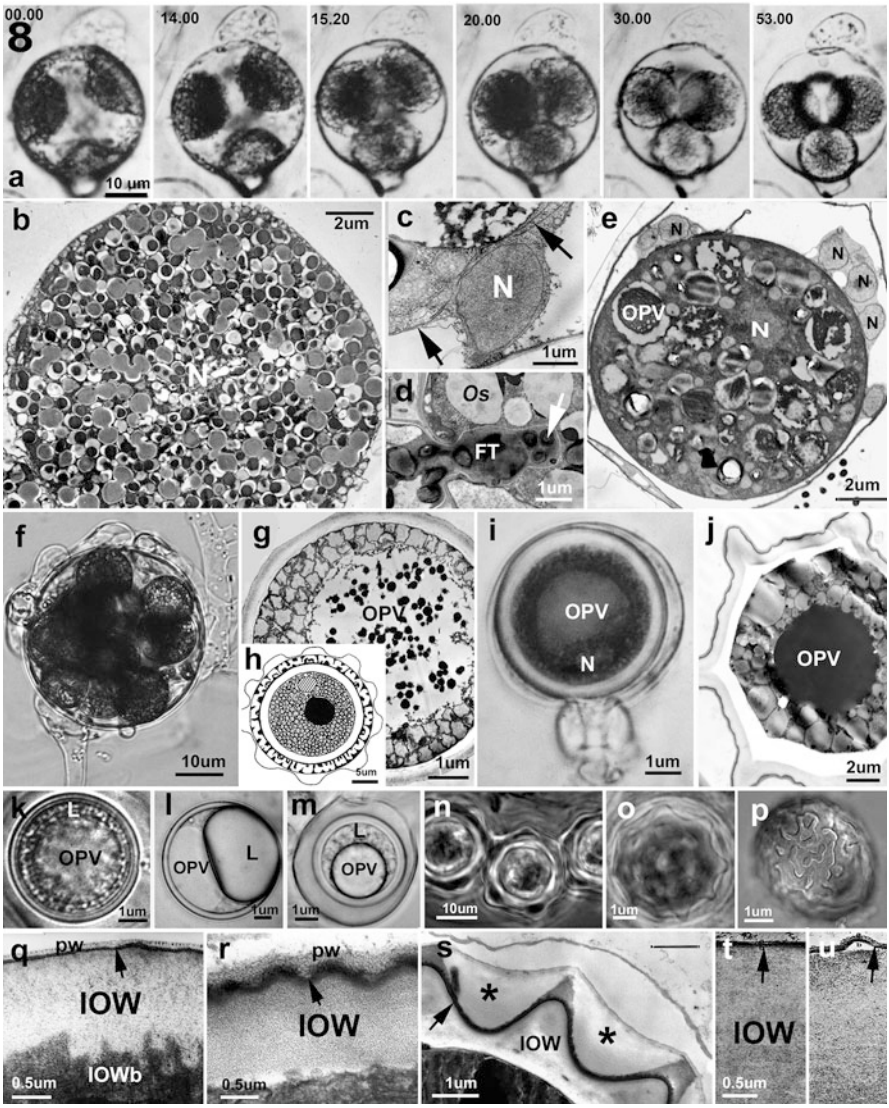

Fig. 8 Sexual reproduction. (**a**–**b**) Differentiating oospheres in *Saprolegnia furcata* (Saprolegniales). (**a**) Timelapse sequence over 53 min showing oosphere formation resulting from the fusion of the tonoplast with the plasma membranes, resulting in naked (unwalled) oospheres that initially swell (<20 min). As the oosphere primary wall forms, the oospheres achieve their final volume (around 30 min) and this is followed by fertilization tube formation from the attached antheridium (53 min). (**b**) TEM section through a newly formed, naked oosphere of S. furcata, showing interspersed lipid and densebody vesicles and central nucleus. (**a**–**b**) From Beakes and Gay (1977),*Trans. Br. Mycol. Soc.* with permission. (**c**–**e**) Differentiationg oospheres in *Myzocytiopsis spp.* (Peronosporales). Detail of a periplasmic nucleus (N) and part of a differentiated oosphere, separated by a double membraned envelope (*arrow*). (**d**) TEM of a developing fertilization tube (FT) extending into the oosphere (Os). The fertilization tube is bounded by a thin wall

In the Saprolegniomycetes, egg (oosphere) differentiation occurs as a result of the fusion of the central tonoplast membrane with the plasma membrane (Fig. 8a) resulting in naked oospheres (Fig. 8b), which within 30 minutes acquire an outer primary oosphere wall (Fig. 8a; Beakes and Gay 1978b). Following fertilization (Fig. 8a, d) thick inner oospore wall layers are accreted below a thin intervening electron-dense layer. In contrast in all Peronosporomycetes, a uninucleate oosphere (Fig. 8e) is cleaved from the cytoplasmic mass, surrounded by an outer periplasmic layer containing supernumerary nuclei (Fig. 8c; Beakes 1981; Hemmes and Bartnicki-Garcia 1975; Stevens 1901), which also contributes to the oospore formation by the the formation of an outer wall of the oospore.

Fig. 8 (continued) (*white arrow*). **e**. TEM of fully differentiated oosphere surrounded by a multinucleate (N) periplasm, typical of all Peronosporomycetes. The central nucleus (N) is surrounded by lipid and coalescing ooplast vesicles (OPV). All from Glockling and Beakes (2006a) *Mycol. Res.* with permission. (**f**) Light micrograph a mature oogonium of *Saprolegnia australis*, showing multiple oospores and diclinously attached antheridia. Beakes and Dieguez-Uribeondo unpublished. (**g**) TEM section through a mature oospore of *S. furcata*, showing central ooplast vacuole (OPV) containing densebody granules, surrounded by a lipid rich peripheral cytoplasm (From Beakes and Gay (1978a) *Trans Br. Mycol. Soc.* with permission) (**h**) Diagram of a mature oospore of *Albugo candida*(Albuginales) showing complex mutli-layered verrucose wall, and rather small ooplast vacuole surrounded by lipid-rich cytoplasm (adapted from Beakes (1981)) (**i**) Mature oogonium of *Phytophthora megasperma* (Peronosporales), showing amphigynous antheridium forming a collar around the the oogonium stalk and single oospore with a homogenous large central ooplast vacuole (OPV) and single zygotic nucleus (N). Beakes unpublished. (**j**) Near media profile of a mature oospore of *Myzocytiopsis venatrix,* showing lipid packed cytoplasm surrounding the homogenous central ooplast vacuole (OPV). Beakes and Glockling unpublished. (**k**) Light micrograph of a near mature centric oospore of *S. furcata* showing ooplast vacuole (OPV) is still homogeneous, but will eventually appear granular due to the dense body granules undergoing Brownian motion. (**l**) Mature eccentric oospore of *Leptomitus* (Leptomitales) showing transleucent ooplast vacuole and single large lipid globule. m. Sub eccentric oospore of *Apodachlya* (Leptomitales) showing homogenous ooplast vacuole (OPV) surrounded by a layer of fairly large lipid droplets (L). (**k**–**m**) Beakes unpublished. (**n**) Chain of stellate oospores of *Chlamydomyzium dictyuchoides* (Atkinsiellales s. lat.), showing punctate thick walls (From Beakes et al. (2014b) *Fung. Biol.* with permission) (**o**) Phase contrast LM of mature oospore of *M. vermicola* (Pythiaceae), showing punctate wall. Glockling and Beakes (2006a), *Mycol. Soc.* with permission. (**p**) DIC light micrograph of mature oospore of *Albugo* "*armoraciae*" (Albuginales), showing complex ornamentation that varies from species to species. Thines unpublished. (**q**) Oospore wall of *Saprolegnia furcata* (Saprolegniales) showing outer exospore wall layer (pw), electron-dense epispore layer (*arrow*) and thick inner endospore wall (IOW), which in this genus has an irregular electron dense inner zone (IOWb). Beakes and Gay (1978b) *Trans. Br. Mycol. Soc*. with permission. (**r**) Mature oospore wall of *Cornumyces* (Saprolegniomycetes), which shows similar layers to above, except for the absence of the inner electron dense zone to the endospore wall. Beakes unpublished. **s**. TEM through mature oospore wall of *Myzocytiopsis vermicola*(Peronosporomycetes) showing that punctate spines are the result of the uneven thickening (*asterisked*) of the endospore layer. The outer electron leucent exospore layer is laid down early in oospore maturation before the formation of the electron dense epispore layer (From Glockling and Beakes (2006a) *Mycol. Res*., with permission) (**t**–**u**) TEM through mature oospore walls of *Ph. megasperma*, fixed with glutaraldehyde and osmium (**t**) and additianlly stained with permanganate (**u**) showing outer electron-dense epispore (*arrowed*) and homogeneous, but finely fibrillar endospore wall (IOW) (From Beakes and Bartnicki-Garcia (1989). *Mycol. Res.*with permission)

Ultrastructure

Mitosis in the Hyphochytiomycota has only been described at the ultrastructural level in *Rhizidiomyces* (Barstow et al. 1989). During prophase the centrioles of unequal length divide and migrate to the poles of the nucleus. During metaphase small polar fenestrae (gaps) develop in the nuclear envelope, allowing the spindle microtubules to span the nucleus. By metaphase the chromosomes are grouped equatorially and vesicles appear and fuse with each other on the poleward side of chromosomes (Barstow et al. 1989). At metaphase the nucleolus is located in a pocket to the side of the chromosomes, after which it disperses completely. During anaphase the intranuclear cisternae migrate ahead of the advancing chromosomes. A perinuclear endoplasmic reticulum and microbodies surround dividing nuclei during anaphase and telophase. During telophase, offspring nuclei are formed by the addition of new envelope to existing membranes and the mid-region of the original nucleus is excluded (Barstow et al. 1989).

This contrasts with the completely closed mitosis described in most Oomycota (Beakes 1980c). Mitosis has been documented at the ultrastructural level in *Albugo* (Khan 1976), *Lagenisma* (Schnepf et al. 1978a), *Olpidiopsis* (Martin and Miller 1986a), *Phytophthora* (Hemmes and Hohl 1973) and *Saprolegnia* (Beakes 1980b, c; Heath and Greenwood 1970a). In most, the nuclear membrane persists throughout mitosis and an intranuclear spindle forms between pairs of polar or sub-polar centrioles, which are usually oriented at 180° (end to end) to each other. Only in *Olpidiopsis varians* are small polar fenestrae reported to form during prophase (Martin and Miller 1986a).

The vegetative thallus in both the Hyphochytriomycota and Oomycota is filled with large somatic vacuoles, which contain osmiophilic inclusion bodies of unknown composition that are often associated with the tonoplast membrane (Figs 2e, 5a, h). Nuclei and other cytoplasmic organelles are distributed throughout the peripheral cytoplasm (Fig. 5a, f, h). In both groups mitochondria have prominent tubular cristae (Fig. 5i), which are a characteristic feature of the chromalveolate lineage (Cavalier-Smith and Chao 2006). In vegetative hyphae and young sporangia in the Saprolegniales, the Golgi dictyosomes are associated with mitochondria and an intervening cisternum of endoplasmic reticulum (ER), a feature shared with many diatoms (Beakes 1989).

During sporangium differentiation in both the Saprolegniales and Peronosporales vacuolar dense body/fingerprint vesicles (DBV/FPV) increase (Beakes 1980a, 1994; Gay and Greenwood 1966; Glockling and Beakes 2006a) and their osmiophilic inclusion bodies become associated with lamellate material of regular periodicity (Fig. 6n–q). In *Phytophthora* phosphorylated glucan derivatives (phospho-mycolaminarin) have been shown to co-localize with isolated FPV (Powell and Bracker 1977). However, no lamellate DBV have been observed in early diverging genera (Beakes and Glockling 2000; Sekimoto 2008; Sekimoto et al. 2008a, b, 2009), which may indicate phosphorylated mycolaminarins are not synthesised by these species, although this needs experimental confirmation.

In most species spore formation involves the division (cleavage) of the multinucleate protoplast into uninucleate spore initials each with a defined complement of organelles. In early diverging genera such as *Eurychasma* (Sekimoto et al. 2008a), *Haliphthoros* (Fig. 5i), *Haptoglossa* (Fig. 5b; Beakes and Glockling 2000), *Olpidiopsis* (Martin and Miller 1986b; Sekimoto et al. 2008b, 2009) and *Petersenia* (Molina 1981) there is a tight association mitochondria around nuclei prior to cytoplasmic cleavage. Cytoplasmic cleavage in the Oomycota follows one of two general patterns (Beakes 1994; Dick 2001). The first, usually referred to as centrifugal cleavage (Beakes 1989, 1994) is found in most early diverging genera (Sekimoto 2008) and Saprolegniomycetes (Beakes et al. 2014a). A central vacuole expands delimiting a peripheral layer of uninucleate initials (Fig. 5h) and spore initial formation is effected by the fusion of the tonoplast with the plasma membrane (Gay and Greenwood 1966; Sekimoto et al. 2008b, 2009), as occurs in oosphere differentiation illustrated in Fig. 8a. In most Saprolegniomycetes flagellum formation occurs after the zoospore initials have differentiated (Beakes 1987; Gay and Greenwood 1966; Glockling and Beakes 2006b).

The second pattern, described as centripetal cleavage (Beakes 1994), predominantly occurs in the Peronosporomycetes (and probably Hyphochytriomycota). The uninucleate zoospore initials are delimited by the progressive disposition of a system of narrow Golgi-derived cleavage vesicles/cisternae, occasionally with additional infurrowing of the plasma membrane as occurs in *Albugo* (Khan 1976, 1977), *Phytophthora* (Hemmes 1983; Hohl and Hammamoto 1967; Hyde et al. 1991a, b) and *Pythium* (Lunney and Bland 1976). This leads to the concurrent, rather than sequential, formation of zoospore initials and flagella (Hohl and Hammamoto 1967; Hyde et al. 1991a, b; Lunney and Bland 1976). As a consequence beating flagella can often be observed in differentiating sporangia or extrasporangial vesicles even before the formation of individual zoospores.

Zoospore Ultrastructure

The ultrastructure of motile cells has traditionally been widely used to provide taxonomically and phylogenetically informative characters in protists and is still important in helping to define clades of chytrid fungi (Powell and Letcher 2014). In the Hyphochytriomycota and Oomycota, zoospores are also a rich source of phylogenetically informative characters (reviewed by Beakes 1987, 1989). Hyphochytriomycota zoospore ultrastructure has been documented for both *R. apophysatus* (Fuller and Reichle 1965) and *H. catenoides* (Barr and Désaulniers 1989; Cooney et al. 1985; Lange and Olson 1979: see Fig. 7f). In common with other members of the stramenopile lineage (Cavalier-Smith and Chao 2006) there is a helically coiled double transitional helix (TH) located just above the basal plate of the flagellum (Fig. 7f). In the Hyphochytriomycota the anterior flagellum is associated with two microtubular rootlets consisting of single (AR1) and doublet (AR2) type, both of which have rib-like microtubules extending from them, providing a cytoskeletal framework for the zoospore (Fig. 7l; Barr and Désaulniers 1989; Beakes et al. 2014a; Dick 2001). In addition, there is a third doublet rootlet (designated as multistranded

root, MS), which originates between the two basal bodies and extends to the spore posterior (Fig. 7l; Barr and Désaulniers 1989).

In the Hyphochytriomycota, the ribosomes in the zoospores are aggregated around the posterior region of the nucleus and are surrounded by a zone of mitochondria (Fig. 7f; Cooney et al. 1985; Fuller 1966; Fuller and Reichle 1965; Lange and Olson 1979). Lipid bodies and microbodies and assorted vesicles, including those containing mastigoneme tubules, are also scattered throughout peripheral zoospore cytoplasm (Fig. 7f). When Hyphochytriomycota zoospores encyst, the axoneme of the flagellum is retracted into the body of the cyst (Fuller and Reichle 1965; Wells 1982). The outer cyst coat is derived from the discharge of structured peripheral vesicles (Fuller 1966) which are similar to the encystment vesicles described in the Oomycota (Beakes 1987, 1989).

The structure and orientation of the four microtubular flagellar rootlets in Oomycota zoospores has been meticulously documented from serial section reconstructions in *Saprolegnia* (Fig. 7m; Barr and Allan 1985; Barr and Désaulniers 1987, 1989; Holloway and Heath 1977b) and *Phytophthora* (Barr and Allan 1985; Hardham 1987) and appears broadly similar to other biflagellate stramenopiles (Anderson et al. 1991; Barr 1981). Most Oomycota zoospores have the expected double TH in the flagellum base (Beakes et al. 2014a; Barr 1981; Dick 2001) although in *Olpidiopsis saprolegniae* it has only a single gyre (Bortnick et al. 1985), and it is apparantly absent in a few species, including the Peronosporomycete *Lagena radicola* (Barr and Désaulniers 1989). Zoospores contain an array of peripheral vesicles (Fig. 7g–j), which upon encystment are discharged to form both a ventral pad of adhesive and the outermost cyst coat layers (Beakes 1983, 1989, 1994; Gubler and Hardham 1988; Lehnen and Powell 1989). In Saprolegniomycetes, this system includes the larger kinetosome-associated (K-bodies) vesicles (Fig. 7h; Beakes 1989; Holloway and Heath 1977b; Randolph and Powell 1992) which upon encystment discharge to form a ventral pad of adhesive material (Burr and Beakes 1994; Lehnen and Powell 1989). In Peronosporomycetes the homologous vesicles are smaller and generally located along the rim of the ventral zoospore groove rather than immediately adjacent to the kinetosomes (Fig. 7j Gubler et al. 1990), and are often morphologically indistinguishable from the dorsal vesicle fraction. Saprolegniomycete genera also contain a second vesicle fraction, which in *Saprolegnia ferax* were called bar-bodies (Heath and Greenwood 1970b), although in other genera such as *Apodachlya* are spherical in profile (Randolph and Powell 1992). Upon discharge the peripheral component of these vesicles give rise to the thin outer electron-dense primary cyst coat (Beakes 1983, 1989; Randolph and Powell 1992). The corresponding vesicles in secondary zoospores of genera such as *Dictyuchus* and *Saprolegnia* contain, respectively, conspicuous tapered spines or boathook spines (Beakes 1983; Burr and Beakes 1994; Heath and Greenwood 1970b) that on release decorate the secondary cyst coat (Fig. 3j). In other genera such as *Apodachlya*, *Aphanomyces*, and *Achlya* the equivalent vesicles are spherical or ovoid in shape and have granular contents rather than tubules or spines and form only the thin outer electron-dense layer to the cyst wall (Beakes 1989). Morphologically similar encystment vesicles also occur in the zoospores of many of

the early diverging oomycetes, including *Eurychasma* (Sekimoto et al. 2008a), *Lagenisma* (Schnepf et al. 1978c), *Haliphthoros* (Overton et al. 1983; Sekimoto 2008), *Haptoglossa* (Beakes and Glockling 2000), *Olpidiopsis spp.* (Sekimoto et al. 2008b, 2009), and *Petersenia* (Pueschel and van der Meer 1985). In contrast, in Peronosporomycetes, the homologous vesicle fraction are the so-called dorsal small vesicles (dsv), which are often morphologically indisguishable from the ventral vesicle fraction, and upon encystment form a structurally diffuse sticky glycoprotein coat (Gubler and Hardham 1988; Gubler et al. 1990).

Mature Oospore Ultrastructure

Following nuclear transfer and fusion (karyogamy) the fertilized oosphere matures into the thick-walled resting zygote, the oospore (Fig. 8g–p; Beakes 1980a; Beakes and Gay 1978a; Hemmes and Bartnicki-Garcia 1975; Tewari and Skoropad 1977). Following fertilisation a thick electron-dense wall layer is layed down (Fig. 8q–u), to which further wall layers may be added both internally from egg cytoplasm and externally from the periplasm (Fig. 8q–u; reviewed by Beakes 1981). This "epispore" layer appears to represent the transition from oosphere to oospore, and after its formation, the mature eggs are much more recalcitrant to TEM fixation (Fig. 8g, j). The overall organization of the cytoplasmic components in mature oospores was described by Dick (1969) and has proven to be a useful taxonomic character. In all species the oospore protoplasm contains a prominent ooplast vacuole (Dick 1969; Fig. 8g–m) derived from the fusion and expansion of the oosphere DBV system (Beakes 1980a; Beakes and Gay 1978a; Beakes et al. 1986; Hemmes and Bartnicki-Garcia 1975; Howard and Moore 1970). This vacuole is usually surrounded by the peripheral cytoplasm containing oil reserves, which may be organized into many small droplets (in centric, subcentric or plerotic oospores; Fig. 8g, h, j) or these may coalesce into a small number of large droplets (as in the eccentric oospores of *Leptomitus*; Fig. 8l). In the genus *Saprolegnia* the mature ooplast vacuole contains small granules which are in constant Brownian motion (granular ooplast; Fig. 8g, k) whereas in the Leptomitales (Fig. 8l; Dick 1969, 1973a) and Peronosporomycete species the ooplast vacuole usually has a uniform refractile appearance (Fig. 8i; Beakes et al. 1986) and appears as a homogenously electron-dense matrix in the transmission electron-microscope (Fig. 8j; Beakes 1981; Beakes et al. 1986).

Oospores are mostly not shed from the oogonium wall which provides an additional protective outer layer to the zygote. It is often thick and multilayered (Beakes and Bartnicki-Garcia 1989; Hemmes and Bartnicki-Garcia 1975) and in many genera can be papillate (e.g in *Chlamydomyzium dictyuchoides*: Fig. 8n; *Sclerospora stellatus*: Fig. 8o) or ornamented (e.g., Fig. 8p; *Albugo ipomoeae-panduratae*; Voglmayr and Riethmüller 2006). In *Saprolegnia* there are often thinner-walled pit regions through which the germ tube hyphae escape. Mature oospore walls are also thick multi-layered structures and contain a large amount of storage carbohydrates (and probably lipids) that are mobilized upon germination (Beakes and Bartnicki-Garcia 1989; Bartnicki-Garcia and Wang 1983). The thick (2–3 μm) innermost endospore wall layer may be multilayered as in the genus *Saprolegnia* (Fig. 8q Beakes and Gay 1978b) or relatively homogeneous as in

Cornumyces (Fig. 8r) and most Peronosporomycete species (Fig. 8t, u; Beakes 1981; Beakes and Bartnicki-Garcia 1989; Hemmes and Bartnicki-Garcia 1975). In the Peronosporomycetes, the outermost oospore wall layer (the exospore layer; Beakes 1981) may at least be partially derived from the residual periplasm (Fig. 8s) and is particularly thick and complex in the Albuginales (Fig. 8h; Stevens 1901; Tewari and Skoropad 1977). In the Albuginales and many Peronosporales species (such as *Peronospora tomentosa*, Fig. 4l and *Myzocytiopsis vermicola*, Fig. 8o; Glockling and Beakes 2006a) the exospore is unevenly thickened, which gives the oospores their ornamented appearance (Fig. 8n–p).

The onset of germination is indicated by the rapid digestion and reabsorption of the thick inner endospore wall (Beakes 1980b; Beakes and Bartnicki-Garcia 1989) followed by the breakdown of the electron-dense ooplast globule material as the central vacuole expands. The broad germ tube hypha is often terminated by a zoosporangium (Ruben and Stangellini 1978). In *Albugo* also the swollen oospore may be converted directly into a zoosporangium, as depicted by Schröter (1893).

Genomic Studies

So far, there are no genome sequences for any Hyphochytriomycota in the public domain, although *Hyphochytrium catenoides* is being sequenced by Tom Richards, University of Exeter, and as part of the ATCC 18717 genome project. Some prelimary data for this organism has been included in publications, exploring horizontal gene transfer into the Oomycota (Richards et al. 2011; Savory et al. 2015). The top ten Oomycota pathogens, which genomes have been sequenced (even though some have not been released to the public domain so far) and which have been extensively studied in molecular plant pathology have recently been reviewed by Kamoun et al. (2015). Six are *Phytophthora* species, with the potato blight pathogen, *Ph. infestans* coming top of the list. The remaining places, were taken by two downy mildews (*Hyaloperonospora arabidopsis* and *Plasmopara viticola*), and a single *Albugo* and *Pythium* (Kamoun et al. 2015). The downy mildew *Hyaloperonospora arabidopsidis* and white blister rust *Albugo laibachii* both infect the model plant *Arabidopsis,* and have provided excellent systems in which to explore host pathogen interactions at the molecular level (Kemen and Jones 2012; Thines et al. 2009a). Much recent effort has been directed at unravelling the molecular basis of pathogenicity in economically important plant pathogenic oomycetes (see reviews by Jiang and Tyler 2012; Thines and Kamoun 2010). However, compared with the Fungi, genetic manipulation of stramenopiles has generally proven difficult and frustrating. With a few exceptions, such as *Phytophthora capsici*, it has been difficult to routinely transform Oomycota (Judelson and Ah-Fong 2009). Gene silencing techniques have often been the only tool available to explore gene functions (Whisson et al. 2009).

Representatives of the phytopathogenic genera *Albugo* (Kemen et al. 2011, Links et al. 2011), *Hyaloperonospora* (Baxter et al. 2010), *Peronospora* (Derevnina et al. 2015), *Plasmopara* (Sharma et al. 2015a), several *Phytophthora species* (e.g., Haas

et al. 2009; Judelson 2012; Tyler et al. 2006), *Pseudoperonospora* (Tian et al. 2011) and *Pythium ultimum* (Cheung et al. 2008; Lévesque et al. 2010) and the fish pathogen, *Saprolegnia parasitica*, have had their full or partial genome sequences released. Comparative genomics is promising to unlock many interesting secrets about these organisms (see Greville-Briggs et al. 2011; Judelson 2012; Lamour et al. 2007; Pais et al. 2013; Seidl et al. 2012; Sharma et al. 2015a, b). Features of genome evolution in the Oomycota, has revealed repeat-driven expansions, deletions, gene fusions and horizontal gene-transfer (Judelson 2012; Haas et al. 2009; Savory et al. 2015; Tyler et al. 2006). One surprising discovery appears to be the extent to which the genomes of oomycetes contain genes derived from other prokaryotes and eukaryotes, suggesting horizontal gene transfer (HGT) from bacteria, fungi and red and green algal endosymbionts (Jiang and Tyler 2012; Maruyama et al. 2009; Richards et al. 2006; Soanes et al. 2007). Genes of green algal ancestry have been discovered in oomycetes (Richards et al. 2011; Jiang and Tyler 2012). This might suggest that the single plastid acquisition-multiple loss interpretation related to evolution of non-photosynthetic organisms, such as Oomycota, from a photosynthetic ancestor needs further evaluation (Dorrell and Smith 2011; Maruyama et al. 2009; Stiller et al. 2009).

Recent genomic studies on non-biotrophic pathogens in genera such as *Aphanomyces* (Gaulin et al. 2007; Krajaejun et al. 2011), *Saprolegnia* (Torto-Alalibo et al. 2005; Wavra et al. 2012) and *Pythium* (Cheung et al. 2008; Lévesque et al. 2010) show these organisms contain a formidable array of glucanase and proteinase encoding genes, which have enabled them to so successfully exploit a wide range of plant and animal substrates (Jiang and Tyler 2012). Genomic studies have also revealed a startling array of pathogenicity factors and effector molecules, which presumably have enabled *Phytophthora* species (Judelson 2012; Lamour et al. 2007; Morgan and Kamoun 2007; Qutob et al. 2002; Sharma 2015a), downy mildew species (Baxter et al. 2010, Derevnina et al. 2015, Sharma et al. 2015a, b), and white blister rusts (Kemen et al. 2011; Links et al. 2011) to become such effective plant pathogens. The independent evolution of obligate biotrophy in the white blister rusts is also reflected by the fact that *Albugo laibachii,* the white blister rust pathogen of *Arabidopsis thaliana* (Thines et al. 2009c), has a much smaller genome compared with obligate parasites in the Peronosporales (Kemen and Jones 2012; Kemen et al. 2011) and has evolved a novel group of CHxC/CxHC effectors that are unique to this clade (Kemen et al. 2011; Links et al. 2011).

Classification

Karling (1977) presents what is probably the most realistic systematic treatment of the Hyphochytriomycota. He questioned Sparrow's (1973) classification that placed emphasis on zoospore cleavage patterns and rejected *Canteriomyces* and *Rhizidiomycopsis* as independent genera. In this account the Hyphochytriomycota have been treated as a phylum in their own right, which may also include the phagotrophic protist, *Pirsonia* (Kühn et al. 2004), which we will consider to be of *incertae sedis*.

The most recent formal systematic account of the Oomycota was by Dick (2001) and is largely based on a critical and scholarly evaluation of morphological characters. Since this account was published there have been many molecular phylogenetic studies on oomycetes (see review by Beakes et al. 2014a). Most of these have compared genes such as those encoding the small (SSU) and large ribosomal subunits (LSU) and the intervening internal transcribed spacer region (ITS), beta-tubulin, NADH and the mitochondrially-encoded cytochrome c oxidase subunit II genes (*cox*2). Some studies have concentrated on higher level taxonomic boundaries and general phylogenic relationships (e.g.; Choi et al. 2015; Dick et al. 1999; Göker et al. 2007; Hudspeth et al. 2000; Lara and Belbahri 2011; Léclerc et al. 2000; Petersen and Rosendahl 2000; Riethmüller et al. 1999; Thines et al. 2008, 2015b), whilst others have been concerned with resolving species clades within the main genera (e.g., *Albugo* Choi et al. 2007, 2008; Ploch et al. 2010; Thines et al. 2009c: *Aphanomyces* Diéguez-Uribeonodo et al. 2009; Levenfors and Fatehi 2004; Lilley et al. 2003; *Haliphthoros* Sekimoto et al. 2007; *Basidiophora* Sökücü and Thines 2014; *Hyaloperonospora* Göker et al. 2004, *Peronospora* Voglmayr 2003; *Peronosclerospora* (Telle et al. 2011), *Phytophthora* Blair et al. 2008; Cooke et al. 2000; Förster et al. 2000; Runge et al. 2011; *Pseudoperonospora* Choi et al. 2005, Runge et al. 2011; *Pythium* Lévesque and de Cock 2004; Martin 2000; *Saprolegnia* Diéguez-Uribeondo et al. 2009; Hulvey et al. 2007; Inaba and Tokumasu 2002; Léclerc et al. 2000; Sandoval-Sierra et al. 2014; Steicow et al. 2013, 2014). As a result of these studies it is now clear that many of the taxonomic changes that were introduced by Dick (1997, 2001) are not supported by molecular data and require substantial revision. It is also becoming apparent that many of the ordinal, family and generic circumscriptions in Oomycota require re-evaluation and that many traditional morphological characters used in taxonomy (such as patterns of asexual spore formation in the Saprolegniceae, patterns of antheridium attachment in *Phytophthora* and conidiophore development in the downy mildews etc.) are not reliable indicators of genetic relatedness.

A revised taxonomic framework of the Hyphochytriomycota and Oomycota based on molecular data is summarised in Table 1. We have refrained from making formal taxonomic descriptions, but will use working names, indicated by “ ” when first used for likely new classes, orders and families. Those taxa that we consider not to be monophyletic and consider are in need of revision are indicated by the ~ before them. We have assumed that the Oomycota form a phylum in their own right and consequently have raised to full class rank the sub-orders proposed by Dick (2001). The placement of the taxa (Rhipidiales, Leptomitales and “Atkinsiellales”) which lie at the cusp of the divergence of the main groups, the Saprolegniomycetes and Peronosporomycetes, have proven particularly problematic and their taxon sampling under-represented, as is the case with many of the smaller marine and holocarpic genera (Table 1).

The majority of Oomycota genera listed in Table 1 fall into one of two major clades with a high degree of statistical support (Fig. 9b). These have been assigned as separate classes the Saprolegniomycetes and Peronosporomycetes (Beakes et al. 2014a, Thines et al. 2015a), which approximate to the galaxies proposed by Sparrow

(1976) and assigned sub-class status by Dick (1997, 2001). Molecular studies have also revealed a number of early diverging basal clades, mostly encompassing marine species (Cook et al. 2001; Küpper et al. 2006; Sekimoto et al. 2007, 2008a, b). However, because of the limited or complete absence of molecular data for many genera in these early-diverging clades, we have refrained from assigning them to new classes at present and therefore they are placed under class(es) *incertae sedis* (Table 1).

Phylum Hyphochytriomycota

Class Hyphochytriomycetes

Order Hyphochytriales

Fuller (1990, 2001) considered that the Hyphochytriomycota consisted of one class (Hyphochytriomycetes), one order (Hyphochytriales), and three families (Anisolpidiaceae, Rhizidiomycetaceae, and Hyphochytriaceae). The Rhizidiomycetaceae have simple monocentric thalli and release their zoospores into a vesicle (Fig. 6b), consisting of 3 genera (Dick 2001; Fuller 2001). The Hyphochytriaceae have polycentric thalli and zoospores differentiate fully within the sporangium and are not released into a transient vesicle and at present also contains 3 genera (Dick 2001; Fuller 2001). Only two Hyphochytiomycota genera, *Hyphochytrium* and *Rhizidiomyces*, have so far been sequenced and together form a well supported clade that is well separated from the Oomycota (Fig. 1b). Recent molecular sequencing has shown that *Anisolpidium* belongs within the Oomycota (Gachon et al. 2015) and will be excluded from the Hyphochytiomycota in this account.

Phylum Oomycota Arx

Basal Class(es) – incertae sedis

Order "Eurychasmales"

The "Eurychasmales" (Table 1, Fig. 9b; Sparrow 1976) are a monotypic order of holocarpic parasites of seaweeds. Although three *Eurychasma* species have been described, most is known about *E. dicksonii* (Fig. 5c) a widespread parasite of filamentous brown seaweeds (Greville-Briggs et al. 2011; Küpper and Müller 1999). In all phylogenetic trees where it is included, *Eurychasma* is the earliest-diverging clade (Küpper et al. 2006; Sekimoto et al. 2008a; Strittmatter et al. 2013).

Order Haptoglossales M.W. Dick

The Haptoglossales (Dick 2001) forms a second early-diverging order (Table 1, Fig. 9b), which may ultimately form a new class together with the Eurychasmales as they both appear to share a common ancestor, but always with long branch separation (Beakes et al. 2006). This monotypic order and family contains a dozen or so species, all of which are parasites of bacterivorous nematodes and rotifers (Beakes and Glockling 1998, 2000, 2002; Glockling and Beakes 2000b, c; Hakariya et al.

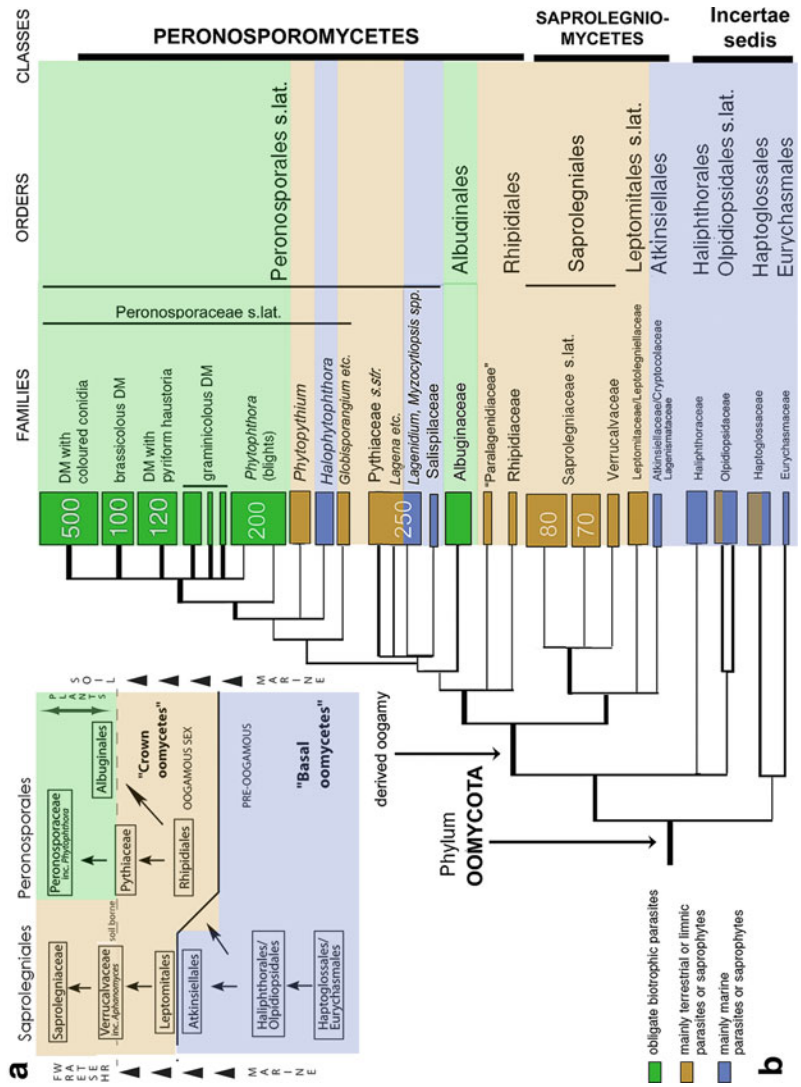

Fig. 9 Oomycete phylogeny diagrammatic summaries. (**a**) Diagram summarizing likely evolutionary path way of the Oomycota, indicating, for illustration, taxonomic clades (Adapted from Beakes et al. (2012). *Protoplasma*, with permission). (**b**) Schematic phylogenetic tree showing main taxonomic clades with an indication of taxon diversity (numbers in *boxes*) and predominant ecology of clades

2002, 2007). *Haptoglossa* (Figs. 2f, 3a, b, 5) is unusual amongst the genera in the early diverging clades of Oomycota in that it is a predominantly terrestrial genus, although *H. heterospora* has also been reported to infect marine nematodes (Newell et al. 1977). This genus produces unique infection 'gun cells' (Figs. 3b, 7q; Beakes and Glockling 1998; Robb and Barron 1982). Recent studies have revealed a number of species, such as *H. erumpens* (Beakes and Glockling 2002) and *H. heteromorpha* (Glockling and Beakes 2000c), which produce several morphological types of gun cells, which suggest they may have evolved to infect multiple hosts. Species within *Haptoglossa* clade also appear deeply diverging (Hakariya et al. 2007) and this genus will require revision.

Order ~Olpidiopsidales s. lat. M.W. Dick

The ~Olpidiopsidales (Fig. 5e–g) as currently defined is a paraphyletic or polyphyletic order of predominantly marine genera that are mostly parasites of marine algae (Fig. 5e–f). This order traditionally encompased three families, the Olpidiopsidaceae, Sirolpidiaceae and Pontismataceae (Dick 2001) but so far only representatives of the first family have been sequenced. The different species of marine *Olpidiopsis* for which sequence data have so far been published fall into two closely related clades, one with *O. porphyrae* and *O. pyropiae* (Klochclova et al. 2015; Sekimoto et al. 2008b) and the second with *O. bostrychiae* and *O. feldmanni* (Fletcher et al. 2015; Sekimoto et al. 2009) (Table 1). However, the type of the genus, *O. saprolegniae,* a freshwater endoparasite of saprolegniaceous water moulds (Bortnick et al. 1985), does not form a monophyletic clade with the marine species (Sekimoto and Inaba, unpublished sequences). This means the marine species will most likely have to be renamed. Recently, Gachon and colleagues (personal communication) have shown that two *Anisolpidium* species (*A. ectocarp, A. rosenvingei*) also forms a discrete clade nested between the marine *Olpidiopsis* spp. and the "Haliphthorales" clade. Therefore, it seems likely that as currently constituted even the genus *Olpidiopsis* is probably polyphyletic and will need significant revision, with new genera names required for the marine species.

Order "Haliphthorales"

The "Haliphthorales" (Figs. 2j–m, 5h) has not been formally described and among others contains the parasites of marine crustacea, *Haliphthoros* and *Halocrusticida* (renamed as *Halodaphnea* by Dick in 1998, but without supporting molecular data). These species can be cultured on agar media, have constricted segmented thalli (Hatai 2012; Hatai et al. 1980, 1992; Sekimoto et al. 2007), and form rather long (often >100 um) hyphal like discharge tubes (Fig. 5j). At present a single order (not as yet not formally described) and family (Haliphthoraceae) contains three or four poorly circumscribed genera (Sekimoto et al. 2007), including the recently described parasite of abalone, *Halioticida* (Maurosa et al. 2009). *Haliphthoros* as currently recognised appears to be a paraphyletic genus, with some "*Haliphthoros*-like" isolates apparently occuring within the crown Oomycota clade close to *Atkinsiella* (Sekimoto et al. 2007; Gachon, personal communication). Clearly much more research is required on this order.

It also seems possible that *Petersenia* and *Pontisma* in the Pontismataceae and *Sirolpidium* in the Sirolpidiacae will also turn out to be related to these two early-diverging marine orders or the basal lineages of the Saprolegniomycetes, currently they are placed as orders *incertae sedis* until sequence data become available (Taxonomic Summary; Table 1). The Ectrogellaceae (Dick 2001; Karling 1981) has been traditionally considered as part of the Saprolegniomycete line (Sparrow 1973, 1976) and also forms 'naked' plasmodial thalli in their diatom hosts similar to *Lagenisma* (Raghukumar 1980 – see below). However *Ectogella* has not yet been sequenced and therefore the Ectrogellaceae must also be considered as a family *incertae sedis*.

Class Saprolegniomycetes Thines et Beakes

The Saprolegniomycetes (Table 1; Fig. 9b), are characterized by the formation of two morphologically distinct generations of zoospore or aplanospore (Figs. 6c, 7a, d), a phenomenon usually referred to as diplanetism (see Dick 2001; Johnson et al. 2002). Fully differentiated zoospores or aplanospores are released directly from the sporangium (Fig. 6g, h). Both zoospores (Figs. 6f–h, 7b, d) and oospores are formed as a result of centrifugal cleavage (Fig. 8a) without the differentiation of a peripheral periplasmic layer of cytoplasm. Saprolegniomycetes are able to synthesize the sterols they require for oogenesis and generally utilize ammonium as a source of nitrogen and may also use organic sulphur (Gleason 1976). We have taken a conservative approach to their taxonomy, recognizing three orders within the class, the "Atkinsiellales", Leptomitales s. lat., and Saprolegniales (Fig. 9, Table 1).

Order "Atkinsiellales" and closely related taxa.

The order Atkinsiellales contains a handful of relatively little studied parasites of marine crustaceans and terrestrial invertebrates and contains two families as defined by Dick (1998, 2001), the Atkinsiellaceae and Crypticolaceae. *Atkinsiella dubia* forms a highly distinct clade (Fig. 16) at the base of the Saprolegniomycetes (Cook et al. 2001; Sekimoto 2008; Sekimoto et al. 2007, Thines et al. 2015a). A second species, *Atkinsiella entomophaga,* a parasite of dipteran larvae described by Martin (1977), was transferred by Dick (1998) to the previously monotypic genus *Crypticola*. The latter had been created for *C. clavulifera,* a parasite of mosquito larvae described by Frances et al. (1989). *C. clavulifera* forms a clade with *A. dubia* in *cox*2 analyses (Deborah Hudspeth, personal communication), which suggests the Crypticolaceae should also be included in this order, although family cirumscriptions require more data.

The diatom pathogen *Lagenisma coscinodisci,* which Dick (2001) placed in its own family, the Lagenismataceae, has been shown to form an early diverging Saprolegniomycete clade with some affinity to *Atkinsiella* (Thines et al. 2015a). We have therefore included this family in the Atkinsiellales (Table 1). The paraphyletic genus *Chlamydomyzium* (Dick 2001), which has both Saprolegniomycete and Peronosporomycete characteristics (Glockling and Beakes 2006b; Beakes et al. 2014), also forms clades amongst these early diverging Saprolegniomycete genera (Beakes et al. 2006; Beakes et al. 2014a). Isolates of the genus *Cornumyces* obtained

from keratin baits appears to be closely related to the nematode parasite *Chlamydomyzium* (Fig. 2o; Inaba unpublished trees) as well as to the Leptomitales clade (Inaba and Hariyama 2006). Dick (2001) also transferred *Lagenidium pygmeaum* to *Cornumyces* in absence of molecular data supporing this. He considered *Cornumyces* might belong in the Leptolegnielliaceae for which there is as yet no supporting sequence data. It is clear that much more work is required on these little studied basal Saprolegniomycetes before their formal taxonomy can be fully resolved.

Order Leptomitales Kanouse

The Leptomitales is a long-standing order that formerly included two families, the Leptomitaceae and Rhipidiacae (Dick 1973a; Sparrow 1960). These were separated into two orders by Dick (2001), and the Rhipidiales are now thought to be members of the Peronosporomycetes (Hudspeth et al. 2003; Thines et al. 2009c). The revised Leptomitales encompasses four small families, the most familiar of which are the Leptomitaceae, which are commonly known as sewage fungi. The Saprolegniomycete characteristics of *Apodachlya* and *Leptomitus* had long been been recognized (Beakes 1987) and sequence data confirms they form an early diverging clade within the class (Petersen and Rosendahl 2000). Recently the anamorphic genus *Blastulidium paedophthorum*, a parasite of freshwater cladocerans, has been confirmed to be in a clade close to *Apodachlya* and *Leptomitus* (Duffey et al. 2015), which confirms Dick's placement in the Leptomitales. To date no sequence data exists for any member of the Ducellariaceae and Letolegnielliaceae that Dick (2001) also included in the Leptomitales. These families contain a number of rarely encountered, holocarpic genera (*Aphanodictyon, Aphanomycopsis Brevilegniella, Ducellaria, Leptolegniella, and Nematophthora*) that have been mainly been documented by Karling (1981).

Order Saprolegniales E. Fisch.

The Saprolegniales is one of the largest and longest-established orders (Sparrow 1960, Dick 1973b) and forms a well-supported monophyletic clade (Dick et al. 1999; Inaba and Tokumasu 2002; Léclerc et al. 2000; Petersen and Rosendahl 2000; Riethmüller et al. 1999; Spencer et al. 2002; Steicow et al. 2014). Beakes et al. (2014a) suggested this order should be divided into three family level clades: a redefined Verrucalvaceae, the "Achlyaceae" and a re-circumscribed Saprolegniaceae sensu stricto. Dick et al. (1999) had introduced the family Leptolegniaceae which encompassed the genera *Aphanomyces, Plectospira* and *Leptolegnia*. Unfortunately, the inclusion of *Leptolegnia* with these other two genera is not well supported by most molecular studies (e.g., Arcate et al. 2006; Léclerc et al. 2000; Petersen and Rosendahl 2000; Steicow et al. 2013, 2014). Furthermore subsequent molecular studies have also shown that the grass pathogens, *Pachymetra* and *Verrucalvus*, which Dick et al. (1988) had placed in their own family, the Verrucalvaceae, also fall within the *Aphanomyces* clade (Hudspeth et al. 2003; Riethmüller et al. 2002; Telle and Thines, unpublished data). Therefore the family name Verrucalvaceae should take precedence over Leptolegniaceae. This clade is characterized by having species

with narrow hyphae and, when formed, relatively undifferentiated sporangia (Fig. 3f). Genera in this clade are predominantly soil-borne, root-infecting parasites, saprotrophs or animal parasites (Fig. 3e–g; Dick et al. 1984, 1988; Diéguez-Uribeondo et al. 2009; Johnson et al. 2002; Levenfors and Fatehi 2004; Lilley et al. 1998). The Verrucalvaceae also includes the nematode-trapping genus *Sommerstorffia* (Spies and Levesque, unpublished sequence data) as well as the recently described rotifer parasitic genus, *Aquastella* (Fig. 3d; Molloy et al. 2014). The genera *Aphanomyces* and *Plectospira* both form clusters (balls) of primary aplanospores (Fig. 6c), a feature shared with *Somerstorffia* (Johnson et al. 2002). All the genera form uni-oosporiate oogonia with more or less plerotic oospores, which in *Verrucalvus* have prominent verrucose ornamentation (similar to shown in Fig. 8n, o; Dick et al. 1988).

Although the branching order of the proposed family clades in the Saprolegniales is not well-resolved statistically, morphological and molecular evidence points to the recircumscribed Verrucalvaceae as the basal family in the Saprolegniales. A comprehensive molecular phylogenetic study of the genus *Aphanomyces* has shown that saprotrophic species, animal parasites and plant pathogens separate into three well supported sub-clades (Diéguez-Uribeondo et al. 2009). Dick et al. (1984) controversially also placed the graminicolus downy mildews in the Verrucalvaceae (Dick 2001) but this is not supported by molecular sequence data (Table 1; Hudspeth et al. 2000, 2003; Léclerc et al. 2000; Riethmüller et al. 1999, 2002; Thines et al. 2008).

The diverse genera of saprotrophic "water moulds" were traditionally all placed in a single family, the Saprolegniaceae containing a dozen or so genera (Table 1; Johnson et al. 2002; Sparrow 1960). Different genera were largely defined by their pattern of zoosporogenesis and asexual spore formation (Fig. 6d–h; Dick 2001; Johnson et al. 2002). However, it seems the best predictor of family-level relationships in the Saprolegniaceae is whether their mature oospores have centric/subcentric (as in Fig. 8k) or eccentric (as in Fig. 8l, m) organization (Léclerc et al. 2000; Spencer et al. 2002; Inaba and Tokumasu 2002). In some analyses (Inaba and Tokumasu 2002) genera with centric or subcentric oospores (*Aplanes, Aplanopsis, Calyptralegnia, Protoachlya, Newbya* and *Saprolegnia*, and possibly *Leptolegnia*) can be separated from those which produce strongly eccentric oospores (*Achlya* s. str., *Brevilegnia*, *Dictyuchus*, *Isoachlya*, and *Thraustotheca*). In the genus *Saprolegnia*, the mature ooplast often contains granules in Brownian motion as a result of the liquifaction of the matrix (Fig. 8k, g). However, even the archetypal water mould genus *Saprolegnia* is apparently not monophyletic, although molecular phylogeny is beginning to help resolve species boundaries (Diéguez-Uribeondo et al. 2007; Hulvey et al. 2007; Inaba and Tokumasu 2002; Sandoval-Sierra et al. 2014). Although we suggested a new family, the "Achlyaceae", might be warranted for the clade containing genera with strongly eccentic oospores, recent publications suggest this may be an an oversimplistic solution (Steciow et al. 2013, 2014; Sandoval-Sierra et al. 2014). Therefore we have decided not to formally split the Saprolegniaceae in this account (Table 1, Fig. 9b). Clearly further work is required before this large and complex family is formally split into well supported families.

Class: Peronosporomycetes M.W. Dick

The Peronosporomycetes are predominantly a terrestrial class. Most have a mycelial fungus-like thallus, although there are holocarpic species and many of the Rhipidiaceae have constricted thalli of determinate growth. Peronosporomycetes have been reported to have a requirement for exogenous sterols to complete oogenesis (Kerwin and Washino 1983) and the non-obligate pathogens of plants are able to utilize sulphate and variable nitrogen sources (Gleason 1976; Dick 2001), while there seems to be a tendency that non-obligate animal parasites and obligate plant parasites have defects in the pathways for the acquisition of inorganic nitrogen and sulfur (Baxter et al. 2010; Kemen et al. 2011; Sharma et al. 2015b). Peronosporomycetes produce only secondary type zoospores (Fig. 7i), which are differentiated within or transiently released into an evanescent extrasporangial vesicle in several genera (Fig. 6m). They have mono-oosporic oogonia in which the single oosphere is surrounded by a layer of periplasm (Fig. 3c, e, i; Dick 2001). Mature oospores often have complex multilayered walls (Fig. 3h, s) and a homogeneous (ooplast) vacuole (Fig. 3i).

Dick (2001) included the orders Rhipidales, Pythiales and Peronsporales within his Peronosporomycotina sub-class. Subsequent molecular studies revealed the white-blister rusts, form a separate basal order (the Albuginales) in their own right (Thines and Spring 2005). Many molecular studies suggest that the order level separation of the Pythiales and the Peronsporales along the lines proposed by Dick (2001) is not supported statistically (see discussion in Beakes et al. 2014a) and some genera, such as *Phytophthora*, were incorrectly placed (Hulvey et al. 2010; Thines et al. 2009a,). Furthermore, unpublished multigene sequencing of genera, is revealing much more diversity amongst the lagenidiaceous and pythiaceous species than has hitherto been suspected (Spies et al. 2014, 2016). However, until more statistically robust multigene sequence data are publically available, as in our previous review, we have adopted the historical position of including all these genera, into a single all-encompassing Peronosporales s. lat. and not suggested new orders and families (Fig. 16, Table 1) to account for a probably paraphyletic Pythiaceae. However, we feel that the recognition of a broad Peronosporales might be preferable over the creation of several new ill-defined orders. At least one clade, that contains the unusual recently-described lagendiaceous mycopathogen of dogs, *Paralagenidium karlingi* (de Grooters et al. 2013), appears to be located between the Rhipidiales and Albuginales and will will probably need to be placed in its own Order and Family (Table 1).

Order Rhipidiales M.W. Dick.

The Rhipidiales consists of a single family, the Rhipidiaceae, containing a small number of saprotrophic genera (Table 1), which often grow on submerged twigs and fruits. Many have determinate, often segmented, thalli with only a few genera showing typical hyphal growth (e.g., *Sapromyces*, Fig. 2p). Some genera, such as *Rhipidium,* have thalli that are anchored to their substrate by rhizoid-like structures (Dick 2001; Sparrow 1960). They typically produce uni-oosporiate oogonia with a

well differentiated periplasm (Sparrow 1960). To date only *Sapromyces elongatus* has been sequenced (Table 1). The phylogenetic placement of this species in phylogenetic trees has proven difficult and varies depending upon the gene sequenced and which other taxa are included in the analyses. Riethmüller et al. (1999) and Petersen and Rosendahl (2000) inferred a position basal to the 'saprolegnian line' in their LSU rDNA analyses, whereas Hudspeth et al. (2000) report it to form the basal clade to 'peronosporalean line'. The COII amino acid sequence derived from from the *cox*2 gene, showed that *Sapromyces* has the same signature amino acid insertion-deletion (indel) sequence (LEF/T) to that found in members of the Peronosporales s.l., and not the YTD indel sequence found in members of the Leptomitaceae (Hudspeth et al. 2000, 2003; Cook et al. 2001). It is clear that much work still needs to be done to resolve the precise relationships between families and genera that appear at the base of both main classes.

Order Albuginales Thines

Traditionally, the white blister rusts, the Albuginales (Fig. 5), were placed together with the downy mildews in the Peronosporales (Beakes 1987; Dick 2001). They are obligate pathogens of angiospermae producing small stalked globose haustoria (Coffey 1975; Mims and Richardson 2002, Soylu et al. 2003). They form blister-like lesions on the leaves (Fig. 4f, i) below the host epidermis in which the basipetal chains of deciduous conidiosporangia are borne on club-shaped sporogenous hyphae (Fig. 4g, h; Heller and Thines 2009). Molecular phylogenetic studies revealed that the white blister rusts form a well supported clade basal to the Peronosporales s. lat. (Fig. 9b; Hudspeth et al.; 2003, Riethmüller et al. 2002; Thines et al. 2009c). This clade has been given its own order designation, the Albuginales (Thines and Spring 2005), containing just one family, the Albuginaceae (Table 1). Members of this family have exceptionally thick, multilayered oospore walls (Stevens 1901; Tewari and Skoropad 1977; Beakes 1981), the outer layers of which appear to be mainly derived from the periplasm. Recent molecular studies have also revealed an unsuspected genetic diversity within this order (Choi et al. 2007, 2008, 2011; Mizaee et al. 2013; Ploch et al. 2010; Ploch and Thines 2011; Rost and Thines 2012; Thines and Voglmayr 2009; Thines et al. 2009c; Voglmayr and Riethmüller 2006) and two new genera, *Pustula* and *Wilsoniana,* have been established based upon conidiosporangium and oospore characteristics (Thines and Spring 2005). These three genera appear to be restricted to specific host orders or subclasses (Thines and Voglmayr 2009). It is also expected that more comprehensive taxonomic re-arrangement of this family will be required as more species and isolates are sequenced.

Order Peronosporales E. Fisch. s. lat

The order Peronosporales s. lat. (Waterhouse 1973) contains a large number of often diverse taxa (Table 1), presently placed in three families, the Salispiliaceae, ~Pythiaceae s. lat. and Peronosporaceae s. lat. (Beakes et al. 2014a). This classification has to be considered provisional, as many lagenidiaceous species have not yet been included in published molecular phylogenies. Within the Peronsporales s. lat.

There have been many published molecular phylogenetic studies on the important plant pathogenic genera (*Pythium*: de Cock et al. 2012; Lévesque and de Cock 2004; *Phytophthora*: Blair et al. 2008; Cooke et al. 2000; Kroon et al. 2004; Martin et al. 2014) and various downy mildew genera (Göker et al. 2003, 2004, 2007; Thines et al. 2009a, b; Voglmayr 2003; Voglmayr et al. 2004). The saprotrophic genera have been less well documented, but there have been accounts of the molecular phylogeny of *Pythiogeton* (Huang et al. 2012), *Phytopythium* (de Cock et al. 2015) and *Halophytophthora* (Nakagiri 2002), but molecular data for the "lagenidiaceaous" holocparic genera is sparse and incomplete (Beakes et al. 2006; Schroeder et al. 2012).

The Salispiliaceae is single genus family which forms a well-supported early-diverging clade in the Peronsporales s. lat. based on concatenated ITS and LSU sequences (Hulvey et al. 2010). They are saprotrophs isolated from salt marshes, with ovoid sporangia and smooth walled oogonia and oospores. However, unpublished trees based on an analysis of 16 genes do not support a basal phylogenetic position of this genus within the Peronsporales s.lat. (see Table 1; Spies et al. 2014 and personal communication). The ~Pythiaceae s. lat. as we have defined it (Beakes et al. 2014a) encompasses more than a dozen genera, including a many of holocarpic genera that were traditionally placed in the Lagenidiales (Table 1, Fig. 9b). However, a recent unpublished multigene analysis of a significant number of isolates identified as *~Lagenidium*, *~Lagena*, *~Myzocytiopsis, Pythiogeton, ~Pythium* and *Salilagenidium* has revealed at least six clades that may ultimately justify family level designation (Spies et al. 2014, 2016, and unpublished trees - summarised in Table 1). There are still a number of Pythiaceous genera, such as *Medusoides* described by Voglmayr et al. (1999) and placed by Dick (2001) in his Pythiogetonaceae, for which no sequence data are publically available. *Lagenidium,* as currently recognised, is a particularly complex paraphyletic or polyphyletic genus, with isolates occuring in several different clades. However, until detailed phylogenies become available, we have retained all of these holocarpic species in a broadly defined ~Pythiaceae s. lat. (Table 1). The genus *Pythium* contains well over a hundred species, most of which have sequence data available (Bedard et al. 2006; Briard et al. 1995; Lévesque and de Cock 2004; Martin 2000; Schurko et al. 2004; Villa et al. 2006). Lévesque and de Cock (2004) recognised 8 clades (A-K) of *Pythium*, some of which are now assigned to new genera (Bala et al., 2010; de Cock et al. 2015; Usuhashi et al. 2010). However, as the relationships of these clades have not been fully resolved, most are subsumed under *Pythium* s. lat. in this review. Species which have simple more or less filamentous sporangia now constitute the genus *Pythium* s. str. (Usuhashi et al. 2010) although some genera, including the animal pathogen *P. insidiosum* cluster with *Pythiogeton* (Huang et al. 2012).

The Peronosporaceae s. lat. family (Table 1, Fig. 9b) includes not only the hyperdiverse downy mildews, but a number of genera that had been been previously included in the Pythiaceae (Dick 2001). These include the genus *Phytopythium* (syn. *Ovatosporangium*, Usuhashi et al. 2010; formerly known as the *Pythium* K-clade, Lévesque and de Cock 2004) described by Bala et al. (2010) and which has been recently monographed by de Cock et al. (2015). A recent multigene analysis also

suggests that two other of the new pythiaceous genera introduced by Usuhashi et al. (2010), *Elongisporangium* and *Globisporangium* might also fall in the Peronosporaceae s. lat. clade (Spies et al. 2014, 2016). The polyphyletic marine genus ~*Halophytophthora* (Ho and Jong 1990; Ho et al. 1992; Nakagiri 2002) contains around 15 species many of which, including the type species, fall into a clade that sits between the *Phytopythium* and the *Phytophthora*/downy mildew assemblage. ~*Halophytophthora* spp. have ovoid to elongate sporangia, often with conspicuous papillar plugs (Nakagiri 2002, Nakagiri et al. 1994) and most show a transient vesiculate discharge of their zoospores (Fig. 6l). All have single-oospored oogonia with paragynous antheridia (Nakagiri 2002, Nakagiri et al. 1994). *Phytophthora* clades are probably paraphyletic with the hyperdiverse downy mildews, which appeared to have evolved from a clade of shoot- and leaf-infecting *Phytophthora* spp. (Cooke et al. 2000, Runge et al. 2011). Most *Phytophthora* taxa (Fig. 4a–e) have sequence data available (Blair et al. 2008; Brouwer et al. 2012; Cooke et al. 2001; Förster et al. 2000; Kroon et al. 2004; Martin and Tooley 2003a, b; Runge et al. 2011; Villa et al. 2006) and fall into 8 to 10 clades (usually referred to as groups). The clades can be broadly separated into two main evolutionary lines, encompassing those species (groups 6–8; Cooke et al. 2000) with non-papillate sporangia (e.g., *Ph. cinnamomi*; Fig. 4c) which are predominantly soil borne root or woody trunk infecting pathogens and those (Groups 1–5; Cooke et al. 2000) which have papillate sporangia (e.g., *Ph. infestans*) that often infect aerial foliage. Traditional morphological characters such as the morphology of the male antheridium and whether species are homo or heterothallic are not good markers of phylogenetic relatedness (Blair et al. 2008; Brouwer et al. 2012; Cooke et al. 2000; Kroon et al. 2004; Runge et al. 2011). In a recent phylogenetic analysis based upon whole genomes, albeit of the very restricted number of five taxa, Seidl et al. (2012) concluded that the downy mildews (represented by *Hyaloperonospora*) were sister to the *Phytophthora* clade rather than embedded within it, with the nonpapillate/semipapillate *Ph. sojae* and *Ph. ramorum* species forming a clade that was sister to the papillate *Ph. infestans* as in the analysis of Runge et al. (2011). In a recent phylogenomic analysis Sharma et al. (2015a) inferred again a sister-group relationship for *Hyaloperonospora* and *Phytophthora*, but also found that *Plasmopara halstedii* was embedded within the latter, highlighting the need for a an expanded taxon samping in future phylogenomic analyses, as the current taxon sampling is probably too low to infer robust phylogenomic trees, despite the generally high to maximum-support observed in these analyses.

The downy mildews (Fig. 4q–v) are a diverse, monophyletic, group currently encompassing 20 genera (Table 1) that are obligate parasites, predominantly of dicotyledons (Göker et al. 2007; Thines et al., 2009a, Thines 2014). Because of their importance as biotrophic plant pathogens they have been extensively studied and sequenced for phylogenetic analyses (Table 1; Göker et al. 2003, Göker et al. 2007; Riethmüller et al. 2002; Sökücü and Thines 2014; Telle and Thines 2012; Telle et al. 2011; Thines et al. 2008, 2009a; Voglmayr 2003). Downy mildews typically produce deciduous conidiosporangia (Fig. 4s) are that born on persistent conidiosporangiophores (Fig. 4u, s, v), although these may be evanescent in the

graminicolous genera *Baobabopsis*, *Eraphthora*, *Peronosclerospora*, *Sclerophthora*, and *Sclerospora* (Fig. 4n–p; Thines 2006, 2009, Telle and Thines 2012, Thines et al. 2015b). *Peronospora* and *Pseudoperonospora* have pigmented conidia and consititute the most species-rich downy mildew clade (Table 1). Features such as haustorium morphology map well onto the molecular clades (Göker et al. 2007; Thines 2006; Voglmayr et al. 2004). Downy mildews with pyriform haustoria (DMPH) form a monophyletic lineage (Fig. 9). Digit-like (hyphal) haustoria (e.g., *Peronospora viciae*; Beakes et al. 1982; Hickey and Coffey 1977, 1978) probably represent the ancestral state and are similar to those formed in *Phytophthora* (Coffey and Wilson 1983). Molecular studies have confirmed that the graminicolous downy mildew (GDM; Fig. 4n–p) genera (*Baobabopsis*, Thines et al. 2015b; *Eraphthora*, Telle and Thines 2012; *Peronosclerospora,* Hudspeth et al. 2003; Shivas et al. 2012; *Sclerophthora,* Thines et al. 2008; *Sclerospora,* Riethmüller et al. 2003) are all related to other downy mildews in the Peronosporaceae sensu *lato.* Three monotypic GDM genera, *Graminivora*, *Poakatesthia* and *Viennotia* (Göker et al. 2003, Thines et al. 2006, Thines et al. 2007) appear to exhibit characteristics intermediate between *Phytopththora* and the downy mildews sensu stricto (Thines 2009).

Maintenance and Cultivation

Saprophytic or facultative parasitic species of Oomycota can be collected very easily from soil and water, and obligate hyperparasites are sometimes found at the same time. Useful sources of information on suitable methods for isolating and culturing aquatic fungi are given by Dick (2001), Fuller and Jaworski (1987), Johnson et al. (2002) and Sparrow (1960). Obligate parasitic downy mildews and white blister rusts must be sought on their known angiosperm hosts, but the less host-specific root parasites can be isolated using various plating and baiting techniques. Reference should be made to papers cited in Karling (1981) for information on the collection of the less-known species of Oomycota. A useful source of information for collecting and maintaining hemibiotrophic species is found in Erwin and Ribeiro (1998).

Typically many species of Saprolegniaceae and a variety of *Pythium* species can be isolated from samples of soil or exposed or submerged mud by placing suitable baits (e.g., 3 or 4 autoclaved hempseeds, sesame seeds, or snakeskin scales) added to sediment slurries diluted with sterile pond water (Dick 2001; Dick and Ali-Shtayeh 1986; Fuller and Jaworski 1987; Sparrow 1960). These dishes should be left undisturbed for 1–3 days at 10°–20 °C. The baits should then be transferred to clean dishes of water and incubated at 10°–20 °C for a further 4–14 days. A wide range (about 40 species) of *Pythium* species has been isolated from soil using a dilution plate procedure (Al-Shtayeh et al. 1986; Dick and Al-Shtayeh 1986). Several species of *Phytophthora* can be isolated by dilution plate techniques using P_{10} PV hymexazol agar (for recipes see Erwin and Ribeiro 1998). Dilutions between 1:30 and 1:100 are recommended for infested soils. The same medium can be used for isolations from infected roots. Since *Mortierella* and *Pythium* are inhibited by hymexazol, the aliquots can be incorporated into the nutrient agar and the washing

stage outlined above is not needed. Incubation is at 25 °C and scanning of plates is carried out after 1–3 days of incubation. Baiting, e.g., with *Rhododendron* and other leaves is a common procedure to isolate leaf-infecting *Phytophthora* species.

For the collection of Rhipidiaceae, in situ baiting techniques are essential. A cage of plastic-coated wire mesh containing fruits (e.g., apples, oranges, tomatoes) is suspended just below the water surface or just above the bottom mud in shallow stagnant or slow-moving water for about 10 days. The fruit is then removed and the fungal pustules examined with a dissecting microscope. Filamentous oomycete saprophytes will also be found. Using selective keratin and chitin baits, species that may be parasites of nematodes and other invertebrates are often selectively isolated (Sally Glockling and Shigeki Inaba, personal communication).

A wide variety of agars is used for culturing these oomycetes, including ones based on glucose, peptone and yeast extract (GYP); glucose, soluble starch, and yeast extract; potato dextrose; potatos and carrots; V8-juice; cornmeal, and others. Agars incorporating up to 10 mg/1 of cholesterol are also used: the carrier for the sterol may be chloroform, ether, or a 1% v/v aqueous solution of Tween 80. Axenic cultures are usually achieved by using several cleansing steps, such as by growing through a Raper's ring. For more details, the reader is referred to Fuller and Jaworski (1987) and Tsoa (1970). Members of the Saprolegniaceae are often stored on infested hemp seeds in distilled water, or on infested hempseeds placed on sterilized dampened filter paper in sterile bottles (Clark and Dick 1974).

Obligate biotrophic species, like the downy mildews and the white blister rusts have so far not been grown on artificial media. There is an account of axenic cultures of graminicolous downy mildews (*Sclerophthora* and *Sclerospora*, cited in Thines 2009), which could apparently not be successfully repeated so far. Other downy mildews and white blister rusts can be maintained in the laboratory by using infected leaves to inoculate detached uninfected leaves or leaf disks of the host species with the spores from the former (e.g., by stamping onto moist leaves or spraying). After inoculation, leaves should be kept dark for 24 hours at moderate temperatures. After that, the inoculated leaves or leaf discs should be kept at 100% relative humidity and at moderate temperatures (10–20 °C depending upon the species) and light quality as close as possible to those encountered under natural field conditions and a regular day-night photoperiod cycle. White blister rusts usually have to be cultivated on whole plants and most do not tolerate high humidity during sporulation.

Evolutionary History

The Straminipila form a well-supported monophyletic clade that is sister to the alveolates (Keeling et al. 2005) within the larger SAR superkingdom (Burki et al. 2007, 2008; Burki and Keeling 2014). In analyses using multiple protein-encoding genes the Oomycota and Hyphochytiomycota appear to form a sister clade to the brown-pigmented photosynthetic algae, the Ochrophyta (Cavalier-Smith and Chao 2006; Rilsberg et al. 2009; Tsui et al. 2006). Together this monophyletic assemblage was sister to a second major heterokont clade which encompasses the fungal-like

Thraustochytrids and Labyrinthulids and the bacteriotrophic bicoecid flagellates (Beakes et al. 2014; Yubuki et al. 2010). It has been estimated that the stem origin of the Ochrophyta was around 571 million years ago (mya) although with a large margin of error (Brown and Sorhannus 2010). The Oomycota and Hyphochytiomycota probably evolved after this, which is consistent with previous molecular clock estimates had suggested the origins of the Oomycota lay somewhere between 524 and 1000 mya (Bhattacharya et al. 2009). Recent molecular clock analyses by Matari and Blair (2014) proposes that the modern pathogenic oomycetes originated around the mid-Paleozoic, approximately 430–400 mya, although they did not include data from any early diverging genera in their analyses.

From earlier single gene analyses, the marine flagellate genus *Developayella* forms the sister clade to the Oomycota (Leipe et al. 1996: Tong 1995), although they have apparently little in common. When Sekimoto (2008) included the 18S sequences derived from assorted unknown stramenopiles from diverse marine ecosytems (Diéz et al. 2001; Massana and Pedró-Alió 2008; Massana et al. 2002, 2004, 2006) in his phylogenetic analyses the heterokont tree topography was markedly altered. An unknown stramenopile clade (lineage 3), consisting of a dozen or so rather deeply branched sequences, formed the sister clade to the Oomycota, although with little statistical support. *Developayella*, clustered in a clade with the flagellate parasitoid *Pirsonia* and the Hyphochytridiomycota and formed the immediate sister clade to the Ochrophyta rather than the oomycetes. Molecular studies have also revealed that most early diverging genera are marine and many are parasites of seaweeds or marine crustaceans (Beakes and Sekimoto 2009; Beakes et al. 2011). This, together with the fact that most of their closest relatives are also marine (Tsui et al. 2006), supports the current view, contrary to that of Dick (2001), that the Oomycota are marine in origin, as saprotrophs or facultative pathogens (Beakes and Sekimoto 2009; Beakes et al. 2012, 2014a).

Molecular studies have confirmed that the Oomycota are monophyletic and have provided a sound framework for hypothesising likely evolutionary pathways within the phylum. A simplified scheme is presented in Fig. 9a. This shows that the evolutionary scheme originally proposed by Bessey (1942), in which the holocarpic Olpidiopsidaceae were evolving prior to the split of the Saprolegniaceae and Peronsporaceae, was remarkably perceptive. We now know that the earliest-diverging clades contain predominantly small, non-mycelial, holocarpic oomycete genera (Beakes et al. 2014a; Karling 1981; Sparrow 1960), none of which have been successfully cultured on artificial media. This suggests this was the likely thallus form of the ancestral Oomycota. Genera in the Haliphthorales, Atkinsiellales s.lat. (*Atkinsiella, Lagenisma*), Leptomitales s.lat. (*Apodachlya, Blastulidium, Chlamydomyzium, Leptomitus*) and Rhipidiales (*Araiospora, Rhipidium, Sapromyces*) all produce extensive, bulbous or constricted thalli (Beakes et al. 2014a), which appears to be the intermediate stage in the evolution of a more-typical branched mycelial thallus that may have occurred about the time, or shortly after, of the Saprolegniomycete-Peronosporomycete divergence. It may have been the development of long, apically extending, hyphal-like discharge tubes in genera such as *Haliphthoros* (Fig. 5j) and *Atkinsiella* that led to the hyphal thallus form, at least in the Saprolegniomycete clade.

Analysis of the host preferences in basal Oomycota also raises the possibility that these organisms might have migrated from the sea to the terrestrial/freshwater environment with their invertebrate or algal hosts. Once on land they may have switched to plant hosts, as evidenced by the morphological similiarity between nematode-infecting species of the genus *Myzocytiopsis*. (Glockling and Beakes 2006a) and the closely related (Spies, personal communication) root-infecting genus *Lagena* (Barr and Désaulniers 1987, 1990). However, it should also be borne in mind that as long as the oomycete communities in marine and estuarian detritus remain largely unexplored (Nigrelli and Thines 2013; Marano et al. 2016), other evolutionary scenarios, such as the multiple independent development of a parasitic lifestyle from saprophytic genera cannot be ruled out. The same is also true for the likelihood of the repeated transition of oomycetes from land to the sea and vice versa (Richards et al. 2012), which has occurred several times within the Peronosporomycetes (Marano et al. 2016; Thines 2014). However, on balance it seems plausible that, at least initially, oomycetes evolved in the sea from holocarpic nutritionally-versatile organisms, many of which were facultative parasites of either invertebrates and or algae.

With the possible exception of the freshwater *Olipidiopsis* spp. (Martin and Miller 1986c), all basal genera lack oogamous sexual reproduction. However, recently a sexual cycle involving conjugation of adjacent cysts or thalli has been reported in *Eurychasma,* although only on some hosts (Gachon et al. 2015), and also occurs in *Anisolpidium ectocarpi* (Johnson 1957), a species now known to be a basal oomycote (Gachon et al. 2015). The recent finding that *Lagenisma*, which also reproduces by means of conjugating meiocysts (Schnepf et al. 1977, 1978a), is a basal Saprolegniomycete (Thines et al. 2015b), could mean that oogenesis may have evolved independently in the two classes of Oomycota. This may also explain the fundamentally different morphological patterns of oosphere formation in the two classes.

A critical evaluation of the fossil evidence for ancient terrestrial oomycetes is given in a recent review by Krings et al. (2011). Stidd and Consentino (1975) describe structures that they suggested represented *Albugo* oospores in the megagametophyte seed tissue of an ancient gymnosperm, *Nucellangium glabrum,* from around 310 mya. However the structures that were described were not conclusively *Albugo* oospores (Krings et al. 2011). A more convincing, though still controversial, *Albugo*-like microfossil appears to be *Hassiella monosperma* from the 412 mya lower Devonian Rhynie chert (Taylor et al. 2006). Structures purported to be small oogonia in *Hansiella* fossils look much more like the small globose haustoria that are typical of the genus *Albugo*. If this fossil is accepted as representing an obligately biotrophic *Albugo*-like pathogen of Rhyniophyte plants, it would mean that the evolution of obligate biotrophy can be traced back nearly 400 mya, which accords with recent molecular clock deductions (Matari and Blair 2014). Obligate symbiotrophy exemplified by the Albuginales, is therefore of ancient origin and must have evolved independently at least twice in the oomycete lineage (Kemen and Jones 2012; Kemen et al. 2011; Thines and Kamoun 2010). All extant white blister rusts are obligate parasites of angiosperms and the latter only diversified from a

common ancestor about 150 mya, even though they can probably be traced back into the Permian. This implies that the white blister rusts have evolved on hosts other than those we know them on today.

Another fossil genus, *Combresomyces*, with spiny papillate oogonia with paragynus antheridia, resembling current-day *Pythium* species, has been recently described associated with the remains of a 300 mya seed fern, *Lygniopteris* (Strullu-Derrien et al. 2010). *Galteriella biscalitheceae* associated with a sporangium of a fern *Biscallitheca,* also from around ca 300 mya, has *Phytophthora*-like amphigynous and paragynous smooth-walled oogonia. Papillate multi-oospored oogonia, reminscent of those found in some present day genera in the Saprolegniaceae have also been found in Rhynie chert deposits from the same time period (Krings et al. 2010). Therefore by the early Mesozoic era, about 300 mya, fossils showing the complete range of oogonium morphologies found in present day genera in the Albuginales, Peronosporales, and Saprolegniales have all been documented and implies that most of the known oomycete diversity had already evolved by then, likely with the exception of the hyperdiverse downy mildews.

The hyperdiverse obligate parasitic downy mildews are thought to have evolved relatively recent from an ancestor belonging to one of the more derived shoot- and foliage-infecting *Phytophthora* clades with papillate sporangia (for an in depth discussion see Runge et al. 2011) and represent the pinnacle of oomycete diversity. Thines (2009) has also discussed a number of traits (indeterminate sporangiosphore development, intracellular hyphal development) in rare graminicolous downy mildew genera, such as *Viennotia* and *Poakatesthia,* that are shared with *Phytophthora* and suggests these may represent relicts of the evolution of downy mildews from *Phytophthora*-like ancestors on Poales.

Finally there appear to be a number of interesting evolutionary parallels between Fungi and Oomycota (Sharma et al. 2015b). The two earliest-diverging oomycete genera *Eurychasma* and *Haptoglossa*, have endobiotic plasmodial thalli and injecting infection mechanism, respectively. These features are mirrored in the early diverging cryptomycete *Rozella* and by microsporidia, respectively (Jones et al. 2011; Lara et al. 2009). The clade (MAST-1) of unknown marine stramenopiles that are the closest to the oomycetes (Sekimoto 2008; Yubuki et al. 2010) may be analogous to the recently described cryptofungal clade that appears to be the sister clade to the Fungi (Jones et al. 2011). This highlights that many phylogenetically critical organisms still remain to be described and we still have little idea what sort of organisms make up unknown stramenopile clades. They are probably being sampled from their zoospores, and it seems possibly that many are parasitoids or parasites. In the future, the systematic application of both genomics and multigene molecular phylogenetic studies should help resolve many of the unresolved evolutionary questions both within oomycetes and to their closest relatives.

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Labyrinthulomycota

14

Reuel M. Bennett, D. Honda, Gordon W. Beakes, and Marco Thines

Abstract

The Straminipila are characterized by their anterior flagellum with tripartite hairs and form a well-supported monophyletic branch of the larger Straminipila/Alveolata/Rhizaria (SAR) superkingdom. This is an account of the molecular systematics and phylogeny of osmotrophic and phagotrophic lineages of the Straminipila, comprising the slime nets and their thraustochytrid allies, as well as some lesser known lineages. The phylum Labyrinthulomycota *s. lat.* contains two main clades, one of which approximates to holocarpic thraustochytrids and the other to the labyrinthulids and aplanochytrids. Together with the flagellate bicosoecids and the protermonads and opalinids, they form a monophyletic clade

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that is sister to the golden-brown algae and Oomycota. The systematics of the Labyrinthulomycota *s. lat.* is still in flux as recent studies employing environmental barcoding have revealed the presence of diverse lineages not branching within genera characterized in terms of their morphology. The current review deals primarily with the two major lineages of the Labyrinthulomycota *s. lat.* and discusses other lineages only briefly, due to the scarce knowledge about these organisms. Characteristics associated with zoosporogenesis and sexual reproduction are discussed in relation to other members of the Straminipila.

Keywords

Amoebae • Amphitremida • Aplanochytrids • Bothrosome • DHA (docosahexaenoic acids) • *Diplophrys* • Eelgrass wasting disease • Ecology • Ectoplasmic net • Labyrinthulida • Marine decomposers • Seagrass wasting disease • Scale coats • *Schizochytrium* • Slime nets • Thraustochytrida • Stramenopiles • Straminipila • Zoospore ultrastructure

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- **Aplanochytriaceae/Aplanochytriidae** (*Aplanochytrium* (including *Labyrinthuloides*))
- **"Stellarchytriaceae/Stellarchytriidae"** (*Stellarchytrium*)
- **Labyrinthulaceae/Labyrinthulidae** (*Labyrinthula*)
- **Oblongichytridiales/Oblongichytriida**
- **Oblongichytridiaceae/Oblongichytriidae** (*Oblongichytrium*)
- **Thraustochytriales/Thraustochytrida**
- **Althornidiaceae/Althorniidae** (*Althornia*)
- **Thraustochytriacae/Thraustochytriidae** (*Aurantiochytrium*, *Botryochytrium*, *Japanochytrium*, *Monorhizochytrium*, *Parietichytrium*, *Schizochytrium*, *Sicyoidochytrium*, *Thraustochytrium*, *Ulkenia*)
- **Amphitremida**
- **Amphitremidae** (*Amphitrema*, *Archerella*, *Paramphitrema*)

●●●●**Diplophrydaceae/Diplophryidae** (*Diplophrys*)
●●●**Amphifilida**
●●●●**Amphifilaceae/Amphifilidae** (*Amphifila*)
●●●**Sorodiplophryidae** (*Fibrophrys*, *Sorodiplophrys*)

Summary classification of major lineages adapted from Tice et al. (2016). It should be noted that the higher level classification needs to be considered provisionally, as the deeper splits within Labyrinthulomycota are largely unresolved (Pan et al. 2017).

Introduction

General Characteristics

The osmotrophic fungus-like members of the kingdom Straminipila are characterized by absorptive nutrition and heterokont biflagellate zoospores. The term stramenopile was first introduced by Patterson (1989) in reference to the "straw hairs" (mastigonemes) that decorate the anterior flagella of this group of organisms (Fig. 1b, e). Dick (2001) pointed out this was an incorrect derivation of the Latin for "straw hair" and that the correct form should be straminipilous. However, Adl et al. (2005) favored the continued use of "stramenopile", the form of the name that is most widely used (Lévesque 2011). It is now apparent that the Straminipila have their evolutionary origins in the sea and that many of the fungal-like organisms seem to be ecologically important and widespread pathogens of algae, animals, and plants (Beakes et al. 2012, 2014; Thines 2014).

Apart from the posteriorly uniflagellate chytrids all of the zoosporic organisms traditionally studied by mycologists can now be placed in the still contentious Straminipila/Alveolate/Rhizaria (SAR) superclade (Burki et al. 2008; Hackett et al. 2007; Reeb et al. 2009).

This account reviews one of the smaller groups within this lineage, the Labyrinthulomycota (predominantly labyrinthulids and thraustochytrids), and updates the pre-molecular account of the group published in the first edition of the Handbook of Protoctista by Porter (1990). Most members of the Labyrinthulomycota are heterotrophic colorless or yellowish protists that absorb nutrients in an absorptive (osmotrophic) or phagotrophic manner. They typically feed saprotrophically (but parasites are known, e.g., Schärer et al. 2007) and are key players in the detrital food web, helping to break down often intractable plant and animal remains and making these substrates more accessible to grazing amoebae and ciliates (Raghukumar 2002; Bongiorni 2012). Many thraustochytrids can also feed phagotrophically (Raghukumar 1992), and some genera such as *Aurantiochytrium* (Fig. 2d) and *Ulkenia* (Fig. 4) have a free-living amoeboid stage. The Labyrinthulomycota are often referred to as "slime nets," which relates to the feature shown by many of the crown genera, the formation of a network of fine, often branching and anastomosing, cytoplasmic threads (Figs. 1c, 2c, and 3a, b, d) that extend into the

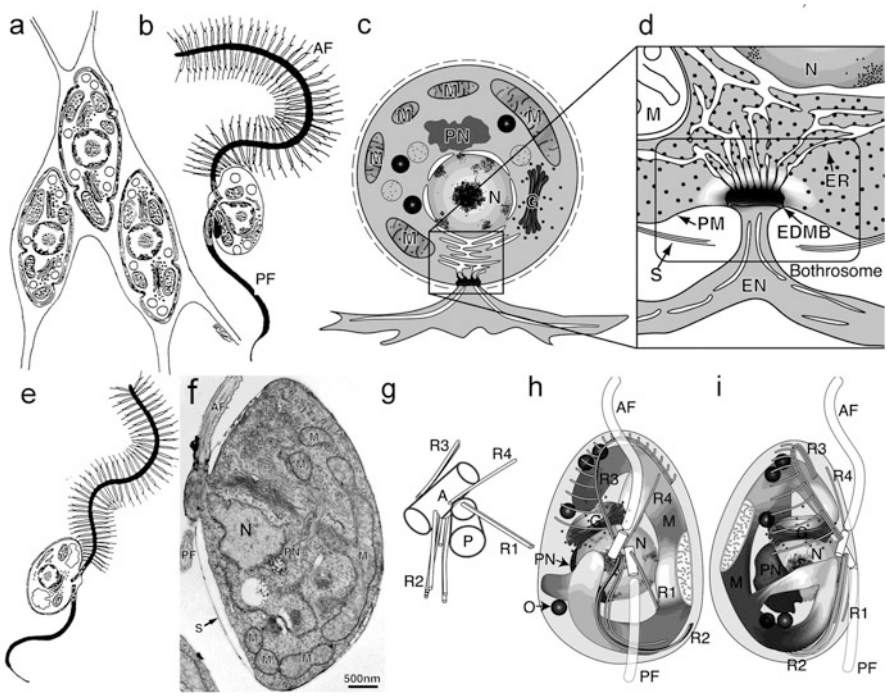

Fig. 1 Morphological features of labyrinthulids and thraustochytrids, part 1. Schematic drawing of *Labyrinthula* showing (**a**) uninucleate spindle-shaped cell bodies, which are coated in scales, containing mitochondria and Golgi dictyosomes and enveloping ectoplasmic net membrane and (**b**) biflagellate zoospore, with mastigonate anterior flagellum (*AF*) and shorter posterior flagellum (*PF*) with tapering terminal acroneme. Both adapted from Porter (1990). (**c**) Schematic drawings of the thraustochytrid *Schizochytrium aggregatum* thallus showing uninucleate (*N*) vegetative thallus and associated Golgi dictyosome (*G*) and surrounding mitochondria (*M*) and other organelles. (**d**) Schematic illustration of the bothrosome showing electron-dense plug material (*EDMB*), ectoplasmic net (*EN*), feeding endoplasmic reticulum (*ER*), plasma membrane (*PM*), and thallus scales (*S*). (**e**) Biflagellate zoospore, *Thraustochytrium* zoospore showing cell body covered in scales and anterior (*AF*) and posterior flagella (*PF*). From Porter (1990). (**f**) Transmission electron micrograph (*TEM*) showing a longitudinal profile of *S. aggregatum* zoospore, showing central nucleus (*N*) associated Golgi body (*G*) and paranuclear body (*PN*). (**g**–**i**) Diagrams of an *S. aggregatum* zoospore. (**g**) Ventral view of the flagellar roots showing the orientation anterior (*A*) and posterior (*P*) kinetosomes and their associated roots R1–R4). (**h**) Ventral and (**i**) right views of zoospore body showing the orientation of organelles and the flagellar apparatus: anterior basal body (*A*); anterior flagellum (*AF*); Golgi body; M, mitochondria; N, nucleus; P, posterior basal body; PN, paranuclear body (G); posterior flagellum (PF); flagellar roots (*R1–4*) (**c**, **d**, **f**, **g**–**i** From Iwata et al. published in Protist http://dx.doi.org/10.1016/j.protis.2016.12.002 Figs. 1, 3, and 5 with permission. All other photographs courtesy of Professor Daiske Honda, Konan University http://syst.bio.konan-u.ac.jp/labybase/index_en.html)

environment from the cell bodies and originating from a unique structure, now generally called the bothrosome (Figs. 1d and 2g, j; Porter 1990; Beakes et al. 2014). These threads provide adhesion to the substrate and absorb nutrients (as in

thraustochytrids) or form trackways along which the cell bodies glide (as in the labyrinthulids; Figs. 1a and 2h, i).

The group also includes a number of unicellular colorless protist genera such as *Amphifila*, *Amphitrema* (Fig. 3e), *Archerella* (Fig. 3f), *Diplophrys* (Fig. 3a–c), and *Sorodiplophrys* (Fig. 3d) that produce fine rhizopodia-like structures (Anderson and Cavalier-Smith 2012; Gomaa et al. 2013; Takahashi et al. 2014). Some such as *Ulkenia* and related genera also have an amoeboid phase (Fig. 2d) in their life cycle (Beakes et al. 2014; Karling 1981; Porter 1990; Yokoyama et al. 2007).

The unusual set of characteristics associated with the Labyrinthulomycota has hampered their taxonomic assignment. As summarized recently (Beakes et al. 2014), labyrinthulids have been assigned to various unrelated groups, such as the Rhizopoda, Mycota, Amoebozoa, and different phyla of the Straminipila, such as Chrysophyta and Oomycota. Based on phylogenetic evidence, as summarized by Gomaa et al. (2013; Fig. 5), the labyrinthulids do not belong to any of these groups and are probably best treated as an independent phylum in the Straminipila, the Labyrinthulomycota, as proposed by Porter (1990).

The recent application of molecular phylogenetic techniques including extensive environmental sampling and sequencing of DNA has, similar to the fungi of the kingdom Mycota (Jones et al. 2011), revealed many, as yet mostly undescribed, and often probably uncultivatable, representatives of this group in diverse marine, freshwater, and terrestrial environments (Collado-Mercado et al. 2010; Diéz et al. 2001; Massana et al. 2002, 2006; Massana and Pedró-Alió 2008; Pan et al. 2017; Richards et al. 2012; Stoeck et al. 2003, 2006, 2007). This methodology has also revealed that several protist genera of previously unclear taxonomic affinity, such as *Amphitrema* and *Archerella*, which were formerly placed together with the filose testate amoebae, also belong in the Labyrinthulomycota (Gomaa et al. 2013; Pan et al. 2017; Tice et al. 2016).

Because of this phylogenetic uncertainty, as with many other protist groups, names in the Labyrinthulomycota have been published both according to zoological (ICZN) and botanical (ICBN/ICNfap) nomenclature. The majority of species within the traditional Labyrinthulomycota have been described by mycologists under the botanical code for nomenclature, whilst many recent changes were suggested under the code for zoological nomenclature and where possible both sets of nomenclature are given in this chapter.

Occurrence

The Labyrinthulomycota appear to be cosmopolitan and were considered to be saprotrophic or only weakly parasitic organisms, ubiquitous in marine and estuarine environments. The morphologically described part of the Labyrinthulomycota consists of a relatively small group of almost exclusively marine genera (Figs. 1b and 3) that typically feed saprotrophically and are an important part of the marine detrital food web (Raghukumar 2002; Bongiorni 2012). However, many thraustochytrids feed bacteriotropically (Raghukumar 2002), and some genera such as

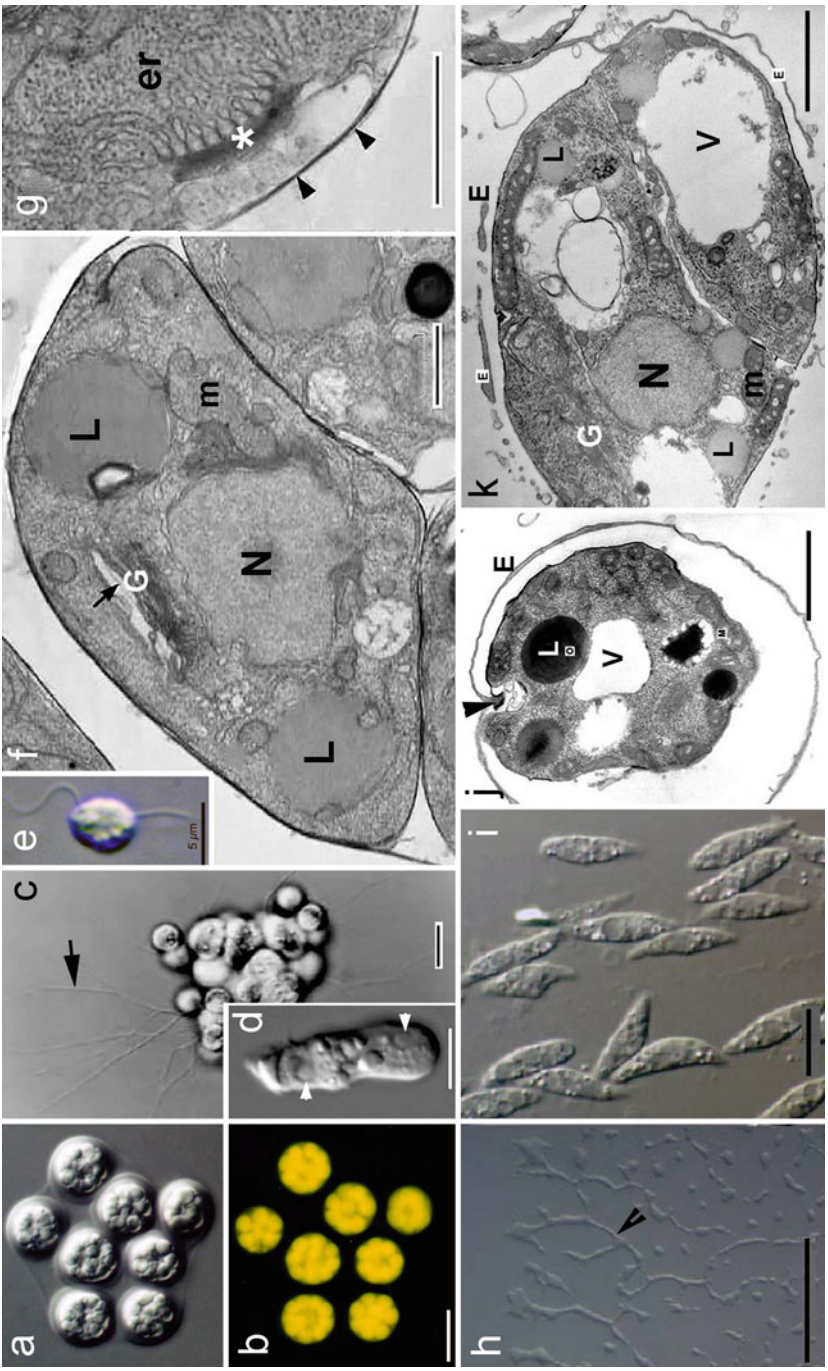

Fig. 2 (continued)

Ulkenia (Figs. 2 and 3h–j) have amoeboid stages engulfing their food. Thraustochytrids can be recovered in large numbers from marine sediments (Bongiorni 2012), including the deep sea (Raghukumar et al. 2001). Labyrinthulids are prevalent living on or within seaweeds and sea grasses, and there is an increasing evidence that they can live as parasites, commensals, or mutualists in plants (Bigelow et al. 2005; Bockelmann et al. 2012) and in other organisms, such as amoebae (Dykova et al. 2008) and mollusk tissues (e.g., Azevedo and Corral 1997).

However, most frequently they have been found associated with the surfaces of benthic algae, marine vascular plants, and detrital sediments (Porter 1990; Raghukumar 2002). Some, such as genus *Althornia*, are part of the free-floating eukaryotic plankton, and many others have been isolated from the marine water column (e.g., Collado-Mercado et al. 2010; Porter 1990), often in association with particulate "marine snow" (Naganuma et al. 2006; Raghukumar et al. 2001; Damare and Raghukumar 2008). Until the turn of the last millennium, the Labyrinthulomycota were considered to be exclusively marine organisms (Porter 1990), but about a decade ago, *Labyrinthula terrestris* has been described as a pathogen associated with turfgrass decline (Bigelow et al. 2005), and molecular studies have revealed an increasingly large number of freshwater members of this phylum (Anderson and Cavalier-Smith 2012; Gomaa et al. 2013; Richards et al. 2012). For instance, the testate protist genera *Archerella* and *Amphitrema* are common components of *Sphagnum* peatland, where they are often made visible by the endosymbiotic Trebouxiophyte algae (Fig. 3e, f) they contain (Gomaa et al. 2013). Many environmental sequences belonging to this clade have been isolated from anoxic sediments, which again suggests the habitats and roles occupied by these organisms is far more diverse than originally thought (Gomaa et al. 2013). Some members of the Labyrinthulomycota are genuine parasites which can have detrimental

Fig. 2 Morphological features of labyrinthulids and thraustochytrids, part 2. (**a**–**f**) Micrographs of the thraustochytrid *Aurantiochytrium limacinum.* DIC phase (**a**) and fluorescent (**b**) images of a colony of showing cells packed with oil globules which fluoresce orange when stained with nile red. (**c**) Colony of vegetative cells growing on agar showing fine branching ectoplasmic net (*arrowed*) emanating from the body cells (scale bar = 10 μm). (**d**) Amoeboid cell showing granular inclusions (*arrowed*) (scale bar = 5 μm) (**e**) Biflagellate zoospore, showing typical ovoid morphology of the Thraustochytridiales (scale bar = 5 μm). (**f**) TEM of vegetative thallus, showing central nucleus (*N*), associated Golgi dictyosome (*G*) and surrounding lipid (*L*) globules and mitochondria (*m*). (Scale bar = 1 μm). (**g**) TEM of bothrosome at the surface of *Aplanochytrium* sp. SEK349 cell. Note the cisternae of endoplasmic reticulum feeding into the plaque of electron-dense plug material (asterisk). (**h**–**k**) Micrographs of *Labyrinthula* sp. AN-1565. (**h**) Branching "slime net" colony growing on surface of agar (scale bar = 0.5 mm). (**i**) DIC micrograph showing spindle-shaped colony cells which migrate along the enveloping ectoplasmic net (not visible) (Scale bar = 10 μm). (**j**–**k**) Transmission electron micrographs showing transverse (**j**) and longitudinal (**k**) sections of thallus cells. Nuclei (*N*) are associated with a single Golgi dictyosome (*G*), and cytoplasm contains lipid globules (*L*), mitochondria (*m*), and vacuoles (*V*). The enveloping ectoplasmic net (*E*) originates from the bothrosome (*arrow*). (Scale bars = 5 μm)

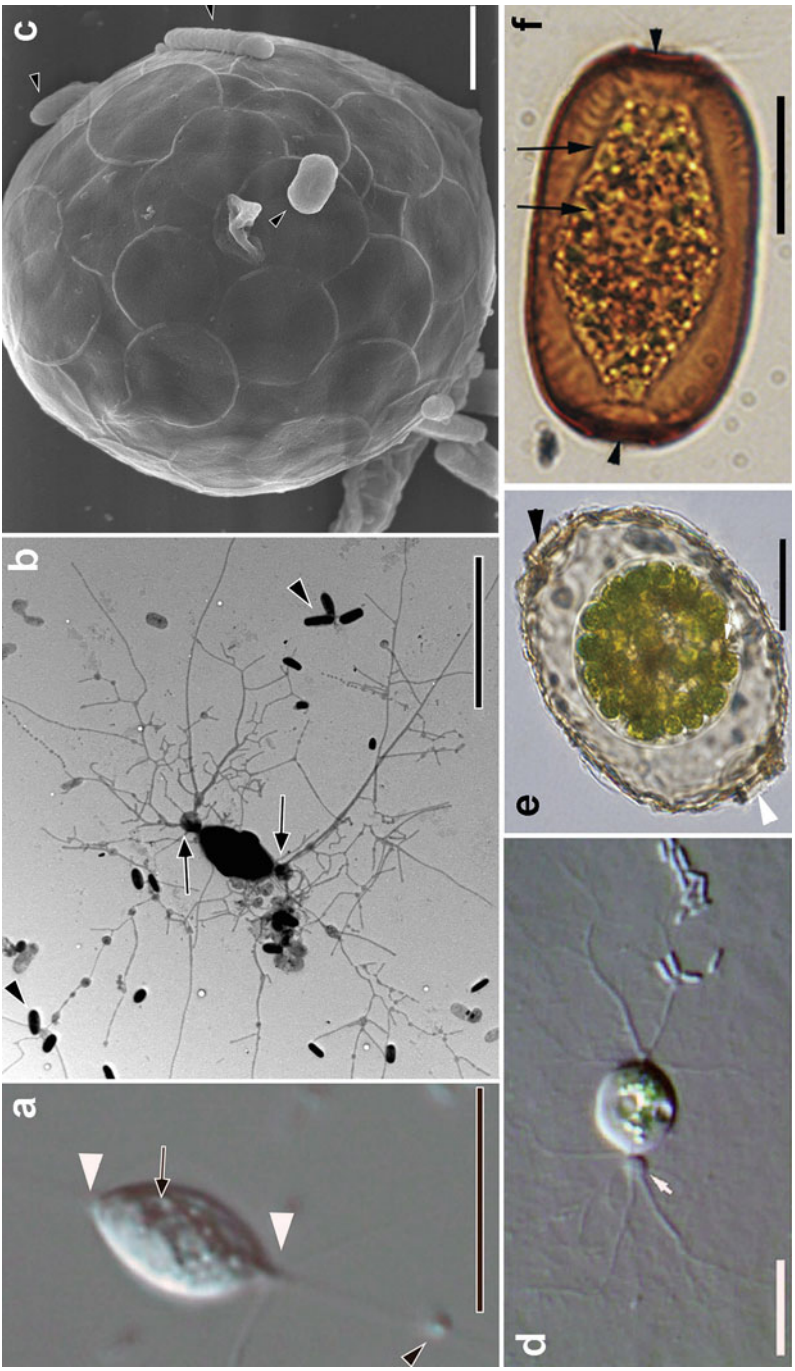

Fig. 3 (continued)

environmental impacts (such as on seagrass beds) and cause diseases of economical and ecological importance (Hatai 2012).

Literature and History of Knowledge

Because they are a small group, there are no dedicated taxonomic monographs on the Labyrinthulomycota, although illustrations of the main thraustochytrid taxa were included in the monograph of simple holocarpic biflagellate fungal-like organisms by Karling (1981). They are also included in the major systematic review of straminipilous fungi by Dick (2001). *Labyrinthula* was first observed by Cienkowski (1867) associated with intertidal algae in the Black Sea. The genus *Thraustochytrium* was first observed by Sparrow (1936), who described *T. proliferum* associated with benthic algae from Woods Hole, Massachusetts, and later monographed zoosporic fungi from various habitats (Sparrow 1960, 1973, 1976). Thraustochytrids were initially included in the oomycetes until the mid-1970s, when ultrastructural investigations revealed significant differences with between them and other biflagellate "zoosporic fungi" of the oomycetes (reviewed by Beakes et al. 2014; Perkins 1976; Moss 1985, 1986; Porter 1990). Physiological aspects of the thraustochytrids were reviewed by Goldstein (1973) and again showed important differences with other biflagellate "fungi." The next significant advances in knowledge came with the advent of molecular systematics. Molecular phylogeny confirmed that labyrinthulids and thraustochytrids were part of the straminipilous lineage as suggested by their ultrastructure (Patterson 1989), although the precise branching order of the various straminipilous clades remained poorly resolved (Leipe et al. 1994). It was not until the advent of multigene analyses based on conserved protein genes that there was a clearer understanding of how the main lineages were related (Tsui et al. 2009; Tsui and Vrijmoed 2012 – see later sections). Only recently have environmental sequencing projects greatly expanded the knowledge on the diversity, habitats, and distribution of Labyrinthulomycota s.lat., as outlined by Pan et al. (2017) (Fig. 5).

Fig. 3 Morphology of the Amphitremida. (**a**–**c**) Micrographs of ***Diplophrys mutabilis*** from freshwater. (**a**) Elongated fusiform cell, showing terminal origin of ectoplasmic elements (*white arrows*) and contractile vacuole (*black arrow*). (Scale bar = 10 μm). (**b**) Whole mount transmission electron micrograph of cell body showing radiating branched ectoplasmic elements from cell poles. Bacteria are also shown (*arrowheads*) (Scale bar = 10 μm). (**c**) SEM image of a lyophilized cell showing circular overlapping cells and attached bacteria. (Scale bar = 1 μm) (From Takahashi et al. 2014 Protist 165: 50–65 Figs. 1b, 2a, and 3a http://dx.doi.org/10.1016/j.protis.2013.10.001 with permission). (**d**) DIC micrograph of an amoeba of coprophilic *Sorodiplophrys stercorea* showing anastomosing pseudopodia, with swellings (*arrowed*) (Scale bar = 10 μm) (From Tice et al. 2016, Fig. 1c *Journal of Eukaryote Microbiology* doi:10.1111/jeu.12311 with permission). (**e**) Brightfield micrograph of *Amphitrema wrightianum*, showing apical shell apertures (pseudostome), and green Trebouxiophyte endosymbionts. (Scale bar = 20 μm) (**f**) Brightfield micrograph of *Archerella flavum*, showing pigmented shell (test) with terminal pores. The protist cell is *arrowed*. (Scale bar = 20 μm) (From Gomaa et al. 2013 Fig. 1. PLoS ONE 8(1) http://dx.doi.org/10.1371/journal.pone.0053046 with permission)

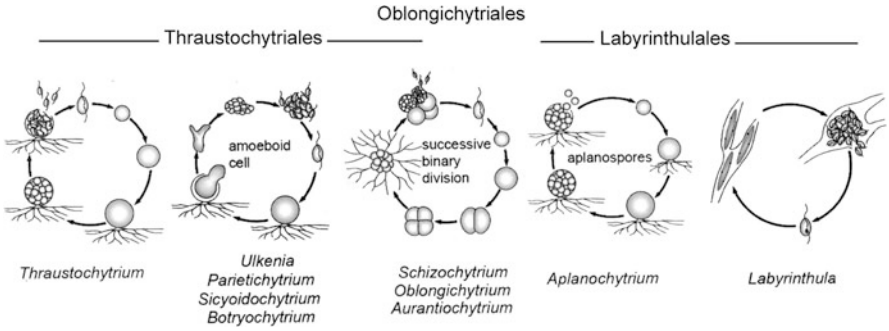

Fig. 4 Life cycles. Schematic drawings summarizing the variations in the life cycles of various thraustochytrid and labyrinthulid species (Adapted from http://syst.bio.konan-u.ac.jp/labybase/index_en.html)

Practical Importance

Thraustochytrids (Figs. 2a–g and 4) play important roles in nutrient cycling in marine ecosystems such as mangroves (Porter 1990; Raghukumar 2002), the open ocean, and sediments (Bongiorni 2012; Collado-Marcado et al. 2010; Kimura et al. 2001; Raghukumar et al. 2001). Although usually present in relatively low cell numbers, because of their large cell size compared with bacterioplankton, they nevertheless still make a significant contribution to the overall biomass of the oceans and probably play a significant role in the "microbial loop" by packaging and recycling nutrients in various communities of marine organisms (Raghukumar and Damare 2011).

Many labyrinthulomycetes (Fig. 2h–k) are parasites that can have a major impact on marine ecosystems or individual species. Muehlstein and Porter (1991) identified a new pathogenic species, *Labyrinthula zosterae,* as the causal agent of the eelgrass (*Zostera marina*) wasting disease. This disease of eelgrass first appeared in the 1930s and was responsible for the destruction of most of the vast subtidal stands of this vascular plant along the Atlantic coasts of North America and Northern Europe. Since larval stages of shellfish, such as oysters, scallops, and shrimp, all depend on eelgrass as a nursery bed, the loss of these stands has also detrimental effects on other ecosystems and the seafood industry. Since the discovery of *Labyrinthula zosterae* as a pathogen causing eelgrass disease, also the endophytic presence of *Labyrinthula* species in eelgrass has been documented, suggesting that various environmental factors might determine the virulence of *Labyrinthula* species (Bockelmann et al. 2012). However, also in terrestrial ecosystems, labyrinthulomycetes might play important, yet mostly unexplored, roles, as evidenced by the species *Labyrinthula terrestris*, the causal agent of a dieback disease of over-irrigated turfgrasses (Bigelow et al. 2005; Craven et al. 2005; Olsen 2007; Douhan et al. 2009) such as those found on coastal golf links and older reports of labyrinthulomycetes in inland habitats with high salinity (Amon 1978).

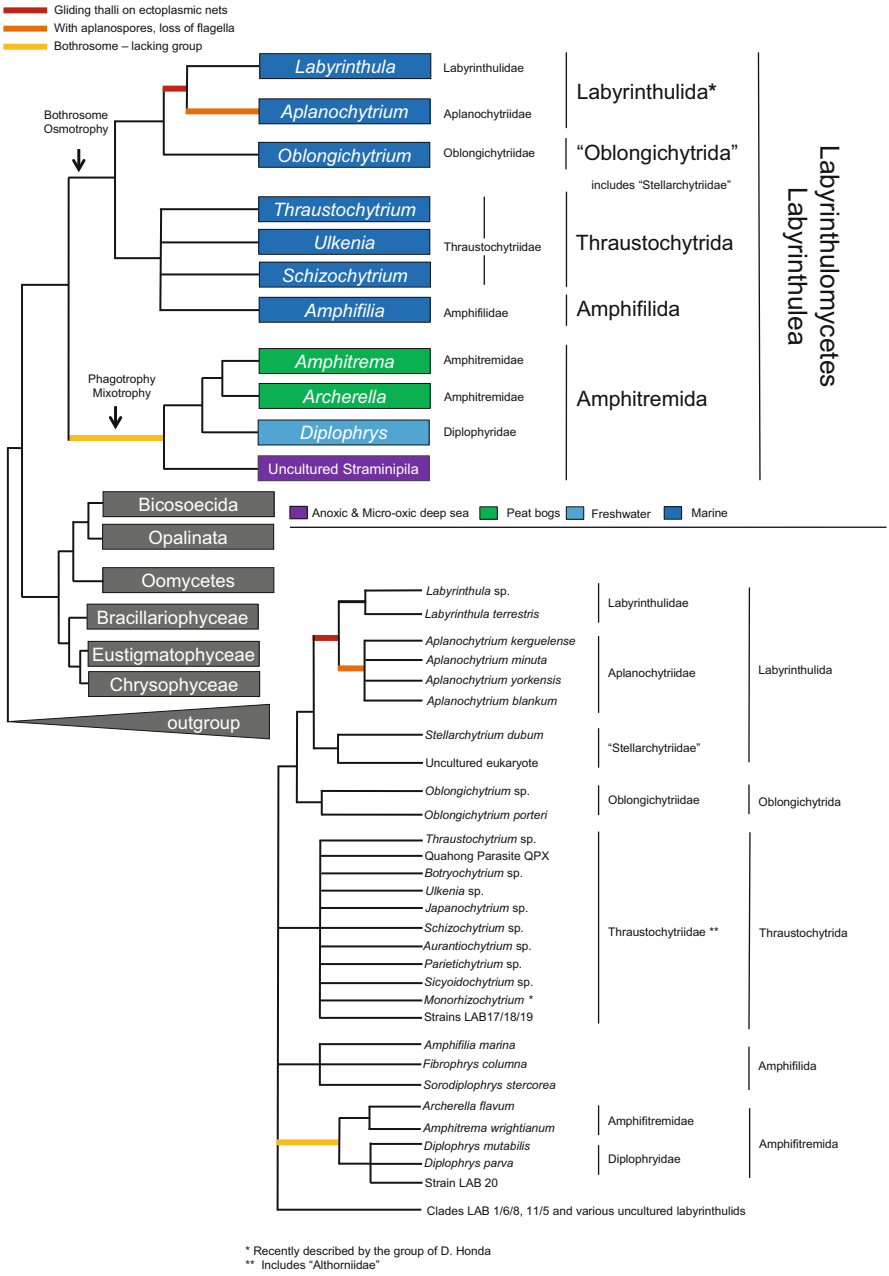

Fig. 5 Phylogeny. Trees summarizing phylogenetic relationships within the Labyrinthulomycota and with other Straminipila. Molecular phylogenetic scheme based on small subunit (SSU) rRNA gene sequences showing phylogenetic position *Archerella* and *Amphitrema* within the Amphitremida. This tree also shows relationship of Labyrinthulomycota and other heterokont

Thraustochytrid infections can cause serious economic losses to commercially reared shellfish. The most well-studied examples include the so-called "QPX thraustochytrid parasite" of the northern Quahog clam (Azevedo and Corral 1997; Lyons et al. 2005, 2007; Garcia-Vedrenne et al. 2013) and *Aplanochytrium haliotidis* infecting abalone (Bower 1987a, b; Bower et al. 1989). Other thraustochytrid species have also been shown to cause mass mortality amongst marine animals such as the nudibranch, *Tritonia diomedea* (McLean and Porter 1987), and cephalopods such as the lesser octopus, *Eledone cirrhosa* (Polglase 1980), and a squid, *Illex illecebrosus* (Jones and O'Dor 1983).

There has been much interest in exploiting marine thraustochytrids for a wide range of products they synthesize (particularly lipids; Fig. 2b), including the production of biodiesel, long-chain omega-3 fatty acids, and exopolysaccharides (Chang et al. 2012). In particular it is hope to culture them as an alternative to fish as a source of polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA), which are important dietary supplements for both animals (Miller et al. 2007) and humans (Kabayashi et al. 2011; Ragukumar 2008). Recently genetic manipulation has been used to improve fatty acid production in thraustochytrids (Kobayashi et al. 2011), and this work has been the main driving force behind sequencing the genome of *Aurantiochytrium* (Liu et al. 2016). Currently, *Schizochytrium* species are used for the commercial production of DHA (Winwood 2013). Squalene, a compound reported to reduce the incidence of coronary heart disease and cancer, accumulates in the thraustochytrid *Aurantiochytrium mangrovei* grown in the presence of the terbinafine (Fan et al. 2010).

Habitats and Ecology

Methodology for Detection and Enumeration Labyrinthulomycetes in the natural marine environment, including the water column, have been documented using various methods. Culture-based methods for determining and quantifying the prevalence of thraustochytrids in nature, using serial dilution and pine-pollen baiting, were pioneered by Gaertner (1968). The direct observation epifluorescence technique described by Raghukumar and Schaumann (1993) is another quantitative method that was considered to be a more sensitive and direct method for detecting and enumerating labyrinthulomycetes. More recently the introduction of molecular techniques involving sequencing of extracted environmental DNA and developing labyrinthulomycete-specific molecular probes has further extended the places where these organisms have been recorded from (Pan et al. 2017). Until now, they have been found in such extreme environments as arctic, subarctic, and antarctic habitats

Fig. 5 (continued) members of the Straminipila. The tree was adapted from Gomaa et al. 2013. PLoS One 8(1) http://dx.doi.org/10.1371/ journal.pone.0053046 with permission. Phylogenetic scheme for Labyrinthulomycota based on partial 18S rRNA sequences, including also environmental sequences. Adapted from Pan et al. 2017.

(Bahnweg and Sparrow 1974; Moro et al. 2003; Naganuma et al. 2006; Riemann and Schrage 1983; Stoeck et al. 2007), oceanic environments of the Indian Ocean (Damare and Raghukumar 2008, 2010), marine sediments (e.g., Bongiorni 2012), saline soils (Aschner 1958; Booth 1971; Bigelow et al. 2005), the deep sea (e.g., Amon 1978; Raghukumar et al. 2001), and shallow water hydrothermal vents (Colaco et al. 2006).

Environmental Tolerances of the Labyrinthulomycota Apparently, labyrinthulomycetes seem to have a wide range of tolerance to different salinity conditions. Some *Labyrinthula* isolates have been found associated with the roots and root hairs of trees in sandy soils irrigated with low salinity (4.3‰) water (Aschner 1958). Labyrinthulids have also been isolated from inland saline soils (Amon 1978). Many thraustochytrids (including isolates of the genera *Thraustochytrium*, *Schizochytriu*, and *Ulkenia*) have been isolated from habitats reflecting a wide range of salinities from weakly brackish waters (3‰) to briny salt evaporation ponds (150‰) indicating they may be thought of as euryhaline organisms (Jones and Harrison 1976). However, none of the species of thraustochytrids that Bahnweg (1979a, b) studied would grow in pure culture above a salinity of 40‰, and *Thraustochytrium pachyder um* appears to be one of the few species so far described that shows growth and zoospore formation at salinities up to 60‰ (Schneider 1981). There are also species and isolates found in habitats of more or less constant salinity and thus might actually be stenohaline.
The ability to withstand other extreme or fluctuating environmental or culture conditions has been reported amongst thraustochytrids (e.g., Banweg 1979a, b; Kuznetsov 1981) – some can apparently resist repeated cycles of drying and freezing (anabiosis). There are reports that thraustochytrids from both frozen arctic coastal soil samples and dried 50-year-old herbarium sheets of marine algae were successfully isolated and grown in culture (Kuznetsov 1981). Isolates of thraustochytrids also survived drying for several days (Jain et al. 2005) or even years (Kuznetsov 1981).

Habitats of Labyrinthulales Species of *Labyrinthula* are found in estuarine and near-shore marine habitats throughout the world associated with (or isolated from) organic detritus, macroalgae, diatoms, and particularly estuarine plants, such as mangroves and other marine vascular plants (Porter 1990). In hanging-drop or other laboratory cultures, the cells of *Labyrinthula* readily colonize a variety of vascular plant and algal tissues. They penetrate the cell walls and appear to decompose the cellular contents. In laboratory culture, labrinthulids are capable of decomposing many different microorganisms as a substrate, including bacteria, yeast, hyphal fungi, diatoms, filamentous algae, and other thraustochytrids (Perkins 1976; Porter 1990). Species of *Labyrinthula* are reliably isolated from submerged moribund or adrift leaves of marine vascular plants and pieces of filamentous or thalloid macroalgae. It has long been believed that healthy algae and marine grasses do not contain *Labyrinthula* cells within their tissues (Porter 1990), although these organisms can be regularly isolated from their tissues and Bockelmann et al. (2012)

reported their endophytic presence. However, *Labyrinthula* is usually not necrotrophic but rather feeds on epibiotic microorganisms and decomposing plant and algal material (Porter 1990). Based on current knowledge, the genus *Labyrinthula* is primarily associated with coastal environments. The genus *Stellarchytrium* associated with starfish has recently been described (FioRito et al. 2016) and represents a case in which an actual organism was found for a group otherwise only known from environmental sequencing, in this case, the LAB1/6/8 clade (Pan et al. 2017).

Habitats of Thraustochytriales Species of thraustochytrids (which includes species now classified in both the Thraustochytriaceae and Aplanochytriaceae) have also been isolated from estuarine and marine habitats throughout the world. Members of the thraustochytrids are able to grow in culture on a variety of plant- and animal-derived substrates (Perkins 1973). For instance, they have been observed growing on the spore cases of vesicular-arbuscular mycorrhizal fungi from barrier sand dunes (Koske 1981). They are generally isolated from decomposing algal and plant material, as well as from sediments, although they may also be found in plankton collected in offshore trails (Damare and Raghukumar 2010). In general, thraustochytrids seem to be mostly surface inhabitants of particulate organic material, primarily saprotrophic in their nutrition. In tropical and sub-tropical areas, mangrove plants (e.g., *Avicennia*, *Bruguiera*, *Kandelia*, and *Rhizophora*) are probably the most well-studied habitat in which to find labyrinthulomycetes (e.g., Fan and Chen 2006; Leaño 2001), where they appear to be primarily as saprobes colonizing the surface of organic detritus.

Thraustochytrids appear to be amongst the initial colonizers of fallen senescent mangrove leaves, alongside oomycetes (Thines 2014; Marano et al. 2016), and thus play an important role in nutrient cycling through exogenous production of their cellulase and xylanase degradation enzymes (Fan et al. 2002; Leaño 2001; Raghukumar 2002; Raghukumar et al. 1994). Thraustochytrids can be recovered in large numbers from marine sediments including from the deep sea (Bongiorni 2012). This group thereby contributes significantly to the biomass in the estuarine or marine environment.

In contrast to labyrinthulids, thraustochytrids appear to grow poorly on living algae and vascular plants. This has been primarily attributed to the presence of secondary metabolites with antimicrobial properties which limit the growth and propagation of these organisms (Raghukumar 2002). However, 7 days after leaf fall, thraustochytrids were found colonizing fallen leaves of *Rhizophora apiculata* (Raghukumar et al. 1995). Labyrinthulomycetes isolated from mangrove areas include *Schizochytrium* sp., *Thraustochytrium* sp., *Ulkenia* sp., and several unidentified strains of *Labyrinthula* sp. and *Aplanochytrium* sp. (Leaño 2001; Leander et al. 2004; Yokochi et al. 2001). The extensive colonization by thraustochytrid thalli on the surfaces of decomposing seaweeds has been noted (Miller and Jones 1983). Thraustochytrids, as epibionts, are probably feeding on other epibiotic microorganisms and decomposing plant and algal material. It is reported that the extent of colonization increased with the rate of decomposition; thus, they are probably saprotrophic followers of labyrinthulids, oomycetes, and

zoosporic fungi. Yokochi et al. (2001) reported not only *Labyrinthula* sp. as a saprobe on *Padina arborescens* and *Sargassum* sp. but also *Aplanochytrium* sp. on *Dictyota cervicornis*, *Chaetomorpha* sp., and *Cladophora* sp. (Leander et al. 2004). *Aplanochytrium minutum* and *Ulkenia visurgensis* were found associated with decaying *Sargassum cinereum* (Sathe-Pathak et al. 1993).

Some thraustochytrids may also be capable of necrotrophic/parasitoid growth on marine invertebrates, particularly mollusks such as nudibranchs (McLean and Porter 1987), squid (Jones and O'Dor 1983), and octopus (Polglase 1980). Thraustochytrids are also regular components of the gut microbiota of certain echinoids (Wagner-Merner et al. 1980) and have been found in a variety of Mediterranean sponges (Höhnk and Ulken 1979) and on the surface mucus of hermatypic corals (Harel et al. 2008). Thraustochytrids themselves may host viruses (Perkins 1976), e.g., herpes-type DNA virus particles (Kazama and Schornstein 1973). As herpes-type viruses are present in some vertebrates and invertebrates, (Segarra et al. 2010; Evans et al. 2017), this raises the possibility that thraustochytrids may be virus vectors for other organisms.

In spite of the many reports of thraustochytrids isolated from numerous substrates and locations, there have been surprisingly few direct observations of thraustochytrids in nature. *Schizochytrium*-like thalli were observed parasitizing colonies of the diatom *Thalassionema* collected from the North Sea (Gaertner 1979). Thraustochytrid-like thalli in Antarctic sediments fixed immediately after collection have been described (Riemann and Schrage 1983). Although diagnostic features were not presented, the thraustochytrids in these samples most closely resemble the genus *Aplanochytrium*.

Parasitic and Symbiotic Relationships Parasitism is another ecological strategy found in a few species of *Labyrinthula*. *Labyrinthula* spp. have been isolated from the marine algae *Chaetomorpha*, *Lyngbya*, *Cladophora*, *Rhizoclonium* (Raghukumar 1987a, b), and several marine vascular plants, such as *Cymodocea*, *Posidonia*, *Spartina*, *Thalassia*, and *Zostera* (Bockelmann et al. 2012; Garcias-Bonet et al. 2011; Stowell et al. 2005), although it is unlikely whether all these are parasitic associations. However, as mentioned previously, *Labyrinthula zosterae* has been identified as the cause of the wasting disease of eelgrasses (*Zostera capricorni*, and *Zostera marina*) resulting in the decline of eelgrass population (Armiger 1964; Muehlstein et al. 1988); and *Labyrinthula terrestris* has been identified as the cause of rapid blight on turfgrasses (Bigelow et al. 2005; Stowell et al. 2005; Craven et al. 2005; Olsen 2007). Garcias-Bonet et al. (2011) studied the occurrence and pathogenicity of *Labyrinthula* sp. in Mediterranean seagrass meadows. They found that their isolates could infect a number of different seagrass genera (*Posidonia, Cymodocea*, and *Zostera*) and indicates their isolate had a broader host range than found in most North American studies which indicated that pathogenicity was host genus-specific (Muehlstein et al. 1988; Short et al. 1993; Vergeer and den Hartog 1991, 1994). *Labyrinthuloides* (now classified as *Aplanochytrium*) *schizochytrops* was commonly isolated from living plants of the seagrass *Halodule wrightii* and was thought to be living as an endophyte, although may also have been responsible for a

brownish discoloration on the host leaves (Quick 1974). The *Schizochytrium*-like thalli parasitizing the diatom *Thalassionema* (Gaertner 1979) were later followed by additional reports of thraustochytrids as diatom pathogens. For example, *Ulkenia amoeboidea* was found capable of infecting a number of diatoms, including *Coscinodiscus* sp., *Grammatophora* sp., *Melosira* sp., *Navicula* sp., and *Nitzschia* sp. (Raghukumar 2006). However, it remains unclear if diatom parasitism constitutes a major ecological niche of Labyrinthulomycota and if these infections have a significant ecological impact.

Many marine invertebrates (e.g., corals, clams, flatworms, sea stars, and sea urchin) have been reported to harbor labyrinthulomycetes, and in some, this relationship may be parasitic, as with *Aplanochytrium haliotidis* on abalone (Bower 1987b) and the QPX thraustochytrid parasite on Quahog clam (Azevedo and Corral 1997; Lyons et al. 2005, 2007). The latter has been most extensively studied as an animal pathogen, and its genome has recently been sequenced in order to try and understand the basis of virulence (Garcia-Verdrenne et al. 2013). Recently a newly recognized species, *Thraustochytrium caudivorum*, was shown to parasitize the marine free-living flatworm *Macrostomum lignano* (Schärer et al. 2007), causing lesions that can lead to the dissolution of the posterior part or even complete animal. Three newly described Labyrinthulales species, *Stellarchytrium dubum*, *Oblongichytrium porteri*, and *Aplanochytrium blankum*, were isolated from dermal tissues of ochre sea stars (*Pisaster ochraceus*) that were exhibiting symptoms of starfish wasting disease (FioRito et al. 2016), although a direct causal relationship with the disease has yet to be established. Thraustochytrids are also capable of necrotrophic growth (perhaps parasitic) on marine invertebrates, particularly mollusks such as nudibranchs (McLean and Porter 1987), octopus (Polglase 1980), and squid (Jones and O'Dor 1983).

Other Labyrinthulomycota appear to have commensal relationships with their hosts such as *Labyrinthula* and *Oblongichytrium multirudimentale* on the coral *Fungia granulosa* (Kramarsky-Winter et al. 2006; Harel et al. 2008) or are saprobic such as *Aplanochytrium minuta* on scleractinian coral mucus (Raghukumar and Balasubramanian 1991). Thraustochytrids are regular components of the gut microbiota of certain echinoids (Wagner-Merner et al. 1980) and have been found in a variety of Mediterranean sponges (Höhnk and Ulken 1979), although details of these relationships are still unknown. Interestingly, thraustochytrids may host viruses (Perkins 1976), and herpes-type DNA virus particles have been described in a *Thraustochytrium* sp. (Kazama and Schornstein 1973). This is the only herpes-type virus to have been found in a host that is not a vertebrate and raises the possibility that thraustochytrids may be virus vectors for other organisms (Porter 1990). However, Labyrinthulomycota also carry RNA viruses of unknown host spectrum (Takao et al. 2005).

Freshwater and Terrestrial Labyrinthulomycota Until the advent of molecular systematics, it had been generally assumed that there were no genuinely freshwater or terrestrial members of the Labyrinthulomycota, although there were historic reports of *Labyrinthula* species infecting the freshwater alga *Vaucheria* (Zopf

1892). Recently it was shown that a number of phagotrophic freshwater protists (Fig. 3) such as the unicellular *Diplophrys parva* and *D. mutabilis* in the Thraustochytriales (Anderson and Cavalier-Smith 2012; Takahashi et al. 2014) and unicellular *Archella flavum* and *Amphitrema wrightianum* (Gomaa et al. 2013) and the sorocarpic *Sorodiplophrys stercorea* (Tice et al. 2016) in the Amphitremida all cluster in the Labyrinthulomycota clade. All of these heterotrophs are characterized by having fine filose pseudopodia. *Diplophrys parva* was isolated from the intestinal tract of a goldfish (Anderson and Cavalier-Smith 2012) and *D. mutabilis* from a freshwater lake (Takahashi et al. 2014), whereas *Archerella* and *Amphitrema* were both free-living protists isolated from wet Sphagnum moss (Gomaa et al. 2013). The coprophilic genus *Sorodiplophrys* was isolated from horse and cow dung (Tice et al. 2016). Environmental sampling has also revealed many more isolates belonging to the Amphitremida and Amphifilidae clades (Fig. 5), including isolates from various terrestrial soils, freshwater ecosystems, and anoxic sediments (Anderson and Cavalier-Smith 2012; Gomaa et al. 2013; Takahashi et al. 2014; Tice et al. 2016).

Characterization and Classification

Thallus (Cell) Morphology and Ultrastructure Members of the Labyrinthulaceae are characterized by forming colonies of spindle-shaped thalli (cells) that are ensheathed in a membranous ectoplasmic network which form a branched track system along which the cells freely migrate (Figs. 1a and 2h, i). Members of the Thraustochytriaceae on the other hand form ovoid or spherical thalli, which are associated with a fine ectoplasmic network of rhizoid (rhizopodia)-like threads (Fig. 3c–i) which act as anchoring and feeding structures (Perkins 1976; Bremer 1976; Moss 1985, 1986; Porter 1990). In terms of size and general appearance, this gives thraustochytrid thalli a superficial similarity to those of hyphochytrids and chytrid fungi (Karling 1981). The planktonic genus *Althornia* lacks rhizoids and absorbs nutrients directly from the environment (Karling 1981; Moss 1986; Porter 1990). The thalli of the Aplanochytridiaceae (now placed in the Labyrinthulales) superficially resemble thraustochytrids (Figs. 3g and 4) but are able to glide slowly along the surface of their ectoplasmic threads (Leander and Porter 2001). A number of previously enigmatic unicellular, sometimes colonial protists with fine-branching rhizopodia often arising bipolarly from the cells (Fig. 3a, b, d, f) have now been included in the Labyrinthulomycota, in a number of newly created families such as the Amphifilidae, Diplophryidae, and Sorodiplophryidae (Anderson and Cavalier-Smith 2012; Takahashi et al. 2014; Tice et al. 2016). In addition, the mixotrophic testate amoeba-like genera in the Amphitremidae, *Amphitrema, Archerella*, and *Paramphitrema* have cells protected in flask-shaped puncate shells (Fig. 3e, f) from which the rhizopodia emanate (Gomaa et al. 2013).

The Labyrinthulomycota have a typical straminipilous cytoplasmic ultrastructure with mitochondria with tubular-vesiculate cristae and prominent Golgi dictyosomes (Figs. 1f and 2f, g, j, k; Perkins 1976; Moss 1985, 1986; Porter 1990; Anderson and Cavalier-Smith 2012; Iwata et al. 2016). The cells usually contain cytoplasmic

vacuoles and oil globules (Figs. 1c and 2a, b, f, j, k). However, an ultrastructural feature that defines the Labyrinthulales and Thraustochytriales is that their ectoplasmic nets originate from the thallus body from a unique endomembrane complex associated with an electron-dense plaque on the plasma membrane (now known as the bothrosome – Figs. 1d and 2g) from which cisternae of endoplasmic reticulum radiate (Perkins 1976; Moss 1985, 1986; Porter 1990; Iwata et al. 2016). Previously this body has also been variously referred to as the sagenogenetosome (Perkins 1976) or sagenogen (Dykstra and Porter 1984). The early stages of the bothrosome complex development and net formation following zoospore settlement have recently been described in *Schizochytrium* by Iwata et al. (2016). This study has shown that the bothrosome forms within minutes of zoospore settlement at the anterior-ventral pole of the cell close to the Golgi body. Immunofluorescence labelling revealed that actin co-localized with newly formed bothrosome co-localized, and that within 18 min of settlement, the ectoplasmic net system had formed, with net filaments rich in actin (Iwata et al. 2016). The ectoplasmic net, unlike the rhizoid system of hyphochytrids and chytrids, is not walled (Figs. 1c, d, and 2j, k) and as well as containing actin, only contains cisternae of endomembrane (Moss 1985, 1986; Porter 1990; Takahashi et al. 2014). However, a classical bothrosome structure does not appear to be associated with slender rhizoids/filopodia of the unicellular protist-like members of the phylum in the Ampifilaceae (e.g., *Amphifila marina* – Dykstra and Porter 1984).

Another major difference between the thalli of the Labyrinthulomycota compared to other straminipilous fungi is that the thallus is surrounded by Golgi-derived ovoid, round or hexagonal scales (Fig. 3c; Perkins 1976; Moss 1985, 1986; Porter 1990) which are not cellulosic but composed of sulfated polysaccharides containing fucose or galactose (Bahnweg and Jäckle 1986; Honda et al. 1999; Moss 1985, 1986). In older thalli layers, scales can form a consolidated wall (Fig. 2f) around the thallus but do not coat the tracks or rhizoids (Perkins 1976; Dykstra and Porter 1984; Porter 1990). Surface scales are also a feature of the planktonic unicellular genus *Amphifila marina* (formerly *Diplophrys* – see Anderson and Cavalier-Smith 2012), which led Dykstra and Porter (1984) to suggest this enigmatic protist had Labyrinthulomycete affiliations. Cells of the freshwater heterotrophs *Diplophrys parva* (Anderson and Cavalier-Smith 2012) and *D. mutabilis* (Takahashi et al. 2014) are also coated in small Golgi-derived capsule-shaped or ovoid scales (Fig. 3c). In contrast, the cells of species in the Amphitremidae are contained in a thick rigid lightly pigmented shell (Fig. 3e, f; Gomaa et al. 2013).

Zoospore Formation and Fine Structure The ways in which these thalli differentiate into motile zoospores and proliferate have been the main defining characteristics (see Karling 1981) of the thraustochytrid genera (Fig. 4), although it now appears that this morphology is poorly correlated with underlying genetic relatedness (Yokohama and Honda 2007; Yokohama et al. 2007; Beakes et al. 2014). In some *Thraustochytrium* species, the whole thallus cytoplasm differentiates into biflagellate zoospores, which are released by the general splitting and disintegration of the thallus wall (Fig. 4; Karling 1981). In other *Thraustochytrium* spp., internal proliferation of

new thalli occurs from cytoplasm cleaved from the basal portion of thallus, concomitantly with the main compartment cleaving into zoospores (Karling 1981; Beakes et al. 2014). Development of the thallus in the genus *Aplanochytrium* is similar except that only non-motile aplanospores are formed (Fig. 4). The genus *Schizochytrium* has thalli which divide by successive bipartitions to form progressively smaller units (Figs. 2c and 4), in which the zoospores ultimately differentiate (Karling 1981). Several genera (*Botryochytrium*, *Parietichytrium*, *Sicyoidochytrium*, and *Ulkenia* – Fig. 4) in the Thraustochytriales have a more complex life cycle in which a free-living amoeboid cell (Figs. 2d and 4) is released from the original parental thallus and which then settles and eventually differentiates into zoospores (Beakes et al. 2014).

Straminipilous zoospores range in size between 3 and 15 μm (Dick 2001) and, as in the Labyrinthulomycota, many are reniform with laterally inserted flagella (Figs. 1b, e, f, and 2e). The straminipilous zoospore has a remarkably conserved overall organization and structure supporting the origin of this clade from a common flagellate ancestor (Tsui et al. 2009; Beakes et al. 2014). The anterior flagellum in all members of the Straminipila is decorated with two parallel rows of tripartite tubular hairs (TTH) and usually four to five times the overall zoospore body length (Fig. 1b, e; Perkins 1976; Porter 1990). The TTH are made of proteins and serve to reverse the flagellum thrust, in effect pulling straminipilous zoospores through the water (Dick 2001). Thraustochytrid, but not labyrinthulid, zoospores are unusual in that the zoospore body is also coated in small scales (Fig. 1e, f; Perkins 1976; Kazama 1980; Porter 1990).

All straminipilous flagellate cells share the same underlying flagellar rootlet system (Fig. 1g–i) which shows a remarkable degree of conservation throughout the lineage (Barr and Désaulniers 1989; Andersen et al. 1991; Dick 2001; Iwata et al. 2016). Zoospores of biflagellate members of the Straminipila have four rootlets, two associated with each flagellum (Andersen et al. 1991; Barr and Allan 1985; Barr and Désaulniers 1987; Iwata et al. 2016). The R3 anterior rootlet is composed of three microtubules, and curves around the anterior end of the zoospore and from which on one side emanate a series of microtubular ribs (Fig. 1h, i; Beakes et al. 2014; Iwata et al. 2016). Labyrinthulomycete zoospores appear to lack the striated fan between the kinetosomes that are a feature of oomycete zoospores (Fig. 1g; Barr 1981; Barr and Allan 1985; Porter 1990; Iwata et al. 2016). Unusually for members of the Straminipila, the Labyrinthulomycota do not have a typical transitional helix (TH) structure associated above the flagellar plate but do have a similarly placed cone-like structure and electron-dense plug (Barr and Allan 1985; Beakes et al. 2014; Cavalier-Smith and Chao 2006). Nuclear division has been investigated by Perkins (1970) and Kazama (1974) and shows similarity to other members of the Straminipila (Beakes et al. 2014).

Sexual Cycle Most members of the Straminipila appear to be diploid organisms that undergo gametic meiosis (Dick 2001; Sims et al. 2006). However, knowledge of the precise timing of meiosis and plasmogamy in the Labyrinthulomycota is still very uncertain (Porter 1990; Beakes et al. 2014). In labyrinthulids, evidence of meiosis

has been found in thalli dividing up to produce flagellate zoospores (Perkins and Amon 1969; Porter 1990), but precisely where syngamy takes place has still not been established. However, epibiotic resting spores are produced by some species of thraustochytrids, although it has not been established if these are the result of sexual reproduction (Karling 1981; Porter 1990).

Classification and Systematics

Even though investigations of the past three decades have revealed new species, cytological details, and development cycles, the complete life cycle of any species of *Labyrinthula* remains to be worked out (Porter 1990; Beakes et al. 2014). However, it has now been established that Labyrinthulomycetes together with the human pathogen *Blastocystis* and the ciliate-like opalinids formed one major, early diverging branch of the Straminipila and that the Hyphochytriomycota, Oomycota, and golden-brown photosynthetic Ochrophyta formed another separate lineage, although both share a common ancestor (Fig. 5).

Prior the era of molecular systematics, the Labyrinthulomycota were divided into two families, the Labyrinthulaceae and the Thraustochytriaceae (Karling 1981; Porter 1990; Dick 2001) within a single order, the Labyrinthulales (or Labyrinthulida). The Labyrinthulaceae contained a single genus, *Labyrinthula* whereas the Thraustochytriaceae had around a dozen genera, mainly defined by thallus morphology and differentiation (Perkins 1976; Karling 1981; Moss 1985; Porter 1990; Dick 2001). The first in-depth molecular systematic study of the group was carried out by Honda et al. (1999) based on SSU rRNA gene sequence comparisons. Their isolates fell into two major clades, which they named the "labyrinthulid phylogenetic group" (LPG) and the "thraustochytrid phylogenetic group" (TPG) (Honda et al. 1999). The LPG clade included *Labyrinthula* and *Aplanochytrium* (syn. *Labyrinthuloides*) in one subclade and *Schizochytrium minutum* and *Thraustochytrium multirudimentale* in another. The TPG clade contained genera such as *Schizochytrium*, *Ulkenia*, as well as many *Thraustochytrium* spp. (Honda et al. 1999). Rather than the straightforward separation of the labyrinthulids and thraustochytrids, these studies revealed for the first time that the labyrinthulids in particular were part of a more diverse monophyletic assemblage that included a number of species that had traditionally been considered to be thraustochytrids. However, the LPG and TPG clades correlated well with the sugar composition of their thallus walls (Honda et al. 1999), with genera in the LPG clade predominantly having fucose and those in the TPG clade having galactose as their major cell wall constituents (Honda et al. 1999). A concurrent study by Leander and Porter (2001) however, suggested there were three major clades within the Labyrinthulomycota. There was an additional clade that included two *Labyrinthuloides* species, *L. yorkensis*, and *L. minuta.* These were subsequently transferred to the genus *Aplanochytrium* in a new family, the Aplanochytriaceae/Aplanochytriidae (Anderson and Cavalier-Smith 2012; Leander et al. 2004), which was sister to the Labyrinthulaceae.

The third clade represented the residual Thraustochytriaceae, containing many of the traditional thraustochytrid genera together with the enigmatic, bothrosome-lacking, planktonic protist *Diplophrys* (now *Amphifila*) *marina* and two isolates of the Quahog clam pathogen (so-called QPX isolates). What these molecular studies also highlighted was that many of the traditional thraustochytrid genera, such as *Schizochytrium, Thraustochytrium*, and *Ulkenia,* which were based on patterns of thallus development, were paraphyletic or polyphyletic (Honda et al. 1999; Leander and Porter 2001; Leander et al. 2004) showing that traditional morphological characters were not good indicators of genetic relatedness. Subsequent studies have led to a radical revision in thraustochytrid nomenclature, with the introduction of many new genera (*Aurantiochytrium*, *Japanochytrium*, *Oblongichytrium*, *Parietichytrium*, *Sicyoidochytrium*, and *Stellarchytrium*) based on combined molecular and biochemical characteristics (Yokoyama and Honda 2007; Yokoyama et al. 2007; FioRito et al. 2016). A recent taxonomic analysis of labyrinthulomycetes phylogenies is shown in Fig. 5 (adapted Gomaa et al. 2013; Pan et al. 2017). The order Labyrinthulales *s. lat.* includes a number of genera (*Aplanochytrium*, *Stellarchytrium*, and some *Thraustochytrium* spp.) that would have previously been placed in the Thraustochytriaceae. Some recent analyses have also separated another, morphologically unremarkable, thraustochytrid-like clade encompassing the genus *Oblongichytrium*, into their own separate family (Oblongichytriidae; Fig. 5; Pan et al. 2017).

The taxonomic subdivision of the Labyrinthulomycota is still in flux and has changed significantly in the last decade as a result of molecular phylogenetic investigations of both the core labryrinthulids and thraustochytrids, but also other groups of heterotrophic protists that are now known to be related. As a consequence of the above taxonomic studies and a series of more recent phylogenetic investigations (Colladao-Mercado et al. 2010; Anderson and Cavalier-Smith 2012; FioRito et al. 2016; Gomaa et al. 2013; Pan et al. 2017; Takahashi et al. 2014; Tice et al. 2016), there seem to be four or five higher-level clades within the phylum (excluding clades only known from environmental sequencing), namely, Labyrinthulales/Labyrinthulida, Thraustrochytriales/Thraustrochytrida, "Amphifilales/Amphifilida," Amphitremidales/Amphitremida, and "Oblongichytriales/Oblongichytrida." However, what is becoming increasingly clear from environmental sequencing is that they are a diverse group of which the vast majority of species still awaits discovery (Worden and Not 2008; Collado-Mercado et al. 2010; Richards et al. 2012; Gomaa et al. 2013; Ueda et al. 2015; Pan et al. 2017). As for most environmental lineages, only partial SSU sequences are available which has been proven to have an insufficient resolution for the deeper splits of the Labyrinthulomycota; it remains unclear, how many of the lineages known only from environmental sequencing can be assigned to the orders given above. In the most comprehensive analysis of environmental sequences currently available, Pan et al. (2017) recognized several additional lineages of the Labyrinthulomycota basal to the known orders or in unresolved positions, which group in four clades, mostly with low to moderate support. These clades, such as the LAB1/6/8 clade containing *Stellarchytrium dubum*, might deserve family- or order-level status once their members have been studied in more detail, e.g., in multigene phylogenies.

Class Labyrinthulomycetes/Labyrinthulomorpha Labyrinthulea? (Lister 1891) Olive ex Cavalier-Smith 1986

Order Labyrinthulales/Labyrinthulida E A Bessey 1950/Doffein 1901

Family Aplanochytriaceae/Aplanochytriidae Leander Ex Cavalier-Smith 2012

A monotypic family formerly included in thraustochytrids. They have typical ovoid to spherical thalli, attached to their substrate by a basal ectoplasmic net that only form non-motile aplanospores. However, unlike members of the Thraustochytriaceae, the thalli are able to slowly glide along the rhizoids. The genus, *Aplanochytrium* (which subsumes the genus *Labyrinthuloides*), contains around half a dozen described genera, but there are probably many undescribed species based on environmental sequencing.

Family Labyrinthulaceae/Labyrinthulidae Haeckel 1868/Cinekowksa 1867

This family contains the classic "slime nets," which form a colony of spindle-shaped thalli that are contained within a branching ectoplasmic network within which the cells migrate. The gliding motility of the cells, which at times is as fast as 100 μm/min, probably driven by a calcium-dependent contractile system of actin-like proteins in the ectoplasmic network (Nakatsuji and Bell 1980). Each cell has a single bothrosome connecting it to the ectoplasmic network. There are around a dozen or so species that have been recognized (Dick 2001). Most are saprotrophs associated with marine debris and decaying macroalgae and marine macrophytes. However, some species have been shown to be the causal agents responsible for the wasting disease of eelgrass (*Zostera*) beds (Muehlstein and Porter 1991) and the turfgrass dieback (Craven et al. 2005).

Family-Level Clade "Stellarchytriaceae/Stellarchytriidae" Undescribed, LAB 1/6/8

This clade, which possibly needs to be described as a new family or even order, is provisionally placed in the Labyrinthulales and contains various lineages known only from environmental sequencing (Pan et al. 2017) and the recently discovered species *Stellarchytrium dubum* (FioRito et al. 2016). *Stellarchytrium dubum* was isolated from diseased starfish, but its role in causing starfish wasting disease still needs to be investigated in detail.

Order Oblongichytriales/Oblongichytrida

Family Oblongichytriaceae/Oblongichytriidae Cavalier-Smith 2012

This monotypic family was first recognized as a result of molecular sequencing by Yokoyama and Honda (2007). It contains around a half-dozen species that were formerly included in the genus *Schizochytrium* based on thallus development. The family name is derived from their slender oblong zoospores they produce rather than the more ovoid zoospores typical of the Thraustochytriales. It appears to form an early diverging clade from the same root as the Labyrinthulales lineage (Fig. 5).

Order Thraustochytriales/Thraustochytrida Sparrow 1973

Even though it has now been split, this still is the largest and most diverse order in the Labyrinthulomycota with the most genera and species. They produce relatively small epibiontic thalli usually attached to substrate by a fine ectoplasmic network of fine-branched anastomosing filaments, which have role in both substrate attachment and feeding. Most are marine organisms that are saprotrophic epibiontic colonizers of a variety of marine detritus, but there are a number of pathogens, mostly of marine invertebrates.

Family Althornidiaceae/Althorniidae Jones and Alderman 1972

This monotypic and monospecies (*A. crouchii*) is the only truly planktonic thraustochytrid as it completely lacks the usual ectoplasmic network. It is also the only genus for which there is at present no sequence data, and therefore its taxonomic placement must be considered as provisional.

Family Thraustochytriacae/Thraustochytriidae Sparrow ex Cejp 1959

Typically thraustochytrids are not colonial but grow by enlargement of cells which develop either into single ovoid or globular thalli or clusters of thalli depending if proliferation takes place before spore formation (traditionally referred to as sori). Within these, either zoospores are differentiated or an amoeboid stage is formed, which are both released by the breakdown of the thallus wall. There are at present six to eight genera within the family, a number of which have been recently created as a result of molecular studies (Yohoyama and Honda 2007; Yokoyama et al. 2007). Genera included in this family are *Aurantiochytrium*, *Botryochytrium*, *Japanochytrium*, *Monorhizochytrium*, *Parietichytrium*, *Schizochytrium*, *Thraustochytrium*, and *Ulkenia*. *Thraustochytrium* is the largest genus with around 20 described species.

Order "Amphitremidales"/Amphitremida Gomaa et al. 2013

Family "Amphitremidiaceae"/Amphitremidae Poch 1913

These organisms were formerly grouped with the testate amoebae. The cells are enclosed with ovoid, cup-shaped, or rectangular punctate shells (Gomaa et al. 2013). Many contain green algal trebouxiophyte endosymbionts and have a mixotrophic nutrition (Gomaa et al. 2013). There are currently three recognized genera, *Amphitrema*, *Archerella*, and *Paramphitrema*. Named species have been isolated from freshwater habitats, such as freshwater wetlands. However, environmental sequencing has revealed many uncultured sequences in a sister group from anoxic and micro-oxic deep-sea sediments.

Family "Diplophrydaceae"/Diplophryidae Cavalier-Smith 2012

This was one of the first of the colorless protist groups that was found associated with the Labyrinthulid clade, although the initial species studied *Diplophrys marina* (Dykstra and Porter 1984) has now been moved to the Amphifilida. The

Diplophryidae *s. str.* are small, largely freshwater heterotrophic protists with colorless spindle- to ovoid-shaped body cells from which a fine network of anastomosing filaments arises in bipolar fashion (Anderson and Cavalier-Smith 2012; Takahashi et al. 2014). The exact order placement of this family is not fully resolved, and it is placed with the Amphitremida on basis of recent phylogenetic investigations (Tice et al. 2016; Pan et al. 2017) although species also share morphological similarities with members of the next order.

Order "Amphifilales"/Amphifilida Cavalier Smith 2012

This is another order of colorless protists that have been phylogenetically elusive. They share many of the morphological characteristics of the genus *Diplophrys* described above, and the new genus was created by Anderson and Cavalier-Smith (2012) to contain the species *Diplophrys marina* which was in a separate clade from freshwater species of that genus. Another member of the family is the genus *Sorodiplophrys*, which had often been placed with dictyostelid amoebae (Tice et al. 2016).

Isolation Procedures

Thraustochytriales Isolation procedures have been summarized by Porter (1990) and are briefly reviewed in this account. Thraustochytrids can be isolated by plating tissue sections on seawater agar, peptone-yeast-glucose seawater agar (PYGSA, approximately 50% seawater), modified Vishniac's medium (KMV), or vegetable juice seawater agar, amended with penicillin and streptomycin to prevent bacterial growth. Small pieces (1 cm^2 or less) of carefully rinsed (e.g., with sterile 50% seawater) tissue sections are placed on agar media and incubated at room temperature for 3 days or until thraustochytrid colonies are visible on the periphery of the tissue samples. Often the bottom surface of the tissue that is in direct contact with the agar is similarly colonized. Slide purification is the easiest method for obtaining an axenic culture of thraustochytrid. This is done by transferring a minute quantity of thraustochytrid cells, often with a fine glass needle, to a drop of sterile water on a slide then serially diluting until few cells or thalli are visible. Individual thalli can then be streaked to another agarised medium used in the isolation process. Baiting samples with pollen, especially from pines, is a method commonly used for isolating chytrids but similarly helpful when isolating thraustochytrids. For pollen baiting, the carefully rinsed substrate is placed in Petri dishes containing sterile-filtered seawater, onto which pollen grains (preferably sterilized) are dispersed. Colonization of pollen grains is usually evident within 2–10 days in the Petri dishes, but these may be held for several weeks if necessary. Thraustochytrid thalli can be observed on pollen grains with a dissecting microscope, ideally at high magnification (60–100×). Individual pollen grains can be transferred with a loop to agar plates or to small drops of sterile seawater from which zoospores, if released, can be picked up and streaked onto agar plates. Alternatively, colonized pollen grains can be transferred in mass to agar plates. Often, especially if a small initial inoculum is used, all of the

colonized pollen grains will have colonies of the same species of thraustochytrid. It has been noted that not all thraustochytrids readily colonize pollen grains; thus, if a synoptic collection is desired, a variety of isolation procedures should be used. Maintenance of thraustochytrids can be achieved by regular subculturing or cryopreservation in 10% glycerol.

Labyrinthulales Several methods for isolating members of the Labyrinthulales have been published (Amon 1978; FioRito et al. 2016; Garcias-Bonet et al. 2011; Yokochi et al. 2001). Moribund (discoloured) but not decomposed seagrass, marsh grass, mangrove litter, and algal fragments collected adrift or recently washed ashore are reliable sources for labyrinthulids. Organic sediments from marine and intertidal aerobic zones and tissues of invertebrate species may also yield labyrinthulids. Successful isolation has been reported with 1% serum seawater agar (SSA) but also with plain seawater agar. Half- to quarter-strength concentration of vegetable juice seawater agar and PYGSA amended with antibiotics (e.g., penicillin, streptomycin, or ampicillin) are similarly useful in isolating *Labyrinthula*. Often the thickness of agar media is minimized to ~2 mm (Yokochi et al. 2001) for ease in observing colonies with an inverted microscope. Similar to thraustochytrids, rinsed plant or algal materials are placed onto agar media and usually incubated at room temperature. Vividly swarming colonies radiating from tissue pieces are usually visible within 7 days of incubation. An agar block containing a swarm of labyrinthula can then be subcultured or cocultivated with marine yeast or bacteria (e.g., *Vibrio*, *Psychrobacter*). This method has been practiced often, since these microorganisms serve as host or food for labyrinthulids. However, maintaining a culture of labyrinthulids is challenging as isolate cessation after subculturing several times occurs, probably because the full life cycle is not concluded under these cultivation conditions.

Evolutionary History

In the absence of any fossil record for this group, all evolutionary speculation has to be based on the evidence of recent molecular phylogenetic studies. All of the osmotrophic fungal-like organisms studied by mycologists, except the plasmodiophorids, fall within the straminipilous branch of the chromalveolate assemblage (Fig. 1a adapted from Tsui et al. 2009; Fig. 1b from Moreira and López-Garcia 2002). The kingdom Straminipila defined by Dick (2001) was often seen as synonymous with the kingdom Chromista (Cavalier-Smith and Chao 2006) which is the name often favored by online taxonomic databases, even though the chromista, as originally defined also contain organisms not belonging to the Staminipila or the SAR supergroup (Burki et al. 2009). However, Dick (2001) argued that because of the non-photosynthetic osmotrophic groups in this lineage that the etymologically correct name Straminipila would be a more appropriate kingdom name, as this would highlight the synapomorphy of a monophyletic group. Nonetheless, a widely adopted form of spelling for this kingdom is

Stramenopila (Adl et al. 2005; Lévesque 2011). Whether cryptophytes and haptophytes and their allies should also be considered as part of a wider supergroup is still debated (e.g., Reeb et al. 2009; Dorrell and Smith 2011).

Hyphochytrids and oomycetes are part of the lineage that shares a common ancestor with the photosynthetic ochrophytes (Tsui et al. 2009; Riisberg et al. 2009; Yubuki et al. 2010). It has recently been suggested that the stem origin of the Ochrophyta was around 571 millions of years ago, (a mean of estimates ranging from 735 to 434 million years ago: Brown and Sorhannus 2010). The Labyrinthulomycota are part of a sister clade, often collectively termed Bigyra which presumably evolved around the same time or only slightly earlier than the other osmotrophic Straminipila. The Labyrinthulomycota, Hyphochytriomycota, and Oomycota, as well as the ochrophyte straminipilous lineages share a common ancestor, which was most likely a photosynthetic mixotrophic marine flagellate (Tsui et al. 2009). The Labyrinthulomycota are part of one major straminipilous line and the Hyphochytriomycota and Oomycota of another (Fig. 5). This explains that whilst there are similarities between the Labyrinthulomycota and the other heterokont osmotrophs, they show much less in common than the other two groups.

The overall relationships between the major groups within the chromalveolate lineage, and the straminipilous groups in particular (see Beakes et al. 2014; Beakes and Thines, this volume), have been investigated using multiple protein-encoding genes (Tsui et al. 2009; Reeb et al. 2009; Riisberg et al. 2009). The statistically well-supported Alveolata kingdom, comprising Apicomplexa, Dinoflagellata, and Ciliata, forms the sister clade to the Straminipila (Keeling 2009). These can be divided into two main lineages: the first encompasses the bacteriotropic flagellate bicosoecids, the protistan gut-inhabiting opalinids (plus proteromonads and *Blastocystis* – equivalent to slopalinids defined by Patterson 1989) and the Labyrinthulomycota (Cavalier-Smith and Chao 2006; Tsui et al. 2009; Riisberg et al. 2009; Reeb et al. 2009), and the second straminipilous clade that includes the osmotrophic Hyphochytriomycota and Oomycota, the photosynthetic Ochrophyta, and a number of phagotrophic flagellates, such as *Developayella* and *Pirsonia* (Beakes et al. 2014). In this account, the Labyrinthulomycota is given phylum rank as in Porter (1990), and an emended Labyrinthulomycota *s. lat.* could be seen as containing other members of the phylum Bigyra (sensu Cavalier-Smith and Chao 2006), thereby replacing it.

The "chromalveolate hypothesis" proposes the red algal origin of the plastid in all chlorophyll c-containing algal groups (reviewed by Keeling 2009). However, recent comparative analyses of genomes of members of the Straminipila have led to the discovery of genes of green algal ancestry in both diatoms (Moustafa et al. 2011) and oomycetes (Richards et al. 2011; Jiang and Tyler 2012). It seems to cast doubt on such a simple "single-acquisition-multiple loss" interpretation (e.g., Maruyama et al. 2009; Stiller et al. 2009; Dorrell and Smith 2011). Theories involving multiple independent chloroplast acquisitions and horizontal gene transfer (HGT) have also been proposed as alternative explanations of the phylogenetic and genomic data (e.g., Martens et al. 2008; Stiller et al. 2009; Baurain et al. 2010). The eyespot in *Labyrinthula* zoospores (Perkins and Amon 1969) resembles those of photosynthetic

Straminipila and may be indicative of the remains of an ancestral chloroplast. However, the fact that such structures have only been observed in the most derived group does perhaps cast doubt on this suggested origin (Tsui et al. 2009). Thraustochytrids produce omega-3 PUFA using a desaturase enzyme that in algae is usually found in chloroplasts (Sargent et al. 1995). If, as suggested by Tsui et al. (2009), it is assumed the ancestor to the straminipilous lineage was a mixotrophic photosynthetic flagellate, then at least two independent plastid losses must have occurred in the straminipilous line, one prior to diversification of the Labyrinthulid clade and the other after the divergence of the ochrophytes leading to the heterotrophic Oomycota and related lineages (Beakes et al. 2014). Alternatively, if plastid loss was deeply seated within the straminipilous line, then an independent reacquisition of a chromistan type plastid must have occurred to give rise to the Ochrophyta as suggested by Leipe et al. (1994). Plastid genes have been widely found in sequenced oomycete genomes (see Lévesque et al. 2010; Jiang and Tyler 2012) but were not reported in the compact 18.8 Mb genome of the anaerobic human gut parasite *Blastocystis* (Doenoeud et al. 2011). The preliminary genome sequence for the thraustochytrid *Aurantiochytrium limacinum* has been recently released (Collier 2012), but preliminary analysis has not so far revealed evidence of genes of plastid origin (Collier personal communication).

The earliest-diverging Labyrinthulomycota clades appear to contain thraustochytrid clades that have still retained the ability of phagotrophic nutrition, which is considered to be the ancestral state (Tsui et al. 2009; Gomaa et al. 2013). The presence of endosymbiotic Trebouxiphyte algae in the cells of members of the Amphitremida indicates this group has retained the ability to feed phagotrophically (Gomaa et al. 2013).

According to the analysis of Tsui et al. (2009), the key evolutionary event in the evolution of the labyrinthulomycetes within the straminipilous lineage was the evolution of the naked ectoplasmic net. The most derived groups are the Labyrinthulids and Aplanochytrids which have lost the ability to feed phagotrophically and rely entirely on osmotrophic nutrition. But also they have evolved gliding movement on the ectoplasmic net. The Aplanochytrids became separated from the Labyrinthulids by the loss of flagella (although it would be interesting to know if flagella genes can be found in their genome) and the acquisition of polygonal scales (Tsui et al. 2009).

What has become apparent in the past decade is that the Labyrinthulomycota are a more diverse assemblage than previously thought (Fig. 5), both in terms of ecological niches they occupy and their morphology than was believed at the time of the last Handbook review (Porter 1990). It is likely, that many more species and hidden genera are yet to be discovered that will provide new insights into the origins and evolutionary develeopment of this enigmatic group of protists.

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Opalinata

15

Martin Kostka

Abstract

The opalinids (Opalinidae: genera *Opalina*, *Cepedea*, *Protoopalina*, *Zelleriella*, and *Protozelleriella*) are highly unusual protists with large cells, multiple flagella, and two to hundreds of nuclei. The name *Opalina* is derived from the iridescent appearance when light reflects on the delicately folded surface of the cells. Opalinids are found exclusively in the intestines of frogs and some other hosts. They form the group Slopalinida together with two related genera of intestinal flagellates, *Karotomorpha* and *Proteromonas*. The former is a tetrakont flagellate that inhabits the intestines of certain amphibians, while the latter possesses only two flagella and is found in a wider spectrum of vertebrate hosts. Both morphology and molecular data suggest that *Karotomorpha* is phylogenetically closer to the opalinids, although both flagellates were traditionally classified in a single family, Proteromonadidae. Molecular data have shown that yet another unusual gut protist is closely related to Slopalinida: the genus *Blastocystis*. Unlike its relatives, it bears no flagella and is usually observed in the form of spherical cells with huge vacuoles. It is quite common in the intestines of many vertebrates (including humans) and invertebrates. Together, these organisms form Opalinata, a diverse assemblage of variously modified unicellular eukaryotes.

Keywords

Opalines • *Karotomorpha* • *Proteromonas* • *Blastocystis* • Gut commensals • Multiple nuclei • Anaerobic mitochondria

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Contents

Summary Classification

●**Opalinata**
●●**Slopalinida**
●●●**Proteromonadidae** (*Proteromonas*)
●●●**Karotomorphidae** (*Karotomorpha*)
●●●**Opalinidae** (*Protozelleriella*, *Zelleriella*, *Protoopalina*, *Cepedea*, *Opalina*)
●●**Blastocystea** (*Blastocystis*)

Introduction

General Characteristics

Opalinata Wenyon, 1926, comprises several types of protists morphologically so distinct that their relationship was recognized only relatively recently. The "core" of Opalinata is formed by opalinids, members of the family Opalinidae Claus, 1874. They are a lineage of unusual unicellular eukaryotes with several conspicuous morphological characteristics. They are quite large (some of them may reach nearly three millimeters) and have multiple flagella and two to many nuclei. The surface of an opalinid cell is arranged in parallel folds. Light interference that occurs on these delicate structures leads to the beautiful opalescence of opalinids when they are observed in reflected light (hence their name). Opalinid genera can be distinguished on the basis of two features: number of nuclei (two vs. numerous) and cell form (cylindrical vs. flattened). Multinucleate genera are *Opalina* Purkinje and Valentin, 1835 (flattened, Fig. 1a) and *Cepedea* Metcalf 1920 (cylindrical, Figs. 1b and 6a), whereas *Zelleriella* Metcalf 1920 (flattened, Fig. 1c) and *Protoopalina* Metcalf, 1918 (cylindrical, Figs. 1d and 6b) have two nuclei. The

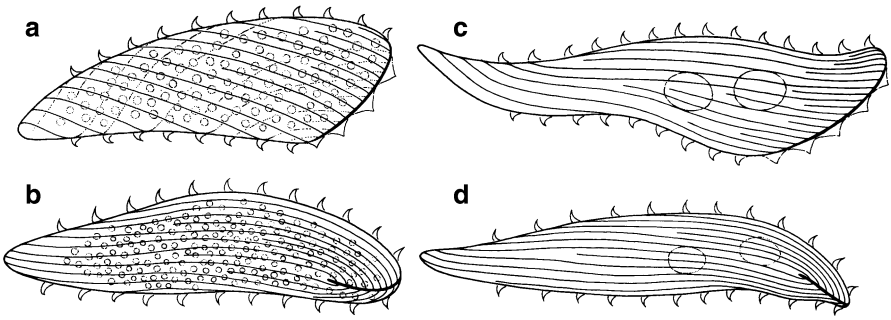

Fig. 1 Schematic drawings of four opalinid genera. *Circles* within the cells represent nuclei; the *lines* represent kineties (rows of flagella). Metachronal waves of beating flagella are symbolized by the waves at the periphery of the cells. The anterior part of cells with falx (*bold line*) points to the right. *Opalina* (**a**) is multinucleate, and its cell body is flat. The kineties run to the cell margin from where they continue on the other side (*dotted lines*). *Cepedea* (**b**) is also multinucleate, but its cell body is circular in cross section. *Zelleriella* (**c**) is binucleate with flat body, either caudate, as seen in the figure, or rounded posteriorly. *Protoopalina* (**d**) is a binucleate genus with cylindrical cells (Figure from Corliss (1989))

most recently erected genus, *Protozelleriella* Delvinquier, Markus, and Passmore, 1991, is similar to *Zelleriella* in appearance but is unique in having a hyaline margin without flagella (Delvinquier et al. 1991b). Two additional genera, uninucleate *Hegneriella* Earl, 1971 and *Bezzenbergia* Earl, 1973 with four nuclei are generally not considered valid. The number of described opalinid species reaches several hundred, but a critical revision of the family would probably lead to a reduction of the number (Sandon 1976).

Based on ultrastructural observations, proteromonad flagellates were recognized as the closest relatives of opalinids. The two genera of this paraphyletic group, *Proteromonas* Künstler, 1883 (Fig. 2a) and *Karotomorpha* Travis, 1934 (Fig. 2b) are represented by several species of rather thin, pointed intestinal flagellates with two or four flagella, respectively. Their Golgi apparatus, nucleus, and single mitochondrion are located in the anterior part of the cell near the kinetosomes. Grassé (1952) included two *incertae sedis* genera among proteromonads, *Dimoerium* Przesmycki, 1901 and *Dimoeriopsis* Hollande & Pesson, 1945. The latter is a parasite of freshwater snail eggs. There are no recent studies of these organisms and their biology and phylogenetic affinities should be rechecked.

The last, quite surprising addition to the group Opalinata was the genus *Blastocystis* Aléxéieff, 1911 (Fig. 3). Its members lack flagella completely and are best known as spherical cells with a large central vacuole and several nuclei since this is how they usually appear in culture. *Blastocystis* is morphologically very different from other members of Opalinata, and its recognition as their sister group was based primarily on phylogenetic analyses of molecular data (SSU rRNA gene sequences). *Proteromonas*, *Karotomorpha*, opalinids, and *Blastocystis* constitute a very interesting monophyletic group of intestinal protists that display extreme morphological disparity, ranging from "normal" flagellates to the complex

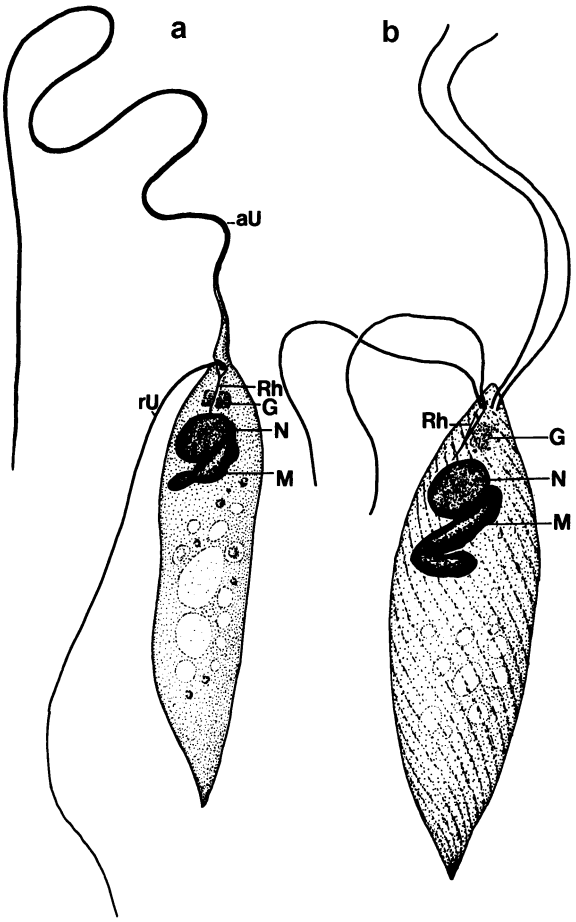

Fig. 2 Schematic drawings of *Proteromonas* and *Karotomorpha*. The cell of *Proteromonas* (**a**) bears one long, thick anterior flagellum (*aU*) and a trailing one (*rU*). The rhizoplast (*Rh*) passes through the Golgi apparatus (*G*) to the nucleus (*N*), behind which lies the mitochondrion (*M*). *Karotomorpha bufonis* (**b**) has two pairs of flagella, a short rhizoplast (*Rh*) running near the Golgi complex (*G*); the nucleus (*N*) is closely associated with a single mitochondrion (*M*). Sinistral surface striation (pellicular folds) is sometimes apparent (Figure from Brugerolle and Mignot (1989))

multiflagellated opalinids, on one hand, and to the morphologically reduced *Blastocystis*, on the other.

Occurrence

All members of Opalinata occur in the intestines of various animals. Opalinids are common inhabitants of the large intestines of frogs of all continents. Some other poikilotherm vertebrates can also be their hosts. *Karotomorpha* is common in some amphibians, while species of the genus *Proteromonas* are commensal in reptiles, urodelan amphibians, and in the caecum of certain rodents. *Blastocystis* has the widest host range: it is found in various mammals (including humans), birds, reptiles, and amphibians but also in some invertebrates such as cockroaches.

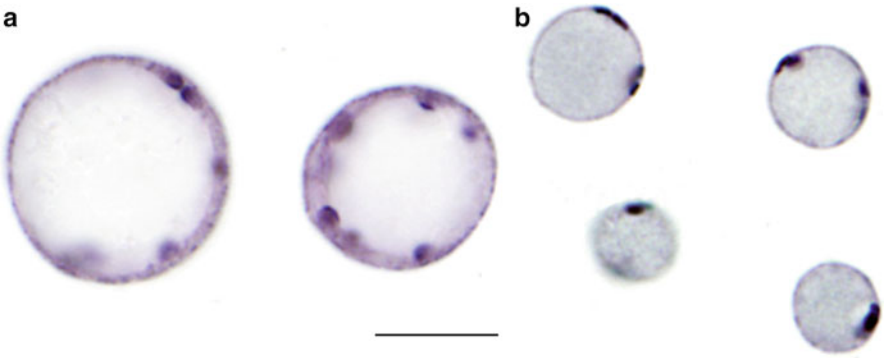

Fig. 3 Microphotographs of protargol-stained vacuolar forms of two cultured *Blastocystis* strains. Both were isolated from chelonians: strain GEEL (**a**) from *Geochelone elegans* and strain PYX (**b**) from *Pyxidea mouhotii*. The scale bar (10 μm) applies to both images. Note the differences in size and number of nuclei. The preparations were stained by Ivan Čepička

No cultures of opalinids are available. However, some frogs maintained in labs (e.g., *Xenopus laevis*) are infected with them, usually with *Protoopalina*. A single axenic culture of *Proteromonas* was established by Kulda (1973) and is available from the Dept. of Parasitology, Faculty of Science, Charles University in Prague, Czech Republic. It is also deposited in the American Type Culture Collection (ATCC PRA-286). *Blastocystis* is quite easily cultured xenically and may be axenized. There are many cultures available in laboratories studying *Blastocystis*; ATCC provides a number of isolates.

Literature

One of the most important early authorities on opalinids was Metcalf, who, among other studies, published two major works (Metcalf 1923, 1940). Later important works include Grassé (1952) and Corliss (1955). Opalinid life cycles and morphogenesis are discussed in Wessenberg (1961). Three very useful modern reviews are Wessenberg (1978), Corliss (1989), and Delvinquier and Patterson (1993). Delvinquier et al. (1991a, b, 1993, 1995a, b, 1998), Delvinquier and Desser (1996), and Delvinquier and Marinkelle (1996, 1997) published a series of papers on opalinid distribution in amphibians throughout the world and described a new genus (*Protozelleriella*).

Proteromonas and *Karotomorpha* are dealt with in detail in the works of Grassé (1952) and Kulda and Nohýnková (1978). Their ultrastructure was studied especially by Brugerolle and Joyon (1975). The phylogenetic affinities of the two genera and opalinids are discussed by Patterson (1985).

Older literature on *Blastocystis* is rather scarce. Extensive critical reviews appeared in the 1990s; the most notable were those published by Zierdt (1991), Boreham and Stenzel (1993), and Stenzel and Boreham (1996). Later, several reviews were published by Tan (2004, 2008). A detailed publication dedicated to

Blastocystis is that of Mehlhorn et al. (2012). Most recent advances are summarized in Clark et al. (2013). As if to outweigh the low number of older publications, a tremendous (and still growing) number of papers on various aspects of *Blastocystis* biology have been published in the last few decades.

History of Knowledge

The first observations of opalinids date back to 1683, when Leeuwenhoek investigated frog feces and saw numerous cells of *Cepedea dimidiata* swimming in his preparation (Dobell 1932). The genus *Opalina* was established by Purkinje and Valentin (1835). The name reflects the iridescent appearance of living cells. Opalinids were studied in detail by several investigators during the nineteenth century, most notably by Zeller (1877). A prominent author of opalinid studies during the first half of the twentieth century was Metcalf, who defined three new genera and described many new species. He concluded that opalinids were primitive ciliates and created the subclass Protociliata within Ciliata to accommodate them apart from true ciliates or Euciliata (Metcalf 1918). This approach was later abandoned by the majority of authors, who regarded opalinids as a unique group related to flagellates, but not to ciliates (e.g., Corliss 1955; Grassé 1952). A number of later studies added to the knowledge of opalinids, for example, those of Wessenberg (1961) and Kaczanowski (1971, 1973). Ultrastructural studies (Noirot-Timothée 1959; Patterson 1985; Pitelka 1956; Wessenberg 1966) provided new data and led to the current hypothesis on opalinid relationships.

The phylogenetic affinities between *Proteromonas* and *Karotomorpha* remained unrecognized for a long time. They were originally classified in distinct lineages of flagellates (bodonids vs. Polymastigidae or Tetramitidae). The first author who suggested a possible relationship between them was Grassé (1929, 1952). Further studies were conducted by Kulda (1961, 1973). An ultrastructural study by Brugerolle and Joyon (1975) further confirmed the evolutionary link between *Proteromonas* and *Karotomorpha* and, together with other works, informed the search for more distant relatives of proteromonads and thus to the discovery of their association with opalinids.

Blastocystis was described from various hosts more than a century ago by Aléxéieff (1911) under the name of *B. enterocola*, although a junior synonym, *B. hominis* (Brumpt 1912), is widely used for human isolates. For some 50 years since its description, *Blastocystis* was mostly overlooked and little studied. Its nature was not well understood – it was usually considered a harmless yeast or even a remnant/cyst of other organisms. The interest in it was reawakened mainly by Zierdt and his collaborators (e.g., Zierdt et al. 1967 and many later publications). He recognized that *Blastocystis* is not a fungus and continued to study its morphology, physiology, biochemistry, etc. His studies initiated further research on *Blastocystis* and many laboratories throughout the world study this organism today. The molecular phylogenetic study of Silberman et al. (1996) established that *Blastocystis* was a relative of slopalinids (represented by *Proteromonas* in the study).

Practical Importance

Although usually abundant in the cloacae of frogs, opalinids do not seem to cause any harm to their hosts. As quite common, yet rather enigmatic organisms, they have played, and can still play, an important role in research in the fields of cell biology, physiology, life cycle, host-symbiont interactions, (co)evolution, etc. *Proteromonas* and *Karotomorpha* may be numerous in the intestines of amphibians and reptiles but also seem to have no harmful effect; they are of no known economic importance.

Blastocystis is common in various hosts and is also one of the most frequently occurring eukaryotes found in the human intestine. Its role in pathogenesis is uncertain. *Blastocystis* is often connected with irritable bowel syndrome and other gastrointestinal symptoms. Some studies suggest a correlation between the presence of *Blastocystis* and these kinds of problems, but other studies indicate there is none (see, e.g., Clark et al. 2013; Poirier et al. 2012; Tan et al. 2010 for reviews and references). Even if there *were* such a correlation, it is still unclear whether *Blastocystis* can actually cause intestinal disorders or is just more efficient in colonization of the altered environment of unhealthy intestine. The whole issue is complicated by the fact that *Blastocystis* in human beings (and in animals, too) is genetically very variable – it is probable that some genetic lineages (subtypes) are more pathogenic than other ones. In some cases, *Blastocystis* was also associated with skin problems such as urticaria (Tan et al. 2010).

Habitats and Ecology

The vast majority of opalinids inhabit posterior parts of the intestine of frogs, but they can also be found in some other amphibians (e.g., Salamandridae, Ambystomatidae). Several species of opalinids were observed in freshwater fish, such as *Protoopalina symphysodontis* in *Symphysodon* (Foissner et al. 1979). They seem to be quite often found in Siluriformes (Sandon 1949). There are also a few marine species of *Protoopalina*: *P. saturnalis* lives in the intestine of the marine fish *Box boops* (Mignot and Molina 1988), while *P. polykineta* occurs in surgeonfish (Grim and Clements 1996) and *P. pomacantha* is found in angelfishes (Grim et al. 2000). Opalinids are occasionally seen in reptiles that presumably acquired them after ingestion of an infected frog (Delvinquier and Patterson 1993).

Because opalinids are so tightly bound to their amphibian hosts, their geographical distribution is dependent on the distribution of frogs. They are thus most diverse in tropical and subtropical regions. There are some patterns in the zoogeography of opalinids – some genera are absent or very rare in some regions: *Zelleriella* in Palaearctic, *Opalina* and *Cepedea* in Australia; conversely, *Protozelleriella* is known only from Africa (Delvinquier and Patterson 1993).

Opalinids themselves can serve as hosts to other protists, namely, amoebae of the genus *Entamoeba* (Chen and Stabler 1936; Stabler and Chen 1936; spelled "*Endamoeba*" in their works). Some metazoan parasites of frogs are predators of

opalinids: Hazard (1941) observed the trematode *Diplodiscus temperatus* feeding on opalines, possibly eliminating them from adult frogs.

Karotomorpha and *Proteromonas* also inhabit the intestines of various vertebrates, where they are usually found intermixed with other gut protists. *Karotomorpha bufonis* is common in certain amphibians, both urodelans and frogs (e.g., *Triturus* spp., *Bufo bufo*). *Proteromonas lacertaeviridis* is a commensal of a wide range of reptiles – not only European lizards of the genus *Lacerta* but also many other lizards, snakes, or even tortoises. Several other species of *Proteromonas*, some (or all) of which might be synonymous to *P. lacertaeviridis*, were described from various reptiles. Urodelan amphibians, for example, *Salamandra salamandra*, may harbor *Proteromonas longifilla*, and other species can be found in the caecum of some rodents, for example, *P. brevifilia* in guinea pigs. Interestingly, Maia et al. (2012) found *Proteromonas* in a few blood and tail tissue samples from reptiles.

Besides being frequently reported from humans, *Blastocystis* can be found in a vast number of hosts including insects (Zaman et al. 1993), amphibians (Yoshikawa et al. 2004), reptiles (Teow et al. 1992), birds, and many nonhuman mammals (summarized in Stensvold et al. 2009; see also Parkar et al. 2010 and Alfellani et al. 2013). It is rather unclear, however, how many *Blastocystis* species there actually are and what their host specificity is. Despite the relatively uniform appearance of vacuolar forms of *Blastocystis* strains isolated from different (or the same) host species, genetic markers (usually SSU rRNA gene sequences) suggest there are multiple lineages that are molecularly divergent and probably ancient. The initial recognition of this hidden diversity led to nomenclatural confusion that made the problem even more difficult. A consensus proposed by Stensvold et al. (2007) recognizes the lineages as subtypes and uses numbers to distinguish between them. Currently, there are 17 subtypes defined (Alfellani et al. 2013), but the number may grow. The host specificity and zoonotic potential of the subtypes is still little known, but an overall picture is slowly emerging as more hosts are screened. Some subtypes are probably more generalist, while others display at least some host specificity. There are possibly human-specific subtypes, as well as examples of human infections accidentally acquired from bird or mammalian hosts (Clark et al. 2013). A number of isolates from poikilotherm vertebrates and invertebrates do not belong to any subtype and form their own lineages.

Characterization and Recognition

Opalinidae

Identification of opalinids is usually not difficult. First, the host is significant. Their host is most often a frog and they are located in the posterior part of the digestive tract. Opalinid cells are medium sized to large and covered with multiple flagella that beat in metachronal rhythms. Metachronal waves of flagellar activity are initiated in the anterior region of living cells and can be seen traveling to the posterior end. The cells are opalescent in reflected light. Two or many nuclei are visible within the cells.

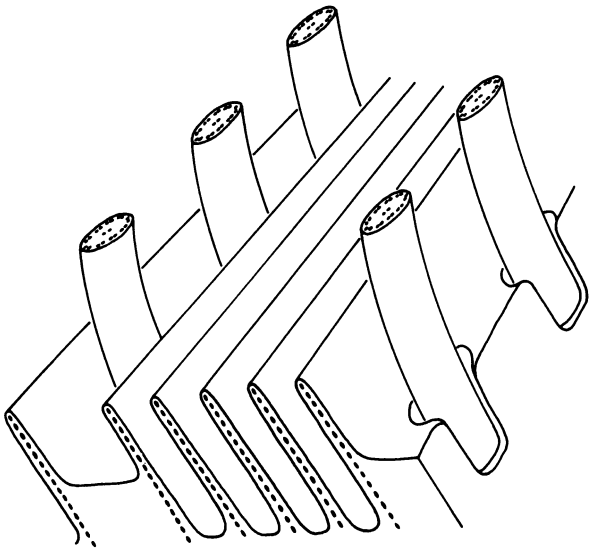

Fig. 4 Schematic representation of surface structure of an opalinid cell. A few flagella in two kineties and the folds between them are shown. The folds are supported by ribbons of microtubules (*dots*) (Figure from Corliss (1989))

Unlike ciliates, which they superficially resemble, opalinids lack any oral structures and their nuclei are not differentiated in micro- and macronuclei.

Morphology and Ultrastructure. The most studied genus of opalinids is *Opalina*. Its cells are characterized as flattened, flexible, elliptical to elongated and with multiple nuclei (Fig. 1a). The biggest specimens can be more than one millimeter long. The cell surface is organized in a complex manner. The flagella (cilia) are arranged in oblique rows (kineties) that run in parallel from the anterior to the posterior end, spiraling around the cell. Kineties arise at an important morphogenetic center, the falx. The falx is a structure composed of several rows of kinetosomes bearing flagella and is located along the anterior end. The falx plays a role in the initiation of flagellar beating. Between the neighboring kineties, the pellicle is heavily folded in several ridges that are parallel with the kineties and supported by ribbons of interconnected microtubules (Fig. 4). The folds themselves are also interconnected by external linkages that stabilize the cortex architecture and ensure regular spacing of cortical ridges (Wooley 2006).

The flagella have the usual $9 \times 2 + 2$ axoneme structure. The detailed ultrastructure of the transitional zone between kinetosomes and axonemes (Fig. 5) is of phylogenetic importance as it is very similar in *Karotomorpha* and *Proteromonas* (Patterson 1985). The bases of flagella are cupped by a membranous pocket. Neighboring kinetosomes within a kinety are connected by an electron-dense connective ("desmos").

The kineties are underlain by bands of microfilaments that are interconnected by additional perpendicular bands arising in regular intervals. Numerous vesicles are located between these lateral microfilament bands, just under the bottom level of the pellicular folds. Interestingly, rows of two vesicular types, spherical and flattened, alternate regularly at this level (Wessenberg 1978). The two types are randomly

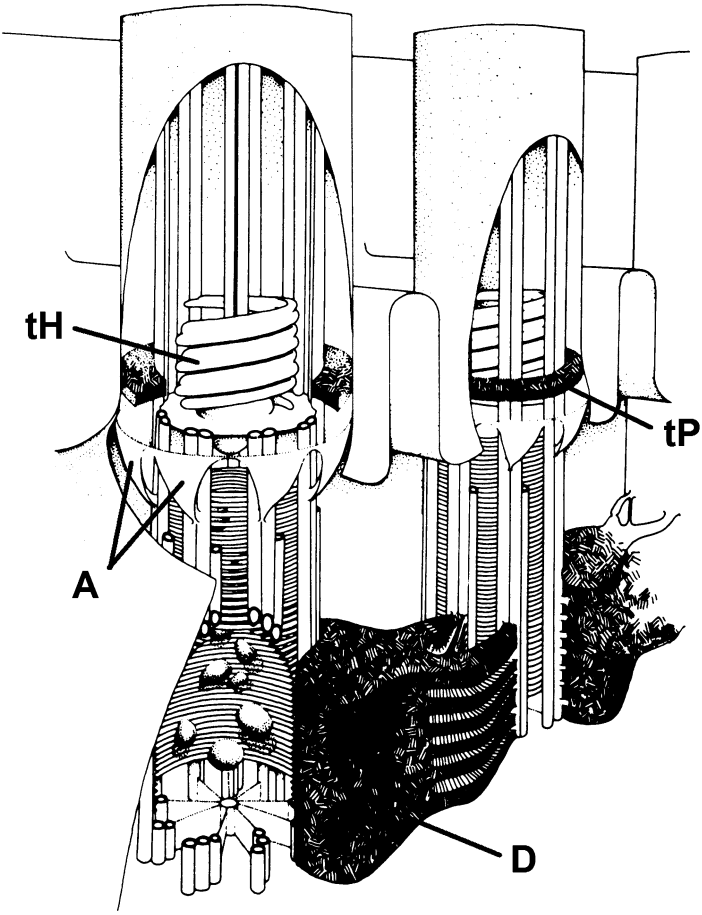

Fig. 5 Schematic drawing of flagellum ultrastructure of opalinids. The flagellar transitional region contains several conspicuous features: double transitional helix (*tH*), transitional plate (*tP*), and nine curved arms (*A*). The advanced basal bodies are interconnected by the desmos (*D*) (Figure from Corliss (1989))

intermixed a little deeper in the cell. The spherical vesicles are coated and are formed at the bottom of the folds via pinocytosis. The flattened vesicles are believed to be exocytic and to compensate for the membrane demand of endocytosis (Delvinquier and Patterson 1993; Grim and Clements 1996). Endocytic vesicles fuse a bit further into the center of the cell to form larger digestive vacuoles (up to 4 μm in diameter).

Golgi complexes occur among the vacuoles, with their concave (trans) face oriented to the surface. In the central part of the cell are numerous ribosomes, mitochondria (formerly known as "Zeller bodies"), and nuclei. The mitochondrial cristae are tubular, and the mitochondria are often accompanied by lipid droplets.

Fig. 6 Microphotographs of two protargol-stained opalinids. *Cepedea* sp. (**a**) was isolated from *Kassina senegalensis*. Note multiple nuclei and kineties originating in the anterior (upper) part of the cell. *Protoopalina intestinalis* (**b**) was isolated from *Bombina bombina*. Its two nuclei are much larger than in *Cepedea* and are visibly connected. Some flagella are faintly apparent around the cells. The scale bar (10 μm) applies to both cells. The preparations were stained by Ivan Čepička

The nuclei of *Opalina* are flattened and 5–7 μm in diameter. Prominent masses of nucleoli are apparent after staining.

The ultrastructure of other opalinid genera does not differ substantially from that of *Opalina*. The falx of the cylindrical genera (*Cepedea* and *Protoopalina*) is parallel rather than perpendicular to the axis of the cell and is shorter. The number of kineties, which twist helically around the cell, is thus lower. The nuclei of the binucleated genera can be much bigger than those of *Opalina* and *Cepedea*: up to 40 μm in *Protoopalina* (cf. Fig. 6a, b). *Protoopalina* and *Cepedea* have a complex, branched network of microfibrillar bundles within the cell (Mignot and Affa'a 1995). Grim and Clemens (1996) report abundant bacterial endocytobionts in *P. polykineta*. *Protozelleriella* is a morphologically unique opalinid. It has a broad, *Zelleriella*-like cell with a thin anterior falx. However, the kineties originating on the falx are very short, forming a central fan-like array surrounded by a hyalinous margin devoid of flagella (Delvinquier et al. 1991b).

Mitosis, Cell Division, and Life Cycle. The nuclear division, chromosomes, and ploidy in *Zelleriella* were extensively studied by Chen (1936a, b, 1948). The nuclear membrane remains intact during mitosis; the mitotic spindle is formed within the nucleus. After the chromosomes are separated near the poles of the dividing nucleus, its central area is constricted and elongated, but the two daughter nuclei can remain

joined by a narrow link for a long time. In *Zelleriella*, cytokinesis often precedes karyokinesis, leading to mononucleated daughter cells whose nuclei will proceed to telophase. Mitosis in other genera is similar, nuclei of multinucleated opalinids divide asynchronously, and mitosis is not strictly dependent on cytokinesis, although the number of nuclei is increased prior to cell division.

Binary fission in *Opalina* remains controversial in some respects: it is unclear whether transverse division (i.e., that cleaves the cell perpendicularly to its longitudinal axis) is a mechanism of actual propagation. Wessenberg (1961, 1978) was one of those who proposed this. The kineties are interrupted in transverse division, however, and for division to be successful, the posterior daughter cell would have to regenerate its falx de novo, which some other authors find dubious (e.g., Delvinquiér and Patterson 1993). Longitudinal division, on the other hand, is common. It is preceded by falx elongation, the falx is then bisected by a cleavage notch, and the cell is divided from the anterior to the posterior end along the kineties, which untwist during the process.

The life cycle of opalinids (Fig. 7) was studied notably by Wessenberg (1961). It is quite complex and is synchronized with the life cycle of the frog host. The best known example is the life cycle of *Opalina*: for the most of the year, the trophonts described above are the only stage found in the rectum of frogs. As the breeding season of frogs draws near, the opalines start to divide without growth (palintomy) producing tomonts and finally small tear-like individuals (progamonts) with a few nuclei. These stages round up and encyst. The cyst are spherical and 20–45 μm in diameter and contain several (most often 4–8) nuclei. They are released with feces into the water, where they remain viable for approximately 3 weeks. Young tadpoles feeding on detritus ingest the cysts. After the excystation in the digestive tract of a tadpole, the released stages – gamonts – divide further to produce unicellular gametes. Meiosis occurs during this process (Kaczanowski 1971). Opalines are anisogamous, producing macro- and microgametes. Both types are slender cells, approximately 40 μm long, with 8–10 kineties. Microgametes are much thinner and a bit shorter than the macrogametes and have a narrow "tail" which may lack cilia and seems to be sticky. They often swim with this part pointed anteriorly to attach themselves to macrogametes. It is not known which point of the life cycle is the stage where the sex of the gametes is determined. After syngamy, zygocysts are formed. These leave the tadpole in its feces and infect other tadpoles feeding on detritus. Upon ingestion and excystation, the sexual processes can repeat. Only in older tadpoles nearing metamorphosis do the excysted stages cease to produce new gametes and instead grow while their nuclei divide without cytokinesis. The resulting cells are "protrophonts" with an axial row of several nuclei. As they grow further, they become wider and flatter and change into trophonts. Some of these early trophonts may switch to palintomy again and produce some new cysts. They have a last chance to infect new hosts, which at this time are becoming young frogs and are already leaving the water. The perfect synchronization of the life cycle of opalinids and their hosts is believed to be achieved in part by an ability of opalinids to properly react to hormonal changes in frogs during the breeding season (El Mofty and Smyth 1964).

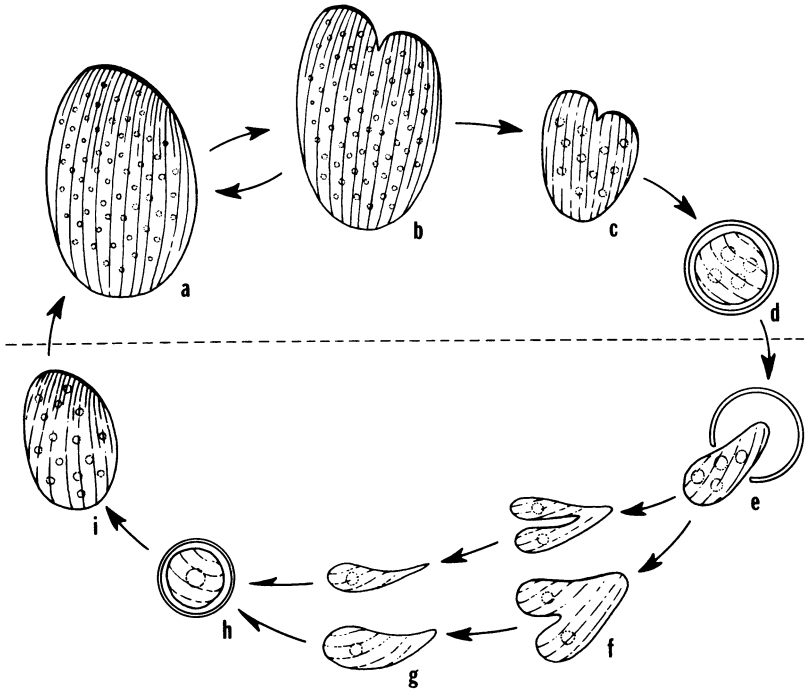

Fig. 7 Diagrammatic and abbreviated life cycle of *Opalina*. Rounded trophonts (**a**) reproduce by longitudinal division (**b**). During the rearing season of the host, they undergo palintomy (**c**) and infective cysts (**d**) are finally released. After excystation (**e**), the gamonts (**f**) divide further, and eventually uninucleate gametes are produced (**g**). They fuse to form a zygote that encysts as a zygocyst (**h**). Excysted stages metamorphose into young trophonts (**i**). Stages **a**–**d** are found in adult frogs, with the infective cysts (**d**) passing out into the water with fecal material. Stages **e**–**i** are found in tadpoles; zygocysts (**h**) are again released into water to be reingested (Figure from Corliss (1989))

Proteromonas and *Karotomorpha*

Assuming that one is studying the intestinal contents of an appropriate host, both *Proteromonas* and *Karotomorpha* are best recognized by their slender cells and agile movement. Their cells typically measure about 15–20 × 5 μm. After Giemsa staining, the number and arrangement of flagella can also help to distinguish them from other flagellates. Fine striations may be visible on stained *Karotomorpha* cells (Fig. 2b).

Morphology and Ultrastructure. Proteromonas (Figs. 2a and 8a) has two apical flagella of different lengths. The longer one (about 40–50 μm) points forward during swimming and is thickened – its axoneme (of the typical eukaryotic structure) is accompanied by a striated fiber attached to one of the microtubular doublets and by additional microfibrils. The second, recurrent flagellum is about 30 μm long. Membranes at the bases of the two flagella are in close contact, forming a gap junction. The two kinetosomes are perpendicular and are interconnected by a short striated

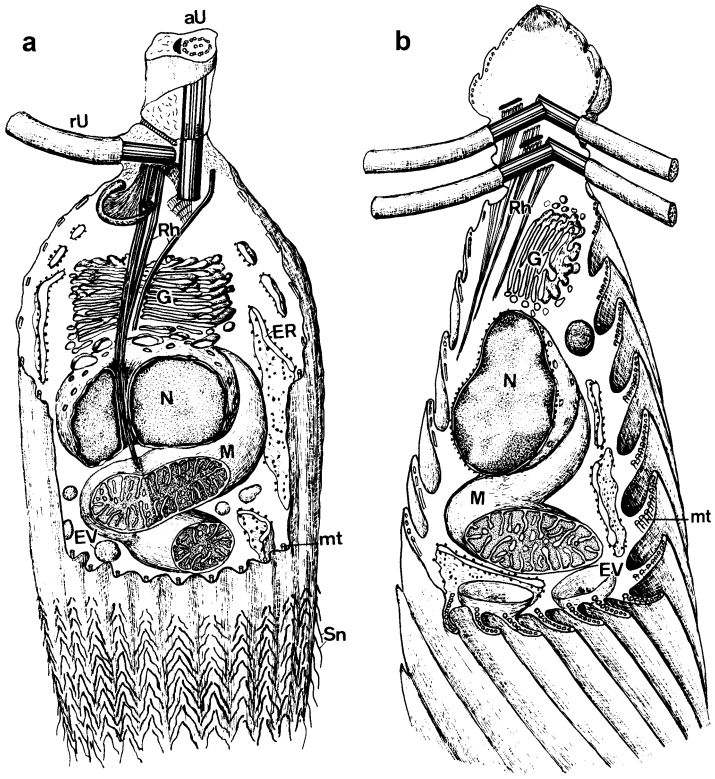

Fig. 8 Diagrammatic reconstruction of the ultrastructure of *Proteromonas* and *Karotomorpha*. *Proteomonas* (**a**) has two flagella; the anterior one (*aU*) is thicker than the posterior one (*rU*) because its axoneme is accompanied by a fiber and fibrils. Between the two flagella, the cell membrane forms a gap junction. The rhizoplast (*Rh*) is a cytoskeletal structure connecting the kinetosomes with the nucleus (*N*) and mitochondrion (*M*). The rhizoplast runs through the Golgi complex (*G*). The surface of the cell is highly folded, with the ridges supported by single microtubules (*mt*). In the posterior part, the cell is covered with fine hairs – somatonemes (*Sn*). Endoplasmic reticulum (*ER*) and endocytic vacuoles (*EV*) are also present in the cells. *Karotomorpha* (**b**) differs mainly in having four laterally pointing flagella and deeper folds supported by ribbons of microtubules (*mt*). Its rhizoplast (*Rh*) does not reach the mitochondrion (*M*) and does not run through the Golgi (*G*). *Karotomorpha* lacks somatonemes. The cell contains nucleus (*N*), reticulum, and endocytic vacuoles (*EV*) (Figure from Brugerolle and Mignot (1989))

fibril. Two additional bundles of microtubules attach to the kinetosome of the posterior flagellum and a dense fiber to the other one (Brugerolle and Joyon 1975). Together, they form the rhizoplast, a structure that runs in the posterior direction through the Golgi complex, passes the nucleus (making a groove in its surface), and ends on the mitochondrion. In *Karotomorpha* (Figs. 2b and 8b), its four flagella point laterally out of the cell and all beat anteroposteriorly. They are arranged in two pairs – each pair is homologous to the two flagella of *Proteromonas*. Kinetosomes of each flagellar pair are again perpendicular and lie in a plane

perpendicular to the cell axis. One kinetosome of each pair (the one homologous to the kinetosome of the posterior flagellum of *Proteromonas*) is associated with the two microtubular bands of the rhizoplast. The rhizoplast of *Karotomorpha* is shorter and does not reach the mitochondrion – it ends near the cell surface at the level of the nucleus. Several delicate features of phylogenetic significance can be found within the transitional region of the flagella of both genera. They include double transitional helix or transitional disk between the peripheral microtubules and the cell membrane, both of which are also present in the proximal region of opalinid flagella.

The cell surface is formed into about 30 shallow ridges in *Proteromonas* and about 20–25 deeper folds in *Karotomorpha* that twist helically around the cell (Brugerolle and Joyon 1975). These surface structures are supported by a cortical cytoskeleton (Fig. 8) in the form of single microtubules, each associated with a microfibril (*Proteromonas*) or ribbons of about ten interconnected microtubules (*Karotomorpha*). In the posterior two thirds of the *Proteromonas* cell, the cortical ridges bear somatonemes that cover the cell surface. Somatonemes are tripartite fine hairs consisting of a bent base, a tubular rod, and a terminal filament. Pairs of somatonemes are anchored to the cortical microtubules at regular intervals. The somatonemes have a similar structure to the mastigonemes associated with the anterior flagellum of typical stramenopile flagellates; the two structures are apparently homologous. Cavalier-Smith (1998) suggests that somatonemes protect the cell surface from larger particles which could either directly damage it or block pinocytosis. The much deeper folds of *Karotomorpha* would then serve a similar role and compensate for the loss of somatonemes.

The anterior region of *Proteromonas* and *Karotomorpha* cells contains three important organelles: the Golgi apparatus, nucleus, and a single mitochondrion. Endoplasmic reticulum is also concentrated here. Cisternae of the Golgi apparatus of *Proteromonas* are ring shaped, since the rhizoplast passes through them. The Golgi plays an important role in the assembly and transport of somatonemes, which are synthesized in cisternae of endoplasmic reticulum, then are transferred to the Golgi, and finally migrate to the cell surface (Brugerolle and Bardele 1988; Brugerolle and Joyon 1975). The nucleus is oval and has a nucleolus and peripheral chromatin. The mitochondrion lies posterior or posterolateral to the nucleus. It is roughly the same size as the nucleus and is surrounded by glycogen particles. The mitochondrial cristae are tubular. The cytoplasm may contain symbiotic bacteria, often near the mitochondrion (Brugerolle and Joyon 1975). The flagellates of both genera feed by pinocytosis; pinocytic vesicles are formed in the posterior part of the cell, among the rows of somatonemes or at the bottom of the cortical folds. Inside the cell, the vesicles fuse to form larger digestive vacuoles.

Cell Division and Life Cycle. The trophozoites of *Proteromonas* and *Karotomorpha* divide longitudinally. Cell division is better understood in *Proteromonas* (Grassé 1926, 1952). It begins with duplication of the kinetosomes and flagella; the rhizoplast is also doubled. The two pairs of kinetosomes then migrate away from each other. Meanwhile, division of the nucleus begins: chromosomes become visible, the nucleus extends perpendicularly to the cell axis, and the spindle forms within it. The membrane of the nucleus remains intact during mitosis.

Rhizoplasts attach to both poles of the dividing nucleus; after karyokinesis, the two daughter nuclei remain associated with the rhizoplasts and through them also with the respective kinetosome pair. The mitochondrion divides during the telophase. Cytokinesis continues from the anterior to the posterior end of the cell. The life cycles of *Proteromonas* and *Karotomorpha* include cysts, which permit transmission from one host to another. The cysts are spherical, have a distinct cyst wall, and contain a single nucleus and a single mitochondrion surrounded with abundant glycogen granules. The rhizoplast is retained near the nucleus. Neither subpellicular microtubules nor flagella (or kinetosomes) were observed in cysts, however, the cysts may contain bacteria (Brugerolle and Joyon 1975).

Blastocystis

Unlike other Opalinata, *Blastocystis* is easily overlooked in fecal samples and may be confused with other objects. It does not move and may be of variable size and morphology. Therefore, molecular methods or cultivation might be preferred in routine diagnostics. The most commonly observed (and most easily diagnosed) form is the vacuolar form (see below).

Morphology and Different Forms. The described variability of sizes and shapes of *Blastocystis* cells is somewhat confusing. It is important to bear in mind the unusual genetic variability among *Blastocystis* isolates, which may account for differences among reports. Moreover, some observed forms may represent culture artifacts or degrading cells (Vdovenko 2010). A single *Blastocystis* strain can alternate between several forms. The best known of them is the vacuolar form (Fig. 3). Cells of this morphotype are spherical and usually have a diameter of several to about 15 μm, although much larger cells (up to hundreds of micrometers in diameter) were also observed (Zierd 1991). Their central vacuole occupies the majority of the cell volume. The vacuole most probably serves a storage function. It is surrounded by a layer of cytoplasm with one or more nuclei, mitochondria, Golgi complexes, and other typical eukaryotic organelles (Stenzel and Boreham 1996). The surface of vacuolar form cells is often covered with a fibrillar layer (surface coat), especially in freshly isolated cells. Although several mechanisms of multiplication were described, binary fission seems to be the predominant (if not the only) reproductive process (Tan 2008). Under certain conditions, granular forms may appear in cultures: these are similar to the vacuolar forms, but contain granules in the vacuole and/or cytoplasm (Dunn et al. 1989). Rarely, other forms were also reported, often from fresh stool samples: avacuolar and multivacuolar forms, with no or multiple vacuoles, and the amoeboid form (Stenzel and Boreham 1996). The latter also appears if *Blastocystis* is cultured on solid agar. Amoeboid cells are irregular in shape, seemingly nonmotile (although producing pseudopodia-like appendages), may or may not contain the central vacuole, and may differ in their ultrastructure (cf. Dunn et al. 1989; Tan and Suresh 2006). *Blastocystis* infects new hosts via small (up to about 5 μm) spherical-to-ovoid cysts (Stenzel and Boreham 1991; Tan 2008; Zaman et al. 1995).

Genomic Data. The growing interest in *Blastocystis* has resulted in the sequencing of several genomes, both mitochondrial (Pérez-Brocal and Clark 2008; Wawrzyniak et al. 2008) and nuclear (Denoeud et al. 2011). Both genomes are relatively small and show considerable reduction in gene number compared to other stramenopiles (Clark et al. 2013). The mitochondrial genome of *Proteromonas* has also been sequenced (Pérez-Brocal et al. 2010). Although its gene content is very similar to that of the *Blastocystis* mitochondrial genome, the two genomes differ strikingly in structure: it is circular in *Blastocystis*, but linear in *Proteromonas*. Because these organisms are anaerobes and the metabolism of their mitochondria is highly modified (Stechman et al. 2008), the mitochondria are often called "mitochondrion-like organelles."

Maintenance and Cultivation

Opalinids can be easily retrieved from the frog intestine in large numbers. They can survive for up to several weeks in various media based on buffered saline solutions that are commonly used for the culturing of intestinal protists (see Delvinquier and Patterson 1993). It seems, however, that long-term cultures of opalinids are quite hard to establish and maintain (e.g., Wessenberg 1978), although several reports of successful cultivation exist. Lwoff and Valentini (1948) established a bacteria-free culture of *Cepedea* in a complex medium containing (among other ingredients) boiled frog liver and autoclaved frog rectal content. Interestingly, during late spring, cysts appeared in their cultures. After the cultures were contaminated by Gram-negative cocci, the opalinids grew better. Cultivation was also achieved by Yang and Bamberger (1953) and Yang (1960), who initially used egg slants overlaid with buffered saline (pH 7.8) supplemented with inactivated serum and antibiotics and later substituted the slants with liver concentrate.

Kulda (1973) established an axenic culture of *Proteromonas lacertaeviridis* at room temperature on Diamond's TYM medium (Diamond 1957) supplemented with inactivated horse serum and a trace of agar. Before reaching the axenic state, proteromonads were for some time (several weeks) cocultivated with a yeast (*Candida* sp.) from the lizard host. They were later separated from the yeasts by repeated migration. Bacteria were eliminated from the primary culture with the use of antibiotics. For short-term purposes, both *Proteromonas* and *Karotomorpha* can survive several hours or days in various saline solutions commonly used for isolation of intestinal flagellates, for example, Ringer's frog solution: NaCl 6.5 g, KC1 0.14 g, $CaCl_2$ 0.12 g, and $NaHCO_3$ 0.20 g in 1000 ml H_2O (Brugerolle and Mignot 1989).

Blastocystis grows well xenically in various media and may be axenized (Tan 2008). For xenic cultures, Jones' medium (Jones 1946) is often used. Axenized strains may be cultured in commercially available Iscove's modified Dulbecco's medium + horse serum (Clark and Diamond 2002). The ability of *Blastocystis* to form colonies on agar plates may be exploited during axenization and cloning (Tan et al. 2000).

Evolutionary History

There is no direct (i.e., fossil) evidence of the evolutionary history of Opalinata. The close link of opalinids to frogs (and also the geographical distribution of opalinid genera) suggests that their main radiation dates back to the Mesozoic. Hypotheses on the evolution within the group are based on morphological data: the number of nuclei (two/many) and cell shape (flat/cylindrical). Because all four possible combinations of the character states are found among opalinids, every evolutionary scenario requires convergence and/or reversal of some of these characters. Generally, the binucleated state is considered primitive and the multinucleated genera are believed to form a monophyletic derived group (Opalininae). Relationships among the three binucleated genera and Opalininae are unclear, but it has been suggested that *Protozelleriella* might be the most primitive representative of Opalinidae, indicating that the cells of *Zelleriella* are primitively flattened. The monophyly of Opalininae is supported by their geographical distribution and several ultrastructural features (Patterson and Delvinquier 1990), as well as by molecular data (Nishi et al. 2005). However, both electron microscopy and PCR-based sequencing were applied to a very limited number of species (and none belonging to *Protozelleriella*!).

Relationships of opalinids to other protists were mysterious for a long time. Their superficial resemblance to ciliates had led nineteenth-century protistologists to place opalinids in this group, although some criticism of this concept appeared early on. Phylogenetic affinities of opalinids were discussed in detail by Metcalf (1918), who erected a new subphylum Protociliata to accommodate opalinids separately from "other," true ciliates. This arrangement satisfied many authors as it reflected both the morphological uniqueness of opalinids and the possibility that they formed a phylogenetic connection between flagellates and ciliates: the presence of two (or many) nuclei, cilia arranged in kineties, and sexual processes in the life cycle of opalinids were long perceived as features that one would expect in a hypothetical ancestor of ciliates (Wessenberg 1978). The debate – whether or not opalinids represent an intermediate stage between flagellates and ciliates – went on for several decades, with the majority of authors deserting the idea of a close affinity between ciliates and opalinids. They thus remained orphaned as an isolated taxon among flagellates (Corliss 1955; Grassé 1952), which is, more or less to say, an isolated taxon among eukaryotes.

The situation changed when substantial electron microscopy data emerged. A key study was that of Patterson (1985), who highlighted that there are several ultrastructural characteristics shared by opalinids, *Proteromonas* and *Karotomorpha*, as already noticed by Brugerolle and Joyon (1975). The arrangement of kinetosomes and associated structures is very similar and another synapomorphy is the folded cell surface, with the folds supported by single microtubules (*Proteromonas*) or microtubular ribbons (*Karotomorpha* and Opalinidae). These and other similarities led Patterson to postulate a close relationship between opalinids and proteromonads and to establish a new order Slopalinida comprising the two groups. The paraphyly of proteromonads was also recognized;

Karotomorpha, with its pellicular folds supported by ribbons of microtubules and more flagella, is more closely related to opalinids than to *Proteromonas*. Some of the details of the kinetosome ultrastructure link slopalinids to other stramenopiles, as do other features of *Proteromonas* in particular (e.g., somatonemes). The molecular phylogenetic study of Silberman et al. (1996) not only supported the monophyly of stramenopiles and the placement of slopalinids among them but also revealed an unexpected relationship between slopalinids and *Blastocystis*. The close relationship between *Proteromonas*, *Karotomorpha*, and Opalinidae was later confirmed by molecular studies based on SSU rDNA (Kostka et al. 2004, 2007, Nishi et al. 2005). Interestingly, the study of Nishi et al. (2005) did not refute a possible link between opalinids and ciliates: phylogenetic analyses of tubulin genes tend to connect the two groups, although probably artefactually. Opalinata relationships are further discussed by Cavalier-Smith (1997, 1998) and Cavalier-Smith and Chao (2006). In these works, *Karotomorpha* and opalinids were treated together in the group Opalinea to the exclusion of *Proteromonas*. In later papers, *Proteromonas* was included (Cavalier-Smith and Scoble 2013; Ruggiero et al. 2015) – the expanded Opalinea group has then exactly the same composition as Patterson's Slopalinida; the group containing only *Karotomorpha* and opalinids was called Opalinida therein.

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Apicomplexa

16

Jan Votýpka, David Modrý, Miroslav Oborník, Jan Šlapeta, and Julius Lukeš

Abstract

The phylum Apicomplexa is a large group of parasitic protists with more than 6,000 described and possibly thousands of undescribed species. All species are obligatory parasites, and potentially every vertebrate and majority of invertebrates host at least one apicomplexan species. More frequently apicomplexans are specialists with rather high host specificity; nevertheless, generalists with low host specificity exist. Many species are highly pathogenic to their host including human and domestic animals and from medical perspective represent the most important eukaryotic parasites. Coccidians are omnipresent in vertebrates, e.g., virtually all poultry and rabbits are infected by several host-specific *Eimeria* spp.;

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theileriosis is responsible for enormous losses in cattle farming; about 20% of global human population is infected by *Toxoplasma gondii*; and, finally, *Plasmodium falciparum* and other *Plasmodium* species cause globally distributed malaria, which kills millions of people in tropical countries.

The phylum Apicomplexa includes morphologically and ecologically diverse protists, such as the gregarines, cryptosporidia, coccidia, haemosporidia, and piroplasms. The life cycle of majority of Apicomplexa involves sexual and asexual multiplication in the parasitized host and an environmentally resilient cyst forms. Transmission strategies are diverse, from direct transmission to intricate cycles in trophic webs between predators and their prey or involving arthropod vectors.

The phylum is highly successful, thanks to morphological and molecular adaptations. The name is derived from two Latin words, *apex* (top) and *complexus* (infolds), and refers to a set of organelles composed from spirally arranged microtubules, polar ring(s), and secretory bodies, such as rhoptries and micronemes. Apical complex structures mediate entry of the parasite into the host cells, where they usually survive inside a parasitophorous vacuole. Most apicomplexans possess a unique organelle called the apicoplast, which is a highly reduced non-photosynthetic plastid, which retains few functions essential for a parasite survival. The phylum evolved from a photosynthetic flagellate, and core apicomplexans form a sister group to a free-living marine and freshwater protists (*Chromera*, *Vitrella*, and *Colpodella*).

Keywords

Alveolata • Apicoplast • Endosymbiosis • Intracellular • Micronemes • Pathogens • Parasites • Protozoa • Rhoptries

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Introduction

General Characteristics

The Apicomplexa (Telosporea, Sporozoa) are parasitic.[1] heterotrophic protists that form uniformly banana-shaped uninucleate stages. Apicomplexans move by gliding motion, and at least one stage is characterized by apical secretory organelles releasing their content through a microtubule-anchored ring. The rod-shaped micronemes and bulk-shaped rhoptries are both essential components typifying the phylum Apicomplexa. A majority of species have complex parasitic life cycles, with alternating asexual and sexual multiplication. They either possess a respiring mitochondrion or a non-respiring mitosome. Apicomplexa contain a multimembranous compartment, now known to be a modified chloroplast, termed apicoplast, acquired via endosymbiosis of a photosynthetic alga. The apicoplast is neither photosynthetically active nor present in all extant members of the phylum; however, if present, it is an indispensable organelle.

The core of Apicomplexa is traditionally divided into three major obligatory parasitic classes (hematozoa, coccidia, and *gregarines*). The sister group of the core apicomplexans has been widely debated over the past 30 years. Colpodellids, free-living predatory protists of previously uncertain status, are now considered a sister group to the monophyletic hematozoans, coccidians, and gregarines.

[1]Parasitism is a type of symbiotic relationship between two different organisms – parasite and host. Three distinct types of parasitism are considered: biotroph, hemibiotroph, and necrotroph. Apicomplexans should be classified as biotrophs and partially as hemibiotrophs. Necrotrophs utilize dead animal tissues as a source of nutrients, while apicomplexans benefit from a prolonged, close association with the living host cells only.

Parasitic apicomplexans have a bad reputation for causing malaria, toxoplasmosis, coccidiosis, and other serious diseases of humans and animals. Recently discovered photosynthetic alveolates *Chromera velia* and *Vitrella brassicaformis* are together with heterotrophic colpodellids closely related to the core apicomplexans.

History of Knowledge

The symptoms of malaria were first described more than 5,000 years ago in Egyptian papyri, and this plague appears in historical records of Greeks and Romans (Cox 2010). Yet the first apicomplexan was spotted by Antony van Leeuwenhoek, who in 1674 observed under his famous microscope coccidian oocysts in the bile of a rabbit. However, thanks to their size, gregarines were the first apicomplexans to attract interest of early protozoologists. The genus *Gregarina* from an insect was described by Leon Dufour in 1828, and they already appeared as a protistan group in the classification of Ernst Haeckel in 1866.

Meanwhile, research on malaria, one of the major scourges of humankind, gathered momentum. In 1880, Charles Laveran became convinced that the pigment in erythrocytes of his patients is a parasite. Within the same decade, Alexander Danilewski discovered several other intracellular parasites in the blood of vertebrates and called them haemosporidians, while Ilya Metchnikow recognized their relationship with coccidians. It was, however, not firmly established until 1897, when Ronald Ross proved that the parasites causing malaria are transmitted by mosquitoes, for which he was awarded the Nobel Prize in 1902. A major contribution to the understanding of the malarial life cycle came also from Giovanni Battista Grassi ("there is no malaria without *Anopheles*"). One more Nobel Prize for research on malaria went to Julius von Wagner-Jauregg, who in 1917 discovered that syphilitic patients can be treated by controlled malaria infection. William Trager is credited for seminal discoveries, such as continuous cultivation of the erythrocytic stages. The last Nobel Prize for research on malaria went in 2015 to Youyou Tu for her discoveries concerning a novel therapy.

The first piroplasmid was described from the blood of cattle during the 1880s epidemic of the Texas cattle fever in the USA. Intracellular blood stages, later included in the genus *Babesia*, were described in 1888 by Victor Babeş. Only a few years later, Theobald Smith and Frank Kilbourne successfully transmitted a related organism to a noninfected cattle via a tick, being first to show that invertebrates can serve as vectors of a parasitic disease. Another Nobel Prize was awarded in 1951 to Max Theiler for his breakthrough studies of the life cycle of *Theileria*. The most widespread apicomplexan, *Toxoplasma gondii*, was first observed in 1908 by Charles Nicolle in a semidesert rodent, the common gundi (*Ctenodactylus gundi*), which was being used for leishmaniasis research in the laboratory of the Pasteur Institute in Tunis (Dubey 2014). Yet it took most of the twentieth century to decipher its intriguing life cycle, because the cat was successfully identified as the host shedding the oocysts only in 1970.

Since their description, the systematics of the apicomplexans had undergone periodical changes. Early influential reviews were published more than a hundred years ago by Labbé (in 1899) and Minchin (in 1903), later followed by the system proposed by Wenyon (in 1926). The pre-electron microscopy era was summarized by Pierre-Paul Grassé (in 1953). The intense studies by electron microscopy in the 1960s and 1970s that resulted in the identification of common ultrastructural features at the apical end prompted Norman Levine to propose the name Apicomplexa for these protists.

Mutual relationships of major groups within the phylum were differently assessed by influential authors, such as Emile Vivier and Isabelle Desportes (in 1980). An exhaustive list of all named species was compiled by Norman Levine (1988), and a recent account of the classification of the parasitic Apicomplexa is reviewed by Frank Perkins et al. (2000). Avian blood parasites have been reviewed exhaustively by Gediminas Valkiūnas (2004).

Practical Importance

Apicomplexans represent an obligatory parasitic lineage with an enormous diversity and more than 6,000 named species infecting invertebrates and mostly vertebrates. Even though under natural conditions most parasitoses are asymptomatic, some Apicomplexa are causative agents of serious human and animal diseases. With no doubt, the main importance rests in the pathogenic character of the species described below (see also Seeber and Steinfelder 2016).

Although the majority of haemosporidians are parasites of wild animals (reptiles, birds, and mammals) exerting only a negligible effect on their hosts, some are responsible for very serious, even fatal diseases. Most notorious are several species of *Plasmodium*, the causative agents of malaria, responsible for enormous human suffering and economic loss in most tropical countries. Human malaria used to be widespread also in the temperate zone, from where it was successfully eradicated after the Second World War. *Plasmodium* is considered one of the most frequent agents of deaths in the history of humankind, even now killing about half a million people annually, particularly children in sub-Saharan Africa (Gething et al. 2011). For good reasons, it is one of the most well-studied protists, yet only a few effective drugs and no fully protective and effective vaccine against human malaria are available.

The east coast fever (theileriosis) and bovine tropical theileriosis in cattle and water buffaloes are caused by *Theileria parva* and *T. annulata*, respectively (Bishop et al. 2004). Several *Babesia* species are responsible for babesiosis of cattle, horses, dogs, and rarely also humans. Poor growth, low milk production, and mortality of infected animals resulted in several efforts to control piroplasmoses. Before implementation of successful eradication programs focused on vectors, the costs of the piroplasmosis were estimated at more than 100 million dollars in direct and indirect annual losses in the USA only. While under control in developed countries, these

diseases still cause serious economic loss in tropical and subtropical countries. Since the eradication of tick vectors is not realistic in most tropical countries, there is a demand for effective control of piroplasmoses by alternative approaches. Vaccines using live attenuated *Babesia bovis* and *B. bigemina* are commercially available and millions of doses of the combined vaccine have been used in the New World and Australia (Jackson et al. 2001). The development of live vaccines against bovine babesiosis was prompted by early observations indicating that cows that recovered from natural *Babesia* spp. infections developed long-lasting immunity. However, vaccines using live *Theileria* parasites, soluble antigen from *Babesia* species (e.g., the vaccine for canine babesiosis was marketed in parts of Europe), or vaccines composed of subunits are being developed or have even reached the stage of clinical trials but have yet to be tested on a large scale.

Toxoplasma gondii, causing toxoplasmosis, is the most widespread protozoan parasite capable of infecting virtually every mammalian (and bird) host species including man, with 15–70% of the human population seropositive (Tenter et al. 2000). Most infections in humans are asymptomatic or mild, even in the acute phase. Yet on the other hand, congenital toxoplasmosis in fetuses can result in serious eye (chorioretinitis) and brain damage (encephalitis and hydrocephalus). Equally important may be the impact of chronic toxoplasmosis on human behavior (Flegr 2007). Neosporosis, caused by *Neospora caninum*, a parasite closely related to *T. gondii*, is found worldwide in dogs, cattle, and other mammals. Relatively recently, *N. caninum* has been implicated as an important cause of abortion in cattle due to congenital infection (Reichel et al. 2013). Numerous *Sarcocystis* species form cystic stages in muscular tissues of various wild animals and under certain circumstances make these hosts more vulnerable to their predators, which represent definitive hosts.

Several *Eimeria* species causing coccidiosis are widespread in poultry farms and represent a major cause of morbidity and decreased weight gain implying economic losses to the industry by direct mortality, decreasing food conversion rate and expenses connected to anticoccidial medication or vaccination. With about 40 billion chickens raised annually worldwide, the disease is estimated to cost upward of 800 million US dollars per annum. Management of coccidiosis through anticoccidial drugs and vaccines using live attenuated *Eimeria* species has critical implications for the poultry industry, while other species negatively affect rabbits and farm ruminants (Allen and Fetterer 2002). *Cystoisospora suis* is the causative agent of an acute diarrhea in piglets. The waterborne *Cyclospora* and *Cryptosporidium* species are important for public health as the causes of diarrhea. Recent Global Enteric Multicenter Study identified *Cryptosporidium* as the second most common pathogen in infants in developing countries (Kotloff et al. 2013). Cryptosporidiosis may cause, under favorable conditions, diarrhea of epidemic proportions even in developed countries. Several *Cryptosporidium* species cause watery diarrhea in humans and are held responsible for gastrointestinal disease and morbidity of HIV-infected patients.

Similar to other infectious diseases and pathogens, several apicomplexan parasites have been introduced to nonnative continents, with their subsequent spreading

through the new areas, as in the case of avian sarcosporidiosis (*Sarcocystis rileyi*) introduced to Europe from North America. Moreover, some etiological agents could be considered as emerging diseases, as in the case of small intraerythrocytic piroplasm *Cytauxzoon felis* in domestic cats or cyst-forming sarcosporidia, *Besnoitia besnoiti*, an emerging pathogen of cattle coursing besnoitiosis mainly in Europe.

Several poorly studied species are also known to infect invertebrates. The most common among them are gregarines, which could inflict serious damage to insect farms or in laboratory colonies. At the same time, various apicomplexan parasites have a potential as agents for biological warfare against the crop, animal, and human pests and vectors, yet for such applications, they have never been put into effect on a large scale.

Habitat and Ecology

Apicomplexans are obligatory parasites, fully dependent on their hosts throughout most of their life cycle. As highly sophisticated parasites, apicomplexans benefit from their prolonged and close association with the host, which they exploit for food, habitat, and dispersal in order to increase their fitness. The act of parasitism reduces host fitness in causing pathology or altering the behavior or social status of the host. In the wild the pathology of most species is low, and the infected hosts usually show no signs of the disease. However, under intensive farming conditions or after the introduction into new susceptible hosts in non-endemic areas, these parasites may cause high morbidity and mortality.

Obligatory Dependence on the Host

The Apicomplexa obtain food (nutrient sources) from the host. Being dependent on a host requires tools and mechanisms to access its metabolites. The apical complex with its repertoire of secretory organelles is the key to the global success of this group of protists. While the apical complex is the unifying morphological feature of the phylum, the means of host exploitation are enormously diverse. By attaching to the host cell via their apical end, gregarines remain extra- or epicellular, with the host cell remaining virtually unaltered. In the case of cryptosporidia, the host cell envelopes the parasite with its flat membrane folds, while the only contact zone between both cells, termed the feeder organelle, is a highly modified interface. Coccidians and hematozoans are intracellular parasites, usually with a complex life cycle, undergoing remarkable morphological transformations allowing them to persist in diverse locations within their hosts. For example, *Plasmodium* is capable of flourishing in both mosquitoes and humans, where it can modify the surface of the infected red blood cells by exporting its proteins through membranes and the lumen of the host cell.

Localization in the Host

In the initial stage of invasion, the motile zoite (Fig. 1) will find the target tissue and establish the infection (Fig. 2). During the development, the intra- and extracellular phases may alternate, although a vast majority of species develop inside of the host cell (Bartošová-Sojková et al. 2015). The life cycle is terminated by a stage resistant to unfavorable conditions that is usually excreted during the host's life, or is released into the environment after its death. Some heteroxenous species do not form any exogenous stages as they are transmitted via ingestion/inoculation by blood-feeding arthropods.

Extracellular parasites. All extracellular species belong to the gregarines. They develop mostly in the digestive tract but can also be found in the respiratory and excretory organs. Even the extracellular gregarines are almost permanently attached to the host cell, this association is being terminated only prior to the extrusion of the parasite into the environment. The epicellular localization represents a transitional form between the extra- and intracellular parasitism. It is characteristic

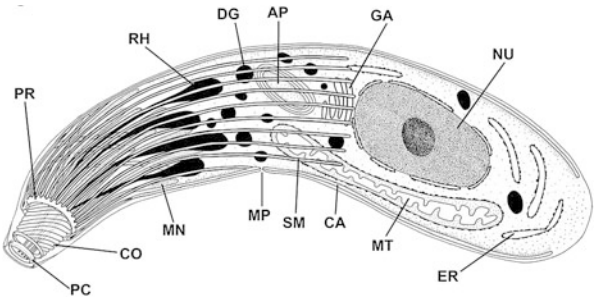

Fig. 1 3D structure of a typical sporozoite or merozoite. *AP* apicoplast, *CA* cortical alveoli, *CO* conoid, *DG* dense granules, *ER* endoplasmic reticulum, *GA* Golgi apparatus, *MN* micronemes, *MP* microporus, *MT* mitochondrion, *NU* nucleus, *PC* pre-conoidal rings, *PR* polar ring, *RH* rhoptries, *SM* subpellicular microtubules

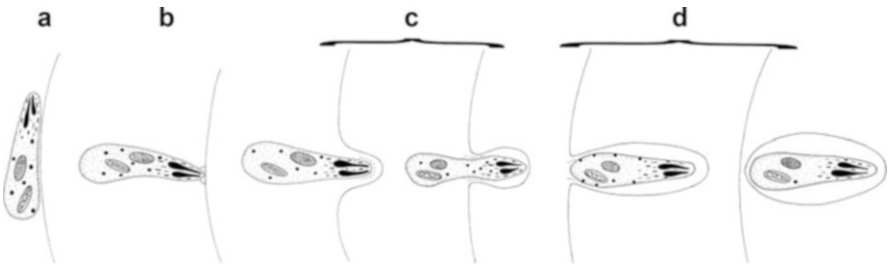

Fig. 2 Invasion of the apicomplexan zoite into a host cell. Primary contact of the zoite without orientation (**a**); attachment followed by the apical reorientation (**b**); induction of the parasitophorous vacuole (**c**); translocation of the zoite into the vacuole (**d**)

for cryptosporidians, which communicate with the host cell via a specialized feeder organelle, which closely resembles the attachment site of some gregarines. Many intracellular species have an extracellular phase in their life cycle, during which a cystic stage is released into the environment, where it awaits ingestion by a new host.

Intracellular parasites. The Apicomplexa are able to invade almost any cell type, *T. gondii* being a prime example of such an indiscriminate strategy. The parasites are either in direct contact with the host cell cytoplasm or are encircled by a "parasitophorous vacuole" formed by components of both the host and parasite cells. Several types of location within the host cell can be distinguished: (i) intracytoplasmic location is typical for most coccidians and hematozoans; (ii) extracytoplasmic location on the periphery of the epithelial cell facing the lumen, during which direct communication with the host cell cytoplasm is maintained, is characteristic for some coccidians of cold-blooded vertebrates; (iii) some coccidians are localized inside the host cell nucleus.

Furthermore, the intracellular stages can be subdivided based on their activity. Usually, upon host cell invasion, the zoite starts to divide and the life cycle proceeds fairly rapidly. However, when the zoite transforms into a dormant stage (dormozoite), the growth becomes arrested, and the stage can persist unchanged for years. It will, however, be awakened by stimuli, such as a change in the health state of the host or by the ingestion of the host by a predator. Another life form, the oocyst, is usually released into the external environment, where it may exist for a long period of time without growth, supporting itself from storage organelles until the next host is encountered.

Multiple Species in One Host

It has been predicted that each multicellular organism hosts at least one apicomplexan species, yet this simplified view is incorrect. Detailed studies of the medically and veterinary important hosts revealed that a single host species may be exploited by more than a dozen of distinct apicomplexan species affecting different host tissues. However, for most host organisms, only an incomplete record exists, keeping the diversity of apicomplexans largely unknown.

Fowl coccidia, the major problem in the poultry industry and the cause of chicken coccidiosis, are an example of such multispecies phenomenon. At least seven distinct *Eimeria* species are found in chicken, each occupying a specific habitat within the gastrointestinal tract (Clark et al. 2016). The most devastating species is *Eimeria tenella*, a parasite of caeca (Sharman et al. 2010). The enormous capacity to propagate is illustrated by the fact that each oocyst of *E. tenella* is theoretically capable of producing 2.5×10^6 oocysts within just 5 days. Besides their specific location within the intestinal tract, individual species invade mucosal cells either at the tips of the villi or in the crypts, while others found the interior of the villi as the most suitable environment. A complete set of economically significant coccidia infecting rabbits along with their specific location with the host is shown in Fig. 3

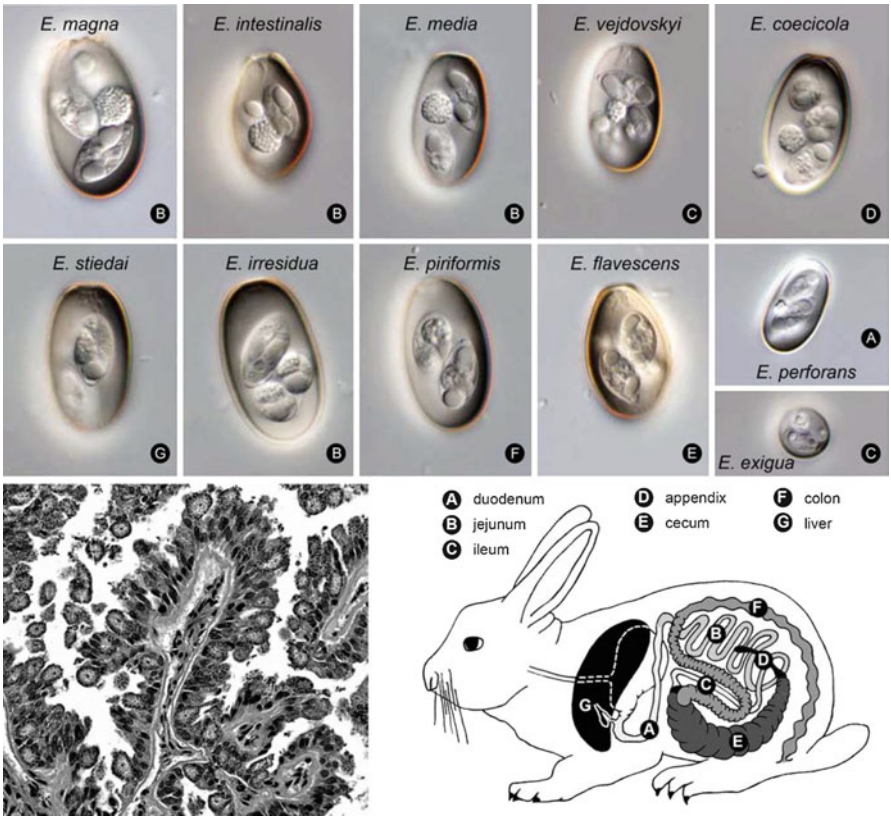

Fig. 3 Multiple *Eimeria* species infecting rabbit. Sporulated oocysts of 11 named *Eimeria* species parasitizing rabbits. Morphologically similar oocysts are distinguished by their size, shape, the presence of the micropyle, and the presence/absence and characteristic structure of the oocyst residuum. Individual species differ in the location in and pathogenicity for the host. The picture in the lower left corner shows proliferative changes in bile ducts with multiple gamogonial stages of *E. stiedae*

(Duszynski and Couch 2013). Thus, each parasite secures its distinct niche within the host organism. *Cryptosporidium* species exploit both the gastric mucosa and the intestinal mucosa. Similarly, human malaria is caused by four distinct worldwide distributed species – *Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* – circulating among humans via the *Anopheles* mosquito vectors; however, at least one more species, *P. knowlesi*, sometimes called the fifth human malarial parasite, which is principally a pathogen commonly found in nonhuman primates in Southeast Asia, may also infect humans (Tenter et al. 2000; Singh and Daneshvar 2013). Each human *Plasmodium* is characterized by a distinct life cycle pattern in the host. By far the most devastating is *P. falciparum*, causing malignant tertian malaria with indefinite multiplication of asexual stages in the red blood cells.

Parasitism and/or Mutualism

The apicomplexans often affect their hosts in highly sophisticated ways. In the two-host life cycles and particularly in those exploiting the predator-prey relationship, the success of the parasite is directly linked to the consumption of the infected prey by the predator. Thus, any mechanism that increases susceptibility to predation enhances the parasite's fitness (Vorisek et al. 1998). On the other hand, (very) low virulence of the parasite for the predator, as often seen in species such as *Toxoplasma* and *Sarcocystis*, can be considered as commensal or even mutualistic rather than parasitic. Since mutualism is a form of coexistence (symbiosis) enhancing the fitness of both partner organisms, it is not surprising that in evolutionary terms the long relationship between coccidians and their hosts frequently developed from parasitism into commensalism or mutualism. For example, *Sarcocystis* (syn. *Frenkelia*) *microti* and *S. glareoli* circulate between buzzards and small rodents, its definitive and intermediate hosts, respectively. While in buzzards it causes no symptoms, large cysts in the brains of rodents make them more vulnerable to the predator. The mechanisms behind such an increased susceptibility do not seem to be associated with cellular pathology, but the parasite most likely changes the social status or behavior of the infected individual. In another example, rats and mice infected with *T. gondii* lose fear of the odor of feline urine. Importantly, humans may also be manipulated by the ubiquitous *Toxoplasma* that may alter our behavior, psyche, and response to certain stimuli (Flegr 2013). However, the extent of alterations during human toxoplasmosis remains controversial.

The development of some monoxenous coccidians seems to follow circadian rhythms (Martinaud et al. 2009). It has been shown that oocysts of *Isospora* are significantly more prevalent in the feces of passerine birds excreted in the afternoon as compared to other parts of the day. Preferential shedding of the environmentally resistant oocysts in the afternoon can be explained by the avoidance of initial and/or prolonged harmful desiccation and UV.

Distribution

The Apicomplexa are virtually omnipresent. As parasites of the majority of vertebrates and invertebrates, they are distributed on all continents, on the ocean floor as well as in the air. Many species produce environmentally resistant envelopes that protect the parasite for months or years, either in the soil or water. *Cryptosporidium* is a typical example of a waterborne pathogen, the oocysts of which contaminate water sources and are passively transmitted to large areas, spreading the disease to new locations. Because of their minute size, these resistant stages can even be passively carried by insects. Blood-feeding arthropods also play a key role in the life cycles of medically and veterinary important hematozoans and piroplasms, the distribution of which follows the geographical range of their vectors. In fact some

estimates consider the Apicomplexa as the most specious group of eukaryotes, predicting the existence of million(s) of species (Pawlowski et al. 2012).

In the past, human colonization of remote places dramatically affected the distribution of many apicomplexan parasites. Transmission of the bird malaria caused by *Plasmodium relictum* is impossible without the compulsory vector mosquito *Culex quinquefasciatus*. Its introduction to Hawaii in 1826 triggered outbreaks of the avian malaria imported to the islands either by exotic birds released in the late nineteenth century or by migratory birds breeding in the Arctic. The total lack of immunity of the endemic Hawaiian birds to malaria caused by thousands of years of the absence of malaria resulted in epidemic mortality bringing some endemic species to the margin of extinction. Similarly, the appearance of avian malaria on the Bermuda Islands is directly linked to human encroachment. A Spanish sailor shipwrecked in 1603 on uninhabited Bermuda reported a total absence of mosquitoes. It is thus likely that the extinction of the endemic passerine birds in Bermuda was caused by the introduction of mosquitoes and alien passerines with malaria that had a similar devastating effect on the resident birds. Bovine babesiosis which has probably haunted farmers since the beginning of livestock production in warm regions of the Old World was later introduced into the New World by early settlers with imported cattle and the first documented reports date from around 1810 in North America.

One of the most successful parasites is undoubtedly *T. gondii* propagated in the form of an asexual clonal population. It is highly prevalent in all warm-blooded vertebrates, its success being supported by several key adaptations of its life cycle. The disease caused by asexually multiplying *Toxoplasma* is in most cases mild and self-limiting, leading to formation of dormant cystic stages in tissues. However, the host will remain an infectious passive carrier for life. Importantly, unlike other cyst-forming coccidians, *Toxoplasma* is able to bypass sexual development. The dormant cysts are capable of inducing an infection in any predator or scavenger munching on the animal tissues containing parasite cysts. The astonishing success of *T. gondii* has been recently explained by unique and ancient North and South American dichotomy of its former population that occurred prior to the reconnection of the Panamanian land and was coupled with a recent global sweep of few clonal populations. More than 95% of isolated strains in North America and Europe belong to just three clonal lineages (Howe and Sibley 1995) that have arisen ~10,000 years ago.

Characterization and Recognition

The Apicomplexa are distinguished by the complex and characteristic organization of the apical part of the invasive stages (the zoites, usually present both in sporozoites and merozoites, which alternate in the life cycle) and by the presence of a small inconspicuous organelle in the cytoplasm of all developmental stages – the apicoplast.

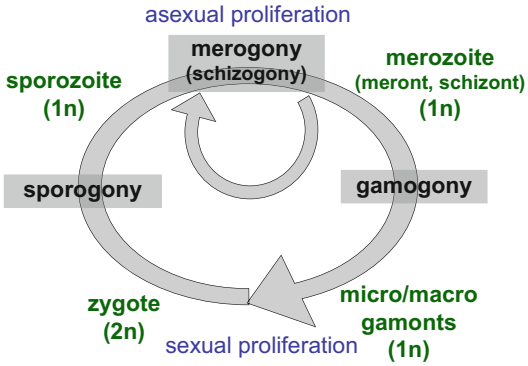

Fig. 4 Schematic drawing of a typical apicomplexan life cycle. See text for explanation

The Life Cycle

The life cycle of apicomplexans is rather complex and comes in several significantly different forms characteristic for main subgroups of the phylum. Its most simple form is known in gregarines, where it is composed of gamogony (the sexual phase) and sporogony (the asexual phase) (Fig. 4) (Ferguson et al. 2008). The life cycle of coccidians and haemosporidians contains asexual multiplication – merogony.

The life cycle usually commences with the release of a sporozoite from the oocyst/sporocyst (see below), an event that often takes place in the intestinal content of the host. The gliding sporozoite has a relatively short time to find a host cell, which it will penetrate by means of its apical complex and thus initiates the infection (Fig. 2). Shortly thereafter, organelles of the apical complex undergo resorption, and the elongate sporozoite transforms into an oval meront that starts growing. Upon reaching a critical size, the meront divides into a dozen to hundreds of merozoites (Fig. 5). These are similar in ultrastructure to the sporozoite and are destined to spread the infection to other host cells, where the cycle proceeds by a new generation(s) of meronts and merozoites. The next phase is characterized as gamogony, since some merozoites are predetermined to become female macrogametocytes, while the rest evolve into male microgametocytes (Fig. 6). The life cycle proceeds by fusion of a small flagellated microgamete with a large and nonmotile macrogamete. This conversion from a haploid into diploid phase is termed sporogony (Figs. 6 and 7) and is characterized by a species-specific number of cell divisions, leading to the formation of sporozoites, usually enclosed in a resistant sporocyst and/or oocyst wall (Figs. 6, 7, and 8). Upon the release of the sporozoites under favorable conditions (Fig. 9), the life cycle is completed, as their function is to transmit the infection into a new host.

Host Cell Invasion and Parasite Multiplication

The Apicomplexa are experts in host cell manipulation and immune evasion. *Toxoplasma gondii*, *Theileria* spp., *Plasmodium* spp., and others secrete different

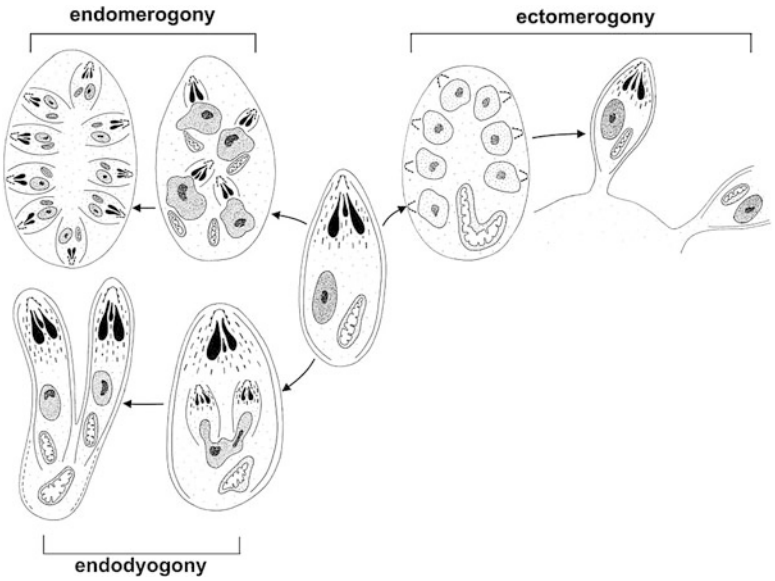

Fig. 5 Schematic drawing of main types of merogonial division. Merogonial division can proceed either via endomerogony (*upper left*), ectomerogony (*upper right*), or endodyogony (*lower left*)

effector molecules into the host cell to reach this aim. Invasion of an apicomplexan into the host cell is a complex action, some parts of which have yet to be elucidated (Baum et al. 2008). Generally, it consists of four phases: (i) primary contact without orientation, (ii) attachment followed by apical reorientation (with the exception of the genus *Theileria*), (iii) induction of the parasitophorous vacuole, and (iv) translocation of the parasite into the vacuole (Fig. 2). Attachment to the host cell via the apical end is followed by establishment of a connection through sequential secretion from the secretory organelles of the parasite. These unique extrusive organelles, represented by few claviform rhoptries, numerous filamentous micronemes, and round dense bodies contain molecules required for the interaction with the host cell (Besteiro et al. 2009). They are deployed in the course of the invasion and play various roles during intracellular development. Apically secreted adhesins from the micronemes are translocated along the parasite length and are shed at the site of the moving junction. This circumferential zone of moving junction is associated with a constriction of the parasite that moves from its apex to the posterior end. The parasite enters the nascent parasitophorous vacuole by capping the moving junction down its body, and components from the rhoptries are secreted into this newly formed compartment (Shen and Sibley 2012). Ultimately, the apicomplexan cell becomes enclosed within a cavity delimited by the invaginated host cell membrane. This protects the parasite against host immune mechanisms. On the other hand, brisk trade of nutrients is in motion among the parasite's surface, inner

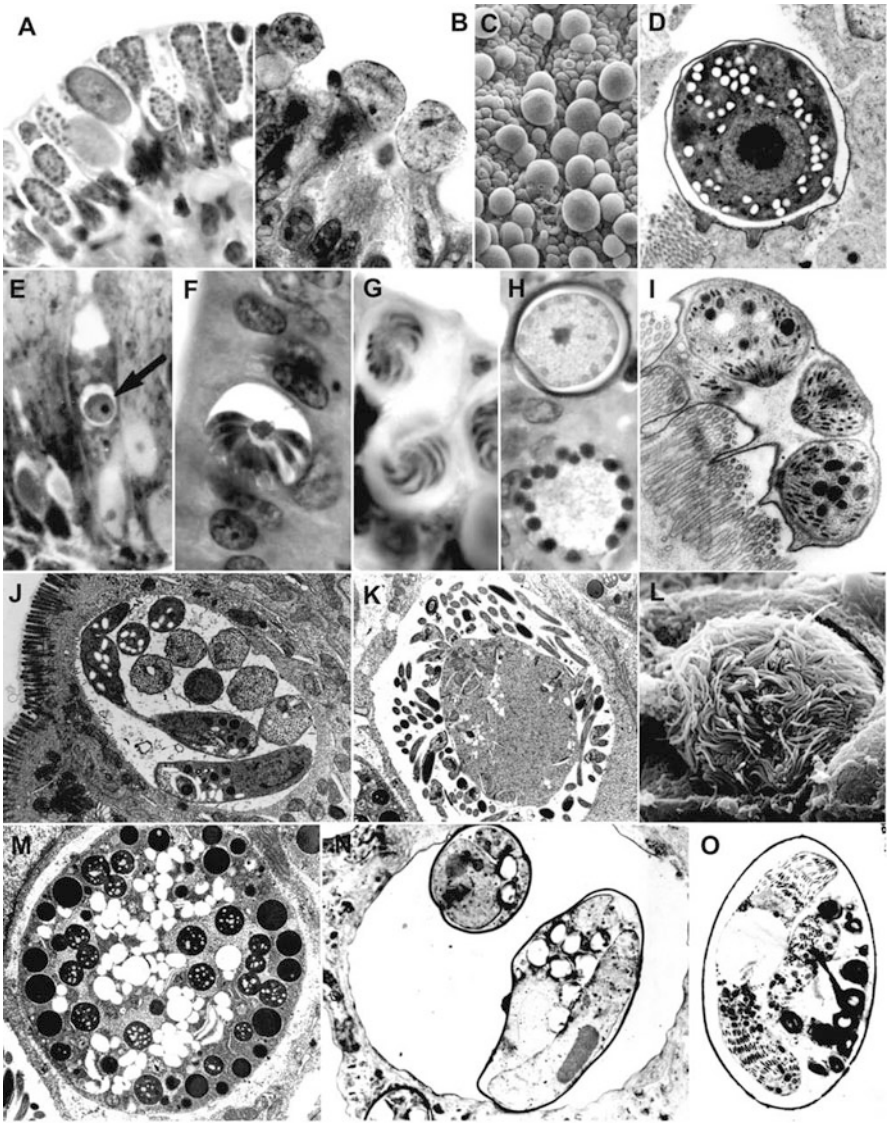

Fig. 6 Representative morphology of coccidian life cycle stages in the intestine, gall bladder, and spleen of various vertebrate hosts. Intestinal epithelium heavily infected by intracellular gamogonial stages of *Eimeria neodebliecki* from a pig (**A**); early gamogonial stages of *Choleoeimeria hirbayah* in the gall bladder of a chameleon; note that the infected cells are displaced toward the lumen (**B**); similar situation showing displaced cells with stages of *Choleoeimeria baltrocki* in the gall bladder of a skink (**C**); early extracytoplasmic meront of *Epieimeria anguillae* from an eel (**D**); young meront of *Goussia bohemica* initiating infection in the goblet cell of a gudgeon (**E**); ectomerogonial division of *Eimeria zuhairamri* from the intestine of a field mouse (**F**); mature microgametocytes of *Eimeria neodebliecki* from a pig, containing prominent flagellated microgametes (**G**); early (*upper* cell) and mature macrogametocytes containing well-visible wall-forming bodies (*lower* cell) and

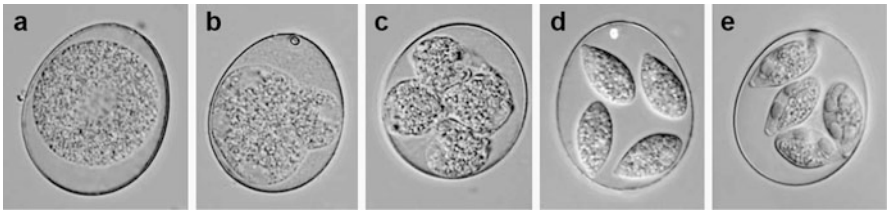

Fig. 7 Sporulation of *Eimeria maxima* from the intestine of a chicken. Unsporulated oocysts are shed with feces into the environment (**a**). When exposed to oxygen in an environment of appropriate humidity and suitable temperature, they undergo sporulation (it takes about 48 h at 25 °C before they become infectious). Upon asexual division through four sporoblasts (**b**, **c**), four sporocysts are formed initially full of granular material (**d**) that during sporulation wanes, until mature infectious sporozoites with remaining sporocyst residuum appear (**e**); note also process of formation of prominent Stieda bodies on poles of sporocysts (**d**, **e**)

membrane of the parasitophorous vacuole, and outer membrane of the infected cell (and thus with the surrounding environment).

Apicomplexan parasites replicate by internal budding termed merogonial division or merogony (schizogony in older literature) to create either two daughter cells (endodyogony) or multiple progeny (endopolygony, multiple synchronized endopolygony, and ectomerogony) that differ mainly in the preservation or loss of the maternal cell (see below) (Striepen et al. 2007). The apicomplexan nucleus divides by cryptomitosis (the nuclear membrane remains intact throughout the process), and karyokinesis occurs without chromosomal condensation.

The Sporozoite

This is the most characteristic stage of the phylum. The sporozoite is invariably elongated with a polar organization of its intracellular structures (Morrissette and Sibley 2002). Its size varies from less than 1 μm to about 25 μm in length. The tapered end is equipped with a conserved and specialized set of structural and secretary organelles labeled the apical complex (Fig. 1). Their extraordinary

Fig. 6 (continued) young oocyst (*upper* cell) of *Eimeria cahirinensis* from a spiny mouse (**H**); extracytoplasmic "spiderlike" meront of *Goussia pannonica* containing three merozoites from a white bream (**I**); numerous mature merozoites of *Eimeria arvalis* from a vole (**J**); mature microgametocyte of *E. arvalis* containing microgametes on its periphery (**K**); mature microgametocyte of *Eimeria vermiformis* from a mouse, containing microgametes on its periphery (**L**); mature macrogametocyte of *E. arvalis*, containing various wall-forming bodies (**M**); sporulating oocyst of *Goussia metchnikovi* from the spleen of a gudgeon, with cross-sectioned sporocysts containing immature sporozoites and large residual body (**N**); sporulated sporocyst of *G. metchnikovi* with a mature sporozoite filled with micronemes and dense bodies (**O**). Histological sections (**A**, **B**, **C**–**H**) and scanning (**C**, **I**) and transmission electron microscopy (**D**, **I**–**K**, **M**–**O**)

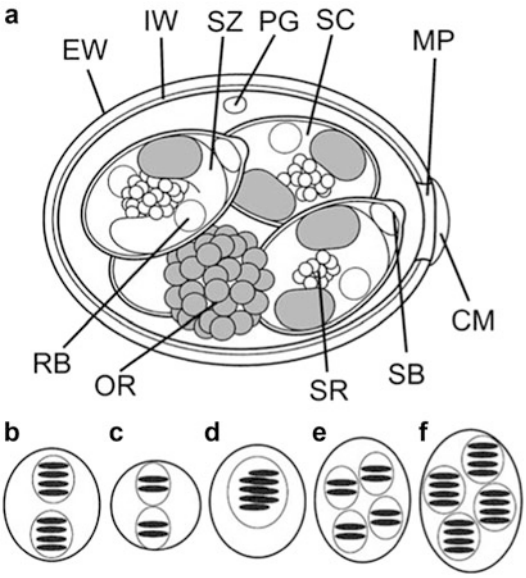

Fig. 8 **Schematic drawing of a typical coccidian oocyst**. *Eimeria* (**a**) *CM* cap of the micropyle, *IW* internal oocyst wall, *EW* external oocyst wall, *MP* micropyle, *OR* oocyst residuum, *PG* polar granule, *SB* Stieda body, *SC* sporocyst, *SR* sporocyst residuum, *SZ* sporozoite, *RB* sporozoite refractile body. Several types of the inner organization of the oocysts are shown: *Isospora*-like (two sporocysts, each with four sporozoites) (**b**); *Cyclospora* (two sporocysts, each with two sporozoites) (**c**); *Caryospora* (single sporocyst, containing eight sporozoites) (**d**); *Eimeria*-like (four sporocysts, each with two sporozoites) (**e**); *Wenyonella* (four sporocysts, each with four sporozoites) (**f**)

combination constitutes the very effective invasion apparatus responsible for an enormous evolutionary success of these parasites. The apical tip contains a polar ring, to which subpellicular microtubules are attached that stretch into the cell. Adjacent to the conoid are one or two apical rings composed of spirally arranged microtubules. The most prominent components of the apical complex are rhoptries and micronemes, secretory organelles full of molecules important for the invasion (see below). This cargo is of protein and lipid nature, specialized for intracellular parasitism. Rhoptries are often very prominent club-shaped organelles, whereas micronemes are usually rather thin, prolonged, and abundant ducts. Dense granules, usually located more distal from the conoid, have also recently been implicated with invasion. The specialized parasitophorous vacuole is formed with their help and its main purpose is to protect the apicomplexan from host attack, while the parasite can still obtain nutrients from the host. The most intensely studied organelle of the sporozoite is the apicoplast (see "Evolutionary History").

Sporozoites, same as all the other apicomplexan stages, also contain standard equipment of the eukaryotic cell, such as the nucleus, Golgi apparatus, endoplasmic reticulum, plasma membrane, and mitochondrion (Fig. 1). At first, the omnipresent

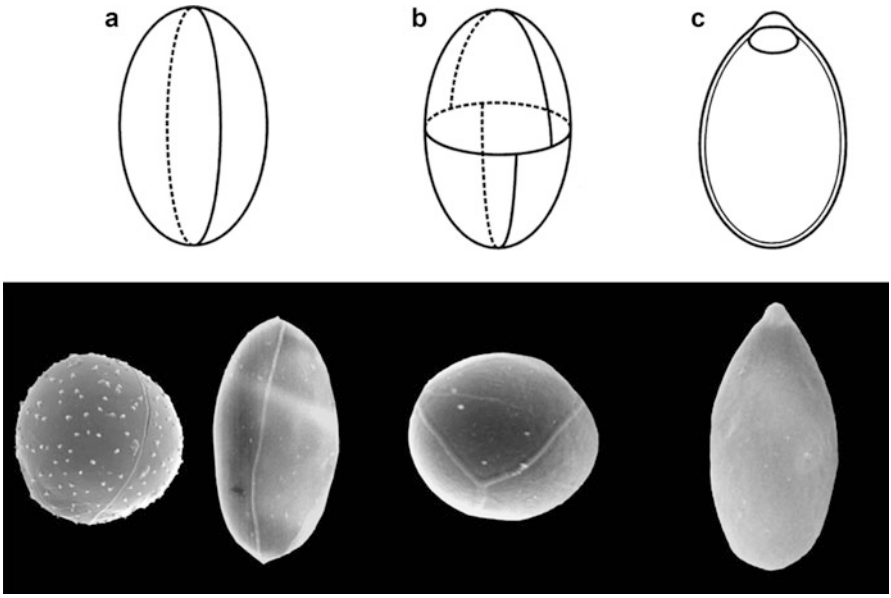

Fig. 9 Excystation structures of coccidian; schematic drawings and scanning electron microscopy of sporocysts. Probably ancestral opening of the sporocyst along a single longitudinal slit (*Goussia janae*, *Aggregata octopiana*) (**a**). Alternative opening by sutures in the sporocyst wall composed of four valves (*Cystoisospora suis*) (**b**). In the eimeriid coccidian (*Eimeria tenella*), sporozoites are released after the plug formed by the Stieda and substieda bodies is dissolved (**c**)

mitochondrion appeared to be missing from the *Cryptosporidium* species. Only recently, it was shown that this organelle is present in an extremely reduced form, termed mitosome, sandwiched between the nucleus and the crystalloid body (Keithly et al. 2005). The position of genus *Cryptosporidium* on the evolutionary tree of Apicomplexa is unresolved largely due to its highly divergent genome and unusual cellular biology (see below).

With the exception of some gregarines, sporozoites enter the host cell, and an intracellular development proceeds. The sporozoites of gregarines also penetrate the cell wall, and the apical portion of the cell develops into a family-specific attachment apparatus. While gregarine sporozoites of the family Ganymenidae and Lecudinidae attach via the so-called mucron, the remaining gregarines develop into a morphologically prominent epimerite, via which they penetrate into the host cell (Figs. 10 and 11). With most of its body extracellular, the sporozoite feeds on the epithelial cells, substantially increases its size, and develops characteristic longitudinal folds that likely propel its movement thru the intestine to other epithelial cells (Fig. 11).

Sporozoites are motile and for host cell invasion utilize gliding motility, which is propelled by the actin cytoskeleton and myosin motors. During the motility phase, micronemes secrete adhesions onto the apical part of the parasite's surface; hence, they are gradually translocated by an actomyosin-based complex. Visualization of the adhesions deposited during a sporozoite's movement can reveal its gliding

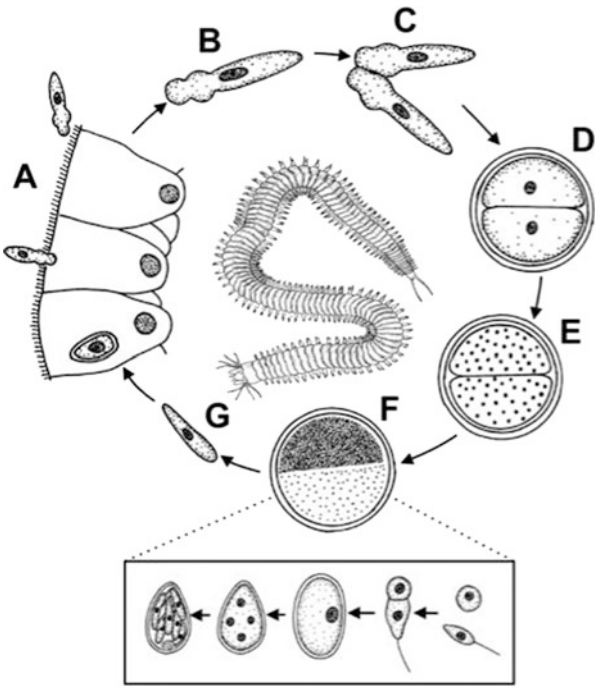

Fig. 10 Schematic drawing of the life cycle of the gregarine *Lecudina* in a Polychaete. Extracellular trophozoite attached to the host epithelium via an epimerit (*A*); free trophozoite (*B*); syzygy (*C*); early gametocyst (*D*); formation of gametes (*E*); cyst filled with separated mature gametes of both sexes (*F*); the inset shows development in the cyst from morphologically distinguishable female and male gametes that upon fusion grow into an oocyst, to a mature oocyst containing infectious sporozoites; free sporozoite initiating new infection (*G*)

movement. In apicomplexans, host cell invasion always initiates by the attachment of the apical end to the host cell, and with just a few exceptions, in the next step, the parasitic cell induces invagination of the plasma membrane. This host membrane transforms into the parasitophorous vacuole enclosing the parasite and subsequently becomes massively altered by the insertion of various proteins and lipids primarily secreted by the rhoptries.

Meront and Merogonial Development

The intracellularly established meront starts intense feeding on the host cell via numerous micropores. In intracellular gregarines and all coccidians, the increase in size is characterized by the accumulation of amylopectin and lipid granules in the cytoplasm and nuclear division(s). The merogonial division (also called schizogony in older literature) may lead to the formation of only two cells within an intact, fully polarized mother cell, a process termed endodyogony (Fig. 5). Nuclear division in the polarized mother cell is followed by the formation of two buds, each composed of newly formed membrane complex and subpellicular microtubules (Striepen et al. 2007). The mature daughter cells finally appear from the mother cell.

However, in the course of more frequent merogonial division through multiple divisions, dozens to thousands of merozoites are formed, their number being usually

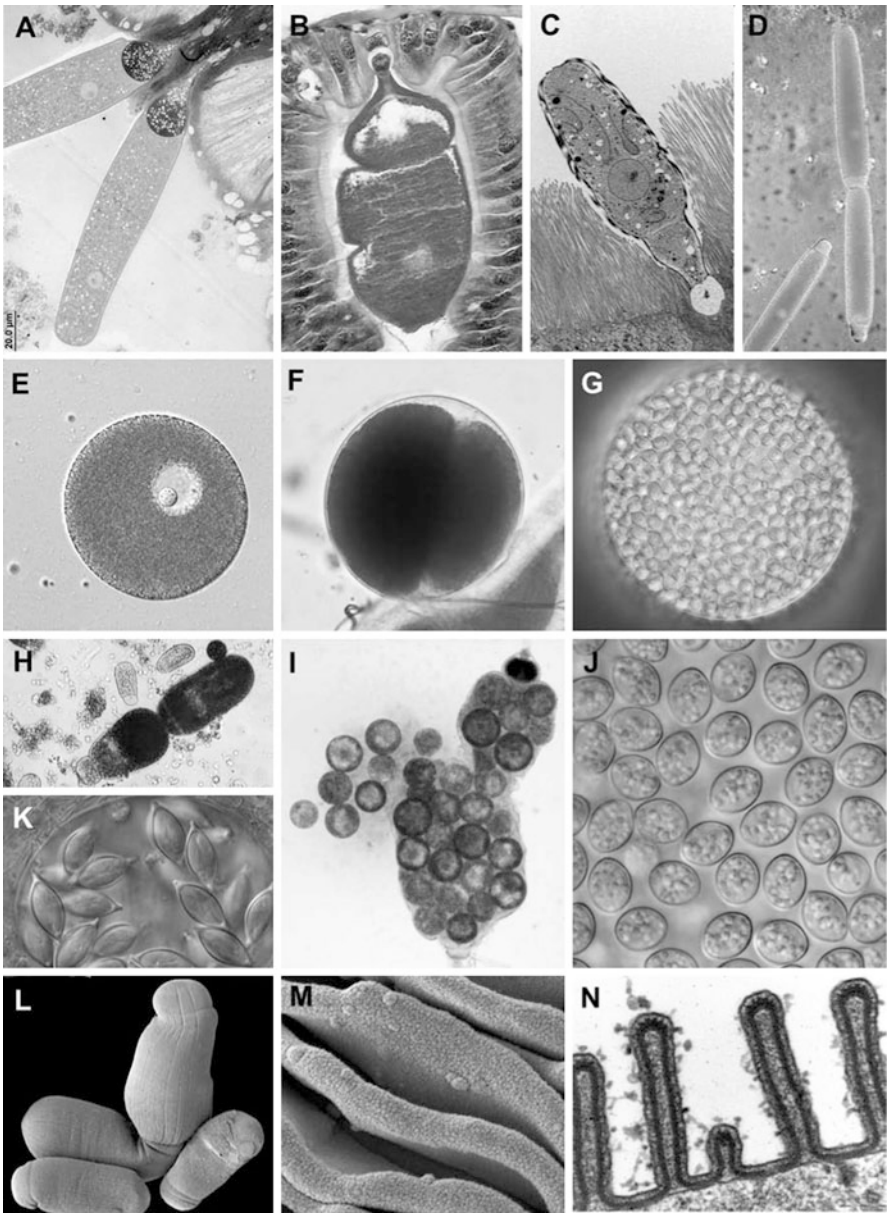

Fig. 11 Representative morphology of life cycle stages of gregarines. Mature trophozoites of *Gregarina polymorpha* (**a**) and *Gregarina garnhami* (**b**) attached to the host cell; mature trophozoite of *Gregarina steini* with a well-developed epimerite, invading a host cell (**c**); caudo-frontal syzygy of *G. polymorpha* (**d**); aseptate gamont of *Ascogregarina chagasi* containing a large nucleus and a prominent nucleolus (**e**); maturing gametocyst of *A. chagasi* with two gamonts separated by partition (**f**) and a gametocyst filled with numerous oocysts (**g**); gamonts of *Gregarina katherina* associated by caudo-frontal syzygy (**h**); sporulated biconical oocysts of *Monocystis* sp. (**i**); flea's

characteristic for a given species. The merogonial division exists either as endomerogony, during which the merozoites are formed within the mother cell, while exomerogony is characterized by merozoites that bud outside of the dividing mother cell (Figs. 5 and 6). The formation of progeny in the merogonial stage usually occurs in the host cell cytoplasm, although this phase of the life cycle may also be located in nucleoplasm of the host cell.

Upon their release from the ruptured host cells, polarized merozoites rely on energy sources that support only a short-time search (usually a few minutes) for a new host cell that they will invade. Following the invasion, the apical complex disassembles, the cell becomes oval and increases in size, the nucleus repeatedly divides, and another generation of merozoites is formed. The number of merogonial generations is usually species specific, ranging from one to a dozen or up to hundreds. Subtle morphological differences often allow assignment of given meront/merozoite to specific generations. The merogonial stages are responsible for rapid proliferation and most of the pathogenicity.

Sexual (Gamogonial) Development

The last generation of meronts enters the sexual phase of the life cycle by evolving into either micro- or macrogametes (Fig. 6); however, in hematozoans and piroplasms, gamonts emerge continuously as a product of specific part of meront populations. Sexual (gamogonial) development results in the production of large numbers of microgametes, or male sexual cells, and a much lower quantity of female macrogametes. Isogamous apicomplexans, haemogregarines and gregarines, where gamonts of each sex are equally numerous, represent exceptions. Young microgamonts contain numerous peripherally arranged nuclei and a homogeneous cytoplasm full of ribosomes, cisternae of endoplasmic reticulum, while lipid inclusions and other granules are small and relatively scarce. During maturation, the nuclei are juxtaposed to the cell surface, and the cell membrane forms dense thickening at the contact site, termed perforatorium anlagen. Mature microgamonts are usually large oval cells with invaginations on their surface. From their periphery, microgamonts emerge, equipped with an apical dense protrusion (perforatorium), one to three prominent flagella, a mitochondrion, and a dense elongate nucleus (Fig. 6). Each flagellum is supported by microtubules in the classical eukaryotic 9 + 2 arrangement (peripheral doublets with two centrally located microtubules).

Fig. 11 (continued) midgut filled with unsporulated gametocyst (**j**) and developing elliptical oocysts of a undescribed gregarine (**k**); two single gamonts of *G. garnhami* and two gamonts associated in caudo-frontal syzygy (**l**); epicytic folds of the apical region of protomerite of *G. garnhami* (**m**); cross-sectioned epicytic folds of *G. garnhami* with a newly formed fold rising between them (**n**). Histological sections (**a**, **b**), light microscopy (**d**, **e**–**k**), and scanning (**l**, **m**) and transmission electron microscopy (**c**, **n**)

The number of microgametes emerging from a single microgamont varies from dozens to thousands.

Early macrogamonts are large oval cells with a central nucleus and prominent nucleolus. The cytoplasm usually contains abundant, concentrically arranged cisternae of endoplasmic reticulum, electron-lucent amylopectin granules, lipid inclusions, electron-dense membrane-bound vesicles of various sizes, and mitochondria (Fig. 6). The amount of amylopectin inclusions as well as various vesicles increases in more advanced stages, and the cytoplasm becomes denser due to high number of ribosomes. Each macrogamont matures into a single macrogamete, which is fertilized by a microgamete, propelled by its flagella. All stages described so far were haploid, but fusion of the nuclei of micro- and macrogametes leads to a zygote, the status of which is diploid.

Sporogonial Development

In most cases fertilization occurs in the same tissue where the merogonial development is located, and this is also the site of the sporogonial phase, or sporogony (Fig. 7), with hematozoans and piroplasms representing exceptions. The zygote forms a protective wall around itself and sporogony, another process of asexual multiplication, yields a sporozoite-filled oocyst. Within the oocyst there are sporocysts, and within these are the sporozoites (Figs. 7 and 8). The oocyst and/or sporocyst wall is composed of several layers which possess a key role in the protection of the infectious and motile sporozoites that in many aspects resemble the merozoites (Figs. 1 and 8) (Belli et al. 2006). The sporozoites are usually released when the sporulated oocyst is eaten by another host. The morphology of oocysts, sporocysts, and sporozoites is one of the key characters of the taxonomy of most apicomplexan groups (Figs. 8 and 9).

Methods of Recognition

Absolute majority of apicomplexans are tiny protists not exceeding dozens of μm in size. They may be recognized with classical light microscopy, which usually suffices for taxonomic diagnosis. Exact species assignment requires the presence of morphologically informative stages. In gregarines and coccidian, these are in most cases trophozoites and oocysts, respectively. The determination of hematozoans is based on the morphology of stages in the blood cells. Thanks to their size reaching hundreds of μm, gregarines such as those infecting seminal vesicles of earthworms can be observed even by the dissecting stereomicroscope. Similarly, the cystic stages of some cyst-forming coccidia can sometimes be observed even with the naked eye, for example, as white nodules in the esophagus of infected sheep (e.g., *Sarcocystis gigantea*) or white fine threads in skeletal muscles (e.g., *Sarcocystis rileyi*).

Diseases caused by apicomplexans have been well recognized prior to the identification of the causative agent. Due to their impact on human health, certain apicomplexans are routinely associated with specific clinical symptoms in given endemic areas. Malaria is a prime suspect in all cases of cyclic fevers in tropical areas. Cryptosporidiosis is globally suspected in travelers' diarrhea. Coccidiosis manifested as diarrhea is always a threat in crowded conditions of intense farming, particularly of young animals.

For observing ultrastructural features, which are sometimes necessary for exact determination, the transmission and scanning electron microscopy are preferred approaches. Morphology is often insufficient, so host specificity is thus considered a leading criterion for species assignment. Completion of the life cycle, observation of the entire development, and elucidation of the host range are in many instances necessary. Public health authorities are particularly interested in the reservoir hosts for the zoonotic species and identification of the complete spectrum of vectors. Increasingly, molecular biology methods based on polymerase chain reaction (PCR), barcoding, and recently also next-generation sequencing (NGS) are being widely applied for detection and identification of apicomplexan species important in human and veterinary medicine. Genetic signatures linked to zoonotic transmission and clinical syndrome play an important role in current epidemiological investigations. In human malarias, fostered by multiple genome sequences and single nucleotide polymorphism (SNP) maps, gene modifications are being linked to antimalarial drugs.

Classification of Apicomplexa

The Apicomplexa comprise five principal working groups: gregarines, haemogregarines, coccidia, hematozoans (malarial parasites), and piroplasms, traditionally grouped into four classes. Besides these dominant groups, there is a myriad of small transitional groups or species, for example, the medically important cryptosporidia forming an independent group. The phylum classification is presented down to the suborder level. We annotate only major families, and for those with single or few representatives, we refer the reader to the work of Perkins et al. (2000), Tenter et al. (2002), and Adl et al. (2012).

The vividly discussed ancestry of the parasitic apicomplexans from predatory non-photosynthetic colpodellids can be found in Adl et al. (2012). Yet it is the photosynthetic chromerid species isolated from stony corals by Moore et al. (2008) that are currently the most favored as the closest living relative to the common ancestor of the phylum. Within this chapter, colpodellids and chromerids are considered "relict apicomplexa", while all the parasitic species are considered "core apicomplexa" (Table 1). The core apicomplexans represent a solid and well-defined group of eukaryotes, in contrary to their sister groups, here represented by, but not exclusive to, the two aforementioned groups. The quest for the basal relict apicomplexan is far from over (Table 1).

Table 1 Classification of Apicomplexa

Phylum: **Apicomplexa** Levine, 1970 emend. Adl et al., 2005	
Core apicomplexans (obligatory parasites)	
Subphylum: **Conoidasida** Levine, 1988	
Class: **Gregarinida** Dufour, 1828	Gregarines
Class: **Coccidia** Leuckart, 1879	Coccidia, haemogregarines
Cryptosporidida *incertae sedis*	Cryptosporidia
Subphylum: **Aconoidasida** Mehlhorn, Peters and Haberkorn, 1980	Hematozoa
Class: **Haemosporidia** Danielewsky, 1885	Haemosporidia (malarial parasites)
Class: **Piroplasmida** Wenyon, 1926	Piroplasms
Relict apicomplexans (free-living)	
Colpodellida Cavalier-Smith, 1993 *incertae sedis*	Colpodellids
Chromerida Moore et al., 2008 *incertae sedis*	Chromerids

Note: Commonly used vernacular names are indicated on the right. Colpodellida are ranked at the same level as Conoidasida and Aconoidasida in Adl et al. (2012)

Phylum Apicomplexa Levine, 1970 Emend. Adl et al., 2005

Apical complex generally consisting of polar ring(s), rhoptries, micronemes, conoid and subpellicular microtubules present at some stage; micropores present at some stage; cilia absent; all species parasitic; about 6,000 named species. The Apicomplexa has become more frequent in recent literature but not exclusive.

Subphylum Conoidasida Levine, 1988

Complete apical complex, including a conoid in all or most asexual motile stages; flagella, where present, found exclusively in microgametes (male gametes); with the exception of microgametes, motility generally via gliding with the possibility of body flexion and undulation of longitudinal pellicular ridges; heteroxenous or homoxenous. This group is not monophyletic. Subdivisions are artificial and unclear at this time.

Class Gregarinida Dufour, 1828 (Syn. Gregarinia Dufour, 1828, Gregarinasina Dufour, 1828)

Mature gamonts extracellular, large; mucron or epimerite, derived from conoid, ordinarily present in mature organism; sexuality usually by syngamy of gamonts; gametes generally very similar; similar number of male and female gamonts produced, zygotes form oocysts within gametocysts; life cycle consists of gametogony and sporogony; parasites of digestive tract or body cavity of invertebrates or lower chordates; generally homoxenous; about 1,800 named species. (*Gregarina*, *Lecudina*, *Mattesia*, *Monocystis*, *Selenidium*).

Class Coccidia Leuckart, 1879 (Syn. Coccidiasina Leuckart, 1879)

Mature gamonts intracellular, small; conoid not modified into mucron or epimerite; syzygy generally absent (if present involves gametes); sexual stages generally very different; different number of male and female gametes; microgametes without flagella; zygote forms oocyst from fertilized macrogametocyte; homoxenous or heteroxenous life cycles consist of merogony, gamogony, and sporogony; parasites of vertebrates and invertebrates about 3,500 names species (Adeleorina Léger, 1911: *Adelina*, *Haemogregarina*, *Hepatozoon*, *Klossiella*; Eimeriorina Léger, 1911: *Caryospora*, *Cyclospora*, *Eimeria*, *Goussia*, *Isospora*, *Lankesterella*, *Neospora*, *Sarcocystis* (syn. *Frenkelia*), *Toxoplasma*; *Aggregata*, *Lankesterella*).

Subphylum Aconoidasida Mehlhorn, Peters and Haberkorn, 1980 (Syn. Hematozoa Vivier, 1982)

Secondarily incomplete apical complex; conoid absent in asexual motile stages (some motile zygotes [ookinetes] contain conoid); formation of macrogametes and microgametes independent; heteroxenous.

Class Haemosporida Danilewsky, 1885

Motile zygote [ookinete] with conoid; flagellated microgametes produced by merogony; oocyst with sporozoites; heteroxenous; parasites of vertebrates; invertebrates serve as vectors, in which sporogony occurs; about 500 named species (*Haemoproteus*, *Leucocytozoon*, *Plasmodium*).

Class Piroplasmida Wenyon, 1926

Conoid and flagella absent in all stages; piriform, round, rod shaped, or amoeboid; no oocyst; sexual stages still uncertain but probably associated with the formation of the large axopodium-like stages; heteroxenous; parasites of vertebrates (in blood cells); ticks serve as vectors; about 200 named species (*Babesia*, *Theileria*).

Gregarines

Gregarines represent an extremely large and highly abundant group of early-branching apicomplexans that exploit exclusively invertebrate hosts, such as annelids, mollusks, nemerteans, phoronids, echinoderms, sipunculids, crustaceans, hemichordates, appendicularians, and insects (Fig. 10). Gregarines have monoxenous life cycles consisting almost exclusively of gamogony and sporogony, since only very few species display merogony. The life cycle of most gregarines commences by the release of young trophozoite from a sporocyst engulfed by the host. The trophozoite generally attaches to the epithelial tissue in the gut lumen of the host and occupies it (Fig. 11). However, some species can be found in coelomic cavities and tissues associated with the reproductive system. After an enormous increase in size, the trophozoite is released from the host tissue into the gut lumen. It transforms into a

gamont that will attach to a partner in a species-specific orientation (head-to-head, tail-to-tail, or head-to-tail) in a process called syzygy (Figs. 10 and 11). In the next step, the gametocyst enclosing both gamonts is formed. Several mitotic divisions inside the gametocyst give rise to hundreds of gametes. Next, the gametes fuse with their partners from the other gamont and produce numerous zygotes. Newly formed sporocyst (oocyst) wall confines each zygote, and subsequent meiosis produces four or more sporozoites per sporocyst (oocyst). Gametocysts filled with mature sporocysts (oocyst) are then released into the environment and ingurgitated by a new host, repeating the cycle. This general scheme has numerous genus- or species-specific modifications, a feature not surprising for organisms displaying such an extreme diversity. The gregarine trophozoites can move and change direction through a mechanism unique among eukaryotes, called gliding motility. This may be accomplished via a cytoskeleton composed of actin and myosin. Gregarines seem to lack the apicoplast, as do the closely related parasitic cryptosporidia.

Gregarines are taxonomically subdivided into three orders: basal archigregarines, advanced eugregarines, and neogregarines. Such a branching order has been inferred from life cycles of these parasites and is, at least to some extent, supported by molecular phylogeny (Leander et al. 2003a, b; Leander 2008; Desportes and Schrével 2013).

Order Archigregarinida Grassé, 1953

This order contains extracellular intestinal parasites of annelids, sipunculids, hemichordates, and ascidians. Their trophozoites are anchored in the host epithelium via the epimerite (or mucron). They are characterized by the absence of septa (aseptate), the persistence of zoite organelles, the pairing of trophozoites (syzygy), and the encystment of gamonts. Sporocysts contain four to eight or even more sporozoites. Archigregarines are parasites of marine invertebrates, with the life cycle completed within the intestinal lumen of a single host. Trophozoites of some species may use myzocytosis-based feeding. This ability, together with the number of infective sporozoites, links archigregarines to colpodellids, free-living biflagellated predators that form a sister group to the parasitic apicomplexans.

Order Eugregarinida Léger, 1900 (Syn. Eugregarinorida Grassé, 1953)

Extracellularly parasitic eugregarines represent the most abundant and best studied group within the class Gregarinida. The trophozoites use epimerite (septate gregarines) or mucron (aseptate gregarines) for their attachment to the host epithelium. Pairing of trophozoites is, same as in Archigregarinida, followed by the encystment of gamonts, producing sporocysts each with eight sporozoites. Eugregarinida

comprises over 1600 species belonging to about 240 genera containing aseptate or septate species.

Order Neogregarinida Grassé, 1953 (Syn. Neogregarinorida Grassé, 1953)

Neogregarines develop intracellularly in the host tissue. After invading the host, they undergo multiple rounds of merogony. The resulting stages known as merozoites spread the infection to other tissues of the host, such as gonads. Usually small gamonts produce a low number of gametes and neogregarinid sporocysts contain eight sporozoites. The order is subdivided into six families of insect parasites.

Cryptosporidia

The genus *Cryptosporidium* was established to accommodate tiny epicellular parasites found in the mouse gastric glands (*C. muris*) and intestine (*C. parvum*). Following their discovery almost 100 years ago, Tyzzer experimentally verified the life cycle and correctly speculated about an autoinfection within the host (Šlapeta 2009). Yet it was only in the 1980s that cryptosporidia were identified as causative agents of cryptosporidiosis, an important waterborne human disease. In 1993 a large waterborne outbreak affected an estimated 400,000 persons in Milwaukee, mostly infected by contaminated water (MacKenzie et al. 1995). The cryptosporidiosis manifests as potentially devastating diarrhea, for which no effective therapy is currently available.

After being released from oocysts in the gastrointestinal tract, the infective sporozoites attach themselves to the host cell membrane and become enveloped by its extended folds (Fig. 12) (Valigurová et al. 2008). A specialized structure called the feeder organelle is formed at the attachment site to facilitate the uptake of nutrients from the host cell by the parasite (Fig. 13). *Cryptosporidium* then undergoes asexual and sexual reproductions, which both have the potential for autoinfection, leading to persistent infection with massive shedding of oocysts in the feces (Fig. 13).

Environmentally resistant oocysts measure 4–8 μm in diameter and are characterized by a single suture at one pole (Fig. 12). *Cryptosporidium* completes the development within a single host, and the oocysts are fully infectious when excreted. The oocysts are spread via host-to-host transmission and indirectly as the waterborne or food-borne pathogens. There are 30 named species affecting virtually all vertebrates. Genotyping of diverse isolates revealed a diverse spectrum of host-specific and zoonotic genotypes. Cattle are considered to be the reservoir for the zoonotic (animal-to-human) transmission.

The traditional classification of *Cryptosporidium* within the coccidians has now been securely rejected, based on comparative ultrastructural and genomic data. The current view holds that the phylogenetic position of cryptosporidia is at the base of the core apicomplexan and gregarine divergence (Morrison 2009).

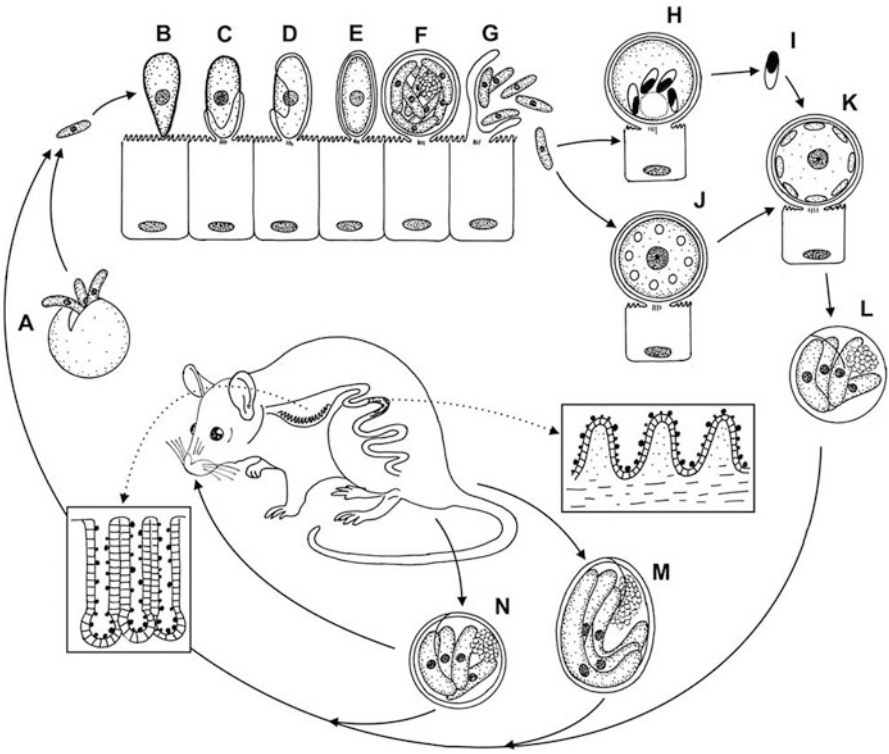

Fig. 12 Schematic drawing of the life cycle of *Cryptosporidium parvum* and *Cryptosporidium muris* in a mouse. Sporozoites are released from a mature oocyst through an open suture (*A*); upon contact with the host epithelium (*B*), sporozoites are enveloped by extended folds of the host membrane (*C–E*); upon epicellularly located merogony (*F*), merozoites are released (*G*) and transform into either microgametocytes (*H*), which produces microgametes (*I*) or macrogametocyte (*J*); upon their fusion (*K*) four sporozoites are formed during sporogony (*L*). Mouse can be infected with either *C. parvum* (*M*) and/or *C. muris* (*N*), confined to the intestine and gastric glands (insets), respectively

Coccidia

Order Eucoccidiorida Léger and Duboscq, 1910

Merogony is present, mostly parasites of vertebrates and less frequently of invertebrates. Besides the order Eucoccidiorida, there are some 20 named species from marine invertebrates classified into separate classes Agamococcidiorida and Protococcidiorida, distinguished by the absence of merogony and/or gamogony, respectively.

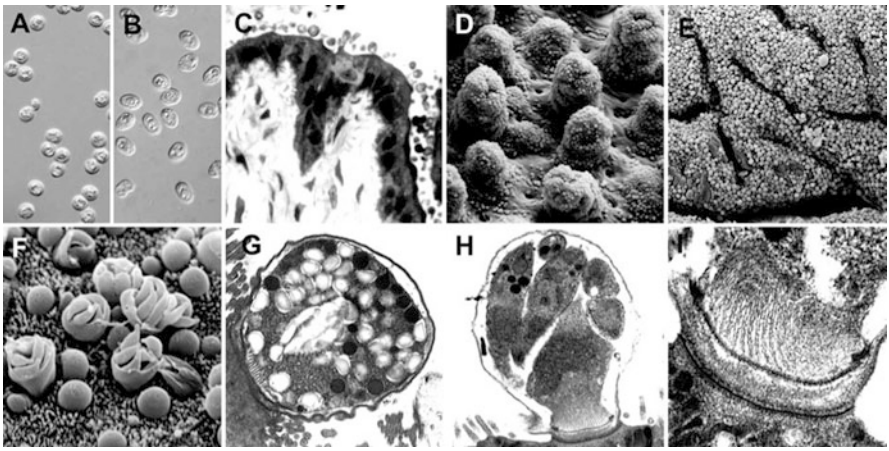

Fig. 13 Representative morphology of cryptosporidian life cycle stages. Spherical oocysts of intestinal *Cryptosporidium saurophilum* (**a**) and ellipsoidal oocysts of gastric *Cryptosporidium muris* (**b**) from a lizard and a mouse, respectively; surface of the intestinal mucosa showing numerous developmental stages of *C. saurophilum* (**c**); surface of the swine intestinal mucosa with prominent villi virtually covered with stages of *C. parvum* (**d**); detail of the gecko intestinal mucosa, heavily infected with *C. saurophilum* (**e**); various developmental stages of *C. saurophilum*, with merozoites undergoing liberation (**f**); macrogamont of *C. parvum* on the surface of an infected enterocyte, revealing the feeder organelle (**g**); developing meront of *C. parvum* (**h**) and detail of the feeder organelle of the same species (**i**). Light microscopy (**a**–**c**) and scanning (**d**–**f**) and transmission electron microscopy (**g**–**i**)

Suborder Adeleorina Léger, 1911

Two groups belong to this suborder: (i) monoxenous coccidians of invertebrates (herein referred to as adelines) and (ii) heteroxenous coccidians cycling between blood-feeding invertebrates (definitive hosts) and various vertebrates (intermediate hosts), usually referred to as haemogregarines. The genus *Klossiella* (Klossiellidae), involving monoxenous coccidia of mammals and reptiles, represents an exception. Phylogenetic studies indicate that entire group is monophyletic, characterized also by several morphological and developmental features. Microgamonts produce usually only one to four microgametes, which associate with the macrogamete in syzygy. Other characteristic features of Adeleorina are the absence of endodyogony and the enclosure of sporozoites in sporocysts and/or oocysts.

So far, there are ~500 named species, almost certainly a great underestimate of the real diversity. Members of the genera *Adelina* and *Adelea* infect mostly insects, whereas *Klossia* is a well-studied coccidium from mollusks. The haemogregarines (Hepatozoidae, Haemogregarinidae, and Dactylosomatidae) comprise several genera, including pathogens of vertebrates, such as *Hepatozoon* from carnivores and reptiles and *Haemogregarina* from fish and turtles (Karadjian et al. 2015). In any case, invertebrates play a role of the definitive host with gamogony in their digestive

system. Then, basically two modes of transmission occur: (i) the inoculative way (*Haemogregarina*, *Dactylosoma*), when the infectious sporozoites enter the vertebrate host during blood feeding, and (ii) alternatively, the parasite is transmitted via the ingestion of an infected definitive (invertebrate) host by the appropriate vertebrate host (*Hepatozoon*, *Haemolivia*, *Karyolyssus*). The latter mode of transmission may even involve a paratenic host (Fig. 14). Regardless of the mode of transmission, the merogonial division of haemogregarines usually takes place in the parenchymatous organs of vertebrates, followed by the formations of infective gametocytes in the circulating red (in the case of *Hepatozoon* also white) blood cells. The next definitive host is infected exclusively by blood feeding (Fig. 14).

Suborder Eimeriorina Léger, 1911

Macrogametes and microgametes develop independently and syzygy is absent. Anisogamous microgamonts produce a large number of flagellated motile microgametes, while the zygote is invariably nonmotile. Sporozoites are always enclosed in a sporocyst. For ~2,500 named species, homoxenous or heteroxenous life cycles have been recorded. Two families comprise species of economic and medical importance.

Family Eimeriidae Minchin, 1903

This family traditionally contains the monoxenous coccidians and arguably is one of the most diversified protist taxa. The formation of environmentally resistant oocysts, usually expelled in host feces, is one of the principal features of Eimeriidae. The general morphology of this easily detectable stage, and especially the numbers of sporocysts and sporozoites within the oocyst, has been widely used to define individual coccidian genera (Figs. 8 and 15). Results of recent phylogenetic studies, however, correlate only poorly with current taxonomy. They also showed that several diagnostic features considered hitherto unique are in fact synapomorphies, shared by several non-related genera.

Life cycle of a typical eimeriid coccidium starts by the ingestion of a sporulated, environmentally resistant oocyst. Following an immediate excystation in the proximal part of the digestive tract, upon invasion of the host epithelia, the sporozoites transform into meronts. These produce numerous merozoites that are consequently released from the ruptured host cell and initiate the next round of merogonial division. Usually there are two to seven asexual generations that differ in the number and morphology of merozoites. The last generation of merozoites eventually becomes intracellular macro- and microgamonts. Macrogamonts can be distinguished by the presence of numerous electron-dense wall-forming bodies, thought to contribute to the formation of the oocyst wall during a later stage of development (Fig. 6). Coccidia of aquatic hosts usually lack this feature, which is attributed to the

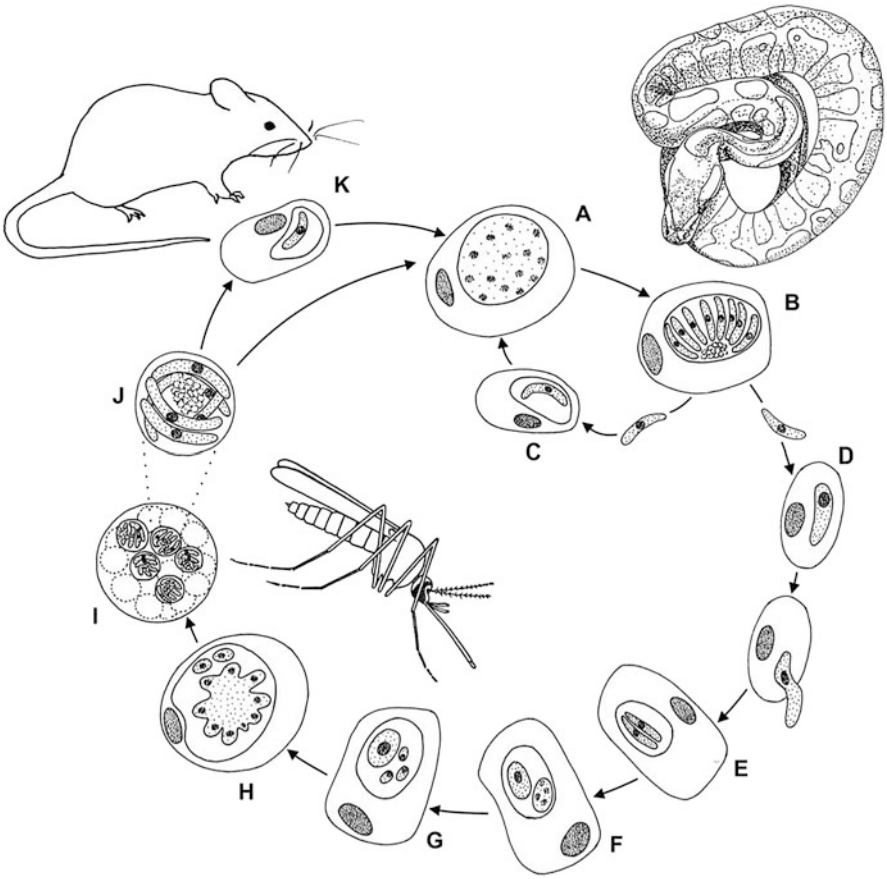

Fig. 14 Schematic drawing of the life cycle of mosquito transmitted *Hepatozoon ayorgbor* in a snake and a mouse. After ingestion of the first intermediate host, merogony takes place in the liver and kidneys of a royal python (*A*, *B*); released merozoites form dormant stages (hypnozoites, *C*) that can probably initiate further merogonial division; merozoites enter red blood cells and transform into gametocytes (*D*); after ingestion by mosquito definitive host, gametocytes enter the fat body cells in host hemocoel, where they associate in pairs in so-called syzygy (*E*); microgamont divides into low number of microgametes (*F*), one of which fuses with the macrogametocyte (*G*) and together form a zygote or young oocyst (*H*); sporoblasts, formed during the asexual division inside the oocyst (*I*), finally develop into sporocysts (*J*); each sporocyst contains several elongated sporozoites and a residual body; an infected mosquito is ingested either by the intermediate host (a phyton) or by the paratenic host (a mouse), in which dormant stages develop (*K*) and wait for the ingestion by a phyton

absence of a prominent oocyst wall in these species. Young oocysts are usually expelled in feces unsporulated and noninfective, as their development is only terminated in the external environment, where further divisions of their contents lead to the formation of sporozoites enveloped by sporocysts (Fig. 7). The entire life cycle is usually completed within 1–3 weeks.

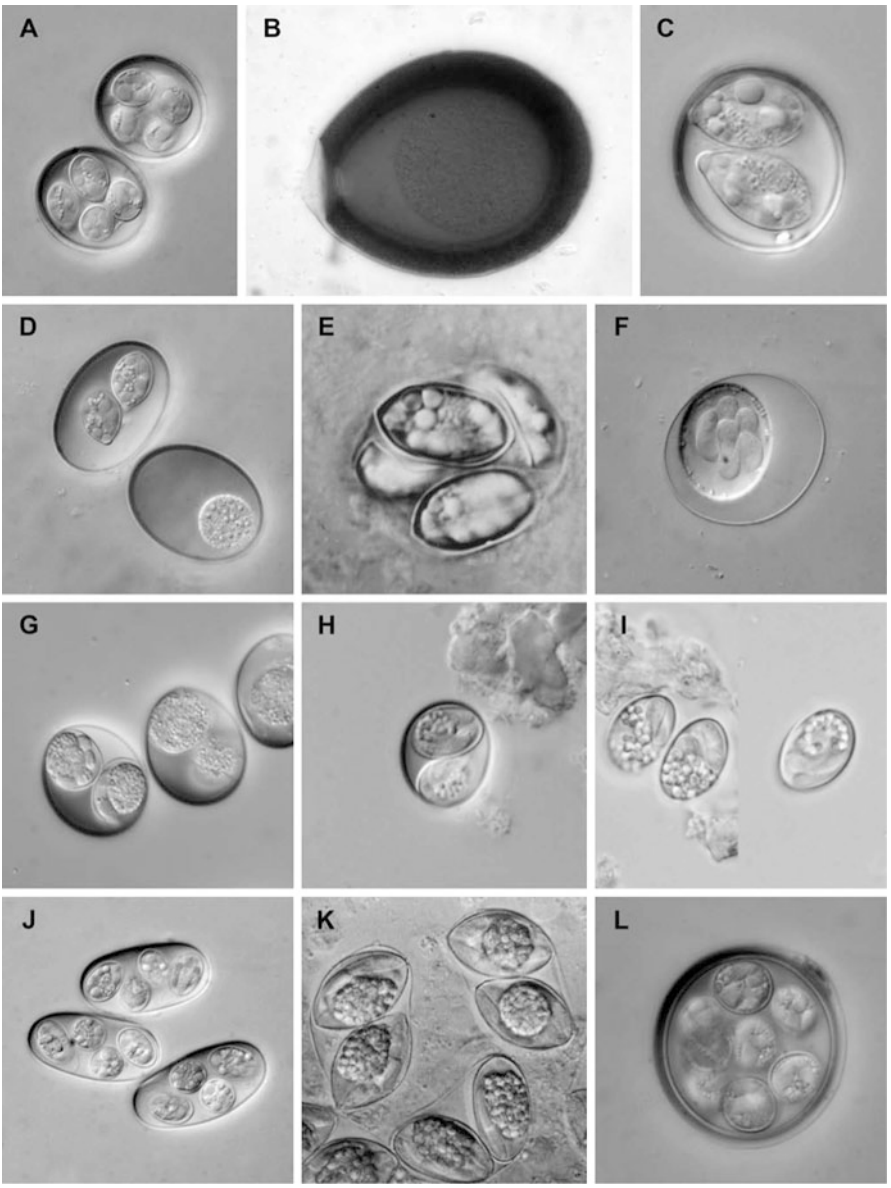

Fig. 15 Representative morphology of sporulated coccidian oocysts from the intestinal content (a–d, f–i, l) or other organs (e, k, j) of various hosts. *Eimeria elephantuli* from a rufous elephant shrew (**a**); giant oocyst of *Eimeria cameli* from a Bactrian camel (**b**); *Isospora* sp. from a passeriform bird (**c**); *Isospora jaracimrmani* from a chameleon (**d**); *Goussia alburni* from the fat body of a perch (**e**); *Caryospora kutzeri* from a kestrel (**f**); *Cystoisospora vulpina* from a fox (**g**); *Toxoplasma gondii* from a domestic cat (**h**); oocyst (*left*) and free sporocyst (*right*) of *Sarcocystis* sp. from a domestic dog (**i**); *Choleoeimeria hirbayah* from the gall bladder of a Yemen chameleon (**j**); *Hyaloklossia lieberkuehni* from the kidney of a green frog (**k**); *Adelina dimidiata* from a centipede of the genus *Scolopendra* (**l**)

Genus *Eimeria*

With ~1,700 described species, this genus is one of the most diversified eukaryotic genera. Traditional definition of the genus is simple and straightforward – it comprises monoxenous coccidians with tetrasporocystic oocysts and dizoic sporocysts (Figs. 7, 8, and 15). However, recent studies based on morphology as well as molecular phylogeny do not support this sensu lato delimitation of monoxenous coccidians. To solve the paraphyly of *Eimeria*, several genera (*Acroeimeria*, *Choleoeimeria*, *Epieimeria*, *Goussia*) have been established. The current view holds that the presence or absence of the Stieda body distinguishes *Eimeria* sensu stricto from other coccidians with *Eimeria*-like oocysts.

The striking diversity of the genus *Eimeria* may be the result of its high host specificity. Poly-infections with several *Eimeria* species are typical for many hosts (ruminants, rodents, lagomorphs, gallinaceous birds), and organ specificity and ecological within-host niche partitioning (Fig. 3) further contribute to the diversity of this genus. Although most *Eimeria* develop in the host intestinal epithelium, bile ducts, kidneys, and even placenta serve as sites of development for some species.

Thanks to features such as direct life cycle, short-generation interval, very high production of oocysts, and intracellular multiplication inside the host, *Eimeria* qualifies as one of the most detrimental parasites of domestic animals. As a result, intensive animal husbandry, especially in the case of domestic fowl, is virtually impossible without efficient control of coccidioses, either by vaccination or anticoccidial medication. However, *Eimeria* has developed drug resistance against most anticoccidials used today which has led to the requirement for an effective vaccine strategy. Attenuated strains of several coccidia were developed and are widely used for vaccination of domestic fowl. Pathogenicity for domestic mammals and birds is mainly caused by high density of animals of the same age in an artificial environment, where the transmission is substantially facilitated, as coccidians of their wild ancestors are usually only mild pathogens.

Other Genera of the Eimeriidae

Monoxenous coccidians of the genera *Caryospora*, *Cyclospora*, *Isospora*, *Tyzzeria*, and *Wenyonella* also exploit vertebrates having life cycle similar to *Eimeria* (Fig. 15). Among them, genus *Isospora,* possessing bisporocystic oocysts with tetrazoic sporocysts and Stieda bodies, is by far the most numerous, comprising ~200 species found mainly avian and reptilian hosts. Exceptionally, some avian *Isospora* (formerly assigned to *Atoxoplasma*) form extraintestinal stages, detectable in the blood cells and parenchymatous organs. In contrast to the simplicity of a typical monoxenous life cycle, some *Caryospora* cycle between snakes and rodents in a rather complicated manner, involving the intestinal merogony and gamogony in the predator and further merogony and gamogony in the connective tissue of the prey.

Despite the fact that many species were described already in the nineteenth century, monoxenous eimeriids of invertebrates (genera *Barrouxia*, *Caryotropha*,

Diaspora, *Dorisa*, *Mantonella*, *Ovivora*) belong to the least known coccidians. So far, no sequences are available from these obscure and neglected organisms.

Family Sarcocystidae Poche, 1913

Traditionally, the family Sarcocystidae comprised heteroxenous coccidians of vertebrates, with the merogony and gamogony located in the intermediate vertebrate prey and final carnivorous predator, respectively. The family accommodates ~350 named species, some of great medical and veterinary significance, with *Sarcocystis* and *Toxoplasma* being the most prominent members. The family is well defined by the unique morphology of its bisporocystous tetrazoic oocysts. The sporocyst wall is composed of four plates joined by sutures that enable the excystation in host's intestine (Fig. 16).

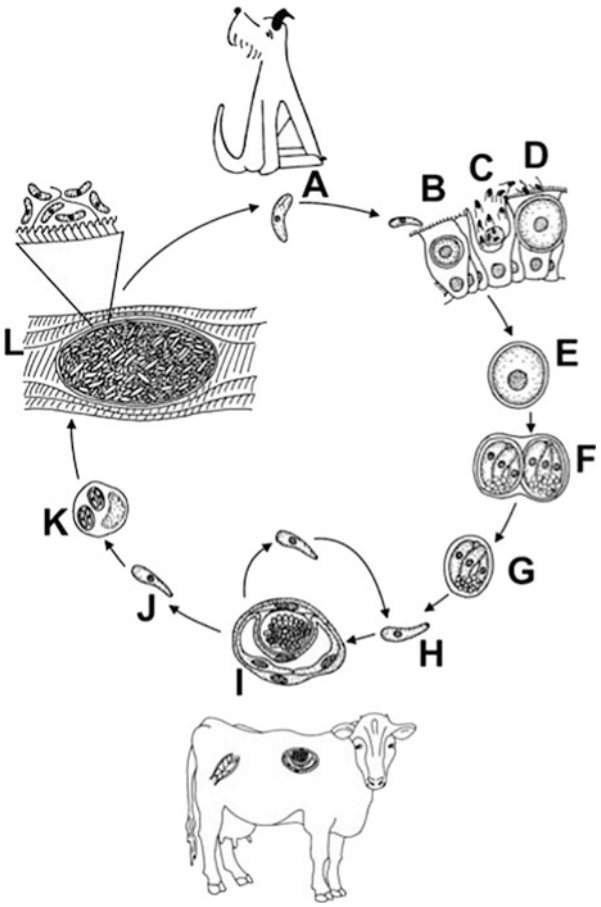

Fig. 16 Schematic drawing of the life cycle of *Sarcocystis cruzi* cycling between cattle and dogs. After ingestion of infected meat by definitive host (dog), cystozoites are released from a tissue cyst during digestion (*A*); cystozoite invades intestinal mucosa, where macrogametocytes (*B*), microgametocytes (*C*), and zygotes (*D*) are formed; unsporulated oocysts (*E*) sporulate in situ (*F*); upon rupture of the oocyst wall, sporocysts are released into the environment and subsequently ingested by the intermediate host (*G*); released sporozoites (*H*) undergo cyclic merogony in endothelial cells (*I*); liberated merozoites (*J*) then spread via macrophages (*K*) to striated muscles, where the tissue cysts develop (*L*); inset shows detail of the cyst wall with invaginations dividing the content into chambers

Phylogenetically, the family is split into two major branches: (i) a well-defined monophyletic assemblage of *Sarcocystis* (including the former genus *Frenkelia*) referred to as Sarcocystidae and (ii) a group consisting of closely related *Toxoplasma*, *Hammondia*, and *Neospora* and somewhat less related *Besnoitia*, *Cystoisospora*, and *Hyaloklossia*. Moreover, recent phylogenetic studies revealed surprisingly close relatedness of some monoxenous coccidians, such as *Cystoisospora* from the intestine of carnivores and *Hyaloklossia* from the kidneys of frogs (Fig. 15), with the above-mentioned heteroxenous genera.

Genus *Sarcocystis*

The most species-rich genus within the Sarcocystidae contains invariably heteroxenous members, cycling between predator (definitive host) and prey (intermediate host). Although the causative agent of macroscopically visible cysts in muscles of various animals was named *Sarcocystis* by Lankester in 1882, the life cycle of these widespread parasites was not deciphered until the 1970s. The definitive host becomes infected by ingestion of meat containing tissue cysts with cystozoites. Directly after that, gamogony takes place in deeper layers of the intestinal mucosa, often close to the lamina propria, and sporulation typically occurs in situ. In most cases, the oocyst wall ruptures in the intestine, and liberated sporocysts are shed in the feces of the definitive host (Fig. 16). Upon ingestion of sporocysts with contaminated food or water by the intermediate host, merogony occurs in its parenchymatous organs, followed by the formation in muscles of tissue cysts, often of macroscopic dimensions and species-specific morphology. Within the cysts, the parasite multiplies by endodyogony or endopolygony, resulting into the formation of metrocytes and later thousands of infectious cystozoites. On the ultrastructural level, the primary wall of the sarcocysts usually bears distinct micro-ornamentation, often with bizarre protrusions (Fig. 17). Numerous invaginations stretching inside the sarcocysts divide its content into chambers, in which cystozoites develop. Cystozoites are characterized by numerous closely packed micronemes (Fig. 17).

The life cycle is based exclusively on predator-prey trophic relationships. The spectrum of definitive hosts comprises carnivorous mammals (especially canids, felids, and marsupials), raptorial birds and owls, and a variety of snakes. Preys of these predators represent intermediate hosts (Fig. 16). For example, dogs (and other canines) are definitive hosts of several *Sarcocystis* species, cycling through goats, sheep, cattle, camels, etc. The so-called dihomoxenous development has been described for *Sarcocystis* affecting lizards in isolated island ecosystems, where the same host species alternatively serves as intermediate and definitive host. In many cases, host specificity of individual *Sarcocystis* species is poorly understood or even unknown. Moreover, more than 50% of species are known only from the intermediate host. At least one species – *Sarcocystis hominis* – is cycling between humans (definitive host) and cattle (intermediate host). Some species possess a remarkable affinity for the central nervous system, where either tissue cysts (species formerly referred to as *Frenkelia*) or meronts (*Sarcocystis neurona* in the brains of its aberrant

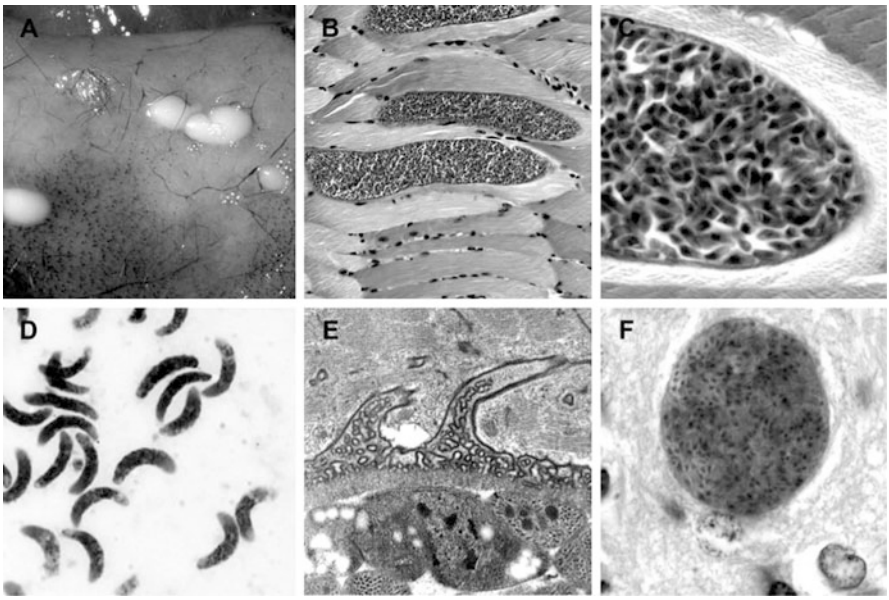

Fig. 17 Representative morphology of sarcosporidian stages in tissues of intermediate hosts. Macroscopically visible cysts of *Sarcocystis dirumpens* from the connective tissue of a rodent (**a**); elongated microscopic cysts of *Sarcocystis dispersa* from the muscles of a mouse (**b**); periphery of mature cyst of *S. dispersa* packed with cystozoites (**c**); cystozoites released from smashed cyst of *Sarcocystis muris* (**d**); species-specific structure of protrusions of primary cyst wall of *Sarcocystis lacertae* from a wall lizard (**e**); characteristic rounded cyst of *Toxoplasma gondii* from brain (**f**). Light microscopy (**a**, **d**), histological sections (**b**, **c**, **f**), and transmission electron microscopy (**e**)

hosts, horses) develop. However, in most cases, clinical significance of *Sarcocystis* infections is generally low both for the definitive and intermediate hosts (Fig. 17).

Genus *Toxoplasma* (and Related Genera)

Since humans are intermediate hosts for *Toxoplasma gondii*, it is understandably the most studied coccidian (Weiss and Kim 2014). The parasite was described already in the beginning of the twentieth century from the brain of a North African rodent (common gundi), but its life cycle remained unknown until 1970, when the domestic cat was identified as its definitive host.

After oral ingestion of sporulated oocysts, asexual multiplication occurs in the intermediate host (Fig. 18). In the so-called "acute" phase, the merogonial development (by endodyogony) occurs in various tissues, leading to the formation of short tachyzoites organized in pseudocysts. This process is repeated many times, ending by the penetration of tachyzoites into the neural (and other) tissues, where cysts are formed. In contrast to the *Sarcocystis* cysts, the tissue cysts of *Toxoplasma* are noticeably smaller and nonseptate (Fig. 17). Inside the cysts, continuous

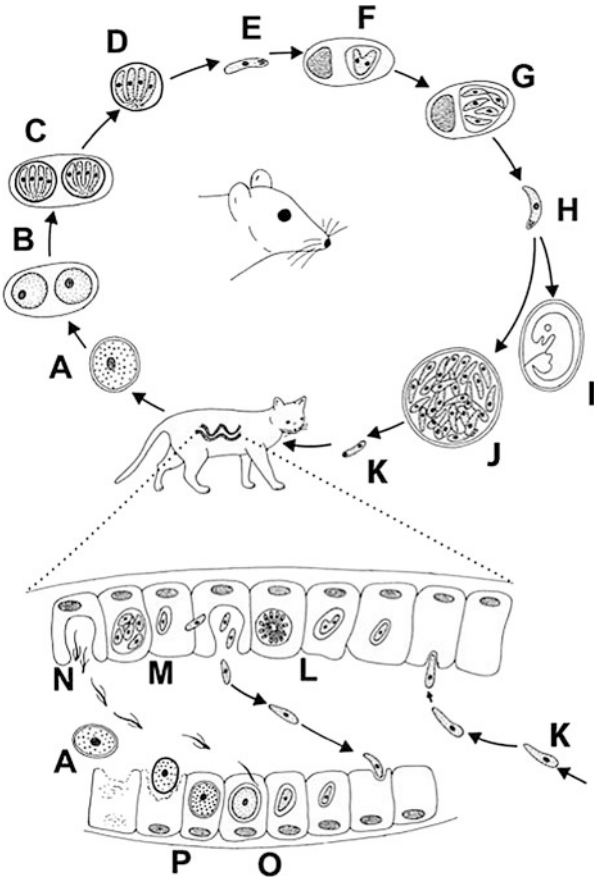

Fig. 18 Schematic drawing of the life cycle of *Toxoplasma gondii* in a mouse and a cat. Unsporulated oocyst in fecal content (*A*); upon sporulation (*B*), two sporocysts each with four sporozoites are formed (*C*); oocyst wall usually ruptures at this stage (*D*); after ingestion by an intermediate host, sporozoites (*E*) invade host cells, where they multiply by endodyogony (*F*), gradually fill the cell (*G*) and form extracellular tachyzoites (*H*); these can be transmitted transplacentally into embryos (*I*); tachyzoites invade new cells and form another generation of tissue cysts (*J*), where bradyzoites are formed (*K*). The inset shows intestinal development infected with bradyzoites (*K*) released from cysts from ingested meal; after several rounds of merogony (*L*, *M*), gamogony takes place (*N*–*P*) and unsporulated oocysts are released from the final host (*A*)

endodyogony occurs, producing prolonged bradyzoites. In this dormant stage, the parasite may survive for years, perhaps even decades. Cat as the definitive host typically acquires the infection by ingesting cysts with bradyzoites in the tissue of the intermediate host. After several merogonial generations in the cat's intestine, gamogony takes place in its intestinal epithelia, and unsporulated oocysts are expelled in feces. Importantly, *T. gondii* can be transmitted among intermediate hosts without involving the definitive one, representing a classical example of opportunism in the transmission mode. For example, humans (as well as any other intermediate host) become infected by several alternative routes: (i) by the ingestion of oocysts from the environment, (ii) by the ingestion of bradyzoites in tissue cysts from meat, (iii) by the transfer of tachyzoites transplacentally, and (iv) rarely by the transmission of tachyzoites in milk (Fig. 18). Along with the domestic cat, wild felids also serve as definitive hosts. When intermediate hosts are concerned, *T. gondii* infects hundreds of mammalian species, less often also birds and rarely some reptiles. Without any doubt, *T. gondii* is the most prevalent parasite of

humankind and one of the most widely distributed parasites of homeotherms in general.

There are two coccidian genera closely related to *Toxoplasma*: *Neospora caninum* exploits dogs as the definitive hosts, while the species of *Hammondia* cycle either through cats or dogs. Only after molecular techniques allowed distinguishing between *T. gondii* and *N. caninum*, the latter turned out to be a potentially serious pathogen of ruminants and dogs. Interestingly, despite its wide distribution and intense research, its life cycle was elucidated only in 1998.

Family Aggregatidae Labbé, 1899

This is a relatively small family (~20 named species) of heteroxenous coccidia from marine invertebrates. The type species *Aggregata eberthi* circulates between crabs and cuttlefish or octopus. Gamogony takes place within the intestine of definitive host (cephalopod), where macroscopically visible oocysts containing thousands of sporocysts are formed. Water contaminated with sporocysts is ingested by crabs, in which extraintestinal merogony occurs. Life cycle is finished by the ingestion of the infected crab by the cephalopod.

Family Lankesterellidae Nöller, 1920

A unique feature of this family is the absence of environmentally resistant oocysts. About 30 named species belonging to the genera *Lankesterella* and *Schellackia* invariably have a heteroxenous life cycle. Frogs and lizards serve as definitive hosts, in the intestine of which gamogony occurs. Oocysts lack sporocysts and harbor variable numbers of sporozoites, which upon exit in situ from the thin-walled oocysts enter the blood cells. The merogony, gamogony, and sporogony of *Lankesterella* occur in the frog's intestine, while sporozoites mature in leeches, which are thought to be principal vectors. *Schellackia* from lizards possesses the same morphological and developmental traits; however, it has only eight sporozoites per oocyst and is transmitted by mosquitoes (Fig. 20).

Haemosporidia

Haemosporidians and piroplasms constitute a phylogenetically well-defined group (e.g., Outlawa and Ricklefs 2011) with obligatory heteroxenous life cycles (Fig. 19). Haemosporidian genera can be distinguished on the basis of the erythrocytic stage morphology, localization of endogenous development in vertebrate host, and the type of invertebrate vector. Merogony of individual species occurs in vertebrate hosts (amphibians, reptiles, birds, and mammals) which serve as intermediate hosts, while sporogony takes place in a broad spectrum of blood-feeding Diptera. Parasites are taken up with the blood meal by the vector, where fertilization occurs and a

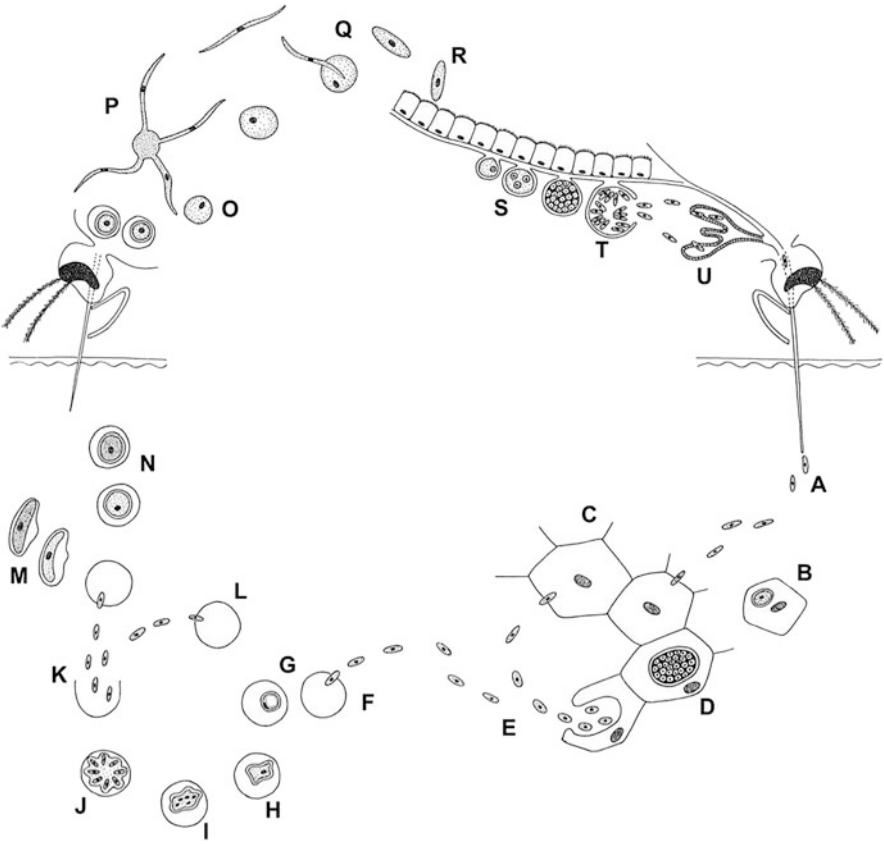

Fig. 19 Schematic drawing of the life cycle of *Plasmodium falciparum* in primate and mosquito hosts. During blood feeding by a mosquito, sporozoites (*A*) are injected into the blood; they enter hepatic cells and either turn into dormozoites (*B*) or active meronts (*C*), which undergo exoerythrocytic merogony (*D*); after release from the liver, merozoites (*E*) invade the red blood cells (*F*); from a characteristic ring stage (*G*), they produce through merogonial division (*H*–*J*), a species-specific number of merozoites (*K*) that either repeat the cycle (*L*) or transfer into gametes; the immature gametes, like other blood stages, have species-specific morphology. Here stages of *Plasmodium falciparum* (*M*) and *Plasmodium malariae* (*N*) are shown; during blood feeding, gametocytes are taken up by another mosquito where they turn into mature macrogametes (*O*) and microgametes (*P*) that copulate (*Q*); the ookinete (*R*) penetrates the intestinal wall and undergoes sporogony (*S*), in the course of which it substantially grows and produces numerous sporozoites (*T*); these invade the salivary glands (*U*) and during the next blood feeding enter another intermediate host

motile zygote (ookinete) is formed. The ookinete actively enters hemocoel by penetrating the midgut wall, rounds up, and transforms into the oocyst. Large oocysts of *Plasmodium* and *Haemoproteus* transmitted by mosquitoes and hippoboscid flies, respectively, subdivide their contents into several sporoblasts, from which hundreds of sporozoites bud off. Haemosporidians with small oocysts

– *Leucocytozoon* (transmitted by black flies) and species of *Parahaemoproteus* (transmitted by biting midges) – produce just one sporoblast with less than a hundred sporozoites. The oocyst ruptures, and the freed naked sporozoites with rudimentary apical complex migrate into salivary glands, where they develop organelles such as rhoptries and micronemes.

Sporozoites injected into the blood of vertebrate hosts by the vectors transform into the exoerythrocytic meronts (Figs. 19 and 20), known to develop most frequently in the liver but found also in the spleen, lungs, kidneys, heart, skeletal musculature, and endothelium of other organs. The megalomeront stage is characteristic of second-generation merogony of *Leucocytozoon*, *Hepatocystis*, and (*Para*) *Haemoproteus*. The prepatent period varies from 2 to 3 weeks. The process of transformation of sporozoites and exoerythrocytic merozoites into trophozoites inside host cells includes a rapid degeneration of the inner double-membrane layer, subpellicular microtubules, polar rings, rhoptries, and micronemes. Within erythrocytes, trophozoites of *Plasmodium* and (*Para*)*Haemoproteus* are localized in the parasitophorous vacuole and absorb host cell cytoplasmic content via a micropyle. Haemosporidians with the intraerythrocytic development (*Plasmodium* and (*Para*) *Haemoproteus*) turn host hemoglobin into a characteristic pigment hemozoin, easily discernible under the microscope. Rapid growth of trophozoites is finalized by the formation of meronts. Members of the genus *Leucocytozoon* depart from the general scheme, as they infect a significantly wider range of host cells, and when infecting erythrocytes digest hemoglobin without the formation of hemozoin granules. Gametocytes of haemosporidians develop only in the blood cells, and individual human malaria species can be distinguished based on their morphology. The life cycle is closed when the gametocytes enter the appropriate vector during blood feeding, where they undergo fertilization and formation of the ookinete (Figs. 19, 20, and 21).

Genus *Plasmodium*

After injection into host blood, sporozoites rapidly attack cells of various inner organs (e.g., hepatocytes in mammals), where the asexual exoerythrocytic division followed by transformation into merozoites occurs. After penetration into erythrocytes, merozoites initiate erythrocytic merogony and develop into meronts. Even though amplification via asexual reproduction in blood cells is not genetically limited in terms of the number of divisions, in each generation a certain number of merozoites develop into macrogamonts (macrogametocytes) and microgamonts (microgametocytes) after entering new erythrocytes. These stages then await ingestion by a mosquito, where each macrogamont matures into a macrogamete, while each microgamont produces six to eight flagellate microgametes (exflagellation) (Fig. 19).

It is often an overlooked fact that only less than 2% of known *Plasmodium* species infect humans, namely, the relatively rare *P. malariae*, *P. ovale*, and simian *P. knowlesi*, followed by *P. vivax* which is responsible for approximately 20% of human malaria worldwide and by far the most pathogenic species *P. falciparum*, which represents the majority of human cases. All four human *Plasmodium* species

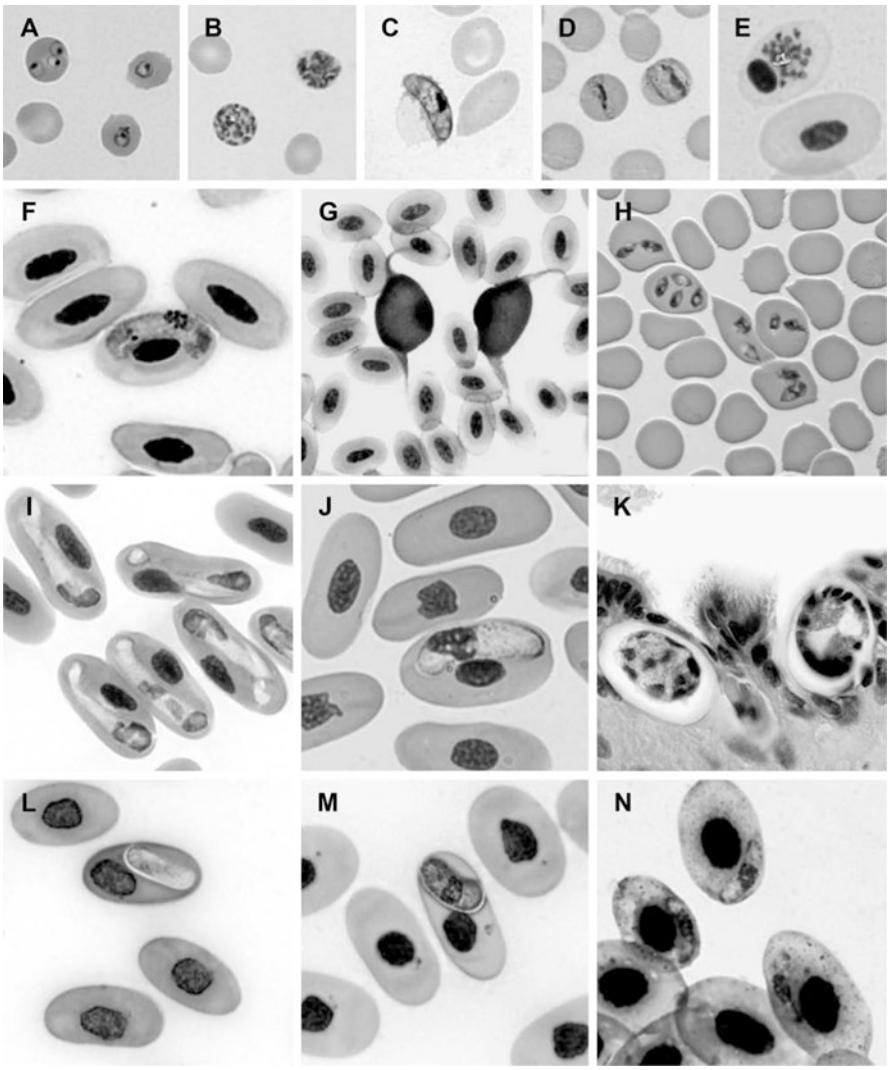

Fig. 20 Representative morphology of apicomplexan stages in the blood cells of vertebrates. Ring stages (**a**), merogonial rosette stages (**b**), and a gametocyte (**c**) of *Plasmodium falciparum* from human blood; characteristic striped meronts of *Plasmodium malariae* from human blood (**d**); *Plasmodium gallinaceum* in an erythrocyte of a fowl (**e**); gametocyte of *Haemoproteus* sp. from an avian host (**f**); gametocyte of *Leucocytozoon* sp. from an avian host (**g**); merozoites of *Babesia canis* from a domestic dog (**h**); gametocyte of *Hepatozoon* sp. from a blue-lipped sea krait (**i**); gametocyte of *Hepatozoon ayorgbor* from a ball python (**j**); merogonial stages of *Hepatozoon* sp. from the lungs of a blue-lipped sea krait (**k**); gametocyte of *Haemolivia mauritanica* from a Greek tortoise (**l**); gametocyte of *Haemogregarina stepanowi* from a swamp turtle (**m**); sporozoite of *Lankesterella minima* from a green frog (**n**). Light microscopy (**a**–**j**, **l**–**n**) and histological section (**k**)

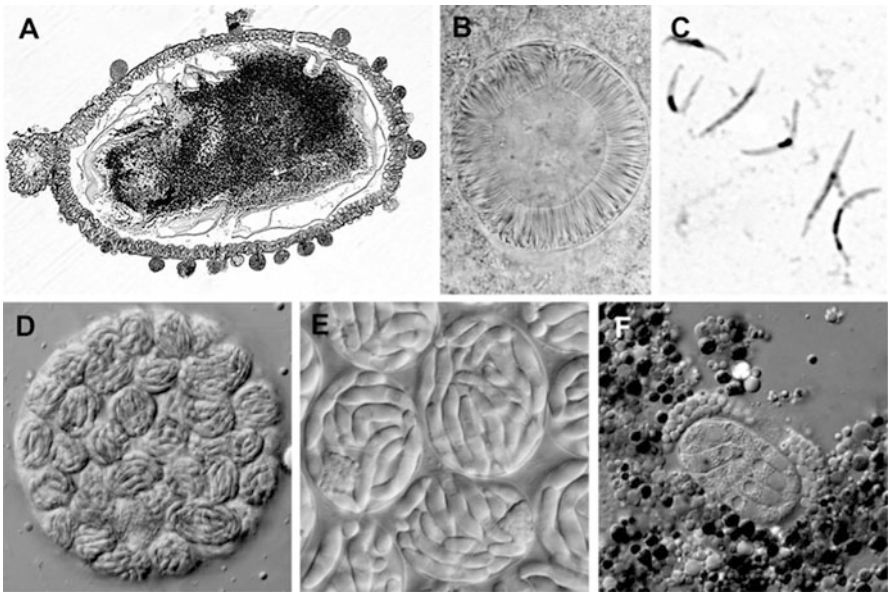

Fig. 21 Representative morphology of apicomplexan stages in the insect vector. The oocysts of *Plasmodium vivax* on the outer intestinal wall of anopheline mosquito female (**a**); the oocyst of *Plasmodium yoelii* with typical wheel-like formation of sporozoites and a central core (**b**, foto J. Vávra) and sporozoites (**c**) from vector salivary glands. Oocyst (**d**) and a detail of sporocysts (**e**) of *Hepatozoon ayorgbor* from the hemocoel of its mosquito vector; sporocyst of *Haemolivia mauritanica* from a hard-bodied tick (**f**). Histological section (**a**), fresh squash preparation (**b**, **d**–**f**), and light microscopy (**c**)

are closely related to various simian species, and *P. falciparum* seems to have been acquired by humans from gorillas only relatively recently (Prugnolle et al. 2011). Lately developed amplification of *Plasmodium* DNA from host feces allowed an insight into the diversity of *Plasmodium* species in African and Asian great apes, sharpening significantly the view on evolution of *Plasmodium* in humans and suggesting some level of cross-species transmission between humans and nonhuman primates. Almost 50 other *Plasmodium* species belonging to three subgenera are transmitted exclusively by anopheline mosquitoes (*Anopheles*) to various mammals, mostly rodents and primates. The remaining five subgenera comprise of more than 40 species attacking birds are transmitted mainly by the *Culex* mosquitoes. In general, avian species do not cause serious diseases in their hosts, with highly pathogenic species, such as *P. gallinaceum* in chicken and *P. relictum* in wild birds, being exceptions. With almost hundred species described to date, reptiles (mainly lizards) host about half of all named *Plasmodium* species. Vectors of *Plasmodium* parasitizing cold-blooded vertebrates are mosquitoes (*Culex*, *Aedes*), phlebotomine sand flies (*Phlebotomus*, *Lutzomyia*), and biting midges (*Culicoides*). Interestingly, so far only two species have been described from amphibian hosts.

Members of the genus *Hepatocystis* (25 species) are considered nonpathogenic and are found in reptiles, birds, and mammals, particularly in bats and monkeys. Phylogenetic studies indicate that *Hepatocystis* represents just an internal group among the mammalian plasmodia. Since oocysts of these less studied haemosporidians develop in the head and thorax of biting midges, the transmission is likely mediated by the ingestion of vectors.

Genera *Haemoproteus* and *Leucocytozoon*

Species of both genera undergo development similar to *Plasmodium* with the following exceptions. Asexual reproduction is limited to the exoerythrocytic merogony that occurs in the endothelial cells (*Haemoproteus*) or hepatocytes (*Leucocytozoon*). A unique feature of the life cycle of *Leucocytozoon* is that huge megaloschizonts develop in host macrophages, producing millions of merozoites. The erythrocytic merogony is absent, and merozoites enter erythrocytes (*Haemoproteus*) or leukocytes and immature erythrocytes (*Leucocytozoon*) only to develop into gametocytes (Fig. 20).

The genus *Haemoproteus* includes over 130 morphologically defined species of avian blood parasites (Valkiūnas 2004; Peirce 2005); however, some reptilian blood parasites are also accommodated within this genus. An absolute majority of species infecting birds belongs to the subgenus *Parahaemoproteus* and are transmitted by biting midges (Ceratopogonidae), whereas sporogony of six named species from the subgenus *Haemoproteus* takes place in hippoboscid flies (Hippoboscidae). The genus *Leucocytozoon* is also confined to the avian hosts and is subdivided into the subgenus *Leucocytozoon* with approximately 40 species transmitted by black flies (Simuliidae) and the monospecific subgenus *Aikiba*, the sporogony of which takes place in biting midges (Figs. 19 and 20).

Piroplasmida

This order is a diverse group of haemosporidians (sometimes called piroplasms or piroplasmids), owing their name to pear-shaped (piriform) intracellular stages formed in the host erythrocytes. Unique morphological features of piroplasmids are the absence of conoid and the reduction of the apical complex to the polar ring. Extreme reduction is characteristic for the family Theileriidae, which lacks subpellicular microtubules, the inner membrane complex, as well as the micronemes. After entering the host cell, piroplasms escape from the parasitophorous vacuole and, with few exceptions (*Theileria buffeli*, *T. separata*), digest host hemoglobin without producing any pigment or other visible residues. Heteroxenous life cycle is composed of merogony taking place in a wide range of mammals (to a lesser extent in birds and reptiles), with gamogony and sporogony occurring in the gut and salivary glands of invertebrates, respectively. So far, only hard ticks (Ixodidae) were identified as vectors, although for the majority of species vectors are yet to be found (Fig. 22).

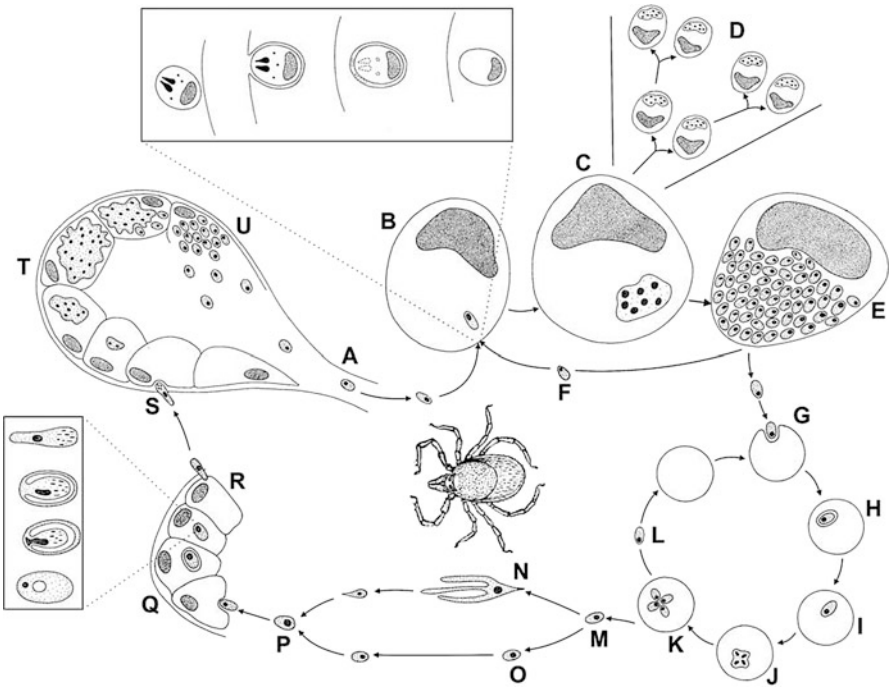

Fig. 22 Schematic drawing of the life cycle of *Theileria annulata* in a cow and a tick. Sporozoite injected with saliva of feeding tick (*A*) in the vertebrate host enters its macrophage (*B*); *inset* shows the invasion process characteristic for *Theileria* without reorientation and escape from the parasitophorous vacuole; merogonial division (*C*) induces a unique clonal expansion of the infected leukocytes (*D*); leukocytes full of merozoites (*E*), although known as Koch's bodies, rupture and released merozoites either repeat the cycle (*F*) or enter erythrocytes (*G*); merozoite also escapes there from the parasitophorous vacuole (*H*, *I*), and multiplication leads to the formation of the Maltese cross composed of four merozoites (*J*, *K*); there can be several merogonial divisions (*L*); upon engorgement during blood feeding by the tick (*M*), gamogony occurs in its intestine, where flagellated microgametocyte (*N*) fuses with macrogametocyte (*O*); motile zygote (*P*) invades epithelial cells, where it transforms into a motile kinete (*Q*); the peculiar transformation is shown in the inset; after traversing, the gut wall (*R*) enters salivary glands (*S*), where sporogony takes place (*T*), producing an enormous number of infectious sporozoites (*U*) terminating the cycle during following blood feeding

Family Babesiidae

In mammalian erythrocytes, trophozoites of the genus *Babesia* usually produce by binary fission two (rarely four) daughter merozoites, which enter new red blood cells (Schnittger et al. 2012). However, development within the invertebrate host is quite complicated and is known for only a few species. After ingestion by a tick, the parasites leave the blood cells and develop into pseudopodia-like gametes (spiky-rayed bodies), which fuse into motile zygote and form a primary kinete. Due to the penetration of the elongated kinetes (vermicules) into numerous internal organs of

the tick, including the ovaries, and in some species, the infection can be passed through transovarial transmission into the next generation, while the ticks can maintain the infection for two or more generations. Further development occurs in the hemocoel and various organs, where *Babesia* produces new secondary kinetes, some of which migrate to the salivary glands. During blood feeding that takes several hours or days, piroplasms rapidly multiply and eventually transform into sporonts and infectious sporozoites. Finally, sporozoites from the tick saliva are injected into the vertebrate host where they directly infect red blood cells and develop into the well-known piriform stages.

The genus *Babesia* contains more than 110 species; several globally distributed species (depending on the range of their tick vectors) are important pathogens of livestock, such as bovines (*B. bovis*, *B. bigemina*, and *B. divergens*), sheep, goats, horses, pigs, dogs (*B. canis*, *B. gibsoni*), cats, and rodents (Uilenberg 2006). Humans can also be accidentally infected with several species (mostly *B. divergens* or *B. microti* of rodents) (Fig. 20). Human babesiosis occurs mainly in the New World where it is a serious disease, especially in immunocompromised and splenectomized persons (Telford et al. 1993; Lobo et al. 2013).

Family Theileriidae

After injection into the vertebrate host, sporozoites enter the T and B lymphocytes or macrophages by a process significantly different from the invasion process known for the other apicomplexans (Fig. 22). Sporozoites as well as merozoites enter into host cells (lymphocytes and erythrocytes) by zippering in from any orientation. Importantly, the invasion does not require reorientation of the parasite's apical end toward the host cell membrane, with the internalization being much slower than in other apicomplexans. The completely surrounded and internalized sporozoites and merozoites release the contents of their secretory organelles (rhoptries and granular bodies), which apparently allows them to escape from the enclosing parasitophorous vacuole into the host cytoplasm. Once established in the host cytoplasm, the parasite grows and differentiates into a multinucleate schizont and, by a remarkable, yet largely unknown mechanism, transforms infected host lymphocytes into immortal cells, which leads to their clonal expansion (Fig. 22). Leukocytes filled with schizonts are called Koch's bodies. The released merozoites invade erythrocytes, where usually another round of division occurs, producing a generation of merozoites, which in turn infect new erythrocytes, particularly in species with limited or missing intralymphocytic multiplication. Multiplication in erythrocytes results in four merozoites forming characteristic tetrads (the Maltese cross), yet some species (*T. parva*) do not multiply in the red blood cells (erythrocytes), their multiple rounds of asexual division being confined only to lymphocytes. Gamogony occurs in the vector's intestine, where gametes fuse to produce a motile zygote. This stage invades epithelial cells, where it transforms into a single motile kinete similar to the haemosporidian ookinetes, and remains there during the development of the tick (trans-stadial transmission). However, unlike in *Babesia*, the kinete does not

multiply and ceases to further develop in the gut but transverses the gut wall and via the celom and hemolymph reaches and consequently penetrates the cells in salivary glands, where sporogony takes place. Feeding of the tick initiates rapid sporozoite development, and in the glandular epithelium, parasites rapidly multiply and produce an enormous number of sporozoites (up to 100,000 per each kinete) that escape into the salivary ducts (Fig. 22).

The two most important species are *T. parva*, which causes the east coast fever in Africa, and *T. annulata*, the causative agents of the tropical or Mediterranean theileriosis (Mans et al. 2015). About 40 *Theileria* species infect mainly ungulates in Africa and Asia but also Australia's marsupials, foxes, and other hosts. Species such as *T. ovis* are pathogenic to sheep and to other small ruminants; *T. equi* is an important pathogen of horses.

The classical difference between the genera *Theileria* and *Babesia* is the absence of extraerythrocytic asexual multiplication (schizogony) in the latter, while schizogony in *Theileria* occurs in lymph nodes and erythrocytes rather than in erythrocytes alone. Despite such a clear distinction, systematic affiliations of several species of piroplasms, even those with economic impact, remain unresolved. Small piroplasms of equines were recently transferred from the genus *Babesia* to the genus *Theileria*. Even more complicated is the case of *Babesia microti*, whose schizogony in lymphocytes and development and transmission in ticks are more similar to *Theileria*. Phylogenetic studies also indicate that the only two named species of the genus *Cytauxzoon* infecting felids including domestic cats represent just an internal group within the *Theileria-Babesia* clades. However, molecular evidence indicates that all these "problematic" parasites differ both from typical *Theileria* and *Babesia*. As in other apicomplexan groups, molecular tools are becoming increasingly important for phylogenetic delineation of the order Piroplasmida (Sivakumar et al. 2014).

Predatory and Photosynthetic Reminiscence of Apicomplexa

Colpodella was first described by Cienkowski in 1865 yet has not found its evolutionary home until the twenty-first century, when insight into its detailed ultrastructure and molecular phylogeny revealed its close relationship with the core Apicomplexa. *Colpodella* is a small (less than 20 μm long; Fig. 23), usually biflagellated free-living predator of other protists and algae, to which it attaches by its anterior tip containing the apical complex, through which it sucks the cellular content of its prey. After feeding, the organism withdraws flagella and forms a cyst. The major component of the apical complex of colpodellids is a pseudo-conoid, composed of an incomplete ring of subpellicular microtubules, micronemes, and elongated organelles reminiscent of rhoptries. All these organelles are considered plesiomorphies common to all apicomplexans. Furthermore, it has been suggested that the gliding motility used by *Colpodella* to penetrate prey cells is very similar to the mode of motility used by apicomplexans to invade host cells (Gubbels and Duraisingh 2012).

Chromerids are photosynthetic algae closely related to apicomplexans, branching in the frame of colpodellids. The group contains just two named species, *Chromera*

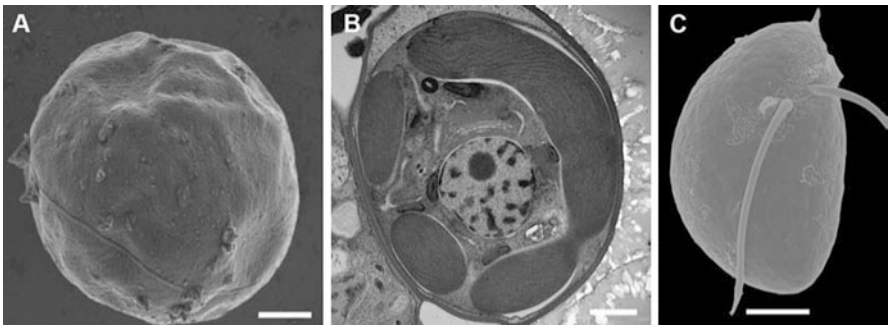

Fig. 23 Morphology of *Chromera velia* and *Colpodella edax*. SEM electromicrograph of *Chromera velia* cell with apparent suture in its cell wall (**a**); TEM electromicrograph of cross-sectioned cell of *C. velia* revealing a giant plastid (**b**); SEM electromicrograph of *Colpodella edax* with two flagella (**c**)

velia and *Vitrella brassicaformis*, isolated from stony corals in Australia (Fig. 23) (Moore et al. 2008; Oborník et al. 2011; Weatherby et al. 2011). The ecology of this organism and the nature of its association with the corals are not fully resolved; however, it has been shown that *C. velia* can infect coral larvae of the genus *Acropora* (Cumbo et al. 2013). The alga hosts a single secondary plastid per cell pigmented by chlorophyll *a*, a novel isoform of isofucoxanthin, and, surprisingly, lacks chlorophyll *c*. Primitive apical complex (pre-conoid) was found in *C. velia*; its presence or absence in *V. brassicaformis* remains to be established (Oborník et al. 2011; Oborník and Lukeš 2013; Portman and Šlapeta 2014; Oborník et al. 2016). Ultrastructural features, noncanonical genetic code in the plastid, and four membranes surrounding the plastid of *Chromera* resembling the non-photosynthetic apicoplast together indicate that *Chromera* possesses characteristics of the relict phototrophic organism leading to the extremely successful phylum Apicomplexa (Fig. 23) (Moore et al. 2008; Janouškovec et al. 2010; Oborník et al. 2016; Woo et al. 2015).

Maintenance and Cultivation

In vitro culture systems represent powerful tools for screening of potential drug candidates. Cultures of apicomplexan parasites such as *Cryptosporidium*, *Eimeria*, *Sarcocystis*, *Neospora*, *Toxoplasma*, *Besnoitia*, *Plasmodium*, *Babesia*, and *Theileria* have been documented, but they are often not productive and capable of sustaining the parasite for only a finite number of replication cycles. The mainstream apicomplexan cell cultures are *Toxoplasma* asexual stages (tachyzoites) in mammalian host tissue cells and *Plasmodium* asexual stages in mammalian red blood cells. The generation of sexual stages is still lacking for *Toxoplasma*, and in vitro animal experimentation is required to fulfill the life cycle (Müller and Hemphill 2013). Cell cultures for *Cryptosporidium* and *Eimeria* remain nonproductive.

Cultivation of any apicomplexan parasite in a good defined cellular system for studies on the proliferative stages is quite complicated because the complex nutritional and environmental characteristics of the host cells are difficult to mimic in vitro. The ultimate goal is to cultivate the parasites in a fully defined medium. In vitro cultivation of apicomplexans is further complicated by the tendencies of most life cycle stages to produce different stages (trophozoites and merozoites transform into merozoites and gamonts, respectively), transfer to a different host, and/or remain as encysted dormant tissue cyst or environmentally resistant oocysts. Only a few examples of life cycle stages have the ability to cycle indefinitely such as those in the mainstream culture systems for *Toxoplasma* and *Plasmodium*.

The availability of cultivation brought many benefits and remains a key research technique for the studies of hematozoans. The asexual stages of *Plasmodium* in the red blood cells are successfully exploited, and a wide array of genetic tools is now available to study malaria in vitro, including stable transfection to study roles of individual genes (de Koning-Ward et al. 2015). Continuous cultivation of *P. falciparum* in a medium containing red blood cells (not fully defined) is fundamental for drug screening and advanced studies of its molecular and cellular biology. Moreover, in 2002, a complete life cycle from sporozoite to sporozoite under in vitro conditions has been achieved for *Plasmodium berghei*, a model malaria species infecting rodents (Al-Olayan et al. 2002; Schuster 2002). Although each parasitic stage requires different cultivation conditions, the tissue culture RPMI-1640 remains the medium of choice, not only for *P. falciparum* and other human malarial parasites but also for piroplasms. However, recent findings have shown that a combination of three commercially available growth media (RPMI-1640, NCTC-135, and IMDM) supplemented with 10% bovine calf serum supports optimally long-term cultivation.

In spite of the fact that certain stages of avian coccidians as well as tachyzoites of *Toxoplasma* or *Neospora* can be readily cultivated in cell cultures, pharmaceutical compounds are still usually tested on parasites collected from experimentally infected hosts that are infected either orally (*Eimeria*), intraperitoneally (*Toxoplasma*), or via arthropod vectors (*Plasmodium*).

Evolutionary History

Origin of Apicomplexa

In the absence of a fossil record, apicomplexan evolution has been inferred from ultrastructural and morphological characters, coevolution with hosts, and molecular phylogenetic analyses. It has been generally supposed that the Apicomplexa first invaded marine invertebrates, as molecular dating places their origin between 600 and 800 million years ago, long before the emergence of vertebrates.

The Apicomplexa belongs to a group named Alveolata, which traditionally consists of three phyla: (i) the almost exclusively parasitic apicomplexans, (ii) the fully heterotrophic ciliates (Ciliophora), and (iii) the facultative photoautotrophic dinoflagellates (Dinophyta), which possess a complex (secondary or tertiary) plastid

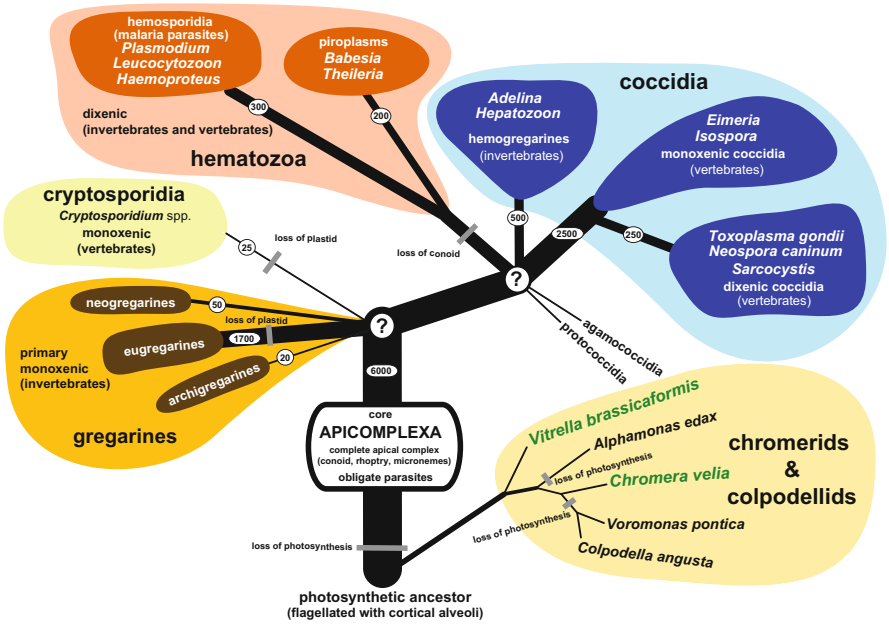

Fig. 24 Hypothetical tree of life of the Apicomplexa. The relationships are derived from morphology, biology, and molecular evolutionary studies based on current state of knowledge. The two major question marks denote the uncertainty of these key radiation events. The key to the common names is provided in Table 1. The branch thickness indicates the relative number of extant species also encircled at the branch

(Fig. 24). From the evolutionary standpoint, the ciliates are early-branching alveolates with the apicomplexans and dinoflagellates constituting an advanced sister groups. Finally, recently discovered chromerids contain coral-associated algae *Chromera velia* (Moore et al. 2008) and *Vitrella brassicaformis* (Oborník et al. 2012) that both appear more closely related to the apicomplexans than to the dinoflagellates (Moore et al. 2008; Janouškovec et al. 2010; Oborník and Lukeš, 2013; Janouškovec et al. 2015; Woo et al. 2015). It has been proposed that the entire group nowadays classified as the supergroup or kingdom SAR (Stramenopila + Alveolata + Rhizaria) (Adl et al. 2012) evolved through secondary and/or tertiary endosymbiotic event(s) between the red alga and a phagotrophic eukaryotic ancestor. However, the exact number of such events remains unknown, with proposals varying from a single endosymbiosis (Cavalier-Smith 1999) to multiple independent endosymbioses for each group of phototrophs (e.g., Falkowski et al. 2004) in the frame of the SAR supergroup. The proposed single secondary endosymbiosis has been dated to ~1.3 billion years ago, about 300–400 million years after the occurrence of the primary endosymbiosis between the heterotrophic eukaryote and cyanobacterium leading to the evolution of the primary plastids known from plants and rhodophytes (Fig. 24).

Although chromerids are closely related to the Apicomplexa, *C. velia* and *V. brassicaformis* do not form sister groups as anticipated but seem to be placed in

unrelated phylogenetic positions in the frame of colpodellids, with *Chromera* being affiliated with the *Colpodella* + *Voromonas* clade, while *Vitrella* is a sister to the genus *Alphamonas* (Gile and Slamovits 2013; Janouškovec et al. 2015; Oborník and Lukeš 2015). This suggests several possible independent losses of photosynthesis in this group. Although chromerids are not as closely related to each other as expected, they are known to possess noncanonical pathway for tetrapyrrole (heme and chlorophyll) synthesis, which is using the heterotrophic C4 route to form aminolevulinate, homologously to apicomplexan parasites and colpodellids (Kořený et al. 2011; Woo et al. 2015; Janouškovec et al. 2015). Since chromerids form relatively long branches, possibly resulting in artifacts in phylogenetic analyses, metabolic synapomorphies in the tetrapyrrole biosynthetic pathway between apicomplexans, colpodellids and chromerids represent one of the most convincing evidence for their common ancestry.

Regardless of the fact that both chromerid algae were isolated from similar environment, they substantially differ in morphology and life cycle. The isolated stage of *C. velia* is a coccoid vegetative cell containing a single large plastid surrounded by four membranes and numerous small mitochondria. Moreover, upon light exposure, large zoosporangia and consequently flagellated zoospores that highly resemble colpodellids are formed in the culture. Autosporangia of *C. velia* contain up to four autospores and zoosporangia up to ten zoospores, whereas the autosporangia and zoosporangia of *V. brassicaformis* are filled with dozens of spores (Oborník et al. 2011, 2012; Oborník and Lukeš 2013). Formation of zoospores in the zoosporangium of *C. velia* ultrastructurally resembles schizogony in Apicomplexa. It represents so far the only known developmental synapomorphy between photosynthetic chromerids and parasitic apicomplexans (Oborník et al. 2016).

Evolutionary Significance of the Apicoplast

The discovery of the apicoplast showed that the evolutionary history of the Apicomplexa is closely associated with the phenomenon of secondary endosymbiosis (Delwiche 1999; Foth and McFadden 2003; Keeling 2013). During this process, a eukaryotic alga was engulfed (or invaded) by a phagotrophic eukaryotic heterotroph and evolved into a multimembraneous complex plastid. Subsequently, this plastid lost its most important function – photosynthesis – and in hematozoans and coccidians, its genome has been reduced to a mere 35 kb circle. At the same time, the ancestral apicomplexan had to switch from autotrophy to heterotrophy, which may have coincided with the evolution of parasitism (Woo et al. 2015). Monophyletic origin of the apicoplast is generally accepted (Denny et al. 1998), yet two distinct lineages that slightly differ in plastid gene order, nucleotide composition, codon usage, and metabolic pathways have been distinguished (Oborník et al. 2002). Secondary endosymbiosis has deeply influenced the apicomplexan evolution by numerous replacements of the secondary host (exosymbiont) nuclear genes by their homologues from all three engulfed algal (endosymbiont) genomes (nuclear,

plastidial, and mitochondrial), likely through endosymbiotic gene transfer. This suggests that a substantial fraction of the apicomplexan genome can be composed of genes obtained from distantly related eukaryotes and their organelles.

However, not all members of the phylum Apicomplexa contain a plastid. This organelle is absent from the genus *Cryptosporidium* (Zhu et al. 2000; Keeling 2004), while its presence in gregarines is yet to be resolved. So far, a multimembraned apicoplast-like structure has been observed in the archigregarine *Selenidium hollandei* (Schrével 1971), whereas the eugregarine *Gregarina niphandrodes* seems to lack both the organelle and its genome (Tosso and Omoto 2007). It is likely that in this group the apicoplast has been lost multiple times, supporting the recent opinion that gregarines form a paraphyletic assembly at the base of the apicomplexan tree. It is plausible that some gregarines and the related genus *Cryptosporidium* lost their apicoplast early in the evolution, well before it became essential, as it is in *Plasmodium* and *Toxoplasma*, where cytosolic pathways were substituted by their plastidial counterparts (Oborník et al. 2009).

Besides predominating parasites, apicomplexans also include free-living marine predators called colpodellids, which use their apical complex for predation instead of parasitism (Leander et al. 2003b). The presence of a plastid in colpodellids has been recently confirmed (Gile and Slamovits 2013). While both photosynthetic alveolates branch within colpodellids, they contain photosynthetic plastids lacking chlorophyll *c*, the hallmark of the chromist and alveolate plastids. Interestingly, the plastid of *C. velia* (but not the one of *V. brassicaformis*) uses the noncanonical UGA triplet to encode tryptophan in the plastid-encoded proteins, which is a synapomorphy with the coccidian apicoplast (Moore et al. 2008). Plastid genomes of both chromerids contain roughly the same number of genes, but they display substantially different sizes and therefore also different levels of genome compaction. While the plastid genome of *C. velia* is linear and ~120 kb long, encoding highly divergent genes, the *V. brassicaformis* plastid genome is circular and compacted into ~80 kb (Janouškovec et al. 2010).

The apicomplexan cell carries unusual mitochondria, which either contain the smallest mitochondrial genome known or lost DNA altogether. While the linear mitochondrial genome of *P. falciparum* is only 5.9 kb long (Suplick et al. 1988; Feagin 1992), the DNA-lacking mitochondrion of *Cryptosporidium* has been reduced to a relic form resembling the mitosomes of microsporidia and diplomonads. Mitochondrial genome of *C. velia* is even smaller than those found in *Plasmodium* and *Toxoplasma*. It contains only two protein-coding genes (conserved *cox1* and highly divergent *cox3*) and fragmented rRNA genes. Consequently, the entire respiratory complex III (ubiquinol: cytochrome *c* oxidoreductase) was lost specifically from *C. velia*; homologously to apicomplexan parasites, *V. brassicaformis* and dinoflagellates, the complex I (NADH: ubiquinone oxidoreductase) is also absent in this chromerid alga. The electron transport function of the complex III is proposed to be substituted by L- and D-lactate cytochrome c oxidoreductases and L-galactono-1,4-lactone dehydrogenase. In contrast to *Chromera*, *Vitrella* still contains complex III; however, the mentioned newly proposed components of the respiratory chain are also present. Phylogenetic analyses showed that

these proteins are mostly of eukaryotic origins and have likely been lost from most of eukaryotic lineages (Flegontov et al. 2015; Oborník and Lukeš 2015).

Nuclear genomes of *C. velia* (194 Mb) and *V. brassicaformis* (73 Mb) were sequenced, and phylogenomic analyses confirmed phylogenetic position of chromerids on the root of Apicomplexa (Woo et al. 2015). It was also shown that massive gene loss (about 3,900 orthogroups) occurred during transition from a phototrophic ancestor to the apicomplexan parasites, while only dozens (80 orthogroups) were acquired. This suggests that the phototrophic ancestor of Apicomplexa already contained most of genes (or their ancestors) which are used for parasitism in apicomplexan parasites (Woo et al. 2015).

Evolutionary Diversity of Apicomplexa

The evolution of the apical complex and gliding motility opened an extremely successful obligatory parasitic niche for the apicomplexans (Portman and Šlapeta 2014; Heintzelman 2015; Keeling and Rayner 2015). The core parasitic Apicomplexa are monophyletic (Fig. 24). There are two alternative schools of thought in respect to relationships among the principal groups. The first school postulates that coccidians represent the ancestral polyphyletic group from which all the other major groups arose independently. This scenario assumes secondary hypertrophy of the gregarine trophozoites, as well as acquisition of extracellularity from primarily intracellular ancestral coccidians. An alternative scenario is that the gregarines are the most ancient paraphyletic group, from which monophyletic hematozoa and coccidia arose. Such a view finds support in the evolutionary relationships with hosts (invertebrates vs. vertebrates) and the complexity of life cycles (single host vs. multiple hosts). Unlike coccidia and hematozoa, gregarines are exclusively parasites of invertebrates and have simple life cycles.

The current knowledge of the gregarine evolution has recently been dramatically challenged by molecular ecology surveys of diverse oceanic and sediment samples (Leander 2008). A large proportion of phylotypes formerly unrelated to any known eukaryotic group have been shown to constitute an assembly of gregarine sequences monophyletic within the Apicomplexa. Their morphological identity remains unknown, but the link between phylotypes represents a challenging issue to be elucidated in the coming decades. Besides these relatively well-defined apicomplexan groups, there is a myriad of organisms of uncertain taxonomic placement, often found in diverse and obscure marine hosts. Organisms classified under agamococcidia (*Rhytidocystis*) from polychetes, protococcidea (*Gemmocystis*) from corals, or even parasites of squids and crabs (*Aggregata*) are just a few examples that challenge even the simplest traditional scenario of apicomplexan evolution.

Due to their effects on human and animal health, members of the monoxenic and cyst-forming coccidia have attracted substantially more attention than the other groups. The evolution of coccidia is traditionally based on the life cycle and number of sporocysts and sporozoites in the environmentally resistant oocysts. Stabilization of the number of sporocysts per oocyst and sporozoites per sporocyst seem to be key

events. However, the modes of excystation are arguably even more informative characters (Jirků et al. 2002). Feline and canine species infecting ruminants as intermediate hosts represent a classical examples of coevolution of the cyst-forming coccidia with their final hosts, where sexual development occurs. The two-host life cycle appears multiple times in coccidian evolution, and it has been hypothesized that homoxeny (single-host cycle) predated heteroxeny (two-host cycle).

The fact that gametogony of hematozoa takes place in vertebrate hosts implies that these parasites have evolved from coccidians of invertebrates rather than vertebrates. The common ancestral host of both avian malarial parasites (*Plasmodium* and *Haemoproteus*) appears to be reptile, and host switches between reptiles and birds are documented quite frequently. By contrast, the host shift from reptiles to mammals was a singular event. On a species level, fascinating recent evolutionary consequences are revealed about malarial parasites affecting humans. It has been postulated that 10,000 years ago a major geographic expansion of malaria took place in Africa. Mechanisms behind this expansion are wide adoption of more efficient agriculture resulting in increased population size and coinciding spread of the *P. falciparum* mosquito vector with climate change in sub-Saharan Africa after the last glacial period. Over the past 40 years, incidence of malaria is rapidly increasing, amplified by the rapid spread of antimalarial resistance, pesticide-resistant mosquitoes, increased population size, poverty, and global warming, all resembling the situation 10,000 years ago. In contrary, recent advances in malaria control led to eradication of significant reduction of impact of malaria in several tropical areas.

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Dinoflagellata

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Juan F. Saldarriaga and F. J. R. 'Max' Taylor

Abstract

Dinoflagellates are a major group of aquatic protists responsible for a major part of marine primary productivity, the creation of coral reefs, marine bioluminescence, and most toxic red tides; indirectly they also cause some human diseases like paralytic shellfish poisoning, ciguatera, etc. They are derived from photosynthetic ancestors and early in their evolutionary history exchanged most of the histones in their nuclei for DVNPs, proteins of putatively viral origin that caused a complete reorganization of chromosomes that includes the loss of the typical eukaryotic nucleosomes and a very marked increase in total amounts of DNA per nucleus. Later on, they acquired other types of DNA-binding proteins, so-called HLPs in at least two waves, possibly lateral transfers from bacteria. Dinoflagellate mitochondrial genomes are some of the smallest known, and the genomes of the ancestral plastid type of the group, the peridinin plastids, are atomized into minicircles with usually one single gene per circle. Roughly half of the dinoflagellates are non-photosynthetic, and the majority of the photosynthetic forms have peridinin plastids. Loss of photosynthesis has occurred repeatedly, but all free-living non-photosynthetic forms remain metabolically dependent on cryptic plastids; complete loss of plastid metabolic activity has only been shown in a few parasitic forms. Several lineages show a marked propensity for reacquisition of photosyn-

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thesis, be it in the form of permanent photosynthetic endosymbionts, kleptochloroplasts, or serial secondary and tertiary endosymbioses that produce cells with a wide variety of plastid types. In a few members of the group, peridinin plastids have become the pigment cup/retinoid of complex eyelike structures, so-called ocelli.

Keywords

Dinoflagellates • Syndinians • MALV • Coral reefs • Bioluminescence • Paralytic shellfish poisoning • Ciguatera • DVNP • HLP • Peridinin • Photosynthesis • Kleptochloroplasts • Tertiary endosymbiosis • Theca • Tabulation • Ocelli

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••**Core dinoflagellates**
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•••**Thecates**
••••**Peridiniales** (e.g., *Peridinium*, *Protoperidinium*, *Heterocapsa*)
••••**The *Symbiodinium* group** (e.g., *Symbiodinium*, *Polarella*, *Borghiella)*
••••**Gonyaulacales** (e.g., *Ceratium*, *Gonyaulax*, *Lingulodinium*)
••••**Dinophysiales** (e.g., *Dinophysis*, *Ornithocercus*, *Amphisolenia*)
••••**Prorocentrales** (e.g., *Prorocentrum*)

Introduction

General Characteristics

Dinoflagellates (Gr. δίνη/díni, to whirl) are an eukaryotic group containing approximately 4,500 species in more than 550 genera, nearly three quarters of the genera and more than half of the species being fossil. Members of the group can be photosynthetic or non-photosynthetic, walled or naked, parasitic or free-living, and very rarely even multicellular. Of the ca. 2,400 living species, 83% are marine, 8% are benthic, 7% are parasitic, and roughly half are photosynthetic (Gómez 2012); several species are also known from snow and sea ice. Numbers of extant species are sure to grow substantially in the future; recent molecular analyses have shown that there are large numbers of undescribed dinoflagellates in environments like marine picoplankton (Moreira and López-García 2002, de Vargas et al. 2015) or as symbionts ("zooxanthellae") in many types of protists and invertebrates like corals or radiolarians (Coffroth and Santos 2005; Brate et al. 2012). The cell periphery, wall, cyst, nuclear, and flagellar features are very distinctive, dinoflagellates show great diversity of form, and some have highly complex internal differentiation.

Occurrence

Dinoflagellates can be found in most aquatic environments, both freshwater and marine, and in intrazoic habitats (see section "Habitats and Ecology"). Principal sources for dinoflagellate cultures include the Provasoli-Guillard National Center for

Culture of Marine Phytoplankton (CCMP, Boothbay Harbor, Maine, USA), the Canadian Center for the Culture of Microorganisms (CCCM, Vancouver, Canada), the CSIRO Collection of Living Microalgae (CSIRO, Hobart, Tasmania, Australia), the Cawthron Institute Culture Collection of Micro-algae (CICCM, Nelson, New Zealand), the Culture Collection of Algae and Protozoa (CCAP, Oban, UK), and the Microbial Culture Collection at the National Institute for Environmental Studies (MCC-NIES, Tsukuba, Japan).

Literature

Because dinoflagellates have been claimed by botanists as algae and by zoologists as protozoa, and the fossil forms by palynologists and micropaleontologists, literature concerning them is widely scattered. The most comprehensive taxonomic reference work is the two-volume contribution by Schiller (1933, 1937, in German) to Rabenhorst's Kryptogamen Flora, although it is now seriously out of date. Examples of more recent English-language taxonomic monographs covering large numbers of species are those by Steidinger and Williams (1970, Gulf of Mexico), Taylor (1976, Indian Ocean), Dodge (1982, British Isles), and Gómez (2003, Mediterranean). The catalogues of genera (Loeblich and Loeblich 1966) and species by Sournia (1973) and Gómez (2005 and 2012) help in tracking down more recently described taxa. The Center for Excellency in Dinoflagellate Taxonomy (CEDiT, http://www.dinophyta.org) provides authoritative information on taxonomic matters; it includes, for example, lists of valid names, sources of first descriptions, etc. The taxonomy of extant and fossil species was unified for the first time by Fensome et al. (1993). A good summary of the biology of the group is presented in Hackett et al. (2004b); papers concerned primarily with the evolution of the whole group include Taylor (2004), Saldarriaga et al. (2004), Zhang et al. (2005), and Bachvaroff et al. (2014).

A small book by Sarjeant (1974) mostly on fossils and volumes edited by Spector (1984) and Taylor (1987) has brought together much general literature. Major reviews have been provided on particular aspects, e.g., Fensome et al. 1993, classification; Granéli and Turner 2006, biology of harmful species; and Coffroth and Santos 2005, zooxanthellae.

History of Knowledge

The largest dinoflagellate, *Noctiluca*, reaches 2 mm in diameter and can be seen with the naked eye as a grayish sphere, luminescent when disturbed. It is not surprising that it was the first dinoflagellate to be described in 1753 by Henry Baker. Several microscopic forms, both freshwater and marine, were discovered by the early Danish microscopist Otto F. Müller in the 1770s and illustrated in 1786. From then on, there was a slow but steady stream of descriptions, most notably by C.G. Ehrenberg who named many protists, particularly those forming microfossils, in the mid-nineteenth

century. Ehrenberg mistakenly believed that they were scaled-down, multicellular animals (the plastids were interpreted to be gonads). Another common misconception was that there was a ring of cilia in the girdle groove (in the position of the transverse flagellum) additional to the trailing longitudinal flagellum, leading to the name "Cilioflagellates" in use until the end of the nineteenth century. The group was first monographed by F.R. von Stein in 1883, at which time 32 genera were recognized (two not attributed to the dinoflagellates today), 26 of which are still in use. He was the first to recognize the taxonomic usefulness of thecal plate patterns in the group. The nomenclatural system for dinoflagellate thecal plates was standardized by C.A. Kofoid in 1907 and 1909, and the "Kofoid System" is still used universally, although its weakness for generic comparisons is becoming recognized (Taylor 1980; Evitt 1985).

Links to marine luminescence were demonstrated by G.A. Michaelis in 1830, and zooxanthellae symbiotic in colonial radiolarians was described and named by Karl Brandt in the 1880s (their dinoflagellate nature was only later recognized by S. Kawaguti in 1944, and they were cultured by H.D. Freudenthal in the 1950s). Parasitic species were studied largely in the early 1900s by Edouard Chatton.

Freshwater species were first monographed by A.J. Schilling at the end of the nineteenth century, with strong contributions on their biology by George Klebs at the turn of the century.

Ecologists gradually became aware of the importance of the photosynthetic members of the group as beneficial, or sometimes harmful, bloom-forming organisms of the phytoplankton. Their frequent causal association with "red tides" became apparent, with massive kills of fish and marine life being recorded with increasing frequency during this century. Their association with paralytic shellfish poisoning (PSP) was recognized by Hermann Sommer and his colleagues in the 1930s, and the link to ciguatera fish poisoning only in the 1970s by T. Yasumoto and colleagues.

The culture of dinoflagellates was pioneered chiefly by Albert Barker in the 1930s. This permitted the physiology and life cycles to be studied more carefully, principally by T. Braarud and his Norwegian colleagues and B.M. Sweeney in America. The latter, together with J.W. Hastings, focused on luminescence and circadian rhythms.

Much of the current ultrastructural knowledge of the group, including the unusual nuclear features, has come from John Dodge in the 1960s and 1970s, with valuable contributions by many others, including J. and M. Cachon, M.-O. Soyer, C. Greuet, K.R. Roberts, G. Hansen, and Ø. Moestrup. Ultrastructural and biochemical data on the dinoflagellate nucleus led to the proposal of the so-called Mesokaryote hypothesis (Dodge 1965), in which dinoflagellates are thought to represent an intermediate kingdom between prokaryotes and eukaryotes. This view was very prevalent until the advent of molecular data.

Dinoflagellates were thought by many to be entirely asexual in reproduction. Early observations by E. Zederbauer and Karl Diwald of apparent sexual fusion were discounted, and it was only careful documentation and observations of H.A. von Stosch in the 1960s that established its occurrence unequivocably in *Ceratium*. The first genetic studies followed later in 1974, using *Crypthecodinium cohnii*,

coincidentally in two different laboratories (C.A. Beam and M. Himes in Brooklyn; R.C. Tuttle and A.R. Loeblich III at Harvard).

The study of fossil dinoflagellates (reviewed by Sarjeant 1974) accelerated in the 1920s and 1930s with studies by O. and W. Wetzel (unrelated) and the growing realization that the fossils were actually cysts rather than thecae, for the most part, and that many of the spiny "hystrichospheres," formerly of unknown affinities, may also be dinoflagellates. This was only clearly established by W.R. Evitt, using careful observation and encystment experiments, and with the excystment of cysts collected from natural sediments by D. Wall and B. Dale during the 1960s. The zygotic nature of resting cysts (most readily fossilizable) only become evident in the 1970s. Later studies on dinoflagellate life cycles and cyst biology have been made by K. Steidinger, M. Montresor, and J. Lewis, among others.

Practical Importance

Dinoflagellates are perhaps best known as causers of harmful algal blooms, as roughly 75–80% of toxic phytoplankton species belong to the group (Cembella 2003). They are frequent causes of "red tides" that may kill fish and/or shellfish either because of toxin production (Table 1) or because of nontoxic effects caused by large numbers of cells in the water (clugging of animal gills, oxygen depletion, etc., e.g., Smayda 1997). Dinoflagellate toxins are among the most potent biotoxins known and accumulated in shellfish or fish cause human diseases like paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), diarrheic shellfish poisoning (DSP), and ciguatera (Lehane and Lewis 2000). They also have been linked to major human health concerns, especially in estuarine environments (*Pfiesteria*). This is significant to coastal aquaculture in that they prevent otherwise productive areas of coastline from being fully exploited. Parasitic species of the genus *Amoebophrya* infect other dinoflagellates, often toxic ones, and have a significant role in ending harmful algal blooms (Velo-Suárez et al. 2013). The syndinian genus *Hematodinium* causes bitter crab disease in 25 species of crustaceans. When infected, crab meat acquires an aspirin-like, bitter taste, and this has large repercussions for crab fisheries (e.g., Meyers et al. 1987; Stentiford and Shields 2005).

Some dinoflagellates (e.g., *Akashiwo sanguinea*) have been used in aquaculture as a preferred food source for larval fish, for example, for anchovies, because they have a higher caloric content per cell than diatoms. Unfortunately they are sensitive to stirring and bubbling, and this, combined with relatively slow division rates (often 0.5 cell divisions or fewer per day), makes them useful only in special cases.

The main ecological importance of dinoflagellates lies elsewhere, though. They are second only to diatoms as marine primary producers, and so are responsible for a very major fraction of marine primary production worldwide. As phagotrophic organisms, they are also important components of the microbial loop in the oceans and help channel significant amounts of energy into planktonic food webs that would otherwise get lost. Dinoflagellates also have a pivotal role in the biology of reef-

building corals: as zooxanthellae, they build symbioses with corals and other animals and protists, and by removing CO_2 from the medium for photosynthesis, they facilitate the deposition of calcium carbonate.

Habitats and Ecology

Dinoflagellates can be found in most aquatic environments including snow, freshwater, marine, or intrazoic habitats.

Comprehensive treatments of their ecology include the chapters by Taylor and Pollingher in Taylor (1987). Reviews on toxic dinoflagellate blooms (e.g., Lundholm and Moestrup 2006) contain numerous references of ecological interest. Pross et al. (2004) provide a good review on palaeobiogeography based on fossil dinoflagellate cysts.

Nutrition

Roughly half the dinoflagellate species are photosynthetic, but completely autotrophic species are very rare (Gaines and Elbrächter 1987; Schnepf and Elbrächter 1992). Photosynthetic dinoflagellates are generally mixotrophic and rely on a combination of photosynthesis and heterotrophic nutrition; the relative importance of the uptake of dissolved organic nutrients, feeding, and photosynthesis for the nutrition of members of the group is unknown. Non-photosynthetic forms can be either free-living or parasitic, and they rely on both osmotrohy and phagotrophy. Prey capture mechanisms in phagotrophic forms vary greatly. Direct phagocytosis occurs in several species. A distinct cell mouth (cytostome) is present in several large phagotrophic genera (e.g., *Oxyrrhis*, *Noctiluca*, *Kofoidinium*, *Erythropsidinium*, *Gyrodinium s.s.*). Other forms, for example, *Protoperidinium*, extend a delicate, pseudopodial "feeding veil" with which they surround portions of diatom chains and other large prey. Digestion then occurs outside of the theca, and only digested material is taken up; the veil is retracted afterward (Gaines and Taylor 1984; Jacobson and Anderson 1992). A third form of feeding, myzocytosis (e.g., in *Paulsenella* spp., "*Katodinium*" *fungiforme*), involves piercing the cell membrane of prey items with a special organelle, the peduncle, and somehow "sucking" the prey cell's contents as if through a straw (Schnepf and Elbrächter 1992). Peduncles, also present in some photosynthetic species, are c shaped in cross section; the details of the mechanism that underlies this mode of feeding are unknown. Parasitic forms can be intra- or extracellular, and they take up nutrients from their host directly.

Only relatively few non-photosynthetic dinoflagellates have been studied in detail using transmission electron microscopy, and several ostensibly non-photosynthetic species have been shown to carry cryptic plastids (e.g., Sparmann et al. 2008). The ratio of photosynthetic versus non-photosynthetic forms in dinoflagellates may well change in the future as more species are investigated in this regard.

Dinoflagellate Phytoplankton

Dinoflagellates are generally considered second only to diatoms in their importance as primary producers among marine plankton. A deceptive impression has built up in the literature that diatoms predominate in colder, and dinoflagellates in warmer, water. A more accurate picture is that diatoms predominate in coastal waters during the most productive periods and also in open waters of high latitudes (arctic, subarctic, circumantarctic). In the nutrient-poor temperate and tropical oceanic regions, all types of plankton are impoverished, with coccolithophorids less so in the former and dinoflagellates less so in the latter. In fact, the greatest concentrations of dinoflagellates (10^7–10^8/l) occur in temperate coastal waters subject to transient periods of vertical stability (Taylor et al. 2008).

Many photosynthetic dinoflagellates behave as annual species. They are generally ecophysiologically diverse and tend to be more specialized to particular habitats/hydrographic regimes than diatoms, for example. For this reason, dinoflagellate blooms tend to be monospecific (Smayda and Reynolds 2003).

Polar waters have relatively few photosynthetic dinoflagellate species (e.g., McMinn and Scot 2005). In temperate coastal and also in freshwaters, dinoflagellates usually bloom in mid- to late summer when sunshine and vertical stability allow strong aggregations to develop at vertical and/or horizontal discontinuities, referred to as clines (e.g., thermocline, nutricline) or fronts. The swimming abilities of the cells (maximum approximately 1 m/h) allow them to resist moderate downward water movements and to occupy compromise positions in the water column relative to light (maximum upward) and inorganic nutrients (maximum downward; Cullen and MacIntyre 1998 and references therein). Subsurface maxima may occur at 1% surface light levels or even less (Anderson and Stolzenbach 1985). In ice-covered lakes, dinoflagellates can accumulate just under the ice if it is not too thick and may bloom early in the season or even in winter.

Daily patterns of vertical migrations are also seen, with the cells rising as far toward the surface as the nutrients allow during the day and downward at night (e.g., *Lingulodinium polyedrum* and *Akashiwo sanguinea* off California or *Ceratium hirundinella* and *Peridinium cinctum* in lakes; Cullen and MacIntyre 1998). *Prorocentrum* spp., *Ceratium fusus*, and *C. furca* tend to predominate in estuarine water. Several of the coastal bloom formers are harmful to marine life or humans when in high concentrations ("red tides"): see Table 1. In higher latitudes (but not polar), the summer community is generally similar but of shorter duration than in warmer temperate waters (e.g., the Bering Sea/Gulf of Alaska relative to southern California or southern Chile compared with Peru; Taylor et al. 2008). Many of the bloom formers overwinter as benthic cysts.

In temperate lakes, the dominants in summer can vary considerably according to many factors, including degree of eutrophy (nutrient level), pH, depth, and surrounding vegetation. Dinoflagellates are represented chiefly by *Ceratium* spp. (especially *C. hirundinella*), when grazing is intense, or *Peridinium* and "*Gymnodinium*" spp. when it is not. In tropical lakes, other protist groups usually predominate, but

Table 1 Examples of toxic dinoflagellates

Species	Toxin	Effect
Alexandrium spp.	Saxitoxins	PSP
Amphidoma spp.	Azaspiracid	Azaspiracid poisoning
Azadinium spp.	Azaspiracid	Azaspiracid poisoning
Cochlodinium polykrikoides	Unknown	Fish kills, smothered corals
Coolia monotis	Cooliatoxin	
Dinophysis spp.	Dinophysistoxin	DSP
Gambierdiscus toxicus	Maitotoxin, ciguatoxin	Ciguatera
Gymnodinium catenatum	Saxitoxins	PSP
Karenia spp.	Brevetoxins	NSP, fish kills
Karlodinium veneficum	Brevetoxins	NSP
Lingulodinium polyedrum	Yessotoxin	
Ostreopsis spp.	Ostreotoxin	
Pfiesteria spp.	*Pfiesteria* toxin	Possible estuary-associated syndrome (PEAS)
Prorocentrum spp.	Okadaic acid, dinophysistoxin	DSP
Protoceratium reticulatum	Yessotoxin	
Pyrodinium bahamense	Saxitoxins	PSP
Takayama spp.	Brevetoxins	NSP
Vulcanodinium rugosum	Pinnatoxins	

Peridinium gatunense is a major dominant in Lake Kinneret, Israel, where it "oversummers" as a benthic cyst (Pollingher 1987).

Tropical nearshore waters are usually diatom dominated, but brief dinoflagellate blooms may occur, and some tropical Atlantic mangrove-lined bays have become famous for persistent blooms of the bioluminescent species *Pyrodinium bahamense* var. *bahamense*; with the development of the shoreline, these blooms have been greatly reduced. Several toxic species bloom in tropical coastal waters. In the oceanic tropics, although a great variety of *Ceratium* spp. are most obviously present, they are not abundant; *Pyrocystis* spp. and in the nanoplankton size range (<20 μm) *Oxytoxum* spp. are usually more abundant.

Dinoflagellate Microzooplankton

Non-photosynthetic forms depend on the presence of their food for nutrition; as might be expected, they are most abundant at the end of blooms of their prey organisms. *Protoperidinium* spp. and *Noctiluca scintillans*, for example, typically

follow diatom blooms. From a biogeographic standpoint, they are most abundant where the latter are. Species of *Protoperidinium* are important in polar waters and are generally coastal in distribution. The effect of non-photosynthetic dinoflagellates on marine (or freshwater) ecosystems is very understudied, but at least in coastal food webs, it can be very large (e.g., Lessard and Swift 1985).

Benthic Dinoflagellates

Dinoflagellates (both photosynthetic and non-photosynthetic) are common inhabitants of benthic sediment habitats, but details of their biology are scarce (Hoppenrath et al. 2014). Early data suggests that benthic marine communities are remarkably similar across locations of similar latitudes, but investigations are too few and geographically restricted to allow for generalized biogeographic conclusions so far. Photosynthetic forms can bloom in benthic habitats; several *Amphidinium* and *Prorocentrum* species may discolor marine sand flats. *Crypthecodinium cohnii* and *Oxyrrhis marina* are often associated with seaweed (brown and green algae, respectively), and the latter also forms intense pink tide-pool blooms. On tropical, bushy seaweeds several toxic species occur, e.g., *Gambierdiscus toxicus*, which adheres to the surface of the weeds and is the ultimate cause of ciguatera (Anderson and Lobel 1987).

Symbioses

Mutualistic Associations

Most zooxanthellae (golden-brown endosymbionts of marine animals and protists) are dinoflagellates. The association between dinoflagellates and reef-building corals was mentioned above, but dinoflagellate endosymbionts inhabit a great number of other invertebrates and protists, for example, many sea anemones, jellyfish, nudibranchs, the giant clam *Tridacna*, and several species of radiolarians and foraminiferans (for a review, see, e.g., Trench 1997). The effect that these associations have on organisms and ecosystems can be massive. They use waste products of their host (e.g., waste nitrogen and phosphorus compounds) as nutrients and release up to 40% or more (possibly more than 90%) of their photosynthate to their hosts, chiefly in the form of glycerol, with smaller amounts as sugars and amino acids. Furthermore, by taking CO_2 from the water for photosynthesis, zooxanthellae facilitate the deposition of calcium carbonate (Marshall 1996) and the production of coral reefs, large foraminiferal skeletons, the massive shells of *Tridacna*, etc.

Dinoflagellate zooxanthellae often belong to the genus *Symbiodinium*, which divides in the coccoid stage and has very transient flagellated stages. But at least seven dinoflagellate genera from four orders have been found in symbiotic associations (Banaszak et al. 1993). For a long time, *Symbiodinium* was considered to be a monospecific genus, but now it is clear that it contains a large cryptic diversity. Coral bleaching is the expulsion/digestion of zooxanthellae in temperature-stressed corals.

Dinoflagellates can also function as hosts of mutualistic symbioses. They may, for example, carry extracellular cyanobacteria ("phaeosomes") that may help fix nitrogen in nutrient-poor oceanic regions, e.g., the dinophysoids *Ornithocercus*, *Histioneis*, and *Citharistes*; other endosymbiotic bacteria are not at all uncommon: Sinophysis and Triposolenia contain for example cyanobacterial endosymbionts. Eukaryotic endosymbionts are also found in many dinoflagellates. *Noctiluca scintillans*, for example, exists in the Pacific in at least two populations: one of them always harbors *Protoeuglena*, a green alga, as an endosymbiont and the other one never seems to contain them. Other noctilucales, for example, *Spatulodinium*, and at least one *Kofoidinium*-like species also contain green endosymbionts (Gómez and Furuya 2007). Two other such endosymbioses that may well be permanent (definitive proof is lacking at the moment) are the genus *Amphisolenia*, which always seems to contain pelagophyte endosymbionts (Daugbjerg et al. 2013), and *Podolampas bipes*, which seems to contain a pedinellid dictyochophyte (Schweikert and Elbrächter 2004). Diatom-carrying dinoflagellates (so-called dinotoms, *Kryptoperidinium*, *Durinskia*, *Dinothrix*, *Galeidinium*, "*Peridinium*" *quinquecorne*, "*Peridiniopsis*" sp.) show a similar situation; they contain (almost) complete diatom endosymbionts and are thus binucleated. Molecular phylogenetic trees put all dinotoms in a clade, and this would seem to suggest that the diatom endosymbiosis occurred before the divergence of the different species. However, things are not that simple: the type of diatom endosymbiont (pennate vs. centric) is different in the different genera (Takano et al. 2008). This situation is very close to being a true tertiary endosymbiosis, but no diatom genes seem to have moved to the dinoflagellate nucleus. True tertiary endosymbioses do exist in dinoflagellates; they involve plastids of haptophyte origin (Patron et al. 2006; Nosenko et al. 2006) and will be discussed in the plastid section below.

Parasitism

Many extant dinoflagellates are parasites (here defined as organisms that eat their prey from the inside, i.e., endoparasites, or that remain attached to their prey for longer periods of time, i.e., ectoparasites), and of those, a majority branch early in the dinoflagellate molecular tree. Syndinians, early-branching parasitic dinoflagellates, are characterized by a plasmodial (multinucleate) stage (references in Cachon and Cachon 1987; Fensome et al. 1993). Core-dinoflagellate parasites on the other hand seem to have originated repeatedly from within the group, and their trophic stages are generally much easier to relate morphologically to the flagellated stages from which they arise. Dinoflagellates can parasitize animal or protist hosts. Ectoparasitic forms show the least modification; they attach to and penetrate the host by a stalklike projection from the sulcus, probably homologous to the peduncle of motile forms. *Chytriodinium* actively penetrates the chorion of crustacean eggs by extraordinary rapid "drilling" movements with its extensible hyposome, while the motile stages of parasites on fish, such as *Piscinoodinium*, *Amyloodinium*, and *Crepidoodinium*, have a pedunclelike organelle with which they penetrate the host. *Blastodinium* inhabits the gut of copepods, maintaining its position by rows of small spines. *Protoodinium*, *Crepidoodinium*, *Piscinoodinium*, and *Blastodinium* retain their plastids while feeding on their zooplanktonic or fish hosts.

Circadian Rhythms

In a number of species, many cellular phenomena are rhythmic, exhibiting daily (circadian) differences. Processes such as bioluminescence, photosynthesis, cell division, and motility have been studied intensively, especially in *Lingulodinium polyedrum* (Sweeney 1987; Akimoto et al. 2004), but it is likely that many other cellular processes are under circadian control and that this cellular "clock" occurs in many – possibly all – dinoflagellates. A key feature of the circadian (about 1 day) control is that the mechanism responsible is endogenous, not directly dependent upon the light-dark cycles, which, however, serve to confer phase to the system (Johnson and Hastings 1986).

Toxins

The toxic species that have caused illness or death of humans or marine fauna, as listed in Table 1, produce two principal types of toxins: (a) water-soluble, small molecular weight substances that block the entry of sodium into the nerves of some animal groups, including humans, and (b) larger, water-, or lipid-soluble compounds that increase membrane permeability to various ions, including sodium and/or calcium. Additionally, there are a few toxic substances such as cholinesterase-like compounds in *Amphidinium carterae* known only from laboratory testing. Toxins in the first group include the saxitoxin complex (saxitoxins, neosaxitoxin, gonyautoxins), heterocyclic guanidines produced by *Alexandrium* species, *Pyrodinium bahamense*, and *Gymnodinium catenatum*, which produce paralytic shellfish poisoning. Saxitoxin, by mass, is 1,000 times more potent than cyanide and 50 times more toxic than curare (Sako et al. 2001). Toxins in the second group are polyether compounds. They include the brevetoxin complex from *Karenia brevis* which kills fish and causes neurotoxic shellfish poisoning, okadaic acid from tropical *Prorocentrum lima*, ciguatoxin and maitotoxin from *Gambierdiscus toxicus*, the dinophysistoxins from *Dinophysis* and *Prorocentrum* spp., pectenotoxin from *Dinophysis*, yessotoxin from *Protoceratium reticulatum* and *Lingulodinium polyedrum*, and azaspiracid from Azadinium spp. and Amphidoma spp. (Van Dolah 2000). They cause ciguatera (Lehane and Lewis 2000), diarrheic shellfish poisoning, and azaspiracid shellfish poisoning. Maitotoxin is one of the most potent biogenic toxins known (Terao et al. 1989).

The functions of the toxins are presently unknown. They do not prevent predation on the producers, and most of their grazers, such as copepods, pteropods, or bivalve mollusks, remain unharmed. However, they can cause massive kills of fish and other marine life (dolphins, manatees, birds, etc.). Toxins produced by benthic dinoflagellates that do not often bloom generally do not cause fish kills: the toxin is taken orally by the fish with its food and is accumulated in the animal's tissues (mostly the liver) where it causes comparatively little damage. Toxins produced by blooming, planktonic dinoflagellates are much more likely to cause fish kills. When the blooms end and the cells die, toxins are released into the water, and fish take the toxin via their

gills, a much more direct way into their bloodstream. In these cases, the effects of the toxin are much more severe. Both brevetoxin and maitotoxin have been shown to accumulate in fish tissues if taken orally, but brevetoxin is more likely to cause fish kills because of the ecology of its producing organism.

Most toxin producers are photosynthetic, but *Protoperidinium crassipes*, producer of azaspiracid, is an exception. Toxicity in benthic coral reef dinoflagellates is a common occurrence (Anderson and Lobel 1987); this is not the case in planktonic dinoflagellates.

Characterization and Recognition

The typical dinoflagellate is a biflagellated eukaryotic unicell, between 10 and 100 μm in length (the extreme range is 2–2,000 μm). One ribbonlike flagellum, the transverse, winds to the left around the cell causing it to turn as well as providing forward thrust. The second flagellum, the longitudinal, beats posteriorly. Although providing some forward thrust (Gaines and Taylor 1985), its principal function seems to be directional (an exception is *Ceratium*). Cell shape is highly variable but is often pyriform.

In most dinoflagellates, the two flagella arise from the side (designated as ventral) and lie in surface grooves: the transverse in the girdle (or cingulum) and the longitudinal in the sulcus (Fig. 1), although its distal portion projects freely behind the cell. This is known as the dinokont condition. If the distal and proximal ends of the girdle do not meet at an equal level at the sulcus, they are said to be displaced. Displacement may be left handed (the most common condition), in which the proximal (left) end is more anterior, or right handed, and the degree is measured in girdle widths, given from the upper edges.

The girdle divides the cell into an anterior body portion, the episome (or epicone), and a posterior hyposome (hypocone). The sulcal groove stops at the posterior of the cell. In athecate (wall-less) cells, there is a thin, anterior extension of the sulcus, the acrobase, which reaches the cell's apex. Acrobases can be straight, sigmoid, or form loops around the apex of the cell.

In a few genera, most notably *Prorocentrum*, the two flagella arise from the anterior (apex) of the cell and are not associated with grooves, although they are differentiated as in dinokonts and beat differently. This is the desmokont condition (Fig. 2).

Flagella

The longitudinal flagellum is relatively conventional in appearance, with few or no hairs (mastigonemes). It may be ribbonlike, and in some, e.g., *Ceratium* (in which it is the main propulsive unit) and *Oxyrrhis*, an accessory fibrillar band may be present, running parallel to the axoneme. It beats with only one or two periods to its wave. In *Ceratium*, it can contract rapidly up to the body.

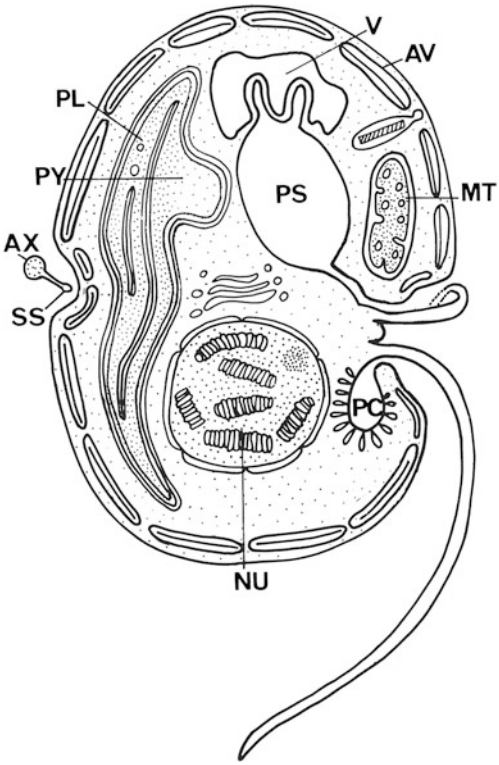

Fig. 1 Longitudinal section through a generalized dinoflagellate (re drawn from Taylor 1980). *AV* amphiesmal vesicle; *AX* axoneme; *MT* mitochondrion; *NU* nucleus; *PC* collecting pusule; *PL* plastid; *PS* sac pusule; *PY* pyrenoid; *SS* striated strand; *V* vacuome

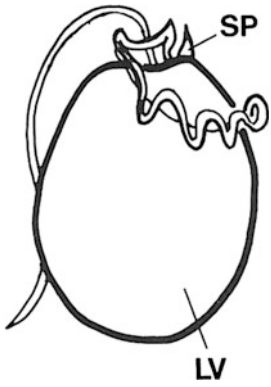

Fig. 2 Flagellar arrangement of *Prorocentrum*. *LV* left valve; *SP* spine

The transverse flagellum (Fig. 3) is generally a wavy ribbon in which only the outer edge undulates from base to tip, due to the action of the axoneme which runs along it. The beat of the axoneme is approximately spiral, but because the ribbon is anchored on its inner edge by an accessory fibrillar band, the striated strand, the

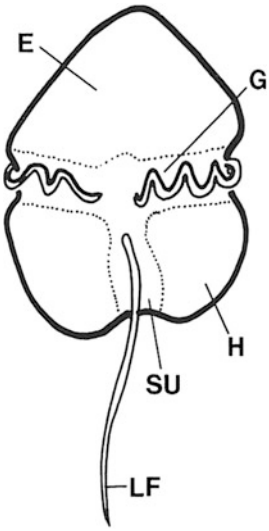

Fig. 3 Flagellar arrangement in a dinokont dinoflagellate seen from the ventral side. *E* episome; *G* girdle (=cingulum); *H* hyposome; *LF* longitudinal flagellum; *SU* sulcus

ribbon forms a travelling ruffle rather than a spiral, the outer advancing faces being inclined forward and downward. The axonemal edge has simple hairs, which can be of varying length. The form of the ruffle as it beats and the hairs act in such a way that there is forward propulsion and also a turning force. Curiously, the cells rotate in the direction of the wave, i.e., always to the cell's left (Gaines and Taylor 1985). Early-branching dinoflagellates (*Oxyrrhis*, the syndinians, and *Noctiluca*) do not seem to have a striated strand in their transverse flagellum.

Amphiesma (Cortex)

The cells may be naked (athecate) or possess a wall (thecate, pelliculate). In a few species of *Oxyrrhis*, *Heterocapsa*, and *Lepidodinium*, very small delicate, star-, or basketlike organic scales occur external to the cell membrane, but in walled dinoflagellates, the close-fitting cellulosic plates which together form the theca are intracellular.

The organization of the outer cortical region of the cell is distinctive. This entire structural complex, regardless of the presence or absence of cellulose plates, is the amphiesma (Morrill and Loeblich 1983; also known as the cortex; Netzel and Dürr 1984). Beneath the cell membrane of the motile cell, a single layer of vesicles is usually present, the alveolae (Fig. 4; the term "alveolus" comes from the ciliate literature, but it is starting to be used in dinoflagellates and apicomplexans to underline the homologous nature of these structures in the three groups). It is within these alveolae (traditionally called amphiesmal vesicles) that the cellulose plates are formed, one per vesicle in thecate (= armored) dinoflagellates. In

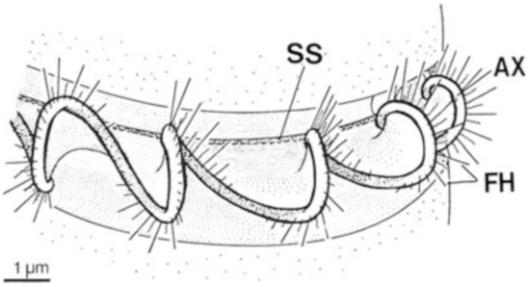

Fig. 4 Detail of the transverse flagellum, modified from Gaines and Taylor (1985). *AX* axoneme; *FH* flagellar hairs; *SS* striated strand

athecate (= naked) species, the vesicles are either empty or contain amorphous material, and the vesicles themselves play a structural role. In some species of *Gymnodinium*, there is a very thin "membrane" within the vesicles that resembles the membranous layer that acts as a plate precursor in *Ceratium* and other more heavily thecate species. The thecal plates usually fit tightly together, the margins often overlapping in a predictable way (imbrication pattern). There is a general trend to overlap from dorsal to ventral and from girdle to pole. The boundaries of the plates are the sutures. Cell growth is permitted by the addition of wall material along some of the margins of the thecal plates. These growth zones, often striated, are termed intercalary bands. In gonyaulacoids, plate growth is usually along only one margin of the suture, whereas it is on both in peridinioids. Pores do not usually occur in the intercalary growth zones. The patterns formed by the thecal plates (tabulation) are of critical importance in taxonomy and are discussed here following the description of other internal components, life cycles, and cysts. Recent molecular phylogenetic trees suggest that thecate dinoflagellates are monophyletic (Janouškovec et al. 2016).

Throughout part or all of the life cycle in some species, there may be a thin continuous fibrous layer, the pellicle, usually lying internally to the alveolae. It consists of cellulose, usually with sporopollenin added to varying degrees. It may form the principal strengthening layer of the amphiesma of athecate genera such as *Ptychodiscus*, *Balechina*, *Sclerodinium*, and *Kofoidinium*. In thecate genera such as *Alexandrium* or *Scrippsiella*, it is present beneath the theca for much of the life cycle and forms the wall of temporary cysts, which are formed rapidly and asexually by the shedding of the theca (ecdysis). Athecate cells with a well-developed pellicle are here termed pelliculate.

Microtubules are also usually present below the vesicles of both thecate and athecate forms, presumably adding some strength to the latter and aiding in morphogenesis. Both microtubular and fibrous (banded, rhizoplast) flagellar roots (portions of kinetids) are present, with sphincterlike collars around the flagellar insertion pockets. Peduncles are tubular structures through which food may be drawn, e.g., in "*Katodinium*" *fungiforme*, *Paulsenella*, *Pfiesteria*, etc.

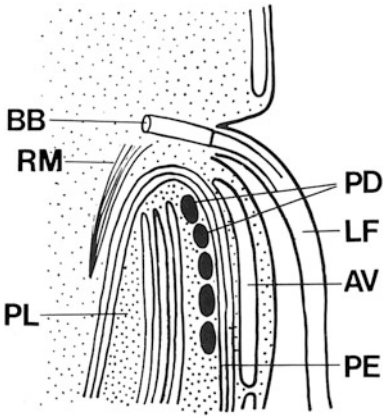

Fig. 5 A typical eyespot, located beneath the longitudinal flagellum (drawn from micrographs by Dodge 1973). *AV* amphiesmal vesicle; *BB* basal body (=centriole); *LF* longitudinal flagellum; *PD* pigment droplets; *PE* plastid envelope; *PL* plastid; *RM* microtubular root

In addition to cholesterol, most dinoflagellate membranes contain a rare 4α,23,24 (R)-trimethyl-5α-cholest-22-en-3-ol, so-called dinosterol, a fossilizing biomarker (Alam et al. 1979); the abundance of dinoflagellate fossils from the Mesozoic onward correlates with levels of derivatives of dinosterols. Early-branching dinoflagellates like syndinians, *Noctiluca*, *Amphidinium*, *Gyrodinium*, and the Kareniaceae lack dinosterol, but the Gymnodiniaceae, *Akashiwo*, and all thecates seem to be able to produce it (Janouškovec et al. 2016).

Ejectile Bodies (Extrusomes)

The most common type of extrusome, of almost universal occurrence in the motile phase, is trichocysts: rod-shaped bodies (Fig. 5) which, when mature, usually lie in the amphiesma perpendicular to the cell membrane. The shaft is a paracrystalline, proteinaceous rod a few micrometers in length, rectangular in cross section. At its distal end, it extends as a group of twisted fibers. The whole is enclosed within a membranous sac, and there is a sheathing material between the rod and the membrane (Livolant 1982a, b). The tip of the sac is in contact with the cell membrane, passing through the amphiesmal vesicles (and thecal plates, if present). The exact mechanism of extrusion is unknown, but it is suspected that the sac ruptures at the contact point at the cell surface, and water entering causes a change in the polymerization of the rod, resulting in an elongation of eight times or more. Trichocysts are formed in the vicinity of the Golgi apparatus (Bouck and Sweeney 1966) and subsequently move to the cell periphery. It appears that most pores in the thecal plates are associated with trichocysts, but this is difficult to establish. Their function is unknown but is assumed to be defensive, excretory, or both. They are most similar to those of ciliates. A less ordered type of extrusome in dinoflagellates is the mucocyst, a simple sac with granular contents, associated with the release of mucoid material.

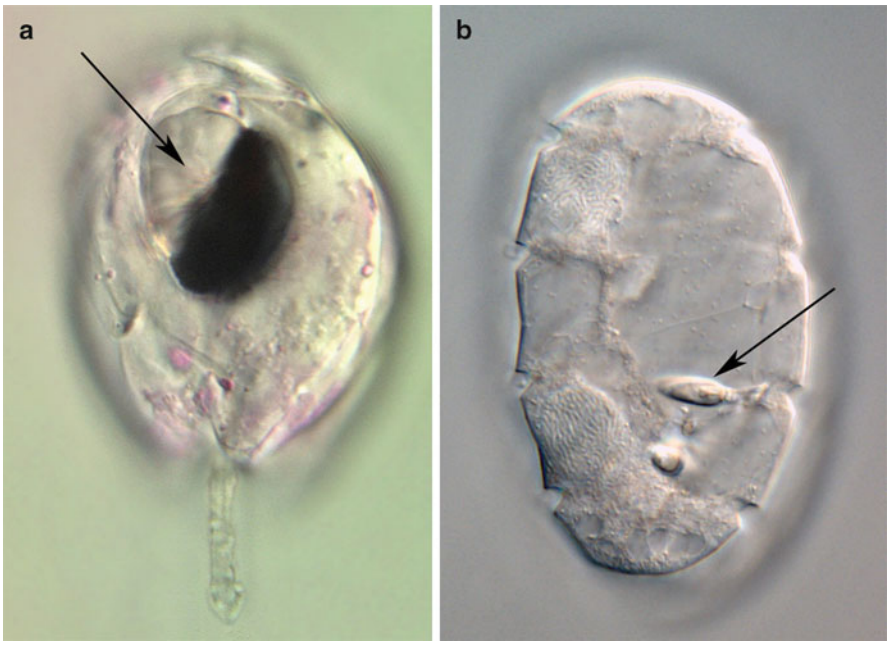

Fig. 6 (**a**) Light micrograph of *Erythropsidinium* sp. *Arrow*: Ocelloid. (**b**) Light micrograph of *Polykrikos kofoidii*. *Arrow*: Nematocyst (Courtesy of Greg Gavelis, Arizona State University)

Much more elaborate extrusomes are found in polykrikoids and warnowiids. These are nematocysts (Fig. 6), named for their resemblance to the stinging organelles of cnidarians (also known as cnidocysts), although their ontogeny differs in a few details (Westfall et al. 1983). Nematocysts are larger than trichocysts and can reach 20 μm in length. They are conical, fluid-filled sacs with a capitate blunt end. Most of the body consists of a large posterior chamber, supported by longitudinal ribs in *Nematodinium*, from which a smaller anterior chamber is isolated; the whole structure is capped by a lidlike operculum. A sharp stylet in the anterior chamber is connected to a tubular filament in the posterior chamber. In *Polykrikos*, it is coiled much like those in cnidarians, and the nematocysts fire by inversion, the stylet driving through the operculum. In *P. schwartzii*, two other structures are invariably associated with the nematocysts: a taeniocyst, which resembles a trichocyst in that it is a solid rod but with more elaborate differentiation (Fig. 6), and a chute with chute organelles, which appears to act as a safe conduit to the exterior when the complex discharges (Westfall et al. 1983). The taeniocyst projects from the cell surface near the kinetosomes. The whole complex originates by coordinated, linked differentiation from Golgi complexes near the nucleus, the primordial forms (anlage) being referred to as the nematogene and taeniogene.

Mitochondria, Golgi Bodies, and Microbodies

Dinoflagellate mitochondria have tubular cristae constricted at the base and arising from the inner membrane. Their genomes are highly unusual (Waller and Jackson 2009): like those of their close relatives, for example, apicomplexans, they encode for only three proteins: cytochrome oxidase 1 (cox1), cytochrome oxidase 3 (cox3), and cytochrome b (cob) as well as ribosomal RNA genes that are fragmented into separate pieces. In dinoflagellates, however, the modification of mitochondrial genomes has gone further than in apicomplexans. For example, all dinoflagellate mitochondrial transcripts need to be edited extensively before translation, and transcripts for at least cox3 need to be trans-spliced (Lin et al. 2002, Zhang and Lin 2005).

Golgi bodies are common, usually near the nucleus; and they may play a role in mitosis, surrounding the zones from which the spindle arises. They give rise to extrusomes. Microbodies are usually present, and some of them seem to be linked with bioluminescence (see below).

Plastids

All dinoflagellates arose from photosynthetic ancestors, and the plastids of a large majority of the photosynthetic members of the group share genetic similarities to the apicomplexan apicoplast and the plastids of chrompodellids like *Chromera* and *Vitrella* (Janouškovec et al. 2015). These so-called peridinin plastids are characterized by triple-membraned (sometimes double-membraned) envelopes, the lack of a girdle lamella, thylakoids usually in groups of unappressed threes, and various types of pyrenoids (Schnepf and Elbrächter 1999). They contain chlorophyll a and c_2 as well as peridinin (a type of carotenoid only found in dinoflagellates), β-carotene, and small amounts of diadinoxathin and dinoxanthin (Jeffrey et al. 1975). DNA-containing areas may be single or multiple, sometimes in prominent "nucleoid-like" regions; they never form a peripheral ring like in some heterokonts (Dodge 1973). In these peridinin-containing plastids, genes appear to exist as minicircles with usually one gene per circle (but two to four in a circle also exist) flanked by a variety of noncoding sequences (Zhang et al. 1999; review in Howe et al. 2008). The absolute number of genes coded in the dinoflagellate peridinin plastids also seems to be much lower than in other algae: while the plastid of cryptomonads, diatoms, and other photosynthetic chromalveolates codes for around 165–185 genes, no more than 16 genes have ever been found in any dinoflagellate peridinin plastid (Green 2004; Nisbet et al. 2004). Some of the missing genes appear to have been moved to the nucleus of the organisms involved (e.g., Hackett et al. 2004a; Bachvaroff et al. 2004), but there are still a number of them that are missing altogether. There are data that suggest that in at least some species, these minicircles may be located in the nucleus, not in the plastids (Laatsch et al. 2004). Peridinin plastids have a bacterial

type of rubisco (evidently a lateral gene transfer) that has a much lower specificity for CO_2 over O_2 when compared to the more common "eukaryotic" rubisco found in other algae (Whitney et al. 1995; Morse et al. 1995). The usual storage products in peridinin dinoflagellates are starch, produced exterior to the plastid, and oils.

In spite of their photosynthetic ancestry, not all dinoflagellates are photosynthetic: roughly half of the members of the group have secondarily lost the ability to photosynthesize and may or may not contain traces of the ancestral plastid. *Oxyrrhis*, *Noctiluca*, and *Crypthecodinium*, for example, contain plastid-targeted proteins even if an organellar plastidial remnant has not been identified, but the syndinian *Hematodinium* appears to have lost all traces of a plastid (Gornik et al. 2015; Janouškovec et al. 2016).

The diversity in types of photosynthesis that exists within dinoflagellates is unparalleled within any group of eukaryotes (Schnepf and Elbrächter 1999), but in this group, it is not always easy to distinguish between true plastids (here defined as organelles that include proteins encoded in their host's nucleus), endosymbionts that have not transferred genes to the host's nucleus but that nevertheless may well be permanent, and other phenomena related to photosynthesis acquisition, for example, kleptoplastidy. The green symbionts in *Noctiluca*, diatoms in the dinotom clade, pelagophytes in *Amphisolenia*, and dictyochophytes in *Podolampas* are (probably) examples of endosymbioses with no genetic transfer to the nucleus (only the dinotoms have been studied in detail in this respect, E. Hehenberger, pers. comm.); at least in dinotoms, this endosymbiosis seems to be permanent. Genetic transfers to the host's nucleus seem to have occurred in at least two lineages that have replaced their peridinin plastids for plastids with completely different origins: the Kareniaceae (*Karenia*, *Karlodinium*, and *Takayama*), which have obtained a haptophyte-derived plastid through tertiary endosymbiosis (Ishida and Green 2002; Patron et al. 2006; Nosenko et al. 2006), and the gymodiniacean genus *Lepidodinium*, which has a plastid derived from a green alga (Watanabe et al. 1991; Minge et al. 2010).

In addition to permanent plastid replacements, non-photosynthetic dinoflagellates may reacquire photosynthesis through the temporary use of plastids from their prey, so-called kleptochloroplasts (stolen chloroplasts; Schnepf and Elbrächter 1999; Janson 2004). Plastids acquired in this way are either eventually digested or lost because of imperfect distribution to daughter cells following division. This is not a rare phenomenon; it has been shown to occur in several eukaryotic lineages like foraminiferans, ciliates, katablepharids, and even animals (sea slugs). In dinoflagellates, kleptochloroplasts have been found in several lineages, for example, *Dinophysis*/*Phalacroma*, *Amylax*, and *Nusuttodinium*, and in an undescribed member of the Kareniaceae, but details are different in the different lineages. *Nusuttodinium* and the undescribed kareniacean use plastids that they take directly from their prey, cryptomonads, and the haptophyte genus *Phaeocystis*, respectively (Onuma and Horiguchi 2015; Sellers et al. 2014). In *Nusuttodinium aeruginosum*, the prey's nucleus and nucleomorph are retained together with the plastid, but as the dinoflagellate lacks the mechanism to initiate the cryptomonad nucleus' division, this is only passed on to one daughter cell after the dinoflagellate's cell division.

Dinoflagellate daughter cells containing cryptomonad nuclei have large, healthy kleptochloroplasts, but in the ones that lack it, the plastids start to degenerate (Onuma and Horiguchi 2015). *Dinophysis* and *Amylax* also have cryptomonad-derived kleptochloroplasts, but they acquire them indirectly by feeding on another kleptoplastidic organism, the ciliate *Mesodinium rubrum*. However, while *Amylax*, like *Mesodinium*, retains the cryptomonad's nucleus and nucleomorph as well as the plastid (Kim et al. 2014), *Dinophysis* seems to digest the cryptomonad nucleus and nucleomorph and retains only the plastid itself. In spite of this, *Dinophysis* kleptochloroplasts can remain viable for at least 10 weeks, a similar amount of time to what is observed in *Mesodinium rubrum*. One possible reason for this is that the *Dinophysis* nucleus contains plastid-targeted genes that may help keep the plastid active; while some of these genes seem to be remnants of the original peridinin plastid of dinoflagellates, others seem to have been obtained from cryptomonads, haptophytes, and other algae (Wisecaver and Hackett 2010). At least one species of *Dinophysis*, *D. mitra*, contains kleptochloroplasts of haptophyte origin (Koike et al. 2005) that may be obtained by preying on kleptoplastidic ciliates like *Tontonia*, *Laboea*, or *Strombidium* (Nishitani et al. 2012).

Eyespots and Ocelloids

No protist group displays so many eyespot types as dinoflagellates (Hansen et al. 2007). Four types (not including ocelloids; see below) have been distinguished, all situated in the sulcal area close to the flagellar roots where they are likely to be shadowed by the proximal part of the longitudinal flagellum. In many dinoflagellates, like in many photosynthetic heterokonts, eyespots consist of osmiophilic, carotene-containing globules inside the plastid, usually as a single or double layer between the plastid envelope and the outermost thylakoids. In some groups, an elongated vacuole that contains brick-like vesicles is located in front of the eyespot but outside the plastid (e.g., the *Borghiella*/*Baldinia* clade; Hansen et al. 2007; Moestrup et al. 2008). In suessialean dinoflagellates, these brick-like vesicles form multiple layers. Another type of eyespot, found in genera like *Esoptrodinium*, *Jadwigia*, *and Tovellia*, consists of osmiophilic globules not bounded by any membrane, floating free in the cytoplasm. And in dinotoms osmiophilic granules are surrounded by three membranes, a situation that has given rise to the hypothesis that this organelle represents the remnant of the original peridinin-containing plastid (see section on "Evolutionary History"). The detailed structure of the eyespot in other dinoflagellates, for example, in non-photosynthetic species like *Oxyphysis oxytoxoides*, is unknown. In *Protoperidinium* species, numerous large carotenoid-like masses occur throughout the cell periphery prior to cyst formation and may act as a reserve material for the wall or for metabolism.

The ocelloid (ocellus) found in the seven genera of the warnowiaceans is a complex organelle showing extraordinary resemblances to metazoan eyes, but at a subcellular level and without any neurological connection to a brain. It consists of four primary components: a darkly pigmented cup called the retinal body; a lenslike,

refractile hyalosome; iris-like rings; and a transparent, cornea-like layer over the lens. The lens is constructed of secretions of unknown material within endoplasmic reticulum and is surrounded by constricting fibers that have been suggested to change the shape of the lens (Greuet 1978, but experimental proof of this is lacking). The "cornea," a transparent layer covering the lens, is composed of mitochondria that extend into a network in the surrounding cytoplasm (Gavelis et al. 2015). The retinal body consists of a cuplike structure containing very precisely aligned membranes backed by a layer of reddish brown to black pigment droplets (Greuet 1978). This retinal body turns out to be a heavily modified plastid: it contains DNA that encodes plastidial genes and dedifferentiates into a plastid of more standard morphology at the end of interphase. The outer membrane of the retinal body of *Nematodinium* is contiguous with that of peridinin plastids that also exist in this cell, and so appears to be a part of a larger netlike plastid. At least some warnowiaceans (e.g., *Nematodinium*) feed on other dinoflagellates, and because the dinoflagellate dinokaryon polarizes light, it has been suggested that function of the ocellus may be to recognize polarized light (Gavelis et al. 2015).

Pusules

In the motile cell, there are usually two specialized vacuoles that arise from ducts that open at the flagellar bases, in addition to the generalized cell vacuolar system (vacuome). These pusules are particularly large in *Protoperidinium*, where they are differentiated into a sac pusule, which can occupy a third or more of the episome, and a collecting pusule, which resembles a cluster of grapes. Each has evaginations, which can be highly elaborate, running close to the vacuome membrane where exchange presumably takes place. Although they resemble water-regulating vacuoles, they do not behave like them. They are most developed in non-photosynthetic marine species. They may be for excretion, uptake, or both (one for each). They do not participate in phagotrophic ingestion, and large particles are usually absent from them. At the ultrastructural level, a flaky material may coat the surface of one of them.

Luminous Organelles

Marine dinoflagellates in at least 18 genera have been documented as being capable of bioluminescence (Poupin et al. 1999); they account for much of the planktonic bioluminescence in oceans. *Pyrocystis noctiluca* and *Noctiluca scintillans* are particularly important in oceanic tropical and coastal temperate regions, respectively. The luminescence occurs as a brief (0.1 s) blue flash (max 476 nm) when stimulated, usually by mechanical disturbance. Flashes have been seen to emanate from individual cytoplasmic bodies ca. 0.5 μm in diameter distributed mainly in the cortical region of the cell (Johnson et al. 1985; Hastings 1986) as pockets that protrude into the main cell vacuole. These so-called scintillons contain luciferase, the main

enzyme involved in dinoflagellate bioluminescence (Nicolas et al. 1985), and luciferin, a tetrapyrrole ring structurally similar to chlorophyll that acts as the substrate to the light-producing reaction. At physiological pHs, (pH 7–8), luciferase is inactive, and luciferin is bound to a protein. Light generation occurs when the pH in the scintillon is lowered to about pH 6, the luciferin is released, and the luciferase takes its active conformation (Hastings 1996). The triggering mechanism for the whole reaction is most commonly mechanical: shearing pressure deforms the cell's plasma membrane, where mechanoreceptors signal a release of calcium ions into the cytoplasm. This forms an action potential across vacuolar membranes in the cell and causes the opening of proton channels in the membrane that release hydrogen ions into the cytoplasm and into the scintillons. The consequent lowering of the pH in the scintillons triggers the light-producing reaction. Luciferin production probably occurs in plastids (cryptic ones in non-photosynthetic dinoflagellates) from precursors repurposed from heme and chlorophyll production (Janouškovec et al. 2016).

Predation on zooplankton by fish and cephalopods is facilitated by dinoflagellate luminescence (Mensinger and Case 1992; Fleischer and Case 1995). The idea proposed to explain this, the so-called burglar-alarm hypothesis, postulates that shearing stress caused by copepod feeding currents trigger dinoflagellate bioluminescence and that this bioluminescence is then used by visual predators like fish and squid to find their zooplankton prey. This, in the end, benefits the dinoflagellates. An alternative possibility is that bioluminescence may startle predators and discourage their feeding (Buskey and Swift 1983).

Luminescent and nonluminescent strains can occur in the same species, e.g., *Alexandrium tamarensis* and *Noctiluca scintillans*.

Dinoflagellate bioluminescence is controlled by circadian rhythms and only occurs at night (e.g., Knaust et al. 1998)

Skeleton

Internal skeletal elements, siliceous in some species, are known in genera of the actiniscaceans and dicroerismaceans. In *Dicroerisma*, there is a single, branching skeleton in the shape of an inverted Y. In *Actiniscus*, the siliceous internal elements are also paired and are star shaped. Basketlike peripheral skeletons are present in *Achradina* and *Monaster*.

Nucleus

The dinoflagellate nucleus is so different from that of typical eukaryotes that it is usually given its own name, the dinokaryon; in the 1960s, the ultrastructural and biochemical differences between dinokarya and typical eukaryotic nuclei were deemed to be important enough to warrant the establishment of an intermediate kingdom between prokaryotes and eukaryotes, the so-called Mesokaryota (Dodge 1965). This view was subsequently disproved by molecular data.

Dinoflagellate nuclei lack nucleosomes (e.g., Rizzo 1991), and the ratio of basic proteins to DNA in them is much lower than in any other eukaryotes (1:10 in dinoflagellates, as opposed to the equimolar ratios found in other eukaryotes). The main basic components in dinoflagellate nuclei are not histones but other types of basic proteins that interact with DNA: so-called DVNP's (dinoflagellate/viral nucleoproteins) that are otherwise only known from a group of large algal viruses (Gornik et al. 2012) and HLPs (histone-like proteins, Wong et al. 2003), which seem to have entered dinoflagellates in two separate waves of lateral transfer from bacterial sources (Janouškovec et al. 2016). Dinoflagellates also contain very high amounts of DNA per cell: 3,000–215,000 Mbp weighing up to 250 pg in a haploid nucleus (in humans those numbers are 2,900 Mbp DNA/cell and 3 pg in a haploid cell). Chromosomes remain continuously condensed and visible during interphase and mitosis, but whereas syndinians have few chromosomes (four in *Syndinium*, Ris and Kubai 1974), some species may have up to 143 (*Alexandrium fundyense*, Oakley and Dodge 1974).

In the so-called core dinoflagellates, chromosomes appear fibrillar, the 3–6 nm fibrils being packed in a highly ordered state (up to six levels of coiling), consisting of arches and whorls (e.g., Dodge 1966; Spector et al. 1981). A prominent nucleolus is also persistent. In those species investigated, there is an unusual substitution (12–68%) of the base thymine by 5-hydroxymethyluracil (Rae 1976).

All nuclear-encoded messenger RNAs investigated in a wide diversity of members of the dinoflagellate lineage (including *Perkinsus marinus*) have been recently shown to be trans-spliced to a universally conserved 22 base pair fragment that is added to their 5′ end (Zhang et al. 2007; Lidie and Van Dolah 2007). In core dinoflagellates, many highly expressed genes are arranged in tandem arrays, a feature that is very rare in eukaryotes (Bachvaroff and Place 2008).

Mitosis

Dinoflagellate mitosis is also unusual. The nuclear envelope persists during mitosis ("closed"), as it does in many other eukaryotes (Raikov 1994). However, with the exception of *Oxyrrhis marina* and several species of the genus *Amoebophrya* (Triemer 1982; Moon et al. 2015), the mitotic spindle is extranuclear and passes through furrows and tunnels that form in the nucleus at prophase (Dodge 1987 and references therein). With the exception of the centrioles in *Syndinium*, there are no obvious spindle pole bodies other than concentric aggregations of Golgi bodies ("archoplasmic spheres"). Some microtubules contact the nuclear envelope, lining the tunnels at points where the chromosomes also contact. The chromosomes usually have differentiated, dense regions inserted into the envelope.

Cytokinesis

The plane of cell cleavage is typically oblique between anterosinistral and posterodextral moieties, passing through the kinetid. In thecate species, the theca may be

shared by the offspring, with synthesis of the missing components (desmoschisis), or the parent theca may be cast off, each offspring forming a complete new theca (eleutheroschisis).

In photosynthetic forms, the time of division is phased; this is controlled by an endogenous (circadian) mechanism (see below). Division typically occurs near the end of the dark period, but in several species, it is phased at other times (Hastings and Sweeney 1964). Division rates are usually relatively slow, many species dividing only once every 2 or more days. *Amphidinium carterae* can divide twice in 1 day. The non-photosynthetic species *Crypthecodinium cohnii* is the most rapidly reproducing dinoflagellate known, dividing three times per day, although parasites may divide faster during sporogenesis via palintomy.

Life Cycle

Most dinoflagellates appear to be haploid, with post-zygotic meiosis. Clearly established sexual fusion is known for only a few species, but, because of its cryptic nature (gametes grossly resembling regular motile cells, slow fusion, occurring at night in photosynthetic species), it is probably widespread.

Syngamy may involve equal (isogamy) or unequal (anisogamy) motile gametes (see Fig. 7). Both heterothallism (no fusion in clonal strains) and homothallism are known. The product of fusion is a tri- or quadriflagellate planozygote (later biflagellate in some), which may remain motile for hours or days. Eventually a nonmotile resting cyst (hypnospore) is formed. After a varying length of time (see section "Cysts" below), excystment occurs. Meiosis, heralded by a peculiar churning and rotation of the nucleus termed nuclear cyclosis, associated with the pairing of homologous chromosomes, may precede or follow excystment and may be accomplished in two conventional, successive divisions (e.g., *Ceratium cornutum*) or possibly one (*Crypthecodinium cohnii*). In some species, the planozygote that emerges from the cyst may again be tri- or quadriflagellate.

In most dinoflagellates, the motile phase (mastigote) is dominant, but in some, most of their life cycle is spent in a coccoid or other nonmotile form. Those living as intracellular symbionts (e.g., *Symbiodinium)* are photosynthetically and reproductively active in the coccoid state (vegetative cyst: see section "Cysts" below). Some marine planktonic forms, such as *Pyrocystis*, live predominantly as greatly inflated trophic cysts, as do the benthic phases of genera like *Halostylodinium*, *Spiniferodinium*, *Cystodinium*, etc. *Thoracosphaera* and *Pfiesteria* are other genera that can divide in the cyst stage. These coccoid life stages usually lack amphiesmal vesicles, trichocysts, and pusules, as well as flagella, and as a consequence are often difficult to identify as dinoflagellates. A continuous, fibrous wall that may be greatly reduced in the symbionts appears to be homologous with the pellicular layer and cyst wall. In the broadest sense, they represent cysts that are metabolically active rather than dormant. Transient mastigote phases occur in these species; they are suspected to be gametes, although no fusion has been seen.

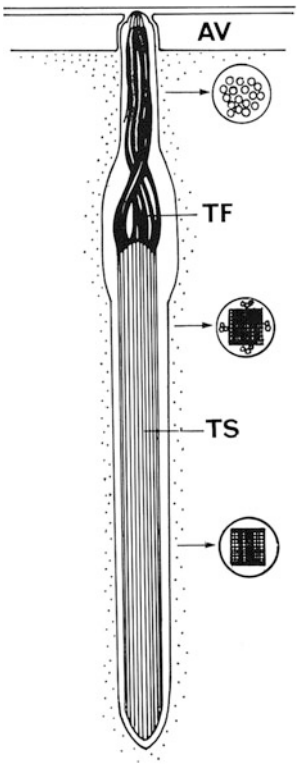

Fig. 7 Longitudinal section of a trichocyst (redrawn from Bouck and Sweeney 1966). *AV* amphiesmal vesicle, *TF* trichocyst fibers, *TS* trichocyst shaft.

Cysts

In dinoflagellates the protozoological term cyst, rather than the approximately equivalent botanical term spore, has been used for nonmotile, continuous walled stages. Fewer than 15% of the living forms are known to form cysts, although the figure is climbing steadily; virtually all fossils appear to be cyst stages; see below. Dale (1983) has reviewed cyst biology, and Fensome et al. 1993 has unified the classification of extant and fossil dinoflagellates.

Cysts can be of several types, according to their roles in the life cycle, and the literature may be confusing because of earlier lack of awareness of this and the lack of standardization of terms. Here, the following are recognized:

1. Resting cyst (resting spore, hypnozygote) –
   A dormant stage, generally resistant to adverse conditions. In several instances (see above), these result from sexual fusion, but it is not known if this applies to most of them. The wall may contain a sporopollenin-like material, additional to cellulose and/or gelatinous material, and may be of several layers. Internally, the contents often shrink (due to loss of water), storage products become polymerized (oils, starch), photosynthetic pigments are gradually reduced, and a large, red-pigmented body is often formed.

2. Temporary cyst (pellicle cyst, ecdysal cyst) –
   In those thecate species with a well-developed pellicular layer (e.g., *Alexandrium* and *Scrippsiella* spp.), the cell may respond to rapid adverse changes by shedding the theca (*ecdysis*), including the outer amphiesmal layers and axonemes, the pellicular layer becoming the cyst wall. In *Pyrophacus* and *Protoperidinium*, this accompanies eleutheroschisis.
3. Trophic cyst (coccoid cells) –
   Nonmotile, usually photosynthetic cells that are metabolically and reproductively active in this phase. Surrounded by a continuous wall homologous with the pellicle (e.g., *Symbiodinium*, *Pyrocystis*, *Spiniferodinium*, *Thoracosphaera*).
4. Digestion cyst –
   This type, in which the organism encysts after feeding, is common in some phagotrophic protist groups but is rare in dinoflagellates. "*Katodinium*" *fungiforme* is an example.

In the first two types, encystment, or the sexual events leading to it, can be triggered by nutrient stress (e.g., nitrogen starvation, the most common experimental method used) or changes in light intensity, photoperiod, or temperature (von Stosch 1964), but other factors are probably also involved. Cyst formation is most commonly observed toward the end of blooms or in the senescent phase of batch cultures.

In many cases, cysts "reflect" the tabulation of the motile cells that gave rise to them by way of ridges or other features like spines, processes, the shape of excystment apertures (archeopyles), etc., that mark the position of thecal boundaries in the motile cells (Fig. 7). This "pseudotabulation" is critical for the taxonomy of fossil taxa.

Excystment will occur after a relatively fixed period at constant temperature. Lower temperature generally prolongs the period. A rapid rise in temperature often triggers excystment. Light may or may not be required. Anaerobic conditions inhibit excystment (see Dale 1983; Pfiester and Anderson 1987 for further details). A residual body, dark brown in color, is often left behind in the empty cyst. It may correspond to an accumulation body or the red body of the cyst (Fig. 8).

Thecal Patterns (Tabulation)

The tabulational patterns formed by the alveolae and the thecal plates contained in them have been used in taxonomy for more than 100 years. Six fundamental types can be recognized (Fig. 9):

1. *Gymnodinoid*. Alveolae are numerous and often hexagonal, the girdle and sulcus being the only clearly distinguishable series. The plates may be too delicate to see or entirely absent. Gymnodinoid tabulations are present in the gymnodiniales and in some members of the distantly related Symbiodiniaceae and Borghiellaceae.

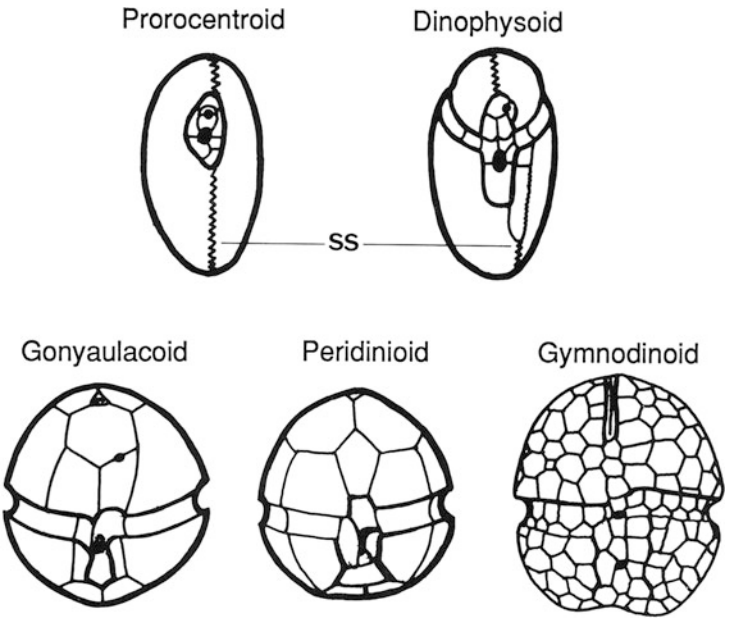

Fig. 8 Basic thecal organizational types (From Fensome et al. 1999)

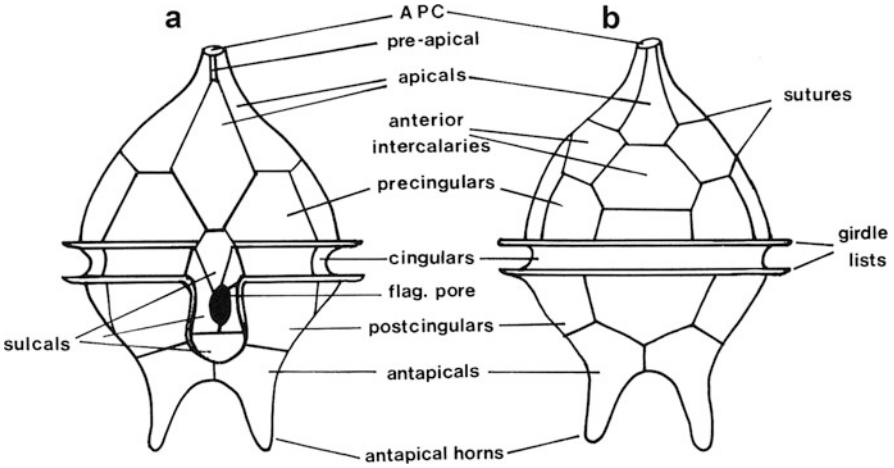

Fig. 9 Thecal plate terminology for a peridinioid or gonyaulacoid taxon. (**a**) ventral view. (**b**) dorsal view

2. *Suessioid*. Amphiesmal vesicles arranged in 6–11 latitudinal series. The number of plates per series, or even the number of series, varies with species. The cingulum is well marked, and it may contain one or two rows of plates. Named after the fossil genus *Suessia*. Extant genera with a suessioid tabulation include

Symbiodinium and *Polarella*, but recent data suggest that fossil Suessiales are not related to *Symbiodinium* and its relatives (Janouškovec et al. 2016).

3. *Peridinioid and Gonyaulacoid*. In these there are five distinct primary latitudinal series termed from apex to antapex/posterior the apicals, precingulars, cingulars (girdle), postcingulars, and antapicals. Plates lying between these series are termed *intercalaries* (anterior or posterior on the epi- or hypotheca, respectively), and those lying within the sulcus are *sulcals*. The midventral epithecal plate often spans both the precingular and apical series. By convention it has been termed the first apical plate (1′). At the apex, an apical pore complex (APC) is often present, consisting of an outer (Po) and inner (Pi) pore plate, and a small pre-apical platelet (Pp) is often present in peridinioids. Apical plates are those that contact the APC. Peridinioid tabulations are defined by a more-or-less symmetrical first apical plate and by the presence of two antapical plates; in gonyaulacoid tabulations, the first apical plate is asymmetrical; and there are two to four fundital plates.
4. *Nannoceratopsioid (fossil only)*. Laterally-flattened cells with a reduced episome. Only cysts are known, and they reflect a sagittal suture dividing the hyposome into right and left halves, like in dinophysoid tabulations. Episomes, however, reflect a gonyaulacoid-peridinioid type of tabulation.
5. *Dinophysoid*. The theca is fundamentally divisible into two halves by a vertical sagittal suture, but a girdle and sulcus are "superimposed" on it, separating an epitheca and hypotheca, and there are small plates on the ventral surface of the epitheca, hypotheca, and in the sulcus around the single large flagellar pore. A simple apical pore is located on the ventral side of the epitheca. The arrangement of the plates varies little within the group, with 18 or 19 being the usual number. Lists (ridges or extensions of the edge of thecal plates) along the girdle and sulcus edges may be prominent and developed to an extraordinary degree in some genera (e.g., *Ornithocercus*, *Histioneis*, and *Citharistes)*, producing bizarre forms, some forming a "phaeosome chamber" from the girdle lists in which extracellular coccoid cyanobacteria occur.
6. *Prorocentroid*. The theca is composed of two large plates, the valves, which join along a toothed margin, the sagittal suture (Figs. 2 and 6). An apical cluster of small platelets of regular arrangement, 8–12 in number (nomenclature in Hoppenrath et al. 2013), surrounds the two pores from which the desmokont flagella arise. The periflagellar platelets lie principally in an excavation of the right valve. A small spine often arises from the periflagellar plate designated as "a" (Taylor 1980).

The plates in each latitudinal series are numbered from the cell's left to right, beginning with the plate closest to the midventral position. This convention, the "Kofoid System," is currently in universal use (Fig. 10). It also uses a notation to designate the series, using primes to indicate the apical (′), precingular (″), postcingular (‴), and antapical (⁗) plates, both when labelling plates on figures and when producing a plate formula. The latter is a listing of the total plates in each series for a species or genus. Thus *Gonyaulax* is represented by Po, Pi, 3′, 2a, 6″, 6C + t, 6S, 6‴, 1p, 1⁗, and *Peridinium* by OP, 4′, 3a, 7″, 5C + t, 6S, 5‴, 2⁗. Cingulars (C),

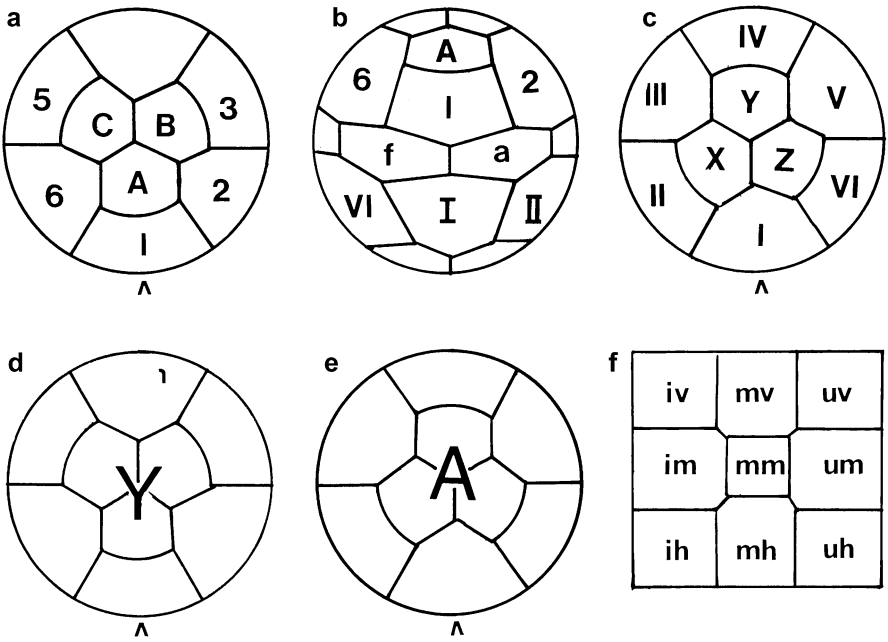

Fig. 10 Model and plate designation used in the Taylor homology system (from Taylor 1980, with modifications by Evitt 1985). (**a**) Polar view; (**b**) ventral view; (**c**) antapical polar view; (**d**) the "Y" arrangement of polar plates relative to the flagellar insertion; (**e**) the "A" arrangement; (**f**) designations for subdivision of a primary plate area (maximum subdivision) using Evitt's modification; *i* initialis; *u* ulter; *m* medialis; *v* vorner; *h* hinter (the latter selected because they are not letters used for whole plates in either system)

sulcals (S), anterior intercalaries (a), and posterior intercalaries (p) are designated by letters. The *t* plate is a small transitional plate between the cingulars and the sulcals at the proximal end of the girdle in peridinioids and at the distal end in gonyaulacoids. Other distinctions between gonyaulacoids and peridinioids include the common occurrence of 6″, 6‴, 1p, and 1⁗ in the former and 2–3a, 7″, 5‴, and 2⁗ in the latter (exceptions being due to apparent suture loss or plate subdivisions); intercalary growth from the overlapping plate margin only in gonyaulacoids versus both sides of a suture; and basic symmetry: the former showing evident torsion, the latter tending to bilateral symmetry.

Although the Kofoid System is usually easy to apply, ambiguities in the attribution of some plates to one series or another can cause problems, resolved by following consensus. This, combined with the mechanical, consecutive numbering, renders the system poor for intergeneric comparisons. Taylor (1980) has introduced a basic model (Figs. 9 and 10) elaborated on by Evitt (1985), consisting of three epithecal polar (A–C), six pre-equatorial (1–6), six equatorial (a–f), six post-equatorial (I–VI), and three hypothecal polar (X–Z) sectors, which represent hypothetical primary plates from which homologous plates can be recognized by

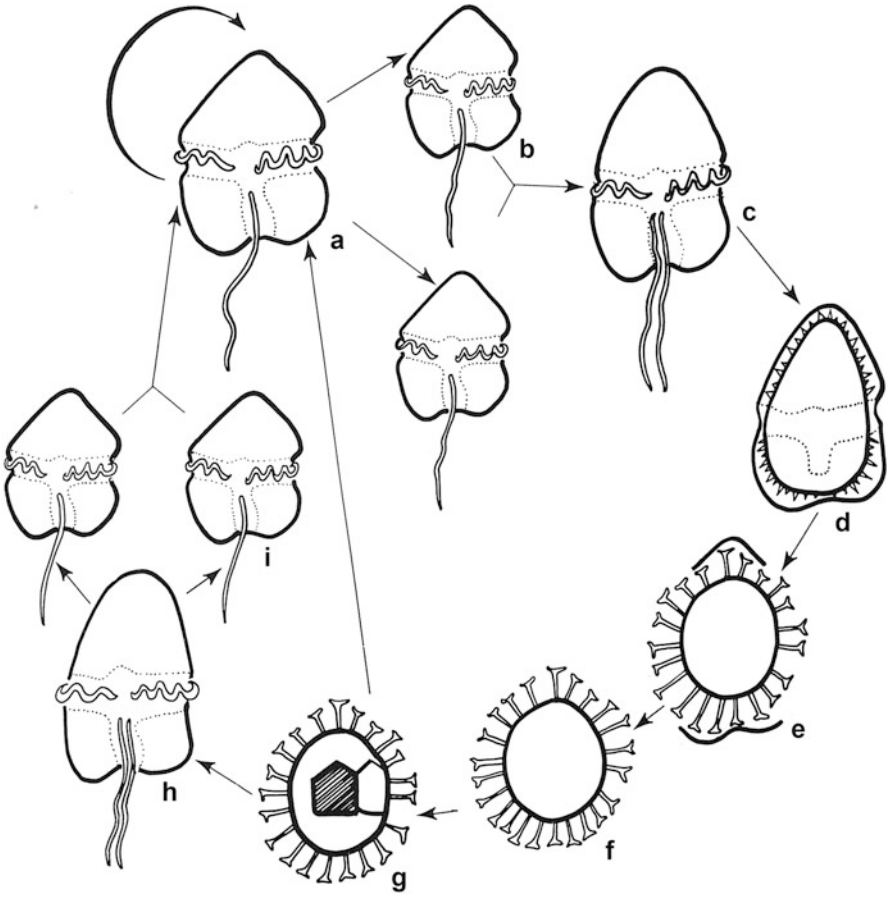

Fig. 11 Common dinoflagellate life cycle (modified from Dale 1983). (**a**) Asexually reproducing motile cell (mastigote); (**b**) gametes (can be iso- or anisogametes); (**c**) planozygote; (**d**) hypnocyst (resting cyst) formation within the theca; (**e**) theca discarded (cysts may be smooth, rigid or spiny); (**f**) dormancy; (**g**) excystment through the archaeopyle; (**h**) meiocytic planozygote; (**i**) meiotic division (**h** and **i** may take place in the cyst and meiosis may involve one or two divisions). Not shown: temporary cysts may be asexually produced from asexually reproducing motile cells (**a**). Pyrocystis and other photosynthetically active amastigotes may be in sexually or asexually produced, pellicle-surrounded "cysts"

assuming subdivisions, suture losses, and plate size and position changes. The first step is to normalize the cell to a sphere, removing obvious plate distortions. Then the primary plates and their sutures are determined by studying the relationships of the plates to each other (see examples given by Evitt 1985).

Cyst walls often exhibit patterns of ridges, spines, or other surface ornamentation, which correspond to the tabulation of the parent theca, although some sutures are often not reflected on the cyst. The pattern discernable on the cyst wall is termed paratabulation and is used extensively in fossil cyst taxonomy (Figs. 11 and 12).

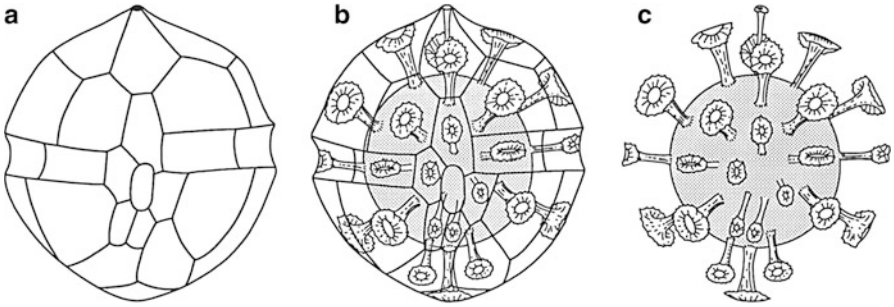

Fig. 12 Development of the fossil cyst species *Hystrichosphaeridium tubiferum* from a hypothetical parent theca. Central body of cyst is shaded (Adapted from Evitt (1985), Fensome et al. (1993). Copyright Micropaleontology Press)

Fossils

If one disregards acritarchs (microfossils with ambiguous morphologies that may or may not be of dinoflagellate origin), a large majority of dinoflagellate fossils consist of cyst stages of forms with gonyaulacoid or peridinoid tabulations (Fensome et al. 1999). Dinoflagellate fossils of other types are rare; they include, for example, suessialean forms, forms with possible dinophysoid affinities (*Nannoceratopsis*), a few Cenozoic gymnodinoid cysts, and fossil chemical traces like dinosterols. In a few cases, some otherwise ambiguous cyst morphologies have been shown to be of dinoflagellate origin through the study of cyst stages of extant forms.

About 15% of extant dinoflagellates produce fossilizable cysts (Head 1996). This does not mean that that was the case in the geologic past, but it does seem to be clear that the fossil record of dinoflagellates is highly incomplete. Nevertheless, certain patterns regarding the evolutionary history of the group can still be recognized.

Fossil dinoflagellates are controversial or absent in strata prior to the early Mesozoic, but quantities of dinosteranes (derivatives of dinosterols, chemical compounds as of yet associated almost exclusively with dinoflagellates) correlate well with some acritarch species' abundance in the Paleozoic (Moldowan and Talyzina 1998). Nevertheless, pre-Mesozoic dinosteranes are unlikely to have originated from dinoflagellates: in extant dinoflagellates, dinosterol is only produced by thecate dinoflagellates and a few of their closest athecate relatives (Janouškovec et al. 2016), and the earliest confirmed (thecate) dinoflagellate fossil is from the mid-Triassic (Fensome et al. 1999). Dinosteranes in early Triassic sediments could be derived from athecate relatives of the thecate clade, but it is unlikely that dinosterol-producing dinoflagellates were present earlier than that. After the mid-Triassic, species diversity increases steadily until the early Cenozoic, and then it declines toward the present day (MacRae et al. 1996). By the mid-Jurassic, practically all major morphological variations of peridinioid and gonyaulacoid forms were already present, and late innovations are very minor. *Nannoceratopsis*, a striking "missing link" between peridinioids and dinophysoids, lived also in the

early Jurassic, as did forms with a suessialean tabulation (already there since the mid-Triassic). Paleontological evidence points to an evolutionary radiation of thecate, cyst-forming dinoflagellates in the late Triassic/early Jurassic that involved early experimentation, stabilization later, and the early presence of "missing links." Whether this evolutionary radiation involved fossil-poor gymnodinoid forms cannot be determined by paleontological data alone.

Maintenance and Cultivation

Dinoflagellates are usually grown in enriched, filtered, and sterilized marine or freshwater. The methods and the media used have been described in detail by Guillard and Keller (1984). The most commonly used media for photosynthetic marine forms are dilutions of Guillard's *f* medium or modifications of Provasoli's *ES*, with Chu's no. 10 for freshwater species. Totally artificial media rarely support vigorous growth, and agar is not suitable for most species. Dinoflagellates are inhibited by strong agitation and prefer light/dark cycles (typically 14:10) to continuous illumination. Many are difficult or impossible to grow axenically (bacteria-free) at present.

Phagotrophic non-photosynthetic species are usually fed smaller, photosynthetic flagellates, with precautions to avoid overgrowth by the latter. Organism-free organic media have been developed for *Oxyrrhis marina* and *Crypthecodinium cohnii*.

Evolutionary History

Molecular evidence shows that the closest relatives of dinoflagellates are apicomplexans and ciliates. These three eukaryotic clades, together with the paraphyletic group that includes their ancestors, the protalveolates (perkinsids, *Colponema*, etc.), form the so-called Alveolates (Cavalier-Smith 1991), one of the best-supported groupings that have emerged from the analysis of molecular phylogenetic data in eukaryotes (e.g., Fast et al. 2002; Cavalier-Smith and Chao 2004 and many others). Morphological data also strongly supports this clade (e.g., Taylor 2004). The closest relatives of alveolates are the stramenopiles (also called heterokonts), the grouping that contains oomycetes, labyrinthulids, opalinids, chrysophytes, diatoms, and brown algae, among others. The relationship between alveolates and stramenopiles is also very well supported with molecular data (e.g., Fast et al. 2001; Harper and Keeling 2003; Hackett et al. 2004a).

The question of whether dinoflagellates evolved from photosynthetic ancestors was answered by the discovery of *Chromera*, a photosynthetic endosymbiont of corals that in phylogenetic trees branches at the base of the apicomplexans (Moore et al. 2008) and whose plastid genes strongly resemble those of the apicomplexans' apicoplast and the peridinin plastids of dinoflagellates (Janouškovec et al. 2010). *Chromera* is only one member of a clade that contains several photosynthetic and many non-photosynthetic members, the so-called chrompodellids, and by comparing

the patterns of the presence, absence, and localization of metabolic pathways involving plastidial elements in chrompodellids, apicomplexans, and dinoflagellates, it was possible to explain the presence of photosynthetic plastids in some members of these groups and not others (Janouškovec et al. 2015). Some non-photosynthetic members of the dinoflagellate lineage have now been shown to contain either plastid-targeted genes or major plastid-associated biosynthetic pathways, e.g., the perkinsozoan *Perkinsus marinus* (Stelter et al. 2007; Matsuzaki et al. 2008; review in Fernández Robledo et al. 2011), *Oxyrrhis marina* (Slamovits and Keeling 2008), *Noctiluca scintillans* (Janouškovec et al. 2016), and *Crypthecodinium cohnii* (Sánchez-Puerta et al. 2007), but in the syndinians, plastids appear to be completely lost (Gornik et al. 2015).

Initially dinoflagellate phylogenetic trees had backbones that were poorly resolved, and so it was difficult to determine phylogenetic relationships of large groups to each other based on this kind of data alone (Daugbjerg et al. 2000; Saldarriaga et al. 2004; Orr et al. 2012); the main value of molecular phylogenetic data was to clarify in-group phylogenies, for example, within groups like calciodinellids, pfiesteriaceans, polykrikoids, or the genera *Symbiodinium* or *Alexandrium*, as well as to underline the differences between groupings of gymnodinoids. More recent phylogenetic studies based on large concatenations of protein sequences (101 genes in Janouškovec et al. 2016) have started to produce phylogenetic trees with better resolved backbones. They suggest that *Oxyrrhis marina* is the earliest branch of the dinoflagellates, followed by the syndinians; whether the Syndiniales are a monophyletic or a paraphyletic group is still unclear. The next group to branch off are the Noctilucales, and the Gymnodiniales build a paraphyletic group that gave rise to thecate dinoflagellates, which are monophyletic. Only a few gymnodinialean lineages are as of yet present in large protein-based trees, but it looks like *Amphidinium* makes the earliest branch after the Noctilucales, followed by the Kareniaceae, the *Gymnodinium* group of families (a single clade that includes *Gymnodinium*, *Togula*, and *Polykrikos*), and *Akashiwo*, the sister group to thecates. The branching order of the thecate groups is not yet clear, but the group includes the Symbiodiniaceae; it looks like the suessioid and gymnodinoid tabulations of the Symbiodiniaceae and Borghiellaceae represent secondary losses of theca (Janouškovec et al. 2016).

Morphological data and palaeontological "missing links" do suggest a close relationship between the four thecate dinoflagellate groups: one theory (unsupported as of yet by molecular data) suggests that the more-or-less symmetric peridinioids arose from gymnodinoids and constitute a paraphyletic grouping that gave rise certainly to the (much more asymmetric) gonyaulacoids, as well as to the *Symbiodinium* group and the dinophysioids (Taylor 2004). The fossil genus *Nannoceratopsis* is a morphological intermediate between peridinioids and dinophysioids (Fensome et al. 1993). The sixth thecate group, the prorocentroids, may have originated from dinophysioid ancestors (Taylor 1980).

A recent study using large phylogenies has suggested that dinoflagellates are primarily a marine group and that transitions to freshwater environments have only happened in a small fraction of the marine lineages (Logares et al. 2007).

Classification

Dinoflagellates have been studied and classified by botanists, zoologists, and paleontologists, and this has resulted in differing taxonomic practices and dual (or even triple) classification schemes. Fensome et al. (1993) unified dinoflagellate classification, and their system builds the scaffolding of the classification system that is presented below. One recent (and very welcome) trend has been the reinvestigation of the type species of large, polyphyletic genera of gymnodinoid dinoflagellates like *Gymnodinium*, *Gyrodinium*, *Amphidinium*, etc., with both ultrastructural and molecular methods (e.g., Daugbjerg et al. 2000; Hansen and Daugbjerg 2004; Flø-Jørgensen et al. 2004). This has enabled a more phylogenetically accurate circumscription of those large genera and has caused a flood of description of new gymnodinoid genera that are not particularly closely related to those types (e.g., *Karenia*, *Karlodinium*, *Takayama*, *Togula*, *Testudodinium*, *Prosoaulax*, *Apicoporus*, *Tovellia*, *Borghiella*, *Baldinia*, *Jadwigia*, etc.). It should be noted, however, that *Gymnodinium*, *Gyrodinium*, *Amphidinium*, etc., are formally still polyphyletic; they contain many species that have not been reinvestigated recently or that have not yet been given new taxonomic placements. Recent papers have used the terms sensu lato and sensu stricto to distinguish between the polyphyletic and the newly defined versions of these genera. In the case of *Gymnodinium*, even the "sensu stricto" version of the genus is still paraphyletic; it has been shown that entire families of dinoflagellates (Polykrikaceae, Warnowiaceae, Actiniscaceae) are descended from it (Hoppenrath and Leander 2007); the corresponding taxonomic changes have not yet been made. In this work, as in much of the primary literature, when there is reason to belive that a species is misclassified into a certain genus, that generic name is given inside apostrophes (e.g., "*Amphidinium*" *longum*).

The classification presented below includes many temporary names and unnamed clades, something that reflects the instability of dinoflagellate classification at the moment. For a more formal classification of the group, see Fensome et al. 1993.

Annex

An informal, annotated classification of living dinoflagellate genera based primarily on molecular data, but using Fensome et al.'s (1993) classification when sequencing data is not available. Note that dinoflagellate classification is currently very unstable, mostly because phylogenies based on small subunit ribosomal genes lack support in many crucial branches.

Perkinsids: Apparently paraphyletic ancestral group to the dinoflagellates. Motile stages have a conoid, micronemes, and rhoptries. External mitotic spindle. Trans-spliced leaders in RNAs from nuclear genes (Zhang et al. 2007), transversal flagellum present in the motile stage of *Parvilucifera prorocentri* (Leander and Hoppenrath 2008). Ancestrally photosynthetic. Inclusion of *Psammosa* in the group seems to render perkinsids paraphyletic, but confirmation of this needs further study (Okamoto et al. 2012).

Perkinsus, *Parvilucifera*, *Psammosa*, *Xcellia*, *Gadixcellia*, *Rastrimonas*?

Dinoflagellates: Eukaryotes lacking nucleosomes and in which histones have been replaced to a large degree by dinoflagellate/viral nucleoproteins (DVNPs); DNA content much higher than in other eukaryotes, chromosomes condensed throughout the life cycle. Ancestrally photosynthetic, with dinokont flagellation (one flagellum takes a transversal orientation), added trans-spliced leaders to nuclear transcripts (Zhang et al. 2007), and an external mitotic spindle (but reversions back to an internal one exist in *Oxyrrhis* and in some species of *Amoebophrya*, Moon et al. 2015).

1. Oxyrrhids: Free-living dinoflagellates with an internal mitotic spindle. Chromosomes continuously condensed, but lacking the fibrillar appearance of core dinoflagellate chromosomes. Molecular data suggests that this monotypic group may be drastically underclassified (Lowe et al. 2005).

Oxyrrhis

2. Syndinians: Parasitic dinoflagellates with at least two life cycle stages: a plasmodial (multinucleate) trophont, and motile, dinokont stages. At least one species has lost all traces of a plastid (*Hematodinium* sp., Gornik et al. 2015); all other described ones are non-photosynthetic. Syndinians may be paraphyletic, but the issue needs more research.

2.1. Ellobiopsids: Trophonts are plasmodial ectoparasites of crustacean zooplankton attached to the host by a nutrient-absorbing rhizoid. Motile stages appear to have dinokont flagella, but this has not been studied in detail. Not always considered to be dinoflagellates; tentatively treated as such here in the absence of nuclear data because of the plasmodial nature of the vegetative stages, because of the apparently dinokont condition of the motile stages, and because molecular data puts the genus *Thalassomyces* within the alveolates with good support, where it weakly clusters with dinoflagellates (Silberman et al. 2004).

Ellobiopsis, *Thalassomyces*, *Parallobiopsis*, *Ellobiocystis*, *Rhizellobiopsis*

2.2. Eduboscquellids and other group 1 alveolates: Most of the members of this group are known only as environmental molecular sequences from the picoplankton of virtually all the world's oceans (de Vargas et al. 2015). Recent data has shown that at least one member of this clade is the genus *Eduboscquella*, a syndinian characterized by a trophont that only becomes multinucleate (i.e., plasmodial) late in its development (as *Duboscquella* in Harada et al. 2007, nomenclatural change in Coats et al. 2012). The fish-egg parasite *Ichthyodinium* also seems to be a member of this group (Skovgaard et al. 2009). Whether the environmental sequences obtained correspond to free-living organisms or to the motile stages of parasites is unknown at present.

Ichthyodinium, *Eduboscquella*, *Dogelodinium*, *Keppenodinium*, symbionts/parasites of radiolarians and phaeodarians (Dolven et al. 2007), and many undescribed species with picoplanktonic life stages in both aerobic and anaerobic environments (Takishita et al. 2007).

2.3. Syndinids and other group 2 alveolates: Another clade whose members are known mostly as environmental sequences from marine picoplankton. A riboclade at the moment, morphological synapomorphies for the group have not been discovered. In molecular trees, there seem to be two distinct groups that correspond to families; a third family exists for which no molecular data has been obtained. Syndinids can be either intracellular or extracellular parasites of copepods, appendicularians, crabs, radiolarians, or other dinoflagellates.

2.3.1. Syndiniaceae: Syndinids in which the trophont consists of a plasmodium with no fixed shape and no internal cavities.

Syndinium, *Hematodinium*, *Merodinium*, *Solenodinium*, *Trypanodinium*

2.3.2. Amoebophryaceae: Syndinids with a wormlike multiflagellated swimming stage, the vermiform.

Amoebophrya

2.3.3. Sphaeriparaceae: Syndinids in which the plasmodial trophont is organized into two segments separated by a sharp constriction, forming an anterior, episome-like region, and a posterior basal disc. Parasitic on appendicularians and radiolarians. No molecular data is available for members of this family.

Atlanticellodinium

2.3.4. Syndinians incertae sedis: *Atelodinium*, *Coccidinium*

3. Core dinoflagellates: Dinoflagellates in which chromosomes are fibrillar in appearance. Mostly free-living, but a few parasitic forms are also known.

3.1. Noctilucales: Dinoflagellates in which trophonts are large and inflated by vacuoles. Only the gametes have a dinokont flagellation and the fibrillar chromosomes that are typical for core dinoflagellates.

Noctiluca, *Kofoidinium*, *Pomatodinium*, *Spatulodinium*, *Leptodiscus*, *Abedinium*, *Cachonodinium*, *Craspedotella*, *Cymbodinium*, *Petalodinium*, *Scaphodinium*

3.2. Gymnodiniales: Paraphyletic group of core dinoflagellates with numerous amphiesmal vesicles arranged non-serially (gymnodinoid alveolar arrangement). Amphiesmal vesicles do not contain thecal plates. Several genera of

this group (e.g., *Gymnodinium*, *Gyrodinium*, *Amphidinium*, *Katodinium*, *Woloszynskia*, *Cochlodinium*) are large and polyphyletic as defined traditionally and are in the process of being reclassified; the classification below only refers to those genera in sensu stricto.

3.2.1. Amphidiniaceans: Benthic or endosymbiotic dinoflagellates with small triangular- or crescent-shaped epicones deflected to the left. Cells dorsoventrally flattened may or may not have chloroplasts.

Amphidinium

3.2.2. Kareniaceans, the "haptophore" lineage: Dinoflagellates with haptophyte-derived plastids and kleptochloroplasts.

Karlodinium, *Karenia*, *Takayama*, *Brachydinium*, *Asterodinium*, *Microceratium*, and the Ross Sea dinoflagellate, an as yet unnamed species from Antarctic ice with haptophyte-derived kleptochloroplasts. *Apicoporus* is related to this clade and may have plastids of very variable sizes, some being not much more than pigmented granules (Sparmann et al. 2008; some cells are entirely unpigmented). These are thought to be peridinin plastids, but no molecular data exists on this.

3.2.3. The *Gyrodinium* s.s. clade: Gymnodinialeans with surface ridges and an elliptical, bisected apical groove. Vesicular chambers around the nucleus. In many species of *Gyrodinium*, there is a tough nuclear capsule either outside of the nuclear envelope or between its two membranes.

Gyrodinium s.s.

3.2.4. Torodiniales: Gymnodinoids in which the episome is much larger than the hyposome and has a hat- or bill-like apical projection. Cells striated longitudinally, vesicular chambers around the nucleus.

3.2.4.1. Kapelodiniaceans: Non-photosynthetic torodiniales with a cap-like apical projection and three rows of vesicles under the rim of the cap.

Kapelodinium

3.2.4.2. Torodiniaceans: Photosynthetic torodiniales with a bill-like apical projection on top of which lies a structure shaped like a counterclockwise inward spiral.

Torodinium

3.2.5. The *Gymnodinium* family group: Molecularly defined grouping of gymnodinoids; many groups have a horseshoe-shaped apical groove running

in an anticlockwise direction and vesicular chambers around the nucleus. Originally conceived as the genus *Gymnodinium* sensu stricto, it later turned out that several families of naked dinoflagellates are contained in the group.

3.2.5.1. Gymnodiniaceans: Paraphyletic family, only definable in a negative way: naked dinoflagellates with no internal skeletons, surface ridges, nematocysts, or ocelli. As defined here, gymnodiniaceans have given rise to polykrikaceans, warnowiaceans, and actiniscaceans.

Gymnodinium s.s., *Paragymnodinium*, *Gyrodiniellum*, *Levanderina*, *Barrufeta*, *Gymnoxanthella*, *Dissodinium*, *Chytriodinium*, *Lepidodinium*, *SpiniferodiniumNusuttodinium*, *Pellucidodinium*, *Pheopolykrikos*, *Togula*, *Syltodinium*/"*Gyrodinium*" *undulans*, "*Cochlodinium*" *polykrikoides*/"*Cochlodinium*" *fulvescens*

3.2.5.2. Polykrikaceans: Pseudocolonial dinoflagellates with half (or a quarter) as many nuclei as zooids. They have the ability to dissociate into pseudocolonies with fewer zooids and just one nucleus. Nematocyst complexes are present. The genus *Pheopolykrikos* is also pseudocolonial (same number of zooids and nuclei), but it is not related to *Polykrikos* in molecular trees (Hoppenrath and Leander 2007).

Polykrikos

3.2.5.3. Warnowiaceans: Dinoflagellates with ocelli, i.e., elaborate light-receiving organelles. Nematocysts also commonly present.

Warnowia, *Erythropsidinium*, *Greuetodinium*, *Nematodinium*, *Nematopsides*, *Proterythropsis*, *Protopsis*

3.2.5.4. Actiniscaceans: Gymnodinialeans with an internal skeleton.

Actiniscus, *Diaster*, *Dicroerisma*

3.2.5.5. Ptychodiscaceans: Naked dinoflagellates in which the pellicle is strongly developed and is the principal structural element in the amphiesma of the motile cell. Few ultrastructural studies, for example, of the nucleus. Probably polyphyletic: *Ceratoperidinium* branches close to the *Gymnodinium* family group in molecular trees, but it is unclear whether the other ptychodiscaceans are related to it.

Tovellia, *Jadwigia*, *Esoptrodinium*, *Opisthoaulax*

3.2.6. Haplozoaceans: Ribbonlike, multicellular dinoflagellates parasitic in appendicularians and polychaetes.

Haplozoon

– Gymnodiniales incertae sedis:
  (a) Genera with uncertain positions in molecular-based phylogenetc trees or whose familiar relationships are unclear: *Akashiwo*, *Ankistrodinium*, *Bispinodinium*, *Moestrupia*, *Testudodinium*. In addition "*Cochlodinium*" *convolutum*/"*Gyrodinium*" *falcatum* makes clades in molecular trees that may represent an undescribed genus.
  (b) Putatively polyphyletic genera with understudied type species: *Cochlodinium*, *Katodinium*, *Woloszynskia*
  (c) Gymnodiniales for which no molecular data exist: *Bernardinium*, *Crepidoodinium*, *Filodinium*, *Gynogonadinium*, *Pavillardia*, *Pyramidodinium*, *Schizochytriodinium*

3.3. Thecates: Dinoflagellates with cellulosic plates inside the alveolae. Primarily with alveolae in a pattern of five or six latitudinal plate series, but these increase in the suessiales and decrease in dinophysiales and prorocentrales.

3.3.1. Gonyaulacales: Thecates in which the first apical plate is asymmetrical and in which there are two to four (usually three) fundital plates (Fig. 10)

3.3.1.1. Cladopyxineans: Gonyaulacales with a partiform tabulation pattern, that is, the first antapical homologue ("Y" plate) contacts the distalmost postcingular plate and in which the posterior sulcal homologue ("Z") is within the sulcus and extends further to the anterior than the posterior intercalary homologue ("X"), thus contacting the first postcingular homologue (Fig. 10). Molecular data are not available for the group.

Cladopyxis, *Acanthodinium*, *Palaeophalacroma*, *Sinodinium*

3.3.1.2. Gonyaulacineans: Gonyaulacales with a sexiform tabulation pattern (Fig. 10), that is, the first antapical homologue ("Y" plate) contacts the distalmost postcingular plate and in which the posterior intercalary homologue ("X") extends further to the anterior than the posterior sulcal homologue ("Z").

3.3.1.2.1. Gonyaulacaceans: Gonyaulacineans with six precingular plates in which the sulcus is more-or-less midventral (may be straight, oblique, or sigmoideal). The antapical outline is more-or-less symmetrical, no dorsoventral compression.

Protoceratium, *Lingulodinium*, *Gonyaulax*, *Acanthogonyaulax*, *Amylax*, *Spiraulax*, *Ataxiodinium*, *Bitectatodinium*, *Halostylodinium*, *Impagidinium*, *Pentadinium*, *Schuettiella*

3.3.1.2.2. Ceratocoryaceans: Gonyaulacineans with five precingular plates and a midventral, L-type sulcus. There is a strong dextral torsion.

Ceratocorys

3.3.1.3. Ceratiineans: Gonyaulacales with at least three horns and in which the first antapical plate ("Y") contacts six or seven adjacent plates including the distalmost postcingular.

Ceratium, *Tripos*

3.3.1.4. "Goniodomineans": Gonyaulacales with a quinqueform tabulation pattern, that is, the first antapical homologue ("Y" plate) does not contact the distalmost postcingular plate. Plate growth occurs only at overlapping plate margins. Note: because of multiple taxonomic and nomenclatural problems (Kretschmann et al. 2015), the generic name *Goniodoma* has been replaced by *Pyrrhotriadinium*. Suprageneric taxon names based on *Goniodoma* (e.g., Goniodomineans, Goniodomaceans, etc.) have not yet followed suit and are given here in quotation marks.

3.3.1.4.1. "Goniodomaceans": "Goniodomineans" in which the principal life-cycle stage is a motile thecate cell.

3.3.1.4.1.1. "Goniodomoideans": "Goniodomaceans" in which the posterior sulcal homologue ("Z") is external to the sulcus and cells are not anteroposteriorly compressed. Dinosporin cysts. Molecular data are not available for the group.

Pyrrhotriadinium, *Pachydinium*

3.3.1.4.1.2. Gambierdiscoideans: "Goniodomaceans" in which the posterior sulcal homologue ("Z") is external to the sulcus and cells anteroposteriorly compressed. No ventral pore.

Gambierdiscus, *Fukuyoa*, *Coolia*, *Ostreopsis*

3.3.1.4.1.3. Helgolandinioideans: "Goniodomaceans" with either of the following characters: tabulation has more than the typical number of plates in at least two plate series or the presence of a smooth cellulosic cyst in the life cycle.

Helgolandinium, *Alexandrium*, *Fragilidium*, *Pyrophacus*

3.3.1.4.1.4. Pyrodinioideans: "Goniodomaceans" in which the posterior sulcal homologue ("Z") and right sulcal homologue are within the sulcus.

Pyrodinium

3.3.1.4.2. Pyrocystaceans: "Goniodomineans" in which the principal life cycle stage is a nonmotile vegetative cyst.

Pyrocystis

3.3.1.5. Gonyaulacales incertae sedis: *Adenoides*, *Heterodinium*, *Crypthecodinium*, *Centrodinium*, *Dolichodinium*, *Goniodinium*, *Peridiniella*, *Planodinium*, *Thecadiniopsis*, *Thecadinium*, *Pseudothecadinium*, *Stylodinium*, *Pseudadenoides*

3.3.2. Dinophysiales: Dinoflagellates with a sulcus, a cingulum, and a sagittal suture that extends the entire length of the cell

3.3.2.1. Dinophysiaceans: Dinophysiales in which the motile cell is never more than three times as long as it is broad. Ventral pore on the ventral episome, and flagellar pore immediately posterior to the cingulum.

Dinophysis, *Phalacroma*, *Citharistes*, *Dinofurcula*, *Latifascia*, *Histioneis*, *Histiophysis*, *Metadinophysis*, *Metaphalacroma*, *Ornithocercus*, *Pseudophalacroma*, *Sinophysis*, *Thaumatodinium*, *Oxyphysis*

3.3.2.2. Amphisoleniaceans: Dinophysiales in which the motile cell is more than four times as long as it is wide. The ventral pore is on the ventral episome, and the flagellar pore is significantly posterior to the cingulum.

Amphisolenia, *Triposolenia*

3.3.3. Prorocentrales: Dinoflagellates with no sulcus or cingulum, apically inserted flagella.

Prorocentrum, *Mesoporos*

3.3.4. The *Symbiodinium* order ("Symbiodiniales," once the taxon is described formally): *Symbiodinium* and several fossil genera have motile stages with seven latitudinal series of amphiesmal vesicles, i.e., a suessioid tabulation, and this feature was used in the past to define the order Suessiales. Nevertheless, the fossil genus *Suessia* has morphological features that distinguish it from extant Symbiodiniaceae (and Borghiellaceae), and is now thought that the two groups are not related (Janouškovec et al. 2016). The term Suessiales should be used for the group that includes *Suessia* and its fossil relatives, not *Symbiodinium*. Several dinoflagellates with a typically gymnodinoid tabulation group strongly with *Symbiodinium* in molecular trees, there is obviously a strong trend within the group to

increase the number of alveolae and reduce the theca. Eyespots in members of this group are associated with one or more rows of brick-like vesicles.

3.3.4.1. Borghiellaceans: Eyespot consists of rows of globules arranged in a single layer within the chloroplast, and a large, narrow vesicle containing a single layer of translucent bricklike structures.

Borghiella, *Baldinia*, "*Woloszynskia*" *pesheri*

3.3.4.2. Symbiodinaceans: Eyespot contains many layers of brick-like structures. No globules inside a chloroplast.

Symbiodinium, *Polarella*, *Protodinium*, *Prosoaulax*, *Pelagodinium*, *Biecheleria*, *Biecheleriopsis*, *Piscinoodinium*, *Haidadinium*, *Ansanella*, *Asulcocephalium*, *Leiocephalium*, "*Gymnodinium*" *natalense*, "*Gymnodinium*" *linucheae*, "*Katodinium*" *fungiforme*

3.3.4.3. "Symbiodiniales" incertae sedis: *Sphaerodinium*

3.3.5. Peridiniales: Thecates in which the first apical plate is roughly symmetrical, and that have two antapical plates placed more-or-less symmetrically about the midventral/middorsal plane (may be fused or subdivided secondarily).

3.3.5.1. Amphidomataceans: Molecularly-defined clade, six or four apical plates.

Amphidoma, *Azadinium*

3.3.5.2. Heterocapsids: Peridiniales with five apical plates, not laterally compressed.

Heterocapsa

3.3.5.3. Glenodinoids: Peridiniales with four apical plates and six postcingular plates.

Glenodinium, *Glenodiniopsis*, "*Gymnodinium*" *impatiens*

3.3.5.4. Peridiniineans: Peridiniales with three or four apical plates and five postcingular plates.

3.3.5.4.1. Peridiniaceans: Peridiniineans with a distinct cingulum of four to six cingular plates (exclusive of a transitional plate that is sometimes present) and with at least one intercingular boundary on the dorsal surface.

3.3.5.4.1.1. Peridinioideans: Peridiniaceans with seven precingular plates and peridinin-containing plastids, and without calcareous cysts (often build cysts of dinosporin). In all likelihood paraphyletic. The apical pore complex may be absent.

Peridinium, *Vulcanodinium*

3.3.5.4.1.2. Dinotoms: Peridiniaceans with diatom-derived plastids

Kryptoperidinium, *Durinskia*, *Dinothrix*, *Galeidinium*, "*Peridinium*" *quinquecorne*, "*Gymnodinium*" *quadrilobatum*, "*Peridiniopsis*" *penardii*, "*Peridiniopsis*" cf. *kevei*

3.3.5.4.1.3. The *Zooxanthella* clade: Symbionts in radiolarians and hydrozoans

Zooxanthella

3.3.5.4.1.4. Endodiniaceans: Endosymbionts in the cnidarian *Velella velella*.

Endodinium

3.3.5.4.1.5. Thoracosphaeraceans: Peridiniaceans with five or six precingular plates that often form calcareous cysts. Preliminary data suggest that they may be paraphyletic, having given rise to the blastodinioids.

Pentapharsodinium, *Duboscquella*, *Duboscquodinium*, *Ensiculifera*, *Calcicarpinum*, *Pernambugia*, *Scrippsiella*, *Brandtodinium*, *Calciodinellum*, *Calcigonellum*, *Calciperidinium*, *Caracomia*, *Follisdinellum*, *Fuettererella*, *Lebessphaera*, *Pentadinellum*, *Praecalcigonellum*, *Wallidinellum*, *Leonella*, *Melodomuncula*, *Posoniella*, *Thoracosphaera*, *Bysmatrum*, *Chimonodinium*, *Theleodinium*, *Bicarinellum*, *Tintinnophagus*, *Aduncodinium*, *Stoeckeria*, *Paulsenella*, *Pfiesteria*, *Cryptoperidiniopsis*, *Luciella*, *Amyloodinium*, *Tyrannodinium*, *Naiadinium*, "*Peridinium*" *aciculiferum*/"*Scrippsiella*" *hangoei*/ "*Peridinium*" *baicalense*/"*Peridinium*" *euryceps*, "*Peridiniopsis*" *niei*, "*Peridiniopsis*" *penardii*

3.3.5.4.1.6. Blastodinioids: Parasitic dinoflagellates living unattached in the gut of copepods and producing a very distinctive trophont. Only the motile stages have an obvious dinokaryon.

Blastodinium

3.3.5.4.1.7. Peridiniopsids: A group of fresh-water dinoflagellates with rDNA sequences similar to those of *Peridiniopsis borgei* from brackish/limnic habitats (Logares et al. 2007).

Peridiniopsis, *Palatinus*, "*Peridinium*" *umbonatum*, "*Peridinium*" *inconspicuum*, "*Peridinium*" *centenniale*

3.3.5.4.1.8. Peridiniaceans incertae sedis: *Ailadinium*, *Amphidiniella*, *Kansodinium*, *Madanidinium*, *Pileidinium*

3.3.5.4.2. Protoperidiniaceans: Peridiniineans with a well-imprinted cingulum with three cingular plates excluding a transitional plate; there are no intracingular boundaries on the dorsal surface.

3.3.5.4.2.1. Protoperidinioideans: Protoperidiniaceans with two antapical plates.

Protoperidinium, *Congruentidium*, *Archaeperidinium*, *Amphidiniopsis*, *Glochidinium*, *Brigantedinium*, *Echinidinium*, *Herdmania*, *Islandinium*, *Minuscula*, *Multispinula*, *Quinquecuspis*, *Stelladinium*, *Trinovantedinium*, *Votadinium*, *Xandarodinium*

3.3.5.4.2.2. Diplopsaloids: Protoperidiniaceans with six precingular and one antapical (=fundital) plate.

Diplopsalis, *Kolkwitziella*, *Boreadinium*, *Diplopelta*, *Diplopsalopsis*, *Dissodium*, *Dubridinium*, *Gotoius*, *Oblea*, *Preperidinium*, *Zygabikodinium*, *Niea*, *Qia*, "*Protoperidinium*" *depressum*/"*Protoperidinium*" *claudicans*

3.3.5.4.2.3. The *Lessardia/Roscoffia* clade: Protoperidiniaceans with five precingular plates.

Lessardia, *Roscoffia*, *Rhinodinium*, *Cabra*

3.3.5.4.3. Podolampaceans: Peridiniineans in which the cingulum is not indented, but is composed of three cingular plates.

Podolampas, *Blepharocysta*, *Gaarderiella*, *Heterobractum*, *Lissodinium*, *Mysticella*

3.3.5.5. Peridiniales incertae sedis: *Chalubinskia*, *Hemidinium*, *Heteraulacus*, *Nephrodinium*, *Oodinium*, *Plagiodinium*, *Protoodinium*, *Sabulodinium*, *Staszicella*, *Thaurilens*

3.3.6. Thecates incertae sedis: *Archaeosphaerodiniopsis*, *Dinosphaera*, *Melanodinium*, *Oxytoxum*, *Thompsodinium*

3.4. Core dinoflagellates incertae sedis: *Actinodinium*, *Adinimonas*, *Apodinium*, *Bargoniella*, *Cachonella*, *Caryotoma*, *Cystodinedria*, *Cystodinium*, *Desmocapsa*, *Desmomastix*, *Dinamoebidium*, *Dinastridium*, *Dinoclonium*, *Dinococcus*, *Geodinium*, *Gloeodinium*, *Glenoaulax*, *Halophilodinium*,

Hypnodinium, *Micracanthodinium*, *Myxodinium*, *Oodinioides*, *Parapodinium*, *Phytodinium*, *Pleromonas*, *Proaulax*, *Pseliodinium*, *Rhizodinium*, *Rufusiella*, *Schizodinium*, *Tetradinium*
Ptychodiscus, *Balechina*, *Berghiella*, *Ceratoperidinium*, *Lissaiella*, *Lophodinium*, *Sclerodinium*, *Amphitholus*, *Achradina*, *Monaster*

Tovelliaceans: Dinoflagellates with a thin theca and an eyespot composed of pigment globules not bound by membranes and not located in a chloroplast. Members of this group also have an apical line of narrow plates, i.e., a small number of narrow thecal plates arranged in a row, level with the cell surface and lined on each side by another row of wider plates.

Genera in dinoflagellate species lists that are not considered to be dinoflagellates by Fensome et al. (1993): *Chilodinium*, *Entomosigma*, *Glyphidium*, *Pelagorhynchus*, *Pronoctiluca* (see Gawryluk et al. 2016), *Protodinifer*

Genera considered to be taxonomic junior synonyms by Fensome et al. (1993), but that have not been formally transferred: *Amphiceratium*, *Aureodinium*, *Biceratium*, *Bourrelyella*, *Branchiophilus*, *Cachonina*, *Caledonidinium*, *Ceratodinium*, *Clathrocysta*, *Clipeodinium*, *Corythodinium*, *Dimastigoaulax*, *Dinoceras*, *Dinopodiella*, *Dinopyxis*, *Discodinium*, *Epiperidinium*, *Exuviaella*, *Gessnerium*, *Gymnocystodinium*, *Hemicystodinium*, *Heteroceras*, *Hirundinella*, *Hyalosaccus*, *Latifascia*, *Lebouraia*, *Leptospathium*, *Manchudinium*, *Melodinium*, *Microtaeniella*, *Murracystis*, *Nectocystis*, *Parahistioneis*, *Parelion*, *Parrocelia*, *Pavillardinium*, *Pentadinium*, *Philozoon*, *Photocystis*, *Phyllodinium*, *Phytodinedria*, *Planinosphaeridium*, *Plectodinium*, *Polysphaeridium*, *Poroceratium*, *Prorocentrum*, *Prodinophysis*, *Proheteroschisma*, *Properidinium*, *Protogonyaulax*, *Pseudoactiniscus*, *Roulea*, *Schillingia*, *Spiraulaxina*, *Sporodinium*, *Steiniella*, *Trochodinium*, *Tuberculodinium*

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Ciliophora

18

Denis H. Lynn

Abstract

The ciliated protists (Phylum Ciliophora) are typically longer than 50 μm in body length and so are conspicuous microbial eukaryotes. There are over 8,000 species of these usually quickly moving protists, which locomote using files of cilia organized on the cell surface. In addition to the files of cilia or kineties on the cell surface, ciliates are also characterized by nuclear dimorphism or the possession of two kinds of nuclei: (1) the micronucleus, which is not transcriptionally active and which is considered the equivalent of the germ line in multicellular organisms and (2) a macronucleus, which is transcriptionally active and which is typically a developmental product of the amplification of the micronuclear or germ-line DNA. The micronucleus participates in conjugation, which is the sexual process of ciliates, and the third major feature to characterize this phylum. Ciliates as large cells are the top predators or heterotrophs in microbial food webs when metazoans are absent. As heterotrophs, they feed upon bacteria, smaller protists, and even other ciliates in ecosystems from the poles to the tropics and from terrestrial soils to the sediments around deep-sea hydrothermal vents. The genus *Mesodinium* includes the only "autotrophic" ciliate species, but many species are mixotrophic, capturing the chloroplasts of prey or hosting autotrophic protists as endosymbionts. Ciliates can also be symbionts of other organisms, ranging from commensals found in the stomachs of ruminants to parasites of fish. Ciliates, such as *Tetrahymena* and *Paramecium*, whose genomes have been sequenced, serve as model organisms for cell and molecular biology.

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Ciliate • Kinetid • *Paramecium* • Phylogenomics • *Tetrahymena*

Contents

Summary Classification

●Ciliophora
●●Postciliodesmatophora
●●●Karyorelictea
●●●Heterotrichea
●●Intramacronucleata
●●●Cariacotrichea
●●●Spirotrichea
●●●Armophorea
●●●Litostomatea
●●●"Conthreep"
●●●●Phyllopharyngea
●●●●Nassophorea
●●●●Colpodea
●●●●Prostomatea
●●●●Plagiopylea
●●●●Oligohymenophorea

Introduction

General Characteristics

The ciliates are undoubtedly one of the easiest groups of protists for the nonspecialist to identify since their typical feature is the presence of files or rows of cilia, known as kineties, on the cell surface. Most also have a cytostome or "cell mouth" around which oral cilia are arranged. Ciliates also exhibit nuclear dualism in which the relatively larger macronucleus is physiologically active, containing thousands of copies of genes, while the relatively smaller, diploid micronucleus is the germ nucleus whose meiotic products are exchanged during conjugation. These general features are found together in no other group of protists. *Stephanopogon* was a disturbingly exceptional ciliate as it appeared to have files of cilia on the cell surface but lacked nuclear dualism, but we now know that *Stephanopogon* is an example of presumed phylogenetic affinity that turned out to be convergence when inspected more closely: its kinetid does not have any typical ciliate fibrillar associates (i.e., no transverse microtubular ribbon, no postciliary microtubular ribbon, no kinetodesmal fiber) (Patterson and Brugerolle 1988; see ▶ Heterolobosea). There are over 1,100 ciliate genera and over 8,000 species included in these genera (Lynn 2008), although some argue that this diversity may represent only 10% of the actual diversity of species (Foissner et al. 2008).

Occurrence

Most species are free-living and found in ponds, lakes, estuaries, saltmarshes, and oceans. They have been collected in almost every conceivable aqueous habitat from Antarctica (Kepner et al. 1999 in Lynn 2008; Song and Wilbert 2000 in Lynn 2008) to hot springs; from small, temporary puddles to lakes and oceans (Kofoid and Campbell 1939); from slightly moistened soils (Foissner 1988a in Lynn 2008) to fresh waters (Beaver and Crisman 1989) and saline waters (Pierce and Turner 1992); and from streams (Cleven 2004 in Lynn 2008) to sewage treatment plants (Curds 1975b in Lynn 2008).

A variety of species is available from culture collections and biological supply houses (Table 1). Those strains kept in culture collections are most likely to have a certified pedigree and should be chosen for experimental work.

Literature

The nonspecialist is advised to consult introductory protozoology books, such as Hausmann et al. (2003), although there is still much of value in older texts, for example, Grell (1973) or Sleigh (1989). Having established an appreciation for the

Table 1 List of ciliate species available from culture collections

Species[a]	Source[b]	Species	Source
Blepharisma americanum CCAP		*Paramecium tredecaurelia* ATCC	
Blepharisma hyalinum CCAP		*Paramecium triaurelia* ATCC	
Blepharisma stoltei ATCC		*Paramecium undecaurelia* ATCC	
		Parauronema acutum ATCC	
Bursaria truncatella BS		*Plagiopyla nasuta* CCAP	
		Potomacus pottsi ATCC	
Cinetochilum margaritaceum ATCC		*Prodiscophrya collini* CCAP	
Chilodonella uncinata ATCC			
Cohnilembus reniformis CCAP		*Sorogena stoianovitchae* ATCC	
Coleps hirtus CCAP		*Spirostomum ambiguum* CCAP	
Colpidium striatum CCAP		*Stentor coeruleus* BS	
Colpoda cavicola ATCC			
Colpoda cucullus ATCC		*Tetrahymena americanis* ATCC, CCAP	
Colpoda inflata ATCC, CCAP		*Tetrahymena asiatica* ATCC	
Colpoda magna ATCC			
Colpoda maupasi ATCC		*Tetrahymena australis* ATCC	
		Tetrahymena bergeri ATCC	
Colpoda steinii ATCC, CCAP		*Tetrahymena borealis* ATCC, CCAP	
Cyclidium glaucoma ATCC, CCAP		*Tetrahymena canadensis* ATCC, CCAP	
Dexiostoma campyla ATCC, CCAP			
Didinium nasutum ATCC, BS		*Tetrahymena capricornis* ATCC	
		Tetrahymena cosmopolitanis ATCC *Tetrahymena corlissi* ATCC	
Euplotes gracilis ATCC		*Tetrahymena elliotti* ATCC	
		Tetrahymena farleyi ATCC	
Euplotes vannus CCAP		*Tetrahymena furgasoni* ATCC	
Euplotes raikovi ATCC		*Tetrahymena hegewischi* ATCC	
Heliophrya sp. ATCC		*Tetrahymena hyperangularis* ATCC	
Ilsiella palustris CCAP		*Tetrahymena limacis* ATCC, CCAP	
Mesanophrys chesapeakensis ATCC		*Tetrahymena lwoffi* CCAP	
Meseres corlissi CCAP		*Tetrahymena malaccensis* ATCC	
Metopus es CCAP		*Tetrahymena nanneyi* ATCC	
Nassula sorex CCAP		*Tetrahymena nipissingi* ATCC	
Opisthonecta henneguyi ATCC		*Tetrahymena paravorax* ATCC	
		Tetrahymena patula ATCC, CCAP	
Paramecium biaurelia ATCC, CCAP		*Tetrahymena pigmentosa* ATCC, CCAP	
Paramecium bursaria CCAP, BS		*Tetrahymena pyriformis* ATCC, CCAP	
Paramecium caudatum BS		*Tetrahymena rostrata* ATCC	
Paramecium decaurelia ATCC		*Tetrahymena setosa* ATCC	
Paramecium dodecaurelia ATCC		*Tetrahymena shanghaiensis* ATCC	
Paramecium jenningsi ATCC			
Paramecium multimicronucleatum ATCC, BS		*Tetrahymena sonneborni* ATCC	

(*continued*)

Table 1 (continued)

Species[a]	Source[b]	Species	Source
Paramecium novaurelia ATCC		*Tetrahymena thermophila* ATCC, CCAP	
Paramecium octaurelia ATCC		*Tetrahymena tropicalis* ATCC	
Paramecium pentaurelia ATCC		*Tetrahymena vorax* ATCC, CCAP	
Paramecium polycaryum ATCC		*Tokophrya infusionum* ATCC	
		Tokophrya lemnarum ATCC	
Paramecium quadecaurelia ATCC		*Trimyema koreanum* CCAP	
Paramecium septaurelia ATCC		*Trimyema shoalsi* ATCC	
Paramecium sexaurelia ATCC		*Uronema marinum* CCAP	
Paramecium sonneborni ATCC		*Vorticella microstoma* ATCC, CCAP	
Paramecium tetraurelia ATCC, CCAP		*Vorticella similis* CCAP	

[a]Some of the species listed are available in several strains or stocks. This listing was prepared from WWW listings of these three culture collections in July, 2013

[b]*ATCC* American Type Culture Collection (www.atcc.org), 10801 University Blvd., Manassas, VA, U.S.A. 20110-2209; *CCAP* Culture Collection of Algae and Protozoa (www.ccap.ac.uk), Scottish Marine Institute, Dunbeg, Argyll, Scotland, UNITED KINGDOM PA37 1QA; *BS* Boreal Science (www.boreal.com), 399 Vansickle Road, St. Catharines, ON, CANADA L2S 3T4

general biology of the phylum, the reader may choose to read Grassé (1984), Hausmann and Bradbury (1996), Jones (1974), and Matthes and Wenzel (1966) or to specialize in any of a number of areas. Lynn (2008) provides a comprehensive account of the literature and the history of ciliatology, and the present chapter is largely a précis of Lynn's monographic work.

More detailed descriptions of particular genera are provided in books on the biology of: *Blepharisma* (Giese 1973); *Paramecium* (Beale and Preer 2008; Görtz 1988); *Stentor* (Tartar 1961); and *Tetrahymena* (Asai and Forney 2000; Collins 2012; Elliott 1973). Details of the physiology and biochemistry of *Tetrahymena* can be found in Hill (1972) and of the developmental biology and genetics of *Paramecium, Tetrahymena, Euplotes*, and other ciliates in Nanney (1980), Frankel (1989), and Beale and Preer (2008).

Several monographs contain review papers that include chapters specifically devoted to the ciliates. These include the general biology of ciliates (Grassé 1984), their systematics (Lynn 2008; de Puytorac 1994), chemical aspects of protozoan biology (Kidder 1967), aspects of the biochemistry and physiology of protozoa (Hutner 1964; Hutner and Lwoff 1955; Levandowsky and Hutner 1980; Lwoff 1951), and selected topics on a wide range of protozoan research topics (Chen 1967–1972).

Specific topics in ciliate biology have been reviewed: extrusive organelles (extrusomes) (Hausmann 1978; Rosati and Modeo 2003 in Lynn 2008); membrane trafficking (Allen and Fok 2000); contractile vacuoles (Allen 2000; Patterson 1980); evolution of cortical microtubular structures (Lynn 1981); somatic function of the micronucleus (Ng 1986); genetics and aging (Smith-Sonneborn 1981); and endosymbionts of *Euplotes* (Heckmann 1983).

Some specific mention should be made of publications on the systematics and ecology of ciliates. Foissner et al. (1994), for example, have published useful keys for freshwater ciliates found in activated sludge plants and other anoxic environments. For a key to species used as biological indicators, see Bick (1972). Curds (1982) and Curds et al. (1983) have provided comprehensive keys to the genera of freshwater ciliates from Britain and other regions. For families of marine ciliates of the northeastern United States see Borror (1973). Foissner et al. (1999) have published a key to limnetic ciliates. Lynn and Small (2002) have provided a broader key to representative genera and species of free-living and symbiotic as well as freshwater and marine ciliates while Jankowski (2007) has reviewed all genera. Berger (2011) is an example of his taxonomic treatments of hypotrich groups while Vd'ačný and Foissner (2012) continue the taxonomic monographs published by the Foissner lab.

History of Knowledge

Antony von Leeuwenhoek was probably the first to observe ciliates. Until the mid-nineteenth century, ciliates were called Infusoria because of their prominence in infusions of vegetation. The early years were spent mainly in descriptive taxonomy. In the nineteenth century, taxonomic research on the protists was expanded by such men as Bütschli, Claparède, Dujardin, Kent, Lachmann, Maupas, and Stein. Stein (1859, 1867 in Corliss 1979) carefully and precisely used the variations in the ciliature of oral and somatic regions of the cortex to establish affinities among taxa. Bütschli (1887–1889 in Lynn 2008) published a comprehensive monograph on the ciliates in which he modified Stein's scheme of classification. Bütschli's classification scheme of the Class INFUSORIA dominated until well into the twentieth century (see Corliss 1974a in Lynn 2008). Kahl (1930–1935) monographed the ciliates, primarily of northern Europe. His encyclopedic work is still authoritative. The name CILIOPHORA was originally proposed by Doflein in 1901. In the mid-1930s, Chatton and Lwoff perfected the "wet" silver impregnation technique, which revealed the pattern of surface and subsurface kinetosomes (basal bodies). The "Chatton-Lwoff" technique revealed details of the cortical patterns and provided information for Fauré-Fremiet's (1950a in Lynn 2008) next revision of ciliate classification, formalized by Corliss (1956, 1961 in Lynn 2008). Basing his analysis primarily on details of the cortex revealed by light microscopy, Jankowski (1967 in Lynn 2008) recognized even more diversity and elevated the number of ciliate orders. The development and use of electron microscopy during the next decade revealed an even more complex picture to systematists (Lynn 2008).

Building on Jankowski's ideas and new ultrastructural information, de Puytorac et al. (1974 in Lynn 2008) and Corliss (1974a, b in Lynn 2008) presented a further revision of what is now recognized as the Phylum CILIOPHORA. Small and Lynn (1981 in Lynn 2008) argued: (1) that these revisions of ciliate classification had been presented with inadequate consideration of their conceptual bases and (2) that more

weight must be placed on the ultrastructural features of the cortex, especially the somatic kinetid (Lynn 1981; Lynn and Small 1981 in Lynn 2008), if phylogenetic affinity was to be recognized. Small and Lynn (1981 in Lynn 2008) proposed a radically new classification system that formed the basis of the revised classifications presented by Lynn and Small (1997, 2002 in Lynn 2008; see section below on "Characterization and Recognition"). Jankowski (2007) has presented a revised system. See Corliss (1979, 1986) and Lynn (2008) for more detailed historical accounts of ciliate systematics.

Practical Importance

The agricultural and medical importance of ciliates relates to their associations with mammals. Large populations of particular species of symbiotic ciliates are found in the digestive tracts of sheep, goats, cattle, pigs, and horses. Although their presence is not essential for the growth of the herbivores, the ciliates most likely stabilize the cellulolytic bacterial populations (Bonhomme 1990 in Lynn 2008; Coleman 1989 in Lynn 2008; Dehority 1993 in Kreier and Baker 1993).

A wide variety of ciliates exploit both freshwater and marine fishes (Basson and Van As 2006 in Woo 2006; Burgess and Matthews 1995b in Lynn 2008; Dickerson 2006 in Woo 2006; Bradbury 1994 in Kreier 1994; Iglesias et al. 2001 in Lynn 2008), presenting economic problems in aquaculture operations only when present in large numbers (Harikrishnan et al. 2010). *Balantidium,* the only endoparasitic ciliate of man, has been reported to cause gastrointestinal infections. These often occur in places where people and pigs cohabit (Schuster and Ramirez-Avila 2008; Zaman 1993 in Kreier and Baker 1993). Numerous ciliates are parasites of invertebrate marine animals (Bradbury 1994 in Kreier 1994). Their effect, from the human perspective, can be defined as harmful, when, for example, populations of the commercially important Dungeness crab are infected (Morado and Small 1995) or rearing of snails for human consumption (Segade et al. 2009) or beneficial, when the infected hosts are the larvae and adults of insects that are vectors of human parasites (Barros et al. 2006 in Lynn 2008; Batson 1983 and references cited therein; Washburn et al. 1988 in Lynn 2008).

Ciliates have been used in a number of practical applications, ranging from the assessment of water quality to their use as model organisms for assessment of the effects of chemicals on metazoans.

Certain associations of ciliates can be used as complex indicators of the quality of the environment (Bick 1972 in Lynn 2008; Foissner 1988; Foissner et al. 1982) and to reveal the complex effects of pollution on the microbiota (Cairns et al. 1972; Tan et al. 2010). Ciliates play an important, perhaps essential, role in the clarification of water during and after sewage treatment (Curds 1969 in Lynn 2008; Fried et al. 2000 in Lynn 2008; Small 1973 in Lynn 2008).

Protists are becoming increasingly popular as bioassay organisms due in part to rising costs of maintaining laboratory animals and increasing pressure from animal welfare groups (Schultz et al. 1978). Ciliates, in particular the *Tetrahymena*

"pyriformis" species complex and *Colpidium campylum*, have been used in bioassays for protein quality (Rølle 1980; Wang et al. 1980), in bioassays to detect toxic substances in aquatic environments (Gilron and Lynn 1996; Gilron and Lynn 1998; Slabbert et al. 1983) and soils (Forge et al. 1993 in Lynn 2008), and as possible models for mammalian cells in assessing the effects of chemicals (Dayeh et al. 2004).

Habitats and Ecology

The comprehensive bibliography of Finlay and Ochsenbein-Gattlen (1982), while dated, should provide the interested reader with a starting point for the literature. Fenchel (1987) provides another focus with some emphasis on the ciliates while Dolan et al. (2013) have provided a thorough and readable overview of the systematics and ecology of tintinnid ciliates, a conspicuous group in the marine plankton.

Habitats

The four main environments where ciliates are obvious include the benthos, especially the marine littoral, terrestrial soils, the plankton, and certain symbiotic associations. Ciliates are also found in some unusual habitats, which will be described as well.

Benthic Habitats. Benthic ciliates have been studied in freshwater, brackish, and marine habitats where they may be found freely swimming over the substrate or attached to it. The community of microbes in aquatic environments attached to rocks, fallen logs, and the like is called the aufwuchs or biofilm.

One of the earliest studies of the benthic ciliates within sediments was Fauré-Fremiet's study (1950c in Lynn 2008) of the interstitial fauna of sandy beaches; he noted that ciliate species may be free-swimming in the interstices or thigmotactic, crawling on grain surfaces. Others are attached to the grains. The distribution of ciliates is affected both by the compaction and the redox potential of the substrate in marine (Fenchel 1969) and salt marsh sediments (Elliott and Bamforth 1975 in Corliss 1979). Ciliates are particularly conspicuous when fine interstices are present and when oxygen tension is low. Azovsky and Mazei (2013) concluded that ~60% marine benthic ciliates species are endemic. Ciliates are common eukaryotic organisms in sediment trap samples off southern California at depths to 2,000 m and in the deep benthos of the Mediterranean Sea (Hausmann et al. 2002 in Lynn 2008), often conspicuous in deep anoxic regions (Orsi et al. 2012; Takishita et al. 2010). They have been found on rock surfaces as well as on the tubes of vent worms in the 21° N hydrothermal vents at depths up to 2,600 m (Small and Gross 1985).

Terrestrial Soils. Ciliates are often conspicuous in damp soils; they are ubiquitous in soil cultures from all parts of the world (Foissner 1998a in Lynn 2008). However, ciliates are usually outnumbered by the testate amebae (Bamforth 1980 in Lynn 2008). Protists in general constitute a relatively small portion of the total biomass in soil (Adl 2003). The species diversity and abundance of ciliates are functions of

geography, season, moisture content, temperature, pH, organic content, and the degree of compaction and abrasion of the soil environment (Foissner 1987, 1997d in Lynn 2008).

The Plankton. Ciliates, conspicuous components of planktonic communities at most times of the year, are found in freshwater (Pace and Orcutt 1981 and Porter et al. 1979, both in Finlay and Ochsenbein-Gattlen 1982), neritic (Beaver and Crisman 1989; Leakey et al. 1994 in Lynn 2008), and oceanic environments (Strom et al. 1993 in Lynn 2008). They are found in small, temporary puddles, tide pools, lakes, rivers, and the major oceans of the world. Because standard zooplankton sampling procedures are unsuitable for soft-bodied ciliates their presence, abundance, and diversity were undoubtedly underestimated until the late 1980s when more appropriate sampling techniques, using water bottles, were adopted. Although loricate tintinnids predominate in the plankton literature (Dolan et al. 2013; Kofoid and Campbell 1939; Heinbokel and Beers 1979 in Coats and Heinbokel 1982), it is now clear that nonloricate ciliates are consistently more abundant when other sampling techniques are used (Lynn and Montagnes 1991 in Lynn 2008; Pierce and Turner 1992). Indeed, ciliates are exceedingly abundant in association with the spring phytoplankton bloom in temperate waters and may flourish at other times of the year in short-term blooms. These blooms can lead to spatial patchiness in the distribution of ciliates that may range from 10s to 100s of meters in size (Bulit et al. 2009).

Symbiotic Associations. Ciliates are found as symbionts in association with a wide variety of species (Bradbury 1996 in Hausmann and Bradbury 1996; Lynn 2008). The most thoroughly studied associations include those with ruminant mammals and related herbivores (Hungate 1978 in Kreier 1978), sea urchins (Levine 1972 in Chen 1972), fish (Hoffman 1978 in Kreier 1978), crustaceans (Ferńandez-Leborans 2001 in Lynn 2008), and a variety of molluscan species (for example, Raabe 1972 in Lynn 2008). The symbiotic relationships are generally thought to be commensalistic, either as endo- or ectocommensals, but ruminant ciliates may be mutualistic (Hungate 1978 in Kreier 1978). Some ciliates found in fish and insects can be classified as parasitic (i.e., harmful to their host): they may be histophagous (tissue-eating; necrotrophic) (Alvarez-Pellitero et al. 2004 in Lynn 2008; Batson 1983; Hoffman 1978 in Kreier 1978).

Ciliates host a variety of microorganisms, including bacteria, mastigotes, chlorellae, and other ciliates. Again, the nature of the symbiotic relationships varies from mutualistic, commensalistic, parasitic, or pathogenic (Ball 1969 in Chen 1969; Berninger et al. 1986 in Lynn 2008; Görtz and Dieckmann 1987 in Lynn 2008; Heckmann 1983; Soldo et al. 1974; Weis and Ayala 1979).

Some Unusual Habitats. The ciliates are not as successful as prokaryotes and mastigotes in exploiting extreme habitats. Nevertheless, species have been described from habitats of temperature extremes: from hot thermal springs and waters near the deep-sea hydrothermal vents at a depth of 2,600 m off the California coast (Small and Gross 1985) to the ice and lakes of Antarctica (Christner et al. 2003 in Lynn 2008; Kepner et al. 1999 in Lynn 2008; Laybourn-Parry et al. 2002 in Lynn 2008; Lee and Fenchel 1972 in Lynn 2008).

Ecology

Three aspects of the ecology of ciliates will be discussed below: the role that ciliates have played in models of ecological theory, the ecology of ciliate communities, and the contribution ciliates make to primary and secondary production.

Ecological Models. Protists/protozoa are excellent experimental organisms for the modelling of ecological theory for several important reasons (Montagnes et al. 2012; Salt 1974).

"These are that if a phenomenon is found to occur in protozoa it has a high probability of being a general one, and that the absence of sexes, age classes, and other characteristics of more complex animals permit certain reactions to be seen in protozoa more clearly than in higher animals." (Salt 1974).

Ciliates share with other protists the properties outlined above by Salt (1974). Being small organisms, they have many generations in a short period of time, and the diversity of "functionally" different species can enable the construction of complex communities. Microcosm experiments with ciliates can be replicated with ease and because these are small and manageable "systems" there can be rigorous to complete control of most abiotic factors.

Gause (1934) was the first to take advantage of the protists in testing and modelling ecological phenomena, both in his studies of predator–prey modelling using *Paramecium caudatum* and *Didinium nasutum* and in his studies of competitive exclusion using *Paramecium aurelia*, *Paramecium caudatum*, and *Stylonychia mytilus*.

Over 30 years later, Salt (1967) modelled the predator–prey interaction between *Woodruffia metabolica* and *Paramecium aurelia*, discovering, among other things, that the predator exhibited a threshold response to prey density rather than a proportional response and that the escape from predation of a portion of the prey population was a result of innate behavioral characteristics of the predator. Salt (1974, 1975 in Salt 1979) investigated predator–prey interactions between two species used by Gause (1934), *Didinium* and *Paramecium*. In these studies, Salt (1974) found that both predator and prey density can act as controls on the capture rate of the predator, that as predator density increases, the size of individual *Didinium* increases while prey capture and/or food intake rates decline (Salt 1975 in Salt 1979), and that "*Didinium* at higher densities are more efficient in the utilization of energy than are those at low densities" (Salt 1979). Li and Montagnes (2015) have used these two species to more deeply explore predator–prey models, concluding predator conversion efficiency and predator mortality, two key model components, can depend upon prey abundance. Luckinbill (1973 in Lynn 2008) concentrated on the prolonged coexistence of predator and prey also using *Didinium* and *Paramecium*. Whereas Gause (1934) and Cooper et al. (2012) found that the prey needed physical refuges or habitat fragmentation to prolong the interaction, Luckinbill (1973 in Lynn 2008) prolonged coexistence by providing a physically homogeneous environment using methyl cellulose to slow both predator and prey locomotion; in this system, if prey growth is restricted and if prey can maintain adequate numbers for survival while simultaneously remaining at low enough

densities to avoid capture, a cycling of the predator and prey populations is achieved. Luckinbill and Fenton (1978) have further explored the relationship between intrinsic rates of increase, frequency of environmental perturbation, and population cycling in bacterivorous ciliates. They demonstrated that populations of fast-growing species track environmental variations more closely and become extinct more quickly than populations of slower growing species.

Gause's initial experiments on competitive ability have been explored in more detail. Natural, rather than experimental, populations of *Paramecium aurelia* were studied in a woodland seepage area (Gill and Hairston, in Gill 1972). Although one stock of *P. aurelia* was apparently competitively excluded, evidence suggests that it was not well adapted to the marginal, highly unpredictable habitat used for the experiments. Further investigating the relationships between intrinsic rates of increase r, saturation densities K, and competitive ability of experimental populations of *Paramecium aurelia*, Gill (1972) concluded that there was "no consistent relationship between r and K and competitive ability, and that simple environmental changes affect competitive ability much less than they affect either r or K." On the other hand, Luckinbill (1979) showed that selection for increased r also increased K of several stocks of *Paramecium primaurelia*. In over six species of bacterivorous ciliates, estimates of r_m (the maximum rate of increase) were also positively correlated with K and negatively correlated with competitive ability (Luckinbill 1979).

Several examples in the recent literature have used ciliates and other protists as model organisms to explore and illuminate aspects of biodiversity and ecosystem function (Giller et al. 2004). Morin and McGrady-Steed (2004) concluded that there was an inverse relationship between species richness and the carbon dioxide flux in microcosms featuring protists, primarily ciliates. Food web diversity and productivity can also strongly influence the composition of bacterial communities in model ecosystems of microbial eukaryotes and thus ultimately influence decomposition rates (Krumins et al. 2006). As a third example, Fukami and Morin (2003) demonstrated that the order in which the ciliate community was assembled had significant impacts on the productivity-diversity relationship. Finally, Limberger and Wickham (2012) showed that diversities and differences among habitats of low connectivity persisted longer compared to habitats with medium and high connectivities.

Assemblages and Communities. Assemblages of ciliates are characteristic, not only of certain habitats but also within the same habitat where predictable assemblages seem to occur at specific seasons or under specific conditions related to biotic and abiotic factors (Bick 1972 in Lynn 2008; Grolière 1978). The apparent predictability of these assemblages has led some investigators to suppose that the many different species have dependent interactions (Cairns and Yongue 1977) although Dolan et al. (2007) concluded that the neutral theory of random colonization could explain the structure of tintinnid communities. There is certainly variability in the appearance of these assemblages both in time (Goulder 1980 in Lynn 2008) and in space (Bulit et al. 2009; Taylor and Berger 1980), driven primarily by resource availability (Galbraith and Burns 2010).

Ciliates are heterotrophs, being either phagotrophs or osmotrophs. Various species can be categorized as bacterivorous, algivorous, carnivorous (Elliott and Bamforth 1975 in Corliss 1979; Fenchel 1968 in Fenchel 1969; Noland and Gojdics 1967 in Chen 1967), or histophagous. Ciliates perform a similar role in soils and aquatic sediments. By grazing on bacterial populations and ingesting plant residues ciliates increase rates of decay and mineral cycling (Fenchel and Harrison 1976; Krumins et al. 2006). Some ciliates in both marine and freshwater habitats can be classified as "autotrophic" or mixotrophic: in these cases they contain algal symbionts or they somehow "steal" the chloroplasts of their algal prey – a phenomenon called kleptoplasty (Johnson 2011; Perriss et al. 1994; Stoecker et al. 1989). Mixotrophy may enable survival in habitats that would be marginal for an obligate heterotroph (Esteban et al. 2010).

In planktonic communities, ciliates are links in the food chains (Sanders and Wickham 1993; Sherr and Sherr 1988). In marine "snow," ciliates are a part of the decomposition food web (Caron et al. 1982). In oceanic regions, perhaps more than 90% of the carbon may be cycled through the protists, including ciliates such as tintinnids and oligotrichs (Lynn and Montagnes 1991 in Lynn 2008). In planktonic food webs, ciliates may be important in the regeneration of some nutrients (Garst and Horstmann 1983; Johannes 1965 in Corliss 1979) but not others (Taylor and Lean 1981 in Garst and Horstmann 1983). In coastal regions, a few ciliates, such as the autotrophic *Mesodinium rubrum*, may even contribute substantially to primary production (Smith and Barber 1979 in Lynn 2008). As links, ciliates are consumed by a variety of other organisms: in the pelagic realm, copepods, jellyfish, and larval fish have been recorded as predators (de Figueiredo et al. 2007; Stoecker and Sanders 1985 in Lynn 2008; Stoecker et al. 1987 in Lynn 2008).

Production. Production is defined as the amount of biomass generated per unit time. There are now many estimates of the contribution ciliates make to both primary and secondary production of biomass. For example, ciliates in littoral sand sediments representing 0.05% of the biomass are estimated to have contributed 15% to the secondary production of the zoobenthos (Burkovsky 1978). In sediments from a freshwater lake, Finlay (1978 in Finlay and Ochsenbein-Gattlen 1982) concluded that production and consumption by benthic ciliates are significant components of the energy flow through the benthos. Tintinnids may constitute more than 25% of the secondary production at certain times of the year (Middlebrook et al. 1987 in Lynn 2008). However, the nonloricate oligotrich ciliates typically "out-produce" tintinnids in a variety of ecosystems (Gilron et al. 1991 in Lynn 2008; Lynn et al. 1991a in Lynn 2008; Montagnes et al. 1988 in Lynn 2008). The record for contribution to **primary** production (as well as high speed swimming) is held by the autotrophic ciliate *Mesodinium rubrum*, which harbors a symbiotic photosynthetic cryptomonad and its chloroplasts (Lindholm 1985). Smith and Barber (1979 in Lynn 2008) recorded photosynthetic rates of 1,000–2,000 mg C m^{-3} h^{-1} for a bloom of this ciliate, matching the most productive phytoplankton. More often, primary production by mixotrophic ciliates is a very minor component (Perriss et al. 1994).

Characterization and Recognition

The Phylum

General Characterization. Ciliates, with rare exceptions (i.e., *Phalacrocleptes*, a suctorian in the PHYLLOPHARYNGEA), have cilia at some stage in their life cycle. Ciliates are dikaryotic; their cells contain macronuclei and micronuclei. In this nuclear dualism, the macronucleus is physiologically dominant, actively synthesizing mRNA and rRNA while the micronucleus, the repository of the genomic DNA, is involved in genetic recombination and sexual phenomena. In most ciliates, the macronuclei contain far greater than diploid quantities of DNA and are considered to be ampliploid (i.e., containing many amplified copies of the ciliate's genome) (Lynn 2008). The micronuclei of ciliates are considered to be diploid, although polyploidy undoubtedly occurs. Nuclear division is closed: the nuclear membranes of both macro- and micronuclei remain intact while the mitotic apparatus separates the DNA. In ciliates of the HETEROTRICHEA, however, most mitotic microtubules are **external** to the macronuclear membrane, a diagnostic feature for this class. Micronuclear chromosomes are attached to microtubules by kinetochores. The macronucleus develops from a micronucleus after conjugation. Initially, the micronuclear chromosomes may be endoreplicated many times to become polytenic. Subsequently, DNA sequences are deleted using a RNA-mediated epigenetic machinery that results in subchromosomal macronuclear DNA molecules that range in length from 2 to 300 kb (Chalker 2008; Juranek and Lipps 2007; Nowacki et al. 2010; Prescott 1994). Telomeric sequences, such as the hexanucleotide CCCCAA, are added by telomerase to the chromosome ends (Blackburn 1992; Blackburn et al. 1983). The macronucleus divides amitotically by an unknown method of segregation, possibly just randomly, of these subchromosomal DNA molecules. The macronuclei of one group of ciliates, the KARYORELICTEA (see below), cannot divide: new macronuclei arise by division and differentiation of micronuclei at each cell division (Raikov 1982; Raikov 1996 in Hausmann and Bradbury 1996).

The ciliature of the body in most ciliates is specialized around the cell mouth or cytostome (Fig. 1). Because of its variability, the pattern and arrangement of this oral ciliature has been the basis for the classification of ciliates for many years (Corliss 1979; Lynn 2008; Lynn and Small 2002). Most ciliates are phagotrophic, ingesting particulate material and/or prey in food vacuoles that are formed at the cytostome. Some ciliates are astomatous and osmotrophic. On completion of the digestive cycle, the food is egested through the cell anus, typically via a well-defined cytoproct.

Other organelles that distinguish one group of ciliates from another are the position and arrangement of contractile vacuoles (Patterson 1980); the type and distribution of such extrusomes as mucocysts and toxicysts (Hausmann 1978; Rosati and Modeo 2003 in Lynn 2008); the presence of stalks, loricae, or other attachment structures; and types of encystment structures (Lynn 2008).

Reproduction, that is, the production of new individuals, occurs by transverse binary fission, also called homothetogenic fission (Lynn 2008): the fission plane

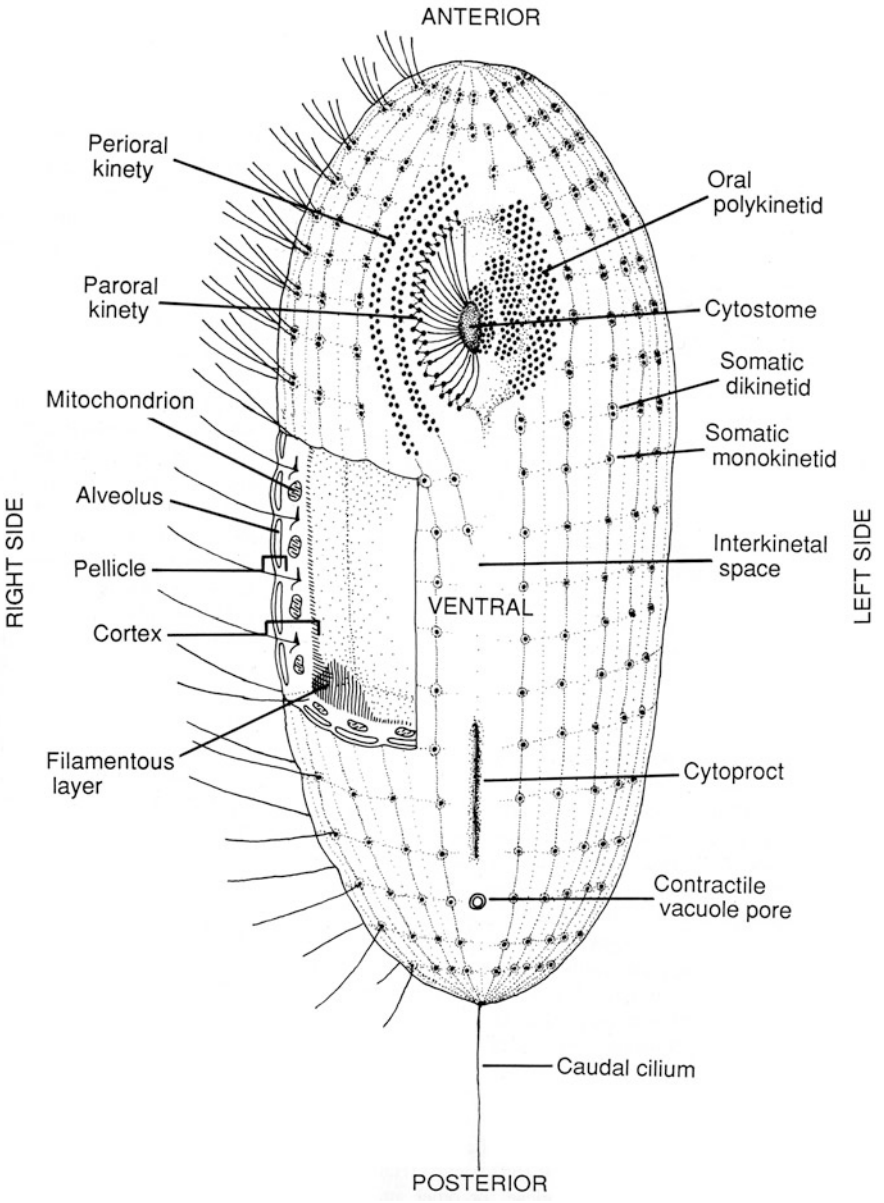


Fig. 1 Schematic figure of the ventral surface of a generalized ciliate. The cortex of a ciliate may be divided into somatic and oral regions. The locomotor units or kinetids of the somatic region are aligned in files called kineties. These kinetids can be dikinetids composed of two kinetosomes and cilia or monokinetids composed of one kinetosome and its cilium. Often a longer caudal cilium is carried posteriorly. Perioral ciliature as specializations of some somatic kineties may border the oral region. In this example, there is a paroral to the *right* of the cytostome and three oral polykinetids to the *left* of the cytostome. The cilia of these organellar complexes have not been illustrated; the

occurs across the longitudinal axis of the kineties and the body. In many taxa, binary fission may be modified so that unequal division occurs.

The sexual process, sometimes seen in field collections or lab cultures as pairing of individual ciliates of complementary mating types, is called conjugation (Miyake 1996 in Hausmann and Bradbury 1996; Nanney 1980; Orias in Collins 2012). Conjugation occurs for minutes or hours to as much as a day or so during which time the partners exchange haploid gametic micronuclei. The conjugating partners of many spirotrich, peritrich, and suctorian species can be quite different in size; in these circumstances, total conjugation or complete fusion of partners may occur. Usually, syngamy is restricted to the fusion of gametic micronuclei, which have undergone meiosis. After syngamy, the partners typically separate. During this process, new macronuclei develop from mitotic products of the zygotic nucleus through a RNA-mediated process using scan RNAs (scnRNA) (see Singh et al. 2014).

Detail of Cell Structure. In a "typical" ciliate, the cortex or the outer 1–2 μm of the cell can be divided into two main regions, the somatic and the oral region (Fig. 1). The somatic region, composed of a "skeletal" support system opposed by the hydrostatic pressure within the cell, functions in locomotion, sensing the environment, attachment to surfaces, and secretion of protective coverings. The oral region functions in sensing, acquiring, and ingesting nutrients. A complicated framework of kinetosomes, microfilaments, microtubules, and other fibers that are collectively called the infraciliature underlies these regions (Figs. 2 and 3a–d).

The infraciliature is comprised of kinetosomes arranged into longitudinal files (rows) called kineties (Fig. 1). Somatic and oral kinetal patterns are characteristic of various groups of ciliates (Lynn 2008; Lynn and Small 2002). The kinetosome is apparently the organizing center for the cortical fibrillar structures: usually two groups of microtubules and a striated kinetodesmal fibril (Figs. 2 and 3a–d) are associated with a parasomal sac. The fibrillar associates of the kinetosome anchor the cilium and provide structural support for the cortex.

Ciliates are bounded by a cell membrane, the plasmalemma (Lynn 2008). The plasmalemma in most ciliates is underlain by unit membrane-bound sacs called alveoli with which are associated a family of cortical proteins, the alveolins (Gould et al. 2008). The alveoli in their turn are subtended by a fibrous layer of varying thickness called the epiplasm, whose component proteins, the epiplasmins, form a complex skeletal network (Damaj et al. 2009) (Fig. 2). The plasma membrane, alveoli, and epiplasm comprise the pellicle, which is part of the cortex (Fig. 2).

Fig. 1 (continued) kinetosomes only have been represented as dots. The cytoproct for egestion and the contractile vacuole pore for osmoregulation are posterior to the oral region, in the interkinetal space. The cut-away portion of the somatic cortex illustrates the sac-like alveoli beneath the plasma membrane, the cortical mitochondria, and the kinetosome with its fibrillar associates. These cortical structures are sometimes separated from the endoplasm by a filamentous layer

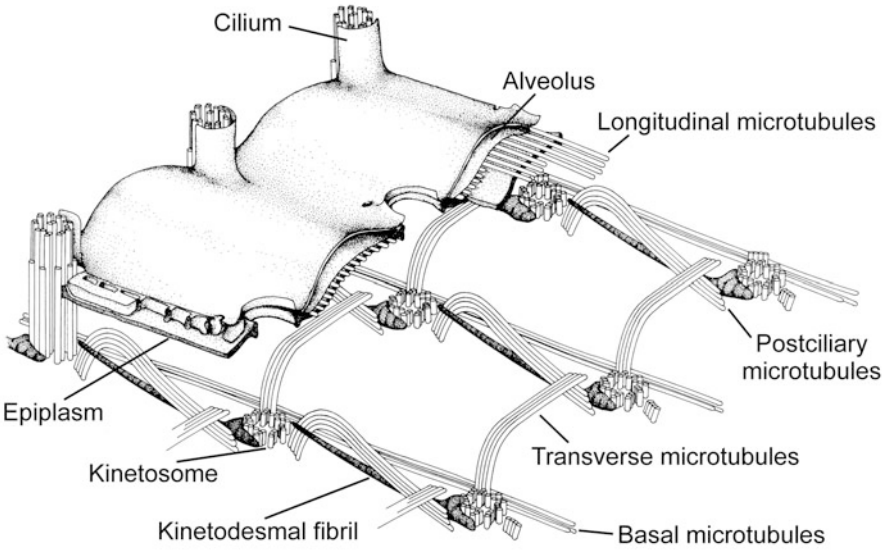


Fig. 2 This is a detailed schematic figure of the generalized somatic cortex of a ciliate. Nine locomotor units or kinetids are illustrated: six are illustrated without cilia and three are illustrated with cilia. The cell surface is covered by a unit plasma membrane (plasmalemma), which is pierced by indentations called parasomal sacs. The cortical alveoli underlie the plasma membrane between kineties and are connected to adjacent alveoli along the kinety. The alveoli, in their turn, may be underlain by microtubules that lie on top of a dense, perhaps fibrous layer called the epiplasm. The epiplasm is pierced by the parasomal sacs and by the kinetosomes. The kinetosomes are associated with three fibrillar structures: a periodically striated kinetodesmal fibril; a laterally directed transverse ribbon; and a posteriorly directed postciliary ribbon. A set of basal microtubules courses beside the kinetosomes but is not directly connected to them

A variety of other organelles is found in the cortex. The majority of the mitochondria with tubular cristae (Fig. 3b, e, g, h) are found in the cortical ridges between kineties, where they are anchored in position by connections to cortical microtubules and to the epiplasm. Mitochondria in some ciliates have transformed into hydrogenosomes (de Graaf et al. 2011), and these are typically intimately associated with symbiotic methanogenic bacteria (Boxma et al. 2005 in Lynn 2008; Fenchel and Finlay 1991a). Extrusomes (Fig. 3e, f, j) are also distributed in the cortex between and within the kineties. There are several types of these exocytotic organelles, which function to aid in the capture of prey, in building the wall in resting cysts, or for some other unknown function (Hausmann 1978; Rosati and Modeo 2003 in Lynn 2008).

The contractile vacuole pore (Figs. 1 and 3g) is a cortical structure that serves as the opening through which the products of osmoregulation or the contents of the contractile vacuole are expulsed (Allen 2000). Egestion takes place through the cytoproct (Fig. 1), usually a slit-like opening in the cortex (Allen and Wolf 1974 in Lynn 2008).

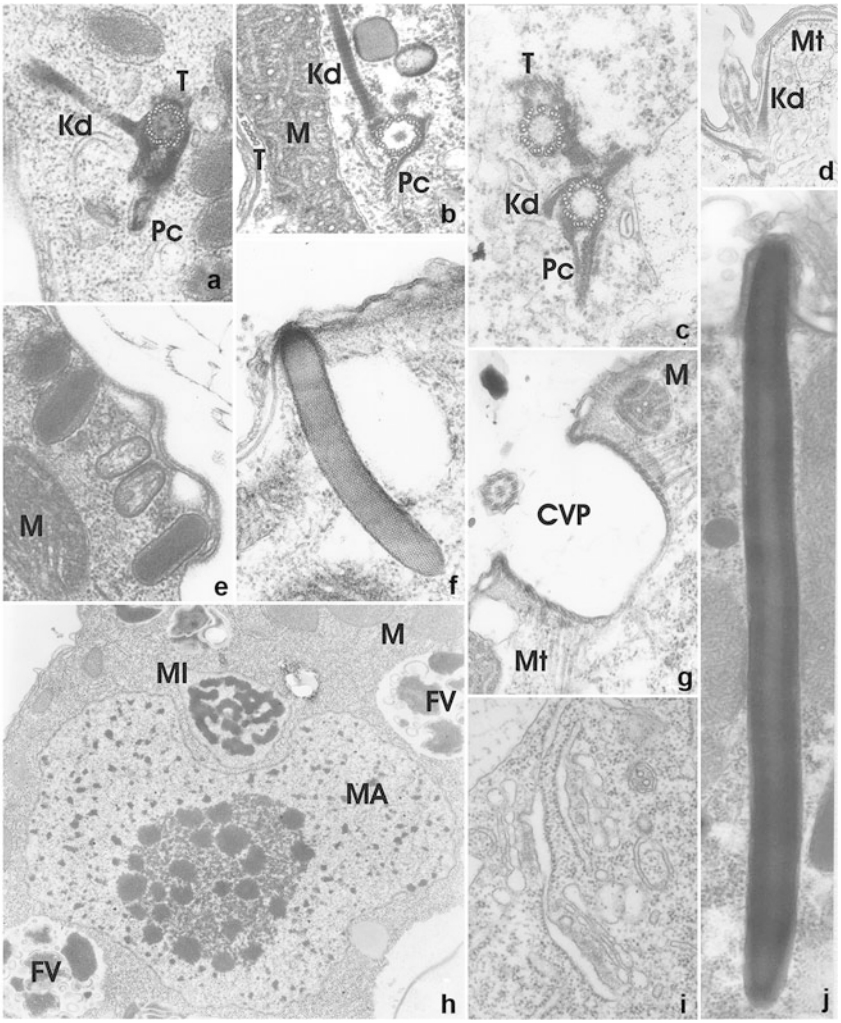


Fig. 3 (**a**–**j**) Ultrastructural features of the Phylum CILIOPHORA. (**a**–**d**) Somatic kinetids of some ciliates. (**a**) *Lepidotrachelophyllum,* a haptorian litostome. ×49,000. (**b**) *Colpidium,* a hymenostome oligohymenophorean. ×50,000. (**c**) *Colpoda,* a colpodean. ×55,000. (**d**) Oblique section through the base of the cilium and a longitudinal section of the tapering kinetodesmal fibril of *Colpoda*. Note the microtubules (*MT*) underlying the cortex with its flattened alveoli beneath the plasma membrane. *Kd* kinetodesmal fibril, *M* mitochondrion, *Pc* postciliary microtubular ribbon, *T* transverse microtubular ribbon. (**e**, **f**, **j**) Extrusomes of several ciliates. (**e**) Mucocysts of *Lepidotrachelophyllum*. ×38,000. (**f**) A mucocyst of *Colpidium*. ×35,500. (**j**) A "mucocyst" of *Ophryoglena.* ×38,000. (**g**) Contractile vacuole pore (*CVP*) of *Colpidium,* an oligohymenophorean. A set of microtubules is embedded in the epiplasm along the wall of the pore while other microtubules (*Mt*) originate in the epiplasm of the wall and extend away from the pore over the surface of the contractile vacuole. *M* mitochondrion. ×20,000. (**h**) Macronucleus (*MA*) and micronucleus (*MI*) of *Colpoda,* a colpodean. Note also the mitochondria (*M*), which have tubular cristae (see also (**e**) and (**g**) above). *FV* food vacuole ×18,000. (**i**) Golgi apparatus of *Colpoda*. ×36,000

The endoplasm of ciliates, in general, has a less obvious organization than the cortex or ectoplasm. The macronucleus and micronucleus (Fig. 3h) are among the largest endoplasmic structures. Food vacuoles are scattered throughout the endoplasm. The Golgi bodies, often called dictyosomes in ciliates and plants, are inconspicuous and scattered throughout the cytoplasm (Fig. 3i). Microtubular ribbons usually originate from cortical kinetosomes and extend into the endoplasm to direct the movement of organelles and vesicles in both directions between the endoplasm and the cortex (Allen and Fok 2000).

Some Life Cycles. The life cycle of a typical ciliate is fairly simple (Fig. 4). In the presence of nutrients, cells grow and reproduce by binary fission to increase the size of the population. As food becomes limiting, some ciliates disperse from the food

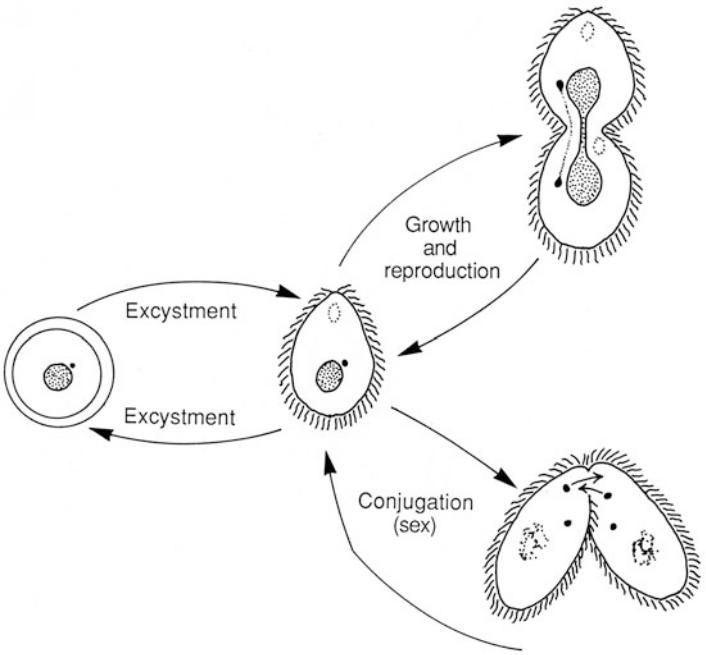


Fig. 4 This figure illustrates a generalized ciliate life cycle, divisible into three phases. The vegetative or asexual reproductive cycle involves feeding, growth, and division by binary fission. Conjugation, the sexual cycle, often stimulated by the depletion of food and the onset of starvation, involves temporary fusion of complementary mating types, meiotic reduction of chromosome number from diploid to haploid, and exchange of haploid gametic nuclei before separation of the partners as exconjugants. If food is present, growth and division ensue; if it is still absent or some other environmental stress such as pH, temperature, toxins, or desiccation stimulates it, the ciliate may enter the encystment-excystment cycle. Cysts may persist for months to years. Ciliates excyst when stimulated by the appropriate environments. According to Goodey (1915), the oldest viable ciliate cysts, two species of *Colpoda,* were more than 38 years old while other protozoan cysts from soils have remained viable for almost 50 years while Shatilovich et al. (2015) have isolated viable cells from late Pleistocene permafrost, 32–35,000 years old

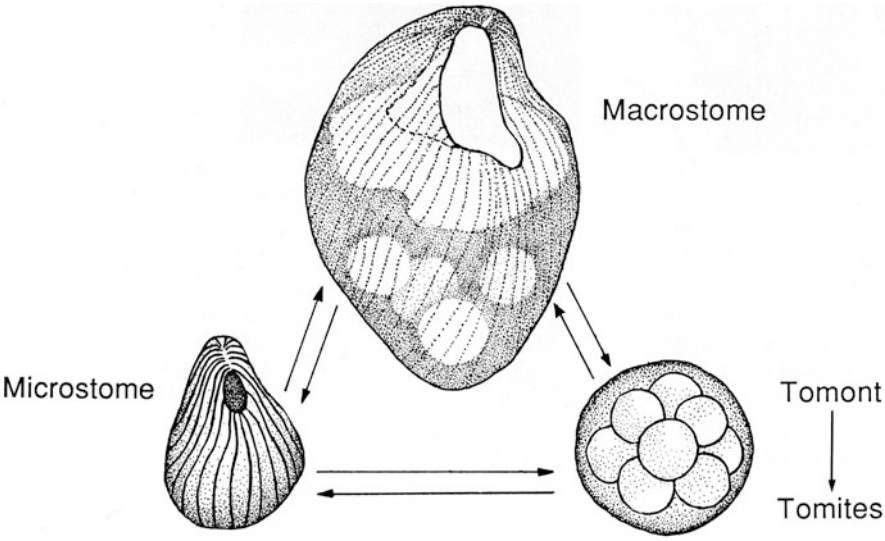


Fig. 5 Life cycle of the macrostome-microstome oligohymenophorean *Tetrahymena vorax*. The microstome form feeds on bacteria. When this food disappears or another suitable ciliate prey is present, some of the population of *T. vorax* are stimulated to undergo a morphogenetic transformation during which their bacteria-feeding oral apparatus dedifferentiates and a new, macrostome oral apparatus designed to capture ciliate prey differentiates. Cell division can occur in a cyst: the divider or tomont undergoes cell division yielding several offspring cells called tomites (After Corliss 1973 in Elliott 1973; Redrawn by S. Alexander)

source and begin to starve. Starvation initiates sexual receptivity in many species. Ciliates conjugate when they encounter complementary mating types. If no mating partner is available, autogamy (self-fertilization) may occur. If nutrients are not discovered, either prior to or subsequent to conjugation or autogamy, many species encyst by secreting a protective wall about themselves. Resting cysts may or may not withstand desiccation.

Some ciliate life cycles are more complicated. The feeding stages or trophonts can be dimorphic. In *Tetrahymena vorax*, one morph is bacterivorous while the other morph feeds on other ciliates (Fig. 5). Depletion of the bacterial population by the microstome bacterivores stimulates some individuals to differentiate as macrostome carnivores that, as cannibals, begin ingesting their siblings or, as predators, other prey ciliates; the presence of the appropriate bacteria stimulates differentiation back to the microstome morph. The life cycles of parasitic species are even more complex. The life cycle of apostome ciliates, for example, is closely linked to the molt cycle of their crustacean hosts. Some apostomes reproduce in the nutrient-rich fluid associated with the shed exoskeleton or exuvium of the host (Bradbury 1996 in Hausmann and Bradbury 1996) (Fig. 6), while other apostomes are parasitoids that kill their krill hosts (Gomez-Gutierrez et al. 2012).

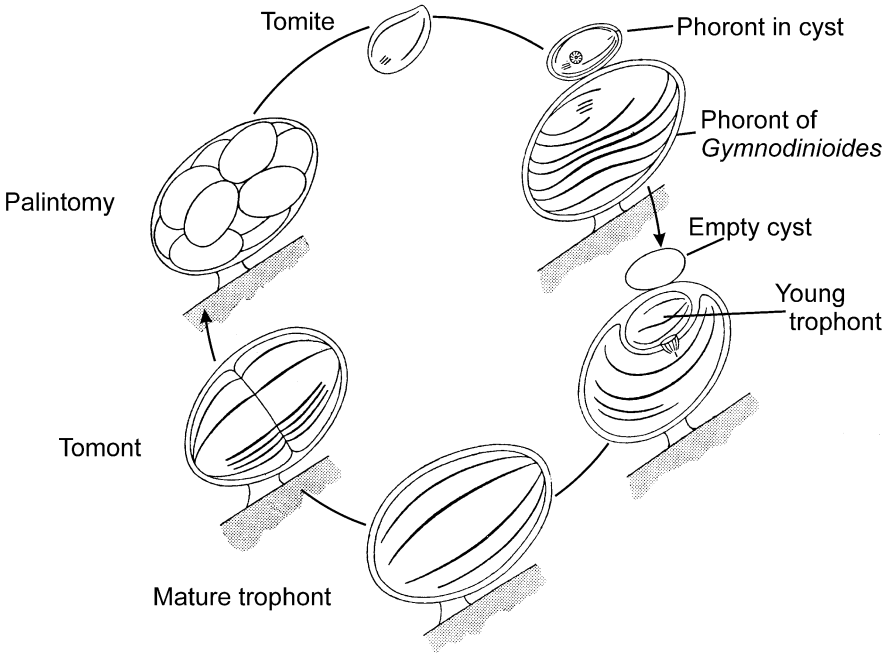


Fig. 6 Life cycle of the predatory apostome Phtorophrya, a "hyperparasite" of the exuviotrophic apostome Gymnodinioides, which itself feeds on the exuvial fluids of its crustacean host. The tomite of Phtorophrya finds a phoront of Gymnodinioides, encysted on the crustacean host's cuticle and attaches to also become a phoront. Phtorophrya then penetrates the phoront of Gymnodinioides, consumes it as a trophont, develops into a tomont that divides by palintomy to produce multiple tomites of Phtoroprhya (Modified from Chatton and Lwoff, 1935a in Lynn, 2008)

Methods and Criteria

Taxa are characterized by morphology: optical and electron microscopy are used. Increasingly, these microscopic approaches are used in conjunction with appropriate biochemical and molecular genetic techniques.

Optical Microscopy. Ciliates can be studied live or fixed and stained by several standard cytological procedures. Studying live ciliates, which requires patience, is facilitated by the combined use of differential interference contrast microscopy and a microcompressor (Skovorodkin 1990; Zinskie et al. 2015) or "slowing agents" such as methyl cellulose or nickel sulfate to retard ciliate movement.

The most informative methods for optical microscopy employ a variety of stains. Silver stains or silver impregnation techniques provide the most information about cortical structures: (1) the protargol or silver proteinate technique provides permanent preparations that reveal the most detail of cortical and subcortical structures including microtubules (Aufderheide 1982; Foissner 1991); (2) the pyridinated silver carbonate method provides either temporary or permanent preparations that

reveal the kinetodesmal fibrils and other finely filamentous cortical and subcortical structures (Fernandez-Galiano 1976 in Augustin et al. 1984; Foissner 1991); and (3) the Chatton-Lwoff silver impregnation procedure provides permanent preparations revealing the pattern of surface structures and kinetosomes (Foissner 1991; Frankel and Heckmann 1968). The only other stain that may be necessary, especially if details of nuclear morphology are needed, is the Feulgen nuclear stain (Foissner 1991) or acridine orange fluorescence stain (Coats and Heinbokel 1982).

Morphological criteria are used to determine ciliate affinities. Features used in taxonomy include, for example, the presence of complex associations of somatic cilia, of few or many oral structures, of loricae, and of stalks (see Curds 1982; Curds et al. 1983; Foissner et al. 1994, 1999; Lynn 2008; Lynn and Small 2002). Species can be determined by mating experiments: mating incompatibility is used as the criterion for a biological species (Lynn and Doerder in Collins 2012; Nanney and McCoy 1976 in Nanney 1980; Sonneborn 1975 in Nanney 1980), but because controlled conjugation is available for so few species, mating tests are not extensively used to identify species. If no mating test organisms exist, quantitative differences can be sought among clones, strains, or species, using multivariate morphometric procedures (for example, Gates 1977; Lynn and Malcolm 1983).

Electron Microscopy. Both scanning and transmission electron microscopy have been used to study ciliates. Preparation of most specimens for scanning electron microscopy is now standardized (Foissner 1991). Preparation of specimens for transmission electron microscopy, especially fixation, is quite varied: for specific methods see references in Lynn (2008).

Features revealed by transmission electron microscopy distinguish between higher taxa. The kinetid – the kinetosome and its microtubular and fibrillar associates – is the fundamental unit of the ciliate cortex. Kinetids of the somatic and oral cortex can be distinguished in the same ciliate (Lynn 2008). The structure and arrangements of the kinetids distinguish one taxon from another (Fig. 7). Clustering techniques have been used on large data sets of ultrastructural characters to determine relationships between taxa (Lynn 1979 in de Puytorac et al. 1984).

Biochemical and Molecular Genetic Techniques. Biochemical criteria restricted to easily cultured, oligohymenophorean ciliates also have been employed to determine relationships among ciliate taxa. DNA hybridization has been used with *Tetrahymena* species (Allen and Li 1974 in Nanney 1980); starch gel electrophoresis of isozymes has been used with species of *Tetrahymena* (Borden et al. 1977 in Nanney 1980) and *Paramecium* (Allen et al. 1983); polyacrylamide gel electrophoresis of cytoskeletal proteins has been used with species of *Tetrahymena* (Vaudaux et al. 1977 in Nanney 1980). However, with the invention of the polymerase chain reaction (PCR), it is now possible, even from single cells, to amplify genes that have been used in various ways to identify species: randomly amplified polymorphic DNAs (RAPDs) have been used to identify species of *Paramecium* (Stoeck et al. 1998 in Lynn 2008) and *Euplotes* (Chen et al. 2001 in Lynn 2008).

A more recent approach is to use mitochondrial genes as "barcodes." The mitochondrial cytochrome *c* oxidase subunit 1 (cox1) "barcode" can identify putative biological species of *Paramecium* (Barth et al. 2006 in Lynn 2008) and

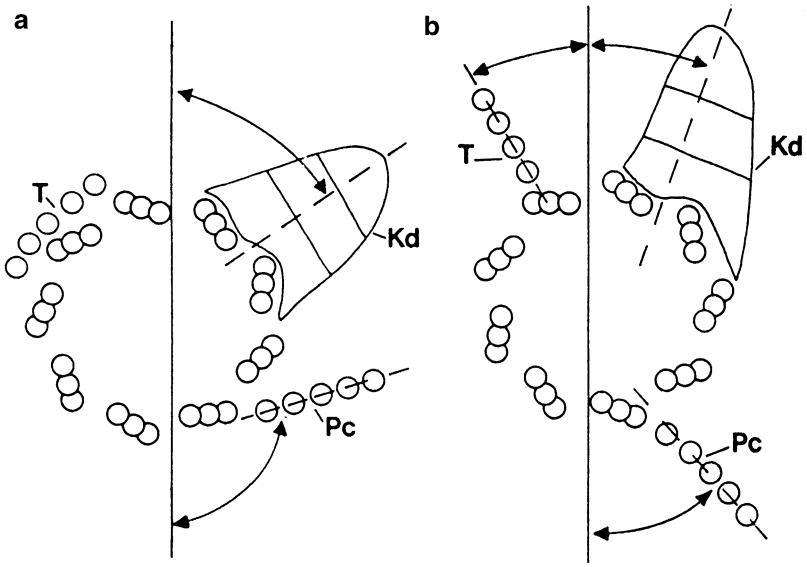


Fig. 7 Schematic figures of two ciliate kinetids illustrate the kinds of characteristics that can be recorded and quantified in comparative studies of cortical ultrastructure. The *central solid line* indicates the longitudinal axis of the kinety. The numbers of microtubules in the transverse (*T*) and postciliary ribbons (*Pc*) can be counted. The relationship of the transverse ribbon to the kinetosome perimeter can be either tangential (**a**) or radial (**b**). With all the radially oriented components, the angle with respect to the kinety axis can be measured and used either quantitatively or semiquantitatively. *Kd* kinetodesmal fibril (After Lynn 1981)

Tetrahymena (Kher et al. 2011). The cox1 barcode is broadly applicable to ciliates (Strüder-Kypke and Lynn 2010) and has been used to infer the existence of cryptic species in genera where mating crosses have not yet been used (Gentekaki and Lynn 2012; Guggiari and Peck 2008). The mitochondrial small subunit rDNA and the apocytochrome b gene also show promise as barcodes to identify cryptic species (Barth et al. 2008; Katz et al. 2011).

Phylogenomic studies are now possible with the annotation of genomes of *Tetrahymena* (Coyne et al. 2008) and *Paramecium* (Arnaiz and Sperling 2011). Gentekaki et al. (2014) published the first phylogenomic analysis of ciliates, using these published genomes along with RNA sequence libraries for representative marine species (see Marine Microbial Eukaryote Transcriptome Sequencing Project – marinemcroeukaryotes.org).

Classification

A Classification Scheme. Several revisions of the classification of the Phylum CILIOPHORA have been presented in the last 15 years: de Puytorac (1994) edited a collaborative revision to genus level with French colleagues; Jankowski (2007) has

presented a revision down to genus; and Lynn (2008) has presented a revision to family level but listing the included genera. These classifications have differences that are discussed by Lynn (2008), and a revised classification is described below, characterizing the major groups only (Table 2).

The somatic kinetid, the most highly conserved structural component of the cortex, is used as a major criterion for this taxonomic scheme, but this is now supplemented by gene sequence data (Fig. 8) (Lynn 1981, 2008). Oral kinetids and their arrangements, which are less conserved, are used to assess more recent common ancestry of the taxa already related by similarities in the somatic kinetid structure. The detailed structure of the somatic kinetid is conserved in many groups and is a more important criterion of relatedness than the number. The total number of kinetids is extremely variable. Mono- and dikinetids can occur in the same subclass or even in the same ciliate cortex, indicating that relatively small heritable changes can change the total number of kinetids, which is a less conservative feature than the number of kinetosomes per kinetid, which in turn is less conserved than the pattern and structure of the kinetid itself (Lynn 2008). Features of the somatic kinetid and the somatic cortex are used to characterize the 11 major groups, called classes by Lynn (2008) (Fig. 9). Orsi et al. (2012) proposed a 12th class, but its independence needs confirmation (cf. Fig. 8). For a more detailed description of the taxa to the family level, see Lynn (2008).

The Major Groups. Several "representative" genera from each class will be use here to illustrate diversity within each class. Because there is so much diversity among genera and because even within a species form and size may change quite dramatically, the term "representative" is an over-generalization.

The first two groups – karyorelicteans and heterotricheans, united primarily by similarities in the somatic kinetids (Fig. 9) and cortex – are placed together in the POSTCILIODESMATOPHORA (Table 2). Many postciliodesmatophorans are highly contractile, possessing similar, presumably homologous, contractile fibrous cytoplasmic structures – the filamentous myonemes, which shorten the cells. The overlapping postciliary microtubular ribbons – the postciliodesmata – extend the cells using microtubule arms that enable sliding of the ribbons on each other and so elongate the cell (Huang and Pitelka 1973 in Lynn 2008).

POSTCILIODESMATOPHORA Gerassimova and Seravin 1976. These ciliates have somatic kinetids whose postciliary microtubular ribbons overlap to form a complex of microtubules that are interconnected by arms – the postciliodesmata.

KARYORELICTEA Corliss 1974. Karyorelicteans (Fig. 10) are thought to represent the ancestral stock of the phylum (see "Evolutionary History", "Fossil Record" and "Phylogeny"). Karyorelicteans possess kinetids with conspicuous kinetodesmal fibrils and postciliary ribbons that overlap to form postciliodesmata (Fig. 9). Their cells contain two to many macronuclei with approximately the micronuclear (diploid) amount of DNA. Their macronuclei arise only by division of micronuclei at the time of cell division. These ciliates are common in estuarine or marine benthic environments.

HETEROTRICHEA Stein 1859. Heterotricheans, because of the similarities of their somatic kinetids (Fig. 9), are thought to have descended from karyorelictean-

Table 2 Classification of the phylum CILIOPHORA[a]

Phylum CILIOPHORA Doflein, 1901
•*POSTCILIODESMATOPHORA* Gerassimova & Seravin, 1976
••**KARYORELICTEA** Corliss, 1974
•••Protostomatida Small & Lynn, 1985
•••Loxodida Jankowski, 1978
•••Protoheterotrichida Nouzarède, 1977
••**HETEROTRICHEA** Stein, 1859
•••Heterotrichida Stein, 1859
•*INTRAMACRONUCLEATA* Lynn, 1996
••**CARIACOTRICHEA** Orsi et al. 2012
••**SPIROTRICHEA** Bütschli, 1889
•••Protocruziidia de Puytorac, Grain & Mignot, 1987
•••Phacodiniidia Small & Lynn, 1985
•••Protohypotrichia Shi et al., 1999
•••Licnophoria Corliss, 1957
•••Euplotia Jankowski, 1979
•••Choreotrichia Small & Lynn, 1985
•••Hypotrichia Stein, 1859
•••Oligotrichia Bütschli, 1887
••**ARMOPHOREA** Jankowski, 1964[b]
•••Armophorida Jankowksi, 1964
•••Clevelandellida de Puytorac & Grain, 1976
••**LITOSTOMATEA** Small & Lynn 1981
•••Haptoria Corliss, 1974
••••Haptorida Corliss, 1974
••••Pleurostomatida Schewiakoff, 1896
••••Cyclotrichiida Jankowski, 1980 *incertae sedis*
•••Rhynchostomatia Jankowski, 1980
•••Trichostomatia Bütschli, 1889
••••Vestibuliferida de Puytorac et al., 1974
••••Entodiniomorphida Reichenow in Doflein & Reichenow, 1929
••••Macropodiniida Lynn, 2008[b]
••***CONTHREEP*** Lynn in Adl et al., 2012[c]
•••**PHYLLOPHARYNGEA** de Puytorac et al., 1974
•••Synhymenia de Puytorac et al. in Deroux, 1978
•••Cyrtophoria Fauré-Fremiet in Corliss, 1956
••••Chlamydodontida Deroux, 1976
••••Dysteriida Deroux, 1976
•••Chonotrichia Wallengren, 1895
••••Exogemmida Jankowski, 1972
••••Cryptogemmida Jankowski, 1975
•••Rhynchodia Chatton & Lwoff, 1939
••••Hypocomatida Deroux, 1976
••••Rhynchodida Chatton & Lwoff, 1939
•••Suctoria Claparède & Lachmann, 1858
••••Exogenida Collin, 1912
••••Endogenida Collin, 1912
••••Evaginogenida Jankowski in Corliss 1979
•••**NASSOPHOREA** Small & Lynn 1981
••••Nassulida Jankowski, 1967
••••Microthoracida Jankowski, 1967
••••Colpodidiida Foissner, Agatha & Berger, 2002 *incertae sedis*

(*continued*)

Table 2 (continued)

•••**COLPODEA** Small & Lynn 1981
••••Platyophryida de Puytorac et al., 1979
••••Bursariomorphida Fernández-Galiano, 1978
••••Colpodida de Puytorac et al., 1974
••••Cyrtolophosidida Foissner, 1978
•••**PROSTOMATEA** Schewiakoff, 1896
••••Prostomatida Schewiakoff, 1896
••••Prorodontida Corliss, 1974
•••**PLAGIOPYLEA** Small & Lynn, 1985[b]
••••Plagiopylida Small & Lynn, 1985
••••Odontostomatida Sawaya, 1940 *incertae sedis*
•••**OLIGOHYMENOPHOREA** de Puytorac et al., 1974
••••Peniculia Fauré-Fremiet in Corliss, 1956
••••Scuticociliatia Small, 1967
••••Hymenostomatia Delage & Hérouard, 1896
••••Apostomatia Chatton & Lwoff, 1928
••••Peritrichia Stein, 1859[d]
••••Astomatia Schewiakoff, 1896

[a]Refer to Lynn (2008) for diagnoses of these taxa and a more complete listing of included families and genera

[b]This taxon, a so-called "ribo-class/group," is based on molecular phylogenetics, primarily the SSUrRNA gene but still lacks a morphological synapomorphy

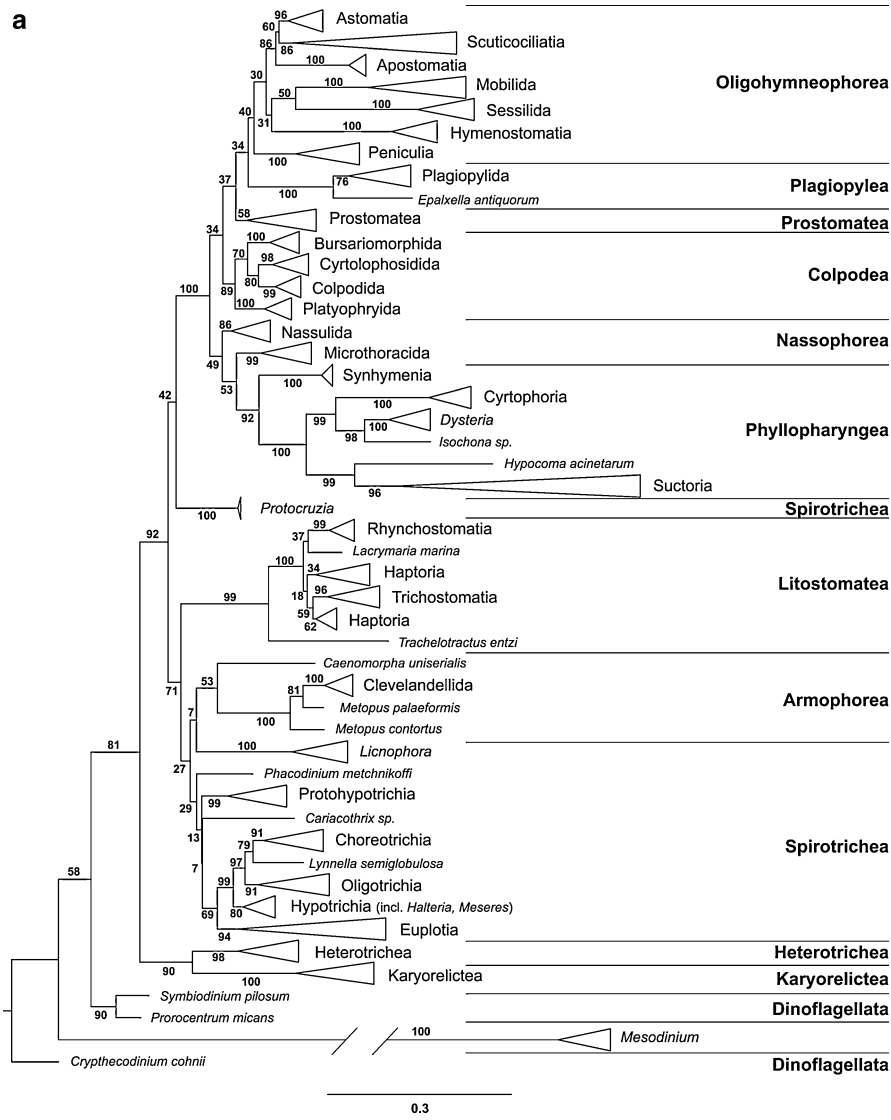
[c]This taxon is another "ribo-class/group," based on molecular phylogenetics of multiple genes (Lynn 2008) but still lacks a morphological synapomorphy. Its name derives from the major included groups (i.e., COLPODEA, OLIGOHYMENOPHOREA, NASSOPHOREA, PROSTOMATEA, PLAGIOPYLEA, PHYLLOPHARYNGEA) and should be pronounced CON-3-P

[d]The Peritrichia are traditionally divided into the Sessilida and Mobilida. Recent molecular phylogenomic analyses have confirmed the monophyly of this group (Gentekaki et al. 2017)

like ancestors (Fig. 10). Heterotrichs have kinetids with postciliary ribbons that overlap to form postciliodesmata and weakly developed kinetodesmal fibrils that often extend slightly posteriorly. The left oral ciliature usually consists of a series of oral polykinetids numerous enough to form a spiral extending out of the oral cavity onto the cell surface (Fig. 10). Their polygenomic macronuclei are capable of division using microtubular bundles that form *outside* the macronuclear envelope – *extramacronuclear* microtubules. This kind of macronuclear division is thought to have arisen independently of that exhibited by the majority of ciliates (see below INTRAMACRONUCLEATA; Lynn 2008). Heterotricheans are found in all habitats described above ("Habitats and Ecology, Habitats").

INTRAMACRONUCLEATA Lynn 1996. The other major division of ciliates is strongly supported by gene sequence data (Fig. 8) (Lynn 2008). There is at present only one significant morphological feature that appears to unite these ciliates: the division of the macronucleus by *intramacronuclear* microtubules – hence the name. Lynn (2008) speculated that a molecular genetic character may ultimately be found that supports this subdivision of the phylum. The remaining major groups are considered intramacronucleates.

SPIROTRICHEA Bütschli 1889. Spirotricheans are a morphologically and genetically diverse class (Figs. 8, 11 and 12). With the exception of *Protocruzia* and *Phacodinium*, replication bands are the specialized morphological feature that accompanies DNA replication in these ciliates (Lynn 2008). *Phacodinium* may have lost this structure as it is placed *within* the spirotrich clade (Fig. 8). On the other hand, *Protocruzia* has a quite unusual macronuclear structure, is only weakly associated with other spirotrichs based on gene sequences (Fig. 8) (Gentekaki



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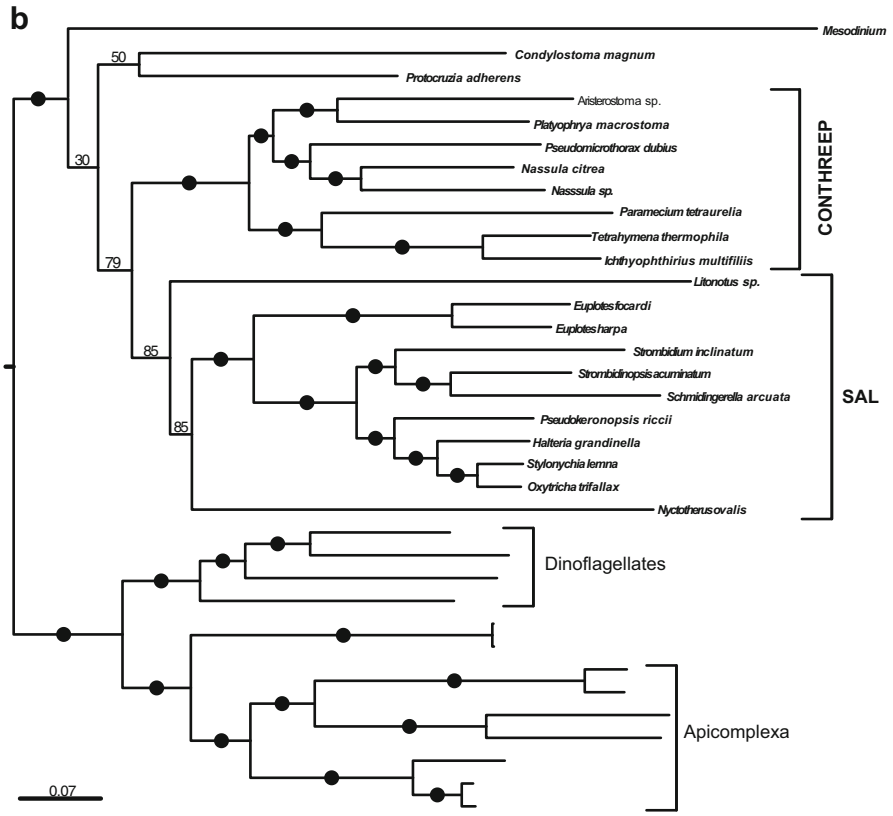

Fig. 8 Phylogenies of the ciliated protozoa based on small subunit rRNA gene sequences (**a**) and on a phylogenomic analysis (**b**). (**a**) A maximum likelihood tree inferred from small subunit rRNA gene data of ciliate species representative of the different classes. The POSTCILIODESMATOPHORA, which includes the Karyorelictea and Heterotrichea, is sister to the INTRAMACRONUCLEATA, which includes the remaining nine major groups. A new class, the Cariacotrichea including *Cariacothrix*, has been suggested by Orsi et al. (2012), but here it is embedded within the Spirotrichea. Dr. Michaela Strüder-Kypke derived this phylogeny using PhyML 3.0 with the GTR (General-Time-Reversible) model with gamma distribution and an estimate of invariable sites. The numbers at the nodes represent the support values for the maximum likelihood analysis. The scale bar represents 30 substitutions per 100 nucleotides. (**b**) A maximum likelihood tree constructed by RAxML using the LG model with empirical frequencies and gamma distribution based on a concatenated alignment of ~120 genes. The *black circles* denote 100% bootstrap support for 1,000 bootstraps (Lynn and Kolisko, unpublished)

et al. 2014), and may in fact represent the type of a new monotypic class of ciliates (Gao et al. 2016; Li et al. 2010). Spirotrichs like *Stylonychia* (Fig. 11) and *Euplotes* (Fig. 12) are typically benthic while *Halteria* (Fig. 11), *Strombidinopsis*, *Tintinnopsis*, *Cymatocylis*, and *Limnostrombidium* (Fig. 12) are typically planktonic, in both marine and freshwater habitats.

The first three groups appear to form the SAL clade, for the first letter in the name of each included group (Fig. 8) (Gentekaki et al. 2014), while the ARMOPHOREA

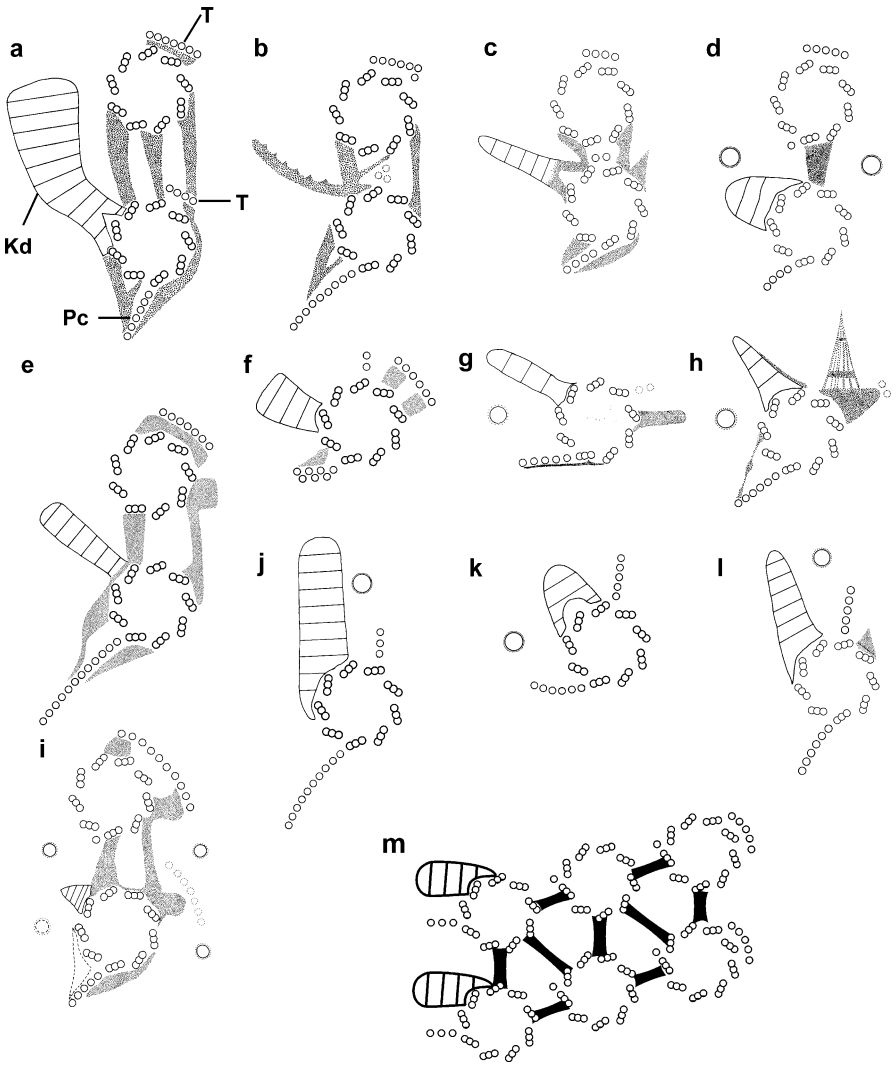

Fig. 9 Schematics of somatic kinetids of genera representative of each major group in the Phylum CILIOPHORA. (**a**) *Loxodes* – KARYORELICTEA; (**b**) *Blepharisma* – HETEROTRICHEA; (**c**, **d**) *Protocruzia* (**c**), *Euplotes* (**d**) – SPIROTRICHEA; (**e**) *Metopus* – ARMOPHOREA; (**f**) *Balantidium* – LITOSTOMATEA; (**g**) *Chilodonella* – PHYLLOPHARYNGEA; (**h**) *Obertrumia* – NASSOPHOREA; (**i**) *Colpoda* – COLPODEA; (**j**) *Plagiopyla* – PLAGIOPYLEA; (**k**) *Holophrya* – PROSTOMATEA; (**l**) *Tetrahymena* – OLIGOHYMENOPHOREA; (**m**) *Plagiotoma* – SPIROTRICHEA. *Kd* kinetodesmal fibril, *Pc* postciliary microtubular ribbon, *T* transverse microtubular ribbon

and LITOSTOMATEA have been proposed to be related based on the lamella-like arrangement of postciliary ribbons that underly the somatic cortex, the so-called Lamellicorticata (Vd'ačný et al. 2010; Vd'ačný et al. 2012).

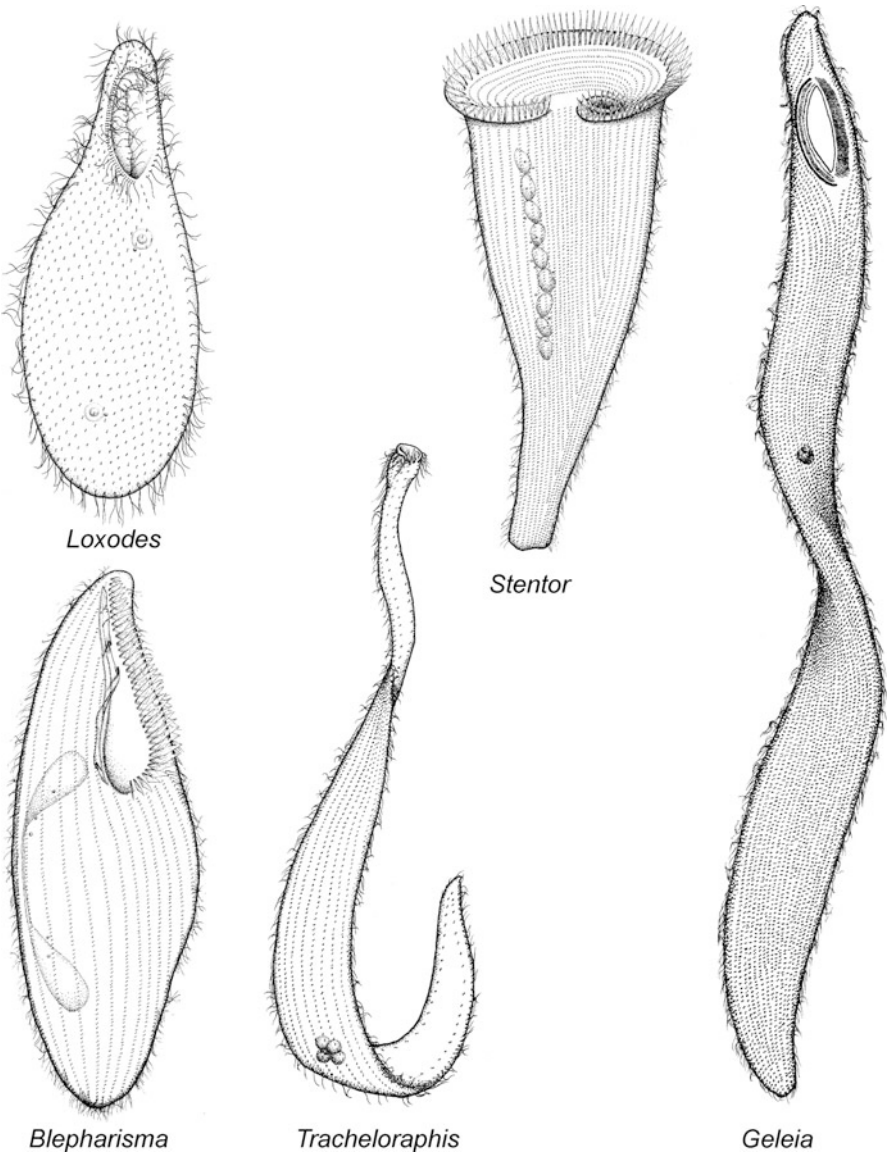

Fig. 10 Representative genera of the POSTCILIODESMATOPHORA. KARYORELICTEA. *Loxodes*, *Tracheloraphis*, and *Geleia*. HETEROTRICHEA. *Blepharisma* and *Stentor*

ARMOPHOREA Lynn 2002. Armophoreans represent one of what have been called "ribo-classes" of ciliates since they were identified as a monophyletic group only by gene sequence data (Fig. 8). In fact, like the spirotricheans, armophoreans are morphologically diverse, both at the cell level *and* at the somatic kinetid level (Figs. 9 and 13). Thus, they can be viewed as "an exception that proves the rule" that

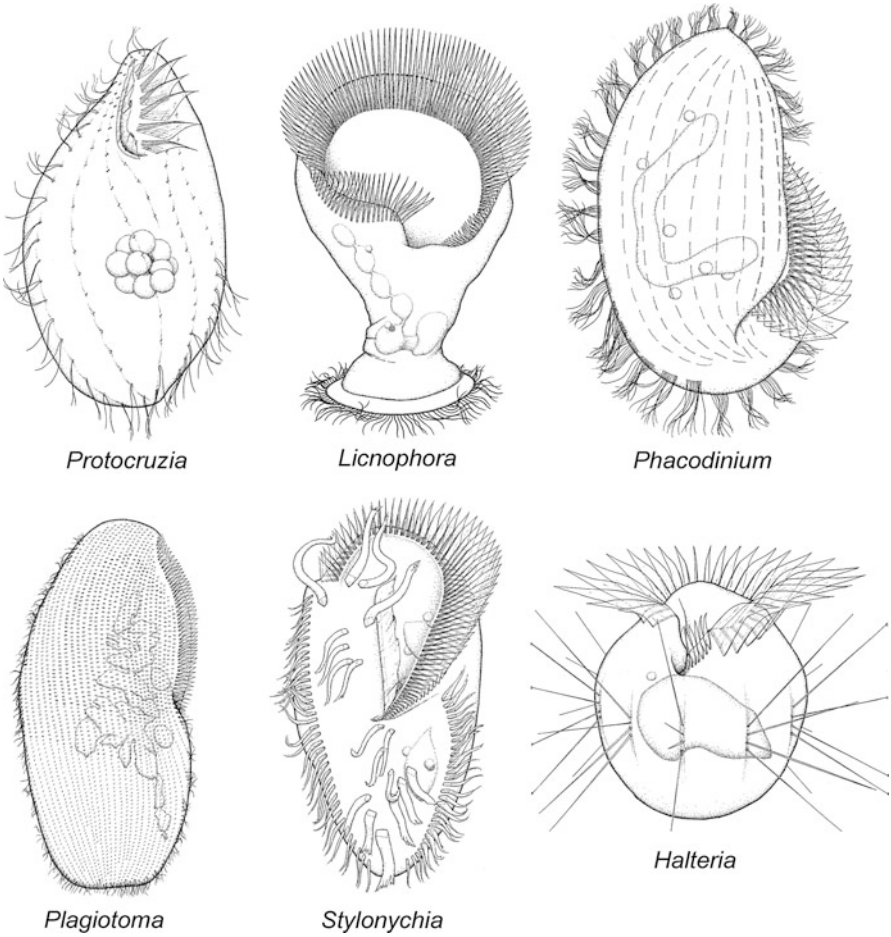

Fig. 11 Representative genera of the SPIROTRICHEA. Protocruzidiidia. *Protocruzia*. Licnophoria. *Licnophora*. Phacodiniidia. *Phacodinium*. Hypotrichia. *Plagiotoma*, *Stylonychia*, and *Halteria*

somatic kinetids generally reflect larger assemblages within the phylum. Armophoreans are typically found in anoxic freshwater and marine habitats, and the gene sequence data may ultimately be corroborated by physiological and biochemical characters since these ciliates all have hydrogenosomes rather than mitochondria. The clevelandellid armophoreans are all intestinal endosymbionts in invertebrates, such as millipedes and cockroachs, and in vertebrates, such as frogs.

LITOSTOMATEA Small and Lynn 1981. The litostomates (Fig. 14) include three rather diverse subgroups of ciliates: the Rhynchostomatia, carnivorous ciliates that use a proboscis for hunting; the Haptoria, principally free-living, carnivorous ciliates; and the Trichostomatia, principally endosymbionts of birds and mammals. These groups have somatic monokinetids with two transverse ribbons – a tangential

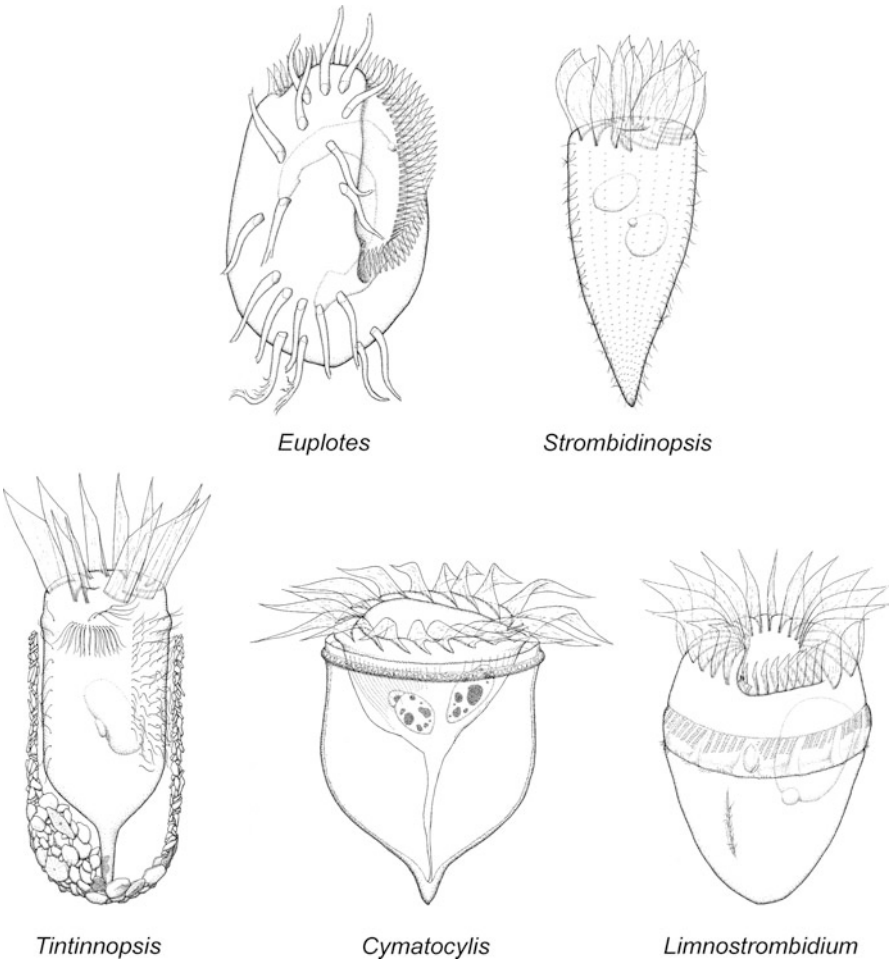

Fig. 12 Representative genera of the SPIROTRICHEA. Euplotia. *Euplotes*. Choreotrichia. *Strombidinopsis*, the tintinnids *Tintinnopsis* and *Cymatocylis*. Oligotrichia. *Limnostrombidium*

one and a radial one, a short, almost laterally directed kinetodesmal fibril, and convergent postciliary microtubules (Fig. 9).

The last six major groups, phyllopharygeans, nassophoreans, colpodeans, prostomateans, plagiopyleans, and oligohymenophoreans are often linked together in molecular phylogenies (Fig. 8) (Lynn 2008). Even though there is no obvious strong unifying morphological feature for this group, it has been named CONTHREEP (pronounced CON-3-P), based on the first letter of the names of the groups that are robustly clustered by genetic features.

PHYLLOPHARYNGEA de Puytorac et al. 1974. Phyllopharyngeans, like the spirotrichs, are a morphologically diverse clade (Fig. 15). However, all have somatic monokinetids with a short, laterally directed kinetodesmal fibril and a

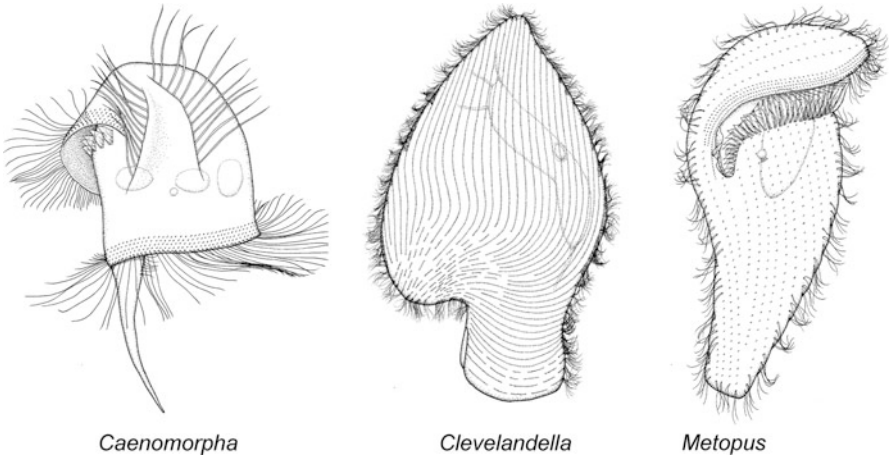

Fig. 13 Representative genera of the ARMOPHOREA. Armophorida. *Caenomorpha* and *Metopus*. Clevelandellida. *Clevelandella*

poorly developed or absent transverse microtubular ribbon accompanied by a fibrous support (Fig. 9). Their postciliary microtubules originate from the kinetosome in a convergent position; subkinetal microtubules course beneath the kinetosomes of a somatic kinety; and the cytopharynx is surrounded by radially arranged microtubular ribbons called phyllae. These ciliates are primarily benthic: chonotrichs and suctoria are sessile on nonbiological surfaces or other organisms. Some are ciliated only during dispersal: for example, the suctorians *Discophrya* and *Acineta* (Fig. 15).

NASSOPHOREA Small and Lynn 1981. The nassophoreans have singly or doubly ciliated somatic kinetids (Fig. 9). Somatic dikinetids have an anterior kinetosome with a tangential transverse ribbon and a posterior kinetosome with an anteriorly directed kinetodesmal fibril, a divergent postciliary ribbon, and often a tangential transverse ribbon. Somatic alveoli are well developed. The cytopharyngeal apparatus, which is similar to that of cyrtophorine phyllopharyngeans, is the cyrtos, a complex microtubular "basket" that functions in ingestion (Tucker 1978). Simple "oral" polykinetids are found adjacent to the cytostome or extending in bands of varying length across the body (Fig. 16). The nassophoreans are commonly free-living, benthic ciliates typically found in fresh, brackish, and salt water, typically feeding on filamentous cyanobacteria.

COLPODEA de Puytorac et al. 1974. The somatic kinetid of colpodeans is strikingly unique (Fig. 9). Colpodeans have dikinetids with a short, laterally directed kinetodesmal fibril and a set of prominent overlapping transverse microtubular ribbons that arise from the posterior kinetosome. Colpodeans clearly demonstrate the range in variability of oral structures while the somatic structures remain invariable (Lynn 2008) (Fig. 17). These ciliates are commonly found in temporary freshwater or soil habitats where they encyst when the environment dries.

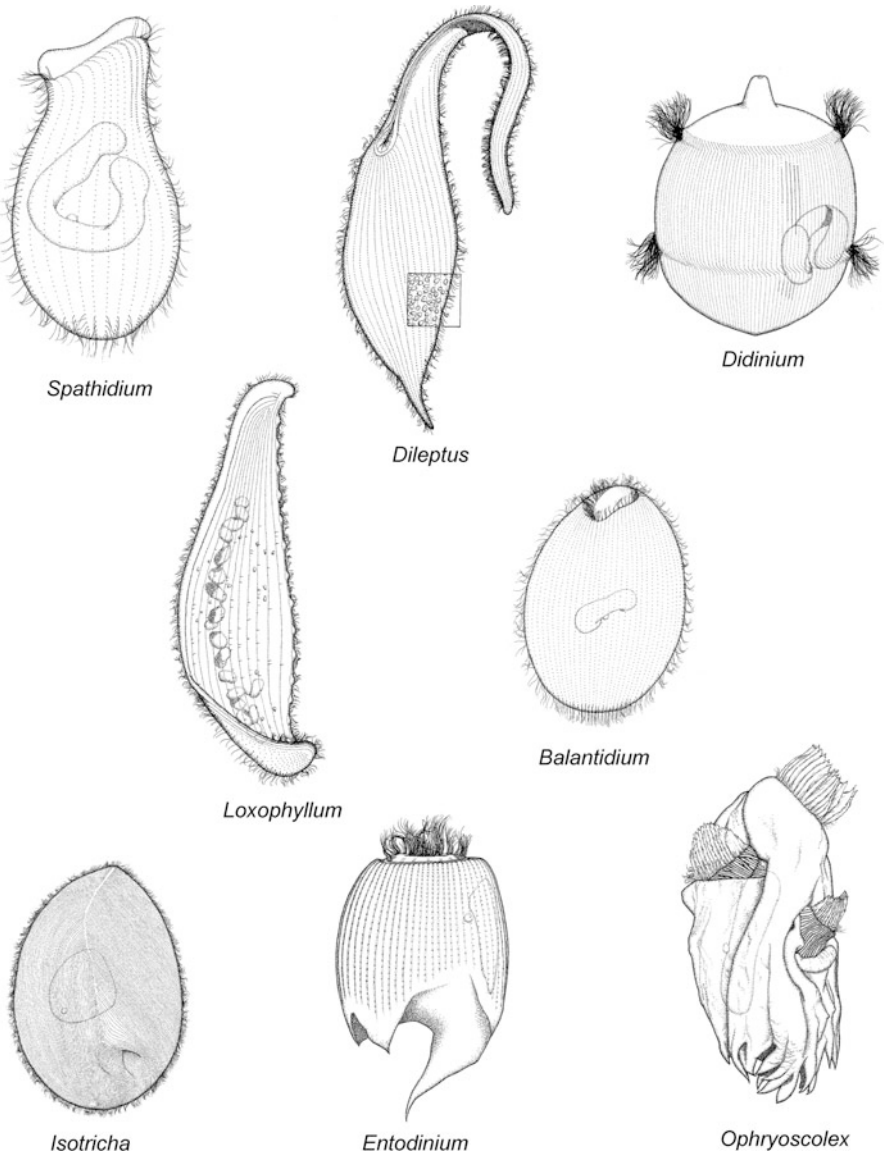

Fig. 14 Representative genera of the LITOSTOMATEA. Rhynchostomatia. *Dileptus*. Haptoria. *Spathidium*, *Didinium*, *Loxophyllum*. Trichostomatia. *Balantidium*, *Isotricha*, *Entodinium*, and *Ophryoscolex*

PROSTOMATEA Schewiakoff 1896. The distinguishing features of the prostomateans include somatic monokinetids with probably a radial transverse ribbon and well-developed, right-anteriorly directed kinetodesmal fibrils (Fig. 9), and the perimeter of the oral area supported by oral dikinetid postciliary ribbons that,

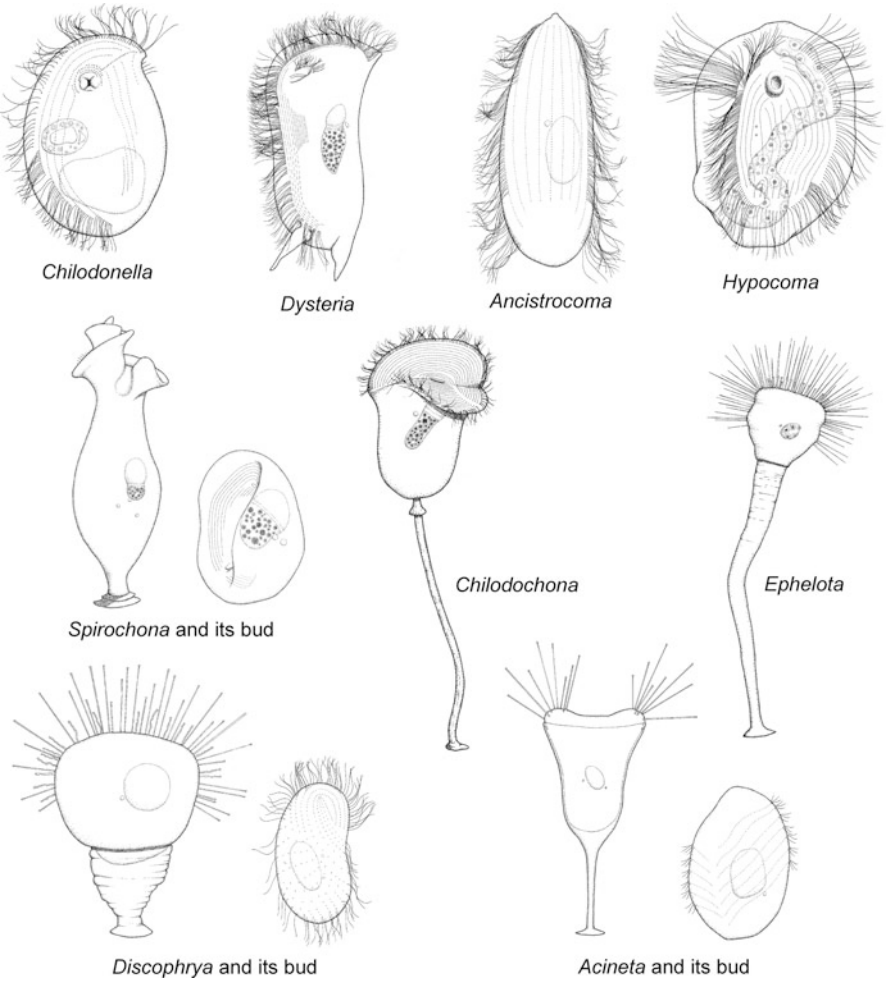

Fig. 15 Representative genera of the PHYLLOPHARYNGEA. Cyrtophoria. *Chilodonella* and *Dysteria*. Chonotrichia. *Chilodochona* and *Spirochona* and its bud. Rhynchodia. *Ancistrocoma* and *Hypocoma*. Suctoria. *Discophrya* and its bud, *Acineta* and its bud, and *Ephelota*. Note how the pattern of the somatic ciliature in the buds of chonotrichs and suctorians resembles that of the cyrtophorians

at least in *Urotricha*, extend from each dikinetid to overlap each other in a circular microtubular band. The prostomateans (Fig. 18) apparently evolved toxicysts independently from the litostomateans, since members of both classes have these organelles. Prostomateans are found in a wide variety of habitats; commonly, they are benthic and most species are marine.

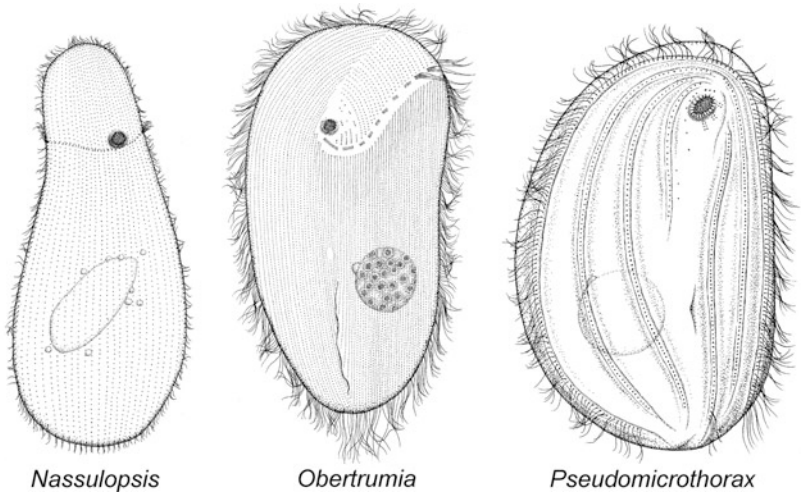

Fig. 16 Representative genera of the NASSOPHOREA. Nassulida. *Nassulopsis* and *Obertrumia*. Microthoracida. *Pseudomicrothorax*

PLAGIOPYLEA Small and Lynn 1985. Like the ARMOPHOREA, the plagiopyleans represent a "ribo-class" as they were recognized as a monophyletic unit based *only* on the sequences of the small subunit rRNA gene (Stoeck et al. 2007 in Lynn 2008). Two groups are now placed in this class – the plagiopylids and odontostomatids, but they are morphologically extremely dissimilar (Fig. 19). Like armophoreans, plagiopyleans are typically inhabitants of anoxic freshwater and marine habitats and have hydrogenosomes, which can be associated with intracellular methanogenic bacteria or with extracellular ectosymbiotic methanogens that increase the ciliate's metabolic efficiency in these habitats. Thus, a unifying biochemical or physiological feature may eventually be discovered to explain this assemblage.

OLIGOHYMENOPHOREA de Puytorac et al. 1974. Oligohymenophoreans are a diverse group morphologically characterized as having a paroral on the right side of the cytostome and typically three oral polykinetids on the left, although their cellular form is quite varied (Figs. 20 and 21). Their somatic kinetids have a *radially* oriented transverse ribbon associated with the posterior kinetosome of a dikinetid or with the monokinetid kinetosome, but the peniculines, such as *Paramecium*, are an exception to this rule (Fig. 9). Postciliary microtubules are divergent and are directed posteriorly, whereas the kinetodesmal fibril associated with the posterior kinetosome of a dikinetid is anteriorly directed and strongly overlapping. The Peritrichia, such as *Vorticella* (Fig. 20), are adapted to sessility and lack somatic kinetids but are related to the other oligohymenophoreans by the pattern of division morphogenesis (i.e., the structure and formation of the oral region). Oligohymenophorean ciliates are common in all habitats described (see above "Habitats and Ecology, Habitats").

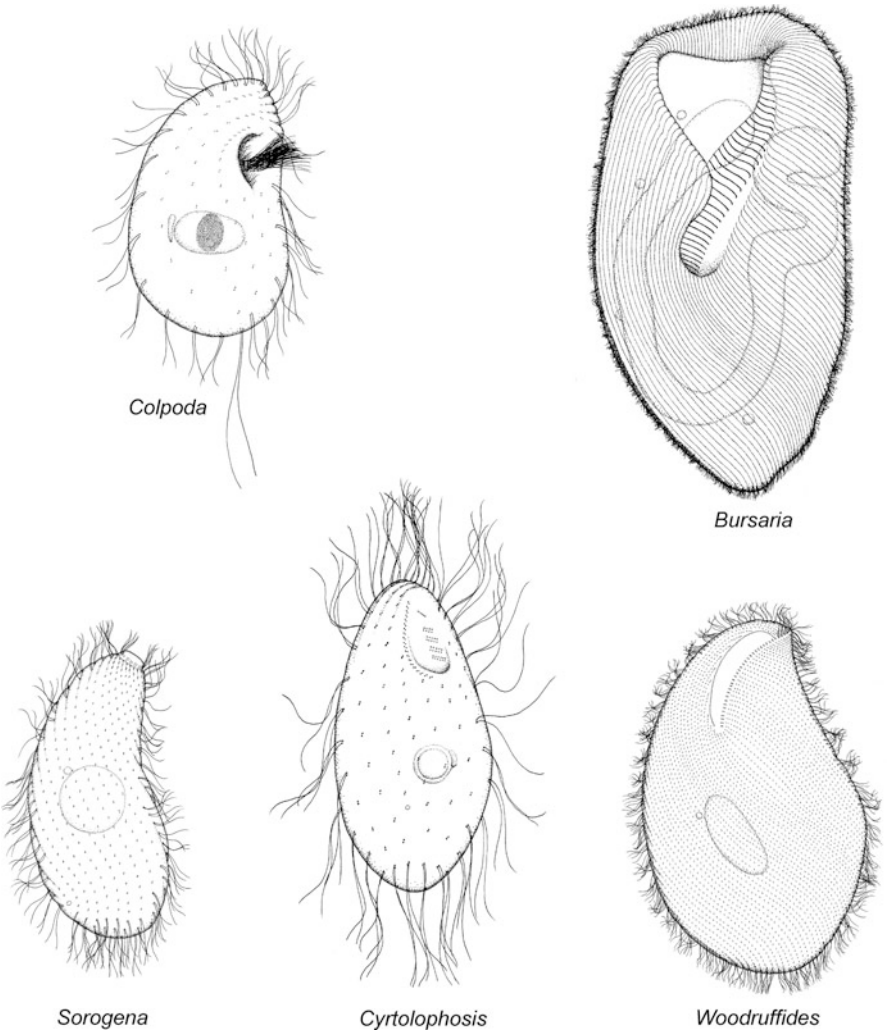

Fig. 17 Representative genera of the COLPODEA. Colpodida. *Colpoda*. Bursariomorphida. *Bursaria*. Sorogenida. *Sorogena*. Cyrtolophosidida. *Cyrtolophosis* and *Woodruffides*

Maintenance and Cultivation

Collection and Isolation from Nature

Collection. Procedures for collecting ciliates vary depending upon the habitat in which the organisms are found (see "Habitats and Ecology, Habitats"). Collection methods for benthic habitats and soils have been outlined by Uhlig (1972), Alabouvette et al. (1981), and Acosta-Mercado and Lynn (2003). Planktonic ciliates

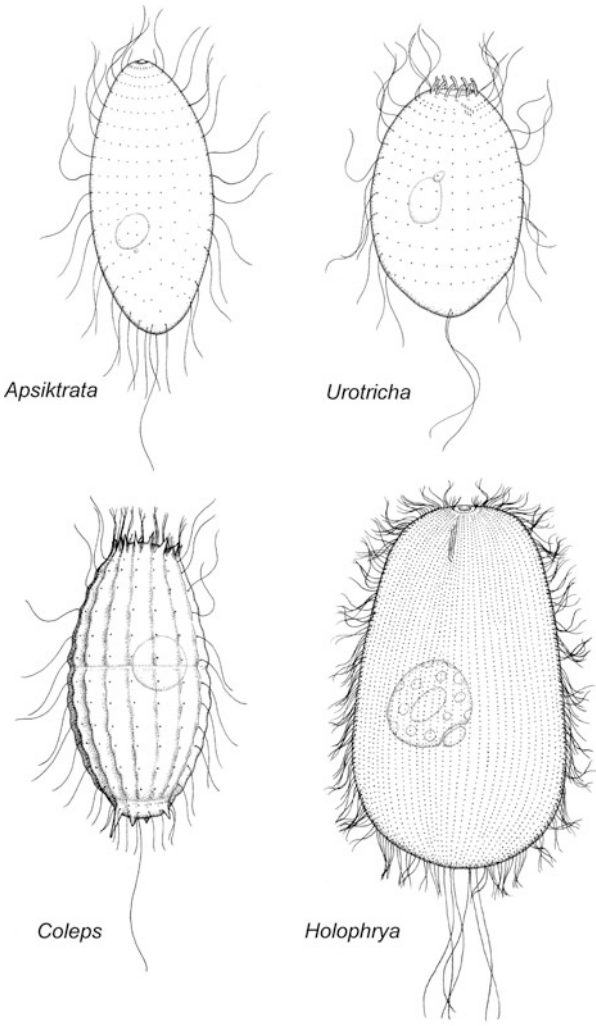

Fig. 18 Representative genera of the PROSTOMATEA. Prostomatida. *Apsiktrata*. Prorodontida. *Urotricha*, *Coleps*, and *Holophrya*

are best collected by using bottles or whole water sampling apparatuses (Montagnes and Lynn 1993).

Enumeration. For enumeration of ciliates, several methods have been published for benthic collections (Dye 1979; Finlay et al. 1979 in Finlay and Ochsenbein-Gattlen 1982; Wickham et al. 2000) and soils (Acosta-Mercado and Lynn 2003). For planktonic collections, counting chambers may be used: the ciliates can be counted alive (Dale and Burkhill 1982) or can be fixed using a concentrated Bouin's fixative and stained using the quantitative protargol stain (Montagnes and Lynn 1993).

Isolation. Field collections can be examined immediately and the species of interest isolated using flame-drawn Pasteur pipettes. Alternatively, the collection can be enriched by the addition of *small* quantities of boiled leaves, seeds, grains,

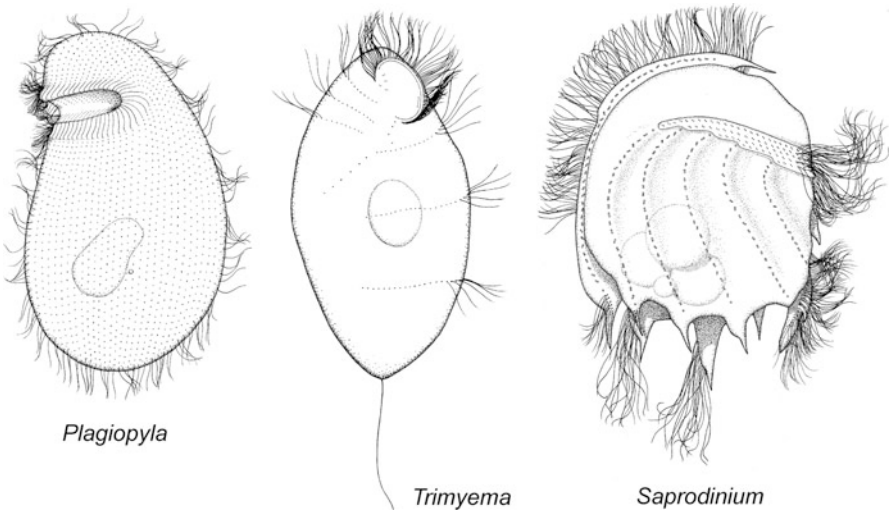

Fig. 19 Representative genera of the PLAGIOPYLEA. Plagiopylida. *Plagiopyla* and *Trimyema*. Odontostomatida. *Saprodinium*

animal tissues, or nutrient media, such as proteose peptone. After a few days, the cultures can be examined and species of interest isolated, usually by hand pipetting single ciliates under a low power microscope (Foissner 1991).

Nyberg (1981) isolated *Tetrahymena* species by enriching field collections with proteose peptone and adding antibiotics to inhibit bacterial growth. Kosinski (1979) described a method of producing axenic cultures by several passages of a bacterized batch culture through sterilized tubes, each transfer being made using sterile hypodermic needles.

Cultivation

Monoxenic Cultures. Many species of ciliates have been grown on a variety of food sources. Observations of what the ciliate of interest feeds upon in nature must be made prior to establishing the organism in culture. The nutritional value of a variety of bacterial species for selected ciliates has been discussed by Dive (1973 in Lynn 2008). There are many variables to consider in order to successfully establish a species in culture (see, for example, Hamilton and Preslan 1969 in Lynn 2008); these become especially complex when culturing planktonic species, such as tintinnids (Gold 1973) or "carnivorous" (ciliativorous) species, that is, ciliates that eat other ciliates.

Axenic Culture. Very few ciliates have been grown in axenic cultures. *Tetrahymena* species were the first, and almost all other axenically grown ciliate species are members of the Class OLIGOHYMENOPHOREA. *Tetrahymena* species are cultured on proteose peptone (Cassidy-Hanley in Collins 2012; Nyberg 1981; Keenan

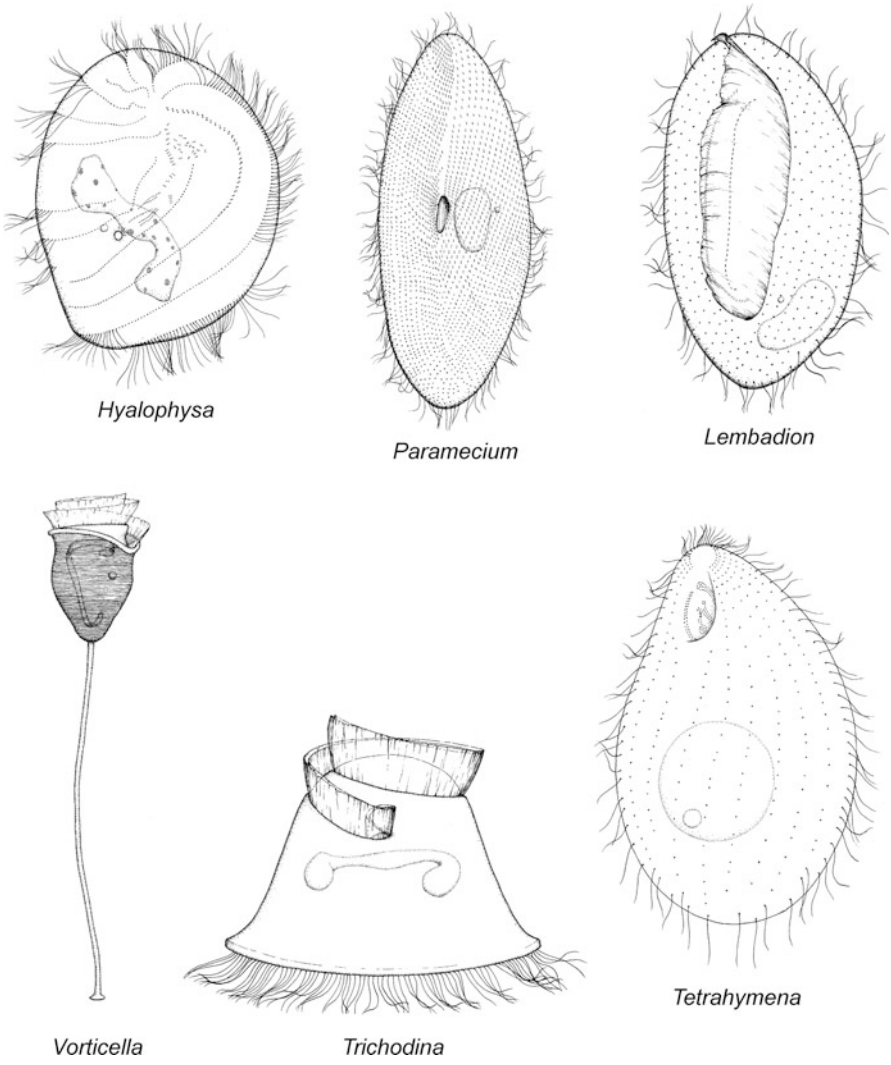

Fig. 20 Representative genera of the OLIGOHYMENOPHOREA. Apostomatia. *Hyalophysa*. Peniculia. *Paramecium* and *Lembadion*. Peritrichia. *Vorticella* and *Trichodina*. Hymenostomatia. *Tetrahymena*

et al. 1978). *Paramecium* species can also be grown on proteose peptone-trypticase media (Fok and Allen 1979; Soldo et al. 1966 in Fok and Allen 1979). *Uronema* and some related scuticociliates are marine forms that have been successfully cultivated axenically on proteose peptone-trypticase media (Iglesias et al. 2003 in Lynn 2008; Soldo and Merlin 1972 in Soldo et al. 1974).

Large-Scale Cultivation. Large-scale cultivation of *Tetrahymena* and *Paramecium* is accomplished by increasing the volume of axenic medium (Thiele et al.

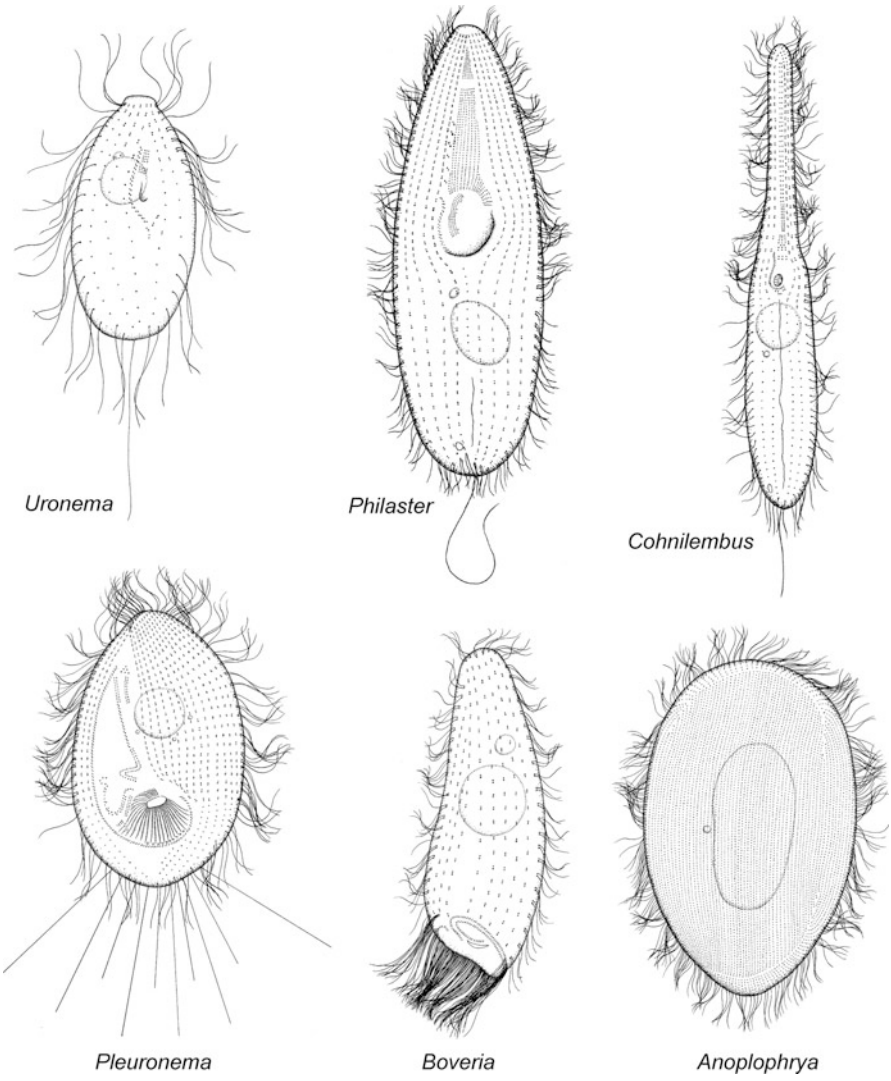

Fig. 21 Representative genera of the OLIGOHYMENOPHOREA cont'd. Scuticociliatia. *Uronema*, *Philaster*, *Cohnilembus*, *Pleuronema*, and *Boveria*. Astomatia. *Anoplophrya*

1980 in Schmidt 1982). Care must be taken that adequate oxygen is provided either by aeration or through having a high surface area to volume ratio in the culture flask.

Large-scale monoxenic cultures must be carefully regulated to ensure maximum growth of the predatory ciliates relative to the food. Several methods have been described for cultivating euplotian and hypotrich ciliates (Laughlin et al. 1983; Schmidt 1982).

Long Term Preservation. Little information exists on methods of long-term preservation of ciliates. A combination of low temperature and cryoprotectant seems to be most successful (Anderson et al. 2009; Cassidy-Hanley in Collins 2012; Krenek and Berendonk 2009). Encystment by some ciliates, such as colpodids, allows for simple, long-term dry storage on vegetation or filter paper.

Culture Media. Media have been devised for the cultivation of a number of species of ciliates. Recipes are listed for two very common media: a medium for bacterized cultures based on an infusion derived from dried cereal grass leaves and a proteose peptone-based medium for axenic culture of some tetrahymenid ciliates (Tables 3 and 4). Two good sources for recipes of a large number of media are an old publication prepared by the Committee on Cultures, Society of Protozoologists (1958), which gives the appropriate media for a variety of species, and the American Type Culture Collection Catalogue of Strains I (1982), in which the species are cross-referenced to the appropriate medium.

Cultivation of anaerobic ciliates began first with those resident in the rumen (Coleman 1969 in Michalowski et al. 1985). Free-living anaerobes, such as the armophorean *Metopus* (Narayan et al. 2007), the plagiopyleans *Trimyema* (Wagener and Pfennig 1987) and *Plagiopyla* (Fenchel and Finlay 1991b), and scuticociliates (Dyer 1989 in Lynn 2008), have now been isolated from various habitats and cultivated using similar techniques.

Table 3 Recipe for proteose peptone-yeast extract culture medium

5–10 g	Proteose peptone (Difco)
5–10 g	Yeast extract (water soluble portion of autolyzed yeast)
2 g	Glucose
1 l	Distilled water
Add the dry ingredients to the distilled water and heat until they are dissolved. Dispense into culture vessels and autoclave to sterilize	
Note: This medium is useful only for axenic cultivation of some tetrahymenid ciliates	

Table 4 Recipe for culture medium based on extract of cereal grass leaves

Preparation of stock infusion of cereal grass leaves	*Preparation of the culture medium*
50 g powdered, dehydrated cereal grass leaves (Sigma) Calcium carbonate (pinch) 1 l distilled water (or, for marine ciliates, ½ strength sea water) Add the cereal grass leaves to the distilled water and boil for 10 min taking care that the suspension does not boil over. Filter the suspension using a Buchner funnel and bring the filtrate back to the 1l volume. Dispense as 10-ml aliquots in screw cap tubes. Autoclave to sterilize and store in the refrigerator	Dilute the stock infusion into 250–1000 ml of distilled water, sterile pond water, or a dilute salt solution; the more dilute the medium, the less populous the bacteria will be. Ciliates may be transferred from nature to this medium. Alternatively, introduce prey bacteria by inoculating the medium with *Aerobacter aerogenes* or other suitable Gram-negative aerobic bacterial prey. Incubate at room temperature for 6–24 h, and then transfer the ciliates to this medium

Evolutionary History

Fossil Record

Ciliates, being soft-bodied organisms, are poorly represented in the fossil record. A number of species have "hard" parts that have the potential to be preserved: *Coleps* species of the PROSTOMATEA secrete internal calcium carbonate structures; *Loxodes* species of the KARYORELICTEA accumulate barium sulfate as conspicuous intracellular granules (Finlay et al. 1983); a variety of tintinnid species of the choreotrich SPIROTRICHEA secrete loricae to which are agglomerated or attached mineral particles from the water column; or the calcium carbonate tests of coccolithophorids (Tappan and Loeblich 1973). The mineral components of *Coleps* and *Loxodes* may be too small and inconspicuous to be recorded as body fossils. Remarkably, whole cells of *Coleps*, *Paramecium*, and *Colpoda* species have been discovered embedded in fossilized amber over 200 million years old (Martín-González et al. 2008; Schmidt et al. 2006).

The loricas of tintinnids, possibly by partial recrystallization of attached coccolith tests, deposited by the tintinnid when alive, and by secondary growth of calcite crystals, have provided a record of the history of this group of ciliates. The tintinnids apparently reached peak diversity during the Jurassic and Cretaceous (Fig. 22; Tappan and Loeblich 1973). Some have argued that fossil tintinnids might be of Proterozoic origins, but this is highly unlikely (Lipps, Stoeck, and Dunthorn in Dolan et al. 2013). Since fossil genera have been placed in present-day families of tintinnids, very little can be said about the rate and degree of divergence of taxa (Lipps et al. in Dolan et al. 2013; Lynn 2008). Lipps et al. (in Dolan et al. 2013) make a strong case that the calpionellids are likely not tintinnids.

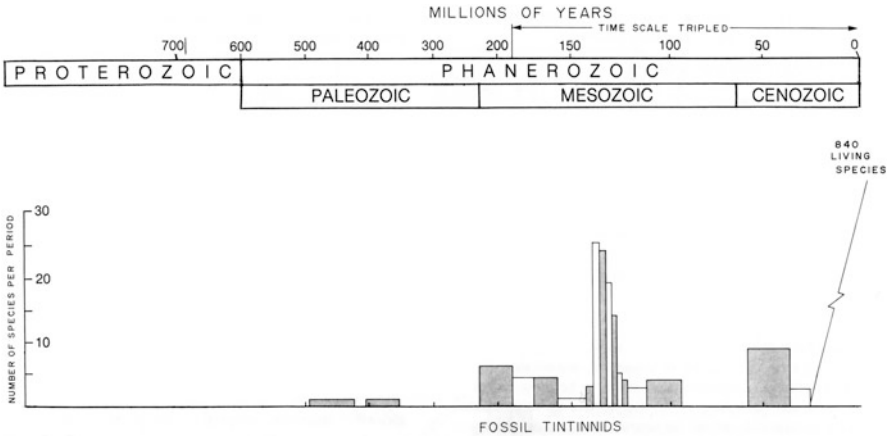

Fig. 22 Total number of species of tintinnids per geological period from their earliest appearance in the Ordovician up to the present. Taxa in the *shaded columns* are likely not tintinnids (After Tappan and Loeblich 1973; Redrawn by K. Wellencamp)

Phylogeny

Phylogeny Within the Phylum CILIOPHORA. The presumed phylogenetic relationships of the major clades of ciliates have been briefly mentioned above (Characterization and Recognition, *Classification*). The most informative data on the adaptive diversification of ciliates is provided by gene sequences, particularly that of the small subunit rRNA gene (Fig. 8a) and now phylogenomic analyses (Fig. 8b) (Gentekaki et al. 2014; Lynn 2008). This is because it is almost impossible to understand how one kind of cortical ultrastructure is related to or transformed into another kind of cortical ultrastructure.

From the molecular genetic perspective, the phylum is unambiguously divided into two major clades (Fig. 8, Table 2). Within the POSTCILIODESMATOPHORA, it has been argued that macronuclear division evolved from a division-less ancestral karyorelictean-like state by the use of *extramacronuclear* microtubules while macronuclear division evolved independently in the other clade – the INTRAMACRONUCLEATA – by use of *intramacronuclear* microtubules (Orias 1991a in Lynn 2008).

How the various groups of intramacronucleates diverged varies somewhat depending upon what gene one uses as the data source (Lynn 2008). The spirotrichs, armophoreans, and litostomes (SAL) appear to be related robustly in phylogenomic analyses, while the remaining six classes form a clade that is often well supported and has been labeled CONTHREEP, an acronym standing for the first letters of the included groups (Fig. 8).

Origin of the Phylum CILIOPHORA. The origin of the Phylum CILIOPHORA is as conjectural as the origin of the groups within it. The putative ancestor could have been a "corticoflagellate": a flagellate with a locomotory dikinetid, cytostome, and with a cortex and infraciliature similar to that of the ciliates (Lynn and Small 1981 in Lynn 2008). There are at least two contemporary taxa that exhibit these features: the dinoflagellates and *Colponema* spp. have dikinetids with ribbons of microtubules associated with each kinetosome, a striated rootlet fibril associated with at least one kinetosome and extending toward the cell surface, and cortical alveoli similar to those of ciliates. Gene sequences confirm the associations of these three contemporary taxa with other alveolates (see ► Dinoflagellata, and ► Apicomplexa).

From this flagellate ancestor, polymerization or an increase in numbers of kinetid units must have occurred to form the ciliary files or kineties. The most plausible model to date has been presented by Eisler (1992). It is imagined that this ancestor had a file of dikinetids, the homologue to the paroral, associated with its cytostome. These paroral dikinetids replicated laterally to produce a file or row of dikinetids to their right, and this would produce the first somatic kinety. Repeated replication of this process would generate multiple kineties that would eventually "cover" the cortical surface (Eisler 1992). It is further proposed that the adoral ciliary structures (i.e., those on the left side of the cytostome) differentiated from somatic kineties adjacent to the left side of the cytostome, a process that often happens in contemporary ciliates but in very diverse ways.

Acknowledgments

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Radiolaria and Phaeodaria

19

Demetrio Boltovskoy, O. Roger Anderson, and Nancy M. Correa

Abstract

Polycystina (~400–800 living species and several thousand extinct forms) and Phaeodaria (~400–500 living species) are exclusively marine, open-ocean planktonic protists, most of which possess elaborate siliceous skeletons. The cytoplasm is divided into an internal part (endoplasm) separated from the external, more vacuolated one (ectoplasm) by a perforated membrane – the central capsule. The Polycystina protrude long and slender cytoplasmic projections (axopodia) supported internally by a rigid central rod (axoneme); while the Phaeodria have a network of peripheral finely interconnected pseudopodia. A few Polycystina are colonial, but most, as well as all Phaeodaria, are solitary, around 40 μm to almost 2 mm in size. Most polycystine species peak in abundance between 0 and 100 m, whereas phaeodarians tend to live deeper, often below 300 m. Polycystines have a rich fossil record dating from the Cambrian and are important for stratigraphic, paleoecologic, and evolutionary studies. The world-wide biogeography and diversity of radiolarians is chiefly governed by water temperature. Radiolarian prey includes bacteria, algae, protozoa, and microinvertebrates. Many surface-dwelling species of Polycystina possess symbiotic algae and photosynthetic

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cyanobacteria that provide nourishment to the host. Some colonial radiolaria reproduce by binary fission of the central capsules. Sexual reproduction of polycystines or Phaeodaria has not been confirmed, but the release of motile swarmers, likely gametes, has been widely documented. In species with a radial symmetry (Spumellaria) shell-growth is centrifugal, whereas in the Nassellaria the internal cephalic elements and the cephalis appear first. Individual longevity is estimated to range between 2 and 3 weeks and 1–2 months.

Keywords
Radiolaria • Polycystina, Phaeodaria, radiolarians

Contents

Summary Classification

●**Rhizaria**
●●**Cercozoa**
●●●**Thecofilosea**
●●●●**Phaeodaria** (=Tripylea)
●●●●●**Phaeoconchia**
●●●●●**Phaeocystina**
●●●●●**Phaeogromia**
●●●●●**Phaeosphaeria**
●●**Retaria**
●●●**Polycystinea**
●●●●**Collodaria** (skeletonless, or with isolated spicules)

●●●●**Spumellaria**
●●●●**Nassellaria**
●●●●**Archaeospicularia** (extinct)
●●●●**Albaillellaria** (extinct)
●●●●**Latenfistularia** (extinct)
●●●●**Entactiniaria** (extinct)

Introduction

General Characteristics

Polycystines and phaeodarians (Fig. 1) are marine protists, previously assigned to the phylum Actinopoda because they both possess elaborate siliceous skeletons surrounding an organic central capsule with pores, from which axopodia emerge in most members of the phylum. However, the phylum Actinopoda is no longer accepted as a valid taxonomic category, and Polycystinea are assigned to the higher-level group Retaria, and Phaeodaria are now assigned to the higher-level group Thecofilosea (Adl et al. 2012). Axopodia are long and slender cytoplasmic projections that protrude radially from the cell and are supported internally by a rigid central rod composed of a shaft of microtubules. Axopodia support a web-like network of sticky cytoplasmic strands of pseudopodia that aid in the capture of prey. Presently, we know that only polycystines possess typical axopodia, whereas Phaeodaria characteristically produce a network of peripheral finely interconnected pseudopodia that arise from two, specialized protoplasmic strands (parapylae) emerging from two pores in the central capsule. In addition there is a more massive cytoplasmic mass that emanates from an aperature (astropyle) resembling the oral aperture of some testate amoebae. Neither the astropyle nor the accessory openings *parapylae* exhibit structures resembling axopodia or fusules (Anderson 1983). All Phaeodaria are solitary, but Polycystinea include some colonial forms. Single cells vary in size from below 40 μm to almost 2 mm (Phaeodaria are usually larger than Polycystinea), but colonies may exceptionally be as long as 3 m (Swanberg 1979).

Occurrence

Radiolarians are present in all major oceans but absent from some marginal seas, such as the White Sea (Bjørklund and Kruglikova 2003). Different species have different depth preferences; polycystines usually peak in abundance between 0 and 100 m and have secondary peaks at various other depths, whereas phaeodarians tend to live deeper, often below 300 m. Both groups are almost entirely restricted to waters with normal open-ocean salinity levels.

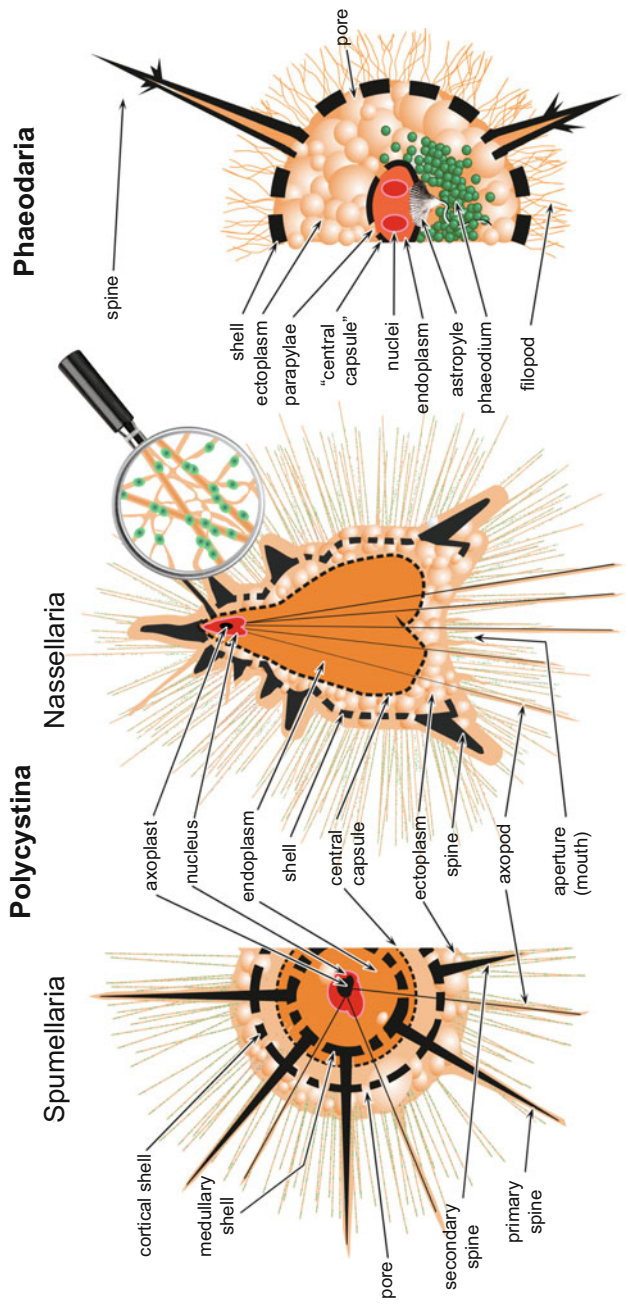

Fig. 1 Schematic diagram of the cellular organization in polycystine radiolarians (Spumellaria and Nassellaria) and phaeodarians. Detail (magnifying glass) shows web-like network of cytoplasmic strands supported by the axopodia and associated symbionts

Literature

The cornerstone of radiolarian studies, including Phaeodaria, is Haeckel's 1887 monograph based on planktonic and sedimentary materials collected by the Challenger Expedition (Haeckel 1887). Kling (1978), Anderson (1983), Petrushevskaya (1986), Anderson et al. (2000), Takahashi and Anderson (2000), De Wever et al. (2001), Afanasieva et al. (2005), Boltovskoy and Pujana (2008), and Nakamura and Suzuki (2015) produced general accounts on radiolarian knowledge. The catalogue assembled by Nigrini and Moore (1979) is still one of the most widely used references for the identification and distribution of the ca. 100 most common recent polycystine species. Other salient references are the works of Petrushevskaya (1967, 1971b) (profusely illustrated descriptions of practically all extant nassellarians and all Antarctic spumellarians); Sanfilippo et al. (1985) (a detailed guide for Cenozoic stratigraphy based on polycystines); Riedel and Foreman (1995) (a catalogue of all the polycystine species described up to 1930); and Boltovskoy et al. (2010) (a compilation of all the distributional data available on Recent polycystines up to 2008).

History of Knowledge

The first description of a living radiolarian is ascribed to Meyen (1834), whereas the first fossil one was recorded by Ehrenberg (1838), who also coined the term "Polycystina". The name "Radiolaria" was first proposed by Müller (1858) to designate planktonic protists with radiating skeletal elements and subsequently used by Haeckel as an informal term encompassing acantharians, polycystines, and phaeodarians (De Wever et al. 2001). The foundation of modern radiolarian studies is Haeckel's (1887) exquisitely illustrated monograph; around the same years several important publications were produced, but interest in the group was limited until the 1950s, when William Riedel and coworkers showed that polycystines could be used for stratigraphic purposes. First stratigraphy, and later paleoecology, fostered radiolarian research, which was particularly active in the 1970s and 1980s (Lazarus 2005; Suzuki and Aita 2011). At present there are about 150–200 specialists that are partially or entirely dedicated to radiolarian studies, over 90% of them with geological-paleontological backgrounds and centered on fossil materials. However, with the advent of modern biological techniques such as electron microscopy and molecular phylogenetic analyses, an increasing number of biologists have begun to elucidate the natural affinities among major groups of radiolaria toward clarifying their taxonomic relations (e.g., Amaral Zettler et al. 1998, 1999; Anderson et al. 1999; Biard et al. 2015; López-García et al. 2002; Polet et al. 2004; Suzuki and Aita 2011).

Practical Importance

During the second half of the twentieth century, studies of polycystines from continuous, well-preserved, mostly Cenozoic sections retrieved by the Deep Sea Drilling

Project (later Ocean Drilling Program, now the Intergrated Ocean Drilling Program) proved the usefulness of these organisms for stratigraphic purposes. By the mid-1970s, a relatively stable tropical zonation had been developed for the Cenozoic (Sanfilippo et al. 1985), and somewhat later several schemes for the polar oceans were proposed (Lazarus 2005). Polycystines are particularly important in Neogene Polar sediments and in red clay bottoms, where carbonate microfossils are largely absent. Polycystine faunas have also been instrumental to the development of paleoenvironmental studies (paleotemperature, paleoceanography, and paleoproductivity), chiefly of open-ocean areas (CLIMAP 1976). Polycystines offer major advantages as material for evolution research: the preservation of almost all species in fossil form, high-resolution chronology, the possibility to sample the entire geographic and chronologic span of the populations, etc. Evolutionary studies of these organisms, in particular speciation and phyletic evolution, have made significant contributions to understanding evolutionary processes in pelagic animals in general. A major limitation in the use of polycystines in all these fields is the problematic species-level taxonomy of the group (Lazarus et al. 2015).

Habitats and Ecology

Geographic Distribution and Biogeography

Horizontal distribution and biogeography. Radiolarians are present in all oceans from the surface to bathypelagic depths, but, with the only known exception of *Lophophaena rioplatensis*, which thrives in the brackish waters of the Río de la Plata estuary at salinities as low as 15.4 PSU (Boltovskoy et al. 2003), they do not tolerate salinities below ca. 30 PSU and are therefore absent from most shelf areas and many inner and marginal seas (e.g., Black Sea, Azov Sea, Caspian Sea, White Sea, Baltic Sea). However, in areas with a narrow shelf where oceanic waters impinge on the shore (e.g. off California, in Norwegian fjords), radiolarians can be collected from the coast. Polycystine densities are usually around 1 cell per liter of water, whereas phaeodarians are normally 15–100 times less abundant (Boltovskoy et al. 1993). Productive, upwelling waters can host 5–10 and up to 70–80 cells/L (Caron and Swanberg 1990). Interestingly, the highest concentrations so far reported (394 cells/L) are those of the monospecific, brackish population of *Lophophaena rioplatensis* in the South American Río de la Plata estuary (Boltovskoy et al. 2003). Polycystine endemicity is generally low, as specific composition changes little with oceanic basin. Even the Arctic and the Antarctic share most of their species (Stepanjants et al. 2004). According to data from 4774 plankton, sediment trap and surface sediment samples compiled by (Boltovskoy et al. 2010), only *Artobotrys borealis* consistently occurs in Arctic and Subarctic waters and has not been recorded in the Antarctic or Subantarctic, but several species are here much more common and abundant than in cold waters of the Southern Hemisphere (*Amphimelissa setosa*, *Artostrobus annulatus*, *Artostrobus jorgenseni*, *Lithomelissa setosa*, *Phormacantha hystrix*, *Plectacantha oikiskos*, *Pseudocubus obeliscus*, *Rhizoplegma boreale*, *Saccospyris conithorax*, and

Siphocampe lineata) (Boltovskoy and Correa 2016a). Species probably restricted to – or at least much more abundant in – the Southern Ocean, include *Actinomma antarcticum*, *Antarctissa denticulata-strelkovi*, *Saccospyris antarctica*, and *Ceraspyris antarctica* (Boltovskoy and Correa 2016a). The other major oceanic climatic belts, defined chiefly by their different water temperature regimes, host dissimilar radiolarian assemblages (Fig. 2), but most of the species occur, albeit sparsely, in more than one area. Within the ranges of normal oceanic conditions (basically salinity), temperature is by far the most important factor in defining polycystine distribution patterns (Boltovskoy and Correa 2016a), followed by nutrients and primary productivity. At ocean basin scales, temperature is also most probably responsible for the fact that polycystine assemblages off the Pacific coasts of Central America differ from the tropical-subtropical ones elsewhere (Fig. 2). In contrast to many open-ocean organisms, whose diversity has been reported to peak at intermediate latitudes (~15–30°N) and drop at the equator (e.g., Foraminifera, Tintinnina, Euphausiacea, and Copepoda), polycystine species numbers are tightly coupled with temperature throughout the entire thermal range of marine waters peaking at the equator (Fig. 3; Boltovskoy and Correa 2016b). In high-latitude assemblages, numerical dominance of a few species is very high, with 1–2 radiolarians often accounting for up to 90% of all the individuals (e.g., *Amphimelissa setosa* in the Atlantic sector of the Arctic and *Antarctissa denticulata-strelkovi* in the Southern Ocean). In warm waters, dominance is much less marked, the most abundant species normally accounting for <5% of the overall inventories in each sample. Throughout the World Ocean, occurrence and abundance of the species are highly correlated: radiolarians recorded in more samples also account for larger proportions of the taxocoenoses analyzed.

Phaeodarian biogeography is still very insufficiently known, but the scarce available evidences suggest that their world-wide patterns are less clearly associated with latitudinal climatic belts. This may be due to the fact that many phaeodarian species are deep-living forms, especially in warm water areas (Nakamura and Suzuki 2015; see below) that inhabit large areas where water temperature is more uniform (Fig. 4).

Vertical distribution. In tropical and subtropical waters polycystines are usually concentrated in the upper 50–100 m (Boltovskoy et al. 2010). Sometimes several discrete maxima are recorded, one at or near the surface and a second one between 50 and 100 m (Kling and Boltovskoy 1995) (Fig. 5). In polar waters, however, peak abundances seem to be associated with deeper and warmer layers, at around 200–400 m and overall polycystine abundances are much lower than in the tropics (Boltovskoy and Alder 1992; Nimmergut and Abelmann 2002; Petrushevskaya 1967) (Fig. 5).

The vertical ranges of most polycystines can be described by the following four patterns: (1) surface (with at least one peak above 100 m), (2) subsurface (around 100 m), (3) intermediate (between 100 and 300 m), and (4) deep (below 300 m) (Fig. 6) (Boltovskoy et al. 2010; Kling 1979; Kling and Boltovskoy 1995). However, worldwide depth zonations cannot be defined in terms of fixed depths because the distribution of radiolarian species is related to water masses which move vertically as well as horizontally. As a result, the same radiolarian species can occupy quite different depth intervals at different locations (Kling 1976). Many cold water radiolarians that

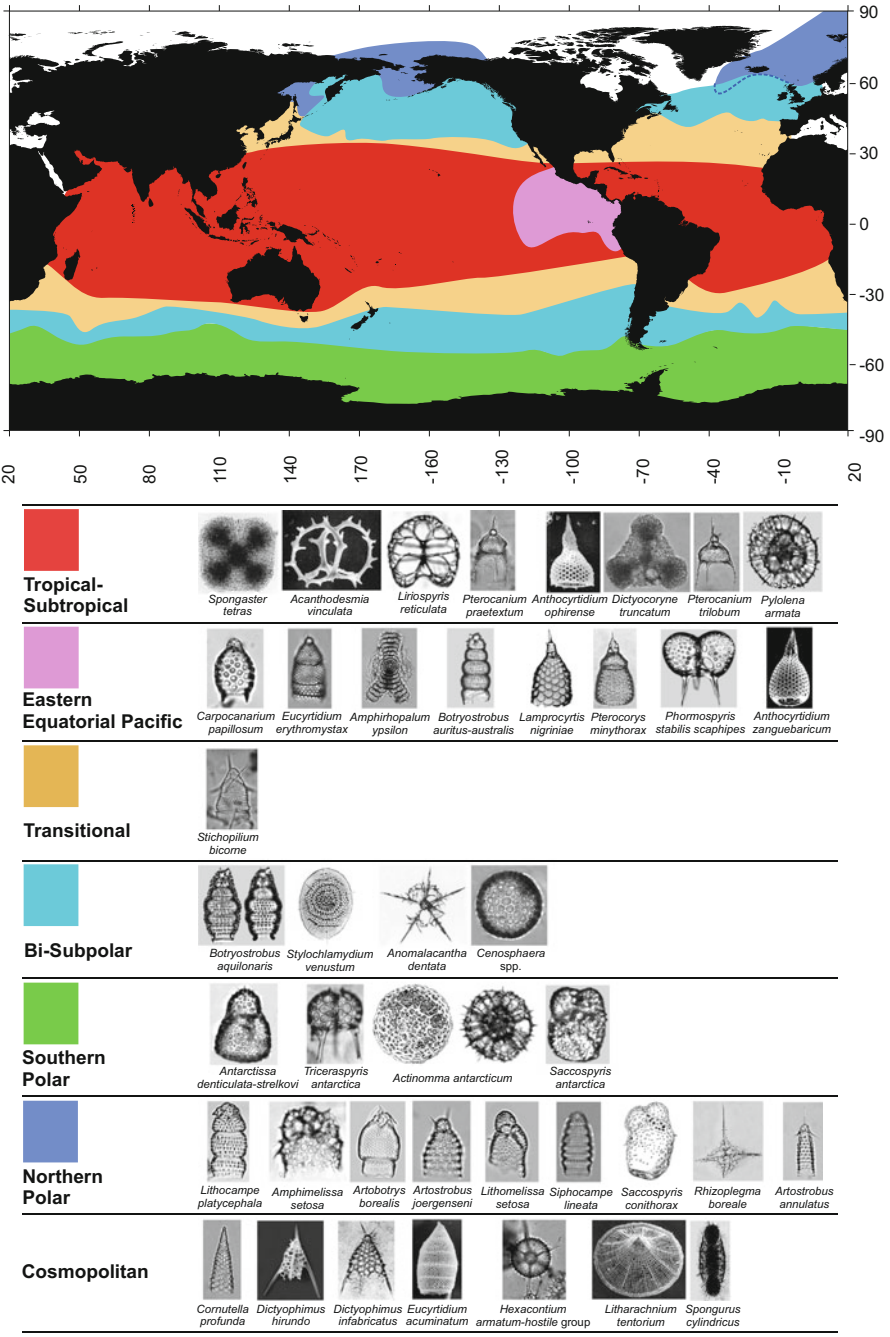

Fig. 2 Biogeographic regions of the World Ocean based on the distribution of polycystines in surface sediments and their most representative species (very few of these are restricted to the provinces indicated) (Simplified from Boltovskoy and Correa (2016a))

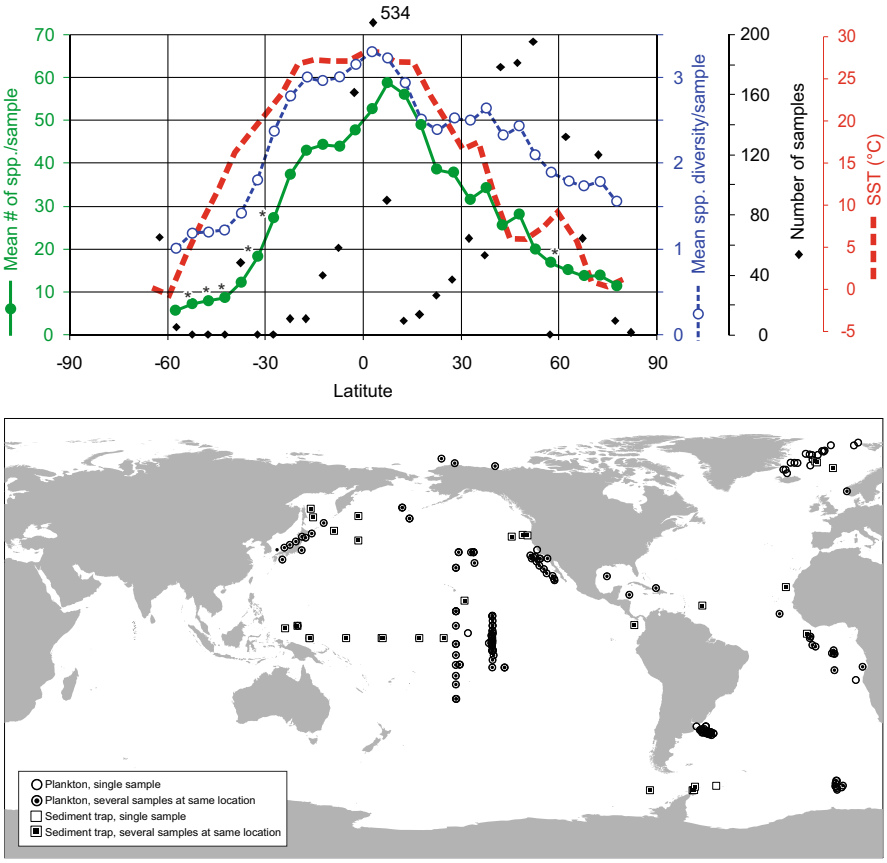

Fig. 3 Numbers of polycystine species, specific diversity (Shannon-Wiener log base 2), and sea surface temperature (SST) in the World Ocean as a function of latitude (pooled data for 5° intervals, 3-point running means, interpolated values are asterisked). Lower panel shows positions of the 2081 water column (plankton and sediment trap) samples used (*diamond symbols* in upper panel). Radiolarian data are based on compilation by Boltovskoy et al. (2010). Temperature curve is representative of mean SST values at the sampling sites involved only (rather than world-wide) (SST data are from Boyer et al. (2013))

inhabit the upper layers at high latitudes submerge with their corresponding water masses and can be found at depth in mid- and low-latitude areas (Boltovskoy 1988; Boltovskoy and Correa 2016b; Casey et al. 1982; Kling 1976).

Vertical changes in radiolarian diversity are more difficult to assess because the living (in situ populations) and dead individuals (i.e., settling shells exported from the upper strata) are seldom adequately differentiated in plankton collections. It is highly probable that protoplasm staining techniques, which are usually applied for these estimates, strongly overestimate the living depth ranges of the species because of the time it takes for the protists' protoplasm to decompose and disappear

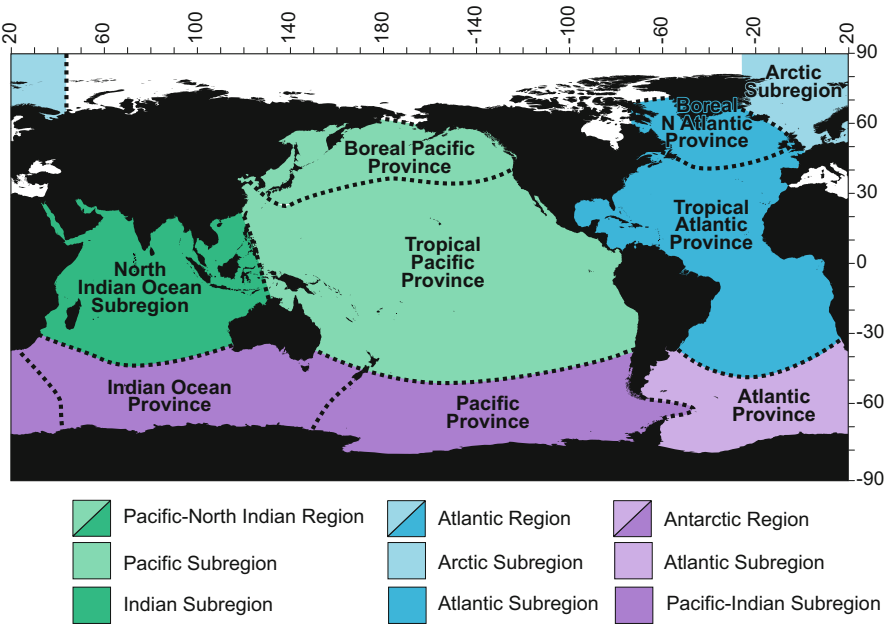

Fig. 4 Biogeographic zonation of the World Ocean based on deep-water Phaeodaria (Redrawn from Reshetnjak (1966))

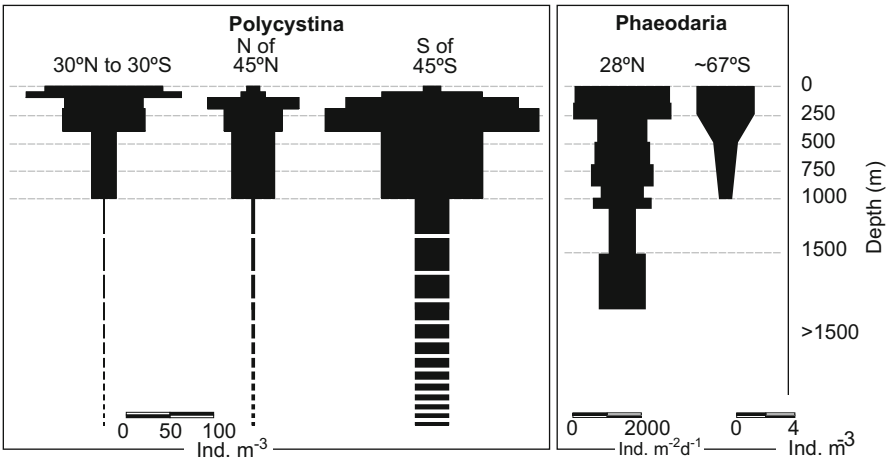

Fig. 5 Vertical profiles of radiolarian abundance in warm water and in cold water areas. Values for Polycystina are based on pooled data from 20 published surveys and our unpublished information from 1145 plankton samples. Values for Phaeodaria are from Gowing (1986) (28°N, North Pacific Central Gyre, sediment trap samples, living specimens only), and from Nothig and Gowing (1991) (Weddell Sea, plankton samples, phaeodarians >0.4 mm only). Notice differences in scales and units

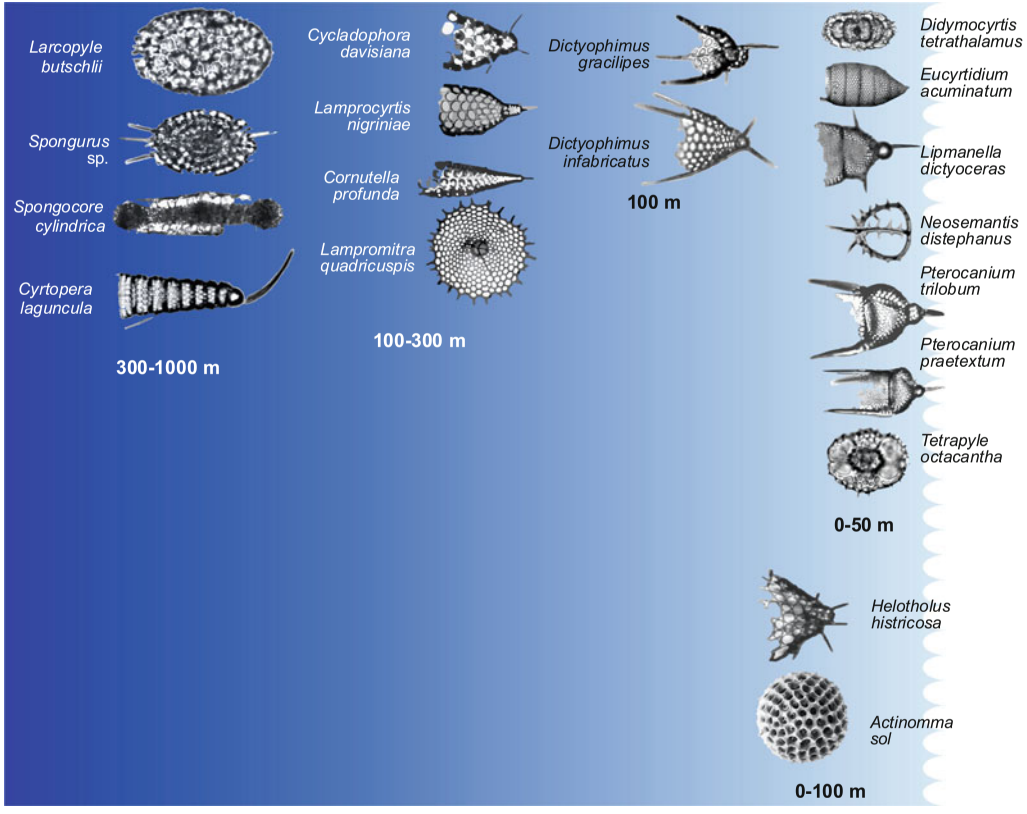

Fig. 6 Vertical distribution of polycystine species, as exemplified by some forms abundant in the northern subtropical Pacific (Based on data from Kling and Boltovskoy (1995))

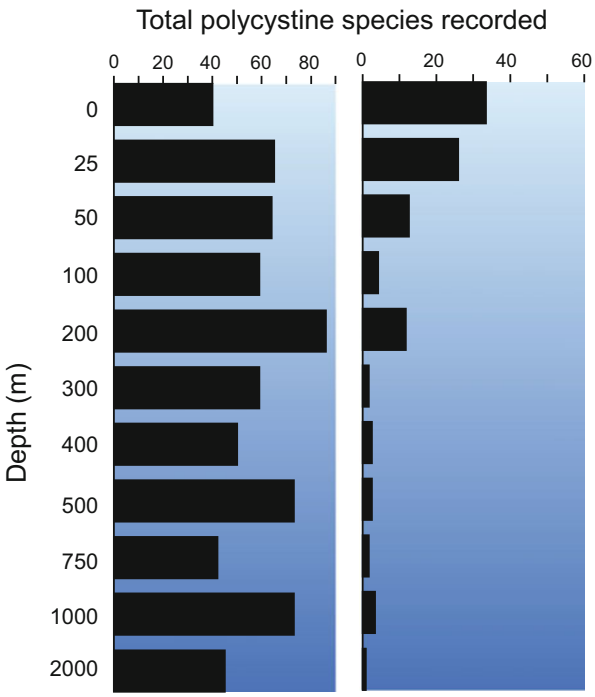

Fig. 7 Total number of polycystine species recorded per sample (*left*) and number of new species added to the inventory of the overlying waters (*right*) in a set of vertical plankton tows performed in the eastern subtropical Pacific. The absence of significant vertical diversity changes (*left panel*) is attributed to the presence at depth of dead, sedimenting skeletons of species whose living ranges are restricted to the upper layers (Adapted from Kling and Boltovskoy (1995))

(Bernhard 1988). The widespread occurrence of large numbers of healthy diatoms, dinoflagellates, and Cyanobacteria at depth >4000 m (Agusti et al. 2015) confirms the assumption that radiolarian sedimentation velocities are fast enough to yield large proportions of stained individuals well below their living depth range. Thus, raw data often show little species richness variation with depth (Fig. 7, left panel). On the other hand, when raw numbers are reinterpreted taking this artifact into account, highest diversities are clearly associated with the uppermost levels (Fig. 7, right panel).

Unlike most other sarcodines, phaeodarians are typically deep-water organisms usually peaking in both abundance and diversity below 200 m (Nakamura and Suzuki 2015), although high concentrations near the surface are not uncommon (Fig. 5). A detailed depth zonation for the area of the Kurile-Kamchatka trench was produced by Reshetnjak (1955, 1966). She concluded that only two (of the 103 species recorded) inhabit the upper 50 m; approximately 30 more have restricted vertical ranges at various depths, while over 50% of the taxa were retrieved from the broad depth interval of 50 to 2000–8000 m. These vertical patterns at a given locale, however, may change significantly because of the dynamics of deep ocean circulation, with species exhibiting quite variable depth ranges over oceanic distances. Vertical profiles in the North Pacific (Kling 1966, 1976) illustrate that species dwelling in near surface water (25 m depth) at high latitudes, are distributed gradually toward lower depths in decreasing latitudes, and dwell at depths

>300 m closer to the equator. Thus, as with the polycystines, depth distributions of phaeodarians on regional scales are not describable in terms of fixed ranges.

Equatorward submergence may account for so-called bipolar distributional patterns characteristic of many radiolarians. High-latitude species could pass under equatorial waters via the Intermediate Water or the Deep Water, to reappear near the surface in the opposite polar or subpolar seas where the adequately colder water temperatures support their growth (Aita et al. 2009; Stepanjants et al. 2006). In the Pacific Ocean, the Intermediate Water circulates in anti-cyclonic gyres that mimic the surface circulation (Reid 1965), thus providing continuity for the water masses and their biological contents. Such bipolar patterns have been described for a number of zooplanktonic species in both the Atlantic (Darling et al. 2000; Pierrot-Bults 1974) and the Pacific Ocean (Alvariño 1965), whereby the north and south polar or subpolar near-surface populations are joined at depths of 800–1000 m across the equator. The fact that these apparently disjoint populations interbreed through their deep-water, tropical and subtropical representatives has been suggested for some protists (Darling et al. 2000).

Radiolarian studies based on sedimentary materials. Because their skeletons preserve in the geological record, studies of extant polycystines have been chiefly based on sedimentary – rather than on planktonic – samples (phaeodarian skeletons very seldom preserve in the sediments). Sediment samples present some advantages but also several important shortcomings (Fig. 8) (Boltovskoy 1994). Whereas polycystine abundances seldom exceed 5 cells per liter in the plankton (Caron and Swanberg 1990), one gram of surface sediments can contain thousands to hundreds of thousands of radiolarian skeletons. Plankton samples yield a snapshot-type image of the composition of the assemblages, which does not necessarily adequately reflect long-term trends. The daily, seasonal, and interannual variability involved is

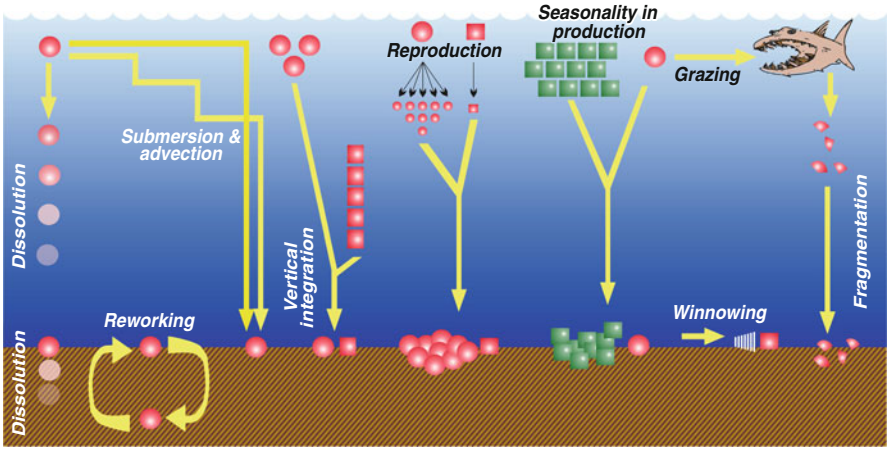

Fig. 8 Schematic diagram of the mechanisms that can distort the sedimentary imprint of the planktonic pattern of fossilizable microplankton in general, and of polycystine radiolarians in particular

smoothed out in the sedimentary record, which may be a welcome trait when general patterns are sought. Further, sedimentary materials are more readily available from the various repositories around the globe than plankton samples. On the other hand, interpretation of the geographic distribution of extant radiolarians on the basis of sediment samples presents several important drawbacks. On their way to the sea-floor and after settling, radiolarian remains dissolve and are grazed upon by various consumers thus breaking their skeletons into unidentifiable fragments. Because more delicate shells are destroyed more readily than the more robust ones, specific skeletal compositions on the bottom and at mid-depths can differ significantly from the living assemblage in the upper water-column. Bottom materials can be reworked after deposition (as a result of which non-Recent deposits, sometimes characteristic of quite dissimilar oceanographic settings, are brought up to the surface layer, or winnowed by bottom currents dislodging settled skeletons and carrying them thousands of kilometers away). Sediments integrate the imprint of near-surface faunas (which are generally associated with surficial environmental traits, as well as with currents and water masses), with the meso- and bathypelagic species whose geographic distribution is uncoupled with upper-water oceanography. The sedimentary distributions of cold-water species tend to show conspicuous equatorward extensions as compared with their planktonic patterns. This distortion is most probably due to the fact that extended survival of the cold water taxa that are expatriated towards lower latitudes is facilitated by submersion (Boltovskoy 1988, 1994; Boltovskoy and Correa 2016a); as a consequence, sediment-derived species-specific ranges may wrongly suggest an enhanced tolerance to gradients in the ecological factors.

Characterization and Recognition

Cell Ultrastructure

Cellular Organization. In broad view, three categories of pseudopod-producing protoctista (including amoebae, Foraminifera and Radiolaria) have been described based on cellular ultrastructure (Anderson 1983): (1) Diffuse, e.g., the naked amoebae without enclosing shells or thecae and a flowing, changeable cell shape, (2) Transitional, including the testate amoebae and foraminifera with a surrounding theca or shell that demarcates a more condensed cytoplasm internally from the web-like, pseudopodial array externally, and (3) Zonal, exemplified by the polycystine Radiolaria with a distinctive porous capsule wall that separates the central, sometimes lobate, intracapsular cytoplasm from the outer, extracapsular, layer of cytoplasm where prey is captured and digested. Interestingly, the Phaeodaria are categorized as transitional since they have a "capsule" with at least one large opening through which the endoplasm protrudes into the ectoplasm, similar to that of testate amoebae. Molecular genetic evidence indicates that Phaeodaria are closely related to testate amoebae within the group Cercozoa (Yuasa et al. 2006) (see below). The

chemical composition of the siliceous skeleton and the test-like capsular wall of Phaeodaria also are similar to that of testate amoebae.

Polycystine Radiolaria. The polycystine Radiolaria include the Spumellaria and Nassellaria (See Systematics). The Spumellaria have a spherical body plan with a centrally located nucleus surrounded by radially arranged lobes of cytoplasm, enclosed by a porous capsular wall (Figs. 1 and 9a, b), (Anderson 1980, 1983; Hollande et al. 1971). Axopodia emanate through pores (fusules) in the capsular wall and protrude radially (Cachon and Cachon 1976a, b). The axopodia support a web-like network of cytoplasmic strands that are sticky and aid in the capture of prey. The external cytoplasm encloses or coats the siliceous skeleton, when present. The Nassellaria have a monoaxial body plan (Anderson 1977), typically an elongated, ovoid, central capsule with a porous plate at the base where the axopodia emerge through closely spaced fusules (Fig. 9c, d). Shafts of microtubules in the axopodia emerge from a conical array of microtubules (podoconus) within the intracapsular cytoplasm (arrow, Fig. 10). Skeletons vary from simple tripods to elaborate, helmet-shaped structures, often with spines or other ornamentation (e.g., Figs. 9c, d and 11). The extracapsular cytoplasm coats the siliceous skeleton, when present, and extends outward as a halo of axopodia and their associated network of rhizopodia, including fine, tapered extensions known as filopodia that are present in Nassellaria and Spumellaria (Fig. 1). In polycystines, digested prey products are transported in small vesicles through the fusules into the intracapsular cytoplasm (Anderson 1977).

Phaeodaria. The ultrastructure of Phaeodaria is distinctly different from the polycystines. The "capsular wall" surrounding the denser endoplasm lacks fusules. There is one large opening (astropyle) containing an emergent massive strand of cytoplasm and two smaller openings (parapylae) with finer strands of cytoplasm (Fig. 9e, f). A large, often darkly colored, mass of partially digested food (phaeodium) is typically located external to the capsule near the astropyle (Figs. 1 and 9e). The continuous, massive strand of cytoplasm in the astropyle provides a pathway for digested prey matter to be carried into the endoplasm as occurs with some testate amoebae and foraminifera (Anderson 1983; Swanberg et al. 1986).

The Skeleton

The skeleton of polycystine Radiolaria, when present, is composed of amorphous silica and is deposited outside of the cytoplasm, but within an enclosing cytoplasmic sheath called the cytokalymma (Anderson 1983). The cytokalymma is a dynamic, living sheath that molds the shape of the silica deposited within it as silicification takes place during skeletal growth. Thus, the species-specific shape of the skeleton is determined by cellular dynamics and undoubtedly is under genetic control. Skeletal morphology is remarkably diverse (Anderson et al. 2000), but species specific.

The two major divisions of the Polycystinea, Spumellaria and Nassellaria, differ in the symmetry of their skeletons. Most spumellarians have a radial or spherical

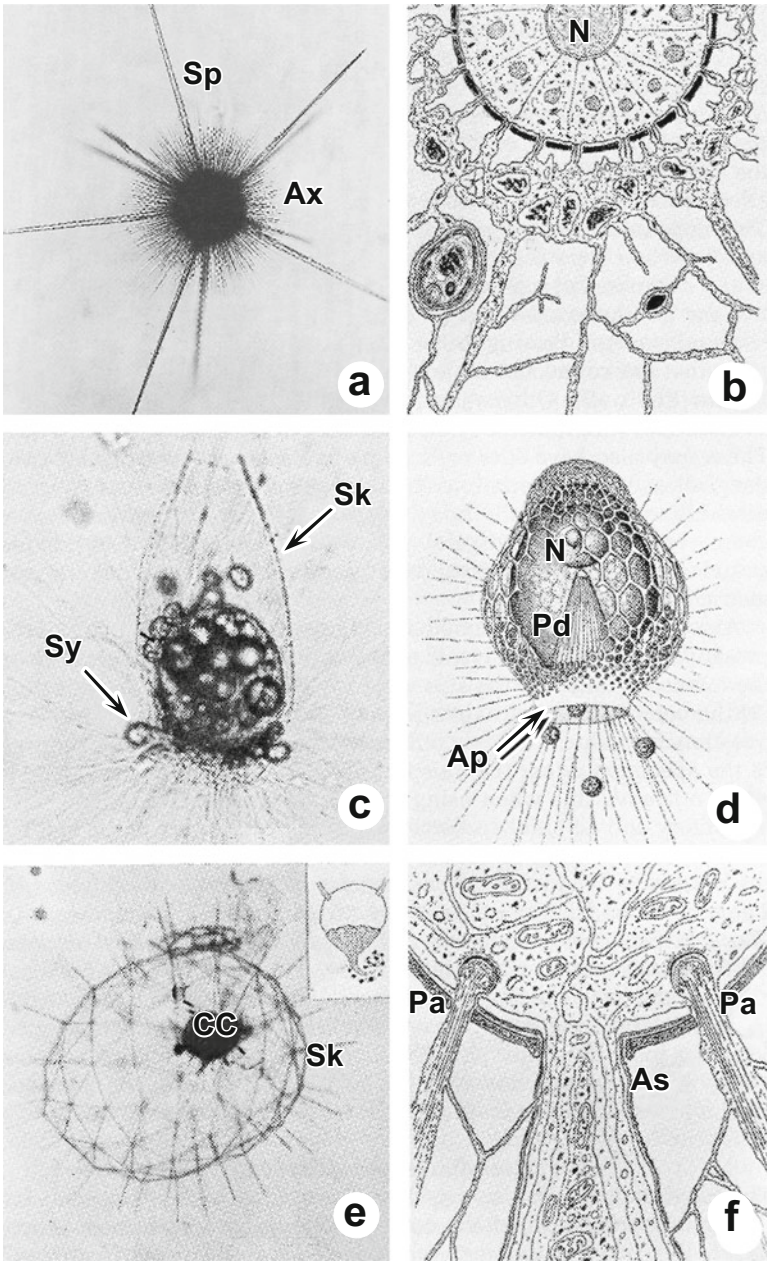

Fig. 9 Comparative morphology and cellular organization of Spumellaria (**a**, **b**), Nassellaria (**c**, **d**), and Phaeodaria (**e**, **f**). (**a**) A living spumellarian with radiating siliceous spines (Sp) and a halo of axopodia (Ax) surrounding the cell. (**b**) A diagram of the cellular organization of a spumellarian showing the centrally located nucleus (N) surrounded by radial lobes of cytoplasm that extend as cytoplasmic strands through pores (fusules) in the dense capsular wall and produce an extracapsular

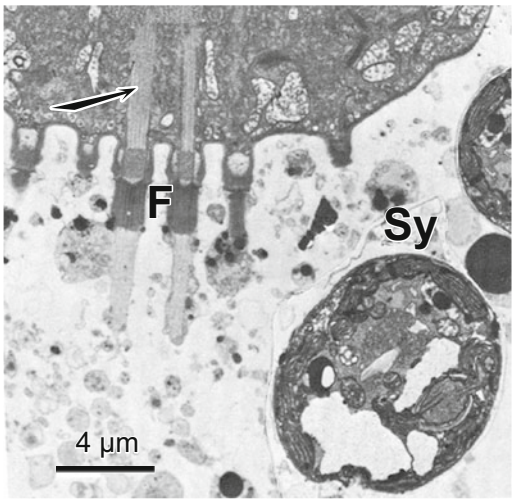

Fig. 10 A transmission electron micrograph of the lower portion of a Nassellarian central capsule showing the shafts of microtubules in the conical podoconus (*Arrow*) that extend from the intracapsular cytoplasm through the pore-like fusules (F) in the capsular wall and protrude outward as axopodia surrounding the cell. Algal symbionts (Sy) are scattered in the peripheral axopodial array (Adapted from Anderson (1983))

symmetry, whereas in nassellarians the body plan usually includes an anteroposterior axis. Figure 11 shows a characteristic, spherical spumellarian whose skeleton comprises several concentric shells. Growth in spumellarians starts with the first, innermost shell and proceeds centrifugally. Nassellarian shells often comprise several sections aligned along an axis. Shell growth starts with the inner, often tripodal, skeleton located inside the first section, or cephalis, and proceeds along the axis to form the thorax, the abdomen, and the postabdominal segments (when present). The wealth of skeletal shapes and morphologies is, however, very ample, including simple spines arranged as a tripod, porous, helmet-shaped skeletons, porous spherical shells, single or multiple concentric geodesic shells composed of rod-like elements joined at nodes in a framework (with or without radially arranged spines), spongiose shells of varied shapes ranging from flattened discs to spheres, and a myriad of other forms, some with ideal geometric shapes (e.g., regular icosahedrons,

Fig. 9 (continued) layer of cytoplasm within a network of rhizopodia. (**c**) A living small nassellarian showing the siliceous skeleton (Sk) forming a conical porous shell (cephalis) surrounding the ovate central capsule. Algal symbionts (Sy) are distributed within the extracapsular rhizopodial network. (**d**) A diagram of the nassellarian cephalis, and a cut-away view of the central capsule showing the nucleus (N) and conical array of microtubules, forming the podoconus (Pd), that extend out of the central capsule as axopodia through the aperture (Ap) at the base of the cephalis. See also Fig. 9. (**e**) A living phaeodarian showing the geodesic siliceous skeletal framework (Sk) surrounding a network of rhizopodia that emerge from a dense central capsule (CC) and the morphology of the central capsule (inset) with a major cytoplasmic strand (astropyle) emerging at the base and two smaller cytoplasmic strands (parapylae) emerging at the opposite pole. (**f**) A diagram of a section of the base of a phaeodarian central capsule showing the emergence of the massive astropyle (As) and smaller parapylae (Pa) projecting outward through openings in the capsular wall (Adapted from Anderson (1983) and J. Cachon et al. (1990))

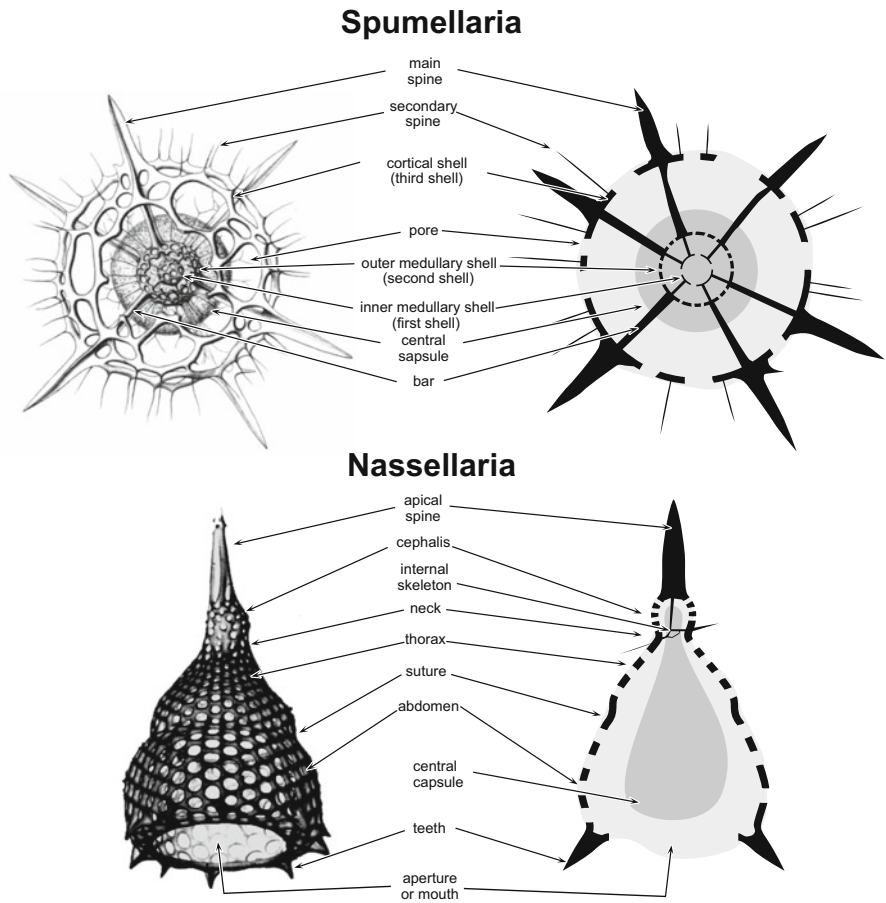

Fig. 11 Scheme of the skeletal elements of the shell of a typical Spumellaria and Nassellaria (Adapted from Boltovskoy and Correa (2014))

dodecahedrons, and octahedrons; Fig. 12) not found in any other living organism (Afanasieva 2006, 2007; Anderson 1983).

The skeleton of Phaeodaria is also composed of amorphous silica but may contain more organic matter than polycystines. The skeletal framework in some species is composed of hollow tubes (e.g., Fig. 9e), not solid rods as in the polycystines. Other species of Phaeodaria have ornate spicules scattered in the external cytoplasm or shells that are either bivalved, resembling small clams or vase-like to pouch-shaped with ornate protuberances around the opening (Fig. 13). Other species have only much branched antler-like spines protruding from a central shell (Fig. 13) (Takahashi and Anderson 2000). However, overall, porous microstructures and basic tubular ultrastructures appear to be common in most of the taxa examined in plankton and sedimentary trap samples from several open ocean locations (Takahashi and Hurd 2007).

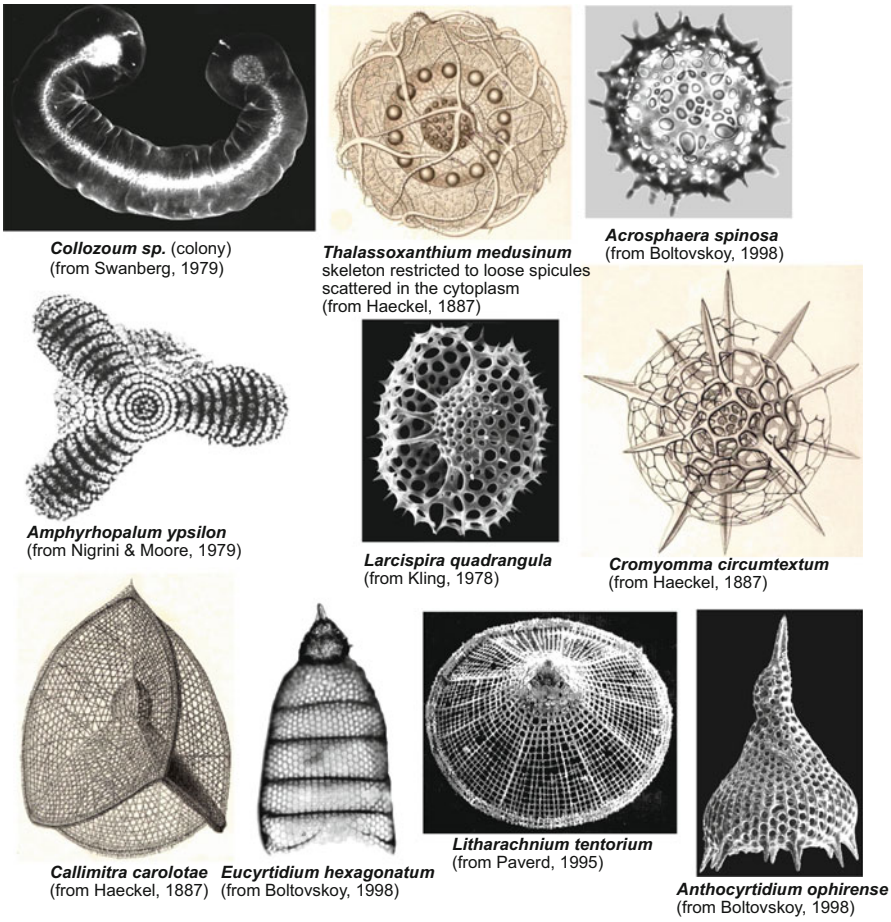

Fig. 12 Representative examples of polycystine species (figures are not to scale)

Feeding, Symbionts, Necrotrophs, and Predators

Feeding. Considerably more is known about the feeding behavior of polycystine Radiolaria compared to Phaeodaria, although our knowledge is still rather limited. Polycystines consume a wide variety of prey including bacteria, algae, protozoa, and microinvertebrates such as copepods and small larvae of marine arthropods. In a rather extensive study of prey observed in SCUBA-collected radiolaria from epipelagic plankton, Swanberg and Caron (1991) noted that a relatively small proportion of captured radiolaria possessed prey (46%), but there was a wide variety of prey consumed including diatoms, tintinnids, and more frequently copepods and their nauplii, or mollusc larvae. Smaller radiolarian species prey largely on bacteria and algae, whereas larger radiolaria also consume small invertebrates (Anderson 1983, 1996; Caron and Swanberg 1990).

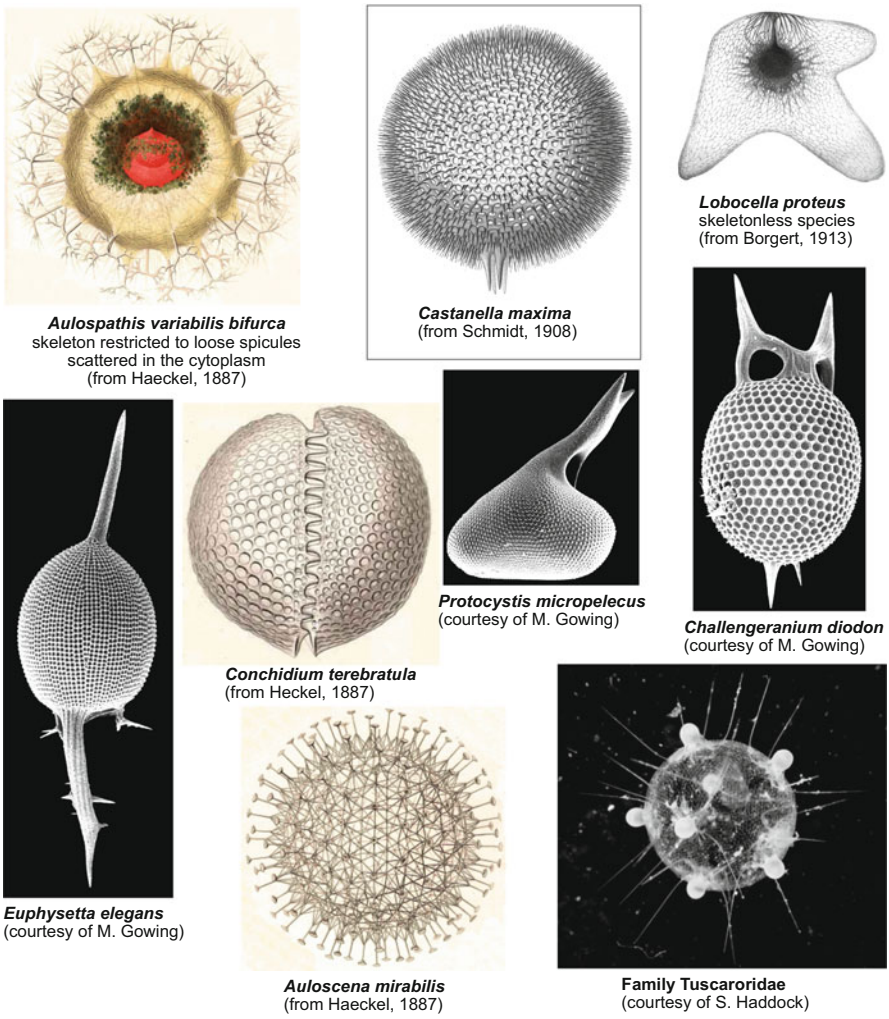

Fig. 13 Representative examples of phaeodarian species (figures are not to scale)

The algal and protozoan prey become snared on the sticky surface of the axopodial array and is engulfed by invagination of the surface membrane to form an intracytoplasmic food vacuole. The food vacuole is converted to a digestive vacuole by secretion of digestive enzymes (Anderson 1996). A much more elaborate mechanism of predation occurs when small arthropods, such as copepods, are consumed (Anderson 1978). The prey becomes entangled within the axopodia and associated rhizopodial network. Eventually, it is surrounded by the rhizopodia that penetrate through weak zones of the prey exoskeleton. Once inside of the host body, the rhizopodia engulf large segments of prey tissue, enclosing them within digestive vacuoles that are carried by cytoplasmic streaming out of the host into the radiolarian

cytoplasm near the central capsule. Small vesicles containing the digestive products are transported through the fusules into the intracapsular cytoplasm where food reserves are stored and major metabolic activities take place. Vacuoles containing undigested, waste material are eventually ejected from the axopodial array by cytoplasmic streaming (Anderson 1983). Among the limited evidence of phaeodarian predation, Swanberg et al. (1986) reported that a mesopelagic, coelographic phaeodarian contained microflagellate and metazoan prey. Copepods and salps also were snared when introduced in the laboratory cultures.

Symbionts. A wide variety of symbionts are sequestered within vacuoles (symbiosomes) including algae and photosynthetic cyanobacteria (Bråte et al. 2012; Probert et al. 2014; Yuasa et al. 2012). Algal symbionts include dinoflagellates (golden yellow), prasinophytes (yellow green), and prymnesiophytes (tawny brown) (Anderson 1978; M. Cachon and Caram 1979; Hollande and Carré 1974). Algal symbionts are highly productive photosynthetically, fixing more carbon than primary producers in an equivalent volume of the surrounding seawater of the Sargasso Sea (Caron et al. 1995). A similar assessment was reported in earlier studies by Khmeleva (1967) in the Red Sea and Gulf of Aden. The symbionts associated with radiolaria, however, account for only a small fraction of the total primary production of the entire water column in the Sargasso Sea studies. The symbionts may provide substantial nourishment to the host. Cytochemical and ^{14}C isotopic tracer studies have shown that the symbionts release organic nutrients that are assimilated by the host and that the host occasionally digests some of them by secretion of enzymes within the normally benign symbiosome vacuoles (Anderson 1983).

Necrotrophs. Dinoflagellate necrotrophs (e.g., *Meriodinium brandti*) infect some species of spumellaria, including colonial radiolaria. *M. brandti* invades the nucleus where it forms a plasmodium (Anderson 1983; Hollande 1974 ; Hollande and Enjumet 1953). Eventually, the *Meriodinium* nuclei divide profusely leading to necrosis of the radiolarian nucleus. The parasite nuclei become segregated from the plasmodial mass to form swarmers with undulipodia and typical dinoflagellate morphology including an epicone and hypocone. They escape from the host to initiate another infective cycle. In other species of *Meriodinium*, the initial proliferation in the nucleus is followed by release of plasmodial fragments that invade the intracapsular cytoplasm and eventually release motile infective swarmers with characteristic dinoflagellate features. The large, skeletonless radiolarian, *Thalassicolla* sp., is parasitized by *Solenodinium fallax*. This dinoflagellate invades the nucleus, forms a plasmodium, and produces tubular inclusions that subsequently emerge from the disintegrating nucleus and protrude into the surrounding intracapsular cytoplasm. The tubules eventually give rise to numerous infective swarmers with typical dinoflagellate morphology. Necrotrophs also have been reported in Phaeodaria, including *Syndinium nucleophaga* (Cachon-Enjumet 1961; Hovasse 1923).

Predators. Our knowledge of radiolarian predators is very limited, but based on digestive tract samples from diverse geographic locations, radiolaria have been detected in tunicates (e.g., salps), crustacea such as copepods, euphausids, and in certain penaeidae, among others. There is some evidence that planktonic

foraminifera prey occasionally on radiolaria. Amphipods have been reported invading and ingesting cells of colonial radiolaria (Swanberg 1979). However, much more detailed analyses of the digestive tract contents of freshly collected predators is needed to verify predatory pressures on radiolaria.

Reproduction, Growth and Longevity

Reproduction. Reproduction in Polycystinea and Phaeodaria has been observed in laboratory cultures. Collodarian Radiolaria (e.g., some colonial Radiolaria) reproduce by binary fission of the central capsules. Sexual reproduction of Polycystinea or Phaeodaria has not been confirmed, but numerous instances of the release of motile swarmers, likely gametes, bearing two undulipodia have been documented (Anderson 1983; Kimoto et al. 2011). Among polycystines, impending reproduction is signaled by contraction of the extracapsular cytoplasm and jettisoning of symbionts and waste matter. The nucleus undergoes repeated division, eventually filling the intracapsular cytoplasm. Each nucleus becomes segregated from the cytoplasmic mass as swarmers that escape through ruptures in the capsule wall. The fate of the swarmers is unknown. Syngamy (swarmer fusion) has not been observed (Anderson 1983; Cachon et al. 1973). Each swarmer contains a vacuolar-bound strontium sulfate (celestite) crystal (Anderson 1983; Hollande and Martoja 1974) enclosed by an organic envelope that appears to control the ultimate shape of the crystal (Anderson et al. 1990). In the phaeodarian *Coelodendrum ramosissimum*, reproduction begins with the disappearance of the phaeodium, followed by degeneration of the capsule and the appearance of small plasmodial spheres in the ectoplasm. Each of the spheres produces hundreds of polynucleated amoeboids that eventually form swarmers with two undulipodia (Borgert 1900, 1909; Cachon-Enjumet 1964).

Growth and Longevity. During the course of maturation, some skeletal-bearing species exhibit a "stair-step" pattern of growth, undergoing one to several days of silica deposition and increase in size followed by plateaus for several days before the next growth phase (Anderson et al. 1989). However, no predictable periodicity of silica deposition has been observed within a given species, and the stair-step curves are highly variable. Further research is needed to document patterns of growth among a wide variety of polycystines. Among the Polycystinea, two processes of skeletal growth and maturation appear to account for all examples of skeletal morphology: (1) Rim growth, commonly found in porous shells, with round to nearly round pores. The pores are formed by deposition of silica on the rims of larger pores that become increasingly smaller in diameter during maturation. (2) Bridge growth, producing geodesic frameworks and latticed shells that are formed by repeated production of rod like elements that grow from one node to another across an opening in the framework, thus producing a skeleton with increasingly more complex design, and in some cases increasingly smaller openings (Anderson 1983). Species with concentric spherical shells construct the innermost, small, primary shell first, typically by bridge growth. Spines elongate from the primary shell and provide scaffolding for the construction of successive larger

surrounding shells, also by bridge growth. Some spongiose skeletal species deposit a small spherical, porous shell initially, followed by very fine bridge growth producing a surrounding meshwork of silica with the characteristic morphology of the species. The skeleton provides protection for the delicate cytoplasmic structures and also supports the axopodia and network of pseudopodial strands radiating from the central cell body, thus permitting efficient capture of prey, including invertebrates such as copepods that may be larger than the radiolarian (Anderson 1978).

Our knowledge of the longevity of radiolaria is limited. Evidence from laboratory maintenance cultures of radiolaria, and inferential data based on environmental observations of the periodic appearance of juvenile and adult stages of radiolarian species, indicates that they live for several weeks to several months before reproducing. Additional research is needed on comparative analyses of life spans of different species and also on the effects of environmental variables on longevity, such as abundance of prey, temperature, and other seasonal and biogeographic factors (Anderson 1983; Casey et al. 1970).

Systematics

Polycystinea. Haeckel (1887) produced one of the earliest comprehensive systems of radiolarian classification describing over 3000 polycystine species, ~2400 of which were new to science. Haeckel's work is still a necessary reference guide, but it does not satisfactorily represent natural relationships because groupings are only based on morphologic similarities, and because the rigidity of these geometry-based diagnoses often ignores the ample intraspecific variability of the radiolarians (Lazarus et al. 2015). Efforts to improve upon the classification schemes inherited from earlier workers have mainly followed two different approaches: cytological data and evolutionary studies. Several authors (Cachon and Cachon 1972a, b; Hollande and Enjumet 1960; Petrushevskaya 1981; Petrushevskaya et al. 1976) proposed revisions which rely heavily on cytoplasmic features, in particular the "nucleoaxopodial complex" (Petrushevskaya 1981). Although these schemes are probably sounder in biological terms, their application to fossil and subfossil materials lacking the protoplasm is problematic, which is one of the reasons for their very limited acceptance among radiolarian workers. Analyses of evolutionary lineages in geological sequences were used as a basis to assess the taxonomic value of key skeletal traits; it was concluded that many of them (e.g., number of segments, number of supplementary concentric spheres, number of feet, number of rays and of equatorial spines in discoidal Spumellaria, and presence and nature of thoracic wings) have little or no suprageneric value. In contrast, several others (especially cephalic structure, but also pore arrangement, shell terminations in Nassellaria, etc.), traditionally considered as of minor value, are conservative through time, reveal evolutionary lineages and, therefore, are relevant for higher-rank divisions (Riedel and Sanfilippo 1986). These results are at least partly in disagreement with the conclusions of the major review by De Wever et al. (2001), who based their classification scheme on the notion that "the farther the skeletal elements are from the first shell, or

initial skeleton, the less important they are for higher level systematics." Riedel (1967, 1971); Petrushevskaya (1971a); (Petrushevskaya 1986); Goll (1968); (Goll 1969); Sanfilippo and Riedel (1970); Dumitrica (1989); De Wever et al. (2001) based on skeletal features alone worked out alternative classifications, either for the entire group or for selected parts of it. Of these, Riedel's (1967, 1971) suprageneric system has become the most widely accepted for extant and Cenozoic radiolarians and is the one adopted herewith with slight modifications. Classification of pre-Cenozoic polycystines follows De Wever et al. (2001).

Phaeodaria. The classification of this group proposed by Haeckel (1862, 1887) has been used by subsequent students with but minor additions. Generic assignments have been followed with few modifications, with the exception of some occasional revisions (Korsun 2011; Nakamura et al. 2015; Nakamura and Suzuki 2015; Reshetnjak 1966), but inconsistent usage (particularly among the family Challengeridae) persists into modern times (Kling and Boltovskoy 1999). The morphology of each family is so distinctive that there has been essentially no controversy as to their taxonomic identity, although lack of discrepancy is probably more a reflection of reduced interest and absence of new research, than of the quality of the information available.

Outline Classification

The classification outlined below incorporates the major higher-order categories defined on the basis of molecular phylogenetic studies, particularly the results of Adl et al. (2012). The classification outline proposed by these authors deliberately omits formal taxonomic categories; for the sake of clarity, we have included them (in parentheses, after the taxon name), as used in traditional classification systems. Adl's divisions within Polycystinea and Phaeodaria are practically identical to those of traditional taxonomy, which seems to be justified by the very scarce information available so far (Ishitani et al. 2012a, b). On the other hand, molecular results obtained with other planktonic protists (e.g., Foraminifera) (Darling and Wade 2008; De Vargas et al. 2004; Seears et al. 2012) suggest that many existing morphospecies include several genetically different organisms with more or less distinct distributional patterns. The few data on Polycystinea published in the last years support this assumption, suggesting that taxonomic assignments based on morphologic features often conflict with genetic molecular studies (Biard et al. 2015; Sierra et al. 2013) and that genetically defined units can differ both morphologically and distributionally (Ishitani et al. 2012b, 2014). It should be stressed that these results do not necessarily imply that traditional, morphological classifications are wrong and those based on molecular data are correct. While molecular studies are undoubtedly a very powerful tool for evolutionary and taxonomic investigations, as any other technique they have important limitations (Decelle et al. 2014). Among other limitations, the use of a single gene to decipher phylogenetic relationships may bias the results, and use of more than one gene often improves the analyses. However, the usefulness of molecular analyses as an additional tool is beyond doubt, not only for addressing taxonomic and phylogenetic issues, including conflicting identifications based on skeletal features (Yuasa et al. 2009), but also for addressing distributional, evolutionary and ecologic problems.

Rhizaria
Cercozoa
Thecofilosea
Phaeodaria (=Tripylea) (Superorder) Siliceous skeleton, when present, may consist of scattered spicules or a well-developed meshwork, but skeletal rods are usually hollow and skeletal material is provided with an organic matrix. Skeletons rarely preserve in sediments. The central capsule normally with one large and two smaller pores. Around 400–500 extant species.
Phaeoconchia (Order) Skeleton formed by two symmetrical valves that can be large and conspicuous (family Concharidae) or small, internal, surrounded by an elaborate meshwork of tubes and spines (family Coelodendridae) (living representatives only, two families).
Phaeocystina (Order) Skeleton absent or formed by loose elements around the central capsule (living representatives only, four families).
Phaeogromia (Order) Skeleton, when present, represented by a globular or ovoidal solid structure with one large opening, often with one or more large radial spines. Very heterogeneous group (a few shelled forms known since the Eocene, 8 families).
Phaeosphaeria (Order) Skeleton usually represented by a large sphere with triangular meshes (living representatives only, 3 families).
Retaria
Acantharia (Subclass)
Polycystinea (Subclass/Superorder) Usually endowed with a siliceous skeleton with solid bars. Cytoplasm divided into two regions: an inner endoplasm and an outer ectoplasm or calymma, separated by a perforated organic membrane, the central capsule. Probably around 400–800 extant species and several thousands of fossil forms.
Collodaria (Order) Solitary or colonial polycystines without a siliceous skeleton or provided with simple or branched spicules scattered in the calymma. (Eocene?-Holocene, four families, all with extant representatives).
Spumellaria (Order) Solitary or colonial (one family only: Collosphaeridae). Shell well developed, with radial symmetry or one derived from it (spiral, discoidal or lenticular biconvex, triaxonic, quadrangular, etc.) or asymmetric. Central capsule with many small pores (Paleozoic-Holocene, 37 families, eight with extant representatives).
Nassellaria (Order) Solitary. Shell represented by several fused spicules only, by a D-shaped ring and associated spines, or by more elaborate mono- or multilocular latticed skeletons. The symmetry of the shell is characterized by the fact that the two extremes of its major axis define two morphologically different poles (Devonian- Holocene, 54 families, seven with extant representatives).
Archaeospicularia, **Albaillellaria**, **Latenfistularia**, **Entactiniaria** (Orders) Fossil polycystines (Cambrian-Triassic, 37 families).

Evolutionary History

Polycystines possess some exceptional traits for their use in evolutionary studies: they appear very early – in the Lower Cambrian (Nazarov 1973; Obut and Iwata 2000), they preserve well in the geological record, they are highly diversified, and they are often very abundant. However, their potential is seriously hindered by the generally poor state of their taxonomy, which affects not only the species concept but also the definition of supraspecific categories and the taxonomic and evolutionary value of most morphologic traits. Thus, with the exception of a few well-researched Cenozoic lineages, our understanding of the evolution of the polycystines is still in an embryonic stage. Modern molecular phylogenetic research, in addition to clarifying the taxonomy of polycystines, has provided additional sources of evidence to trace their origins and divergences during the evolution of this group in relation to other taxa in the tree of life (Ishitani et al. 2012a; Sierra et al. 2013).

Although some authors have presented debatable evidence of links with benthic ancestors (Petrushevskaya 1986), the origin of radiolarians is uncertain. Until the Permian, their diversification was moderate, but in the Triassic, many new families appeared and from there on the number of extinctions was roughly balanced with that of new forms. For the Paleozoic, over 600 polycystine species (80 genera) have been described, suggesting a speciation rate of about 1–2 species per million years (Vishnevskaya and Kostyuchenko 2000). For the Mesozoic, this rate soars to over 10 species per million years; the total number of Mesozoic species described is around 2500 (Vishnevskaya and Kostyuchenko 2000), including the first multisegmented nassellarians, the appearance of twisted spines in spumellarians, etc. In agreement with most other organisms, polycystines show a strong diversity drop around the Cretaceous-Tertiary boundary, recovering in the Eocene (Sanfilippo et al. 1985; Vishnevskaya and Agarkov 1998). In the Cenozoic, the number of polycystine species varies around 400–800. The skeletons of these species are conspicuously lighter than those of most pre-Cenozoic forms, presumably due to the competition for dissolved silica with the diatoms (Lazarus et al. 2009). The longevity of most Cenozoic species ranges around 1–5 Ma (before going extinct or changing sufficiently to be identified as a different species) (Sanfilippo et al. 1985). Thus, despite the fact that polycystines represent potentially useful evolutionary and stratigraphic tools, taxonomic inconsistencies and the scarcity of specialists hinder their extensive use in these fields.

Maintenance and Cultivation

Continuous, reproducing cultures of radiolaria have not been established in the laboratory, probably due to the particular environmental requirements of the earliest growth stages. However, juvenile radiolaria that are collected by gentle drift tows using nets, or captured in hand-held small jars by SCUBA divers, can be maintained in laboratory culture (Anderson 1992). Individual radiolaria are retrieved from the sample using pipettes fitted with a rubber bulb and a tip with a large opening. The

radiolarian is gently deposited in glass culture dishes or small vials containing seawater, freshly collected from the sampling site. Symbiont-bearing species are illuminated by fluorescent lights and temperature is maintained by surrounding the dishes with recirculating water from a constant temperature bath set at a temperature equivalent to the sampling site. Algal cultures established in the laboratory, including planktonic diatoms, dinoflagellates, and other small protists, provide a source of protistan prey. Small droplets are introduced into the culture vessels, but only sparingly and at intervals of several days to avoid fouling the culture dishes with overgrowth. Small crustacea or young nauplii of brine shrimp (*Artemia*) reared in the laboratory are suitable additional prey for larger species. In some cases, the freshly collected, unfiltered, seawater from the sample site contains sufficient prey to sustain growth of illuminated radiolarian cultures if the seawater is replaced with freshly collected seawater every several days. No additional prey are required, especially if the cultures are illuminated. The larger radiolaria can be observed using a high-power dissecting microscope. Inverted microscopes with long-distance objective lenses can be used for more detailed visualization of smaller floating radiolaria, preferentially maintained in small culture vials with optically clear flat bottoms.

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Chlorarachniophytes

20

Patrick J. Keeling

Abstract

The chlorarachniophytes are a group of single-celled phototrophic, mixotrophic eukaryotes in marine environments. They are most common in tropical and temperate seas. The group is primarily studied due to their evolutionary history. Chlorarachniophytes acquired photosynthesis by secondary endosymbiosis, where an amoeboflagellate host took up a green algal symbiont and retained it. The symbiont is distinguished by having retained a relict nucleus, or nucleomorph, which has been intensively studied to help elucidate the process of organelle origins by endosymbiosis. Historically, work on the nucleomorph was an important clue suggesting that secondary endosymbiosis played a role in the distribution of photosynthesis and plastids in eukaryotes. More recently, a number of genomic and cell biological studies, in particular focusing on gene flow within the cell and protein targeting, have further contributed to our understanding of organelle integration during endosymbiosis. The host component is now known to be a member of the Cercozoa and can include amoeboid, flagellate, and cyst stages, various species having any combination of one or more stages in the life cycle.

Keywords

Endosymbiosis • Nucleomorph • Green alga • Amoeba • Cyst • Flagellate

Contents

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Summary Classification

●**Chlorarachniophyceae**
●●***Amorphochlora***
●●***Bigelowiella***
●●***Chlorarachnion***
●●***Cryptochlora***
●●***Gymnochlora***
●●***Lotharella***
●●***Norrisiella***
●●***Partenskyella***

Introduction

The chlorarachniophytes are a small group of tropical to temperate marine amoeboflagellates with chlorophyll *a*- and *b*-containing chloroplasts. They have attracted the attention of biologists primarily due to their complex cell biology and evolutionary history, stemming from the fact that they acquired their green chloroplasts by secondary endosymbiosis and have retained a vestigial nucleus of the engulfed alga, now called a nucleomorph (McFadden et al. 1994; Archibald 2007). All known chlorarachniophytes are phototrophic and can possess from one to several chloroplasts, each associated with a nucleomorph. The host cells may be found as amoebae, some plasmodial with individual cells linked by a network of reticulopodia, as thick-walled coccoid cells, or as highly motile uniflagellated zoospore. In some genera, all three cell types are found, although one type is the dominant trophic stage, whereas in other genera, only two or one of the cell types have been observed (Ishida et al. 2007). The endosymbiont is known to be derived from a green alga, and the host is a member of the Cercozoa (Cavalier-Smith 1999; Rogers et al. 2007).

The type species for the group is *Chlorarachnion reptans*, originally described by Geitler (Geitler 1930) and also the first species to be investigated in detail by light microscopy (LM), electron microscopy (EM), and pigment composition analysis

(Hibberd and Norris 1984). *Bigelowiella natans* has since emerged as the best-studied species, and now most of our information comes from this organism.

History of Study and Literature

For many years after the discovery of *C. reptans*, there remained little literature on chlorarachniophytes (Hibberd 1990). The suggestion that they originated by secondary endosymbiosis led to new interest in the group, and early work proving this and preliminary characterization of the nucleomorph both led to a surge in reports on the group, in particular on *B. natans*. Recent work has focused on genomics (McFadden et al. 1997a; Williams et al. 2005; Gilson et al. 2006; Rogers et al. 2007; Curtis et al. 2012), molecular evolution (Archibald et al. 2002, 2003; Takishita et al. 2005; Burki et al. 2007), protein trafficking (Rogers et al. 2004; Gile and Keeling 2008; Hirakawa and Ishida 2010; Hirakawa et al. 2009, 2010, 2011a, 2012a, b), and on the description of new species. Many genomes are now available, especially from *B. natans*, but the emerging model for cell biology is *Amorphochlora amoebiformis*, due to the creation of a transient transfection system that has been used with green fluorescent protein (GFP) markers (Hirakawa et al. 2009). Most information on the cell structure, life history, and habitat is found in the formal descriptions of the 14 species described to date (Hibberd and Norris 1984; Calderon-Saenz and Schnetter 1987; Ishida and Hara 1994; Ishida et al. 1996, 2000, 2011b; Moestrup and Sengco 2001; Dietz et al. 2003; Ota et al. 2005, 2007a, b, 2009a, b, 2011, 2012). There is a large number of review articles on chlorarachniophytes, almost all focusing on molecular biology and endosymbiosis, due to the presence of the nucleomorph and its importance to understanding genome reduction and the endosymbiotic history of plastids (McFadden and Gilson 1995; Gilson et al. 1997; Gilson and McFadden 1997, 2002; McFadden et al. 1997a; Gilson 2001; Archibald and Keeling 2002; Cavalier-Smith 2002; Archibald 2007; Ishida et al. 2007).

Characterization and Recognition

General Characteristics

Although there is much variation between members of the group, there are three common life history stages in chlorarachniophytes: amoeboid, coccoid, and zoospore. In some species all three stages have been observed, whereas in others only one or two stages have been observed, and where more than one is known, the dominant trophic stage can vary. Characteristics common to different stages are discussed here, and stage-specific characteristics will be discussed in turn.

Cells contain a single nucleus (with the exception of a giant amoeboid/coccoid stage of *Gymnochlora dimorpha*/*Lotharella reticulosa* (Ota et al. 2011, 2012)), which divides by open or semi-open mitosis. Mitochondria display typical tubular cristae and are dispersed throughout the cell. Extrusomes have been observed in

several species. Cells contain numerous Golgi bodies, some associated with the pyrenoid and the pyrenoid-capping vesicle. The pyrenoid-capping vesicle is bounded by a single membrane and contains a homogeneous material that has been shown to react with antibodies specific to β-1,3-glucans, which are the primary carbohydrate storage product (McFadden et al. 1997b). The chloroplast lacks starch (Hibberd and Norris 1984), so all carbohydrate storage seems to be carried out in this form by the host. The lipid composition of chlorarachniophytes has also been examined and found to be unlike that of other algae (Leblond et al. 2005).

The chloroplast is the best-studied structure of chlorarachniophytes. All cells contain one or more bilobed, peripheral, chlorophyll *a*- and *b*-containing chloroplasts, typically with a central, inwardly projecting pyrenoid that is closely surrounded by a capping vesicle. Chloroplast lamellae are usually composed of one to three thylakoids, and a girdle lamella is absent. Each chloroplast is bounded by four membranes that may appear closely appressed or as two pairs separated by a space. The outermost membrane is derived from the endomembrane system of the Cercozoan host but is smooth and is not directly connected to the rough endoplasmic reticulum (ER), as is the case in several other algal groups with secondary plastids. The second membrane is derived from the plasma membrane of the green algal endosymbiont. The space between the outer pair and inner pair (which corresponds to the cytoplasm of the green alga) is sometimes referred to as the periplastid space or periplastidial compartment. The inner pair of membranes is derived from the chloroplast envelope. Protein targeting to the plastid has been examined in some detail and is mediated by a bipartite leader consisting of a signal peptide followed by a transit peptide-like (TPL) sequence. The signal peptide directs the protein to the endomembrane system, and the TPL directs it across the remaining three membranes (Hirakawa et al. 2009, 2010, 2012a). Proteins cross the last two membranes using a fairly conventional plastid translocon complex (TOC and TIC systems: Hirakawa et al. 2012a), but how proteins cross the membrane derived from the endosymbiont plasma membrane remains mysterious. The characteristics of the leader that mediate this process are understood, but the mechanism is unknown: currently it seems unlikely that chlorarachniophytes use symbiont ERAD-like machinery (SELMA) including Der1 proteins (Hirakawa et al. 2012a), which is used by red algal secondary plastids (Hempel et al. 2009).

The periplastid space contains a dense homogeneous matrix including many visible ribosomes equivalent in size to eukaryotic cytosolic ribosomes (Hibberd and Norris 1984; McFadden et al. 1994). The periplastid space is generally only a thin layer around most of the chloroplast, but around the base of the pyrenoid or within a wedge-shaped invagination in the pyrenoid, the space is enlarged and contains the nucleomorph, the relict nucleus of the green algal endosymbiont (Fig. 1i). The nucleomorph is small, bounded by a double membrane with pores (Hibberd and Norris 1984; Ludwig and Gibbs 1989; McFadden et al. 1994), and has been shown in all examined species to contain three small, linear chromosomes amounting to 330–1133 kbp of DNA (Gilson and McFadden 1999; Silver et al. 2007; Ishida et al. 2011a). Like the plastid, the periplastidal space also lacks sufficient nucleomorph-encoded genes for function (Gilson et al. 2006), and now a

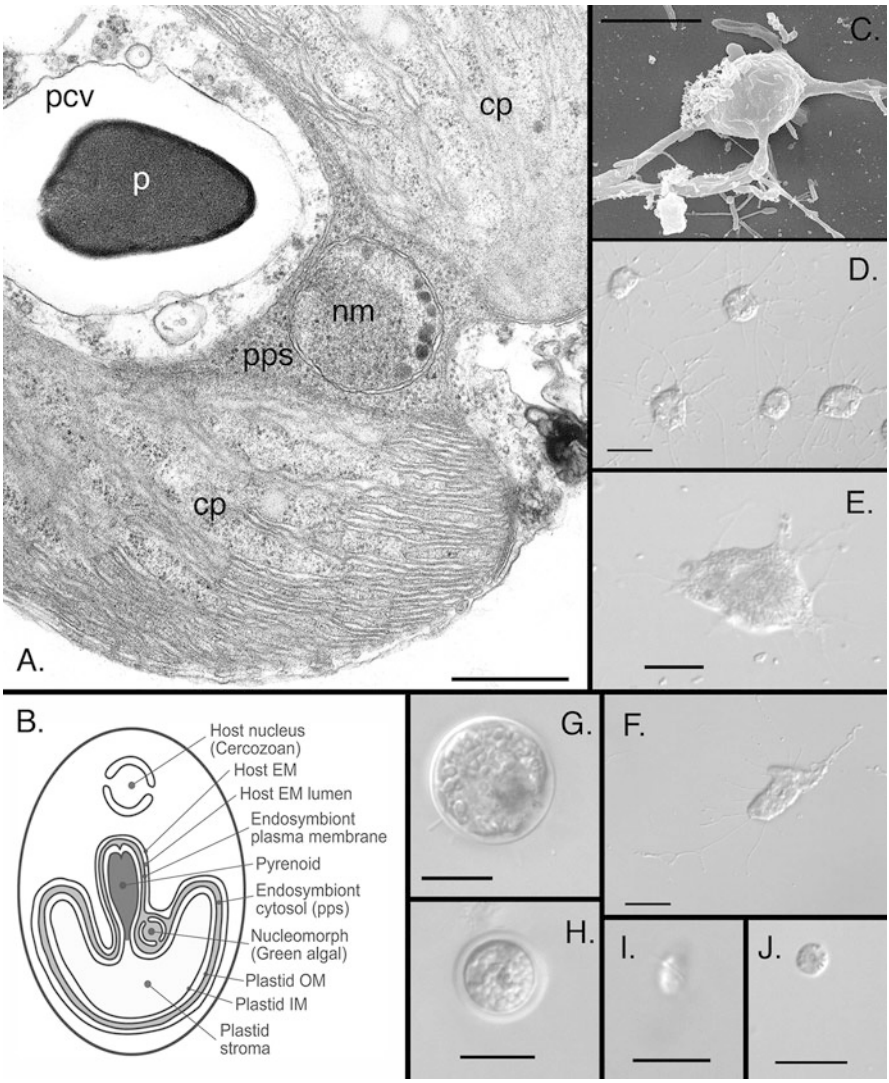

Fig. 1 Morphology and diversity of chlorarachniophytes. (**a**) Transmission electron micrograph of *B. natans* showing the nucleomorph (*nm*), periplastid space with eukaryotic size ribosomes (*pps*), two lobes of the chloroplast (*cp*), and bulbous pyrenoid (*p*) with pyrenoid-capping vesicle (*pcv*). (**b**) Schematic diagram of chlorarachniophyte plastid. Labeled to the right are the three genome-containing compartments (host and endosymbiont nucleus and plastid – the mitochondrion is not shown), the pyrenoid (the pyrenoid-capping vesicle is not shown), and the various membranes and compartments of the complex plastid, including their evolutionary origin (abbreviations are: *EM* endomembrane, *pps* periplastid space, *OM* outer membrane, *IM* inner membrane). (**c**–**f**) Examples of the amoeboid stage: a scanning electron micrograph of *G. stellata* showing cell body and emerging filopodia is shown in (**c**), and light micrographs of amoeboid stages are shown for *G. stellata* (**d**), *Lotharella amoeboformis* (**e**), and *Chlorarachnion reptans* (**f**). (**g**–**h**) Examples of the coccoid stage from *Lotharella vacuolata* (**g**) and *Chlorarachnion reptans*

number of nucleus-encoded periplastid-targeted proteins have been identified. Direct evidence for targeting is only available for three such proteins, histones H2A and H2B (which are targeted to the nucleomorph: Hirakawa et al. 2011a) and EFL (Gile and Keeling 2008). Targeting of these proteins is mediated by a bipartite leader resembling the plastid-targeting leader, except that the TPL portion is distinguished by a net neutral/negative charge, and some proteins have a hydrophilic tail enriched with lysine and aspartic acid residues (Hirakawa et al. 2009, 2010). A large group of potentially periplastidal compartment (PPC)-targeted proteins were identified in the nuclear genome with similar characteristics (Curtis et al. 2012).

Amoeboid Stage

Amoeboid cells (Fig. 1c, e–h) are roughly isodiametric, angular cells found in benthic environments, ranging from 8 to 20 μm (not including the filopodia). From each cell radiate several filose pseudopodia, in *C. reptans*, *L. polymorpha*, *L. vaculolata*, and *L. reticulosa* apparently fusing to form a network of reticulopodia (Hibberd and Norris 1984; Dietz et al. 2003; Ota et al. 2005, 2012) but in others remaining distinct and unconnected (Calderon-Saenz and Schnetter 1989; Ishida et al. 1996, 2000; Ota et al. 2007b). Movement on surfaces is very slow and in some species nonexistent (Ota et al. 2007a), although cytoplasmic streaming can readily be seen in the pseudopodia. Contents of filopodia are mostly restricted to microtubules and vesicular and granular material; mitochondria are the only organelles occurring in the reticulopodia. The amoeboid cells are phagotrophic, engulfing a variety of motile and nonmotile eukaryotes and prokaryotes in the pseudopodia, which may develop large ingestion vesicles. Uptake of prey species has been described for *C. reptans* as differentiated, and some species are taken up preferentially and digested quickly whereas others are resistant to digestion (Hibberd and Norris 1984). Most digestion takes place in pseudopodia (Hibberd and Norris 1984) but has infrequently been observed in the cell body (Ishida et al. 1996). Amoeboid cells may divide or give rise directly to zoospores or coccoid cells, depending on the species.

Walled Coccoid Stage

Coccoid cells (Fig. 1d–e) are also found in benthic environments and are spherical cells 5–15 μm in diameter with a firm wall of variable thickness composed of

Fig. 1 (continued) (**h**) where the thickened wall is apparent. (**i**–**j**) Examples of the zoospore stage from unnamed strain (CCMP622) of the *Lotharella* clade (**i**), in which the helically coiled flagellum can be seen, and *Bigelowiella natans* (**j**). Scale bars on all LM and SEM parts are 10 μm. Scale bar on TEM scale bar is 500 nm (TEM is courtesy of Ø. Moestrup, SEM is courtesy of S. Bowser, and all differential interference contrast light micrographs are by P. Keeling)

multiple layers (Ishida and Hara 1994; Ota et al. 2005). They are often found in older cultures where they probably act as a resting stage or cyst (Hibberd and Norris 1984; Ota et al. 2007b), but in some species, the main vegetative cell type is walled or walled amoebae with short pseudopodia can extending from the wall (Ishida and Hara 1994; Dietz et al. 2003; Ota et al. 2005, 2007a). These have more irregularly shaped chloroplasts than in the amoeboid stage, a laterally positioned nucleus, and contain a large number of vesicles with contents similar in appearance to those of the pyrenoid-capping vesicles. Coccoid cells may divide or give rise directly to the amoeboid stage and also to zoospores via a tetrad division, depending on the species.

Zoospore Stage

Zoospores (Fig. 1a–b, i) are small, planktonic, pyriform, ellipsoid, or ovoid cells ranging from 4 to 24 μm long × 3–7 μm wide. The cytoplasm often has a distinct granular appearance at the anterior end. Zoospores are uniflagellate, although ultrastructural investigation of *B. natans* has revealed a vestigial second basal body (Moestrup and Sengco 2001). The flagellum typically has a hair point and fine lateral hairs and is anchored by a root system consisting of a microtubular component and a second root (Hibberd and Norris 1984; Moestrup and Sengco 2001). During swimming, the flagellum is wrapped helically around the cell body within a concavity. Swimming is rapid – about 100 μm per second for *C. reptans* and faster for smaller cells. In some species the zoospore may become temporarily amoeboid, with the anterior end forming one or more blunt pseudopodia (Hibberd and Norris 1984; Moestrup and Sengco 2001). Zoospores may divide or give rise directly to the amoeboid or coccoid stages, depending on the species.

Reproduction and Life Cycle

The division of the nucleus, nucleomorph, and plastid has all been examined, as has the order of events in *B. natans* where the order of division is pyrenoid, nucleomorph, chloroplast, and finally the nucleus (Moestrup and Sengco 2001). In nuclear division, the envelope breaks down but fragments of it remain, and mitosis is otherwise not unusual (Moestrup and Sengco 2001). Separation of the daughter cells, on the other hand, can be by very unusual means, including a variation on cytoplasmic streaming in *B. longifolia* and *L. vacuolata* (Ota et al. 2005, 2007b). The nucleomorph divides amitotically: no chromosomal condensation or microtubules have been observed. Rather, the inner membrane invaginates and joins to form a barrier, after which the outer membrane invaginates and the two daughter nucleomorphs are separated (Ludwig and Gibbs 1989). Sexual reproduction is poorly understood in chlorarachniophytes, but gametes and sexual reproduction have been reported in zoospores of *C. reptans* (Grell 1990) and amoebae of *Cryptochlora perforans* (Calderon-Saenz and Schnetter 1989).

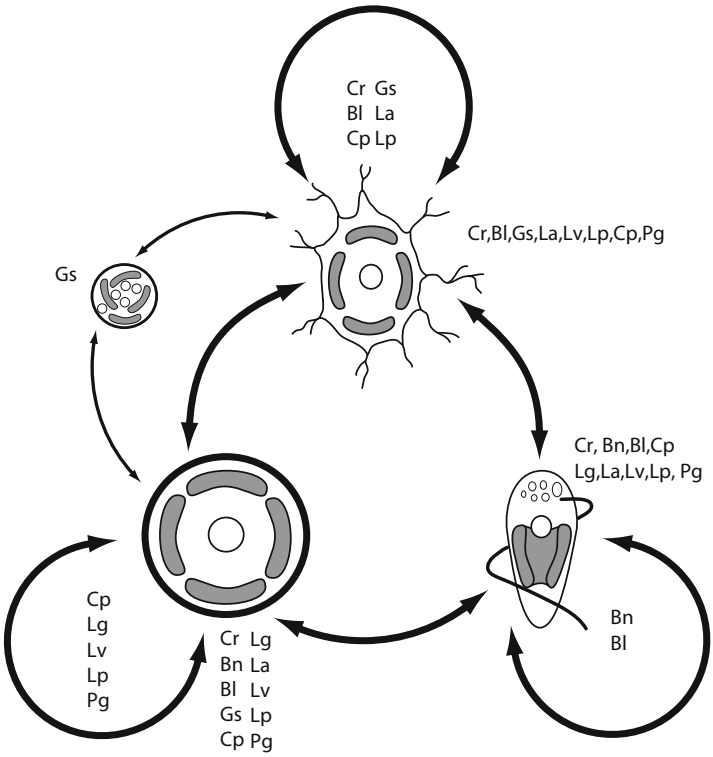

Fig. 2 Life history stages common to chlorarachniophytes. Amoeboid, coccoid, and zoospore states are illustrated, with *arrows* representing transitions (between stages) or vegetative division of a stage. Initials adjacent to stages or within vegetative division loops represent the genus and species where that stage or that vegetative division has been observed (see Classification section for all full genus and species names). *G. stellata* is also known to form a plasmodial form that divides through synchronous cytokinesis, shown parallel to the transition between amoeboid and coccoid stages. All transitions have not been observed in all species, but each transition is known in at least one species

As described above, there are three main life history stages, and in some species all three are known whereas in others one or two are absent. Life history stages have been documented from members of all eight genera, although direct observation of transformations between the various stages is lacking for some species. Available data on which life history stages are present in which genera are summarized in Fig. 2 and Table 1, and see Ishida et al. (2007) for review.

Classification

Chlorarachniophytes are currently classified as members of the Cercozoa, as described below. Currently only 14 species and one variety have been formally described, distributed in 8 genera (Table 1). Classification schemes have relied

Table 1 Summary of characteristics used to classify the six genera of chlorarachniophytes

Genus	Main vegetative stage	Amoeboid	Coccoid	Zoospore	Pyrenoid structure	Nucleomorph location
Chlorarachnion	Amoeboid	+	+	+	Nucleomorph-embedded	Embedded in pyrenoid
Bigelowiella	Zoospore	+/−	+/−	+	Shallow slit	Near pyrenoid base
Lotharella	Amoeboid/ Coccoid	+/−	+	+	Deep slit	Near pyrenoid base
Gymnochlora	Amoeboid	+	−	−	Tubular invaginations	Near pyrenoid base
Amorphochlora	Amoeboid	+	+	+	Deep slit	Near pyrenoid base
Norrisiella	Coccoid	−	+	+	Shallow slit	Near pyrenoid base
Partenskyella	Coccoid	+	+	+	No pyrenoid	Inside chloroplast cup
Cryptochlora	Coccoid	+	+	+	?	?

on pyrenoid shape, location of the nucleomorph, presence or absence of different life history stages, and molecular phylogeny (see Ishida et al. 2007 for review).

Chlorarachnion reptans (Hibberd and Norris 1984) is the type species and only described species of *Chlorarachnion*. It includes all three cell types in its life history and is also distinguished by the location of its nucleomorph within the pyrenoid slit. The genus *Bigelowiella* includes *B. natans* (Moestrup and Sengco 2001), the model species for most chlorarachniophyte cell and molecular biology, and *B. longifolia* (Ota et al. 2007b). *B. natans* has only been observed as zoospores (although they can form pseudopodia (Moestrup and Sengco 2001)), whereas a true amoeboid stage is known in *B. longifolia* (Ota et al. 2007b). There are currently six known species of *Lotharella*: *L. vacuolata* (Ota et al. 2005), *L. globosa* (Ishida and Hara 1994), *L. globosa var. fortis* (Hirakawa et al. 2011b), *L. polymorpha* (Dietz et al. 2003), *L. oceanica* (Ota et al. 2009b), and *L. reticulosa* (Ota and Vaulot 2012), some of which are primarily coccoid and some primarily amoeboid, but all share a pyrenoid with a deep slit and a nucleomorph positioned at the base of the pyrenoid. *Amorphochlora amoebiformis* was originally described as *L. amoeboformis* (Ishida et al. 2000) but was transferred to a new genus based on molecular phylogenetic evidence (Ishida et al. 2011b). *Gymnochlora stellata* and *G. dimorpha* are the described species of *Gymnochlora* (Ishida et al. 1996; Ota et al. 2011), and *Norrisiella sphaerica* is the only described species of *Norrisiella* (Ota et al. 2007a). Both are distinguished from other genera by pyrenoid and nucleomorph characters. *Partenskyella glossopodia* is the only species of *Partenskyella* and is distinguished by a complete absence of a pyrenoid (Ota et al. 2009a). A last genus comprises the enigmatic and poorly described species *Cryptochlora perforans*, which is a mixotrophic species that is attracted to damaged algal *thalli* that it can physically penetrate and feed upon. It has been classified as a chlorarachniophyte based on similarities in life history complexity and plastid characteristics (Calderon-Saenz and Schnetter 1987, 1989). Unfortunately, it has not been described at the ultrastructural or molecular levels, so its exact relationship to chlorarachniophytes is not completely clear.

The relationships between chlorarachniophyte genera are not at all clear from morphological characters, but molecular phylogenies based on genes from the nucleus, nucleomorph, and mitochondria all support the currently analyzable species as being distinct, as well as genera-level distinctions. Phylogenies generally support an overall picture shown in Fig. 3. There is a consistent and well-supported close relationship between the genera *Bigelowiella* and *Norrisiella*, both of which are in turn related to *Chlorarachnion*, with *Partenskyella*, *Lotharella*, and *Gymnochlora* forming an unresolved radiation at the base of the group (Gilson and McFadden 1999; Ishida and Cavalier-Smith 1999; Silver et al. 2007; Ota et al. 2009a; Ota and Vaulot 2012).

Molecular bar codes have been established and tested for all chlorarachniophyte species available in culture (Gile et al. 2010). Nucleomorph ribosomal RNA intergenic spacer (ITS) sequence was found to provide good resolution at the species

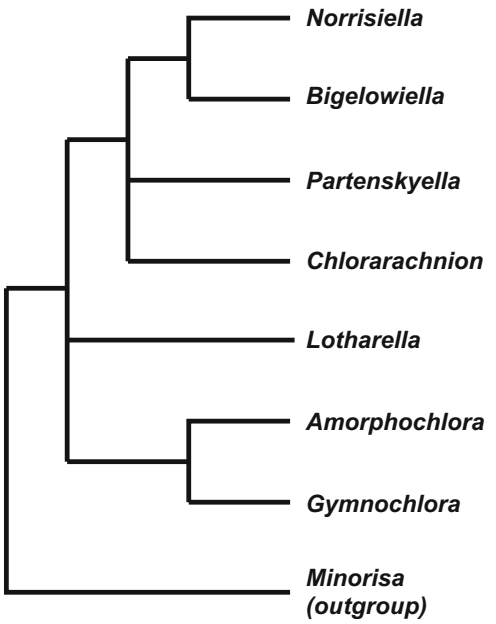

Fig. 3 Phylogeny of described chlorarachniophytes. A schematic molecular phylogeny predominantly based on analyses of SSU rRNA, and the DNA bar code marker ITS is shown

level, at least for the few genera with multiple species, and was subsequently used to characterize new isolates (Hirakawa et al. 2011b; Ota and Vaulot 2012).

Maintenance and Cultivation

Many species grow easily but slowly in a variety of marine media (see Hibberd and Norris 1984; Ishida et al. 2000; Moestrup and Sengco 2001; Ota et al. 2007a, b). Primarily amoeboid and coccoid species mostly accumulate in masses on surfaces, while primarily flagellated forms can be grown to high densities by shaking or aeration. Currently over 30 strains are available from several culture collections, the largest collection of strains being at the Provasoli-Guillard National Center for Culture of Marine Phytoplankton.

Genomics

The unique evolutionary history and current complexity of chlorarachniophytes has led to several genomic and comparative genomic projects. Currently complete genomes for all four compartments, plastid, mitochondrion, nucleomorph, and nucleus, have been sequenced for the model species *B. natans* (Gilson et al. 2006; Rogers et al. 2007; Curtis et al. 2012). Organelle genomes, proteomics, and surveys of gene expression have also been carried out for a number of species (Williams et al. 2005; Slamovits and Keeling 2009; Hopkins et al. 2012; Tanifuji et al. 2014;

Suzuki et al. 2015), all revealing a model for the effects of endosymbiotic integration and nuclear genome compaction. The nucleomorph genome is severely reduced with only about 300 tightly packed genes but still retains over 800 introns that are all compacted, nearly all to 18–21 bp in length (Gilson et al. 2006; Slamovits and Keeling 2009). Nuclear genome organization is conventional but revealed a large number of genes derived by horizontal gene transfer, as well as genes originating by endosymbiotic gene transfer from the endosymbiont (Archibald et al. 2003; Gile et al. 2008; Hirakawa et al. 2011a; Curtis et al. 2012). The nucleus is haploid and nucleomorph diploid (Hirakawa and Ishida 2014).

Evolutionary History

The unique combination of characters found in chlorarachniophytes led to much speculation and confusion about their possible evolutionary origin in early studies (Geitler 1930; Hibberd and Norris 1984; Grell 1990; Hibberd 1990), particularly before it was understood that they are a symbiotic fusion of a colorless amoeboflagellate and a green alga (Whatley and Whatley 1981; Cavalier-Smith 1982). Now this endosymbiotic origin of chlorarachniophytes has been demonstrated beyond any doubt by ultrastructural studies and characterization of the nucleomorph genome (Ludwig and Gibbs 1989; McFadden et al. 1994; Gilson et al. 2006). While the nucleomorph and its genome have been retained, many endosymbiont genes were moved to the host nucleus (Deane et al. 2000; Archibald et al. 2003; Gile and Keeling 2008; Hirakawa et al. 2011a; Curtis et al. 2012) and many other features simply lost, for example, Golgi bodies, mitochondria, locomotary organelles, and carbohydrate storage, during the integration of chlorarachniophyte plastids.

Even after the endosymbiotic origin of chlorarachniophytes was well established, however, the origin of both the host and the endosymbiont continued to be controversial. The presence of chlorophyll *b* immediately suggested a link to green algae (Hibberd and Norris 1984), but numerous theories about which kind of green algae were put forward (Sasa et al. 1882; Cavalier-Smith et al. 1994; Van de Peer et al. 1996; Ishida et al. 1997; Ishida and Cavalier-Smith 1999). Current data only suggest it is a member of the ulvophyte-trebouxiophyte-chlorophyte complex (Rogers et al. 2007; Turmel et al. 2009). The evolutionary history of the host was considerably more obscure since chlorarachniophytes do not share any obvious defining morphological feature with any other group. Molecular data have shed considerable light on this, however, and consistently show the chlorarachniophyte host to be part of a large and diverse group of flagellates, amoebae, and amoeboflagellates, called Cercozoa (Cavalier-Smith 1999). There are currently no structural characteristics that uniquely unite all Cercozoa, but phylogenies based on all genes that have been examined individually or as large concatenates (Bhattacharya et al. 1995; Cavalier-Smith and Chao 1997; Keeling et al. 1998; Keeling 2001; Longet et al. 2003; Nikolaev et al. 2004; Takishita et al. 2005; Burki et al. 2007, 2012), as well as the presence of unique insertion/deletions in polyubiquitins and

rRNA (Cavalier-Smith and Chao 1997; Archibald et al. 2002), all consistently show Cercozoa to be monophyletic group that includes chlorarachniophytes, likely as an early-branching subgroup. Cercozoa, in turn has been shown to be part of an even larger group called Rhizaria, which also includes a number of mostly amoeboid lineages such as foraminiferans, acantharians, and polycystines (Sierra et al. 2012). Altogether, Rhizaria consistently branch as sisters to the alveolates and stramenopiles in large multigene phylogenetic analyses (Burki et al. 2007, 2012). The closest cercozoan sister group to the chlorarachniophytes in current phylogenies appears to be the pico-heterotroph, *Minorisa*, one of the smallest known eukaryotes (del Campo et al. 2013).

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Phytomyxea

21

Simon Bulman and Sigrid Neuhauser

Abstract

Phytomyxea are endoparasites of Plantae or heterokont hosts. They are distributed between two orders, the Plasmodiophorida and Phagomyxida. Several phytomyxids cause the formation of large galls on their hosts, but many are only visible via light microscopy of roots, hyphae, or phytoplankton. PCR and sequencing from environmental DNA samples is beginning to reveal many new phytomyxid lineages. Phytomyxids persist in the environment as thick-walled, uninucleate resting spores. These germinate to produce zoospores which locate hosts via the propulsion of heterokont flagellae. Cell penetration by encysting zoospores is via a distinctive projectile-like extrusome. Intracellular growth forms are multinucleate, unwalled protoplasts termed plasmodia. A synapomorphy for the class is cruciform mitotic division at metaphase. Plasmodiophorids occupy a phylogenetically distinct position from most other plant parasites. They cause several economically important diseases such as clubroot of Brassicaceae and powdery scab of potatoes. Plasmodiophorids also act as vectors of damaging plant viruses.

Keywords

Plant parasites • Heterokont parasites • Intracellular • Extrusome • Virus vector • Cruciform divisions • Plasmodiophora

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Summary Classification

●**Phytomyxea**
●●**Plasmodiophorida** (*Plasmodiophora*, *Spongospora*, *Woronina*, etc.)
●●**Phagomyxida** (*Phagomyxa*, *Maullinia*, etc.)

Introduction

General Characteristics

Organisms in the class Phytomyxea are endoparasites of plants or heterokonts. They are distributed between two orders, the Plasmodiophorida, to date found in soil/freshwater, and Phagomyxida, found in marine ecosystems. Phytomyxids persist in the environment as thick-walled, uninucleate resting spores. Upon resting spore germination, the released zoospores locate hosts propelled by smooth heterokont flagellae. Cell penetration is via a distinctive projectile-like extrusome. Intracellular growth forms are multinucleate, unwalled protoplasts termed plasmodia. A synapomorphy for the class is cruciform mitotic division at metaphase. Plasmodiophorids cause several economically important plant diseases – either directly or as vectors of viruses.

Literature and History of Knowledge

The first phytomyxid genus was erected by Woronin in 1877 to accommodate *Plasmodiophora brassicae*, the organism causing clubroot of cabbage. Literature

published in the early 1900s was based largely on light microscopy (Cook 1933). Much of the phytomyxid species discovery occurred during this period, with knowledge summarized in important monographs by Karling (1942, 1968). Ultrastructural and karyological studies predominated into the 1990s, leading to a greatly increased understanding of phytomyxid lifecycles (Dylewski 1990; Braselton 1995; Bulman and Braselton 2014). Considerable research during this period was carried out on the oomycete parasite *Woronina* and on the *Veronica* parasite, *Sorosphaerula veronicae*, whereas little has been published on these organisms in subsequent decades. Continuing research is centered upon phytomyxid species that cause diseases of economically important crops, while reports on other phytomyxids are infrequent. Physical identification of new phytomyxid species, such as the recently described parasite of grapes *Sorosphaerula viticola* (Kirchmair et al. 2005), is rare. With greater focus on marine ecosystems, a small number of new phagomyxid parasites have been discovered (Maier et al. 2000; Schnepf et al. 2000; Goecke et al. 2012). An accessible and important source of information on the plasmodiophorids remains the Plasmodiophorid Home Page (http://oak.cats.ohiou.edu/~braselto/plasmos/).

Beginning with first the DNA-based studies (Mutasa et al. 1993), techniques of molecular biology are now driving large changes to our understanding of phytomyxid biology and diversity. Molecular tools were initially developed for pathogen detection (Buhariwalla et al. 1995), delineation of populations and species within *Polymyxa*, and assessing the presence of plant viruses associated with phytomyxids (Legreve et al. 2002; Kanyuka et al. 2003; Smith et al. 2013). Molecular phylogenetic analyses succeeded in finding a taxonomic home for the phytomyxids in Rhizaria (CavalierSmith and Chao 1997; Archibald and Keeling 2004; Nikolaev et al. 2004; Burki et al. 2010) and confirmed the close relationship of Phagomyxida with Plasmodiophorida (Bulman et al. 2001; Neuhauser et al. 2014). While identification of new phytomyxid lineages in broadly targeted culture-independent studies is not common, such works do suggest a diversity of phytomyxids not yet seen with the naked eye or by microscopy (Takishita et al. 2005, 2007). Importantly, anonymous DNA sequencing coupled with targeted PCR amplification is revealing many new lineages of plasmodiophorids (Neuhauser et al. 2014). Studies of phytomyxid genomes, which were initially slowed by the obligate biotrophic nature of these protists, began with small collections of DNA sequences (Bulman et al. 2006, 2007; Siemens et al. 2009). Early utilization of next-generation sequencing techniques provided a better understanding of the phylogenomics of plasmodiophorids (Burki et al. 2010). A complete mitochondrial DNA sequence from *Spongospora subterranea* (Gutierrez et al. 2014) and the first complete phytomyxid genome sequence, from *P. brassicae* (Schwelm et al. 2015a), have been published. The greatest amount of research continues to be focused on *P. brassicae* infection of the model plant *Arabidopsis thaliana* (Devos et al. 2006; Siemens et al. 2006; Malinowski et al. 2012). Biochemical and cellular characterization of this interaction is accelerating based on new genomic data (Feng et al. 2010; Ludwig-Muller et al. 2015). The first technique for the genetic transformation of *P. brassicae* has recently been published, potentially opening up greater opportunities for characterization of gene function in this parasite (Feng et al. 2013).

A node of clubroot research has developed as a response to the large losses caused by the disease in the Canadian canola industry (Hwang et al. 2012).

Practical Importance

The best studied phytomyxids infect important agricultural food plants worldwide. *Plasmodiophora brassicae* causes clubroot in Brassicaceae (Dixon 2009). *Spongospora subterranea* causes powdery scab of potato (Merz and Falloon 2009). *Spongospora nasturtii* causes crook root disease in watercress (Claxton et al. 1996). *Polymyxa graminis* infects the roots of grasses such as wheat, oats, and rice, and *Polymyxa betae* grows in the roots of sugar beets. Phytomyxids are known to transmit about 20 plant viruses, all but one of which are non-enveloped, positive polarity, single-stranded RNA viruses (Rochon et al. 2004). *Polymyxa* species do not directly cause disease but instead transmit a range of damaging viruses (Kanyuka et al. 2003; Rochon et al. 2004). Viruses transmitted by *P. graminis* include *wheat spindle streak mosaic virus*, *oat golden stripe virus*, *rice necrosis mosaic virus*, and *peanut clump virus*. *Polymyxa betae* transmits viruses including beet *necrotic yellow vein virus*, the cause of sugar beet rhizomania. *Spongospora subterranea* and *S. nasturtii*, respectively, transmit *potato mop-top virus* and *watercress yellow spot virus*. The resting spores of plasmodiophorids remain viable in soil for many years, and there are few pesticides available for control of diseases caused by plasmodiophorids. Once soils become infested with resting spores, it is difficult to continue cropping of the susceptible host.

The ecological roles of phagomyxids in marine environments are a matter of increasing interest but as yet have been little studied (Neuhauser et al. 2011).

Habitats and Ecology

As obligate biotrophs, phytomyxid distribution follows that of their hosts (Table 1). Phytomyxids are mostly not available from culture collections. To date, Plasmodiophorida are found in terrestrial and freshwater environments where they parasitize plants and heterokonts such as *Phytophthora* and *Pythium* spp. Both *P. brassicae* and *S. subterranea* are found worldwide in soils where Brassicaceae and potatoes are grown. *Polymyxa*, *Sorosphaerula*, and *Ligniera* species are common soilborne plant parasites, found widely in arable and natural environments. Several phytomyxids including *Spongospora nasturtii*, *Tetramyxa* spp., *Sorodiscus callitrichis*, and *Membranosorus* spp. parasitize aquatic vascular plants. *Sorodiscus karlingii* infects charophyte algae. Phagomyxida so far discovered are marine parasites of heterokonts, including diatoms and brown algae.

Since the retirement of prominent researchers in this field, knowledge of sites to collect many phytomyxid species is restricted. Records of non-crop-infecting phytomyxids are increasingly sporadic and often in the realm of citizen science. Species of *Tetramyxa*, *Sorodiscus*, *Membranosorus*, and, especially, *Octomyxa* are

Table 1 A selection of the most common and consistently reported phytomyxids together with information on host taxa

Species[a]	Host and location	Citation
Plasmodiophorida		
Ligniera verrucosa	*Veronica*, *Beta*, *Chenopodium*, *Bromus*, and *Festuca* spp. (P, A)	Miller et al. (1985)
L. junci[7]	Especially *Juncus* spp., but many wild and cultivated plants (P, A)	Neuhauser and Kirchmair (2009)
L. pilorum	*Poa annua*, *Bromus inermis* (P, A)	Barr (1979)
Membranosorus heterantherae	*Heteranthera dubia* (P, A)	Forest et al. (1986)
Octomyxa achlyae	*Achlya glomerata* (H, O)	Dylewski (1990)
O. brevilegniae	*Brevilegnia linearis*, *Geolegnia inflata*. (H, O)	Dylewski (1990)
Plasmodiophora brassicae [10]	Brassicaceae plants (P, A)	Dixon (2009)
P. bicaudata	Internodes of *Zostera nana* (P, A)	den Hartog (1989)
P. halophilae	*Halophila* spp. (P, A)	Marziano et al. (1995)
Polymyxa graminis[1 and 6]	Many cultivated and wild grasses including sorghum, oats, wheat (P, A)	Vaianopoulos et al. (2007)
P. betae[5]	Many plants including *Beta vulgaris*, *Chenopodium* spp. (P, A)	Barr (1979)
Sorodiscus callitrichis	Stems of *Callitriche* spp. (P, A)	Robbins and Braselton (1997)
S. karlingii	*Chara contraria*, *C. delicatula* (P, C)	Cook (1933)
Sorosphaerula veronicae[4]	Roots and stems of *Veronica* spp. (P, A)	Miller (1958)
S. viticola[3]	Grapes (*Vitis* sp.) (P, A)	Neuhauser et al. (2009)
Spongospora subterranea[9]	Roots and tubers of *Solanum* spp. (P, A)	Merz and Falloon (2009)
S. nasturtii[15]	*Nasturtium officinale*, *N. microphyllum*. (P, A)	Claxton et al. (1996)
S. campanulae	*Campanula rapunculoides.* (P, A)	Cook (1933)
S. cotulae	*Cotula australis* (P, A)	Karling (1968)
Tetramyxa parasitica	*Ruppia*, *Zannichellia*, *Potamogeton* species (P, A)	Braselton (1990)
Woronina pythii[13]	*Pythium* spp. (H, O)	Dylewski (1990)
W. glomerata	*Vaucheria* spp. (H, YGA)	Dylewski (1990)
W. leptolegniae	*Leptolegnia caudata*. (H, O)	Karling (1981)
W. cokeri	*Pythium* spp. (H, O)	Robbins and Braselton (1997)
Env[2]	Glacier forefield soil, Austria	Neuhauser et al. (2014)
Env[8 and 12]	Fynbos soil, South Africa	Neuhauser et al. (2014)
Env[11, 14, 16]	Volga soil, Russia	Neuhauser et al. (2014)
Phagomyxida		
Phagomyxa algarum	*Ectocarpus mitchellae*, *Pylaiella fulvescens* (H, BA)	Karling (1944)
P. bellerocheae	*Bellerochea malleus* (H, D)	Schnepf et al. (2000)
P. odontellae	*Odontella sinensis* (H, D)	Schnepf et al. (2000)

(*continued*)

Table 1 (continued)

Species[a]	Host and location	Citation
Maullinia ectocarpii	*Ectocarpus siliculosus* (H, BA)	Maier et al. (2000)
Maullinia sp.	*Durvillaea antarctica* (H, BA)	Goecke et al. (2012)
Env (2)	Anoxic sediments	Takishita et al. (2005) and Takishita et al. (2007)
Plasmodiophora diplantherae	Stem galls *Halodule wrightii* (P, A)	Braselton and Short (1985)

H Heterokontophyta, *P* Plantae, *A* Angiosperm, *C* Charophyta, *O* Oomycota, *D* diatom, *BA* brown algae, *YGA* yellow green algae. Infections of green plants occur in roots unless otherwise stated
[a]Numbers in superscript indicate phylogenetic clades from Neuhauser et al. (2014). A subset of phytomyxid clades that have only been detected via environmental sequencing (Env) is also shown. A citation specifically focused on the organism in question, or a review article giving such information, is also provided

currently little studied. Although the distribution of many phytomyxids is reportedly limited, some studies of herbarium samples suggest a broader geographical distribution than previously recognized (Forest et al. 1986; den Hartog 1989).

Anonymous DNA sequencing techniques have revealed new phagomyxid lineages in anoxic marine/saline environments (Takishita et al. 2005, 2007) and plasmodiophorid lineages from geographically widespread soil and rhizosphere sites (Lesaulnier et al. 2008; Bass et al. 2009; Neuhauser et al. 2014). Although anonymous phytomyxid sequences have not so far been associated with specific hosts, the low diversity of sequences in any one sample, and greater abundance in rhizosphere versus bulk soil, implies a close relationship with plant hosts (Neuhauser et al. 2014). Cloning of anonymous sequences also suggests that oomycete-infecting phytomyxids are diverse and widely distributed in soils (Neuhauser et al. 2014).

Characterization and Recognition

By far the majority of phytomyxid life-cycle research has been carried out on plasmodiophorids such as *P. brassicae* (Kageyama and Asano 2009), *S. veronicae* (Miller 1958), and *W. pythii* (Dylewski 1990), rather than phagomyxids. The most recognized phytomyxid life cycle has a bipartite format; a composite life-cycle scheme, most strongly drawn from plant-infecting Plasmodiophorida, is presented here (Fig. 1).

Penetration of Host

Phytomyxids persist over time through environmentally resistant resting spores. These germinate to produce heterokont *primary* zoospores that exhibit a cyclotic

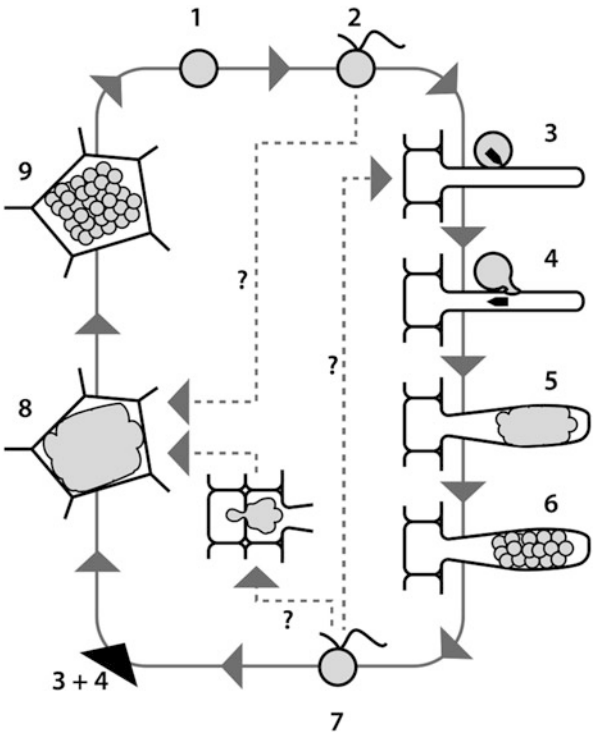

Fig. 1 A Phytomyxid life-cycle scheme drawn mostly from plasmodiophorid infection of crop plants. Variations to this life cycle may occur in some species such as among the marine phagomyxids. *1*, environmentally resistant resting spore; *2*, biflagellate primary zoospore; *3*, location of host cell by zoospore and commencement of encystment – for many plasmodiophorids, primary infection occurs in root hairs; *4*, cell penetration by Stachel followed by zoospore contents; *5*, development of multinucleate plasmodium; *6*, multilobed structure containing zoosporangia; *7*, secondary zoospore – reinfection of host cells occurs by encystment as in *3* and *4*, or via a myxamoeboid phase (dashed lines to 8); *8*, secondary plasmodium; *9*, resting spores or aggregates thereof. Dashed lines indicate uncertainty about direct progression to secondary infection mediated via primary zoospores or generation of new cycles of primary infection by secondary zoospores

swimming motion. On encountering the host, zoospores retract their flagellae and begin a characteristic infection process termed encystment. An infection apparatus develops within the zoospore, consisting of a tubular cavity (Rohr) containing a bullet-like structure (Stachel), with one end oriented in the direction of the host wall (Keskin and Fuchs 1969; Aist and Williams 1971). The Rohr rapidly contracts, and the Stachel penetrates the host wall followed by the unwalled, uninucleate protoplast of the parasite, which is presumably forced out by turgor pressure created by the expansion of a large vacuole in the encysted zoospore (Fig. 1).

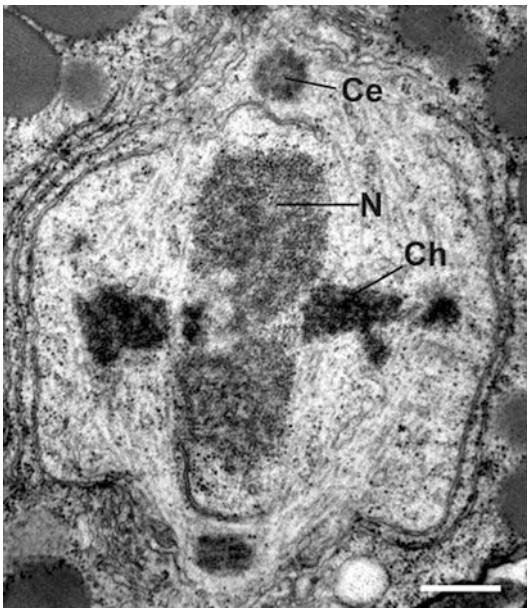

Fig. 2 Transmission electron micrograph of cruciform division in sporogenic plasmodium of *Plasmodiophora brassicae* on Chinese cabbage (*Brassica rapa*). *N* nucleolus, *Ce* centriole, *Ch* chromatin. Scale bar = 0.5 μM. Photograph James Braselton, Plasmodiophorid Homepage

Sporangial Plasmodia

Inside infected cells, the uninucleate protoplast matures into a zoosporangial plasmodium, with a 9–24 nm host-plasmodiophorid interface (Aist and Williams 1971; Braselton and Miller 1975; Miller and Dylewski 1983a). Synchronous mitotic divisions yield a multinucleate plasmodium (Dylewski and Miller 1983). These cruciform divisions are the major synapomorphy defining phytomyxids (Braselton and Miller 1975). At metaphase, chromatin aligns at the equator of the nucleus, perpendicular to the elongating, persistent nucleolus. A symmetrical cross is formed that can be seen by light microscopy (Fig. 2) (Dylewski et al. 1978; Garber and Aist 1979).

After the mitotic divisions, the plasmodium cleaves into a thin-walled multicelled structure in the infected cell (Fig. 3). Zoospore formation occurs as the protoplasm within each zoosporangium cleaves. *Secondary* zoospores from zoosporangia may be released outside of the host, into adjacent cells, or into the same cell. Conspicuous exit tubes may be formed between the zoosporangia and adjacent host cells (Littlefield et al. 1998).

Sporogenic Plasmodia

The sporogenic phase culminates in the formation of thick-walled resting spores (Fig. 4). At the cessation of sporogenic division, cleavage furrows appear and meiosis begins (Dylewski and Miller 1984). Nucleoli begin to disperse during

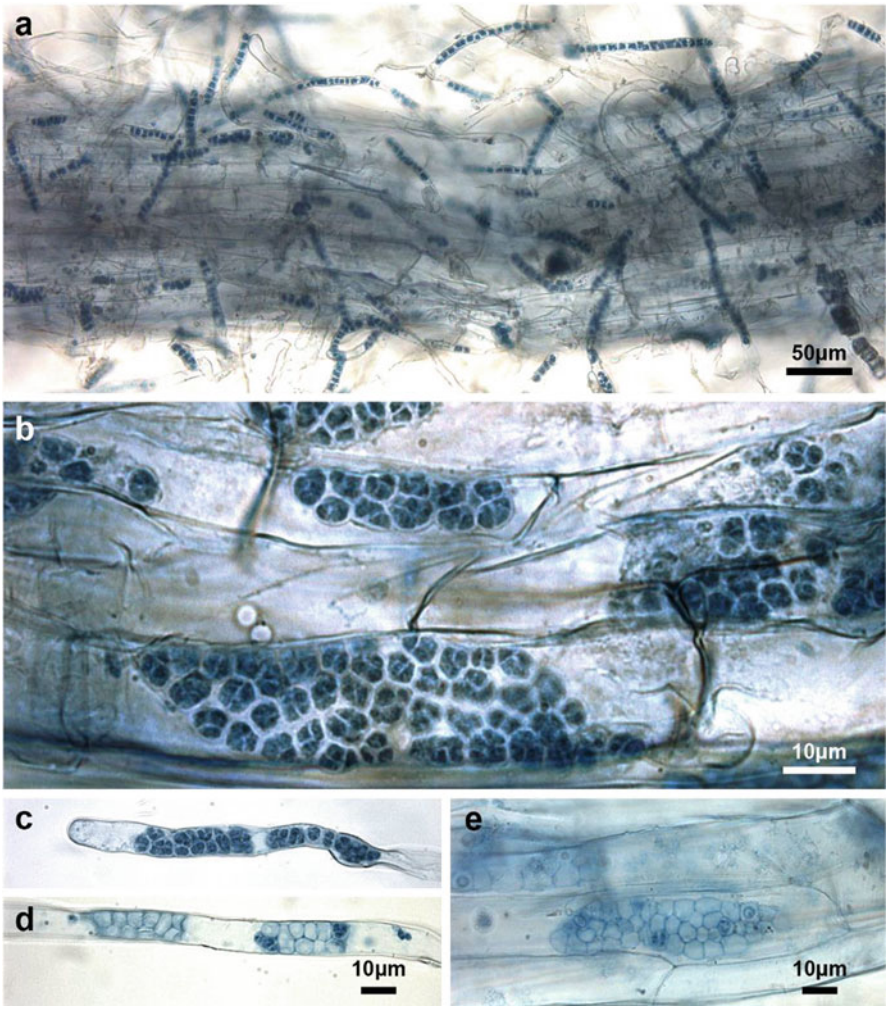

Fig. 3 *Spongospora subterranea* zoosporangia in trypan blue stained potato roots. (**a**) root with heavily infected root hairs; (**b**) infected root epidermal cells; (**c**) root hair with zoosporangia; (**d**) and (**e**) root hair and epidermal cells containing empty zoosporangia following zoospore release. Photographs Richard Falloon

prophase of meiosis I, rendering the nuclei less obvious in the plasmodium. It is believed that chromosome number is halved as a result of meiosis during resting spore formation (Dylewski 1990). Eventually, each nucleus is partitioned, forming binucleate resting spores. One nucleus presumably undergoes degeneration because all mature resting spores become uninucleate (Dylewski and Miller 1984).

Many phytomyxids have resting spores arranged in aggregate bodies called sporosori. For example, *Sorodiscus* sporosori are usually composed of two closely

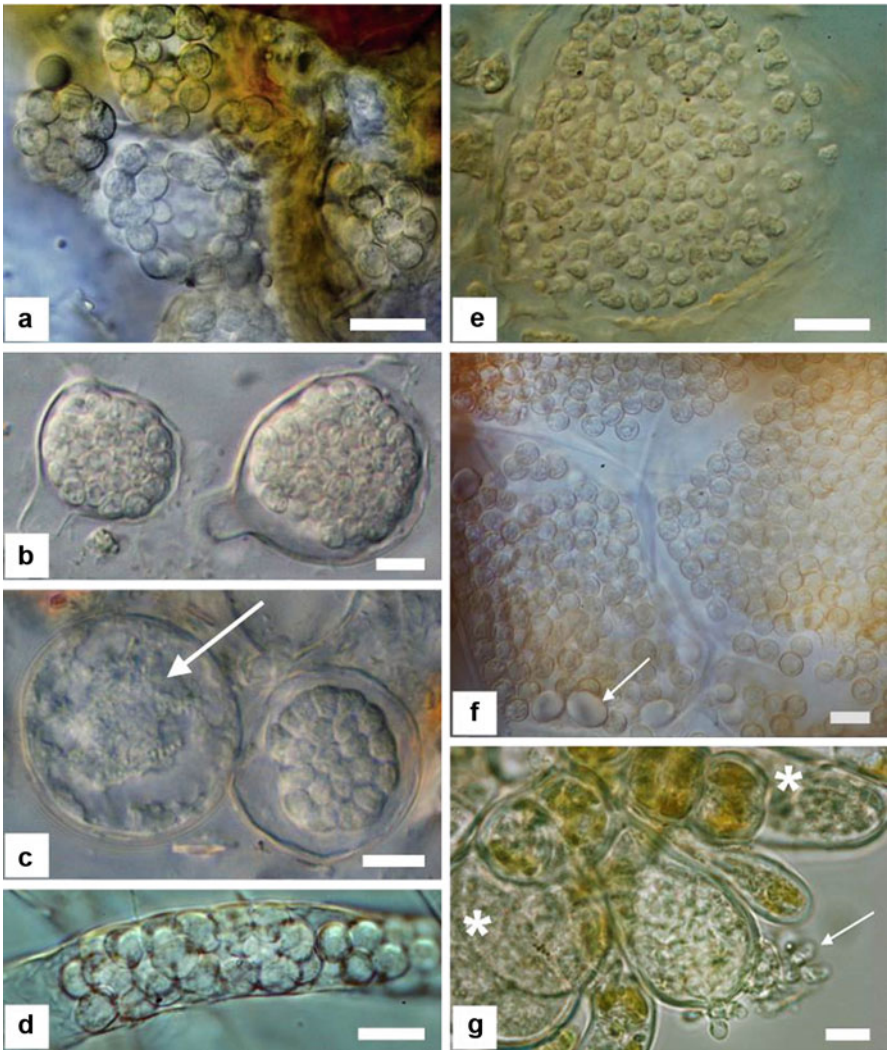

Fig. 4 Morphology of resting spores from selected phytomyxids: *left column*, Plasmodiophorida; *right column*, Phagomyxida. (**a**) *Sorosphaerula viticola*: hollow sporosori in the roots of *Vitis* sp. (**b**) *Woronina pythii*: resting spores in *Pythium* sp. (**c**) *W. pythii* in *Pythium* sp.: lobose plasmodium, just starting to develop into resting spores (*arrow*); right mature resting spores. (**d**) *Ligniera junci*: resting spores in the root hairs of *Juncus effusus*. (**e**) *Maullinia* sp. resting spores in *Durvillaea antarctica*. (**f**) *Plasmodiophora diplantherae*: resting spores in enlarged cells of *Halodule* sp. *Arrow*: starch grains. (**g**) *Maullinia ectocarpii*: hatching zoospores (*arrow*) from an enlarged infected cell of the host *Ectocarpus fasciculatus*. *Plasmodia in enlarged host cells. Scale bar = 10 μM. Photographs Sigrid Neuhauser

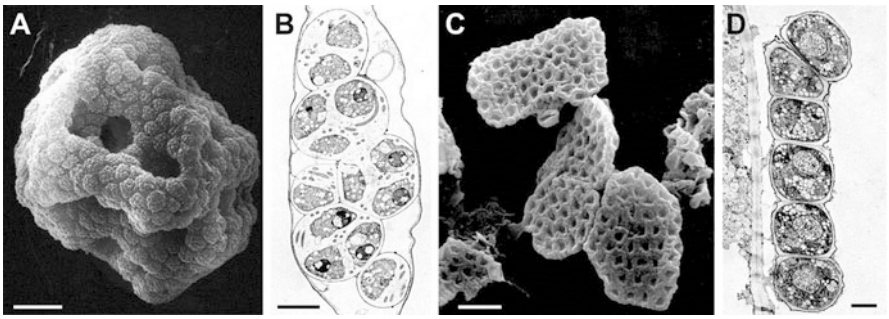

Fig. 5 (**a**) Scanning electron micrograph of *Spongospora subterranea* sporosorus showing individual spores with punctate outer surface ornamentation. Scale bar = 10 μM. Photograph Ueli Merz. (**b**) Transmission electron micrograph of *S. subterranea* secondary zoospores in zoosporangia. Scale bar = 3 μM; (**c**) Scanning electron micrograph of *Membranosorus heterantherae* sporosori. Scale bar = 8 μM. (**d**) Transmission electron micrograph of a sporosorus of *M. heterantherae*. Scale bar = 1 μM. (**b**–**d**) Photographs James Braselton, Plasmodiophorid Home Page

pressed layers of resting spores, whereas those of *Membranosorus* occur in a single layer usually lining the inner periphery of the host cell (Fig. 5). *Spongospora subterranea* sporosori are particularly large and distinctive; approximately 200–700 resting spores are aggregated into spongelike structures of variable size (Falloon et al. 2011) (Fig. 5).

Sporogenic plasmodia become more abundant as the host ages. In several plasmodiophorid species, sporangial and sporogenic plasmodia are distinguishable by their occurrence in separate tissues. In *P. brassicae*, sporangial plasmodia develop in root hairs and epidermal cells, whereas sporogenic plasmodia are found in the root cortex and stele. In *S. veronicae*, sporosori are produced only in galls on shoots and not during root infections. In *S. subterranea*, zoosporangia occur soon after infection in root epidermal cells, while sporosori are formed later in root galls and tuber lesions. Although sporangial development typically precedes sporogenic development, both stages can be seen in the same tissue early in *Polymyxa* infection (Ledingham 1939). Primary zoospores may be capable of initiating both sporangial and sporogenic plasmodia in *P. brassicae* (Mithen and Magrath 1992; McDonald et al. 2014). The biochemical and developmental factors that determine the transition to sporogenic growth are not known. In cultures, the state of the culture medium appears to have an influence on the development path of *Woronina* plasmodia (Miller and Dylewski 1983b).

Sporogenic development is associated with growth of hypertrophic plant galls characteristic of several plasmodiophorid diseases. While sporogenic development is considered to be initiated via secondary zoospore infection, there are persistent

reports of direct penetration of the root cortex. Indeed, a myxamoeboid stage is nearly an accepted part of the plasmodiophorid life cycle, despite the exact nature of this stage remaining unclear (Mithen and Magrath 1992; Claxton et al. 1996; Kobelt et al. 2000; Asano and Kageyama 2006). Few genes encoding proteins with cellulose-binding domains, which might be involved in the cell wall modification needed to penetrate into new plant cells, were detected in the *P. brassicae* or *S. subterranea* transcriptomes (Schwelm et al. 2015b).

Nutrition

There are some unanswered questions about the degree of phagotrophy in phytomyxids. As the genus name suggests, ingestion of host material has been reported as a feature of nutrition in *Phagomyxa* (Karling 1944; Schnepf et al. 2000). On the other hand, during early sporangial growth of plasmodiophorids, pseudopodial-like extensions of protoplasm grow outward and partially surround host organelles and cytoplasm, but it has generally been agreed that these fail to completely surround host cytoplasm and that there is consequently no phagotrophic nutrition (Dylewski 1990).

Karyogamy

The occurrence of karyogamy in phytomyxids is not well understood. Protoplasm fusion was claimed to occur between haploid secondary zoospores or between the nuclei in plasmodia, prior to resting spore formation and the onset of meiosis (Ingram and Tommerup 1972). Potential karyogamy in sporogenic *P. brassicae* plasmodia has also been reported (Buczacki and Moxham 1980).

Atypical-Host Infection

An unusual feature of plasmodiophorids is their appearance in a wide range of hosts beyond those in which they complete a full life cycle. Primary plasmodia have been observed in the roots of such atypical plant hosts, with little or no evidence for progression to secondary plasmodia. For example, *S. subterranea* and *P. brassicae* have been observed in many plant species other than their respective *Solanum* and Brassicaceae hosts (Ludwig-Muller et al. 1999; Qu and Christ 2006). A model for atypical host infection is provided by the *Polymyxa-Arabidopsis thaliana* interaction (Desoignies et al. 2010). Frequent host shifts have occurred during the evolution of phytomyxids; whether these host shifts are related to promiscuous host infections at primary stages remains to be investigated (Neuhauser et al. 2014).

Maintenance and Cultivation

Collection and Isolation

Phytomyxids are "isolated" from soil or water samples through infection of their hosts. Plasmodiophorids may be collected from wild sources or from hosts deliberately planted in infested potted soils. Plant-infecting species of *Plasmodiophora* (Fig. 6), *Spongospora*, *Sorosphaerula*, *Sorodiscus*, and *Tetramyxa* produce obvious galls or hypertrophies that are easily collected from infected plants. *Spongospora subterranea* is most frequently collected from sporosori-filled scabs on potato tubers (Fig. 6). *Ligniera* and *Polymyxa* species must be found by microscopically examining the roots of hosts, which is time-consuming due to the lack of external symptoms (Fig. 4).

Woronina and *Octomyxa* spp. are typically attracted to hosts growing on seeds added to water or water amended with soil. Samples may be baited with specific oomycetes if available. Oomycete-infecting plasmodiophorids are then detected by light microscopy (Fig. 4).

Phagomyxid species are identified through microscopic surveys of marine heterokont hosts. *Phagomyxa odontellae* and *P. bellerocheae* are found infecting diatoms in marine phytoplankton samples (Fig. 7) (Schnepf 1994; Schnepf et al. 2000). *Maullinia* spp. can be collected from galls on marine brown algae macrophytes (Fig. 8). Resting spores were observed for *Maullinia* infecting *Durvillaea antarctica*, raising the possibility that this species may be maintained in a viable form within collections (Goecke et al. 2012).

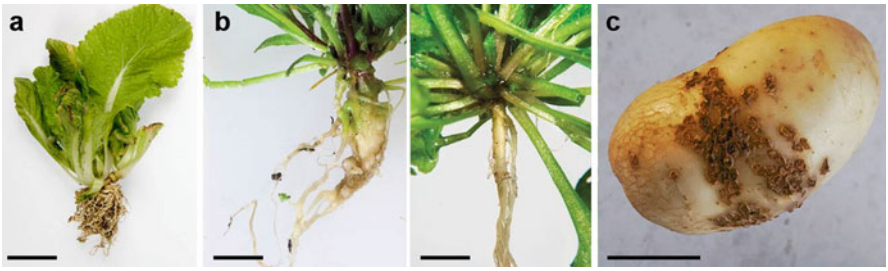

Fig. 6 Plasmodiophorid plant infections. (**a**) Chinese cabbage (*Brassica rapa*) plant showing heavy clubroot symptoms (*Plasmodiophora brassicae* infection). Scale bar = 10 cm; (**b**) *Arabidopsis thaliana* Columbia-0 plants with (*left*) and without clubroot infection. Scale bars = 1 cm. Photograph Robert Lamberts/Simon Bulman; (**c**) Potato tuber with severe symptoms of powdery scab (*Spongospora subterranea)*. Scale bar 3 cm (Photograph Robert Lamberts/Richard Falloon)

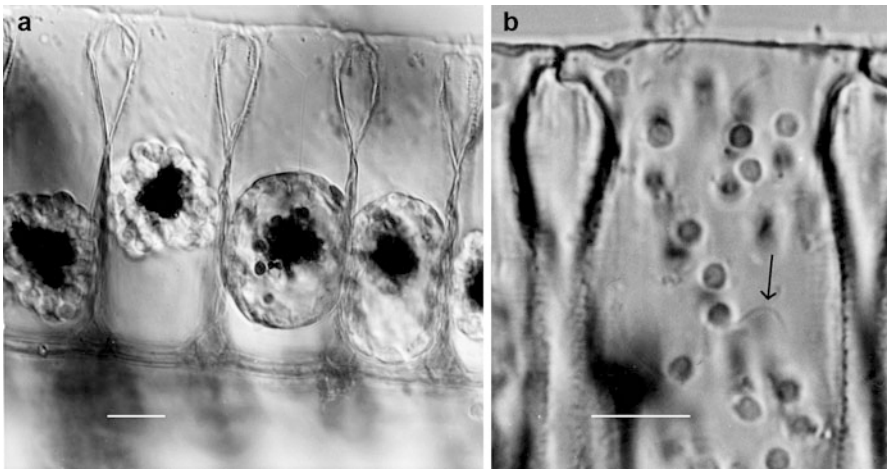

Fig. 7 *Phagomyxa bellerocheae* infecting the diatom *Bellerochea malleus*. (**a**) Plasmodia containing secondary zoospores. (**b**) Released zoospores with whiplash flagellae (*arrowed*). Scale bars 10 μM. Photographs Eberhard Schnepf

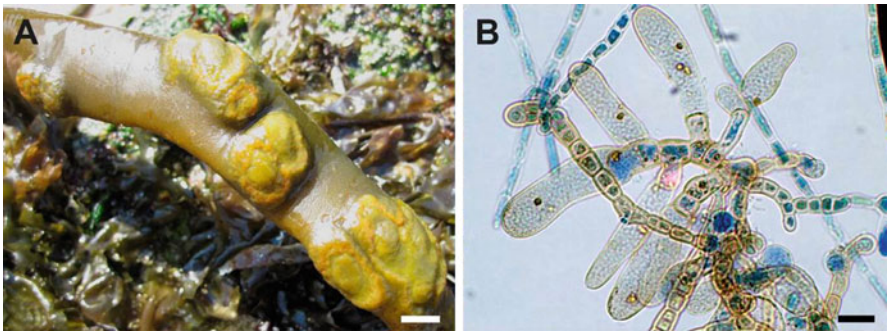

Fig. 8 *Maullinia*. (**a**) Gall-like structures on infected *Durvillaea antarctica* fronds from central Chile. Scale bar = 1 cm. Photograph Franz Goecke; (**b**) Type slide at the NHM London (registration number: 2000:2:29:1) showing zoosporangia of *Maullinia ectocarpi*. Scale bar = 10 μm. Photograph Sigrid Neuhauser

Cultivation

Studies of the interactions between phytomyxids and their hosts are most tractable for *P. brassicae* which infects the model plant *Arabidopsis thaliana* (Fig. 6). For manipulating *P. brassicae*, spore suspensions are prepared by maceration and filtering of decayed galls (Castlebury et al. 1994). For new plant infection, the suspension is applied to soil surrounding plant seedlings. Temperature and pH are important for disease progression, with 20 °C and pH <7 being typical conditions for maximal

P. brassicae growth. Clubroot galls or *P. brassicae* spore suspensions may be kept frozen in a viable state for at least 3 years.

Sporosori samples from *S. subterranea* are prepared by scraping scabs from potato tubers followed by air drying and sieving. A solution-culture assay (Merz 1989) has been adopted for studying the potato-*S. subterranea* interaction.

Although phytomyxids cannot be cultured in the absence of their hosts, several publications have detailed the establishment of dual cultures of plasmodiophorids with plant cells. *Plasmodiophora brassicae* and *S. subterranea* have been grown in hormone-induced callus/cell cultures (Buczacki 1983; Asano and Kageyama 2006; Bulman et al. 2011). *Plasmodiophora brassicae*, *S. subterranea*, and *P. betae* have been grown with *Agrobacterium*-induced in vitro hairy root cultures (Mugnier 1987; Qu and Christ 2007).

Dual cultures of plasmodiophorids and oomycete hosts in water and "soft" agar media have been established for *W. pythii* and *W. cokeri* (Miller and Dylewski 1983a). Resting spores of *W. pythii* may be dried on filter paper and germinated by rehydration after up to 14 months at 6 °C (Miller and Dylewski 1983a). Laboratory co-cultures of *Maullinia ectocarpii* with a range of brown algae macrophytes have been established under controlled conditions (Maier et al. 2000).

Evolutionary History

Classification

Phytomyxids are likely to be at least 400 million years old based on fossil records (Taylor et al. 1992). For a long period, their taxonomic position was unstable, oscillating between fungi, slime molds, and protozoa (Barr 1981). The first ribosomal DNA sequence from *P. brassicae* provided evidence for a relationship between plasmodiophorids and Cercozoa (Cavalier-Smith and Chao 1997; Castlebury and Domier 1998). Assembly of sequences from a greater diversity of protists has confirmed that this grouping with Rhizaria (Nikolaev et al. 2004; Bass et al. 2005, 2009) and that Phagomyxida belong in Phytomyxea (Bulman et al. 2001). Plasmodiophorid polyubiquitin sequences were shown to have an unusual amino acid insertion, as do those from Cercozoa and Foraminifera (Archibald et al. 2003; Archibald and Keeling 2004). Phytomyxids fall within the subphylum Endomyxa that includes a mixture of free-living and parasitic organisms including vampyrellid amoebae (predators), *Filoreta* (bacterivores), Ascetosporea (parasites of marine invertebrates), and *Gromia* (Bass et al. 2009). Phylogenomic studies have mostly indicated that Endomyxa is a distinct clade (Burki et al. 2010; Sierra et al. 2013; Cavalier-Smith et al. 2015). The exact phylogenetic position of Phytomyxea relative to other endomyxans remains to be finalized, although ribosomal phylogenies point to vampyrellids as close relatives (Bass et al. 2009).

Phytomyxea genera were historically designated by the aggregation of resting spores in sporosori and by ultrastructure, with less emphasis placed on host affiliations. However, *S. nasturtii* was raised to species rank partly on the basis of its

significantly different host to *S. subterranea* (Dick 2001), while *W. cokeri* was moved to the genus *Woronina* harboring other oomycete parasites (Robbins and Braselton 1997). Across the last century, a large number of phytomyxids were described; many of the reported species appear to have been synonyms or were doubtful taxa, as reviewed in Karling (1942; 1968). A summary of some of these taxa is presented in Table 1. Although each phytomyxid genus could once be uniquely identified by spore arrangement and ultrastructure (Dylewski 1990), it is now clear that neither feature provides a firm framework for understanding within-group relatedness. As with the overall phylogenetic position of Phytomyxea, the internal relationships of the group have been radically altered by the advent of DNA techniques. Ribosomal small subunit RNA phylogenies showed large evolutionary distances between plasmodiophorid species that were largely indistinguishable by morphology. For example, *Spongospora subterranea* and *S. nasturtii* were found to be phylogenetically remote from one another (Bulman et al. 2001). Even more strikingly, *Plasmodiophora diplantherae* was shown to be a phagomyxid rather than plasmodiophorid (Neuhauser et al. 2014). Anonymous DNA sequencing coupled with specific PCR has now revealed many new distinct lineages, especially within Plasmodiophorida (Neuhauser et al. 2014); a selection of these environmental lineages is listed in Table 1. An intermixed cluster of *Polymyxa*, *Sorosphaerula*, and, to a lesser degree, *Ligniera* species was confirmed (Neuhauser et al. 2014). This group of genera appears ripe for taxonomic revision based on a combination of ecological and DNA data. Anonymous DNA sequences also indicated a significant diversity of lineages in the *Woronina* clade (Neuhauser et al. 2014). It will be highly informative to use molecular techniques to link these *Woronina*-like lineages with their, presumably, oomycete hosts. Phagomyxid lineages in marine ecosystems await exploration with the techniques of molecular ecology.

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Paramyxida

22

Robert J. G. Lester and P. Mike Hine

Abstract

Paramyxidans are obligate parasites of marine invertebrates. They produce a characteristic delicate spore with one cell within a second cell. In some genera, these are enclosed within a third cell and even a fourth cell. Life cycles are generally unknown. In two *Marteilia* species that are major pathogens of oysters, there could be an alternate host in the life cycle.

Keywords

Paramyxida • *Marteilia* • *Marteilioides* • *Paramarteilia* • Oyster pathogen • Aber disease • QX disease

Contents

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Summary Classification

●**Paramyxida**
●●***Paramyxa*** (*P. paradoxa, P. nephtys*)
●●***Eomarteilia*** (*E. granula*)
●●***Marteilia*** (*M. refringens, M. sydneyi, M. cochillia, M. chungmuensis*)
●●***Paramarteilia*** (*P. orchestiae, P. canceri, P. branchialis*)

Introduction

The Paramyxida currently comprise a small group of species that differ from other protistans in that their spores consist of several cells, one inside another, that arise from internal cleavage within a mother cell (Fig. 1). Paramyxidans are also commonly referred to as paramyxeans, a reflection of the unclear taxonomic status of the group (Ward et al. 2016). Known paramyxidans all develop within marine invertebrates: polychaetes, crustaceans, or mollusks.

Thirteen species are recognized (Table 1). There are at least 15 other records of paramyxidan-like organisms in marine invertebrates including an enigmatic form in a tunicate (Choi et al. 2006; Carrasco et al. 2012). Most records of the phylum are from Eurasia (Ward et al. 2016) though there are reports of paramyxids from Florida

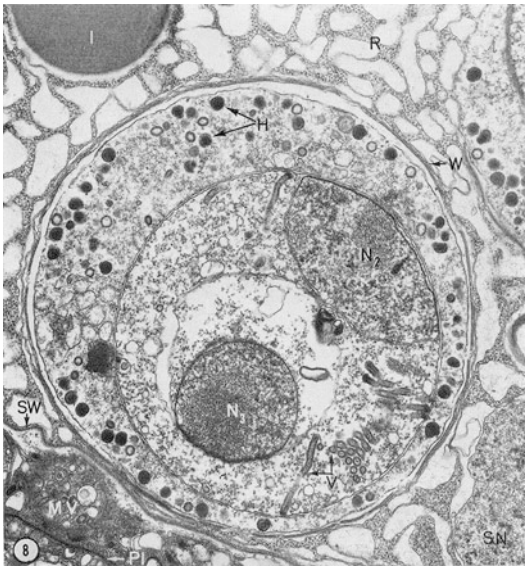

Fig. 1 *Marteilia sydneyi* maturing spore showing the three sporoplasms, the nuclei of the two innermost ones being visible. *H* haplosporosomes, *I* refringent granule, *MV* multivesicular body, N2 and N3 nuclei of cells C4 and C5, *PL* wall of primary cell (C1), *R* reticulated cytoplasm, *SN* sporont (C2) nucleus, *SW* sporont wall, *V* vesicles, *W* spore wall (From Perkins and Wolf 1976)

Table 1 Recognized paramyxidan species

		Sporulation	
Paramyxidan	Host	Organ	References
Paramyxa paradoxa	*Poecilochaetus serpens*	*Gut*	Chatton (1911)
Paramyxa nephtys	*Nephtys caeca*	*Gut*	Larsson and Køie (2005)
Eomarteilia granula[a]	*Ruditapes philippinarum*	*Dig.gl.*	Itoh et al. 2014
Marteilia refringens[a]	*Ostrea edulis*	*Dig.gl.*	Grizel et al. (1974)
Marteilia cochillia	*Cerastoderma edule*	*Dig.gl.*	Carrasco et al. (2013)
Marteilia christenseni	*Scrobicularia piperata*	*Dig.gl.*	Comps (1983 [1985])
Marteilia lengehi	*Saccostrea cucullata*	*Dig.gl.*	Comps (1976)
Marteilia sydneyi[a]	*Saccostrea glomerata*	*Dig.gl.*	Perkins and Wolf (1976)
Marteilia octospora	*Solen marginatus*	*Dig.gl.*	Ruiz et al. 2016
Paramarteilia orchestiae	*Orchestia gammarellus*	*Testis*	Ginsburger-Vogel and Desportes (1979b)
Paramarteilia canceri	*Cancer pagurus*	*Systemic*	Feist et al. (2009)
Marteilioides chungmuensis	*Crassostrea gigas*	*Ovary*	Comps et al. (1986)
Marteilioides branchialis	*Saccostrea glomerata*	*Dig.gl.*	Anderson and Lester (1992)

Dig.gl. digestive gland
[a]Associated with mass mortalities

(Moyer et al. 1993) and Mexico (Grijalva-Chon et al. 2015). The Florida parasite destroyed local scallops suggesting it was an exotic introduction, and the *M. refringens* in Mexico probably originated from Europe. Three records of paramyxidans from the ovaries of oysters within ports but not elsewhere suggest that their distributions can be extended by shipping (Becker and Pauley 1968; Wolf 1977; Hine and Thorne 2000).

Paramyxidans develop within tissues of invertebrates both inside and outside tissue cells. Sporulation typically occurs in the digestive epithelium or gonad cells of the host. Three species, *Marteilia refringens*, *M. sydneyi*, and *M. (*syn. *Marteilioides) chungmuensis*, have had and continue to have devastating effects on oyster industries around the world. Most of what we know about the biology of paramyxidans come from studies on these three species.

Habitats and Ecology

The best known species, *Marteilia refringens,* sporulates within the tissues of *Ostrea edulis*. Infections occur throughout the summer. Sporulation takes place at temperatures over 17 °C, much of the epithelium of the digestive gland is destroyed and 50–90% of the oysters may die. The paramyxidan infection is the cause of "Aber

Disease" in *O. edulis* in Western Europe. As a result of its pathogenicity and that of *Bonamia ostreae*, a haplosporidian, *O. edulis* has been replaced by *C. gigas* in the most of the European oyster industry. The paramyxidan also infects and sporulates in *Mytilus edulis, M. galloprovincialis, Solen marginatus, Chamelea gallina*, *Ostrea stentina*, and possibly other *Ostrea* species. In *Mytilus* spp. it causes little significant mortality, apparently depending on the species and strain of host (Berthe et al. 2004). Infections in *M. galloprovincialis* inhibit gonad regeneration after the first spawning (Villalba et al. 1993). It shows considerable variability, the strain commonly found in mussels having been regarded as *M. maurini* (see Lopez-Flores et al. 2004, 2008).

Marteilia sydneyi develops in *Saccostrea glomerata* in Australia. The epithelium of the digestive gland becomes swollen with sporulating parasites, and heavily infected oysters die within 6 weeks (Wolf 1979). In southeast Queensland and northern New South Wales, the parasite infects over short periods in late summer (Lester 1986; Rubio et al. 2013). To avoid "QX Disease," as the infection is known, farmers empty estuarine leases in December (late spring) and restock in April (early autumn). In mid-New South Wales, epizootics are less regular but destroy most of the year's production when they occur.

Marteilia chungmuensis develops within the ovary of *C. gigas, Crassostrea nippona,* and *Saccostrea echinata*. In Japan it causes large nodules to form in the gonad of *C. gigas* rendering the oysters unmarketable (Ngo et al. 2003) (Fig. 2). Oysters develop disease only in the summer; low temperature inhibits the development of the infective stage in winter, although it is present all the year (Tun et al. 2008a). Infected oysters spawn later in the year than normal, and infected oocytes are sterile (Tun et al. 2008b). Those oysters that survive the winter lose their infection as they change to males. Like oysters with *M. refringens*, those infected by *M. chungmuensis* become watery from depleted glycogen reserves, though the paramyxidan is not very pathogenic (Tun et al. 2008b).

Paramarteilia orchestiae in the testes of male gammarids result in the crustaceans becoming females or intersex forms (Ginsburger-Vogel 1991). Crabs infected by the systemic *Paramarteilia canceri* are lethargic and have shrunken internal organs (Feist et al. 2009).

Fig. 2 *Crassostrea gigas* with ovarian swellings caused by infection with *Marteilioides chungmuensis* (Photo N. Itoh)

Characterization and Recognition

Life Cycle

In *Marteilia refringens*, *M. sydneyi*, and *M. chungmuensis*, initial stages occur within the tissues of the gills, palps, to a lesser extent mantle, and anterior gut. After entry the paramyxidans divide, and bicellular stages are liberated into the surrounding connective tissue and hemolymph spaces. Following systemic dissemination, the parasite infiltrates the target organ. In *M. sydneyi* the outer cell forms a nurse cell beneath the epithelial cells of a digestive tubule. Within this cell, daughter cells and eventually sporonts develop (Figs. 3, 4, and 5). A nurse cell has not been reported from *M. chungmuensis*, which develops in the ovary (Itoh et al. 2004).

Spores, enclosed within a propagule (sporont) singly or in pairs or groups depending on the species, are released with oyster feces or, in the case of *M. chungmuensis*, enclosed in an ovum and released through the genital canal (Tun et al. 2008b). The next stage in the life cycles is not clear. Though Comps and Joly (1980) found what appeared to be young stages of *M. refringens* in *M. galloprovincialis* after exposing them to a homogenate from infected *O. edulis*, the life cycles have been generally considered to be indirect. Attempts by Balouet et al. (1979) and others to infect oysters with *M. refringens* by feeding or injecting infected homogenized digestive gland, by cohabitation with infected stock or by introduction of oysters into the field after a disease event, failed to produce infections detectable by histology. Lester (1986) made the same observations with *M. sydneyi*. These negative results, together with the poor survival of *M. sydneyi* spores in seawater (Roubal et al. 1989; Wesche et al. 1999) compared to the long periods of

Fig. 3 Proposed development of *Marteilia sydneyi* in *Saccostrea glomerata*. (***A***) Initial replication in the gill and palp epithelium. (***B***) Disseminating cell enters subepithelium of digestive tubule to form nurse cell Nc. (***C***) Daughter cells (*Dc*) internally cleave primary cells (*Sc*) which then internally cleave 8–16 secondary cells (sporonts) each containing two multicellular spores (From Kleeman et al. 2002)

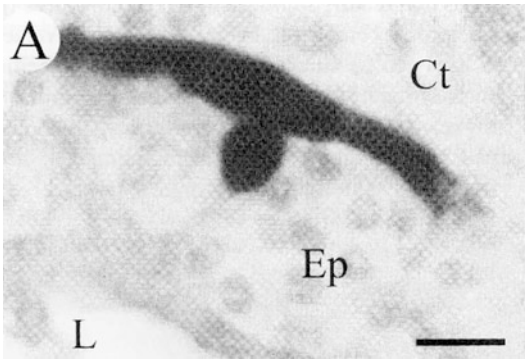

Fig. 4 *Marteilia sydneyi* nurse cell under digestive epithelium (*Ep*) of *Saccostrea glomerata* (ISH, from Kleeman et al. 2002). *Ct* connective tissue, *L* tubule lumen; bar = 5 um

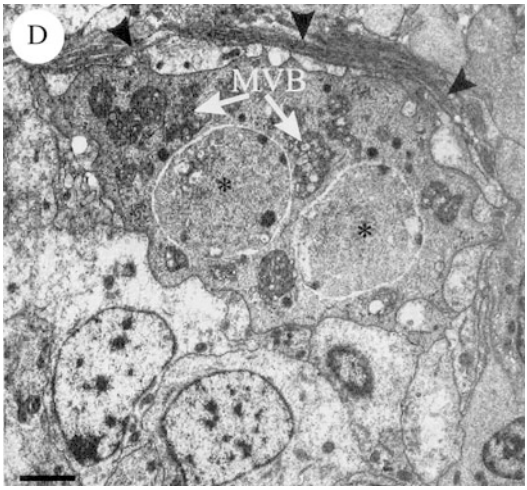

Fig. 5 *Marteilia sydneyi* nurse cell containing two daughter cells (*) (From Kleeman et al. 2002). *Arrow heads* basal membrane of tubule epithelium, *MVB* multivesicular bodies; bar = 1 um

a year or more between disease outbreaks, led to a search for an alternate host. A non-specific DNA probe SMART2 has enabled researchers to visualize paramyxeans in putative alternate hosts particularly copepods (Audemard et al. 2004; Carrasco et al. 2008; Boyer et al. 2013; Arzul et al. 2014) and polychaetes (Adlard and Nolan 2015).

It is not certain that an alternate host is required. In a Mediterranean lagoon, infections by *M. refringens* were common in mussels, found by histology, and confirmed by PCR but were not detected, by histology, in oysters in the same area (Arzul et al. 2014). On the east coast of Australia, using PCR and in situ hybridization, *M. sydneyi* was shown to be present in *S. glomerata* in areas where it had never been detected by histology and which had no history of the disease (Adlard and Wesche 2005). Lester (1986) found that oysters with overt infections could survive the summer and may have been able to carry the disease through the winter. These

results have moved the research focus to the conditions that precipitate the rapid and extensive sporulation that kills the oysters.

High temperature has been associated with high prevalence of a number of paramyxidans. In northern France *M. refringens* is most prevalent in *O. edulis* during the summer when the temperature is over 17°C (Balouet et al. 1979; Audemard et al. 2004). In the Mediterranean it is abundant, in *M. galloprovincialis*, in early and late summer (Boyer et al. 2013). Prevalence of *M. chungmuensis, in C. gigas,* increased in summer (Imanaka et al. 2001), and mortality associated with *Marteilia conchillia*, in *Cerastoderma edule*, occurred when temperatures were up to 29 °C (Carrasco et al. 2015), though *Eomarteilia granula* in *Ruditapes philippinarum* was detected (by histology) more frequently in winter (Itoh et al. 2014).

For *M. sydneyi*, onset of disease has been linked to high temperatures and sudden changes in salinity (Lester 1986; Green et al. 2011; Rubio et al. 2013). Under normal conditions *S. glomerata* appear to be able to kill sporulating parasites and survive. Low salinity has been shown to inhibit defense mechanisms, but as low salinity is not invariably associated with disease other factors must be at play. Immunosuppression in *S. glomerata* has been demonstrated to occur with a range of factors such as extremes of temperature, physical agitation, magnesium chloride, and starvation. The environmental stressors needed to trigger a lethal outbreak of QX disease are currently unknown (Raftos et al. 2014).

Selective breeding of *S. glomerata* for QX resistance has been successful. Dove et al. (2013) found up to 72% survival by progeny in a QX outbreak after four generations of selection versus 3% survival in controls. Dang et al. (2011) observed 80% survival after five generations versus 14% in controls. The resistance appears to be from an enhanced ability to kill *M. sydneyi*, rather than block its entry, a capacity apparently linked to the high number of granulocytes in the hemolymph of resistant oysters (Dang et al. 2011). However, even with genetically bred resistant oysters, the resistance can be overwhelmed (Dang et al. 2013), and the continued susceptibility of local oysters in an area endemic for the disease suggests that the natural development of resistance may involve the loss of another capability (Thompson et al. 2015).

Paramarteilia orchestiae in amphipods of the genus *Orchestia* and an unknown paramyxidan in the amphipod *Echinogrammarus marinus* appear to be vertically transmitted (Ginsburger-Vogel 1991; Short et al. 2012).

Ultrastructure

During the sporulation of paramyxidans, primary cells (C1) bud endogenously to form secondary cells (C2-sporont), in which tertiary cells (C3) bud to form the outer spore wall in which further cells bud (C4–C6) (Fig. 6). Primary cells, tertiary cells of most species, and some spore cells (Larsson and Køie 2005) contain haplosporosomes, but secondary cells do not (Figs. 1, 6, and 7). Haplosporosomes have an external and an internal unit membrane, may contain DNA (Perkins 1968), have glycoproteins in the core and membranes with more glycoproteins in the outer membrane than the inner, and the outer has a lipid component (Azevedo and Corral

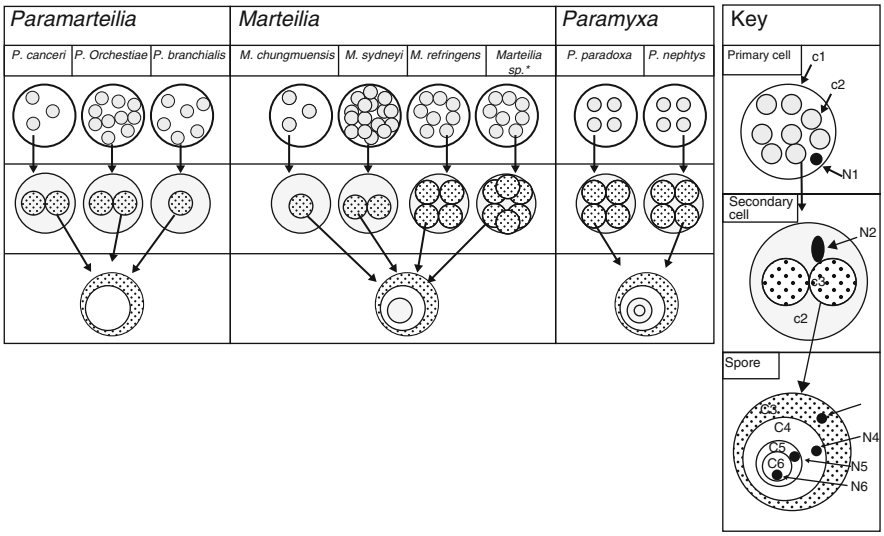

Fig. 6 Proposed division of the genera based on the number of cells in the spore. Marteilia sp.* is *Marteilia* sp. of Lopez and Darriba (2006). Numbered nuclei are shown in the key on the right (From Feist et al. 2009)

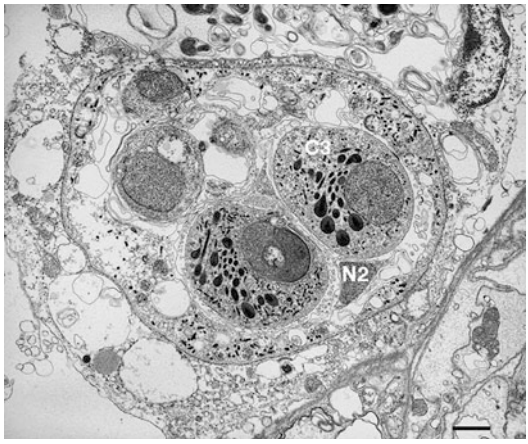

Fig. 7 *Paramarteilia canceri* early pansporoblast (sporont) containing secondary cells and tertiary cells (*C3*) with typical electron-dense bulbous haplosporosomes. The nucleus of the secondary cell (*N2*) is constrained to the periphery of the secondary cell and assumes a triangular appearance on cross section. Bar = 0.5 um

1985). In some haplosporidians, the outer membrane fuses with the parasite plasma membrane permitting exocytosis of the haplosporosome (Hine et al. 2002). Endogenous budding appears to occur by the linking up of cytoplasmic vesicles to form membranes (Perkins and Wolf 1976; Ginsburger-Vogel and Desportes 1979a, b; Anderson and Lester 1992; Larsson and Køie 2005). Primary and secondary cells become compressed during sporogony, the nuclei often appear triangular in cross section (Fig. 7).

The Primary Cell (C1)

Primary stem cells are often amoeboid (Comps et al. 1986; Kleeman et al. 2002) and contain many ribosomes, smooth endoplasmic reticulum (sER), and haplosporosomes, in all species. They may develop dendritic extensions used to nourish the cell (Kleeman et al. 2002). Multivesicular bodies (MVBs) have been reported from *Marteilia christenseni, M. sydneyi, Paramarteilia branchialis*, and *Paramarteilia* spp., but not *P. paradoxa, P. nephtys,* or *M. refringens* and may be involved in haplosporogenesis (Perkins and Wolf 1976). Mitochondria occur in the primary cells of *P. paradoxa* and *M. sydneyi* but have not been reported in other species. *Marteilia refringens* and *M. christenseni* primary cells have cytoplasmic striated platelike inclusions (Grizel et al. 1974; Comps 1983; Longshaw et al. 2001).

The Secondary Cell (C2) or Sporont

Secondary cells have many ribosomes and vesicles and may have mitochondria (Perkins and Wolf 1976; Ginsburger-Vogel and Desportes 1979b; Desportes 1981; Comps 1983; Anderson and Lester 1992), centrioles (Ginsburger-Vogel and Desportes 1979a; Comps et al. 1986; Larsson and Køie 2005; Feist et al. 2009), or cytoplasmic refringent granules (Perkins and Wolf 1976; Comps 1983; Itoh et al. 2014). Haplosporosomes are absent, but in *M. branchialis* (Anderson and Lester 1992), and possibly *M. chungmuensis* (Fig. 7 in Comps et al. 1986) they develop after nuclear division but before tertiary cell formation. In *P. nephtys* and *P. canceri*, there are perinuclear masses of dense material resembling those seen in early haplosporogenesis in haplosporidians (Larsson and Køie 2005; Feist et al. 2009; Hine et al. 2002). The C2 of *M. chungmuensis* and C3 of *M. branchialis* are encircled by layers of sER (Comps et al. 1986; Anderson and Lester 1992). In *M. refringens* and *M. sydneyi*, C2 persists as the wall of the propagule which is released from the oyster and which contains refringent granules and spores.

The Tertiary Cell (C3) or Spore Wall Cell

Tertiary cells usually contain ribosomes, sER, vesicles, and haplosporosomes, but seldom mitochondria (Perkins and Wolf 1976), and form the outer layer of the spore. Exceptions are *P. paradoxa* in which haplosporosomes only occur in C1 and C4 (Desportes 1981), and *P. nephtys* in which haplosporosomes occur in C1, C4, and C5 (Larsson and Køie 2005). Large haplosporosomes occur in C3 cells of *P. orchestiae* (see Ginsburger-Vogel and Desportes 1979b), and *P. canceri,* and in the latter are associated with many MVBs (Feist et al. 2009) (Figs. 7 and 8). In *P. nephtys*, after degeneration of C1 and C2, C3 forms a sac around the spores from material released

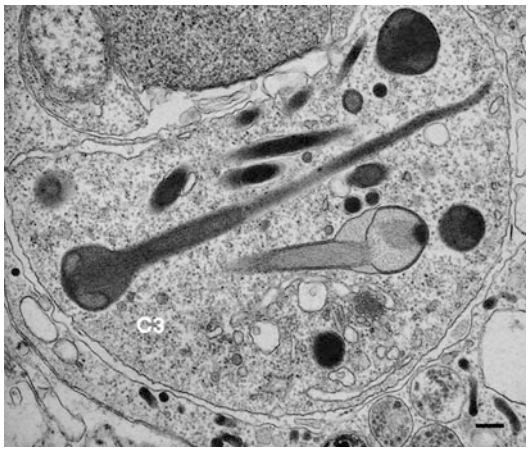

Fig. 8 *Paramarteilia canceri* tertiary (*C3*) cell with bulb-ended haplosporosomes. Bar = 0.2 um

from regularly arrayed ridges on its surface (Larsson and Køie 2005). The spore wall of *P. paradoxa* is underlain by a cytoskeleton of microtubules (Desportes 1981), possibly associated with cell division. Its spores lie in sacs comprising the residual membrane of the sporont (Desportes 1981). Subsequent shrinkage and appearance of fibrous material results in the formation of striated projections on the spore wall. The C3 of *E. granula* (Itoh et al. 2014; Ward et al. 2016) has a thick external wall, unlike other genera. In *M. refringens* central thin laminated structures are present within the C3 (Grizel et al. 1974). In *M. sydneyi* the C3 produces a thick layer of concentric membranes that surrounds the spore cells (Perkins and Wolf 1976). In *M. christenseni* the C3 forms a double membrane containing fine granular osmophilic material (Comps 1983).

Spore Cells (C4–C6)

C4 cells, sometimes called intermediate cells (Grizel et al. 1974; Perkins and Wolf 1976; Desportes 1981; Comps 1983; Larsson and Køie 2005), and their subsequent endogenous cells are rich in ribosomes and sER. They may include mitochondria and haplosporosomes, although these vary between species. In *E. granula, M. sydneyi*, and *M. christenseni*, flattened vermiform vesicles occur in intermediate cells, but only in C3 of *M. refringens* (Perkins and Wolf 1976; Comps 1983; Grizel et al. 1974). Larsson and Køie (2005) state that C4 and C5 of *P. nephtys* have haplosporosomes, which are spherical in C4 and rodlike in C5. The sporoplasms of *M. refringens* contain central thin laminated structures (Grizel et al. 1974). Otherwise, C4 onward are uniformly described as being small and dense with ribosomes, sER, and haplosporosomes and the nuclei having prominent nucleoli. The nuclei are usually round and dense, but in the inner sporoplasm of *P. paradoxa* they are horseshoe shaped (Desportes 1981).

Taxonomic Position of Paramyxidans

The taxonomy of the Paramyxida has frequently changed (Berthe et al. 2004), and the group has been recognized as a phylum (Desportes and Perkins 1990), separate from the Haplosporidia and the Myxosporea (Berthe et al. 2000), although they and haplosporidians have also been regarded as separate orders within the phylum Cercozoa (Cavalier-Smith and Chao 2003a, b). The relationship of paramyxidans with haplosporidians and myxosporeans has been studied because all three groups possess prominent cytoplasmic haplosporosomes (paramyxidans, haplosporidians) or sporoplasmosomes (myxosporeans), and myxosporeans, like paramyxidans, divide by endogeny. The occurrence of endogeny in the Paramyxida has led to the suggestion that they are intermediate between protists and multicellular organisms (Desportes 1984). Paramyxidan haplosporosomes resemble those of haplosporidians in the disappearance of haplosporosome-like bodies from early vegetative cells, to be reformed in the spore, and in the occurrence of invaginations in the nuclear surface and perinuclear dense granular material (Feist et al. 2009). Haplosporosomes in haplosporidian spores and sporoplasmosomes of myxosporeans develop from similar membranous structures. There is no apparent role for haplosporosomes in any of the three groups, except perhaps release and the lysis of surrounding cells in some haplosporidians and myxosporeans.

The suggestion by Cavalier-Smith and Chao (2003a, b) that *Marteilia refringens* is a haplosporidian is not supported by ultrastructure, spore formation, spore structure (Hine et al. 2009), or analysis of the small subunit ribosomal gene sequence (Berthe et al. 2000). Paramyxidans divide by endogeny and possess centrioles (Ginsburger-Vogel and Desportes 1979a), while haplosporidians do not undergo endogeny and appear to lack centrioles, although a reticulated structure in some haplosporidians may be a degenerate centriole (Hine et al. 2002).

Taxonomy Within the Paramyxida

The taxonomy of paramyxidans has been based on the pattern of endogenous cleaving of the primary or stem cell, giving rise to secondary cells, which become sporonts when further cleaving occurs to produce spores. However, the taxonomy is confusing as some authors follow cleaving patterns, primary cells giving rise to secondary cells that develop to tertiary cells, etc., while others regard the cleaving within secondary cells as sporulation, the secondary cell being a sporont. There is also confusion as to whether a primary stem cell gives rise to a single secondary cell within a cytoplasmic vacuole (Desportes 1981), and primary cells are less common than would be expected (Berthe et al. 2004), given current interpretations of development.

Definitions of genera have included:

Paramyxa Chatton, 1911: Primary cell produces two to four secondary cells or sporonts. Each sporont produces four tetracellular spores.

Paramyxoides Larsson and Køie 2005: Primary cell produces two to four secondary cells or sporonts. Each sporont produces four tetracellular fusiform spores.

Paramarteilia Ginsburger-Vogel and Desportes, 1979b: As *Marteilia* spp. but producing a bicellular spore.

Eomarteilia (syn. *Marteilia*) *granula* Itoh, Yamamoto, Kang, Choi, Green, Carrasco, Awaji, Chow 2014: Primary cell gives rise to eight secondary cells (sporangia), each containing four spores, comprising innermost, intermediate, and outermost cells.

Marteilia Grizel, Comps, Bonami, Cousserans, Duthoit and Le Pennec, 1974: Secondary cells (sporanges) each give rise to eight cells.

Marteilioides: (Comps et al. 1986): Production of one tertiary cell from each secondary cell. Differentiation of a tricellular spore from the sporont.

Paramyxa and *Paramyxoides* are similar in having two to four secondary cells, each of which produces four tetracellular spores, and both are parasites of the digestive tracts of polychaetes. However, *Paramyxoides* was distinguished from *Paramyxa* because the latter has rod-shaped spores with plugs at each end and the innermost cell (cell 4) contains haplosporosomes, while the former has fusiform spores, and cells 4 and 5 contain haplosporosomes (Larsson and Køie 2005). There is no formal definition of the genus *Marteilia* or the species *M. refringens,* only microscopic observations on tissue tropism and development, which showed that the secondary cell (sporont) produces eight cells (Grizel et al. 1974), while the congeneric *M. sydneyi* sporont produces 8–16 cells (Berthe et al. 2004). There is also disagreement over whether the secondary cell (Berthe et al. 2004) or the tertiary cell is the sporont (Desportes and Perkins 1990). The genus *Marteilioides* is defined as producing tricellular spores (Comps et al. 1986), but the congeneric *M. branchialis* produces bicellular spores (Anderson and Lester 1992).

A recent review of eukaryote taxonomy (Adl et al. 2012) defines the Paramyxida as having a bicellular spore, consisting of a parietal cell and one sporoplasm, without an orifice. The definition needs to be broadened to reflect the variation in sporogony observed in *Paramyxa* and *Paramyxoides,* which have tetracellular spores and *Marteilioides* with tricellular spores. In describing the stages, terms such as sporont, sporoplasm, and spore suggest function. When the function is unknown, C1–C6 have been used.

A proposal has been made to revise the taxonomy of paramyxidans with emphasis on the number of cells comprising the spore, the number of cells produced by secondary cells (sporonts), and the shape of the spores (Feist et al. 2009) (Fig. 6). This proposal gives emphasis to the spore cell number, the number of secondary cells, and similarity in host group and tissue tropism, resulting in only three genera. An analysis of known 18S sequences was not quite congruent with this classification. Ward et al. (2016) concluded that *Marteilia granula* was distinct from other *Marteilia* species and proposed the genus *Eomarteilia* for this species. Their analysis also suggested that the species *Marteilia chungmuensis* was close to the *Paramarteilia* clade despite having a more complex spore and suggested that *Marteilioides* should be retained for *M. chungmuensis*. No 18S sequence is currently available for *Marteilioides branchialis*. As 18S sequences are not invariably good discriminators of genera, it would be useful to have sequences from other parts of the genome to clarify relationships.

Classification

Paramyxida (Chatton 1911)

Sporulation results from series of internal cleavages within an amoeboid stem cell that germinates spores in tissues of invertebrate animals. Development characterized by production of offspring cells that remain inside the parent cell. Spores consist of several cells enclosed inside each other. Parasitic. Four genera in proposed revision of the phylum (Feist et al. 2009):

Paramyxa Each secondary cell produces four tertiary cells (spores), each of which contain three additional cells. Sporulate in polychaetes. The genus comprises two species, *P. paradoxa* and *P.* (*Paramyxoides*) *nephtys.*

Eomarteilia Each of the eight secondary cells produces four spores composed of three cells. Sporulation in *Ruditapes philippinarum.* Currently a monotypic genus, phylogenetically basal to *Marteilia* spp.

Marteilia Secondary cells produce variable number of tertiary cells, each of which contain a further two cells. Sporulate in mollusks. The genus comprises *M. refringens, M. sydneyi,* and *M.* (*Marteilioides*) *chungmuensis* (but see note above).

Paramarteilia Secondary cells produce variable number of tertiary cells, each of which contains a further single cell. Sporulate in mollusks and crustaceans. The genus comprises *P. orchestiae, P. canceri*, and *P.* (*Paramarteilia*) *branchialis.*

This scheme proposes that from the number of cells in the spore, *Paramyxoides* is congeneric with *Paramyxa*, that *M. branchialis* be transferred to the genus *Paramarteilia,* and that *M. chungmuensis* is a species of *Marteilia*.

P. paradoxa and *P. nephtys* are more similar to each other than to other known genera in their pattern of sporogony and in having elongated rather than spherical spores. However, in *P. paradoxa* the cytoskeleton, rodlike spores with terminal plugs in which transverse section are rosette like (Desportes 1981) and differ considerably from the ridges in the spore wall of C3, the sacs around spores, their fusiform shape and elongated striated projections in *P. nephtys* (see Larsson and Køie 2005). Retention of separate genera may be valid but as Larsson and Køie (2005) observe, they are probably con-familial.

In *Paramarteilia* spp., the C3 of *P. orchestiae* have large dense osmiophilic bodies (Ginsburger-Vogel and Desportes 1979b), but, in the C3 of *P. canceri,* bacilliform haplosporosomes develop from MVBs, and large elongated haplosporosomes with bulbous heads are present (Feist et al. 2009) (Figs. 7 and 8). In the C3 of *M. branchialis*, large (>300 nm in diameter) dense osmiophilic bodies and haplosporosomes are present (Anderson and Lester 1992), suggesting they may be congeneric with *Paramarteilia* spp. However, the C3 of *M. branchialis* (Anderson and Lester 1992) is encircled by layers of sER similar to those of *M. chungmuensis* (see Comps et al. 1986) and unlike other genera making the placement of *M. branchialis* in the genus *Paramarteilia* less certain.

Eomarteilia is distinguished by the thick wall in C3 and flattened vesicles in C4.

It appears that *Marteilia* spp. may be distinguished from other paramyxidan genera by the presence of flattened vermiform vesicles associated with the plasma

membrane of C3 in *M. refringens* or intermediate cells of *M. sydneyi* and *M. christenseni* (Grizel et al. 1974; Perkins and Wolf 1976; Comps 1983). It is unclear whether *M. chungmuensis* possesses similar vesicles, although the text of Comps et al. (1986) mentions "des trabécules vermiformes opaques aux électrons," none are visible in the electron micrographs.

Maintenance and Cultivation

These organisms require host cells in order to develop. The required cell lines are not yet available. Though Ginsberger-Vogel and Carre-Lecuyer (1976) were apparently able to infect gammarids by implanting tissue from an infected gammarid, it has not yet been possible to infect oysters in the laboratory by this method (Balouet et al. 1979; Lester 1986) or even to have early infections thrive in already infected oysters (Tun et al. 2008a; Lester unpubl.), possibly because the environmental stressors and/or nutritional requirements of the target tissues are not met in laboratory tanks.

Evolutionary History

The evolutionary history of paramyxidans depends on whether they belong in an order within the cercozoans (Cavalier-Smith and Chao 2003a, b) or are an independent phylum not closely related to other eukaryotes (Berthe et al. 2000). If the former is the case, they are rhizarians, a group comprising radiolarians and foraminiferans which have an abundant fossil record extending back to the early Cambrian. The fossil record of the cercozoan *Difflugia* extends back to the Neoproterozoic, 1,000–542 ± 1 million years ago (Finlay et al. 2004). However, while molecular studies have shown a close relationship between cercozoans and foraminiferans in the amino acid sequences of their α-tubulin, β-tubulin, and actin (Takashita et al. 2005), in their polyubiquitin (Archibald et al. 2003), and SSU rRNA (Berney and Pawlowski 2003) genes sequences, cercozoans are morphologically diverse, and no morphological feature distinguishes them from other protists. Their position within the Cercozoa is uncertain because they differ from all other protists in their SSU rRNA gene sequences (Berthe et al. 2000) and because they are multicellular (Desportes 1984). As they are soft bodied, there is no paramyxidan fossil record.

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Haplosporidia

23

Carlos Azevedo and P. Mike Hine

Abstract

Haplosporidian species (phylum Haplosporidia Caullery & Mesnil, 1899) are a small group of four genera of sometimes pathogenic protozoan parasites usually with uninucleated spores. They are widely distributed in marine and freshwater invertebrates, although their status in Africa, much of Eurasia and Central and South America, is largely unknown. They infect molluscs including commercially important bivalves and other molluscs, annelids, crustaceans, ascidians, trematodes, turbellarians, and probably many invertebrate groups. The morphology, development, and ultrastructure of the four haplosporidian genera (*Haplosporidium*, *Minchinia*, *Urosporidium*, and *Bonamia*) are described using light, scanning, and transmission electron microscopy. Several new haplosporidian species have recently been described based on their genetic sequences, spore morphology, and ornamentation. Ultrastructural organization of the spores and the origin of the spore wall ornamentation are discussed. This phylum contains 52 described species and several unnamed species reported in the four genera. Life cycle stages involve exosporulation of the endosporoplasm to form multinucleate plasmodia and sporoblasts giving rise to the spores that are described from some species. The phylogenetic analysis based on SSU rRNA strongly supports that the genera *Minchinia*, *Urosporidium,* and *Bonamia* are monophyletic, while

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the genus *Haplosporidium* is paraphyletic. The taxonomic positions and affinities between these genera within phylum Haplosporidia are discussed. The negative economic impact on the commercially important infected hosts is reported.

Keywords
Haplosporidia • Protozoa • Parasites • *Haplosporidium* • *Minchinia* • *Urosporidium* • *Bonamia* • Life cycle • Ultrastructure • Taxonomy • Phylogeny

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Introduction

General Characteristics

The phylum Haplosporidia (syn. Aplosporidia Caullery & Mesnel, 1899; Ascetospora Sprague, 1979; or Balanosporidia Sprague, 1979) is a protozoan group that infects several tissues/organs of different species of some invertebrate groups. These include molluscs (Arzul and Carnegie 2015), such as bivalves (Bower and McGladdery 2003; Burreson and Ford 2004), gastropods (Azevedo 1984; Azevedo et al. 2006; Burreson 2001; Vea and Siddall 2011; Ituarte et al. 2014), chitons (Ball 1980), arthropods (Newman et al. 1976; Larsson 1987; Dyková et al.

1988; Lipa and Hokkanen 1991; Marchand and Sprague 1979; Perkins 1975; Bower and Meyer 2002; Stentiford et al. 2004, 2013; Winters and Faisal 2014), annelids (Ormières 1980), platyhelminths (De Turk 1940; Zaika and Dolgikh 1963), echinoderms (La Haye et al. 1984), tunicates (Ormières and de Puytorac 1968), and other groups (see below). Some haplosporidians infect parasites in clams, cockles, oysters, and other invertebrates (Perkins 1971, 1979; Perkins et al. 1975; Carballal et al. 2005; Le et al. 2015).

They appear to infect mainly commercially important molluscs from freshwater, brackish, and marine environments, but this reflects research effort to date, and it is likely that they are ubiquitous in other invertebrate groups. Haplosporidians occur in different regions of the world (Azevedo 1984; Perkins 2000; Bower and Meyer 2002; Hine and Thorne 2002; Bower and McGladdery 2003; Burreson and Ford 2004; Ituarte et al. 2014), although their status in many regions (Africa, Central Eurasia, Central and South America) is largely or totally unknown. They are sometimes responsible for significant mortality in their hosts (Haskin et al. 1966; Diggles et al. 2002; Hine and Thorne 2002; Hine et al. 2002b; Renault et al. 2002; Burreson and Ford 2004; Cranfield et al. 2005; Bearham et al. 2008a, b, c).

Historically, the phylum Haplosporidia contained three genera: *Haplosporidium* Caullery & Mesnil, 1899; *Minchinia* (Lankester, 1895) Labbé, 1896, and *Urosporidium* Caullery & Mesnil, 1905 (Figs. 1a–c, 2a–i, and 3a–c). However, electron microscope and molecular phylogenetic studies support the inclusion of the genus *Bonamia* Pichot et al. 1980 within the phylum Haplosporidia (Fig. 3d, e) (Hine and Jones 1994; Hine et al. 2001; Reece et al. 2004; Carnegie et al. 2006). Another genus *Mikrocytos* Farley et al. 1988 comprised two species, *Mikrocytos mackini* and *Mikrocytos roughleyi*, which are not known to form spores, but *M. roughleyi* was later reclassified as a haplosporidian, *Bonamia roughleyi* (Carnegie et al. 2000; Cochennec-Laureau et al. 2003). However, a subsequent molecular study could not identify the organism as a separate species from *B. exitiosa* (Carnegie et al. 2014), and the disease, "winter mortality" with which *M. roughleyi* was associated, cannot be attributed to *B. exitiosa/roughleyi* clade (Engelsma et al. 2014; Spiers et al. 2014).

The phylum Haplosporidia at the present consist of 52 species in four genera (*Haplosporidium*, *Minchinia*, *Urosporidium*, and *Bonamia*) and several unnamed species. They form spores with an uninucleated endosporoplasm surrounded by the spore wall that has an orifice (micropyle) and a complex membranous system named spherulosome (formerly named spherule) (Azevedo and Corral 1985, 1989). The orifice is either covered by a hinged operculum on the other face of the orifice or occluded by a lingula that covers the inner edge of the orifice (Perkins 1971). Haplosporosomes are cytoplasmic inclusions of haplosporidian spores that characterize this taxonomic group. These structures are randomly distributed throughout the cytoplasm and are delimited by a membrane and characterized as being electron-dense organelles with variable morphology (Figs. 2d, h, i, and 3a, c). The spores of the genera *Haplosporidium* and *Minchinia* are similar, possessing an orifice covered by a hinged operculum differentiated from spore wall, differing in the presence or absence of "ornaments" attached to the spore wall (Fig. 2a–d). The lack of uniform terms to describe these structures has caused some taxonomic confusion (Azevedo

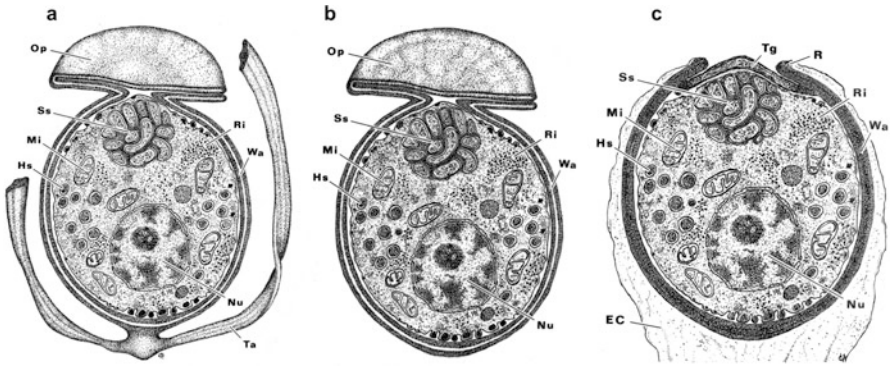


Fig. 1 Schematic drawings showing the morphological and taxonomic differences among the spores of the three genera of the phylum Haplosporidia: (**a**) *Haplosporidium lusitanicum* showing a spore with two tails, as ornaments of the spore wall, and the operculum; (**b**) *Minchinia* sp. showing a spore with the operculum without any ornaments; (**c**) *Urosporidium* sp. showing a spore with the apical orifice of the spore closed by a flap of wall material and the epispore cytoplasm extending posteriorly into an ephemeral tapering extension. *Abbreviations*: *Op* operculum, *Ss* spherulosome, *Mi* mitochondria, *Hs* haplosporosomes, *Ta* tail, *Nu* nucleus, *Wa* spore wall, *Ri* ribosomes, *EC* epispore cytoplasm, *R* rim, *Tg* lingula

2001; Azevedo et al. 2006; Burreson 2001; McGovern and Burreson 1990; Burreson and Reece 2006).

At the present, it is difficult to characterize and define Haplosporidia. Some species, particularly those of crustaceans (Newman et al. 1976; Dyková et al. 1988; Bower and Meyer 2002; Stentiford et al. 2004, 2013; Nunan et al. 2007) and some *Bonamia* spp. (Pichot et al. 1980; Hine et al. 2001), lack spores, and bodies similar to the characteristic haplosporosomes are not recognizable in some species (Bower and Meyer 2002; Stentiford et al. 2004).

Literature and History of Knowledge

Over a century ago, two French researchers, Maurice Caullery and Félix Mesnil in 1899, created the genus *Aplosporidium* to contain the species *A. scolopli* and *A. heterocirri*, two sporozoan parasites of marine annelids. They proposed the genus *Aplosporidium* that was considered an incorrect transliteration according to the Article 32 of the International Code of Zoological Nomenclature. Later, the correct transliteration was applied and thereby altered to the new name *Haplosporidium* (Sprague 1963b). The phylum Haplosporidia consists of four genera: *Haplosporidium*, *Minchinia*, *Urosporidium*, and *Bonamia* (Burreson and Ford 2004). The status of the two major genera, *Haplosporidium* and *Minchinia*, continues to be confused and is a source of disagreement between some authors (Azevedo et al. 1999, 2003; Perkins 2000; Burreson 2001).

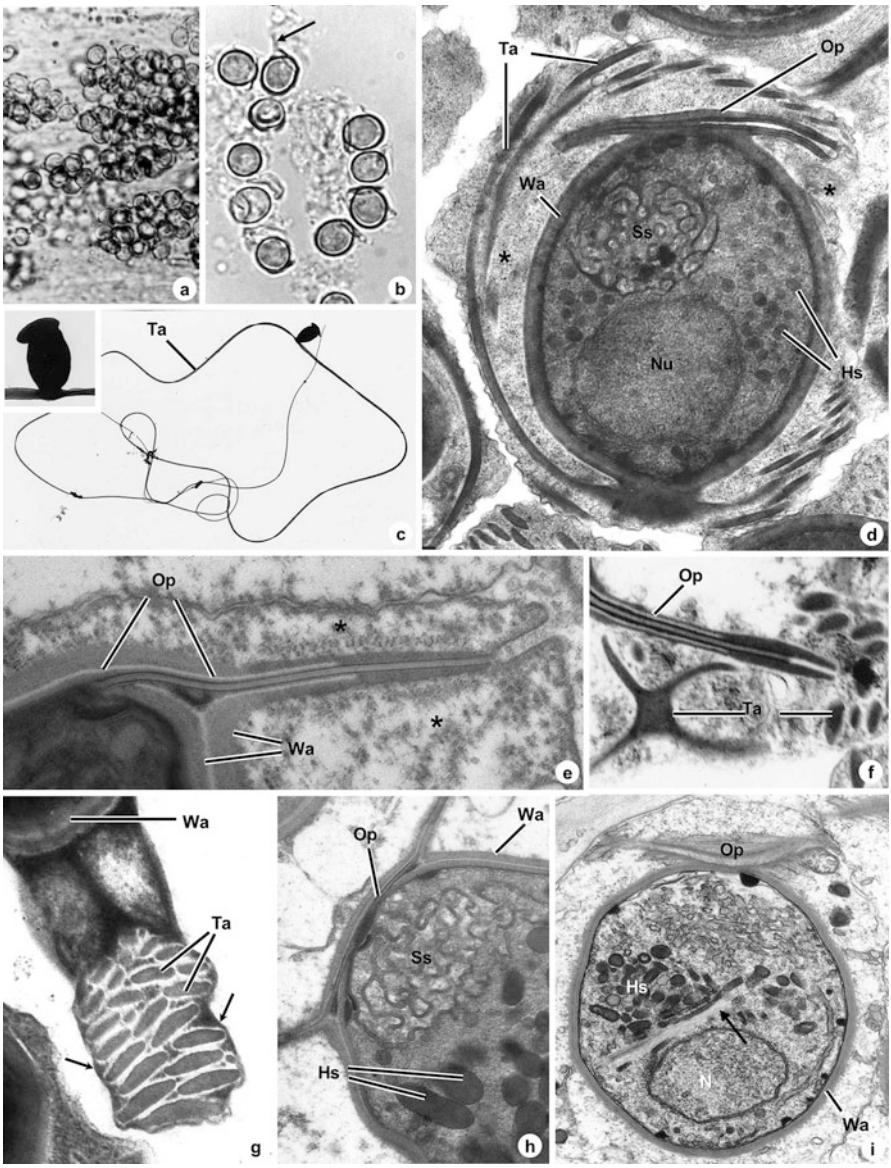


Fig. 2 Morphological and ultrastructural aspects of the spores of haplosporidian species: (**a**, **b**) Light microscopy observations of *Minchinia tapetis* spores showing epispore cytoplasm (*arrow*); (**c**) and inset: Two aspects of the spores of *Haplosporidium* sp. observed in TEM, showing two long tails (Permission of Allen Press); (**d**) Ultrastructural aspect of a longitudinal section showing the different organelles and structures of the *Haplosporidium* sp. spore; (**e**) TEM showing a section of the apical region reporting the operculum, spore wall, and exosporoplasm of a spore of *H. lusitanicum* (Courtesy of Springer); (**f**) Part of an operculum and transverse sections of tails of *H. lusitanicum* under TEM; (**g**) TEM of transverse sections of the tail when surrounded in the immature spore; (**h**) TEM of the apical region of the *H. lusitanicum* spore showing the operculum,

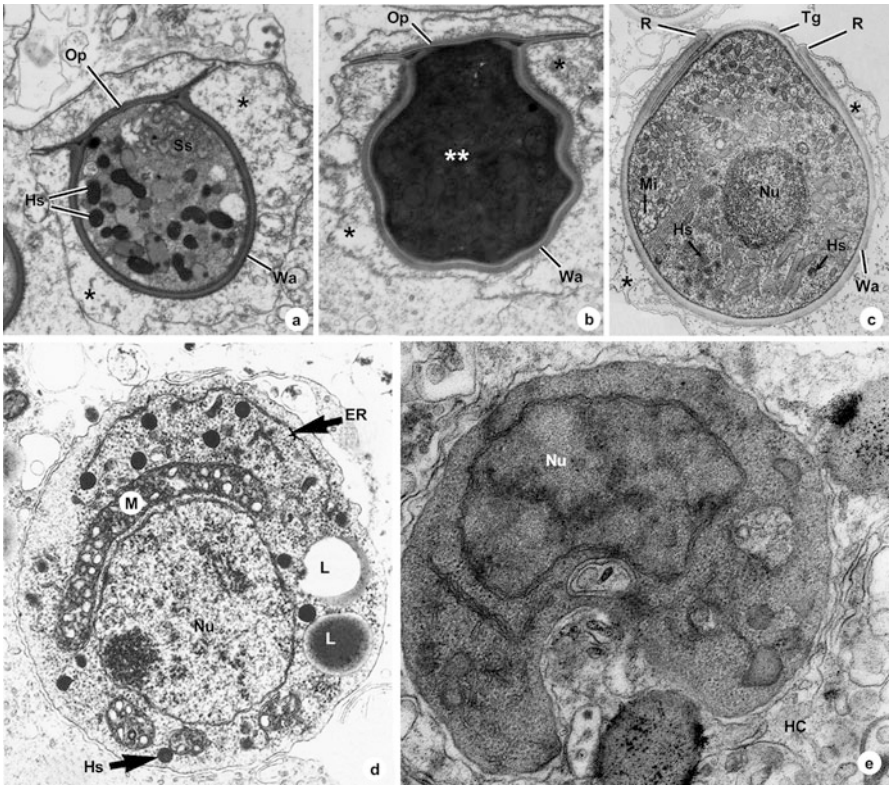


Fig. 3 Morphological and ultrastructural aspects of some life cycle stages of haplosporidian species: (**a**) Immature spore of *Minchinia tapetis* showing the surrounding external epispore cytoplasm, the wall, the operculum, and the endoplasm with the spherulosome and haplosporosomes (Courtesy of Springer); (**b**) A spore of *M. tapetis* showing a densification of the endospore, the spore wall, the operculum, and the surrounding epispore cytoplasm (Courtesy of Springer); (**c**) A spore of *Urosporidium crescent* (Courtesy of F. O. Perkins); (**d**) The uninucleate infective stage of *B. exitiosa* showing the central nucleus with a nucleolus, mitochondria, spherical haplosporosomes, endoplasmic reticulum, and lipid droplets; (**e**) Detail of a spore of *Bonamia* enclosed in a hemocyte showing the nucleus and the cytoplasmatic organelles (Courtesy of Elsevier). *Abbreviations*: (*) external epispore cytoplasm, *Wa* spore wall, *Op* operculum, *Ss* spherulosome, *Hs* haplosporosomes, (**) endospore, *M* mitochondria, *ER* endoplasmic reticulum, *L* lipid droplet, *HC* hemocyte, *Nu* nucleus, *R* rim, *Tg* lingula

Fig. 2 (continued) spore wall, spherulosome, and haplosporosomes; (**i**) A spore of *H. armoricanum* showing the wall, the anastomosing spherulosome beneath the hinged lid, a basal nucleus, and elongated, axe-head-shaped haplosporosomes in between. The bundle of microfilaments is a common feature of unknown function in the spores of some *Haplosporidium* spp. *Abbreviations*: *Ta* tails, *Op* operculum, *Ss* spherulosome, *Hs* haplosporosomes, *Nu* nucleus, *Wa* spore wall, *EC* epispore cytoplasm, (*) exosporoplasm, (*arrows*) exosporoplasm membrane

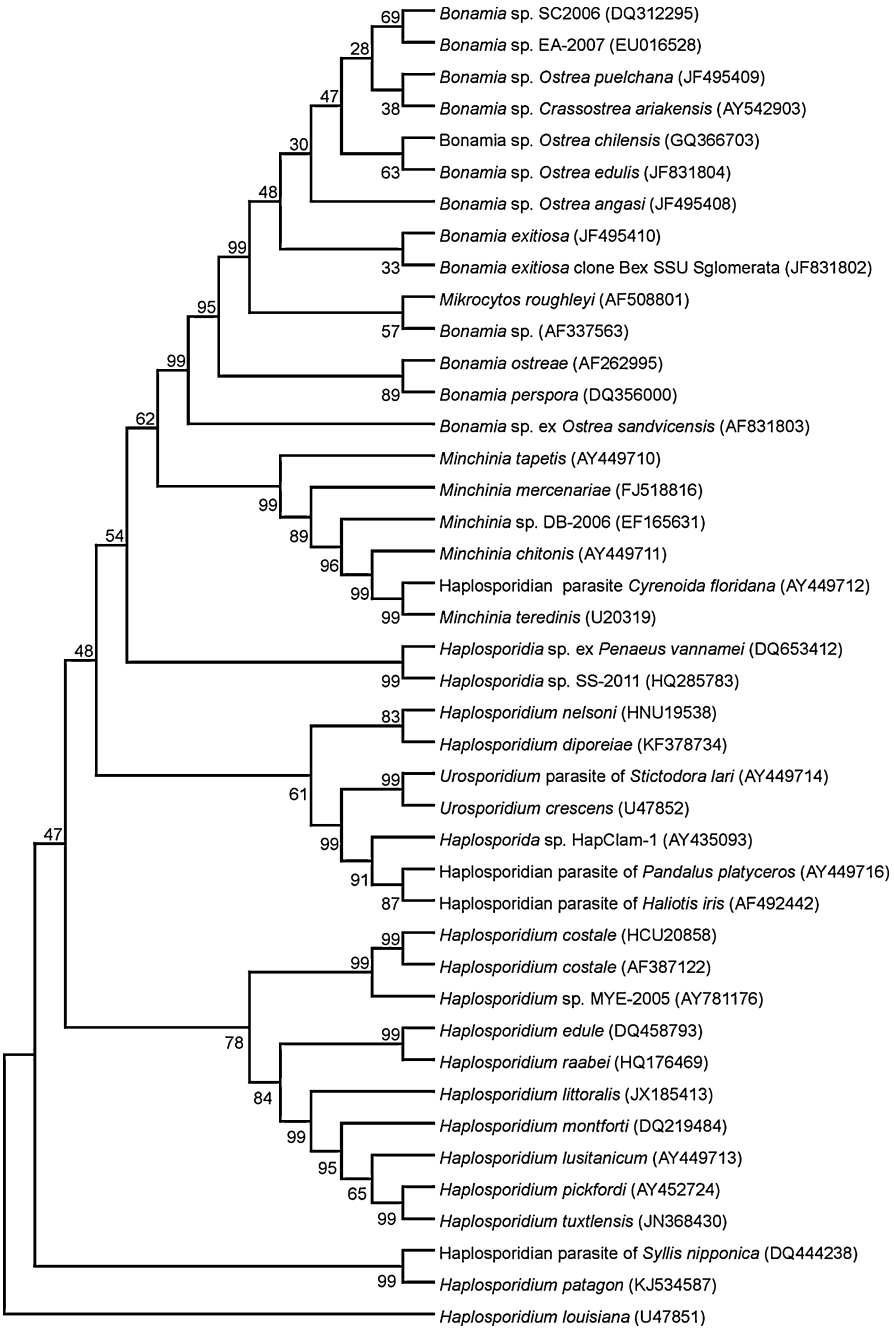
Much attention has been given to spore ornamentation (variously termed filaments, tails, projections, extensions, folds, wrappings, bifurcated slender projections, and epispore extensions) in the different descriptions (Perkins and van Banning 1981; Azevedo and Corral 1985; McGovern and Burreson 1990; Comps and Tigé 1997; Azevedo et al. 1999; Burreson 2001; Hine and Thorne 2002; Azevedo et al. 2003, 2006; Carnegie et al. 2006; Bearham et al. 2008a; Molloy et al. 2012). They were regarded as the principal taxonomic feature distinguishing the genera *Haplosporidium, Minchinia,* and *Bonamia perspora* (Burreson 2001; Burreson and Reece 2006), but a molecular study (Burreson and Reece 2006) showed no correlation between ornamentation and phylogeny, and it appears that they are not taxonomically important. This is not surprising given that *Haplosporidium* is a paraphyletic genus (Flores et al. 1996; Burreson and Ford 2004; Hine et al. 2007), containing *Haplosporidium*-like orphan species, such as *H. parisi* (Ormières 1980) and *H. ascidiarum* (Ciancio et al. 1999) that are ultrastructurally different and probably belong in separate genera. Unfortunately the type *Haplosporidium*, *H. scolopli*, is inadequately described, there being no molecular tools or electron microscopy in 1899, and until rediscovered and adequately described, other *Haplosporidium*-like species cannot be distinguished from it. Consequently, generalizations cannot be made about the spore ornamentation of all current *Haplosporidium* spp. The spores of *Minchinia* spp. are devoid of ornaments (Azevedo et al. 1999; Burreson and Reece 2006). However, an unnamed *Minchinia* sp. has epispore cytoplasmic extensions, unattached to the spore wall and comprising microtubules (Comps and Tigé 1997), but these structures are considered to be ephemeral, disappearing during the spore maturation process (Azevedo et al. 1999; McGovern and Burreson 1990).

Phylogeny

Several studies on haplosporidians include phylogenetic trees (Reece and Stokes 2003; Burreson and Ford 2004; Reece et al. 2004; Azevedo et al. 2006; Carnegie et al. 2006; Nunan et al. 2007; Molloy et al. 2012; Burki et al. 2013; Stentiford et al. 2013; Engelsma et al. 2014; Ituarte et al. 2014; Sierra et al. 2015) which differ in details depending on how they are constructed (Table 1). A study using group-specific PCR primers on eDNA samples revealed several distinct novel clades, novel lineages within known clades, and seasonal and habit-related patterns in assemblage composition (Hartikainen et al. 2014). Planktonic stages and host-free stages were also detected, in these highly divergent and diverse lineages.

These trees are consistent in (a) the basal position of the spot prawn (*Pandalus*) parasite (SPP) and New Zealand abalone parasite (Reece and Stokes 2003; Reece et al. 2004; Hartikainen et al. 2014); (b) more derived but intermediate positions of *Urosporidium* spp., *H. nelsoni*, and *H. louisiana*; (c) the integrity of *Minchinia* spp. and *Bonamia* spp., which are sister clades (Engelsma et al. 2014; Hartikainen et al. 2014); and (d) the phylogenetic closeness of gastropod *Haplosporidium* spp. A "core" group of *Haplosporidium* spp. comprises *H. lusitanicum*, *H. pickfordi*

Table 1 Maximum likelihood tree of the SSU rRNA sequences of different species of phylum Haplosporidia. Numbers on the branches are bootstrap percent values on 500 replicates trees. There were a total of 1712 positions in the final dataset. GenBank accession numbers in parentheses after the species name



(Fig. 4g), *H. tuxtelensis*, *H. montforti*, and *H. littoralis*, while *H. edule* and *H. raabei* cluster together (Hartikainen et al. 2014). *H. nelsoni* is not included in the "core" group.

Phylogenetically, *Bonamia perspora* and *B. ostreae* are more closely related to each other than to *B. exitiosa*, while a *Bonamia* sp. in *Dendostrea sandvicensis* in Hawaii is basal to the other *Bonamia* spp. (Hill et al. 2014).

According to recent phylogenetic studies, rhizarian (supergroup Rhizaria) parasites evolved from homoplastic processes occurring both in the animal and plant lineages. The first comprises the Ascetospora, intracellular parasites that infect marine invertebrates and that form a monophyletic clade in which all haplosporidian species are included (Burki et al. 2013; Sierra et al. 2015).

Practical Importance

Haplosporidians infect and cause disease in commercially important oysters, such as *Crassostrea virginica* (Perkins 1968, 1969; Ford and Haskin 1982), *Crassostrea gigas* (Comps and Pichot 1991; Renault et al. 2002), *Ostrea edulis* (Pichot et al. 1980; Hine et al. 2007), *Ostrea chilensis* (Hine 1991; Cranfield et al. 2005; Lane et al. 2016), *Ostrea puelchana* (Kroeck and Montes 2005), and *Saccostrea cucullata* (Hine and Thorne 2002; Bearham et al. 2008b). They also infect pearl oysters (Hine and Thorne 1998; Bearham et al. 2008a), clams (Azevedo 2001; Ford et al. 2009), cockles (Azevedo et al. 2003), mussels (Comps and Tigé 1997), and abalone (Diggles et al. 2002; Azevedo et al. 2006, 2007; Balseiro et al. 2006). In economic terms, *C. virginica* (Ford and Haskin 1982), *O. edulis* (Tigé et al. 1986), and *O. chilensis* (Cranfield et al. 2005) have suffered the biggest impacts. There may be indirect impacts, such as the discoloration of nematodes infected with *Urosporidium spisuli* in clams, making them unattractive to consumers (Perkins et al. 1975).

There is evidence that haplosporidians of clams, *M. tapetis* and *M. mercenaria*, may be associated with epizootics (Hartikainen et al. 2014).

Commercially important shrimp, *Penaeus vannamei* (Dyková et al. 1988; Nunan et al. 2007), and a crab (Newman et al. 1976) are also infected with haplosporidians, with losses in *P. vannamei* culture in Indonesia estimated at >US$ 5 m over 5 years (Utari et al. 2012).

Characterization and Recognition

General Appearance

Phylogenetically Basal Haplosporidians

The phylogenetically basal haplosporidian of the spot prawn (*Pandalus*) parasite (Reece et al. 2004) infecting and castrating *Pandalus platyceros* comprises multinucleate plasmodia that divide into uninucleate trophonts, some of which contain a

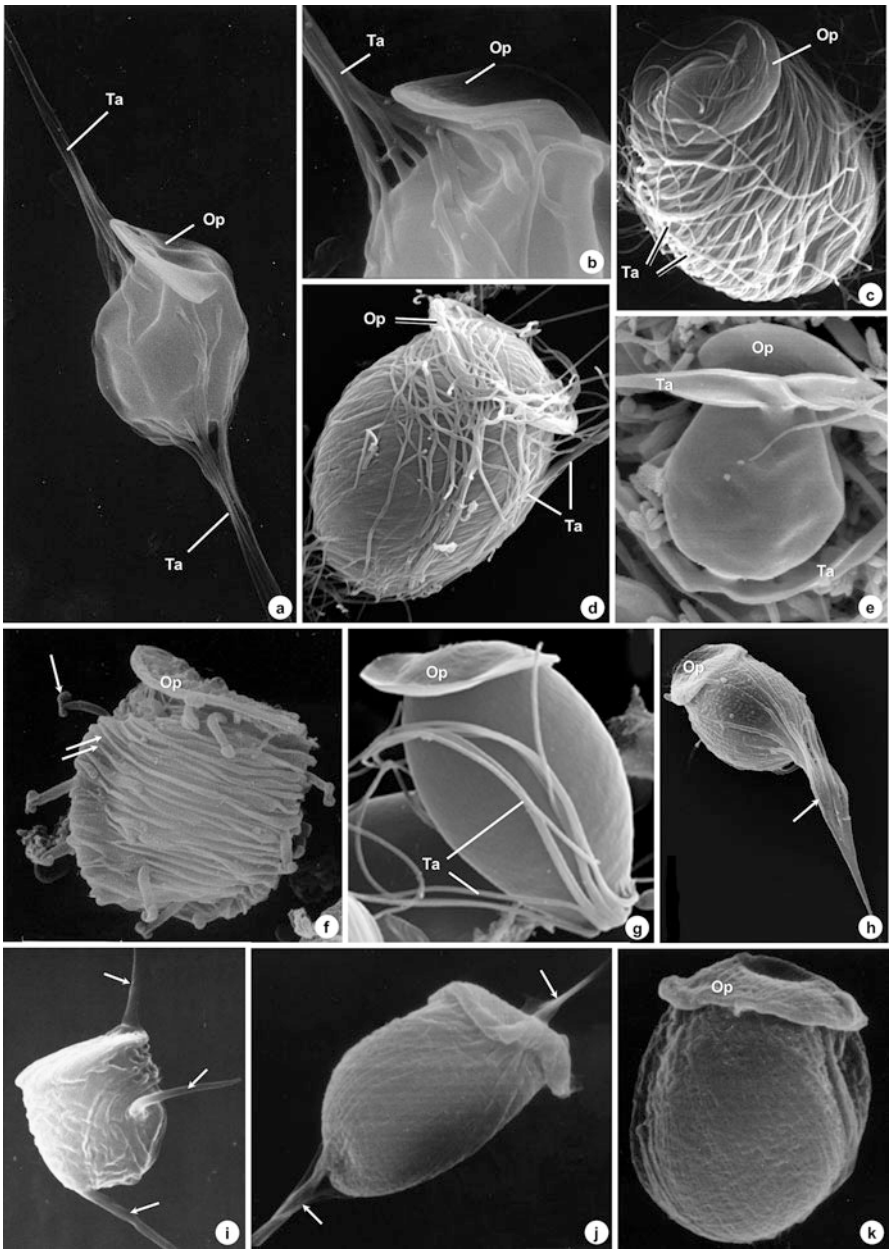


Fig. 4 Scanning electron micrographs of aspects of spores of some haplosporidian species: (**a**, **b**) *Haplosporidium armoricanum* showing some details of the external morphology evidence of tails and operculum (Courtesy of Springer); (**c**) *H. louisiana* showing a complex network of the tail and operculum (Courtesy of E. M. Burreson); (**d**) *H. nelsoni* (Courtesy of E. M. Burreson; Permission of Allen Press); (**e**) *H. montforti*: spore showing the tails and operculum (Courtesy of Elsevier);

dense basophilic inclusions. Ultrastructurally single-membraned peanut-shaped bodies containing tiny tubules cluster around the nucleus; there are plasmalemma extensions resembling ectoplasmic nets and large round to oval cytoplasmic inclusions, sometimes with a light periphery and darker core. Characteristic haplosporosomes are not present (Bower and Meyer 2002).

Conversely, the phylogenetically basal New Zealand abalone parasite (Reece and Stokes 2003) infecting *Haliotis iris* (Diggles et al. 2002; Hine et al. 2002b), has characteristic haplosporosomes formed from material in indentations on the nuclear surface, which is processed through Golgi to the *trans*-Golgi network where haplosporosomes form. These then either pass to the plasma membrane where the outer haplosporosome membrane fuses with the plasma membrane to release the core, or they are degraded in autophagic-crinophagic vacuoles in the cytoplasm. Released cores may also be internalized and degraded in these vacuoles (Hine et al. 2002b). Spores are unknown, observations being restricted to one outbreak of disease in one abalone farm. It is noticeable that currently molecular phylogenies place the spot prawn parasite and the New Zealand abalone parasite together at the base of haplosporidian phylogeny, despite them being so different that other than basic eukaryotic structure and organelles they appear to have nothing in common.

Haplosporidium – The principal characteristics of this genus are spores with an apical-hinged operculum (Fig. 2c–f) and a variety of extensions externally (tails, filaments, extensions, wrapping, folds, and epispore extensions) (Fig. 2c, d, g) formed by the same material of the spore wall. The number of ornaments is variable according to the different species. The internal uninucleated endosporoplasm contains a spherulosome (structure formerly designated by the name "spherule"), generally located at the apical region of the spore, several haplosporosomes, and mitochondria (Fig. 2h, i). However, a parasite of shore crabs (*Carcinus maenas*), *Haplosporidium littoralis* (Stentiford et al. 2013) does not appear to form spores (Stentiford et al. 2004). The occurrence of ultrastructural features reported up to 2009 has been compared (Fig. 2i) (Hine et al. 2009).

Haplosporidium louisiana (Sprague 1963b) (Figs. 4d, e) infects crabs, *Panopeus herbstii* in the USA, and a very similar species, *Minchinia cadomensis* (Marchand and Sprague 1979) infects crabs, *Rhithropanopeus harrisii* in France. They are probably conspecific and regarded as *H. louisiana.* This species is phylogenetically basal to other *Haplosporidium* spp. (see above) and differs from them in

Fig. 4 (continued) (**f**) *H. edule*: spore showing the external morphology organized with several folds and some small tails (Courtesy of Elsevier); (**g**) *H. pickfordi* showing the tails and the operculum (Courtesy of E. M. Burreson, Permission of John Wiley & Sons); (**h**) *Minchinia tapetis* spore showing the basal epispore cytoplasm (*arrow*); Courtesy of E.M. Burreson); (**i**) *Minchinia teredinis* showing tree ephemeral epispore cytoplasm (*arrows*) (Courtesy of E. M. Burreson); (**j**) *Minchinia tapetis* spore showing the ephemeral epispore cytoplasm (*arrows*) before to attain a complete maturation (Courtesy of Spring); (**k**) *Minchinia tapetis* mature spore without epispore cytoplasm (Courtesy of Springer). *Abbreviations*: *Ta* tails, *Op* operculum, (*arrow*) epispore cytoplasm

haplosporogenesis occurring in plasmodia from amorphous electron-dense masses formed by nuclear membrane-bound Golgi, which contain membrane-bound vesicles that by budding into the cytoplasm acquire a second membrane to form classic haplosporosomes (Perkins 1975, 1979). Spores are ~12 × 8 μm, with a spherulosome which produces striated formative bodies from which haplosporosomes form by budding.

Haplosporidium nelsoni parasitizes *Crassostrea virginica* and *Crassostrea gigas*, the former in eastern North America and the Gulf of Mexico, the latter in western North America, Europe (France, Spain, Ireland), and east Asia (Japan, China, South Korea) which was probably the origin of North American and European infections.

Haplosporidium costale infects oysters (*Crassostrea virginica* and *Crassostrea gigas*) on the eastern coast of the USA (Perkins 1969), France (Comps and Pichot 1991), and China (Wang et al. 2010).

Haplosporidium lusitanicum (Azevedo 1984) parasitizes the gills and visceral tissues of *Helcion pellucidus* (Mollusca, Gastropoda). Spores are ellipsoidal ~3.0 × 2.1 μm and surrounded by a proteinaceous wall ~0.1 μm thick (Figs. 1a, and 2d, e, h, i). In the basal region, the wall is thicker and gives rise to two long tape-like proteinaceous filaments (or tails), each ~112 μm long (Fig. 2c, d, g). The apical zone of the spore wall is modified into a complex opercular system covering a circular orifice (micropyle) ~0.5 μm in diameter (Fig. 2d, e, h).

Haplosporidium armoricanum (Azevedo et al. 1999) (syn. *Minchinia armoricana* (van Banning 1977) parasitizes the oyster *Ostrea edulis.* It was transferred to *Haplosporidium* on the basis that it has filaments originating from the spore wall. The ellipsoid spores are about 5.0 μm long and 3.1 μm wide with an operculum and two long epispore cytoplasm extensions (ECE) attached eccentrically at opposite ends of the spores (Figs. 2i, 3a, and 4b). The base of each ECE is attached to the spore wall by a bundle of 9–13 cylindrical fibers arising from the spore wall (Figs. 2i, 4b, and 5a). Each of these filaments is about 130 μm long. Spores may have a spherulosome and Golgi attached to the nucleus (Hine et al. 2007).

Haplosporidium montforti (Azevedo et al. 2006, 2007) infects the connective tissue, gill, digestive gland, and foot muscle of the abalone, *Haliotis tuberculata*, imported from Ireland and experimentally grown in Galicia, Spain. The spores are spherical to slightly ellipsoidal (2.4 ± 0.5 × 2.3 ± 0.6 μm). The apical pole of the spore wall is modified into a complex opercular system covering a circular orifice that measures about 0.5 μm across. The operculum is connected to the spore wall by a hinge. The spore wall is about 110 nm thick, with four filaments (or tails) (20–28 μm long). The cross-sections through the base of these filaments are T-like and X-like (Figs. 2f, 4e, and 5b). Internally, the uninucleated endosporoplasm contained typical haplosporidian structures, such as haplosporosomes, a spherulosome, and mitochondria with vesicular cristae (Fig. 5b).

Haplosporidium edule (Azevedo et al. 2003) parasitizes the digestive gland tissues of the cockle *Cerastoderma edule* in Galicia (northwest Spain) and has tape-like filaments (Figs. 4f and 5c). The spores are ovoid to ellipsoidal ~3.2 × 2.2 μm with the apical wall modified into a complex opercular system covering the micropyle. The spore wall 0.1–0.12 μm thick is composed of three

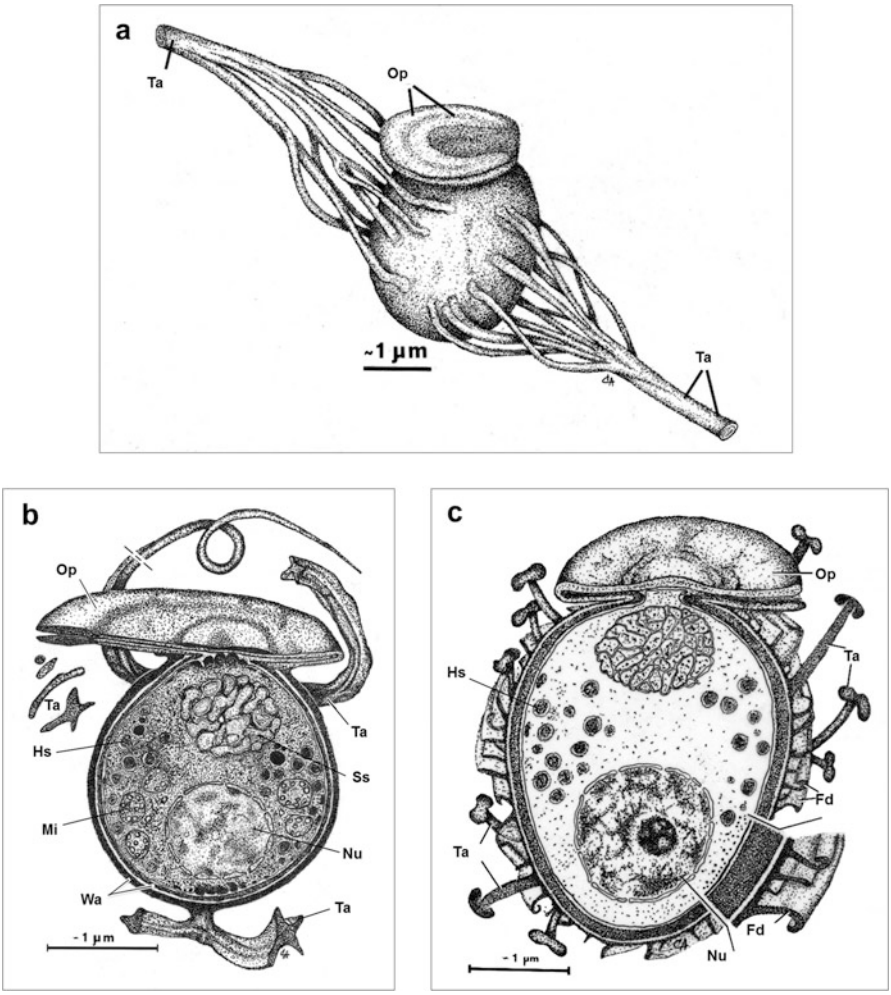


Fig. 5 Schematic drawings of spore of some haplosporidian species showing the external and internal organization: (**a**) *H. armoricanum* (Courtesy of Elsevier); (**b**) *H. montforti* (Courtesy of Elsevier); (**c**) *H. edule* (Courtesy of Springer). *Abbreviations*: *Op* Operculum, *Ta* Tails, *Ss* Spherulosome, *Hs* Haplosporosomes, *Mi* Mitochondria, *Wa* Spore wall, *Nu* Nucleus, *Fd* Folds

layers. The outermost has numerous folds surrounding the periphery of the spores. Several slender projections having two short opposite, dilated branches emerge between the folds (Fig. 4f). The inner endoplasm contains similar structures as those described in *H. lusitanicum*.

Haplosporidium littoralis (Stentiford et al. 2013) is a parasite of green crabs (*Carcinus maenas*) that is only known from multinucleate plasmodia that divide by cytokinesis to form uninucleate stages that undergo karyokinesis to form more plasmodia (Stentiford et al. 2004). Haplosporosomes are arrayed around the nuclear

membrane. Not only spores, but even the initial stages of sporogenesis, are not observed. Haplosporidians of blue crabs (*Callinectes sapidus*) (Newman et al. 1976) and *Penaeus vannamei* (Dyková et al. 1988; Nunan et al. 2007) also comprise uninucleate stages and multinucleate plasmodia with haplosporosomes arrayed around the nuclear membrane, with no evidence of sporogenesis. Therefore, *H. littoralis* and the two unnamed species may represent a group of crustacean haplosporidians that appear to be different from *H. louisiana*, which has spores.

Minchinia – This genus is characterized by having mature spores without any ornaments. The endosporoplasm of *Minchinia* structurally resembles *Haplosporidium* spp., having a spherulosome, haplosporosomes, and mitochondria (Fig. 3a). The immature spores when observed free may present an epispore (exospore) cytoplasm forming an ephemeral extension that disappears in the final phase of spore maturation (Fig. 4h–k).

Minchinia tapetis (Azevedo 2001) (formerly described as *Haplosporidium tapetis*) was described parasitizing the clam *Tapes decussatus* gill. The internal organization of *Minchinia* spore was similar to that *Haplosporidium*, however, without spore ornamentations (Figs. 3a, b, and 4k). The immature spores may show the presence of an ephemeral surrounding epispore cytoplasm that disappears during the final phase of maturation (Fig. 4j, k).

Urosporidium – The spores of this genus are characterized by flask-shaped spores, with a cap covering the orifice (Figs. 1c and 3c). The spores of different species are spherical to ellipsoidal or oval, measuring 3.0–5.5 μm in width and 3.0–6.0 μm in length. Some spores have extrasporal ornamentations deriving from the epispore cytoplasm (Le et al. 2015). Epispore extensions may be fibrous (Anderson et al. 1993; Le et al. 2015), have a dense core (Perkins et al. 1975), have ribbons and extensions (Carballal et al. 2005), or contain microtubules (Ormières et al. 1973). The endosporoplasm of these spores is similar to those of *Haplosporidium* spp. (Fig. 1a–c), except the equivalent of a spherulosome may occur as scattered cisternae throughout the sporoplasm (Perkins 1971; Perkins et al. 1975) or as parallel cisternae underlying the plasma membrane, the spherulosome having no fixed position in relation to the apical orifice (Anderson et al. 1993).

Bonamia – The cells of *Bonamia* spp. measure 2–5 μm in diameter and are intrahemocytic within the connective tissue of the mantle and gills and in the vascular sinuses near the digestive gland, intestine, and stomach (Fig. 3d, e). The cells of *B. ostreae* are smaller (2–3 μm), and in tissue smears the nucleus appears eccentric, whereas in *B. exitiosa* smears, the nucleus is central, giving a "fried egg" appearance.

This genus comprises three species which infect oysters, of which one species, *B. perspora*, forms spores and the other two species, *B. ostreae* and *B. exitiosa*, are not known to form spores. Another organism, originally described as *Mikrocytos roughleyi* (Farley et al. 1988), was transferred to the genus *Bonamia* (Cochennec-Laureau et al. 2003), but this is probably not a valid classification, and it may be a form of *B. exitiosa* (Carnegie et al. 2014). *B. perspora* infects small oysters, *Ostreola equestris* (type host), in North Carolina, USA (Carnegie et al. 2006).

Bonamia ostreae infects *Ostrea edulis* on the eastern and western coasts of the USA and is present in several Western European countries (France, Ireland, Italy, the

Netherlands, Portugal, Spain, and the UK). It has recently been reported from New Zealand (Lane et al. 2016), which is the first report from the Southern Hemisphere. It is thought to have originated from eastern North America, moved to the US west coast in infected oysters, and then moved from there to France in infected oysters in 1978–1979, resulting in massive European epizootics. *B. ostreae* has probably also infected *O. chilensis* (Grizel et al. 1983), *O. puelchana* (Pascual et al. 1991), and *O. angasi* (Bougrier et al. 1986) when introduced live into France. It can establish mild infections in *C. ariakensis*.

Bonamia exitiosa infects *Ostrea chilensis* (type host) in New Zealand (Fig. 3d), *O. angasi* in Australia, *O. puelchana* and *O. stentina* in Argentina, *O. stentina* from SE USA and the Mediterranean, *O. edulis* in Spain, and a related species parasitizes *O. chilensis* in Chile. It also infects *Crassostrea ariakensis* at the port of Morehead City Bay in North Carolina, USA (Audemard et al. 2014), but only in the vicinity of the port (Bishop et al. 2006). This finding and the origin of an epizootic of *B. exitiosa* in an oyster (*O. puelchana*) next to a bunkering wharf for international shipping in San Antonio Bay, Argentina (Kroeck and Montes 2005), strongly suggests that this parasite has been spread by international shipping.

Ultrastructure

The Haplosporidia were originally regarded as spore-forming species (Fig. 1a–c), but *B. ostreae* and *B. exitiosa* were reported infecting oysters in the absence of spores. Despite this, their uninucleate and multinucleate stages containing typical haplosporosomes closely resemble the presporogonic stages of spore-forming haplosporidians, and they are recognized as such (Carnegie et al. 2000). Uninucleate and multinucleate stages with typical haplosporosomes, but without spores, have also been reported from crabs (Newman et al. 1976) and shrimps (Dyková et al. 1988). Two other crustacean-infecting haplosporidians, the phylogenetically basal spot prawn parasite (Reece et al. 2004) and *H. littoralis*, which is phylogenetically close to *Haplosporidium* spp. from gastropods (Stentiford et al. 2013), also do not appear to form spores, but *H. littoralis* has dense vesicles among which some may have an inner membrane suggestive of haplosporosomes (Stentiford et al. 2004), and the basal spot prawn parasite lacks haplosporosomes (Bower and Meyer 2002).

Plasmodial Stages

Uninucleate stages (Fig. 3d) contain a usually central nucleus, with or without intranuclear microtubules, Golgi that often arises from the nuclear membrane (Perkins 1968, 1969, 1971, 1975; Perkins et al. 1975; Hine 1991; Hine and Wesney 1992, 1994a; Hine et al. 2001, 2007, 2009; Carnegie et al. 2006) and is associated with a *trans*-Golgi network from which haplosporosomes form (Perkins 1968, 1969, 1975, 1979; Hine 1991; Hine and Wesney 1992, 1994a, b; Hine et al. 2001; Carnegie et al. 2006), mitochondria, and smooth endoplasmic reticulum. In *H. nelsoni* dense

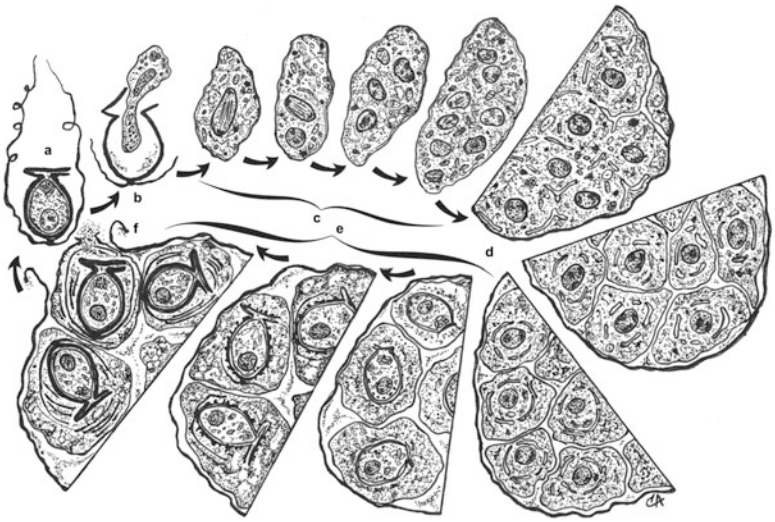


Fig. 6 Schematic drawing of the life cycle (spores → plasmodium → sporontes → sporoblasts → to 100 spores) of *Haplosporidium lusitanicum:* (*a*) free mature spore; (*b*) excystment of the spore thought the micropile; (*c*) sequential phases of the plasmodial development after penetration on the host, characterized by sequential nucleokineses originating a plasmodium with some hundreds of nuclei; (*d*) development of several cisternae around the nuclei, each of one give rise to a nucleate cell (sporont); (*d–f*) sporoblasts within the sporocyst and sequential phases of sporogenesis during which each sporoblast gives rise to a spore, when mature is released from the sporocyst by rupture of its wall

cytoplasmic formative inclusions give rise to haplosporosomes (Perkins 1968, 1979). In haplosporidians infecting crustaceans, the haplosporosomes may cluster around the nucleus (Newman et al. 1976; Dyková et al. 1988; Stentiford et al. 2013). The nucleus divides by binary fission (Figs. 6 and 7c), sometimes to form diplokarya (Fig. 7d). (Perkins 1968, 1969, 1971), and multiple divisions result in a multinucleate plasmodium (Azevedo et al. 1985) (Figs. 6 and 7a–e). Formation of diplokarya may indicate the beginning of sporogony.

The multinucleate plasmodia undergo irregular multiple fission (plasmotomy) to yield daughter cells – the sporonts (Figs. 6 and 7a–e). In the next phase, a very irregular membranous system and some Golgi complexes begin to differentiate in the cytoplasm, among the plasmodial nuclei. Each nucleus and a portion of surrounding cytoplasm are encircled by a limiting membrane which arises from fusion of Golgi vesicles (Fig. 8a, b). In sporonts, several hundred sporoblasts are thus formed (Fig. 8c, d). Sporoblastogenesis is characterized by a gradual thickening of the sporoblast membrane which becomes the spore wall (Fig. 8d and inset). During the thickening of the wall, the pre-operculum appears and later differentiates into an operculum, while in the endosporoplasm, the spherulosome and haplosporosomes begin their formation. Sporogenesis occurs during the development of the spore wall, and in spores with ornaments, their development begins. Later, each sporoblast differentiates into immature spores (Fig. 8e) (Azevedo et al. 1985, 2007). Spore

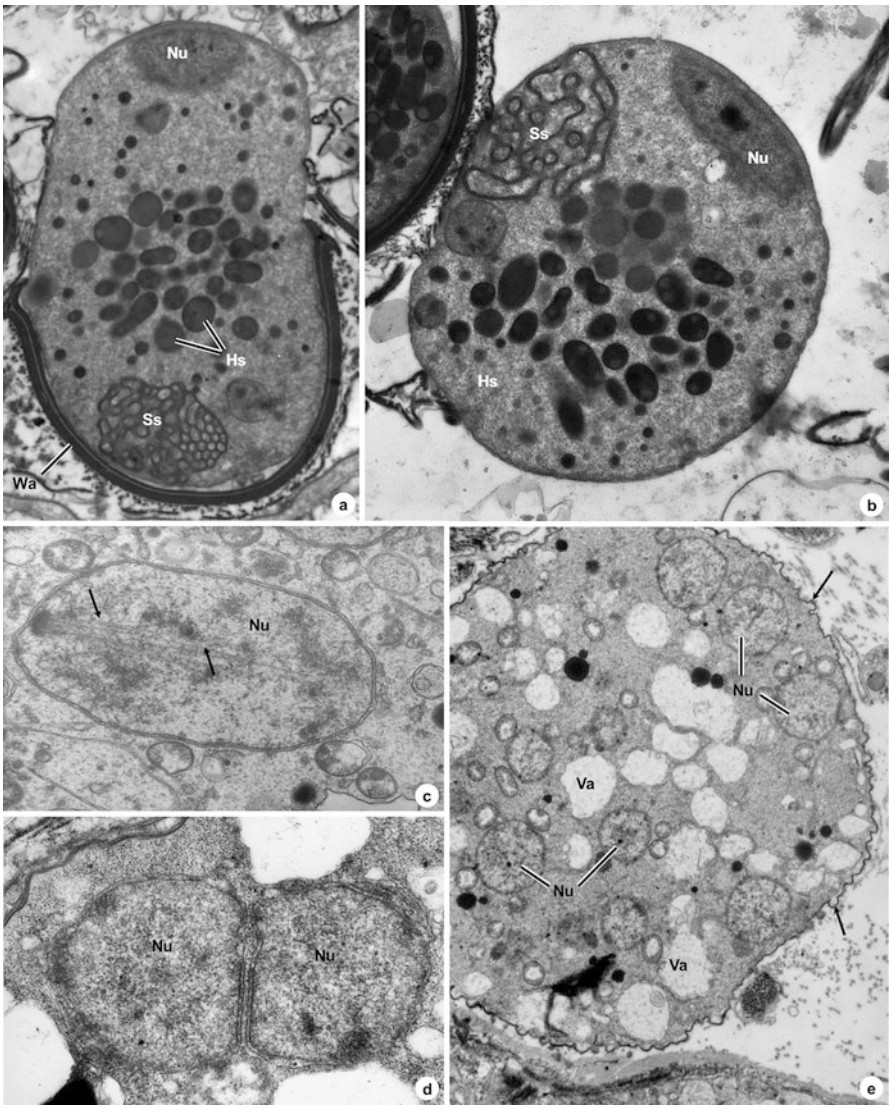


Fig. 7 Phases of the development of the life cycle of a haplosporidian species: (**a**) Spore excystment showing the wall showing the typical structures of the endosporoplasm as the spore wall, nuclei, haplosporosomes, and spherulosome (Courtesy of Elsevier); (**b**) Free endosporoplasm (amoebula ou amoeboid stage) after liberation from spore wall (Courtesy of Elsevier); (**c**) Amoebula nucleus showing a phase of mitotic division. The nucleus, completely surrounded by nuclear envelope, shows some microtubules in longitudinal section, attached to a spindle pole body (Courtesy of Springer); (**d**) A binucleated plasmodial (diplokaryon stage) cell showing each nucleus containing bundle microtubules (Courtesy of Springer); (**e**) Sporont (plasmodium) with an external membrane bound (*arrows*) showing several nuclei and vacuoles. *Abbreviations*: *Wa* spore wall, *Nu* nucleus, *Hs* haplosporosomes, *Ss* spherulosome; *arrows* microtubules, *Va* vacuoles

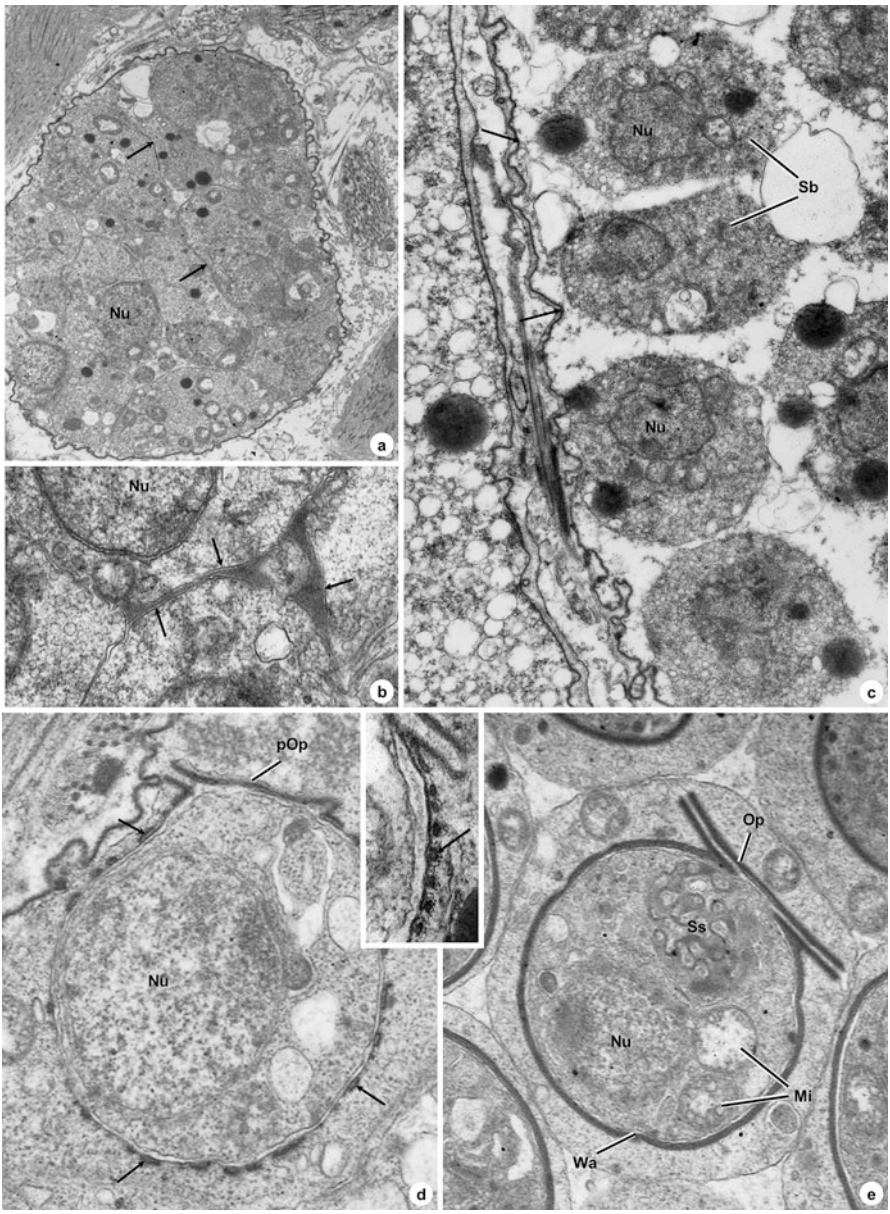


Fig. 8 Sequential phases of evolution of the life cycle of haplosporidian species: (**a**) Sporocyst in initial process of sporoblast cells, showing the formation the membranes that give rise to sporoblast plasmalemma (*arrows*); (**b**) Detail of part of a sporocyst showing the initial process of sporoblast formation with evident differentiation of the peripheral sporoblast membrane (*arrows*); (**c**) Late stage of sporoblast formation. The sporoblasts are surrounded by an irregular sporocyst wall (*arrows*); (**d**) Aspect of the initial process of spore wall formation showing several blisters of dense material (*arrows*):

maturation occurs during the degradation of the episporoplasm, rupture of the sporocyst, and liberation of the mature spores (Fig. 6).

The spores of the paraphyletic *Haplosporidium* spp., *Minchinia* spp., and *Bonamia perspora* are similar (Burreson and Ford 2004; Carnegie et al. 2006), having an oval to ellipsoidal shape, with an apical pore covered by a hinged operculum. The spores range in size from about 3–8 × 2–5 μm. The spore ornaments are not of taxonomic significance (Burreson and Reece 2006), except that *Minchinia* spp. lack spore ornaments and epispore cytoplasm is never attached to the spore wall. Internally, the endosporoplasm contains haplosporosomes, mitochondria, the spherulosome, and Golgi equivalent. *Urosporidium* spp. have spores without an operculum but instead an internal flap of wall material covering the spore wall orifice. The spore wall does not have ornaments (Figs. 1c and 3c).

When the mature spores are free or within the same host (Perkins 1975, 1979; Ball 1980; Desportes and Nashed 1983; Azevedo 1984; Hine and Thorne 2002), excystment of endosporoplasm occurs through the micropyle of the spore (Figs. 6 and 7a) (Azevedo and Corral 1989).

Life Cycle

The life cycles of spore-forming species are unknown despite attempts to determine the life cycle of *H. nelsoni* (Burreson et al. 1997). The developmental cycle of *H. lusitanicum* (Fig. 6) may represent the sequential phases in the majority of haplosporidian species, but there is no evidence that *H. lusitanicum* transmits directly between chiton (*Helcion pellucidus*) hosts. *Bonamia perspora*, unlike other *Bonamia* spp., forms spores, and *H. littoralis,* unlike other spore-forming *Haplosporidium* spp., does not. While utilization of more than one host has its advantages (dispersal, survival, reservoir hosts), it also has the disadvantage of relying on all hosts to be available. Direct transmission by species that do not form spores is of benefit when the host species is abundant, with contiguous populations, but not when hosts are scattered or the parasite has to be dormant to survive.

Life cycles may have a degree of plasticity, depending on environmental conditions. New Zealand *B. exitiosa* has large amoeboid stages (Hine and Wesney 1994a), not reported from other *B. exitiosa*, and diplokaryotic stages and plasmodia with slightly thickened membranes suggestive of early sporogony, although further development has not been observed. Under unfavorable conditions, *H. littoralis* may form spores but under favorable conditions may not need to sporulate and may

Fig. 8 (continued) inset – details of the process (Courtesy of Elsevier); (**e**) An immature spore showing a nucleus, pre-spherulosome, mitochondria, and the spore wall with the operculum (Courtesy of Elsevier). *Abbreviations*: *Sb* sporoblasts, *Nu* nucleus, *Ss* pre-spherulosome, *Mi* mitochondria, *Wa* spore wall, *pOp* pre-operculum, *Op* operculum

transmit directly. Even when sporulation occurs, direct uninucleate or plasmodial transmission may also be possible.

An environmental DNA (eDNA) study (Hartikainen et al. 2014) found evidence of *H. edule*, *M. tapetis*, and *B. exitiosa* in planktonic samples, suggesting these species may utilize plankton in their life cycles, although *B. exitiosa* also transmits directly (Hine et al. 2002a). *B. ostreae* also infects planktonic larvae of its oyster host (Arzul et al. 2011). The eDNA study found similar and novel haplosporidian sequences in benthic and planktonic samples from Europe, Panama, and South Africa (Hartikainen et al. 2014), suggesting ubiquitous infection in invertebrates including zooplankton globally.

Systematics

Overall Phylogenetic Position

The protistan phylum Haplosporidia is composed of histozoic parasites of great variety of freshwater and marine invertebrate.

Systematics

The taxonomy of the phylum Haplosporidia is not well yet established. In this document, it was decided to present one of the most consensual taxonomies of this group that includes the Haplosporidia:

Protista (kingdom)
Rhizaria (supergroup)
Cercozoa
Ascetospora
Haplosporidia Caullery & Mesnil, 1899 (phylum)
Haplospora Caullery, 1953 (class)
Haplosporida Caullery & Mesnil, 1899 (order)
- Haplosporiidae (family)
  - *Haplosporidium* (with 34 species)
  - *Minchinia* (with 6 species)
  - *Bonamia* (with 3 species)
- Urosporiidae (family)
  - *Urosporidium* (with 9 species)

Evolutionary History

There is no fossil record of haplosporidians. However, molecular phylogenies show that haplosporidians are cercozoans (Cavalier-Smith and Chao 2003), related to Foraminifera and Radiolaria, which evolved in the early Cambrian (Pawlowski et al. 2003). Like foraminiferans and radiolarians, the basal haplosporidian SPP

(Reece et al. 2004) has ectoplasmic extensions. Haplosporidians may also therefore date back to the Cambrian.

List of the Haplosporidian Species

Aplosporidium Caullery & Mesnil, 1899 (name later altered to *Haplosporidium*)

- *A. scolopli* Caullery & Mesnil, 1899 (later altered to *Haplosporidium scolopli*)
- *A. heterocirri* Caullery & Mesnil, 1899 (later altered to *Haplosporidium heterocirri*);

Anurosporidium Caullery & Chappellier, 1906 (later altered to *Urosporidium*)

- *A. pelseneeri* Caullery & Chappellier, 1906 (later altered to *Urosporidium pelseneeri*)

Haplosporidium Caullery & Mesnil, 1899

1. *H. scolopli* (Caullery and Mesnil 1899) (formerly described as *Aplosporidium*)
2. *H. heterocirri* (Caullery and Mesnil 1899) (formerly described as *Aplosporidium*) (altered to *Minchinia* and later newly transferred to the genus *Haplosporidium*)
3. *H. marchouxi* (Caullery and Mesnil 1905)
4. *H. potamillae* (Caullery and Mesnil 1905)
5. *H. vejdovskii* (Caullery and Mesnil 1905)
6. *H. limnodrili* (Granata 1914)
7. *H. nemertis* (Debaisieux 1920)
8. *H. caulleryi* (Mercier and Poisson 1922)
9. *H. mytilovum* (Field 1924)
10. *H. ascidiarum* (Duboscq and Warrant 1923)
11. *H. cernosvitovi* (Jírovec 1936)
    - *H. tapetis* (Vilela 1951) (later transferred to *Minchinia*)
12. *H. pickfordi* (Barrow 1961)
13. *H. costale* (Wood and Andrews 1962)
14. *H. louisiana* (Sprague 1963b) (*syn. H. cadomensis* Marchand and Sprague 1979) (formerly described as *Minchinia*)
15. *H. nelsoni* (Haskin et al. 1966) (formerly described as *Minchinia*)
16. *H. tumefacientis* (Taylor 1966)
17. *H. simulii* (Beaudoin and Wills 1968)
18. *H. macobdellae* (Jennings and Gibson 1968)
    - *H. cadomensis* (Marchand and Sprague 1970) (*Syn. H. louisiana* Sprague 1963b) (formerly described as *Minchinia*)
19. *H. prostomae* (Gibson and Moore 1979)
20. *H. parisi* (Ormières 1980)

21. *H. comatulae* (La Haye et al. 1984)
22. *H. lusitanicum* (Azevedo 1984)
23. *H. gammari* (Larsson 1987)
24. *H. meligethi* (Lipa and Hokkanen 1991)
25. *H. armoricanum* (Azevedo et al. 1999) (formerly described as *Minchinia*)
26. *H. edule* (Azevedo et al. 2003)
27. *H. montforti* (Azevedo et al. 2006)
28. *H. hinei* (Bearham et al. 2008)
29. *H. occulta* (Bearham et al. 2008)
30. *H. tuxtlensis* (Vea and Siddall 2011)
31. *H. raabei* (Molloy et al. 2012)
32. *H. littoralis* (Stentiford et al. 2013)
33. *H. patagon* (Ituarte et al. 2014)
34. *H. diporeiae* (Winters and Faisal 2014)

Minchinia Labbé, 1896

1. *M. chitonis* (Lankester, 1885) Labbé, 1896
   - *M. heterocirri* (Caullery and Mesnil, 1905) (formerly described as *Aplosporidium*) later newly transferred to genus *Haplosporidium*)
2. *M. dentali* (Arvy 1957)
   - *M. louisiana* (Sprague 1963) (later transferred to *Haplosporidium*) (Sprague 1963)
   - *M. nelsoni* (Haskin et al. 1966) (later transferred to *Haplosporidium*)
   - *M. costale* (Perkins 1969) (later transferred to *Haplosporidium*)
   - *M. armoricana* (van Banning 1977) (later transferred to *Haplosporidium armoricanum*)
   - *M. cadomensis* (Marchand and Sprague 1979) (later transferred to *Haplosporidium*)
3. *M. teredinis* (Hillman et al. 1990)
4. *M. tapetis* (Azevedo 2001) (formerly described as *Haplosporidium* Vilela, 1951)
5. *M. occulta* (Bearham et al. 2008)
6. *M. mercenariae* (Ford et al. 2009)

Urosporidium Caullery & Mesnil, 1905

1. *U. fuliginosum* (Caullery and Mesnil 1905)
2. *U. pelseneeri* (Caullery and Chapellier 1906) (formerly described as *Anurosporidium*)
3. *U. crescens* (De Turk 1940)
4. *U. tauricum* (Zaika and Dolgikh 1963)
5. *U. constantae* (Howell 1967)
6. *U. astomatum* (Menke 1968)
7. *U. jiroveci* (Ormières et al. 1973)

8. *U. spisuli* (Perkins et al. 1975)
9. *U. cannoni* (Anderson et al. 1993)

Bonamia (Pichot et al. 1979)

1. *B. ostreae* (Pichot et al. 1979)
2. *B. exitiosa* (Berthe and Hine 2003) (formerly described as *B. exitiosus*) (Hine et al. 2001)
3. *B. perspora* (Carnegie et al. 2006)

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Cryptophyta (Cryptomonads)

24

Kerstin Hoef-Emden and John M. Archibald

Abstract

Cryptomonads are small (~5–50 μm) biflagellate protists found in diverse freshwater, brackish, and marine habitats. They are characterized by a distinct cellular asymmetry and the presence of extrusive organelles called ejectosomes. Many cryptomonads are photosynthetic; their plastids are diverse in pigmentation and coloration. Plastid-bearing cryptomonads are noteworthy in their possession of a "nucleomorph," a residual nucleus of secondary endosymbiotic origin. Members of the cryptomonad genus *Goniomonas* lack plastids and ingest bacteria for nutrition. Mixotrophic cryptomonads may also exist, and loss of photosynthesis has given rise to colorless, heterotrophic, leucoplast-bearing species on multiple occasions. Cryptomonad taxonomy was traditionally based on morphology and now includes consideration of ultrastructural features such as the cell shape, the periplast structure, the type of cell invagination present (furrow-gullet system), the flagellar apparatus architecture, and the presence-absence of pyrenoids. However, molecular sequence data suggest that morphology is of limited taxonomic utility at the level of species identification. Cellular dimorphisms have been found within clonal cultures, supporting the notion that cryptomonads are capable of sexual reproduction. Approximately 20 genera and >100 species of cryptomonads have been described, although their true diversity and abundance in nature is unknown.

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Contents

Summary Classification

- **Cryptophyceae**
- - **Cryptomonadales** (e.g., *Chroomonas*, *Cryptomonas*, *Geminigera*, *Guillardia*, *Proteomonas*, *Rhodomonas*)
- - ***Goniomonas***

Introduction

General Characteristics

The cryptomonads (= cryptophytes = Cryptophyceae) are a delineated protist phylum. Most species are photosynthetic and motile (Figs. 1, 2, and 3); palmelloid forms are also known. The latter tend to form colonies invested in multiple mucilaginous sheaths. Some are known to form thick-walled cysts (Fig. 4). Cryptomonads are easily recognized with their flattened asymmetric cells, distinctive swimming motion, refractile ejectosomes (a kind of extrusome), and distinctive ultrastructure (Figs. 1, 5, 6, 7, 8, 9, 10, 11, and 12). Cryptomonad plastids contain chlorophylls *a* and c_2 as well as a proteinaceous phycobiliprotein as a second light-harvesting complex and are unusual in that a remnant nucleus of endosymbiotic origin lies in close association with the organelle (Fig. 11; see "Electron Microscopy" section below).

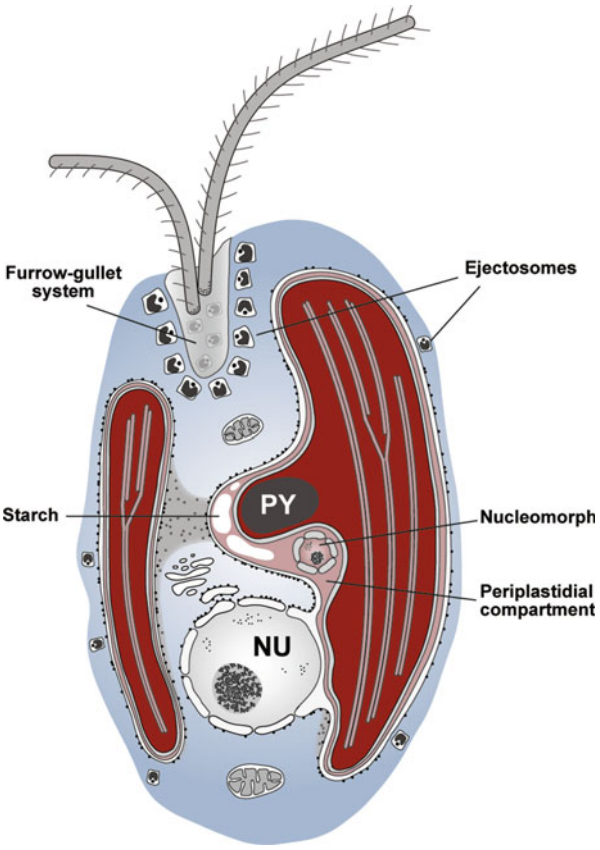

Fig. 1 Drawing of a generic cryptomonad cell. Most cryptomonads possess a single bilobed plastid surrounded by four membranes. The outermost plastid membrane is continuous with the endomembrane system and is studded with ribosomes. Abbreviations: *NU* nucleus, *PY* pyrenoid

It is often difficult to distinguish different cryptomonad genera from one another. Many taxonomically informative characters require electron microscopical methods and/or spectrophotometric analysis. Unidentified dimorphic life histories in some genera have resulted in an inconsistent systematics, and a lack of species-specific characters hampers identification of species by morphology. In several cryptomonad genera, species identification may be possible only with molecular signatures.

Occurrence

Cryptomonads are ubiquitous in marine, brackish, and freshwater habitats (Klaveness 1988), though it is currently not possible to determine the distribution of cryptomonad taxa on a global scale. Recent research has shown that even in well-sampled regions and habitats, the true diversity of cryptomonads has not yet been uncovered (von der Heyden et al. 2004; Hoef-Emden 2007; Lane and Archibald 2008; Shalchian-Tabrizi et al. 2008). Cryptomonads tend to be quite fragile; their numbers may therefore be underestimated in field collections.

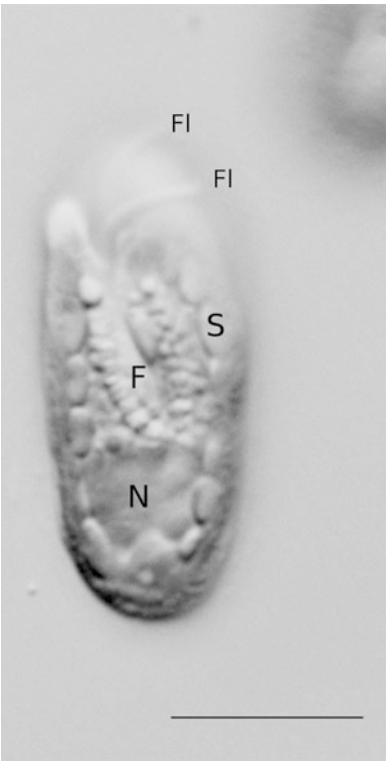

Fig. 2 Differential interference contrast (Nomarski optics) picture of *Cryptomonas borealis*, epitype strain CCAC 0113. Image focused on the ventral side of the cell, showing the elongate open furrow and approximate insertion site of the two flagella. The elliptical platelets lining the cell periphery are starch grains. Cell embedded live in ultra-low gelling agarose. Scale bar is 10 μm and is also valid for Figs. 3 and 4. *N* nucleus, *Fl* flagella, *S* starch grain

Literature

No exhaustive monographs are available for cryptomonads. Traditional morphology-based classification schemes for cryptomonads are inconsistent (Deane et al. 2002; Hoef-Emden et al. 2002; Hoef-Emden and Melkonian 2003; Hoef-Emden 2005, 2007; Lane and Archibald 2008). Most of the literature addressing cryptomonad species or genera has relied on morphological characters alone and is therefore outdated. The most recent comprehensive keys for species identification were assembled by Huber-Pestalozzi (1950; freshwater taxa, in German), Butcher (1967; marine taxa), and Starmach (1974; freshwater taxa, in Polish). Although some genera have subsequently been revised or synonymized, classification schemes of cryptomonad genera based on ultrastructural characters nevertheless provide a good overview of cryptomonad cell biology (Clay et al. 1999; Kugrens and Clay 2002; Novarino 2003).

History of Knowledge

The name-giving genus *Cryptomonas* was erected by Ehrenberg in 1831. He described the first cryptomonad species in 1832 (Ehrenberg 1832), and figures

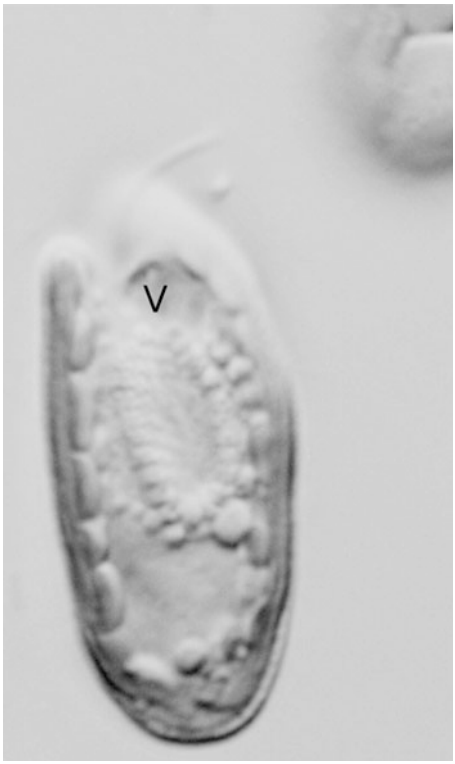

Fig. 3 *Cryptomonas borealis*, epitype strain CCAC 0113. Same cell as in Fig. 2, but with plane of focus in the cell middle, showing the ejectosome-lined gullet. *V* contractile vacuole

were published as colorized copper-plate engravings 6 years later (Ehrenberg 1838). The Ehrenberg Collection, which includes C. G. Ehrenberg's original watercolor drawings, manuscripts, and specimens, is maintained at the Museum für Naturkunde of the Humboldt University at Berlin (http://www.naturkundemuseum-berlin.de/en/collections/palaeontology/ehrenberg-collection/, last accessed 2016-01-15; Lazarus and Jahn 1998).

Practical Importance

Although cryptomonads have not yet been exploited commercially on a large scale, they are nontoxic, are easy to mass-cultivate, contain polyunsaturated fatty acids among other lipids, and are therefore used as food for copepods in fish farming (Brown et al. 1997; Knuckey et al. 2005). Cryptomonad biliprotein pigments may be used as fluorescent dyes (Telford et al. 2001; Sekar and Chandramohan 2008). Govorunova et al. found genes for channelrhodopsin proteins with seven transmembrane helices in the genome of the model cryptophyte *Guillardia theta* (Govorunova et al. 2015). When expressed in human embryonic kidney cells, these rhodopsins proved to be anion-specific (Cl^-) light-gated channels that can be used as

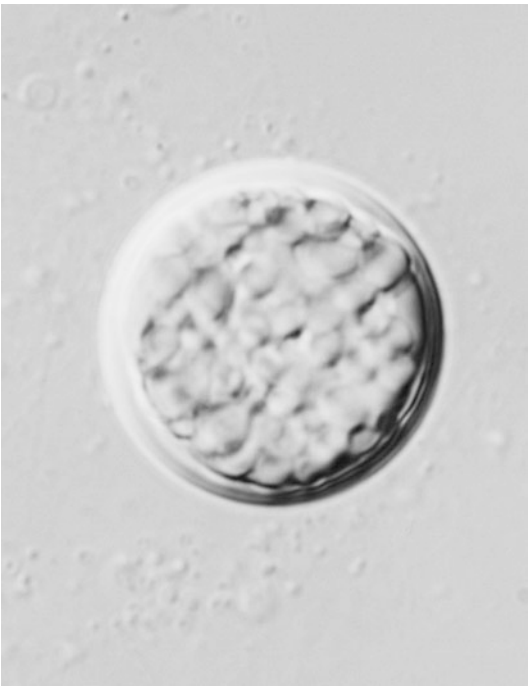

Fig. 4 Differential interference contrast (Nomarski optics) picture of *Cryptomonas curvata*, cryptomorph strain CCAC 0006. Image shows resting cyst with thick cell wall from a starved culture. Cell embedded live in ultra-low gelling agarose

optogenetic tools in neuroscience research for the hyperpolarization of cells. Cryptophyte channelrhodopsins may thus prove useful as antagonists of chlorophyte Ca^{2+}-specific channelrhodopsins, which have already been established as tools to depolarize neural cells.

Habitats and Ecology

Photosynthetic cryptomonads belong to the phytoplankton community of essentially every body of water one can imagine, be it marine, brackish, or limnic; only the phagotrophic and aplastidic genus *Goniomonas* seems to be benthic (Skuja 1948; Klaveness 1988; Patterson and Simpson 1996; Bernard et al. 2000). Cryptomonads have been found in cold, temperate, and tropical waters; in offshore and coastal water samples; in rock pools, tide pools, freshwater lakes, ponds, puddles, and rain barrels; and even in snow and hypersaline lakes (Butcher 1967; Javornický and Hindák 1970; Klaveness 1988; Hill 1991a; Alcocer et al. 1998; Garibotti et al. 2003; Hoef-Emden 2007; Phlips et al. 2008). It is almost impossible to draw a water sample that does not contain at least a few cryptomonad cells. Cryptomonads are present throughout the year and are often reported as one of the most prominent or even the dominant algal group at certain times of the year (Alcocer et al. 1998; Garibotti et al. 2003; Phlips et al. 2008). Species of the genus *Cryptomonas* probably represent the most abundant

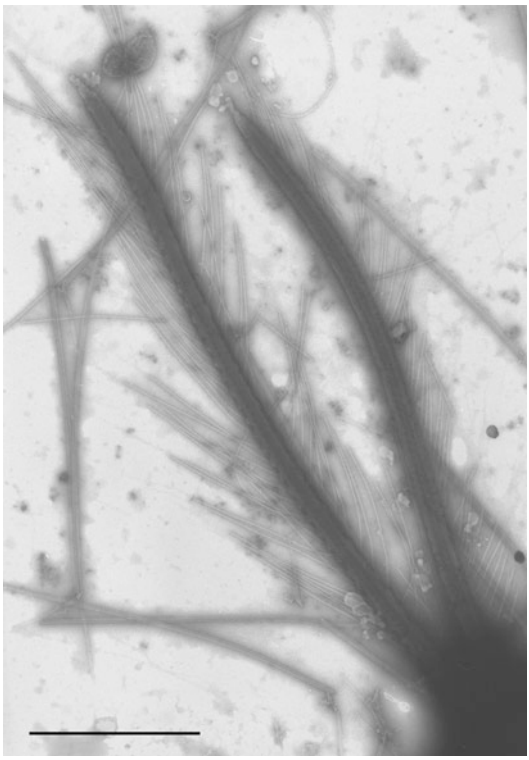

Fig. 5 Whole mount preparation of *Cryptomonas commutata* flagella (cryptomorph strain CCAC 0109). The longer flagellum has two opposite rows of flagellar hairs, the shorter one only one row. The straight structures scattered across the image are discharged ejectosomes. Uranyl acetate stain, negative contrast. Scale bar = 2 μm

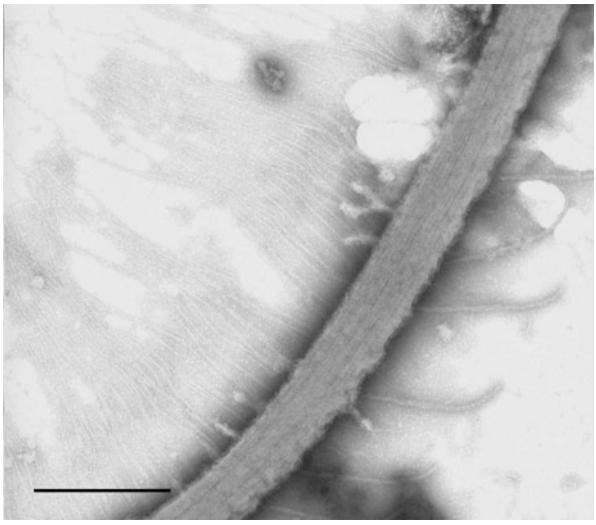

Fig. 6 Whole mount preparation of a *Goniomonas truncata* flagellum (strain M0871). One row of curved "spikes" runs along the flagellum, on the other side, many thin and long fibrils are visible. Uranyl acetate stain, negative contrast. Scale bar = 0.5 μm

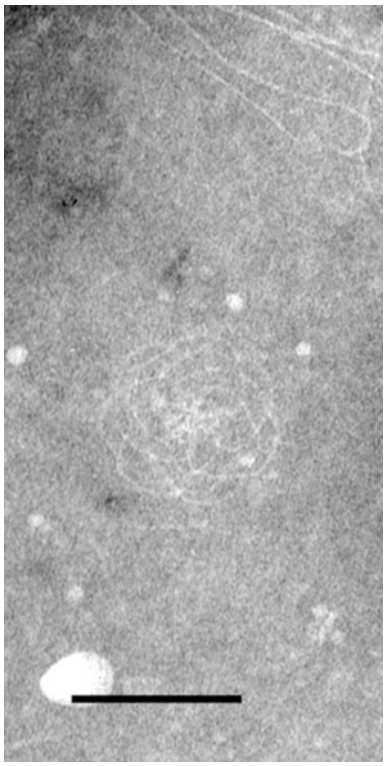

Fig. 7 Whole mount preparation of a Rosulate scale of *Rhodomonas* sp. strain CCAC 1090 B. Thin fibrils from the tip of a nearby flagellum are visible on the *top right*. Stained with methylamine tungstate, negative contrast. Scale bar = 125 nm

cryptomonads in freshwater, often forming dense populations close to the chemocline of lakes. They have been reported to migrate vertically in the water column down to the anoxic environment at night and up to the epilimnion during the day (Gasol et al. 1992; Gervais 1997; Camacho et al. 2001). Cryptomonads constitute a major component of aquatic food chains as nontoxic prey for planktonic ciliate, dinoflagellate, or copepod predators (Pedrós-Alió et al. 1995; Weisse and Kirchhoff 1997; Roberts and Laybourn-Parry 1999; Tirok and Gaedke 2007). Vertical migration and the formation of palmellae – accumulations of flagellated cells embedded in mucus – may represent predator avoidance strategies (Klaveness 1988). However, it has been hypothesized that cryptomonads stay close to the chemocline at night to take up nutrients such as phosphorus (Camacho et al. 2001). To survive unfavorable environmental conditions, *Cryptomonas* species may produce globular and thick-walled cysts as resting stages (Lichtlé 1979, 1980). Cryptomonads not only suffer from predation but can also be invaded by intracellular parasites (Brugerolle and Mignot 1979; Ettl and Moestrup 1980; Brugerolle 2002).

Photosynthetic cryptomonads are important primary producers in aqueous habitats, not only due to their ubiquitous presence and abundance but also as low-light specialists. Using biliproteins as light-harvesting complexes, compensation points of cryptomonad photosynthesis have been found at light intensities below 30 μmol

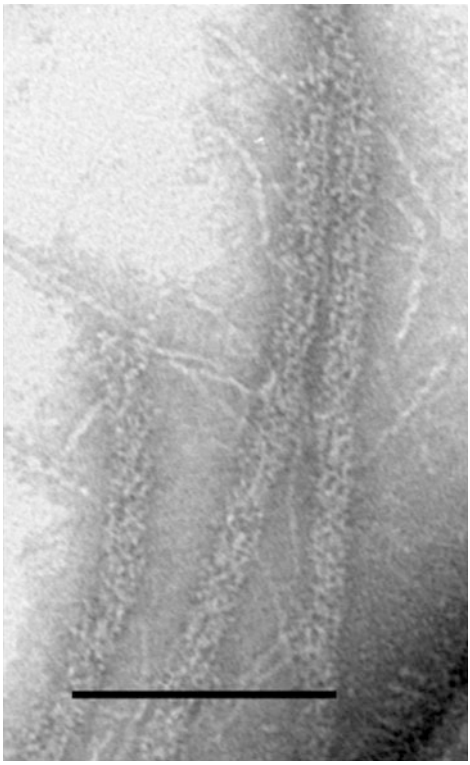

Fig. 8 Whole mount preparation of a "spiked" flagellum of *Goniomonas truncata* strain M0871. The granular surface structure of the "spikes" resembles that of flagellar hairs of plastid-containing cryptomonads. Short fibrils are attached to the "spikes." Uranyl acetate stain, negative contrast. Scale bar = 100 nm

photons m^{-2} s^{-1} (Gervais 1997; Hammer et al. 2002). In addition, the absorption spectrum of the biliproteins fills the "green gap" between the blue and red light absorption maxima of the chlorophylls. This enables cryptomonads to utilize the green light passing through layers of other algae (Doust et al. 2006).

Several reports indicate that plastid-containing cryptomonads may be mixotrophic. A marine strain of *"Chroomonas" salina* (subsequently renamed to *Rhodomonas salina*) was shown to be capable of growth in darkness at extremely high glycerol concentrations of 0.5 M (Antia et al. 1969). The observation that loss of photosynthesis has evolved at least three times independently from photosynthetic *Cryptomonas* ancestors further supports the notion of mixotrophy in photosynthetic cryptomonads (Hoef-Emden 2005). At least some strains of *Cryptomonas paramecium* (formerly subsumed under the genus *Chilomonas* together with the two other unrelated leucoplast-containing lineages; Hoef-Emden and Melkonian 2003; Hoef-Emden 2005) can grow axenically in media with organic additives (e.g., strain CCAC 0056; refer to the websites of the culture collections CCAC and CCAP for media recipes). While no cytostome has yet been observed in plastid-containing cryptomonads (unlike the phagotrophic genus *Goniomonas*), uptake of bacteria has been observed (Tranvik et al. 1989). According to Kugrens and Lee, bacteria entered the cells of a freshwater *Chroomonas* via the contractile vacuole (Kugrens and Lee 1990).

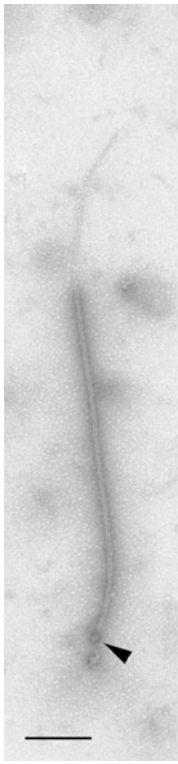

Fig. 9 Whole mount preparation of a detached flagellar hair of a *Cryptomonas obovoidea* flagellum (strain CCAC 0106 B). Flagellar hairs from the longer flagellum are attached to the axoneme by a globular structure (*arrow head*). Hairs from the shorter flagellum do not possess a globular attachment site and have a shorter shaft and two unequal filaments instead of only one terminal filament. Uranyl acetate stain, negative contrast. Scale bar = 250 nm

Other studies refute bacterivory or consider these bacteria to be merely endocytic (Schnepf and Melkonian 1990; Gervais 1997). Bacteria, however, may settle on the surface of *Cryptomonas* cells causing modifications in cell shape (Klaveness 1982).

Some cryptomonads are highly adaptable to different salinities. A *Chroomonas* species from South African habitats has been reported to grow in marine as well as freshwater media (Meyer and Pienaar 1984a). A vacuolar region in the apex of these cells transformed into a contractile vacuole in freshwater and vice versa. A more detailed study by Hoef-Emden (2014) confirmed most of the observations of Meyer and Pienaar. However, the contractile vacuole did not stop operation at levels of marine salinity; it just worked more slowly and with a small diameter (Hoef-Emden 2014). All close relatives of the African isolate were euryhaline and in one representative culture of the clade, CCAP 978/08, the osmolyte produced under brackish to marine conditions proved to be floridoside, an osmoprotectant known otherwise from red algae (Hoef-Emden 2014).

Several phagotrophic dinoflagellates – *Dinophysis* spp., *Nusuttodinium acidotum*, *N. aeruginosum*, and *Pfiesteria piscicida* – and the ciliate *Myrionecta rubra* (formerly *Mesodinium rubrum*) mimic a photosynthetic lifestyle by engulfing cryptomonads and taking advantage of their photosynthetic organelles for days at a time before completely digesting them (Schnepf et al. 1989; Fields and Rhodes

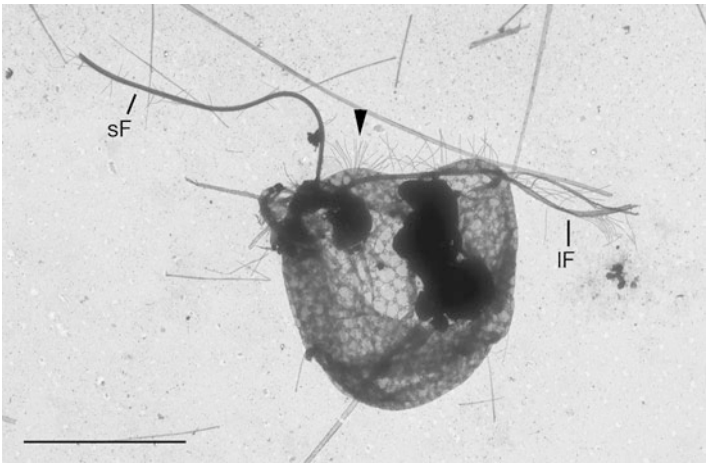

Fig. 10 Whole mount preparation of an isolated inner periplast component (IPC) of cryptomonad strain CCAP 979/35 (cryptomorph of either *Cryptomonas curvata* or *C. pyrenoidifera*). The IPC consists of poly- to hexagonal plates with ejectosome discharge holes between the corners of the plates. The dark globules inside of the periplast are starch grains. The IPC has been isolated with a microtubule-stabilizing buffer usually used to isolate cytoskeletons. The axonemes of the two flagella are therefore sometimes preserved during the isolation procedure. The axoneme of the shorter flagellum (*sF*) is sparsely covered by some of the remaining flagellar hairs. Almost all hairs are still attached to the axoneme of the longer flagellum (*lF*). The tuft of hairs at the base of the longer flagellum is also still present (*arrow head*). Fragments of discharged ejectosomes are scattered around the IPC. Uranyl acetate stain, negative contrast. Scale bar = 10 μm

1991; Lewitus et al. 1999; Gustafson et al. 2000; Takishita et al. 2002; Minnhagen and Janson 2006; Onuma and Horiguchi 2015). This phenomenon is referred to as kleptoplastidy. *Dinophysis* spp., *Pfiesteria piscicida*, and *Myrionecta rubra* form blooms under optimal environmental conditions, which is especially problematic in the case of *Dinophysis* spp. and *Pfiesteria piscicida*, as they produce dangerous toxins (Rao et al. 1993; Burkholder and Glasgow 1997).

Characterization and Recognition

Light Microscopy

Cell sizes of plastid-containing cryptomonads range from below 5 μm (*Hemiselmis* species) up to 50 μm (*Cryptomonas curvata* campylomorphs). The cells show a marked asymmetry and may be twisted along their longitudinal axis. The apex often is shifted to the left side of the cell (Figs. 2 and 3), whereas the two unequal flagella insert subapically or laterally and to the right side of the cell in the vestibulum of an invagination, the furrow-gullet system (Figs. 2 and 3; Klaveness 1985; Clay and Kugrens 1999; Hoef-Emden and Melkonian 2003). The furrow-gullet system defines

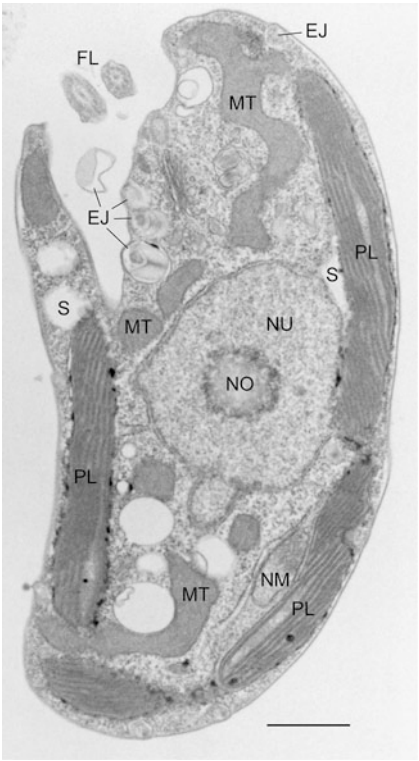

Fig. 11 Transmission electron micrograph of the cryptomonad *Guillardia theta* strain CCMP327 sectioned approximately longitudinally. The flagella (*FL*) and one of the large gullet-associated ejectosomes (*EJ*) are shown in the cross section. Periplast-associated ejectosomes are also apparent. Starch (*S*) deposits are located in the periplastidial compartment and, depending on the plane of section, can be difficult to distinguish from lipid-containing vesicles in the cytoplasm. Additional abbreviations: *NU* nucleus, *NO* nucleolus, *NM* nucleomorph, *PL* plastid, *MT* mitochondrion. Scale bar = 1 μm

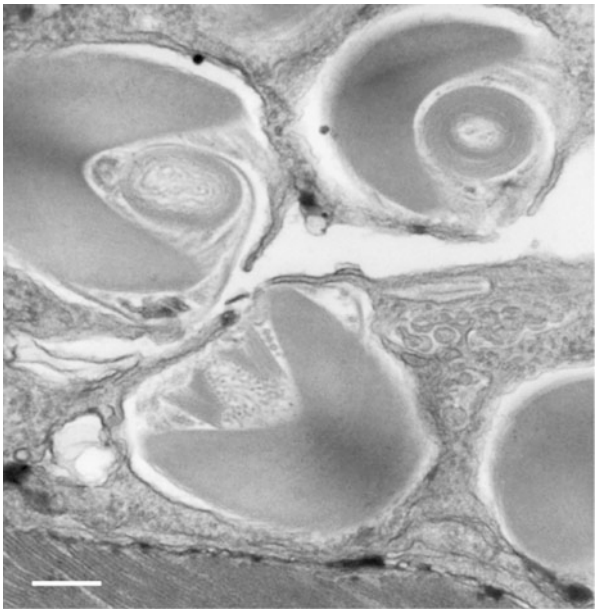

Fig. 12 Transmission electron micrograph showing large gullet ejectosomes of *Cryptomonas* sp. strain CCAP 979/52. Scale bar = 200 nm

the ventral side of the cryptomonad cell. In the cross section the cells have a rounded to elliptical outline. In *Cryptomonas* the broad side views usually correspond to ventral and dorsal sides of the cell. Large explosive organelles, termed ejecto- or ejectisomes, line the furrow-gullet system (Figs. 2 and 3). Although the term ejectisome was introduced by Anderson (1962) and is still seen in the literature; the corrected spelling ejectosome is used throughout this text (since both parts of the term are of Greek origin, the letter "o" must be used to connect them).

One striking feature of cryptomonads is the astounding variety of colors they exhibit. Due to the presence of different types of blue or red biliproteins (albeit with only one type per cell/clonal culture; Hill and Rowan 1989), in combination with chlorophylls *a* and c_2 and the carotenoid alloxanthin, cryptomonad plastids may display shades from chocolate brown to olive brown, bright brown, tomato red, brick red, blue green, and sky blue (Butcher 1967; Clay et al. 1999). Cryptomonads usually contain only one H-shaped plastid per cell. In most cryptomonad genera, the bridge connecting the two plastid lobes is broad and contains a single pyrenoid, whereas it is very thin or – perhaps prior to cell division – absent in *Cryptomonas* species (Hollande 1942; Ettl 1980; Klaveness 1985; Hill 1991a, b; Hoef-Emden and Melkonian 2003). In genera/species with two separate plastids or with very thin connections between the plastid lobes, pyrenoids are either absent or distributed pairwise at the inner sides of the two lobes (Taylor and Lee 1971; Hill 1991a; Hoef-Emden and Melkonian 2003). In some *Chroomonas* species, an eyespot consisting of carotene globules is present in the plastid close to the pyrenoid (Erata et al. 1995; Clay et al. 1999). Cryptomonads mainly use starch as their energy storage material, but lipids can also be used (Pringsheim 1968; Santore 1985; Deschamps et al. 2006). Whereas lipid vesicles are found in the cytosol, starch is produced in the periplastidial compartment (see Electron Microscopy section below). Under suboptimal growth conditions, the starch grains accumulate on the surface of the plastid and – due to a more or less regular pattern – may be mistaken for many small plastids or even periplast plates in surface view (Fig. 2; Pringsheim 1944).

In most cryptomonads, the longer flagellum serves as an anteriorly directed locomotoric flagellum, which pulls the cell behind. The cell rotates about its longitudinal axis during forward swimming, emphasizing the characteristic compression and asymmetry. Upon irritation, the forward swimming motion may be interrupted by abrupt directional changes accompanied by sharp backward jerking movements (first noticed by Ehrenberg 1838). The latter may be the result of discharge of ejectosomes.

Several cryptomonad species form palmellae (Pringsheim 1968; Klaveness 1985). A palmella is an agglomeration of flagellated cells embedded in mucus, which may grow to macroscopically visible size. The flagellates divide within the mucus, but may escape from it easily (Pringsheim 1968). In the genus *Chroomonas*, palmellae may be quite robust and virtually undissolvable. In a study of osmotolerance in *Chroomonas* species thriving in tide pools, it was hypothesized that these palmellae may also serve to protect against desiccation (Hoef-Emden 2014). Flagellar insertion sites in the embedded cells are retracted into the gullet region, resulting in a branched gullet (Meyer and Pienaar 1984a). In members of the

genus *Cryptomonas*, cells can be mobilized from palmellae simply by placing a cover slip on a drop of culture. Some species (in *Cryptomonas* seemingly only cryptomorphs; see Taxonomy section below) may produce cysts as resting stages upon starvation and/or high light intensities (Lichtlé 1979, 1980; Hoef-Emden and Melkonian 2003). A thick cell wall consisting of concentric layers protects the globular starch-laden cyst, which shows no internal structures such as furrow-gullet system or ejectosomes (Fig. 4). Even after several months in nutrient-depleted cultures, cysts may still germinate after being transferred to fresh culture medium (Lichtlé 1979, 1980; Hoef-Emden and Melkonian 2003).

The aplastidic and phagotrophic genus *Goniomonas* differs in cell shape, organization of the cell, and swimming behavior from plastid-containing cryptomonads (see also Electron Microscopy). *Goniomonas* cells are markedly flattened with an obliquely truncate anterior. Parallel to the truncate anterior, a single straight line or ring of ejectosomes traverses the apical part of the cell (Larsen and Patterson 1990; Lee et al. 2005). Light microscopically visible striations on the cell surface may correspond to periplast plates (Hill 1991c). Goniomonads engulf bacteria and usually they prefer gliding along substrates to swimming freely in the water column.

Electron Microscopy

Cell periphery. Cryptomonads lack true cell walls. The cells are bounded by the periplast, composed of proteinaceous inner and surface layers that sandwich the plasma membrane that can be best examined by freeze-fracture techniques and scanning electron microscopy. The inner periplast component (IPC) may consist of hexagonal, polygonal, rectangular, or elongate plates or may be continuous and sheetlike (Fig. 10 shows polygonal IPC plates). The surface is covered by congruent plates, rosulate scales, and/or a fibrous coat (the surface periplast component = SPC; Table 1; Fig. 7). In sectioned material, the IPC appears either as a separate layer or closely appressed to the plasma membrane; retention of the external layer is sensitive to the fixation procedure. Evidence from freeze-fracture studies of members of the genus *Cryptomonas* (Brett and Wetherbee 1986) indicates that there are two types of fibrous coats, one of which is associated with rosulate body scales. Scales have also been observed in association with flagella (Pennick 1981; Santore 1983). The periplast plates tend to decrease in size toward the posterior of the cell; they do not extend into the vestibulum or the furrow-gullet region.

Furrow-gullet system. The cryptomonad furrow-gullet system can be usually localized by the presence of large ejectosomes (see below) lining this cell invagination (Figs. 2, 3, and 11). The identification of the type of furrow-gullet system in most smaller cryptomonads requires electron microscopical methods. Among other characters, different types of furrow-gullet systems have been used to differentiate genera (Table 1; closed tubular gullet vs. slit-like opening along entire length of cell invagination = furrow vs. combination of furrow and gullet vs. shallow groove). In the genus *Cryptomonas*, which possesses a furrow extending into a more or less pronounced tubular gullet, the furrow can open and close (Kugrens et al. 1986;

Table 1 Cryptomonad genera

Genus	Type of biliprotein	Position of the nucleomorph	Furrow-Gullet system	IPC	SPC	Rhizostyle	Other features
Chroomonas	PC630 or PC645	Free	Gullet or branched gullet	Longitudinal rows of stepped rectangular plates or hexagonal plates	Fibrils, plates	None[1]	Thylakoids traverse pyrenoid matrix, eyespot may be present
Cryptomonas[2]	PE566 or leucoplast	Free	Combination	Polygonal plates (CR) or sheet (CA)	Fibrils, rosulate scales	Non-keeled (CR) or keeled (CA)	Dimorphic; deeply bilobed plastid, ev. with one or more pairs of pyrenoids
Falcomonas	PC569	Free	Furrow	Polygonal plates	Plates with fibrillar margins, few rosulate scales	No information available	Pyrenoid bisected by periplastidial cytoplasmic extension
Geminigera	PE545	Embedded in nucleus	Combination	Sheet	Fibrils, rosulate scales	Keeled	Deeply bilobed plastid with two pyrenoids
Goniomonas	No plastid	-	Groove + cytopharynx	Large longitudinal plates	Longitudinal plates	Non-keeled, short	Flagella with "spikes" and long fibrils
Guillardia	PE545	Free	Gullet	Sheet	Large longitudinal plates	Non-keeled[3]	
Hanusia	PE545	Free	Furrow	Sheet	Rosulate scales	Keeled	
Hemiselmis	PC615, PC630, PC577 or PE555	Free	Short gullet	Hexagonal plates	Hexagonal plates, fibrils	None[1]	Thylakoids traverse pyrenoid matrix

(*continued*)

Table 1 (continued)

Genus	Type of biliprotein	Position of the nucleomorph	Furrow-Gullet system	IPC	SPC	Rhizostyle	Other features
Komma	PC645	Free	Gullet	Hexagonal plates	Hexagonal plates, rosulate scales	No information available	No thylakoids in pyrenoid matrix
Plagioselmis	PE545	Free	Furrow	Hexagonal plates	Hexagonal plates, tail without plates	Non-keeled	
Proteomonas	PE545	Free	Furrow	Polygonal plates (1n), sheet (2n)	Fibrils, rosulate scales (1n + 2n)	Non-keeled (1n), keeled (2n)	Dimorphic
Rhinomonas	PE545	Embedded	Gullet	Polygonal plates	Polygonal plates, fibrils	None[1]	
Rhodomonas	PE545	Embedded	Combination	Rectangular plates	Fibrils, rosulate scales	Keeled	
Storeatula	PE545	Embedded	Gullet	Sheet	Fibrils	Keeled	
Teleaulax	PE545	Free	Furrow	Sheet	Fibrils, rosulate scales	Keeled	

Notes: Genera sorted in alphabetical order. Ultrastructural characters and biliproteins according to Mignot (1965), Mignot et al. (1968), Dodge (1969), Roberts et al. (1981), Grim and Staehelin (1984), Santore (1984; *Rhodomonas* = *Pyrenomonas*), Brett and Wetherbee (1986); Hill and Wetherbee (1986, 1988, 1989, 1990), Wetherbee et al. (1986); Kugrens and Lee (1987, 1991), Hill (1991a, b, c), Novarino et al. (1994), Deane et al. (1998), Clay and Kugrens (1999), Clay et al. (1999); Hoef-Emden and Melkonian (2003), Novarino (2003), Hoef-Emden (2008), Lane and Archibald (2008). Potential candidates for alternative morphotypes according to phylogenetic analyses: *Rhinomonas*, *Rhodomonas*, and *Storeatula* (synapomorphy of this clade = nucleomorph embedded in pyrenoid matrix); *Geminigera*, *Plagioselmis*, and *Teleaulax* (Deane et al. 2002; Hoef-Emden et al. 2002; Hoef-Emden 2008). (1n), *Proteomonas* haplomorph; (2n), *Proteomonas* diplomorph; combination, slit-like opening (furrow) extending into a closed tubular gullet; embedded, nucleomorph embedded in pyrenoid matrix; *IPC*, inner periplast component; keeled, keeled rhizostyle (posteriorly directed microtubular root of the flagellar root system) with winglike appendages; *PC*, phycocyanin; *PE*, phycoerythrin; *SPC*, surface periplast component. Some additionally described features such as ligulae or other details of furrows or midventral bands have not been listed. [1]According to Clay et al. (1999); [2]includes the synonymized "*Campylomonas*," "*Chilomonas*," "*Cryptochrysis*," and "*Pseudocryptomonas*" (Hoef-Emden and Melkonian 2003; Hoef-Emden 2005, 2007); [3]according to Gillott and Gibbs (1983), but keeled according to Clay et al. (1999)

Melkonian et al. 1992). The cells probably achieve this by contracting two bands of centrin that run longitudinally along the left and right margins of the furrow and may correspond to the rim fibers of Gillott and Gibbs (Gillott and Gibbs 1983; Melkonian et al. 1992). Furrows may differ in the presence/absence of delicate structures such as vestibular ligulae or rim folds (Kugrens et al. 1986). In all cryptomonads, freshwater, brackish, or marine, a contractile vacuole appears to occupy the apical pole of the cell and empties into the vestibulum (Fig. 3; Patterson and Hausmann 1981; Hoef-Emden 2014).

Ejectosomes. Cryptomonads possess unique explosive organelles, termed ejectosomes (Figs. 2, 3, 11, and 12). Small ejectosomes are located underneath the periplast, either at the corners between IPC plates or equally distributed in strains with sheetlike IPCs (Fig. 11). Larger light microscopically visible ejectosomes line the furrow-gullet system (Figs. 2, 3, and 11). An undischarged ejectosome is a tightly coiled, tapered ribbon that is wound with the wider end toward the outside; a smaller coil is attached to it and lies in the depression of the larger one (Morrall and Greenwood 1980; Fig. 12). Prior to release, ejectosomes are enclosed within vesicles. When discharged, the ribbon unfurls, with the shorter segment forming a beaklike tip on the longer. The edges of the ribbon tend to curl inward, producing circular and c-shaped profiles in the cross section. Yamagishi et al. examined the proteins of purified ejectosomes and found sequence similarities to the product of *reb*B, a component of the ejectile R-bodies in the gammaproteobacterium *Caedibacter taeniospiralis*, which lives as an endosymbiont in the ciliate *Paramecium* (Yamagishi et al. 2012). The ejectosome proteins, termed ejectisins, proved to be extremely resistant to high concentrations of detergent, to freezing and thawing cycles, and to reducing chemicals (Ammermann et al. 2013).

Flagella and flagellar apparatus. The slightly unequal flagella of plastid-containing cryptomonads emerge near the base of the vestibulum and bear rows of bipartite flagellar hairs closely resembling and possibly being related to stramenopile mastigonemes. Flagellar hairs of cryptomonads consist of a shaft with thin terminal filaments, but do not have a proximately tapering basis like mastigonemes. Instead, the flagellar hairs of the longer flagellum are attached to the axoneme with a globular structure, whereas the flagellar hairs of the shorter flagellum are not and seem to detach more easily (Hibberd et al. 1971; Figs. 8 and 9). Kugrens et al. observed six different patterns of distribution of flagellar hairs in cryptomonad strains (Kugrens et al. 1987). Some strains showed the "classical" two rows of flagellar hairs on the longer flagellum, whereas the shorter one bore a single row (Fig. 5). In other strains, each flagellum was covered by only one row of flagellar hairs, sometimes together with thin filaments, or only the longer flagellum had one row of hairs. According to Kugrens et al. (1987), two flagella with only one row of flagellar hairs have been observed in *Cryptomonas* strains with a sheetlike IPC (campylomorphs; see Taxonomy below), whereas the classical two-row/one-row combination was found in *Cryptomonas* strains with periplast plates (cryptomorphs) and in some *Chroomonas* species (Kugrens et al. 1987; Fig. 5). This indicates a correlation of flagellar hair pattern with the alternating morphotypes in *Cryptomonas.* A tuft of flagellar hairs was present on a swelling near the base of the longer flagellum in a cryptomonad

strain with "classical" flagellar hair pattern (Hibberd et al. 1971; Fig. 10). In addition to the flagellar hairs, fine filaments and rosulate scales 12–14 nm in diameter may be associated with the flagellar surface (Pennick 1981; Santore 1983; Fig. 7).

The axoneme has a typical 9 + 2 arrangement; fibrous or amorphous material is sometimes present on the side of the axoneme. The transition region, located external to the cell body, is composed of two to several platelike partitions (Gillott and Gibbs 1983; Santore 1982; Roberts et al. 1981; Roberts 1984; Hibberd et al. 1971; Mignot et al. 1968). The central pair of axoneme microtubules terminates at the distal partition, which extends out to the plasma membrane. One to three proximal partitions may be present; these span only the central portion of the axoneme. There is no evidence of a transitional helix such as those found in other chlorophyll *c*-containing algal classes (Hibberd 1979). The basal bodies are oriented at a slight angle to each other and are connected by two bands, at least one of which is striated (Mignot et al. 1968; Roberts et al. 1981; Gillott and Gibbs 1983). In addition to these connections, some components of the flagellar rootlets pass between the basal bodies (Gillott and Gibbs 1983; Roberts et al. 1981; Roberts 1984).

The most prominent flagellum-related structures are the rhizostyle and the compound rootlet. The rhizostyle is a posteriorly directed microtubular structure. It originates alongside one of the basal bodies and extends deep into the cell, passing near the nucleus in some species, although no physical connections with the nuclear envelope have been found (Gillott and Gibbs 1983; Roberts et al. 1981). In many cryptomonads, e.g., a colorless *Cryptomonas* sp. (Roberts et al. 1981; Mignot et al. 1968), *Rhodomonas* sp. (Mignot et al. 1968), and *Hanusia phi* (Gillott and Gibbs 1983), each of the rhizostyle microtubules bears a winglike lamellar projection. These characteristic wings are absent in other taxa, e.g., *Guillardia theta* (Gillott and Gibbs 1983). In the genera *Proteomonas* (Hill and Wetherbee 1986) and *Cryptomonas* (Hill 1991a; alternative morphotype described as a distinct genus *Campylomonas*), the absence or presence of winglike extensions is correlated with alternating life histories. In both genera, the morphotype with a sheetlike periplast contains a rhizostyle with winglike extensions, whereas in the morphotype with polygonal periplast plates, the rhizostyle is without wings (Hill and Wetherbee 1986; Hill 1991a).

The compound rootlet is made up of microtubules associated with a striated fibrous band. The microtubules of the compound rootlet originate near the rhizostyle and pass between the basal bodies; the striated fiber component attaches to the opposite side of both basal bodies. This rootlet extends laterally, passing between the vacuolar region and the plasma membrane. Two other microtubular structures are commonly associated with the basal bodies. One microtubular rootlet, which may be very short, originates near the rhizostyle and extends anteriorly. A second microtubular structure, variously termed the lateral (Gillott and Gibbs 1983) or curved (Roberts et al. 1981) rootlet, extends dorsolaterally. A mitochondrion-associated lamellar rootlet has been found in several *Cryptomonas* strains (Roberts et al. 1981; Roberts 1984). Anti-centrin antibodies have been used to label four different parts of the *Cryptomonas* cytoskeleton (Melkonian et al. 1992). Apart from the rim fibers of

the left and right furrow margins, centrin was observed to run as a thin band along the microtubular rhizostyle, to interconnect the two basal bodies, possibly corresponding to the striated band of Gillott and Gibbs (1983), and to connect the basal bodies to the vestibulum (Melkonian et al. 1992).

The fluid dynamics of flagella in diverse protists has been examined and modeled mathematically. In flagella with two opposite rows of stiff flagellar hairs, a sinusoidal beating pattern in plane with the flagellar hairs results in a reversal of thrust (Sleigh 1991). Thus, the flagellum pulls the cell behind. In cells with only one row of stiff flagellar hairs attached to the longer flagellum, this system does not work. In Brennen and Winet (1977), a *Chilomonas* species (i.e., a heterotrophic *Cryptomonas* with a sheetlike IPC) was observed to possess a helical flagellar beat, which may account for the presence of a flagellum with only one row of stiff flagellar hairs. The differences between the flagellar root systems, i.e., a long rhizostyle with winglike extensions versus a shorter non-decorated rhizostyle, thus may be related to different arrangements of stiff flagellar hairs and beating modes of the longer flagellum.

Nucleus. The cryptomonad nucleus occupies much of the antapical half of the cell. It contains both light and darkly staining regions, the latter often closely associated with the nuclear periphery (Fig. 11). A nucleolus may or may not be visible. The outer membrane of the nuclear envelope is contiguous with the outermost plastid membrane (Fig. 1), as is the case for many other chlorophyll c-pigmented algae (see below).

Plastid and periplastidial space. Cryptomonad plastids are unusual in both their pigment composition and ultrastructure. They contain a complex plastid derived by secondary endosymbiosis. Four membranes enclose the plastid: the inner pair ensheathes the plastid stroma and the outer pair forms the plastid endoplasmic reticulum (Fig. 1). The four-membrane configuration is common in chlorophyll *c*-containing algae. However, unlike other groups of algae, the outer membranes are not uniformly appressed to the plastid membranes. In the cryptomonads, there is an expanded space between the plastid endoplasmic reticulum and the plastid envelope on its inward face (Figs. 1 and 11). This periplastidial compartment contains 80S ribosomes, starch grains, and the nucleomorph. The nucleomorph is an unusual, double-membrane-bounded structure that contains a fibrillogranular region and dense bodies (Fig. 11). The nucleomorph has been shown to be a vestigial nucleus belonging to a red algal endosymbiont (see "Evolutionary History" section below). That hypothesis is supported by the demonstration of nucleoprotein within this organelle with cytochemical staining (Gillott and Gibbs 1980), fluorescence (Ludwig and Gibbs 1985), and, more recently, genome sequencing (Douglas et al. 2001; Lane et al. 2007; Tanifuji et al. 2011; Moore et al. 2012). The position of the nucleomorph within the cell varies: it can reside between the pyrenoid and nucleus (e.g., *Cryptomonas*) or be embedded within a groove in the pyrenoid (e.g., *Storeatula*) (Clay et al. 1999).

The thylakoids of cryptomonads are most often paired (Fig. 11), although single thylakoids as well as large stacks have also been observed. They do not, however, form structured grana. Pyrenoids are usually covered by a cap of starch, but the pyrenoid matrix and starch grains are separated by the two inner plastid membranes.

In *Chroomonas* and *Hemiselmis* species, thylakoids traverse the pyrenoid matrix (Santore 1982, 1987; Meyer and Pienaar 1984a; Hill 1991b). The Cryptophyceae are characterized by the presence of chlorophylls *a* and c_2, one type of either a red or a blue phycobiliprotein, carotenes, and alloxanthin as a major xanthophyll (Pennington et al. 1985; Lichtlé et al. 1987; Hill and Rowan 1989). Only one type of biliprotein is present in a single species. The chlorophylls *a* and c_2 operate as photosynthetic pigments in a light-harvesting complex that is – as in other algae and in land plants – embedded in the thylakoid membrane contacting the photocenters from the side of the thylakoids (Lichtlé et al. 1987; Kereïche et al. 2008). The biliprotein is not part of phycobilisomes as in red algae, glaucophytes, or *Cyanobacteria*, but comprises a fully functional low-molecular-weight second light-harvesting complex inside of the thylakoid lumen (Gantt et al. 1971; Lichtlé et al. 1987; Vesk et al. 1992; Doust et al. 2006).

Mitochondrion. Cryptomonads contain a single, large vermiform or branched mitochondrion, which extends throughout the cytoplasm (Fig. 11) and contains flattened fingerlike cristae (Santore and Greenwood 1977; Roberts et al. 1981; Roberts 1984; Hill and Wetherbee 1986).

Members of the genus Goniomonas. *Goniomonas* differs from all other cryptomonads in numerous important aspects. These include a different cell shape and ultrastructure, the lack of a plastid, a benthic lifestyle, and a phagotrophic mode of nutrition. *Goniomonas* has distinctly flattened cells with a groove running along the truncate anterior. The flagella insert apically in the vestibulum of the groove and a conical infundibulum or cytopharynx advances close to the groove into the cell (Mignot 1965; Kugrens and Lee 1991; Kim and Archibald 2013). Whereas in plastid-containing cryptomonads, the broad side mostly corresponds to a ventral or dorsal view, i.e., the cells are dorsoventrally compressed, *Goniomonas* cells are typically compressed in a left-right lateral plane. The small side of the truncate anterior thus corresponds to the ventral side; the opposite side closer to the basal bodies corresponds to the dorsal side, whereas the broad views with cytopharynx or flagellar insertion represent the left or right sides, respectively. The structure of the flagellar apparatus supports this notion. The non-winged rhizostyle originates from the basal body closer to the presumed dorsal side (Mignot et al. 1968). However, instead of passing by the nucleus as in the plastid-containing cryptomonads, the comparably short rhizostyle tapers off and vanishes anteriorly and dorsally close to the nucleus (Mignot et al. 1968). Similar to the plastid-containing cryptomonads, a striated band connects the two basal bodies (Mignot et al. 1968). A larger microtubular root originating from the basal body closer to the ventral side follows the cytopharynx into the cell (Mignot et al. 1968). Some additional rootlets accompany the groove margins. Equivalents to the dorsolateral or the compound striated/microtubular roots of the plastid-bearing cryptomonads have not been found (Mignot et al. 1968). The large ejectosomes along the groove and the small ejectosomes underneath the periplast are similar in structure to the ejectosomes of the plastid-containing cryptomonads (Mignot 1965). The *Goniomonas* periplast consists of elongate plates (Kugrens and Lee 1991). The nucleus is found dorsally and posteriorly (Mignot 1965; Kugrens and Lee 1991; Kim and Archibald 2013). The nuclear

envelope is continuous with the endoplasmic reticulum (Mignot 1965), and food vacuoles with ingested bacteria are scattered in the cytoplasm (Mignot 1965; Kugrens and Lee 1991). In cells of the freshwater species *Goniomonas truncata*, a contractile vacuole empties into the distal part of the groove (Mignot 1965). Very long thin filaments cover both flagella of *Goniomonas truncata*; one flagellum bears one row of curved spikes (Kugrens et al. 1987; Figs. 6 and 8). The similarities in granular structure of the shafts of flagellar hairs and spikes indicate that the spikes may be highly modified flagellar hairs (Fig. 8). Overall, the differences in flagellar surface structure between *Goniomonas* and plastid-containing cryptomonads may be due to differences in lifestyle (i.e., benthic vs. pelagic).

Reproduction and Life Cycle

Cryptomonads normally reproduce asexually by mitosis and cytokinesis of the motile cells. Although neither meiosis nor fertilization has been reported in any cryptomonad, some researchers have presented direct or indirect evidence for sexual reproduction in this class, and meiosis-related genes were found in the nuclear genome of *Guillardia theta* (Curtis et al. 2012). Friederike Wawrik provided the first reports of cryptomonad cell fusions, including the formation of zygotes in field samples (Wawrik 1969, 1971, 1979). An ultrastructural study describing cell fusion in a *Chroomonas* strain is also available (Kugrens and Lee 1988). The described processes of cell fusion differ markedly. In a *Cryptomonas* species, Wawrik observed the formation of a thin tube between the apices of two cells, the addition of a second tube resulting in a ring formation, and then transformation into a globular zygote (Wawrik 1969). In the same publication, she described the fusion of two cells at the apex without tube or ring formation in a second *Cryptomonas* species (Wawrik 1969) and a similar process in *Chroomonas acuta* from an Austrian field sample 2 years later (Wawrik 1971). In a later publication and in the study of Kugrens and Lee, however, the cells of an unnamed *Cryptomonas* species and of a strain of *Chroomonas acuta* were shown to have fused asymmetrically (Wawrik 1979; Kugrens and Lee 1988). One cell touched the midventral side of another cell with its antapical tip, leading to cell fusion. All reports, however, were congruent in that the gametes remained motile during the fusion process, resulting in four-flagella stages prior to formation of the zygote.

Observations of cellular dimorphisms in clonal cultures lend further support to the notion of sexual reproduction in cryptomonads (Hill and Wetherbee 1986; Hoef-Emden and Melkonian 2003). Microspectrophotometric measurements of relative nuclear DNA content indicated that the two morphotypes of *Proteomonas sulcata* may correspond to haploid and diploid stages (Hill and Wetherbee 1986). Phylogenetic trees inferred from DNA sequences of nuclear and/or nucleomorph ribosomal operons have provided indirect evidence that dimorphic life histories may be an ancestral character state in plastid-containing cryptomonads (Deane et al. 2002; Hoef-Emden et al. 2002; Hoef-Emden and Melkonian 2003; Hoef-Emden 2007; Majaneva et al. 2014). Apart from the revised genus *Cryptomonas*, within at least

two additional distantly related clades, genera defined on the basis of ultrastructure do not form monophyletic groups and are instead intermingled with one another.

Cryptomonads continue to swim during the mitotic division cycle. Divisions occur predominantly during the second half of the night (Oakley and Dodge 1976; Oakley and Bisalputra 1977; McKerracher and Gibbs 1982; Meyer and Pienaar 1984b; Perasso et al. 1992). Progress of cytokinesis is delayed or arrested completely upon exposure to light during microscopical observation (Oakley and Bisalputra 1977; Meyer and Pienaar 1984b). Only 10 min may suffice to go through mitotic cell division (Oakley and Bisalputra 1977). Peculiarly, the cell polarities of the daughter cells invert during cytokinesis. The former apex of the mother cell turns into the antapices of the daughter cells (shown for a *Cryptomonas* and a *Komma* strain; Perasso et al. 1993). During this process, the mother cell divides longitudinally, starting with a cleavage furrow anteriorly at the ventral site, which extends to the apex. The two basal bodies and flagellar apparatuses migrate along the two future ventral sites, following the growing cleavage furrow down to the former antapex until they reach the new apices of the daughter cells. Thereafter the cells separate completely (Perasso et al. 1993).

The ultrastructural details of mitosis have been examined in several cryptomonad strains (Oakley and Dodge 1976 [*Chroomonas salina* = *Rhodomonas salina*]; Oakley and Bisalputra 1977 ["*Cryptomonas*" sp. the authors examined a marine strain, but *Cryptomonas* is known today to be exclusively freshwater]; McKerracher and Gibbs 1982 [*Cryptomonas* sp. θ = *Guillardia theta*]; Meyer and Pienaar 1984b [*Chroomonas africana*]; Meyer 1987; for changes in taxonomy see below). The plastid divides prior to mitosis by constriction of the bridge connecting the two lobes of the organelle; both daughter plastids stay attached to endoplasmic reticulum (ER) (McKerracher and Gibbs 1982). In a *Chroomonas* species with an eyespot, the carotene globules entered the pyrenoid prior to plastid division and became segregated into two groups upon dissection of the pyrenoid matrix (Meyer 1987). Cryptomonads do not contain probasal bodies, thus, at first basal bodies and flagella duplicate. According to Perasso et al. (1992), the mature older basal body belongs to the longer dorsal locomotoric flagellum. Upon duplication, the ventral trailing flagellum matures to a locomotoric flagellum, whereas both new basal bodies give rise to new trailing flagella and associated flagellar roots (Perasso et al. 1992). The nucleus migrates to the apical part of the cell, and thereafter the nuclear envelope disintegrates (Oakley and Dodge 1976; Oakley and Bisalputra 1977; Meyer and Pienaar 1984b). During metaphase, a plate of condensed chromatin penetrated by small tunnels for spindle microtubules forms, but no individual chromosomes have been observed. The metaphase plate separates into two anaphase plates that move into the vicinity of the plastid-ER complexes, where during telophase the nuclear envelopes reassemble. Mitotic spindle formation starts at approximately the same time as the basal bodies duplicate. Microtubules originate from amorphous material and flagellar rootlets surrounding the basal bodies and grow toward the nucleus (Oakley 1978). The two flagella pairs move apart, each one associated with a Golgi apparatus (McKerracher and Gibbs 1982). At metaphase, the spindle adopts the shape of a compact barrel (Oakley and Bisalputra 1977; Oakley 1978; McKerracher

and Gibbs 1982; Meyer and Pienaar 1984b). The microtubules of the mitotic spindle either traverse the metaphase plate through the tunnels or contact the chromatin without visible kinetochores. At telophase, the microtubules disappear. In *Chroomonas africana* and *Guillardia theta*, fragments of the nuclear envelope were observed to border the mitotic spindle at its longitudinal sides and remain in contact with the plastid-ER complexes during mitosis (McKerracher and Gibbs 1982; Meyer and Pienaar 1984b). Cytokinesis in cryptomonads starts in meta- or anaphase apparently without participation of microtubular structures (Oakley and Dodge 1976; Oakley and Bisalputra 1977). Instead a thin layer of amorphous material has been observed underneath the periplast at the cleavage site (Oakley and Bisalputra 1977).

Nucleomorph division has been examined in *Guillardia theta*, two photosynthetic *Cryptomonas* strains, the leucoplast-containing *Cryptomonas paramecium* strain CCAP 977/2a, and in a marine strain assigned the species name *Plagioselmis punctata* (McKerracher and Gibbs 1982; Morrall and Greenwood 1982). In *Guillardia theta*, nucleomorph division takes place after duplication of the basal bodies (McKerracher and Gibbs 1982). Nucleomorphs undergo a closed mitosis, i.e., the nucleomorph envelopes remain intact. Prior to segregation the nucleomorph constricts in the middle adopting the shape of a dumbbell (McKerracher and Gibbs 1982). Both studies agree in that no microtubules appear to be involved in mitosis of the cryptomonad nucleomorph. Nevertheless, genes for α-, β-, and γ-tubulins have been found in the nucleomorph genome, indicating that some cytoskeletal elements are required in the periplastidial compartment (Keeling et al. 1999). Alignment and partitioning of electron-dense globular to rod-shaped particles – putative heterochromatic regions – seem to be steered by thin fibrillar material observed to connect the particles among each other and to the nucleomorph envelope (Morrall and Greenwood 1982). During mitosis of the nucleomorph, the elongated electron-dense particles arrange in parallel displaying a paracrystalline structure in the cross section (Morrall and Greenwood 1982). The complete absence of a mitotic spindle indicates that the mechanisms underlying nucleomorph division may be quite different from a "normal" mitosis. Nevertheless, some similarities to red algal nuclei still seem to exist, such as the presence of electron-dense particles and the fibrillar material embedded in the chromatin (Morrall and Greenwood 1982). This is potentially significant, given that the cryptomonad nucleomorph and plastid are derived from a red algal endosymbiont (see below).

Hirakawa and Ishida examined ploidy levels of the four different genomes in *Guillardia theta*. Whereas the nuclear genome of the model cryptophyte was haploid, the nucleomorph was tetraploid. Multiple genomic copies were found in the mitochondrion (ca. 24–43) and in the plastid (ca. 130–260) (Hirakawa and Ishida 2014). The process of DNA synthesis in these complex cells must be strictly choreographed. In the host cell nucleus of *Pyrenomonas helgolandii* strain SAG 28.87, DNA synthesis was shown to take place during the light until the onset of darkness and lasted 8–10 h (Sato et al. 2014). The nucleomorph DNA was doubled 2–4 h after the start of the dark period within ~2 h. The plastid divided first, followed by the nucleomorph. Mitosis of the nucleus occurred thereafter, followed by division

of the two outer plastid membranes and cytokinesis (Sato et al. 2014). Concerning the synthesis of cryptophyte organellar DNA, results consistent with those of previous studies in embryophytes were found. DNA synthesis of mitochondria and plastids was not synchronized, but rather found to occur throughout the cell cycle (Sato et al. 2014).

Taxonomy

As in most protist groups, the first cryptomonad genera and species were described using field material and light microscopically visible morphological characters, assuming that morphological characters were stable and differences in morphology would reflect species limits. Even prior to the establishment of electron microscopical methods, examinations of clonal cultures raised doubts concerning consistency in cryptomonad systematics (Butcher 1967; Pringsheim 1968; Klaveness 1985). Pringsheim examined several clonal strains of the freshwater genus *Cryptomonas* and expressed concern over a lack of distinct morphological characters and almost continuous transitional forms obviating the establishment of a reliable morphospecies concept (Pringsheim 1968). Butcher and Pringsheim emphasized the unreliability of color for classification (*Rhodomonas* = red cryptomonads, *Chroomonas* = blue cryptomonads, *Cryptomonas* = brownish cryptomonads with a combination of furrow and gullet; Butcher 1967; Pringsheim 1968). These authors observed that cell colors can change dramatically in a culture upon starvation. Butcher therefore abandoned cell color as a genus-specific criterion and entirely reorganized cryptomonad classification using the structure of the furrow-gullet system as major diagnostic criterion (Butcher 1967). As a consequence, cryptomonads formerly classified as *Rhodomonas* were reassigned to the genera *Cryptomonas* or *Chroomonas.* Several studies, however, demonstrated that at least eight different types of biliprotein can be produced by cryptomonads and that each clonal strain contains only one type, which is stable within a culture (Hill and Rowan 1989; Glazer and Wedemayer 1995; Hoef-Emden 2008). Therefore, Butcher's classification did not hold, but still causes some confusion in naming of strains in older literature and in culture collections.

In the 1960s, electron microscopical techniques developed and were used to unveil the fine-scale features of cryptomonad ultrastructure. As a consequence, genera within the cryptomonads have been distinguished by their type of biliprotein, the number and shape of their plastids, the position of the nucleomorph, pyrenoid structure, flagellar apparatus, morphology of the furrow-gullet system, and ultrastructure of the periplast (starting with Santore 1984; Clay et al. 1999; Kugrens and Clay 2002; Novarino 2003). Since most of these characters have been examined in clonal cultures, they are deemed to be stable. However, in 1986, a clonal strain, described as a new genus and species *Proteomonas sulcata*, was found to possess two different morphotypes (Hill and Wetherbee 1986). Both morphotypes shared the same type of biliprotein, phycoerythrin 545, but differed significantly in ultrastructure. The larger cells had sheetlike inner periplast components and keeled

rhizostyles, whereas the inner periplast components of the smaller cells consisted of hexagonal plates, and the rhizostyles of these cells were non-keeled. Microspectrophotometric measurements showed that the nuclei of the smaller cells contained only half of the DNA of the larger cells. Hill and Wetherbee therefore termed the different morphotypes diplomorph and haplomorph and suggested the presence of a dimorphic life history with sexual reproduction (Hill and Wetherbee 1986).

Molecular phylogenetic analyses using nuclear and nucleomorph ribosomal RNA genes demonstrated inconsistencies in several of the ultrastructurally defined cryptomonad genera. Genera were para- or polyphyletic, or strains assigned to two different genera proved to be genetically identical or almost identical in phylogenetic analyses (e.g., *Rhinomonas* and *Storeatula* strains or *Cryptomonas* and *Campylomonas*; Marin et al. 1998; Deane et al. 2002; Hoef-Emden et al. 2002; Hoef-Emden and Melkonian 2003). The genus *Cryptomonas* proved to be restricted to freshwater, to encompass only species with phycoerythrin 566 or with leucoplasts, and to be dimorphic (Hoef-Emden and Melkonian 2003). The situation in *Cryptomonas* and "*Campylomonas*" was similar to *Proteomonas sulcata*. *Cryptomonas* is a morphotype with polygonal periplast plates, whereas *Campylomonas* corresponds to the diplomorph with a sheetlike inner periplast component (Hill and Wetherbee 1986; Hoef-Emden and Melkonian 2003). Since DNA content of the *Cryptomonas* cells has not been determined and since the two morphotypes do not always differ in size, the terms crypto- and campylomorph have been adopted, instead of haplo- and diplomorph (Hoef-Emden and Melkonian 2003). Similar pairs of "genera" likely representing different morphotypes within a life cycle can be found in other cryptophyte clades: *Rhinomonas* and *Rhodomonas* (both with IPC plates) versus *Storeatula* (sheetlike IPC; Majaneva et al. 2014) and *Plagioselmis* (IPC plates) versus *Geminigera* and *Teleaulax* (sheetlike IPC; Shalchian-Tabrizi et al. 2008). Previous reports and evidence for dimorphism in several genera suggest that the biological species concept does apply to cryptomonads, although the inductors of sexual reproduction are unknown (see Reproduction and Life Cycle section). As of June 2015, only two genera, *Cryptomonas* and *Hemiselmis*, have been examined in greater detail by combining light and electron microscopical methods with molecular phylogenetic analyses and have been revised accordingly (Hoef-Emden and Melkonian 2003; Hoef-Emden 2007; Lane and Archibald 2008). In all three revisions, Pringsheim's observation of a low resolution of morphological characters at species level could be confirmed (Pringsheim 1968; Hoef-Emden and Melkonian 2003; Hoef-Emden 2007; Lane and Archibald 2008). In both *Cryptomonas* and *Hemiselmis*, genetic diversity appears to outweigh morphological diversity, resulting in cryptic species complexes probably encompassing several biological species. In *Cryptomonas*, morphological characters could also be misleading due to differences in the cell shapes within a strain and among strains that are genetically identical even in the highly variable internal transcribed spacer 2 of the nuclear ribosomal operon (Hoef-Emden 2007).

Problems with defining the boundaries of a genus have also been encountered in *Chroomonas*. By tradition, *Chroomonas* was said to contain a PC630 or PC645 biliprotein, to have a tubular gullet, and to be surrounded by a periplast consisting of

rectangular plates (Clay et al. 1999). An SEM examination of the original specimen of the type species, *Chroomonas nordstedtii*, revealed a periplast consisting of hexagonal periplast plates, which raises doubts over the current genus description (Novarino 2003). In addition, the genus *Chroomonas* proved to be paraphyletic in phylogenetic analyses with *Hemiselmis* and *Komma* being nested within it (Hoef-Emden 2008, 2014).

Various cryptomonad genera and their characters are listed in Table 1 according to their current classification status.

Maintenance and Cultivation

Cryptomonads are present in most samples taken from still waters. If cryptomonad cells are rare, enriching a sample with culture medium often helps to increase cell numbers. Since cryptomonads have no cell wall only palmella-forming taxa have proven capable of growth on agar. The isolation method of choice is the capillary technique. Capillaries are produced by heating glass Pasteur pipettes over a flame, stretching them and breaking them at their thinnest point. With these capillaries, single cells are identified and isolated under a – preferably inverted – microscope and washed by transferring each cell repeatedly into fresh drops of medium, ultimately placing the cell in a vessel with culture medium (a titer plate with culture medium-filled wells or small petri dishes). This method is difficult or impossible to apply if the cells are of nanoplanktonic size. If cryptomonads occur at a greater abundance, serial dilution techniques may be successful to obtain clonal cultures. Otherwise fluorescence-activated cell sorting (FACS) can be used to sort photosynthetic cryptomonads according to the fluorescence maximum of their biliprotein (Sensen et al. 1993). FACS is expensive but efficient and can be used to establish axenic cultures (Surek and Melkonian 2004).

Most cryptomonads can easily be grown in one of the standard culture media, such as WARIS and BBM (freshwater) or ASP-12, ESM, f/2, and ASP (marine; see recipes on the CCAC, CCAP, CCMP, NIES, and SAG websites). Cryptomonads need vitamins, but a standard cocktail containing vitamin B_{12}, niacin amide (nicotinic acid amide), biotin, and thiamine will suffice for most strains. Axenic cultures of photosynthetic freshwater cryptomonads may not grow without soil extract in the culture medium (1 mL per L may suffice). Sodium glycerophosphate and soil extract are often required additives for marine isolates. The phagotrophic *Goniomonas* species are more difficult to isolate and maintain. Success probably depends on whether or not their prey organisms, e.g., bacteria, are transferred by accident together with the cells and are able to grow in the culture medium. Otherwise prey has to be provided together with the culture media. Leucoplast-containing *Cryptomonas* species may be less difficult. They can be grown at first in biphasic soil-water media with a piece of pea or lentil, but some also grow in WARIS with soil extract, at least if they are not axenic. Axenic strains require some organic additives, such as a pea or lentil or bacterial growth media.

Many marine cryptomonad strains grow equally well in marine or brackish water media, but differences in temperature optima have to be considered for different isolates. Arctic or antarctic isolates often do not survive warming up to even 15 °C, whereas isolates from warmer regions may not divide at lower temperatures. Thus, when isolating cells for establishing new cultures, growth temperatures should at first approximate those of the respective habitat. Experiments concerning temperature tolerance may be done subsequently. Cryptophytes generally prefer larger surface to depth ratios in culture vessels, i.e., cultures become denser in petri dishes than in small Erlenmeyer flasks or glass tubes. Although the delicate nature of cryptomonad cells can cause problems, they can be easily grown at a large scale in aerated mass cultures. Rates of cell division and achievable cell densities, however, differ considerably among strains. Small-celled *Cryptomonas* and *Hemiselmis* strains divide at a faster rate than large *Cryptomonas ovata* cells or the campylomorphs of *Cryptomonas curvata. Chroomonas* strains that are extensively palmelloid do not lend themselves to cell counting and may grow comparably slowly, yielding low-density cultures.

Evolutionary History

Cryptomonads appear to be absent from the fossil record, presumably because they lack silica- or calcium carbonate-containing surface structures amenable to preservation. Attempts to discern how the different cryptomonad lineages relate to one another and, more generally, where cryptomonads belong on the eukaryotic tree of life have traditionally relied on morphological, ultrastructural, and biochemical data. With the advent of gene and whole genome sequencing, molecular approaches to cryptomonad systematics are now also widely used. Given that plastid-containing cryptomonads acquired photosynthesis by secondary endosymbiosis, a process whereby a non-photosynthetic eukaryote engulfs an unrelated eukaryotic phototroph and retains its plastid (Gould et al. 2008; Archibald 2012), the origin of both the host and endosymbiont components of cryptomonad cells must be accounted for.

The cryptomonad host cell has been allied with a wide variety of algae over the years, including the glaucocystophytes, dinoflagellates, and rhodophytes (see Edwards (1976) and references therein for early views on large-scale algal systematics). Cavalier-Smith (1986) placed the Cryptophyta in the kingdom Chromista together with the Haptophyta and Heterokonta, based on their shared presence of chlorophyll *c*-pigmented plastids and similar plastid ultrastructure. As is the case in cryptomonads, the haptophytes and photosynthetic heterokonts have plastids that are surrounded by four membranes. In each of these three groups, the outermost plastid membrane is contiguous with the host cell's endomembrane system, an arrangement historically referred to as the chloroplast (or plastid) endoplasmic reticulum (Gibbs 1979). Cavalier-Smith (1986) proposed that the cryptomonad, haptophyte, and heterokont plastids are the product of a single secondary endosymbiosis in their common ancestor, and that these three groups are each other's closest relatives. Molecular phylogenetic analyses of plastid and (in the case of cryptomonads)

nucleomorph gene sequences have shown convincingly that cryptomonad, haptophyte, and heterokont plastids are each red algal in origin (e.g., Douglas and Penny 1999; Douglas et al. 2001; Sánchez Puerta et al. 2005; Oudot-Le Secq et al. 2007).

Cryptophytes inherited several traits from the red algal ancestor of their plastids. The periplastidial space surrounding the complex cryptophyte plastid probably corresponds to the former cytosol of the engulfed red alga, since it contains starch grains and eukaryotic ribosomes. The starch metabolism in this compartment is also powered by UDP, as in red algae (Viola et al. 2001; Deschamps et al. 2006). Floridoside (α-D-galactopyranosyl 1 → 2′-glycerol) is the red algal equivalent to sucrose in Viridiplantae: it serves as the major soluble carbon pool and acts together with its relative isofloridoside as an osmolyte to counter salt stress (Hagemann and Pade 2015). In two algae with complex plastids of red algal ancestry, the stramenopile alga *Poterioochromonas malhamensis* (isofloridoside) and the cryptophyte *Chroomonas* sp. CCAP 978/08 (floridoside), these red algal-derived products have been shown to serve as osmoprotectants (Kauss 1981; Hoef-Emden 2014).

Many of the genes of the former red alga have been transferred to the nucleus, e.g., the *cpe*A genes for the α subunits of the biliprotein (Broughton et al. 2006). Cells with complex plastids require a sophisticated addressing system to correctly target nucleus-encoded proteins to their respective compartments or membranes and the targeting systems present in the model cryptophyte *Guillardia theta* and the model diatom *Phaeodactylum tricornutum* are compatible with each other. Gene products that have to cross five membranes such as the α subunit of the biliprotein (the plastid ER membrane, periplastidial membrane, outer plastid membrane, inner plastid membrane, and finally thylakoid membrane) depend on the presence of an N-terminal tripartite topogenic signal (Gould et al. 2007). Targeting of such cryptophyte proteins was demonstrated in the transformable *P. tricornutum.* First, the signal peptide directs translation through the plastid-ER membrane. An AXAF motif at the cleavage site between the signal and transit peptide marks the protein for import into the plastid lumen. If a twin arginine motif follows the transit peptide, the protein will end up in the thylakoid lumen (Gould et al. 2007). Since only some biliprotein α subunits possess a twin arginine motif, Gould et al. hypothesized that the two plastid-encoded β subunits, one α subunit with a twin arginine motif and another without, obtain their tetrapyrrole chromophores in the plastid stroma and come together to form the biliprotein. The fully assembled biliprotein is then imported via a twin arginine transporter into the thylakoid, guided by the twin arginine motif in one of the subunits. As in other plastids of primary and secondary endosymbiotic origin, cryptophyte plastids use TIC (*t*ranslocator of the *i*nner *c*hloroplast membrane) and probably also TOC (translocator of the outer chloroplast membrane) for protein import. The nature of the transporter for the periplastidial membrane has provided insight into the processes of integration between two eukaryotic cells. Specifically, it was identified as being derived from the ERAD-L of the red alga (*e*ndoplasmic *r*eticulum-*a*ssociated *d*egradation system for *l*uminal proteins). Instead of exporting defective proteins from the ER for degradation, its

direction of transport was reversed and now serves to import proteins into the periplastidial space. The modified ERAD-L was termed SELMA (*s*ymbiont-derived *E*RAD-*l*ike *ma*chinery; Gould et al. 2007).

A specific relationship between the plastids (and by extension, the host cells) of chromists and alveolates (i.e., dinoflagellates, apicomplexans, and ciliates) has also been proposed (Cavalier-Smith 1999). Many dinoflagellate algae as well as some apicomplexan parasites (e.g., the malaria parasite *Plasmodium*) possess plastids of red algal ancestry, and the so-called "chromalveolate" hypothesis posits that their red algal plastids share a common origin with those of cryptomonads, heterokonts, and haptophytes. A wide range of molecular, biochemical, and ultrastructural data has been brought to bear on this question, including consideration of plastid ultrastructure and pigmentation (see above), the nature of the plastid protein import apparatus in the organisms in question, and comparative analyses of plastid and nuclear genes and genomes from a broad range of photosynthetic and non-photosynthetic taxa (Sánchez Puerta and Delwiche 2008). It remains to be seen whether some or all of the plastids in cryptomonads, haptophytes, heterokonts, dinoflagellates, and apicomplexans share a common endosymbiotic origin (Baurain et al. 2010; Archibald 2012; Keeling 2013; Gould et al. 2015). Large-scale phylogenomic analyses of nuclear genes refute the hypothesis of a monophyletic origin of cryptomonads and haptophytes with the other chromalveolate lineages (e.g., Patron et al. 2007; Burki et al. 2008, 2012; Baurain et al. 2010). Comparative phylogenomic analyses and statistical analyses of gene sets indicate that the complex plastids of heterokonts, cryptomonads, and haptophytes may be the products of serial endosymbioses (Baurain et al. 2010; Stiller et al. 2014). The precise origin(s) of the red algal endosymbiont(s) in the three chromist lineages is unclear, as no particular modern-day rhodophyte lineage has emerged as an obvious sister of the plastids of cryptomonads and other chlorophyll *c*-containing algal groups.

Cryptomonads may also be specifically related to katablepharids, a ubiquitous lineage of marine and freshwater heterotrophic flagellates. Katablepharids lack plastids, but they share vague similarities with cryptomonads, including the presence of both large and small ejectosomes (the former being associated with the feeding apparatus; they differ from cryptomonad ejectosomes in that no small ribbon is attached to the large one) and two thick, unequal, subapically inserted flagella (Lee and Kugrens 1991; Lee et al. 1991). Molecular phylogenies are consistent with a distant relationship between cryptomonads and katablepharids (e.g., Okamoto and Inouye 2005; Okamoto et al. 2009; Burki et al. 2012; Yabuki et al. 2014). Under a model of serial endosymbiosis, the phagotrophic cryptomonad *Goniomonas* and the related katablepharids were ancestrally non-photosynthetic, i.e., they never possessed plastids. In contrast, under the chromalveolate hypothesis, these lineages would have evolved from plastid-bearing lineages and lost their photosynthetic organelles secondarily.

Within cryptomonad diversity, there is a clear distinction between lineages with plastids (e.g., *Chroomonas*, *Cryptomonas*, *Guillardia*, *Rhodomonas*) and those without, the latter being represented by the genus *Goniomonas* (Fig. 13). Beyond the presence or absence of a plastid, these two cryptomonad types differ from one

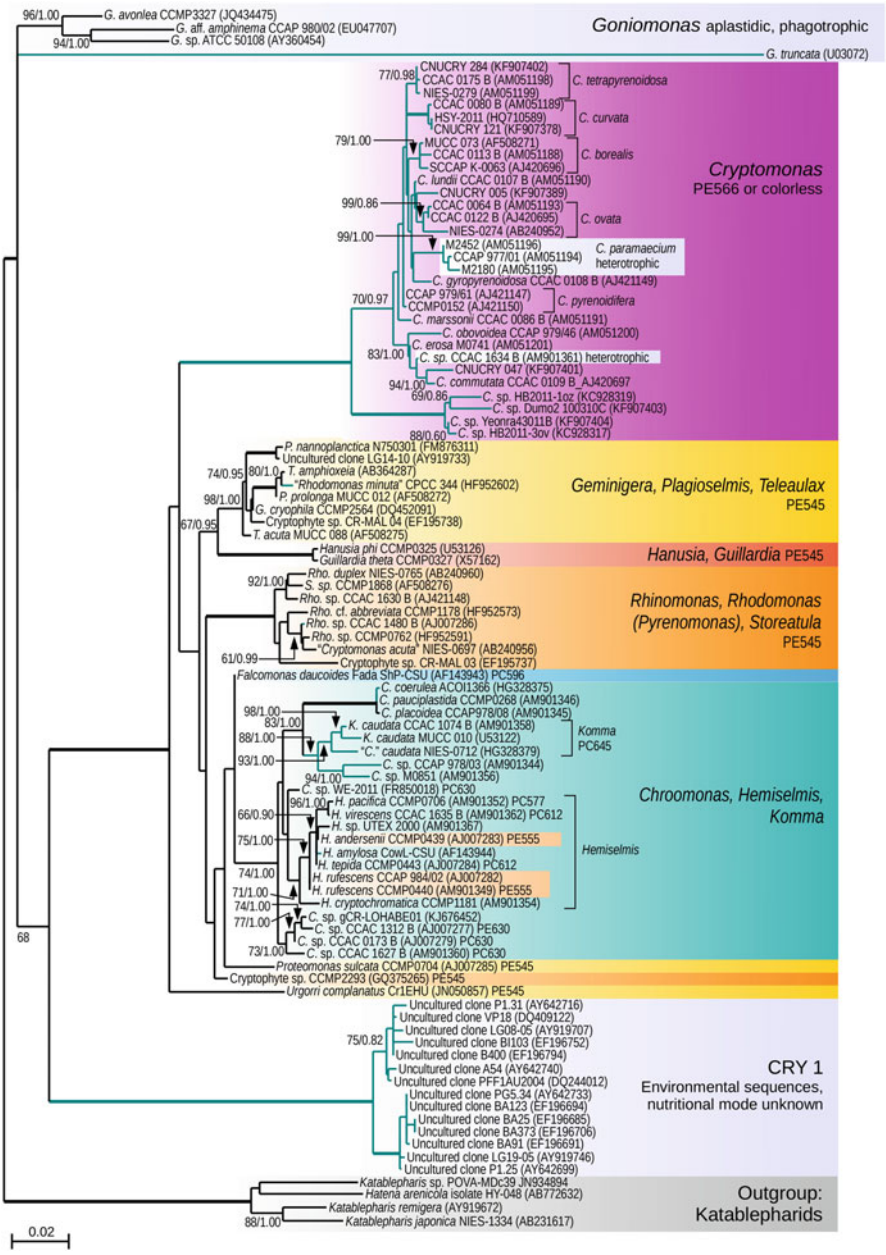

Fig. 13 Rooted maximum likelihood tree of the cryptomonads inferred from nuclear small subunit ribosomal (SSU rRNA) genes. The closest related sister group of the cryptomonads, the katablepharids, have been used as an outgroup. The plastid-containing cryptomonads form a monophyletic clade. In most subclades of the plastid-containing cryptomonads, the type of biliprotein is congruent – although not clade specific – with the respective subclade. The

another in terms of their ultrastructure (see "Characterization and Recognition" section above), and molecular sequence analyses (e.g., McFadden et al. 1994; von der Heyden et al. 2004; Kim and Archibald 2013) strongly support the hypothesis that *Goniomonas* is evolutionarily distinct from all other cryptomonads (Fig. 13).

Cryptomonad biliproteins originate from the phycobilisomes of the former red algal endosymbiont and have proven to be useful evolutionary markers within the group. Phycobilisomes consist of three types of biliproteins. Phycoerythrins and phycocyanins are organized in disk-shaped tri- or hexaheterodimers and are interconnected by linker proteins to form rods (Adir 2005). Together with three central units of allophycocyanin, several rods comprise one phycobilisome. These high-molecular light-harvesting complexes transfer energy to the photosystems from the outside the thylakoids (Adir 2005). In cryptomonads, the phycobilisomes have disassembled and disappeared almost entirely in the course of evolution. Only one type of biliprotein, the formerly peripheral phycoerythrin, has been retained (Apt et al. 1995). Its structure changed to an $\alpha\alpha'\beta\beta$ heterodimer and it moved into the lumen of the thylakoids (Gantt et al. 1971; Vesk et al. 1992; Glazer and Wedemayer 1995). The genes for the α subunits have been transferred to the nucleus, whereas the gene for the β subunit is still plastid-encoded (Jenkins et al. 1990; Douglas and Penny 1999; Broughton et al. 2006). The β subunit is highly conserved in amino acid sequence, despite the highly modified structure, localization, and function of the cryptomonad biliprotein. The α subunits, however, are so diverged that their origin has remained elusive (Apt et al. 1995; Wilk et al. 1999). The different types of biliproteins in cryptomonads evolved by exchange of the linear tetrapyrrole chromophores. Thus, the so-called cryptomonad "phycocyanins" are by origin phycoerythrins that mimic blue phycocyanins (Glazer and Wedemayer 1995). Eight types are currently known, three types of phycoerythrin (plus one slight modification) and five types of phycocyanin (Hill and Rowan 1989; Hoef-Emden 2008). They are named according to the approximate wavelengths of their respective absorption maxima.

Currently, seven distinct evolutionary lineages are known within the plastid-containing cryptomonads, two of which are represented by only one culture (*Proteomonas sulcata* and *Falcomonas daucoides*; Fig. 13). Of the different types of biliprotein, the phycoerythrins are largely congruent with clades in phylogenetic trees, but mostly not clade specific (Marin et al. 1998; Deane et al. 2002; Hoef-Emden et al. 2002; Hoef-Emden and Melkonian 2003; Fig. 13). The orange-red phycoerythrin 545 is found in four different lineages (Fig. 13). The purple

Fig. 13 (continued) phycocyanins are an exception. In several clades, mixtures of two to three genera indicate inconsistencies in systematics probably caused by dimorphic life histories. 104 taxa, 1450 positions, evolutionary model GTR + CAT with final gamma optimization, support values from *left* to *right*, ML bootstrap (1000 replicates), posterior probabilities from Bayesian analysis (six million generations, GTR + Γ6); *bold* branches, 100% BS support and PP 1.00. *Turquoise* branches represent freshwater taxa. Abbreviations: *PC* phycocyanin, *PE* phycoerythrin. Scale bar = substitutions per site

phycoerythrin 566 is exclusive to the genus *Cryptomonas*, although several independent lineages with leucoplasts have evolved within this group (Hoef-Emden and Melkonian 2003; Hoef-Emden 2005). Phycocyanins are found in only two lineages, *Falcomonas daucoides* and a highly diverse clade comprising the genera *Chroomonas*, *Hemiselmis*, and *Komma* (Hoef-Emden 2008; Lane and Archibald 2008; Fig. 13). In the latter clade biliproteins displayed a complex pattern of evolution in phylogenetic trees (Hoef-Emden 2008; Lane and Archibald 2008). Within the genus *Hemiselmis*, a third type of phycoerythrin, PE 555, has evolved from phycocyanin 615 (Hoef-Emden 2008; Lane and Archibald 2008). X-ray crystallography and 2D electronic spectroscopy demonstrated structural and functional differences between types of biliproteins that may be congruent with cryptophyte lineages. In a *Rhodomonas* and a *Chroomonas* species, the biliprotein was found to be in a closed conformation, i.e., the two boat-shaped subunits (αβ and α′β) are closely attached at their inner sides, allowing for energy transfer by quantum coherence. In contrast, in examined *Hemiselmis* species, the two subunits do not interact in this manner, hampering this kind of energy transfer (Collini et al. 2010; Harrop et al. 2014). It is possible that open-state biliproteins are a synapomorphy of the *Hemiselmis* clade.

Ultrastructural characters that are related to dimorphic life histories such as, e.g., the type of periplast, are not congruent with clades in phylogenetic trees in at least three cryptophyte lineages (*Cryptomonas*, the *Rhinomonas/Rhodomonas/Storeatula* clade, and the *Geminigera/Plagioselmis/Teleaulax* clade) (Marin et al. 1998; Deane et al. 2002; Hoef-Emden et al. 2002; Hoef-Emden and Melkonian 2003; Shalchian-Tabrizi et al. 2008; Majaneva et al. 2014; Fig. 13). Members of the *Rhinomonas/Rhodomonas/Storeatula* clade share a synapomorphic feature, i.e., their nucleomorphs are found in a periplastidial tongue embedded in the pyrenoid matrix (Hill and Wetherbee 1989; Hill 1991a). Another synapomorphy may be shared by the phycocyanin-containing genera *Chroomonas*, *Hemiselmis*, and *Komma.* In examined representatives of these taxa, thylakoids traverse the pyrenoid matrix (Santore 1982, 1987; Meyer and Pienaar 1984a; Hill 1991b).

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Haptophyta

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Abstract

Haptophyta are predominantly planktonic and phototrophic organisms that have their main distribution in marine environments worldwide. They are a major component of the microbial ecosystem, some form massive blooms and some are toxic. Haptophytes are significant players in the global carbonate cycle through photosynthesis and calcification. They are characterized by the

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haptonema, a third appendage used for attachment and food handling, two similar flagella, two golden-brown chloroplasts, and organic body scales that serve in species identification. Coccolithophores have calcified scales termed coccoliths. Phylogenetically Haptophyta form a well-defined group and are divided into two classes Pavlovophyceae and Coccolithophyceae (Prymnesiophyceae). Currently, about 330 species are described. Environmental DNA sequencing shows high haptophyte diversity in the marine pico- and nanoplankton, of which many likely represent novel species and lineages. Haptophyte diversity is believed to have peaked in the past and their presence is documented in the fossil record back to the Triassic, approximately 225 million years ago. Some biomolecules of haptophyte origin are extraordinarily resistant to decay and are thus used by geologists as sedimentary proxies of past climatic conditions.

Keywords

Biogeochemical cycles • Coccoliths • Ecology • Evolution • Fossil record • Haptophyta • Morphology • Ocean acidification • Phylogeny

Contents

Summary Classification

●**Haptophyta**
●●**Pavlovophyceae**
●●●**Pavlovales** (e.g. *Pavlova*, *Rebecca*)
●● **Coccolithophyceae** (=*Prymnesiophyceae*)
●●●**Phaeocystales** (*Phaeocystis*)
●●●**Isochrysidales** (e.g. *Isochrysis*, *Emiliania*)
●●●**Coccolithales** (e.g. *Coccolithus*, *Syracosphaera*)
●●●**Prymnesiales** (e.g. *Prymnesium*, *Chrysochromulina*)

Introduction

The division Haptophyta is a group of unicellular algae that are predominately marine, although there are a few freshwater and terrestrial records. Haptophyte nutrition is mainly phototrophic, but many exhibit phagotrophy and some are exclusively heterotrophic. Haptophytes usually appear yellow-brown at high population densities (in culture or blooms) because of accessory carotenoid pigments. Most haptophytes occur as solitary motile or nonmotile forms, but a few form colonies or short filaments. The cells are usually covered with scales of varying degrees of complexity ranging from elaborate calcified structures termed "coccoliths" that are usually visible in light microscopy to ornamented unmineralized organic scales, many of which can only be observed in electron microscopy. Scales and coccoliths are used in species identification (e.g., Bendif et al. 2011, Chrétiennot-Dinet et al. 2014, and http://ina.tmsoc.org/Nannotax3). Motile forms usually possess two equal (isokont) or subequal flagella, which can beat similarly (homodynamic) or differently (heterodynamic). The two flagella are similar in microanatomy and do not have superficial structures except in the class Pavlovophyceae, where they are markedly different in length and are covered by knob scales and fine hairs. Haptophytes possess a unique structure called a haptonema, a filiform organelle associated with the flagella, but different in structure. The name of the division is based on the name of this organelle (from the Greek, hapsis meaning touch).

The Haptophyta includes some 80 extant genera with approximately 330 species in two classes, the Coccolithophyceae (Prymnesiophyceae) with around 76 genera and 318 species (Jordan et al. 2004) and the Pavlovophyceae with 4 genera and 13 species (Bendif et al. 2011). Its members often form an important component of oceanic and coastal plankton and several species are known to form blooms; some of them toxic. Coccolithophores have been recognized in sedimentary rocks and ocean sediments since the early nineteenth century (Ehrenberg 1836) from their calcified scales called "coccoliths" (Huxley 1858). Haptophyte diversity probably peaked in the past, as documented in the coccolith fossil record that extends back to the late Triassic, ca. 225 million years ago.

Habitats and Ecology

Habitats

Haptophytes are found worldwide as common components of coastal and oceanic habitats, and most genera have representatives in the marine plankton. They constitute a considerable percentage of the numbers and biomass of nanoplankton (2–20 μm) (Marchant and Thomsen 1994; Thomsen et al. 1994; Masquelier et al. 2011) as well as in the picoplankton (<3 μm) (Thomsen 1986; Liu et al. 2009; Jardillier et al. 2010; Kirkham et al. 2011). Few estimates have been made, however, of the fraction of production attributable to haptophytes, partly because of the difficulty in identifying and quantifying them in preserved samples. Molecular methods have been instrumental in revealing haptophyte diversity and distribution. Environmental sequencing of clone libraries have repeatedly shown a large diversity including haptophyte clades with no cultured representatives (e.g., Moon-van der Staay et al. 2000; Liu et al. 2009; Shi et al. 2009; Cuvelier et al. 2010), an indication of a large hidden biodiversity in the group. The use of high-throughput sequencing (HTS) has revealed unprecedented haptophyte diversity in marine waters with most of the OTUs (operational taxonomic units) with best match to an environmental sequence, of which some may represent novel taxa from class to species levels (Bittner et al. 2013; Egge et al. 2015a).

Many species are euryhaline, such as the pavlovophyte *Diacronema vlkianum* and the toxic and economically important *Prymnesium* species, *P. parvum* (Green 1975; Green and Hibberd 1977; Green et al. 1982; Larsen 1999). It also grows epipsammically in sand and may thrive in ponds, lakes, and river basins with slightly elevated salinity (Edvardsen and Imai 2006; Johnsen et al. 2010; Southard et al. 2010). Filamentous and palmelloid forms of taxa such as *Ruttnera* and *Chrysotila* (see Andersen et al. 2014, 2015 for update on taxonomy) occur in the splash zone of marine cliffs and are found on damp, usually basic substrates, such as chalk cliffs, and limestone walls (Green and Parke 1975b). Coccolithophores in the related genera *Jomonlithus*, *Ochrosphaera*, and *Hymnenomonas* are typically found in nearshore coastal zones and estuaries.

Only a few genera and about 15 species have been reported from freshwater environments (Preisig 2002; Nicholls 2014). Freshwater records include reports of *Hymenomonas roseola* (Manton and Peterfi 1969), several *Prymnesium*, and *Chrysochromulina*. Freshwater phylotypes belonging to both classes have been revealed by 18 S rDNA environmental sequences (Shalchian-Tabrizi et al. 2011). Some Prymnesiales thrive in and under ice (Hällfors and Niemi 1974; Takahashi 1981).

Several species of *Phaeocystis* occur as symbionts of the widespread and abundant zooplanktonic acantharians (Decelle et al. 2012), and haptophytes have also been found in symbiosis with polycystine radiolarians (Febvre and Febvre-Chevalier 1979; Anderson et al. 1983) and foraminifers (Gast et al. 2000). A haptophyte with affinities to *Braarudosphaera* harbors a nitrogen-fixing cyanobacterium (Thompson et al. 2012). Several members of Prymnesiales and the motile stage of *Emiliania*

huxleyi and *Coccolithus pelagicus* phagocytize bacteria, artificial particles, or other algae (Parke et al. 1955; Jones et al. 1994; Tillmann 1998; Rokitta et al. 2011). Many coccolithophores, for example, species of *Balaniger*, *Ericiolus*, *Pappomonas*, and *Trigonaspis*, have been reported to be heterotrophic (Marchant and Thomsen 1994; Thomsen et al. 1994, 1995). Growth of some haptophytes is stimulated by the uptake of dissolved organic compounds (Pintner and Provasoli 1968; Antia 1980).

Biogeography

Investigations of the distribution and community structure of coccolithophores have been conducted since Lohmann (1912), who was probably the first to examine the oceanic distribution of extant coccolithophores. In Winter et al. (1994), rough biogeographic coccolithophore zones were established largely based on two pioneering oceanic transect studies in the Atlantic (McIntyre and Bé 1967) and the Pacific (Okada and Honjo 1973). Hagino and Young (2015) reviewed recent literature and defined eight coccolithophore floras based on the dominance of one (*E. huxleyi*, *G. oceanica*, or *U. irregularis*) and common occurrence of accompanying taxa. Their compilation supports the latitudinal zonal distributions of the Atlantic (McIntyre and Bé 1967; Winter et al. 1994), but highlights that floras of the equatorial Pacific are also subdivided along east–west gradients, likely corresponding to nutrient content (Hagino and Young 2015).

Distinct floral assemblages are found in the subarctic, temperate, subtropical, tropical, and subantarctic biogeographic zones, largely associated with major ocean current systems. Species diversity generally increases from polar to subtropical–tropical regions, where distinct vertical zonations are often related to (permanent) stratification and deep light penetration of oligotrophic surface waters. The majority of species are restricted to either an upper photic (0–80 m) or a lower photic (120–220 m) zone. The intermediate zone (80–120 m) may contain species from the assemblages above and below. Holococcolithophores are often found in relation with (seasonal) surface water stratification (Cros and Estrada 2013).

The lower photic zones are dominated by *Florisphaera profunda* (with occurrences of *Algirosphaera* spp. and *Gladiolithus* spp.) in temperate to tropical waters, whereas these deeper-dwelling taxa are absent in subpolar–polar waters (Hagino and Young 2015). Placolith-bearing taxa occur in the upper and intermediate photic zones. *Emiliania huxleyi* dominates the upper photic zone in all temperate to polar waters, as well as the equatorial Atlantic and eastern equatorial Pacific. Assemblages with *Umbellosphaera irregularis* are found in the oligotrophic Atlantic subtropical gyres and western equatorial Pacific. Notably, *Gephyrocapsa*-dominated assemblages have been, to date, only reported from the equatorial Indo-Pacific regions and western Pacific coastal waters (Hagino and Young 2015).

The open waters of the Arctic host *Coccolithus pelagicus*, *Calciopappus caudatus*, *Algirosphaera robusta*, and *Emiliania huxleyi* (Winter et al. 1994), and several representatives from the partially calcified genera, such as *Pappomonas*,

Papposphaera, and *Wigwamma*, are also present (Thomsen et al. 1991; Thomsen et al. 1994). The latter group is most abundant in coastal Arctic waters and is sometimes even observed in ice (Marchant and Thomsen 1994; Winter et al. 1994).

In Antarctic waters, common species of the Papposphaeraceae include *Papposphaera*, *Pappomonas*, *Trigonaspis*, and *Wigwamma* (Marchant and Thomsen 1994; Winter et al. 1994), and more than 20 species have been identified including *E. huxleyi* and several species of *Gephyrocapsa*. The abundance and diversity of Southern Ocean coccolithophores decreases poleward (Findlay et al. 2005). The presence of considerable concentrations (>1000 cells/L) of *E. huxleyi* in all sectors of the Southern Ocean and far south of the Antarctic Polar Front (Winter et al. 2014) affirms the eurythermal character of this species.

Less is known about the distribution of members of the Prymnesiales, such as *Chrysochromulina, Chrysocampanula*, *Haptolina*, *Dicrateria*, and *Prymnesium* species, because species identification usually requires electron microscopy examination or genetic methods, and in many instances species delimitation is difficult. Investigations of the Prymnesiales have been carried out in most regions of the world (Leadbeater 1972; Leadbeater 1974; Estep et al. 1984; Hoepffner and Haas 1990; Gao et al. 1991; Marchant and Thomsen 1994; Jensen 1998; LeRoi and Hallegraeff 2004; LeRoi and Hallegraeff 2006), but with present knowledge of species delimitation and their geographical distribution, a reasonable biogeography of the different Prymnesiales species cannot be outlined. Many species such as *Chrysocampanula spinifera*, *Chrysochromulina acantha*, *C. leadbeateri*, *C. simplex*, *C. mantoniae*, *Dicrateria rotunda*, *Haptolina ericina*, and *H. hirta* are reported from areas stretching from the sub-Arctic to the Antarctic and would in this sense be considered cosmopolitan. It has been claimed by de Vargas et al. (2007) that species we recognize as cosmopolitan and display a high genetic and/or ecological plasticity are in fact sibling species within morphological superspecies. The morphological variation in many species as for instance *C. leadbeateri* and *C. simplex* is considerable, and the different morphotypes may in the future prove to constitute sibling species or even different species. Very few of the species of this order have been reported to have their distribution restricted to certain geographical areas of the world. Of those that have, *Chrysochromulina tenuisquama* has only been reported from the southern part of the Atlantic Ocean (Estep et al. 1984), *C. elegans* has been reported only from the southern part of the North Atlantic and Scandinavian waters (Estep et al. 1984; Jensen 1998), and *C. throndsenii* has so far only been recorded from Scandinavian waters (Eikrem 1996; Jensen 1998).

In the Phaeocystales *Phaeocystis globosa* is recognized in the North Sea and English Channel and in temperate waters worldwide; *P. pouchetii* is common along the Norwegian coast, in the Barents Sea, and in Northern Pacific; *P. antarctica* is usual in the Antarctic; and *P. jahnii* is known from the Mediterranean Sea. All of these species have a colonial phase in their life cycle. *Phaeocystis cordata* described from the Mediterranean and *P. scrobiculata*, which has been reported from temperate and subtropic regions, are only known as flagellates (Lange et al. 1996; Medlin and Zingone 2007).

Species of the Pavlovales genera *Diacronema*, *Rebecca*, *Pavlova*, and *Exanthemachrysis* are commonly found in nearshore coastal environments (Carter 1937; Butcher 1952; Droop 1953; Van der Veer 1979), and reports from oceanic environments are scarce. *Diacronema vlkianum* has been recorded a few times from ponds and lakes in Europe, including the British Isles (Preisig 2002).

In a seasonal study of haptophyte diversity (assessed by OTU composition) in Skagerrak (Egge et al. 2015b), a strong seasonal pattern was revealed. Pavlovales representatives were only present in the summer and early spring communities and were dominated by Phaeocystales and Prymnesiales reads, together with environmental sequences that suggest a new haptophyte class. In summer and fall, reads representing coccolithophores and *E. huxleyi* in particular were frequent. Some taxa, e.g., *Phaeocystis cordata* and *Chrysochromulina simplex*, were detected all year.

Ecological Impacts of Bloom-Forming Haptophytes

Haptophytes thrive in both oceanic low-chlorophyll recycling systems and in high-biomass new-production systems, but only few species form intense blooms over large areas of the ocean. *Emiliania huxleyi* and *Gephyrocapsa oceanica* are by far the most prominent bloom formers. They can create blooms in temperate and boreal regions that cover very large areas, for instance, >200,000 km^2 in the Bering Sea (Sukhanova and Flint 1998) or about 250,000 km^2 in the North Atlantic (Holligan et al. 1993; Malin et al. 1993). Whereas cell densities of blooms in the North Sea, Western English Channel, North Atlantic, or the Bering Sea are usually below 6×10^6 cells/L (Holligan et al. 1983; Malin et al. 1993; Sukhanova and Flint 1998), higher densities have been recorded in the Black Sea (up to 30×10^6 cells/L; Mihnea 1997) and in Norwegian fjords (up to 115×10^6 cells/L, Berge 1962).

The conditions for such blooms are created in the course of seasonal succession cycles, typically after diatom blooms when waters have low amounts of macronutrients, especially silicate, prohibiting further growth of diatoms (Townsend et al. 1994; Nanninga and Tyrrell 1996). In these low-nutrient regimes that become more stratified as the season progresses, *E. huxleyi* can typically thrive and outgrow other phytoplankton. This ecological success may be attributed to key physiological traits identified in most or all *E. huxleyi* strains. Photosynthesis, for example, seems not to be inhibited by high irradiances. Cells can grow with near-maximal rates at intensities equal to or exceeding surface sunlight at 1000–1700 μmol photons m^{-2} s^{-1} (Nielsen 1995; Nanninga and Tyrrell 1996). An efficient phosphate acquisition system enables high affinity uptake of inorganic phosphate but also allows scavenging of organically bound phosphates (Riegman et al. 2000; Xu et al. 2010). *Emiliania huxleyi* tolerates nitrogen deprivation much better than diatoms (Löbl et al. 2010). The efficient metabolic recycling of organic N and the direct malate oxidation by malate-quinone oxidoreductase seem to be additional features that increase *E. huxleyi*'s competitiveness in oligotrophic environments (Rokitta et al. 2014). To overcome trace metal limitation and especially that of iron,

E. huxleyi is able to substitute Fe-containing enzymes by functionally equivalent forms that bind other metal cofactors (Read et al. 2013). Although *E. huxleyi* usually does not form blooms in Fe-limited regions (the Southern Ocean), these adaptations certainly broaden its ecological scope and explain its abundance and persistence in contemporary oceans.

Blooms of *E. huxleyi* are unique in their optical properties. As a bloom progresses, coccoliths or entire coccospheres are shed (Balch et al. 1991; Paasche 2002) and scatter the incoming light, turning surface waters turbid and milky, a phenomenon visible from ships and remote sensing satellites (Holligan et al. 1983; Balch et al. 1991). At high concentrations in surface water, the average light intensity is increased due to the scattering of light by the $CaCO_3$ crystals. With depth, however, the irradiance is strongly attenuated (Balch et al. 1991; Holligan et al. 1993; Tyrrell et al. 1999). Increased light and heat trapping in the surface layers can cause a further decrease in the depth of the euphotic zone and increase the stratification (Balch et al. 1991; Tyrrell et al. 1999).

Blooms of *E. huxleyi* can sustain considerable populations of zooplanktic herbivores and are, because of high growth rates, usually not "top-down" regulated by grazing (Nejstgaard et al. 1997). Instead, mature blooms grow into nutrient starvation and are typically lysed by specific viruses (Castberg et al. 2002). Phycodnaviruses, like animal viruses (Mackinder et al. 2009), take control of cellular machinery, replicating and inducing cell lysis and viral burst within 2–3 days (Kegel et al. 2010). This top-down regulation of E. *huxleyi* blooms enables the transition to the next stage of ecological succession (Martínez et al. 2007). *Emiliania huxleyi* exhibits a haplo-diplontic life cycle and the haploid stage is a scale-bearing, non-calcified cell that possesses flagella. The distinct morphology and striking resistance to viral attacks (Frada et al. 2008) point toward an important ecological function of life cycling: growing diploid populations sporadically produce haploid cell stages, and this "background population" of haploid individuals can function as a new founding population in the case of a virally mediated termination of the parental diploid bloom, a phenomenon termed the "Cheshire cat escape strategy" (Frada et al. 2008; Frada et al. 2012). The distinct genetics of the haploid phase and consequentially also the distinct morphology and metabolism of the haploid phase are also thought to extend the species' ability to occupy new ecological niches (Rokitta et al. 2011).

The biogeochemical impacts of such coccolithophore blooms on the global carbon cycle are well studied. As phytoplankton, coccolithophores contribute to primary production and to the export of organic matter to the deep sea. Whereas this process, termed the *organic carbon pump*, causes a net drawdown of CO_2 from the atmosphere into the ocean, the production and export of calcium carbonate has the opposite effect on air/sea CO_2 exchange, causing a net release of CO_2 to the atmosphere (Rost and Riebesell 2004). This counteracting effect on the ocean–atmosphere CO_2 flux is referred to as the *carbonate counter pump*. As coccolithophores contribute to both carbon pumps, the drawdown of CO_2 caused by organic carbon production is partly compensated by the release of CO_2 via calcification. Primary production by coccolithophores is therefore a smaller sink

for CO_2 when compared to a non-calcifying production system, such as a diatom bloom (Robertson et al. 1994). In cases of calcite overproduction, a phenomenon typical for *E. huxleyi* blooms growing into nutrient limitation can become a net source of CO_2 and effectively release CO_2 into the atmosphere (Purdie and Finch 1994). $CaCO_3$ also acts as a "ballast" mineral that increases the transfer efficiency of organic matter from surface waters to depth (Klaas and Archer 2002). It has been shown that coccoliths in fecal pellets and marine snow enhance their sedimentation rate (Buitenhuis et al. 1996).

Estimates of global calcium carbonate production ranges from 0.64 to 2 Gt C per year (Morse and Mackenzie 1990; Milliman 1993; Westbroek et al. 1993; Wollast 1994; Milliman and Droxler 1996). Global annual marine primary production are ~45–50 Gt C (Antoine and Morel 1996; Field et al. 1998; Carr et al. 2006), of which 5 ~ 10 Gt C are exported to the deep sea (Laws et al. 2000; Palmer and Totterdell 2001; Henson et al. 2011). A significant part is driven by the ballast effects derived from $CaCO_3$ created by *E. huxleyi* and *G. oceanica*, but also other heavily calcified, non-blooming coccolithophore species, such as *Calcidiscus leptoporus* or *Coccolithus pelagicus*, may contribute substantially to $CaCO_3$ vertical fluxes (Baumann et al. 2004; Ziveri et al. 2007).

Phaeocystis is a cosmopolitan mucilage-producing genus whose species may occur in high concentrations in both northern and southern hemispheres (Veldhuis et al. 1986; Moestrup and Thomsen 2003; Schoemann et al. 2005), and the species *Phaeocystis globosa*, *P. pouchetii*, and *P. antarctica* (Edvardsen and Imai 2006; Medlin and Zingone 2007) are important bloom formers. Off the British coast and along the coasts of Netherlands and Germany, *P. globosa* regularly blooms in late spring or early summer, producing mucilage in sufficient quantity to clog fishing nets. High winds cause the decomposing mucilage to foam and accumulate on beaches and shores where it is considered a nuisance for recreational beach use (Moestrup 1994). Blooms of *Phaeocystis* may cause harmful effects on animals and *P. pouchetii* may be toxic to cod larvae (Aanesen et al. 1998; Hansen et al. 2004). *Phaeocystis* blooms have influenced herring migration through the North Sea (Savage 1930).

Phaeocystis and other haptophytes are known to be prodigious producers of dimethylsulfoniopropionate (DMSP) (Keller et al. 1989), the precursor compound to dimethyl sulfoxide (DMS), a climate-active gas that induces atmospheric cloud formation and thus may affect planetary albedo. *Phaeocystis* species produce DMSP that is cleaved into acrylic acid and DMSP, but is not considered toxic to animals (Schoemann et al. 2005). It is unclear whether the observed harmful effects derive from toxin production, the toxic nature of breakdown products as blooms decays, or deoxygenation of the water during bloom decays.

The controversial CLAW hypothesis (Charlson et al. 1987; Quinn and Bates 2011) proposed that this DMS-cloud albedo system would represent a negative feedback acting to stabilize climate. Several studies on *Phaeocystis* and *E. huxleyi* in culture and in natural blooms have contributed significantly to our current understanding of DMSP production and cycling (Keller et al. 1989; Malin and Steinke 2004). Many haptophytes, both calcifying and non-calcifying species,

produce high amounts of DMSP (Holligan et al. 1983; Holligan et al. 1993; Malin et al. 1993; Malin and Steinke 2004), and this compound has typical properties of a compatible solute/osmolyte (Kirst 1996) but may also have additional physiological roles (Stefels 2000), e.g., acting as a mitigator of oxidative stress (Sunda et al. 2002). Several environmental factors (nutrients, irradiance, and temperature) alter intracellular DMSP content; while little DMSP is produced in exponential growing cultures, nutrient limitations or stress causes unbalanced growth and increased DMSP cell quotas (Stefels and van Boekel 1993; Sunda et al. 2002). Grazing or viral lysis releases DMSP to the water column (Malin et al. 1993). Despite a large marine DMSP pool, only a very small proportion is ever emitted as DMS to the atmosphere, the rest being recycled within the marine food web or transported to deeper waters (Malin and Steinke 2004). *Phaeocystis* is one of few microalgal genera found to be able to enzymatically convert DMSP into the antibacterial compound acrylic acid and the volatile compound DMS (Stefels 2000). Already Sieburth emphasized the antibiotic properties of acrylic acid produced by *Phaeocystis* to the intestinal bacterial flora of animals (Sieburth 1961).

Widely reported from coastal and inland saline waters in Britain, Denmark, the Netherlands, Israel, Norway, China, and North Africa (Holdway et al. 1978; Moestrup 1994; Edvardsen and Paasche 1998; Edvardsen and Imai 2006; Johnsen et al. 2010) as well as lakes and river basins in southern United States (Baker et al. 2007; Southard et al. 2010), *Prymnesium parvum* causes serious economic losses because of its association with extensive fish kills. It produces toxins with ichthyotoxic, cytotoxic, neurotoxic, antibacterial, and allelopathic activity (Shilo 1981; Meldahl et al. 1994; Edvardsen and Imai 2006; Graneli et al. 2012), which act on biological membranes. The ichthyotoxic effect is assumed to be caused by increased permeability in fish gills resulting in disturbed ion balance and possibly in higher susceptibility to any toxic agents in the water, including other components of the *P. parvum* toxins (Yariv and Hestrin 1961; Ulitzur and Shilo 1966). Several different compounds have been ascribed the toxic effects of *P. parvum* such as proteolipids (Ulitzur and Shilo 1970), glycolipids (Kozakai et al. 1982), hemolysins (Kozakai et al. 1982), polyethers (Igarashi et al. 1996), prymnesins −1 and −2 (e.g., Igarashi et al. 1995; Igarashi et al. 1996), and recently fatty acid amides (Bertin et al. 2012a; Bertin et al. 2012b). Toxins produced by *Prymnesium polylepis* show the same type of toxicity as *P. parvum* including toxicity to the brine shrimp *Artemia* (Yasumoto et al. 1990; Meldahl et al. 1994). In 1988 an extensive bloom of this species occurred in the Skagerrak, northern Kattegat, west coast of Sweden, and coastal waters of Southern Norway (Dahl et al. 1988; Granéli et al. 1993). Both natural fauna and flora were affected severely, and 900 metric tons of caged fish were killed (Gjøsæter et al. 2000). A number of additional haptophytes, such as members of *Chrysochromulina*, *Haptolina*, *Phaeocystis*, and *Chrysotila* (*Pleurochrysis*), have been associated with fish kills or with other toxic or allelopathic activities (Edvardsen and Paasche 1998; Edvardsen and Imai 2006). Several species of *Chrysotila* and *Jomonlithus littoralis* have been shown to be toxic to the brine shrimp *Artemia salina.* Local blooms of *Chrysotila* species have been reported with some harmful effects (Houdan et al. 2004b), and also *Chrysochromulina*

parva has been associated with fish kills (Hansen et al. 1994). A bloom (5–9 million cells L^{-1}) of *C. breviturrita* was reported to have produced lake-wide obnoxious odors in Ontario and New Hampshire, North America (Nicholls et al. 1982).

Coccolith Function and Calcification

Despite intensive research on coccolithophores, the ecophysiological function of calcification is not yet understood (Young 1994; Paasche 2002; Brownlee and Taylor 2004). It has been hypothesized that the coccosphere serves to protect against grazing and/or virus attack, but as yet no experimental proof of these hypotheses has been reported (Harris 1994; Young 1994). Coccoliths could optimize the light interception by the algal cell (Young 1994), or calcification could provide a way of dissipating absorbed light energy to avoid photodamage under nutrient limitation (Paasche 2002), but these hypotheses also remain unproven. It was suggested that cells use the coccosphere to regulate their buoyancy, because controlled sinking might provide means of escaping high irradiances or reaching depths with higher nutrient concentrations (Young 1994; Balch et al. 1996). However, calculated rates of sinking vary significantly among authors and are numerically negligible compared to the wind-driven mixing and wave turbulence. It therefore seems unlikely that a coordinated sinking in response to environmental situations is a reasonable evolutionary purpose of calcification.

There is some support from a theoretical basis for a function of calcification as a carbon-concentrating mechanism (CCM). $CaCO_3$ precipitation releases CO_2 or protons in the course of calcification according to the following reactions:

$$Ca^{2+} + 2HCO_3^- \rightarrow CaCO_3 + CO_2 + H_2O$$

$$Ca^{2+} + HCO_3^- \rightarrow CaCO_3 + CO_2 + H^+$$

Consequently, CO_2 derived from calcification can in principle be used in photosynthesis, or protons could be used in the conversion of HCO_3^- to CO_2 for the same purpose. In either case, this may provide the calcifying cell with a mechanism to access HCO_3^-, which represents the largest pool of inorganic carbon in seawater. Such functional coupling between calcification and photosynthesis was first proposed by Sikes et al. (1980) and has since then been discussed by various authors (Nimer and Merrett 1993; Anning et al. 1996; Buitenhuis et al. 1999).

Experimental data, however, suggest that calcification in coccolithophores is neither a prerequisite for efficient photosynthesis nor is it particularly effective in mitigating CO_2 limitation. The latter is indicated, for example, by the fact that photosynthesis continues unaffected when calcification ceases (Paasche 1964; Herfort et al. 2002; Trimborn et al. 2007), and non-calcifying haploid cells of *E. huxleyi* can photosynthesize as efficiently as or even more efficiently than the diploid calcifying ones (Rost and Riebesell 2004). Moreover, non-calcifying cells of *E. huxleyi* are equally capable of direct HCO_3^- utilization for photosynthesis, which

implies that HCO_3^- utilization is not tied to calcification (Trimborn et al. 2007; Rokitta and Rost 2012). Ultimately, calcification appears unsuited to prevent CO_2 limitation in *E. huxleyi*, which is indicated by observations that the rate of photosynthesis often decreases with decreasing CO_2 concentration despite a concomitant increase in calcification rate (Riebesell et al. 2000; Berry et al. 2002; Hoppe et al. 2011).

The removal of Ca^{2+} ions from the cytoplasm is a prime necessity of literally all organisms to prevent cytotoxic effects of uncontrolled Ca^{2+} signaling. Calcification in coccolithophores may thus represent a form of "irreversible removal" from the cell (Young 1994). Recent data indeed show that, in contrast to non-calcifying phytoplankton, coccolithophores can tolerate massively increased [Ca^{2+}] and maintain fitness even at concentrations 4–5fold higher than in today's oceans. Most strikingly, strains that were earlier described as "non-calcifying" started to re-calcify after ~2 weeks in high Ca^{2+} medium. Hence, the key evolutionary advantage of intracellular calcification may lie in the removal of potentially toxic Ca^{2+} ions, thus enabling cells to have a better control on the transient regulation of cytoplasmic enzyme activity (Müller et al. 2015).

Calcification and Ocean Acidification

Several future emission scenarios predict an increase from currently 400 to more than 750 µatm CO_2 for the year 2100 (IPCC scenario IS92a). As the ocean takes up CO_2, concentrations of dissolved inorganic carbon increase and the pH of seawater decreases. This ocean acidification (OA) is expected to intensify, so that pH levels will have dropped by 0.4 units relative to preindustrial values by the end of this century (Wolf-Gladrow et al. 1999). Ongoing OA will decrease saturation states for $CaCO_3$ minerals. Whereas only "true" undersaturation will cause dissolution, a lowered saturation state can already to some extent affect the biological mineralization processes, simply because $CaCO_3$ precipitation under low pH is thermodynamically less favored (Ridgwell and Zeebe 2005).

Specific differences in the sensitivity to OA do exist (Langer et al. (2006)). In *Calcidiscus leptoporus*, an optimum curve was observed with maximum calcification rates at present-day CO_2 levels, but calcification rates did not vary significantly with pH in *Coccolithus pelagicus*. In both species, photosynthetic carbon fixation rates remained constant at CO_2 levels ranging between 150 and 920 µatm. Most of our current understanding of the processes and sensitivities of calcification and photosynthesis derives from studies on *E. huxleyi* and the closely related species *G. oceanica*. Both belong to an evolutionary very young lineage of rather atypical coccolithophores in terms of structure, physiology, and ecology (Sáez et al. 2004; de Vargas et al. 2007). Different OA response patterns have also been observed between different strains of *E. huxleyi*, with strains largely showing negative or no effects on calcification at pCO_2 values expected for the end of the century (Langer et al. 2009). Overall, most studies have observed negative responses in growth, positive or no responses in biomass production, and negative or no responses in calcification under

high CO_2, leading in most of the studies to a lowered PIC:POC ratio under OA (Zondervan 2007; Hoppe et al. 2011). When assessing possible responses of this group to global change, the intraspecific variability and the diversity within coccolithophores have to be acknowledged, including the different life cycle stages (haploid and diploid) because they display different morphologies and modes of calcification or no calcification and thus may differ in their sensitivity to ocean acidification.

The intracellular mechanism of calcification by coccolithophores was for a long time not well understood (Brownlee and Taylor 2004), despite the wealth of information on the effects of environmental conditions on coccolith production (see review in Paasche 2002). Recently molecular techniques have yielded insights into the functions of calcification-related genes and their responses to environmental change (Mackinder et al. 2010; Rokitta et al. 2012; Rokitta and Rost 2012). Unlike other calcifying organisms, where calcification occurs in extracellular fluids, calcite precipitation in coccolithophores takes place in intracellular vesicles (at least in the case of heterococcoliths) and hence is under control of the cell. It is therefore surprising that calcification in *E. huxleyi* shows such a strong dependency on seawater carbonate chemistry, similar to that observed in Foraminifera and corals (Gattuso et al. 1998; Wolf-Gladrow et al. 1999; Riebesell et al. 2000).

Coccolithophores have been at the center of debate about the consequences of ocean acidification. Numerous findings from laboratory and mesocosm studies (Riebesell et al. 2000; Zondervan et al. 2002; Delille et al. 2005; Hoppe et al. 2011), suggest that calcification by *E. huxleyi* will be reduced in response to OA. In most studies, the decrease in calcification was accompanied by an increase in biomass production (Hoppe et al. 2011), which has been attributed to an intracellular reallocation of acquired carbon away from the impacted calcification toward photosynthesis (Rokitta and Rost 2012). Under OA, multiple parameters of the carbonate system change, which affect the different physiological processes in various ways. The cell-integrated changes in calcification and photosynthesis under elevated pCO_2 have typically been ascribed to the decrease in carbonate ion concentration and thus calcite saturation levels (Cyronak et al. 2015), but more recent and systematic investigations could show how exactly the particular processes are affected by the isolated parameters of the carbonate system. To elucidate the effects of the different parameters of the carbonate system, Bach and coworkers have uncoupled the carbonate chemistry in their experiments (Bach et al. 2011; Bach et al. 2013). They were able to show that growth, biomass production, and carbon acquisition are primarily controlled by CO_2 supply at low (subsaturating) levels, whereas at higher concentrations, they are adversely affected by the concomitant decrease in pH. Further (also transcriptomic) evidence suggests that calcification of scales varies with HCO_3^- supply and is impaired by low pH (Bach et al. 2013). Despite some differences between coccolithophore species and strains, these authors derived a unifying mathematical concept that robustly describes the dependence of calcification rates on carbonate chemistry speciation (Bach et al. 2015).

The precipitation of $CaCO_3$ and the consequential shift in carbonate chemistry inevitably cause acidosis in the cytoplasm of the cells. This is prevented by rapid H^+

effluxes via voltage-gated H^+ channels that are situated in the plasma membranes of many eukaryotes. The increased acidity under OA causes a less steep proton gradient, therefore impairing channel gating and leading to overall higher $[H^+]$ in the cytoplasm of cells (Suffrian et al. 2011; Taylor et al. 2011). The impairment of calcification leads to an intracellular reallocation of inorganic carbon toward the competing reaction, biomass production. This happens in such way that OA often does not significantly affect production of total carbon, i.e., biomass and calcite (Zondervan et al. 2002; Feng et al. 2008; Langer et al. 2009). This effect of carbon reallocation from calcification toward photosynthesis seems to be dependent on light intensity (Rokitta and Rost 2012). Under high light conditions, cells can mitigate and prevent the effects of OA, whereas under limiting light intensities, the reallocation effects are large. This modulation of OA effects by light intensity is accompanied by metabolic reconstellations, in which reductive, anabolic pathways, for example, may synthesize glucans and fatty acids to be upregulated over oxidative, catabolic pathways such as glycolysis and respiration (Rokitta et al. 2012). Cellular redox levels appear to be the informational "hub" that mediates the modulation of OA responses by integrating light intensity into cellular organic carbon metabolism.

In general, a reduction in the degree of calcification is assumed to put coccolithophores at an ecological disadvantage, suggesting a rather "grim future" for this phytoplankton group. This view is derived mostly from short-term acclimation studies over several generations. However, there are also new lines of evidence regarding the midterm and long-term adaptability to OA. Lohbeck et al. (2011) have grown monoclonal cultures of *E. huxleyi* under OA scenarios for ~500 generations and observed that these strains were still affected by OA, but exhibited higher calcification rates than unadapted strains when confronted with OA. This implies that evolution occurring at timescales of months may help to maintain calcification as a cellular function under global change. Data from present-day phytoplankton assemblages and sediment cores covering the last 40,000 years indicate, however, that changes in carbonate chemistry impose a massive selective pressure on the degree of calcification and induce floristic shifts from heavily calcified to less calcified species and strains with increasing CO_2 levels (Beaufort et al. 2011). In the Chilean upwelling system, a rare but distinct over calcified morphotype of *E. huxleyi* thrives under permanently low pH conditions, suggesting that this species is in general able to cope with and adapt to changing ocean acidity.

Characterization and Recognition

Cell Shape and Organization

Unicellular haptophytes exhibit a variety of cell shapes (Fig. 1). In the Coccolithophyceae, cells may be more or less spherical as in *Dicrateria* (Fig. 1e) and many coccolithophores, e.g., *E. huxleyi* and *C. pelagicus* (Figs. 1j and 7a). Saddle-shaped species are confined to the *Chrysochromulina* species (*C. campanulifer*a and

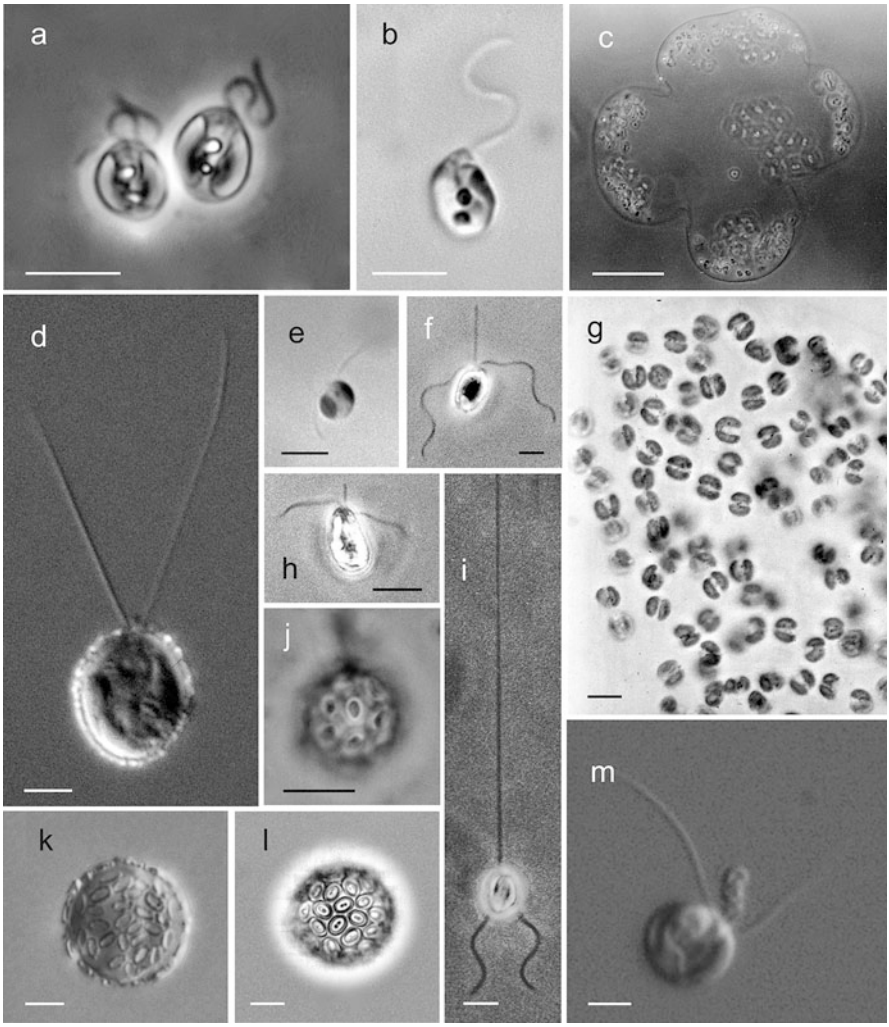

Fig. 1 Light micrographs of representatives of the Haptophyta. (**a**, **b**) Pavlovophyceae, (**a**) *Pavlova*, (**b**) *Rebecca*; (**c**–**m**) Coccolithophyceae, (**c** and **g**) *Phaeocystis pouchetii* colonies; (**d** and **k**) *Chrysotila carterae*; (**e**) *Dicrateria rotunda*; (**f**) *Prymnesium polylepis*; (**h**) *Prymnesium parvum*; (**i**) *Chrysochromulina simplex* with extended haptonema; (**j**) *Emiliania huxleyi*; (**k**) *Chrysotila carterae*; (**l**) *Coronosphaera binodata*; (**m**) *Chrysochromulina campanulifera* with coiled haptonema. Scale bars 5 μm Figs. **a**–**b**, **d**–**m**; Fig. **c**, 100 μm

C. rotalis, Fig. 1l) in which the flagella and haptonema (the appendages) are inserted ventrally. Elongate forms are found in *Prymnesium* (Fig. 1f, h), *Haptolina*, and *Chrysocampanula* and some coccolithophores such as *Calciopappus caudatus* and *Syracosphaera pirus*. Many species such as *Haptolina brevifila*, *Dicrateria rotunda* (Fig. 1e), the motile stage of *E. huxleyi*, and *Calyptrosphaera sphaeroidea* have their

appendages inserted in a polar position, sometimes in association with clusters of spine scales (coccoliths) as can be seen in *Michaelsarsia elegans*. Clusters of spine scales may be located both at the apex and antapex as in *Calciosolenia murrayi* and *Acanthoica quatrospina. Ophiaster hydroideus* has spines only at the antapex. Spines may also cover the entire cell body as in *Haptolina ericina* and *Rhabdosphaera clavigera*. In *Prymnesium*, *Isochrysis*, *Chrysoculter*, and *Chrysotila* species, the appendages are usually inserted sub-anteriorly. The appendages may arise from a depression as in *Corymbellus aureus* and the large, amoeboid cells of *Prymnesium pigrum.*

In the class Pavlovophyceae, cells may be variously shaped, but are almost always flattened with the appendages arising sub-anteriorly on the ventral side. In *Diacronema lutheri*, the insertion of the appendages is almost in the center of the ventral face (Green and Hibberd 1977; Bendif et al. 2011). Species of *Isochrysis*, *Ruttnera*, and the Pavlovophyceae have an asymmetric appearance that is accentuated by the possession of only one plastid, whereas most members of the Haptophyta appear to have two (rarely four, *Dicrateria inornata*). There are a few instances of colonial, stalked, and filamentous stages within the Coccolithophyceae. In *Phaeocystis* (Fig. 1c, g), several species form palmelloid colonial stages. *Phaeocystis* are commonly observed as gelatinous bladders that, in their younger condition, are roughly spherical and up to a few millimeters in diameter, but which later become irregular in form and may be several centimeters in their longest dimension. The cells themselves are small (approximately 3–8 μm diameter) and are arranged in a layer at the periphery. They are separated from each other and with no apparent connection between them. The exact nature of the colorless bladder is not known, but from its physical properties, it seems the surface has some of the elastic properties of a membrane and the matrix, while holding the cells in position, is apparently semiliquid, dispersing readily once the bladder is punctured (Kornmann 1955; Zingone et al. 2011). Benthic palmelloid forms are also known from cultures (Gaebler-Schwarz et al. 2010).

Motile colonies are seen in *Corymbellus aureus* in which the flagellated cells are attached to each other laterally and the colony has an annular shape (like a doughnut ring). In the motile colonies of *Prymnesium radiatus*, the cells are attached to each other at the posterior end and the colony has a ball shape with the flagella extending outward (Sym et al. 2011).

Ruttnera lamellosa and *Chrysotila stipitata* form benthic colonies made by concentric layers (*Ruttnera*) or stalks (*Chrysotila*) of gelatinous material. The motile cells of *R. lamellosa* are asymmetrical and *Isochrysis*-like, but after they have settled, they become spherical with a thick, lamellate mucilage sheath (Andersen et al. 2014). The benthic "Apistonema" stage of *Chrysotila* has cell walls composed of organic scales in a cementing substance and forms branched filaments. The motile cells bear coccoliths (Pienaar 1994). Nonmotile palmelloid cells have been reported in a number of species. Parke et al. (1955) described palmelloid cells in cultures of *Chrysochromulina* species, and nonmotile mucilage-embedded stages are known in *Ochrosphaera* (Fresnel and Probert 2005) and several species of *Pavlova* (Bendif et al. 2011).

Flagella, Transition Zone, and Flagellar Roots

In the Pavlovophyceae, the two flagella are markedly unequal, the more posteriorly inserted one is much shorter than the anterior flagellum. In *Rebecca salina*, the short flagellum is reduced to a short stump, detectable only with electron microscopy. The longer flagellum in the Pavlovophyceae usually carries a covering of fine hairs and small, dense, knob-like scales (Fig. 6a). The fine hairs do not resemble the tubular hairs (mastigonemes) of the heterokont algal groups in their structure and consist only of a fine non-tubular thread (Green 1980; Green and Hori 1994; Bendif et al. 2011). The knob scales (Fig. 6a) are often regularly arranged on the longer flagellum as in *Pavlova gyrans*, but in *Diacronema lutheri*, they are irregularly arranged and they are totally absent in *Exanthemachrysis* and *Diacronema vlkianum* (Bendif et al. 2011).

Coccolithophyceae flagella when present are more or less equal and naked. In flagellated species, two flagella are common, but *Chrysochromulina quadrikonta* has four (Kawachi and Inouye 1993).

The flagellar action may be homo- or heterodynamic. In Pavlovophyceae, the longer flagellum is directed forward with respect to the direction of swimming and beats with a sinuous S-like beat. The shorter flagellum is directed posteriorly, away from the cell body, and beats with a stiff, inflexible action. In swimming Coccolithophyceae cells, the flagellar pole may be in front with the flagella moving along the cell surface, or posterior with the flagella directed away from the cell because the smooth flagella always push the cell. The action of the flagella may be homodynamic and display an undulating motion as in *Chrysochromulina*, *Haptolina*, and many Coccolithales or be heterodynamic as in *Chrysocampanula* and many *Prymnesium*.

The axoneme consists of the usual "9 + 2" arrangement of microtubules except where reduction has taken place as in the short posterior flagellum of *Rebecca salina* in which the axoneme consists only of a ring of nine single microtubules (Green 1976). At the proximal end of the normal flagellum, there is a short transition region in which there are two transverse partitions, the more distal often appearing rather diffuse and thickened in the center. The central pair of axoneme microtubules stops at this partition. The double partition structure appears to extend throughout the phylum (Green and Hori 1994), having been recorded in *Chrysochromulina* (Eikrem and Moestrup 1998), *Haptolina* (Eikrem and Edvardsen 1999), *Prymnesium* (Manton 1964b; Green and Hori 1990), *Phaeocystis* (Parke et al. 1971), and *Dicrateria* (Green and Pienaar 1977), although in species of *Isochrysis* (Hori and Green 1991), *Hymenomonas*, and *Chrysotila* (*Pleurochrysis*) only the proximal partition is present. A helical band is located distally to it in the flagella in *Chrysotila carterae* (Beech and Wetherbee 1988) and *Hymenomonas coronata* (Roberts and Mills 1992). Tiers of tubular rings may also be present proximal to the axosome (Green and Hori 1994; Billard and Inouye 2004). In *Prymnesium*, tubular rings are present in addition to the double partition structure (Birkhead and Pienaar 1994, 1995). Some Pavlovales appear to have one partition only in the posterior flagellum (Green and Hibberd 1977). *Chrysoculter rhomboides* and *Diacronema vlkianum* are

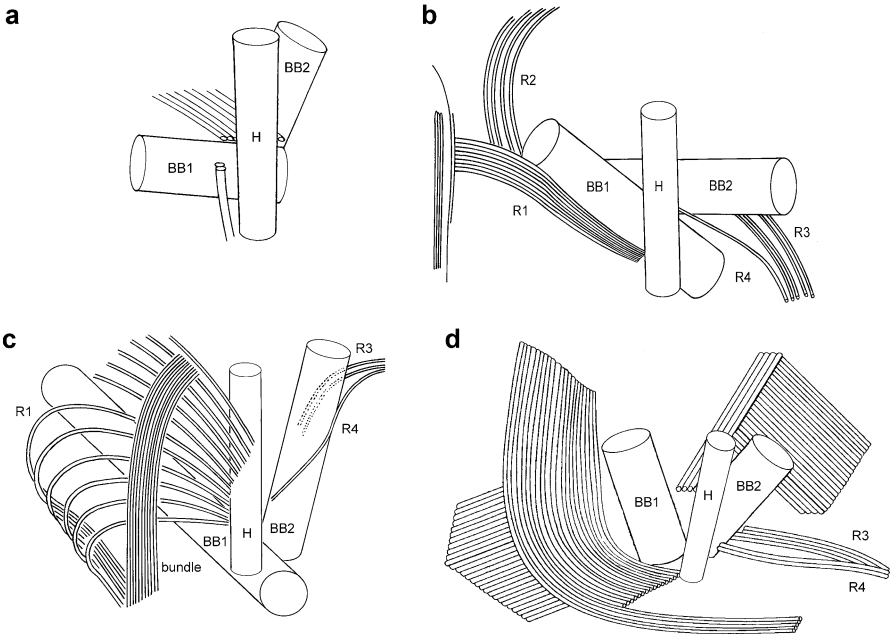

Fig. 2 Schematic three-dimensional reconstruction of the flagellar apparatus with microtubular roots of the Haptophyta: (**a**) *Diacronema*; (**b**) *Chrysochromulina scutellum*; (**c**) *Prymnesium palpebrale*; (**d**) *Chrysotila*

unusual in that the shorter posterior flagellum has a swelling on the side adjacent to the cell body (Green and Hibberd 1977).

The flagella bases and flagellar roots anchor the flagella within the cell and are components of the cell's cytoskeleton as well as being involved in cellular functions, such as mitosis. Both microtubular (Fig. 2) and fibrous flagellar roots are recorded in the Haptophyta and the microanatomy of the flagellar apparatus is believed to offer phylogenetic information because its morphology appears to evolve very slowly. Coccolithophyceae flagellar roots R1 and R2 are associated with the left flagellum and R3 and R4 with the right flagellum (see Eikrem and Moestrup 1998). R1 originates close to the base of the haptonema. The roots are numbered in a clockwise direction starting at the left flagellum with R1 (Green and Hori 1994). Over generations, the right flagellum and its basal body develop into the left flagellum. The left flagellum is termed the mature flagellum and it corresponds to the longer flagellum; the right flagellum is shorter and will transform into a mature flagellum in the next generation (Beech et al. 1988).

Inouye and Pienaar showed that the coccolithophores, *Umbilicosphaera foliosa*, and a species of *Chrysotila* (*Pleurochrysis*) have a complex root system with some components consisting of as many as two hundred microtubules (Inouye and Pienaar 1984; Inouye and Pienaar 1985). Such roots have been termed compound roots and consist of a sheet of microtubules and a closely packed bundle of numerous

microtubules. The bundles are termed crystalline roots (CR1, CR2) and are associated with the roots (R1, R2) of the basal body of the left flagellum (Fig. 2d). Some species have crystalline roots in both R1 (termed CR1) and R2 (termed CR2), whereas others have only one of them (Billard and Inouye 2004). Crystalline roots (Fig. 2c) are also found in *Isochrysis* (Hori and Green 1991) and *Prymnesium* (Birkhead and Pienaar 1994; Birkhead and Pienaar 1995).

Syracosphaera pulchra (Inouye and Pienaar 1988), *Algirosphaera robusta* (Probert et al. 2007), and the saddle-shaped species of *Chrysochromulina* seem to lack crystalline roots, and the R1 of saddle-shaped *Chrysochromulina* species (Fig. 2b) often consist of less than ten microtubules (Moestrup and Thomsen 1986; Eikrem and Moestrup 1998; Jensen and Moestrup 1999). In non-saddle-shaped Prymnesiaceae species, they may number more than 20 and form a broad sheet of microtubules.

The cytoplasmic tongue (Beech and Wetherbee 1988) is a complex formed by some microtubules of the R1 sheet and the fibrous root originating on the left basal body. It is delineated by an extension of the peripheral endoplasmic reticulum and extends deep into the cell in a narrow space of cytoplasm (Billard and Inouye 2004) in several *Chrysotila* species (Gayral and Fresnel 1983; Beech et al. 1988; Fresnel and Billard 1991) and *Prymnesium palpebrale* (Birkhead and Pienaar 1995). In *Prymnesium nemamethecum*, it is present, but reduced (Birkhead and Pienaar 1994).

R2 may be compound, but only a few microtubules constitute the sheet of R2, and it originates between the basal bodies close to the left basal body. Possible homologies between the complex and simple systems have been suggested (Inouye and Pienaar 1984; Moestrup and Thomsen 1986; Green and Hori 1994). Roots R3 and R4 are never compound and consist of only a few microtubules. R3 originates from the right side of the right basal body and R4 from the left. Accessory and connecting fibers link the flagellar bases and the haptonema base. They may appear striated or nonstriated in electron micrographs of thin sections (Green and Hori 1994; Pienaar 1994). The fibrous root associated with the cytoplasmic tongue is the most prominent.

The flagellar roots found in the Pavlovophyceae are different. Two microtubular roots, R1 and R2, with only a few microtubules are accompanying the basal body of the short flagellum. A conspicuous fibrous root is associated with the basal body of the long flagellum extending deep into the cell close to the nucleus. Accessory and connecting fibers are present (Green and Hori 1994).

Haptonema

In its most extreme form, the haptonema is very long, often many times the diameter of the cell body in length as in *Chrysochromulina rotalis* (Fig. 1i). In *C. strobilus*, the extended haptonema may be up to 100 μm in length, the cell diameter being only approximately 8 μm. The reduced haptonema of *Isochrysis* and *Chrysotila* is very short (1–2 μm) and barely detectable with light microscopy. In *Dicrateria rotundata*, reduction is more extreme such that the haptonema is represented by at most a small

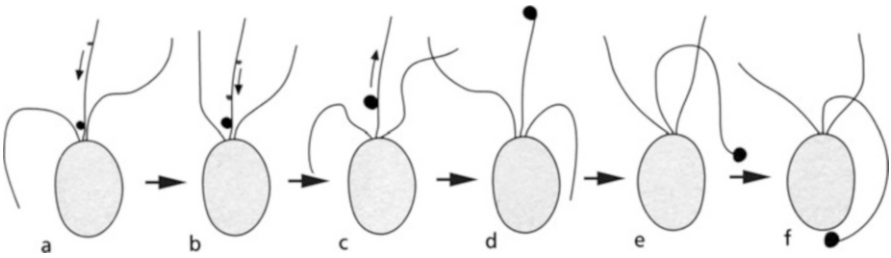

Fig. 3 Schematic interpretation of nutrient particle uptake studies in *Haptolina hirta* by Inouye and Kawachi (1994) from Graham and Wilcox (2000): (**a**–**b**), food particles captured by the distal part of the haptonema and transported along the haptonema surface to aggregate in a larger particle on the proximal part; (**c**–**d**) food particle aggregates move to the haptonema tip; (**e**–**f**) haptonema manipulating the food aggregate to be deposited on the posterior cell surface where it will be engulfed

elevation between the flagella. Between these extremes may be seen a variety of haptonema lengths. In some taxa, the haptonema coils (*Haptolina ericina*, *Chrysochromulina campanulifera*), whereas in others (*Prymnesium parvum*, *Phaeocystis pouchetii*), the haptonema flexes, but without any organized pattern of movement.

The haptonema may function as a feeding organelle (Inouye and Kawachi 1994; Kawachi and Inouye 1995), and the process is demonstrated in Fig. 3. In swimming cells of *Haptolina hirta* with the haptonema directed forwardly and the flagella alongside the cell, prey particles adhere to the haptonema. They are transported to a point ca 2 μm from the base where an aggregate of prey is formed. The aggregate is moved to the tip of the haptonema and the haptonema bends to deliver it to the surface of the rear end of the cell where it is phagocytized into a food vacuole.

In both long and short haptonema-bearing species, the cell may attach to the substrate (slide, cover glass) either at the tip or, in the case of a long haptonema, by any point along its length. Attached haptonema may show gliding or sliding movements in a sinuous manner, whereas unattached, extended haptonema may demonstrate bending or flicking movements. Coiling often occurs at contact with an obstacle. The most spectacular movements, however, are seen in the coiling and uncoiling of long haptonema of *Chrysochromulina* species. Coiling is a very rapid process and may take only 1/60–1/100th of a second, whereas the uncoiling process is much slower and may take between 2 and 10 s. Coiling is usually initiated from the haptonema tip, and the direction and orientation of coiling and number and diameter of gyres is predetermined (Parke et al. 1971; Leadbeater 1971a; Inouye and Kawachi 1994). Coiling is believed to be the result of an influx of Ca + from the medium in which the cells live and into the lumen of the haptonema (Gregson et al. 1993; Inouye and Kawachi 1994).

The fine structure of the haptonema (Fig. 4) is quite unlike that of the flagella. The plasmalemma of the free part typically encloses a fenestrated cylinder of endoplasmic reticulum (ER) within which there is a ring of 6 or 7 single microtubules.

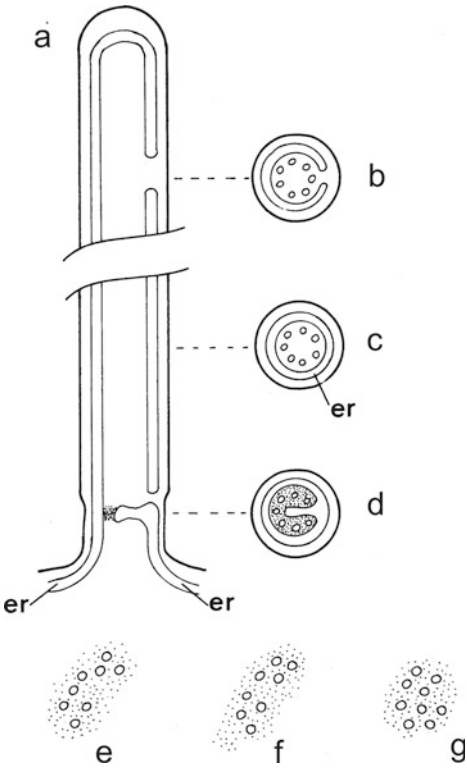

Fig. 4 Schematic view of a haptonema: (**a**) longitudinal section with endoplasmic reticulum, microtubuli not shown; (**b**–**d**) transverse section at indicated positions showing endoplasmatic reticulum and microtubuli; (**e**–**g**) microtubuli within the cell. Abbreviation: *er*, endoplasmic reticulum

The plasmalemma of the haptonema is confluent with that of the cell body, and the ER is continuous with the peripheral ER of the cell. Toward the proximal end of the haptonema, the ring of microtubules becomes reoriented into an arc facing one of the flagella, with the ER cisterna lying on the outer curved side of the arc. Immediately beneath this level, the ER once again forms a complete cylinder, but with a flat-ended, fingerlike component projecting inward; in transverse section, the projection appears to be capped by the arc of microtubules. On entering the cell, additional microtubules appear and further rearrangements of the microtubules take place to form two superposed arcs of 4 microtubules each, followed in some cases by the addition of a ninth microtubule (Manton 1964b; Moestrup and Thomsen 1986; Billard and Inouye 2004).

Reduced haptonema are known in many Coccolithophyceae species, such as the short bulbous haptonema of many *Chrysotila* species (Manton and Peterfi 1969; Billard and Inouye 2004). In *Isochrysis* and *Ruttnera*, the haptonema is reduced to a small protrusion containing ER profiles and only three microtubules, increasing to four within the cell. In *Emiliania huxleyi* and *Dicrateria inornata*, there is no trace of a haptonema (Klaveness 1972; Green and Pienaar 1977).

In the Pavlovophyceae, the free part of the haptonema is short and the number of microtubules reduced (only one to four microtubules), although the number

increases to seven or eight inside the cell (Green and Hibberd 1977; Gayral and Fresnel 1979; Inouye and Kawachi 1994). The ER is strongly fenestrated and may be reduced to a small vesicle on one side of the organelle (Green 1980).

Haptonemal roots have been described in a few species of the Pavlovophyceae where they appear as fibrous structures originating at the proximal end of the haptonema microtubules (Green and Hori 1994). Haptophyte haptonema microtubules may also be attached to one of the kinetids by a fibrous connection (Manton 1964a; Moestrup and Thomsen 1986; Green and Hori 1994).

Plastid, Nucleus, Golgi Apparatus, Pyrenoids, and Stigma

Haptophyta cells normally possess 1–2 plastids containing three thylakoid lamellae and there is no girdle lamella (Fig. 5). Pyrenoids may be immersed within the plastid and penetrated by one or a few pairs of thylakoids (Fig. 5c), but in some genera, they may bulge from the inner face of the plastid. The plastid and pyrenoid are surrounded by endoplasmic reticulum confluent with the nuclear envelope (Fig. 5b, c), the nucleus itself usually lying close to the plastid (Pienaar 1994). Coccolithophyceae cells usually lack a stigma, but an autofluorescent substance has been associated with the flagella in several species (Kawai and Inouye 1989).

A stigma or "eyespot" consisting of a single layer of lipid globules occurs in many Pavlovophyceae (Fig. 5d), although its position is variable. It is absent in *Diacronema ennorea* and *Rebecca helicata*. In some, it lies on the inner face of the

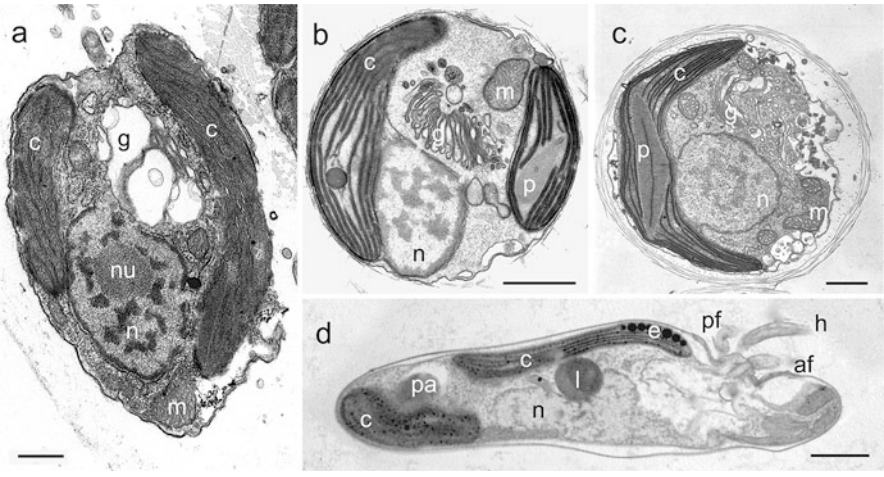

Fig. 5 Ultrastructure, TEM sections. (**a**) *Phaeocystis antarctica*, (**b**) *Chrysochromulina throndsenii*, (**c**) *Calyptrosphaera sphaeroidea*, (**d**) *Diacronema noctivaga*. Abbreviations: *c*, chloroplast; *n*, nucleus; *nu*, nucleolus; *pf*, posterior flagellum; *af*, anterior flagellum; *h*, haptonema; *p*, pyrenoid; *l*, lipid droplet; *m*, mitochondrion. Scale bars 5 μm (Image of *Phaeocystis* by courtesy of Adriana Zingone, *Calyptrosphaera* by Dag Klaveness and *Diacronema* by Madhi Bendif)

plastid close to the anterior flagellum, but in *Diacronema lutheri* and *D. vlkianum*, it is found on the outer face of the plastid lying beneath the shorter flagellum (Green 1980; Van Lenning et al. 2003; Bendif et al. 2011).

The mitochondrion has tubular cristae and is probably single and highly reticulated (Beech and Wetherbee 1984). The Golgi apparatus consists of a single dictyosome (Fig. 5b) and lies between the nucleus and the kinetids. In sections, the cisternae are arranged in a fan formation with the edges of the cisternae closer and less dilated on the side of the stack nearer the kinetids. The central parts of several adjacent cisternae may be inflated in a manner that seems to be unique to members of the Coccolithophyceae (Manton 1967; Hibberd 1980; Pienaar 1994) and may be concerned with carbohydrate polymerization during scale formation (Romanovicz 1981).

Storage Metabolites, Lipids, and Sterols

The primary storage metabolite of most members of the Haptophyta is assumed to be the water-soluble 1–3 glucan chrysolaminarin, as demonstrated for *Phaeocystis globosa* motile cells (Janse et al. 1996) and *Emiliania huxleyi* (Vårum et al. 1986). In the Pavlovales, another 1–3 glucan, the solid paramylon, commonly found in the euglenoids, has been identified by X-ray analysis (Kreger and Van der Veer 1970). Lipid bodies are also commonly present in the Haptophyta. Lipids and sterols found in the Haptophyta have been reviewed by Conte et al. (1994) and Marlowe et al. (1984). Many Isochrysidales species are unusual as they produce long-chain polyunsaturated C_{36} fatty acids and C_{37}–C_{39} sterols and diverse other neutral lipid compounds that can be collectively referred to as "polyunsaturated long-chained (C_{37}–C_{39}) alkenones, alkenoates, and alkenes" (PULCAs) (Eltgroth et al. 2005). The relative composition of the latter compounds, their degree of unsaturation, and their ^{13}C and ^{2}H signatures are often well correlated with environmental parameters such as temperature, salinity, and also CO_2 concentration (Marlowe et al. 1984; Pagani 2002; Van der Meer et al. 2007). Additionally, they are comparably resistant to digenesis in sediments (see Rontani et al. (2013) for a recent review), which makes PULCAs a tool widely used by geologists for paleo-reconstructions of temperature, salinity, and CO_2 (Conte et al. 1998; Pagani 2002; Beltran et al. 2007; Liu et al. 2008). These genera also have particularly high concentrations of the sterol 24-methylcholesta-5,22E-dien-3-ol, which occurs as well in some other coccolithophores but has not been recorded in the Prymnesiales taxa examined and only occurs in low concentrations in the Pavlovophyceae. Other sterols occurring in significant concentrations, though not universally distributed throughout the class, include cholest-5en-3-ol (cholesterol), 24-methylcholest-5-en-3-ol, 24-ethycholesta-5,22E-dien-3-ol, and 24-ethylcholest-5-en-3-ol. Well-known PULCA producers include the Isochrysidales species *Emiliania huxleyi*, *Gephyrocapsa oceanica*, and *Isochrysis galbana* (Conte et al. 1998); PULCA production has also been observed in the benthic lacustrine *Ruttnera* (Sun et al. 2007). Biochemically, these compounds derive from specialized cellular lipid

synthetic pathways, possibly similar to polyketide synthesis, as can be interpreted on the basis of genomic evidence (Read et al. 2013) and gene expression patterns (Rokitta et al. 2011). PULCAs are typically deposited in cytoplasmic liposomes and may function as sinks of cellular reductive energy and carbon; this latter function may especially be important in situations when growth is arrested, under nutrient starvation (Eltgroth et al. 2005). In experimental mesocosm blooms, cells of *E. huxleyi* accumulated up to 6 pg. PULCA cell^{-1} during exponential phase growth, a significant portion of the cellular biomass (Benthien et al. 2007). In addition, PULCAs appear also to serve as energy stores as they can be degraded in the dark (Eltgroth et al. 2005).

Pigments

Haptophytes have high pigment diversity. Chl *a* and the accessory pigments divinyl protochlorophyllide (MgDVP), Chl c_2, diadinoxanthin, diatoxanthin, and β,β-carotene comprise the common haptophyte pigments (Van Lenning et al. 2004). The haptophytes can then be divided into eight pigment types based on the content of nine chl *c*-type pigments (DV-Chl c_{PAV}, MV-Chl c_{PAV}, DV-Chl c_3, MV-Chl c_3, MgDVP, Chl c_2, Chl c_1, Chl c_2-MGDG$_{Ehux}$, Chl c_2-MGDG$_{Cpoly}$) and five fucoxanthin derivatives (Unk-Fx, 4kFx, Bfx, HFx, 4kHFx) (Van Lenning et al. 2003; Van Lenning et al. 2004; Zapata et al. 2004). Among the coccolithophores, the pigment profiles follow large phylogenetic groups and/or ecological preferences (Van Lenning et al. 2004). The pigment profiles of the class Pavlovophyceae agrees with SSU rDNA phylogenies and some ultrastructural features (Van Lenning et al. 2003). The pigment types within the non-calcifying order Prymnesiales do, however, not appear to correlate with phylogenetic groups (Zapata et al. 2001; Edvardsen et al. 2011).

The fucoxanthin derivative 19-hexanoyloxyfucoxanthin (HFx) was suggested as a pigment marker to determine the contribution of haptophytes to phytoplankton assemblages (Everitt et al. 1990), but was later found to be absent in the coastal families Isochrysidaceae, Pleurochrysidaceae, and Hymenomonadaceae and to be present in some dinoflagellates with plastids of haptophyte origin such as species of *Karenia* and *Karlodinium* (Berger et al. 1977), as well as in the ochrophytes *Dictyocha speculum* (Daugbjerg and Henriksen 2001) and *Pseudochattonella farcimen* (Edvardsen et al. 2007). The chemotaxonomic approach should be based on the combined use of chlorophylls and carotenoids to define pigment types (Seoane et al. 2009) and validated by microscopy or molecular species identification.

Scales

The unmineralized scales of the Coccolithophyceae (Fig. 6d–q) are composed of microfibrils (see Leadbeater 1994 and references therein) usually arranged in two layers where the proximal face (facing the cell membrane) has a radial pattern of

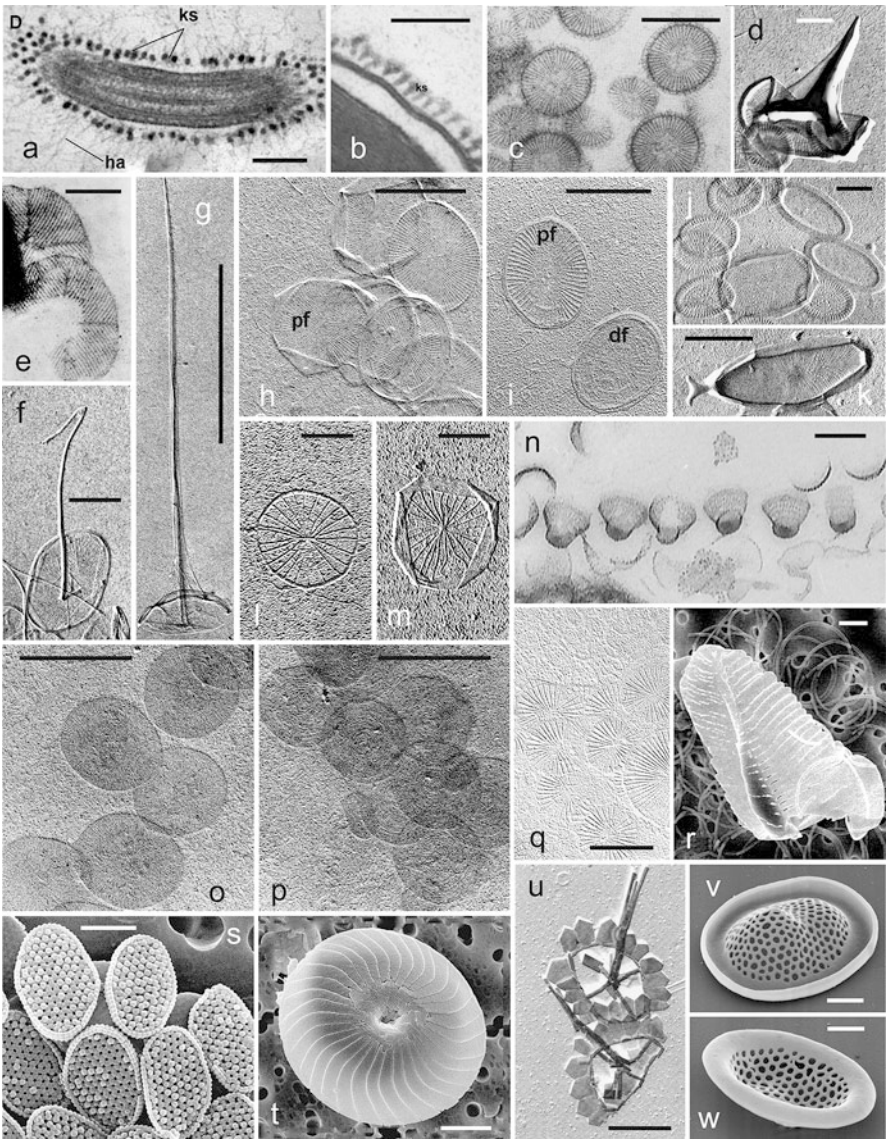

Fig. 6 Scales and coccoliths in Haptophyta. (**a**–**b**) Pavlovophyceae: (**a**) *Pavlova pinguis*, knob scales on flagella; (**b**) cell surface with knob scales in *Rebecca salina*. (**c**–**w**) Coccolithophyceae: (**c**–**d**) *Phaeocystis*, (**c**) small and large body scales; (**d**) *Prymnesium polylepis*, spine scale aberrant type. (**e**) *Emiliania*, body scale covering flagellate stage; (**f**–**g**) *Haptolina hirta*, (**g**, **h**) body scales; *H. fragaria*, (**i**) body scales; *Chrysochromulina scutellum*, body scales; (**j**, **k**) *Prymnesium polylepis*, (**j**) flat body scales, (**k**) scale with fishtail like extension. (**l**, **m**) *Dicrateria rotunda*: (**l**) flat body scale, (**m**) body scale with raised rim; (**n**) *Chrysochromulina campanulifera* cup scales. (**o**, **p**) *Calyptrosphaera sphaeroidea*: (**o**) body scale proximal side, (**p**) body scale distal side; (**q**) *Chrysochromulina simplex* body scales; (**c**) *Ceratolithus cristatus* nannolith-ceratolith. (**s**, **t**) *Calcidiscus leptoporus*: (**s**) holococcoliths, crystallolith (*Crystallolithus rigidus* stage), (**t**)

microfibrils, often arranged into quadrants (Fig. 6e, h), whereas the distal face is more variable with spiral and interwoven patterns that are common and modifications often form spines, cylinders, or cup-shaped structures (Fig. 6d, g, n). The distal face may have marginal thickenings as in the plate scale of *Chrysochromulina campanulifera* or more substantial rims that can be inflexed as in *Prymnesium polylepis* (Fig. 6j) or upright as in outer layer scales of *Chrysochromulina throndsenii* and *Dicrateria rotunda* (Fig. 6m). The scales may be arranged in one or more layers and with scales bearing spines (*Haptolina hirta*) (Fig. 6f, g), cups (*C. campanulifera*) (Fig. 6o), or cylinders (*C. microcylindra*) constituting the outer layer when present. Scale form and ornamentation are important taxonomic characters at the species level. In *Prymnesium neolepis*, both organic scales and silicified scales (Fig. 6v, w) are present (Yoshida et al. 2006).

Scales occur in a variety of structures from apparently simple plates, such as those found in *Isochrysis* (Green and Pienaar 1977), *Chrysotila*, and the motile stage of *Emiliania huxleyi* (Fig. 6e), to scales formed into spines that can be elaborated as in the alternate stage of *Prymnesium polylepis* (Fig. 6d) and sometimes of considerable length as in *Haptolina ericina* (9–15 μm long). Spines are often formed by hypertrophy of the distal face of the scale, either wholly or in part, such as the long spines of *H. ericina* (Manton and Leedale 1961) or *Chrysochromulina mantoniae* (Leadbeater 1972). Some spines are closed as in *Haptolina* (Fig. 6f, g) whereas others such as those covering *H. ericina* and *Chrysocampanula spinifera* are open-ended tubes (Manton and Leedale 1961; Pienaar and Norris 1979). Lateral development of the scales is shown in the authentic stage of *Prymnesium polylepis* in which there are a variety of oval plate scales, some of which have fishtail extensions at one end (Fig. 6j, k).

The scales are usually distributed over the entire cell surface, and where there is more than one type of scale, these may be in discrete layers. In *C. campanulifera*, the cuplike scales form a distal layer overlying the plate scales (Manton and Leadbeater 1974), and in *Phaeocystis globosa* (Fig. 6c) and *Dicrateria rotunda* (Fig. 6l, m), the scales with erect rims overlie the scales without such rims. However, the scales are not always evenly distributed and examples of this may be seen in the spine scales of *Chrysochromulina mantoniae*, which occur in clusters at the poles of the ovoid cells, or the very small scales found only on the haptonema in *Isochrysis* species (Green and Pienaar 1977) and in *Prymnesium nemamethecum* (Pienaar and Birkhead 1994).

Fig. 6 (continued) heterococcolith, placolith; (**u**) *Papposphaera sarion*, heterococcolith, pappolith; (**v**, **w**) *Hyalolithus neolepis*, silicified body scales. Abbreviations: pf, proximal face; df, distal face. Scale bars Figs. **a**–**c**, 0.2 μm; Fig. **d**, 1 μm; Fig. **e**, 0.2 μm; Fig. **e**, 1 μm; Fig. **f**, 0.5 μm; Fig. **g**, 10 μm; **h**–**i**, Fig. 0.5 μm; Fig. **j**–**k**, 1 μm; Fig. **l**–**n**, 0.2 μm; Figs. **o**–**p**, 1 μm; Fig. **q**, 0.5 μm; Figs. **r**–**s**, 1 μm; Fig. **t**, 2 μm; Figs. **u**–**w**, 1 μm. TEM images of knob scales in *Pavlova* and *Rebecca,* by courtesy of Madhi Bendif, *Emiliania* and *Calyptrosphaera* scales by Dag Klaveness and pappolith by Helge A. Thomsen. SEM images of holococcolith, placolith, and ceratolith, by LLuïsa Cros. Silicified scales, by courtesy of Masaki Yoshida

The cells in the filamentous "Apistonema" stage of Chrysotila have several layers of tightly packed scales (Leadbeater 1971b; Pienaar 1994).

Pavlovophycean scales are small spherical or clavate dense bodies (6 a, b) known as "knob scales," produced in the Golgi body and found particularly on the longer flagellum, although in some species they may also be found also on the cell body (Fig. 6b) as in *Pavlova gyrans* and occasionally on the haptonema, for example, in *Diacronema lutheri*. Such scales are usually smaller and sparser than those of the flagellum. The latter are often arranged in regular rows longitudinally, each row being slightly displaced relative to those adjacent. In a few species, however, the flagellar knob scales are disposed randomly on the flagellum. Most members of the Pavlovophyceae lack a distinct cell covering, but nonmotile cells may be invested with layers of mucilage (Green 1980; Leadbeater 1994; Bendif et al. 2011).

Scale Composition and Formation

Unmineralized Coccolithophyceae scales consist largely of complexed carbohydrates and some protein (Allen and Northcote 1975; Klaveness and Paasche 1979; Romanovicz 1981; Leadbeater 1994). The structural aspects of scale formation were first reviewed by Hibberd (1980) and Romanovicz (1981). The Golgi body is the site of scale formation. Scales are released to the cell surface by fusion of the plasmalemma with the cisternal membrane. Scales are often composed of four distinct components where the proximal radial microfibrils are to a large extent composed of sulfated polysaccharides, whereas the distal spiral microfibrils contain cellulose and protein. A glycoprotein covers the spiral microfibrils and there is also an amorphous layer composed of acidic polysaccharides. The radial fibrils are laid down before the spiral fibrils and then the amorphous matrix is added. In the Pavlovophyceae microfibrillar, scales are unknown (Leadbeater 1994).

Coccoliths

The coccolithophores possess external calcified ($CaCO_3$ as calcite) scales termed coccoliths (Figs. 6s–v and 7a–i). There are many living and fossil forms exhibiting an astounding variety in morphology and an extensive literature exists on the subject (see, e.g., Tappan (1980), Kleijne (1993), and Jordan et al. (2004)). The characteristics of these structures are briefly outlined here. Coccoliths have been classified into two main types, heterococcoliths and holococcoliths, based on coccolith ultrastructure and morphology, and there are a number of terms in use to describe both the overall form of the coccolith and its crystal structure (Jordan et al. 1995; Young et al. 1997). Nannoliths are a third group that differs from both holo- and heterococcoliths in structure and architecture (Jordan et al. 1995; Billard and Inouye 2004). Coccospheres are made up of multiple coccoliths and may be multilayered and monomorphic as in *Emiliania huxleyi* (Fig. 7a), have two layers of

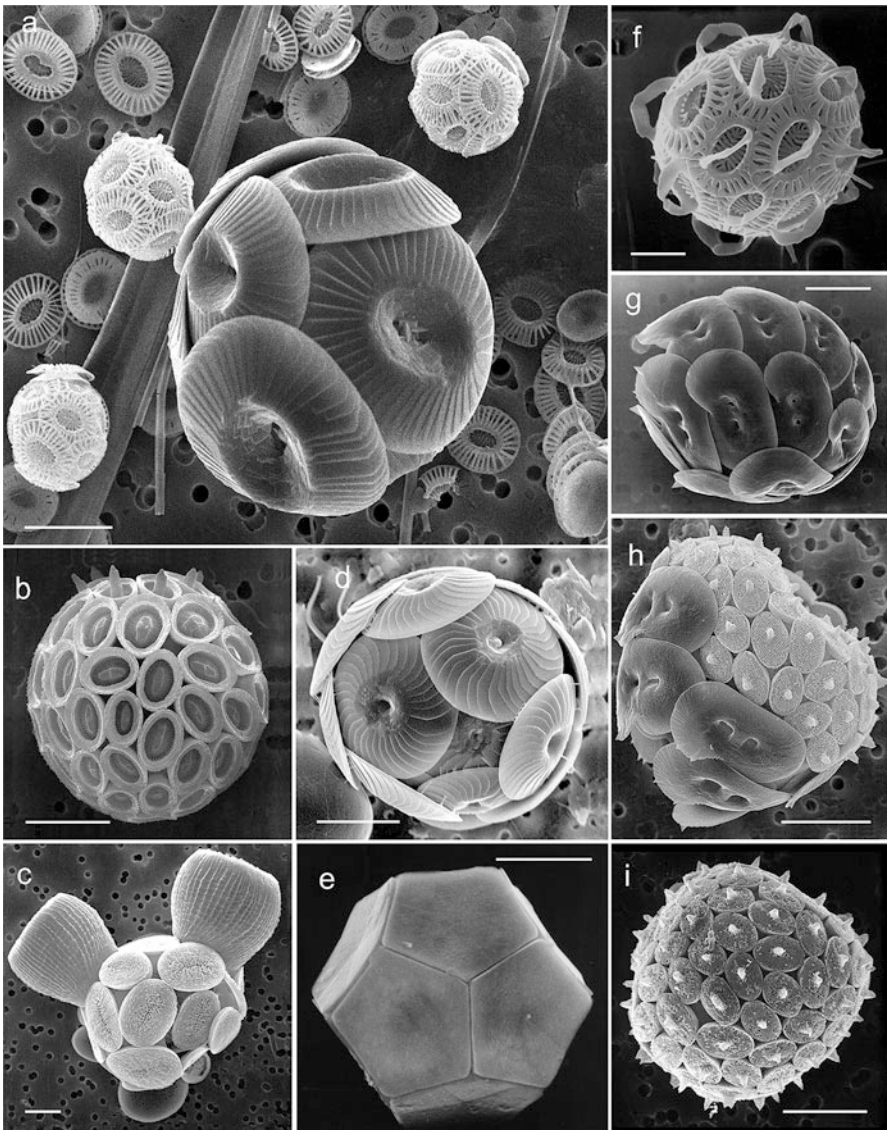

Fig. 7 SEM graphs of Coccolithophores: (**a**) *Coccolithus pelagicus* and *Emiliania huxleyi* (arrows). (**b**) *Coronosphaera mediterranea*: (**c**) *Scyphosphaera apsteinii*. (**d**) *Calcidiscus leptoporus*: (**e**) *Braarudosphaera bigelowi*. (**f**) *Gephyrocapsa ericsonii*: (**g**, **h**, **i**) *Helicosphaera carteri*, (**h**) combination cell, (i) *Syracolithus catilliferus* stage. Scale bars 5 μm. SEM images of *Coccolithus* and *Emiliania* by courtesy of L. Luïsa Cros and J-M. Fortuño. *Coronosphaera*, *Scyphosphaera*, *Gephyrocapsa*, and *Helicosphaera*, by courtesy of LLuïsa Cros. *Braarudosphaera*, by courtesy of Karen R. Gaarder

morphologically different coccoliths (dithecate) as in most *Syracosphaera* species, or have two types of coccoliths within one layer (dimorphic and monothecate) (Fig. 7b).

Heterococcoliths are elaborate interlocking structures composed of multiple strongly modified calcite crystals (Figs. 6u, v and 7a–g). The crystallographic orientation of subvertical "V-units" and sub-radial "R-units" (Young et al. 1992) is key to understanding heterococcolith ultrastructure and phylogenetic relationships (especially when dealing with their fossil record). There are several types of heterococcoliths, such as placoliths (Figs. 6u and 7a, d, f), caneoliths (Fig. 7b), cribriliths, lopadoliths (Fig. 7c), helicoliths (Fig. 7g, h), and pappoliths (Fig. 6v). Pentaliths (Fig. 7e) and ceratoliths (Fig. 6s) are considered nannoliths.

Holococcoliths are non-interlocking structures composed of rhombohedral crystallites of uniform size (ca. 0.1 μm in diameter) that cover the cell surface. Each holococcolith is made up of numerous identical calcite elements in the form of minute rhombohedral or hexagonal prisms (Figs. 6t and 7h, i). A variety of holococcolith types exist including crystalloliths (composed of rhombohedra arranged evenly on an organic baseplate in *Crystallolithus hyalinus* stage of *Coccolithus pelagicus*), calyptroliths (cap-shaped coccoliths of hexagonal crystals in *Calyptrosphaera*), and zygoliths (elliptical rings with crossbars and a distal knob) in *Helladosphaera cornifera*.

Coccolith Formation

Detailed observations of coccolith formation are possible in ultrathin sections of fixed cells in transmission electron microscopy, and complimentary information is provided by biochemical studies. The mechanisms of intracellular formation of heterococcoliths have been most extensively studied in *Emiliania huxleyi* (Wilbur and Watabe 1963; Klaveness 1976; Van Der Wal et al. 1983; Van Emburg et al. 1986; Figs. 9 and 10) and *Chrysotila* (Manton and Leedale 1969; Outka and Williams 1971; Van Der Wal et al. 1983; Marsh et al. 2002), with additional information available from a few taxa such as *Coccolithus braarudii* (Manton and Leedale 1969), *Umbilicosphaera sibogae* (Inouye and Pienaar 1984), *Syracosphaera pulchra* (Inouye and Pienaar 1988), and *Algirosphaera robusta* (Probert et al. 2007). By comparison, little information is available concerning holococcolith formation.

The heterococcoliths (Fig. 8b, c) of *E. huxleyi* are synthesized intracellularly in a system of vacuoles derived from the Golgi body, consisting of a coccolith vesicle (cv) enclosing the growing coccolith and a reticular body (rb) with anastomosing tubes (Fig. 8a). Inside the coccolith vesicle, coccolith production occurs through two discrete processes, controlled nucleation of crystals and their subsequent growth (Young et al. 1999). Crystal nucleation of a "proto-coccolith rim" occurs around the rim of the baseplate scale. These crystals subsequently grow in various directions to form complex crystal units. Crystal growth is regulated by a coccolith-associated

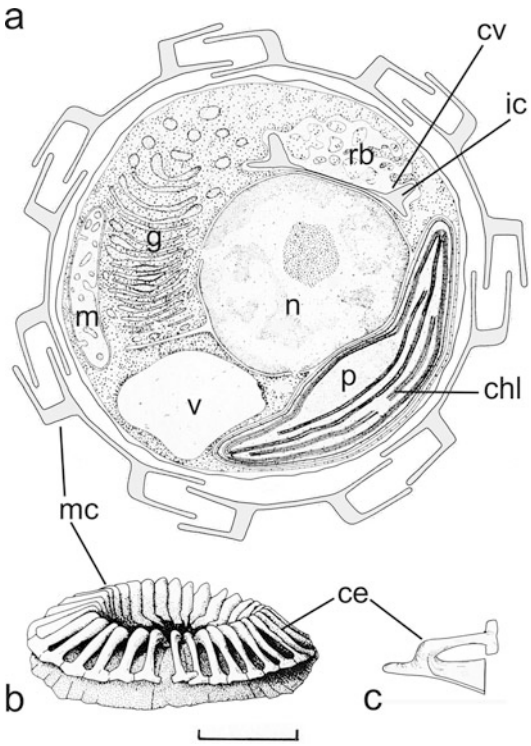

Fig. 8 Schematic drawing *Emiliania huxleyi*: (**a**) transverse section of whole cell showing the chloroplast (*chl*), coccolith vesicle (*cv*), Golgi body (*g*), immature coccolith (*ic*), mature coccolith (*mc*), mitochondrion (*m*), nucleus (*n*), pyrenoid (*p*), reticulate body (*rb*), vacuole (*v*), (**b**) single mature coccolith showing elements arranged in an oval ring, (**c**) calcite element (*ce*) of a coccolith. Scalebar 1 μm

polysaccharide (CAP) that has been located in the cv-rb system at four different sites: the outline of the membranes, fine threads inside the lumen, the baseplate, and a thin film surrounding the $CaCO_3$ crystals. The CAP is a highly complex soluble acidic polysaccharide containing at least 13 different monosaccharide residues, including uronic acids, mono- and dimethylated sugars, and sulfate esters (Fichtinger-Schepman et al. 1981). It has been shown that this CAP can inhibit crystal growth (Borman et al. 1982) and influence crystal morphology by site-specific attachment to crystallographic steps (Henriksen et al. 2004). It is therefore thought that this polysaccharide has a regulatory function in the inhibition, termination, and therefore modeling of crystal growth. The regulation of coccolith shape is also thought to be dependent on the morphology of the membrane of the coccolith vacuole. Using various inhibitors, Langer et al. (2010) experimentally demonstrated that cytoskeletal microtubules and actin filaments play a role in coccolith morphogenesis in *E. huxleyi*, presumably by determining the shape of the coccolith vesicle. After it is completed, the coccolith is transported to the cell membrane and exuded to the extracellular coccolith cover. In the motile scale-bearing form of *E. huxleyi*, the scales are formed in the Golgi apparatus, but do not calcify, although a cv-rb system is present (Klaveness 1972).

In contrast to *E. huxleyi*, *Chrysotila carterae* scales and coccoliths are produced in the trans (maturing) part of the Golgi apparatus. Granular elements termed

coccolithosomes are formed in cisternae at the cis side of the Golgi. They appear to be transported to vesicles containing calcifying scales where they disintegrate as the formation of a $CaCO_3$-associated matrix proceeds. Coccolithosomes contain high concentrations of calcium and polysaccharide (Van Der Wal et al. 1983). Three types of polysaccharide have been identified in *C. carterae* (PS1, PS2, PS3; (Marsh et al. 2002)). PS1 and PS2 bind calcium and form coccolithosomes, with PS2 probably playing an important role in the nucleation of the proto-coccolith ring, as shown by the fact that mutant *C. carterae* cells deficient in this polysaccharide show very little calcification (Marsh and Dickinson 1997). During coccolith growth, PS3 is located between the crystals and the vesicle, and it is believed to be involved in shape regulation, because *C. carterae* cells not expressing PS3 produce a proto-coccolith ring that does not develop further (Marsh et al. 2002).

Comparison of observations of heterococcolith formation in other taxa reveals a similar overall pattern with calcification occurring in Golgi-derived vesicles and commencing with nucleation of a proto-coccolith ring of simple crystals around the rim of a precursor baseplate scale, followed by growth of these crystals in various directions to form complex crystal units. However, significant diversity is also evident, with, for example, coccolithosomes only being observed in *Chrysotila*, the reticular body being unique to *E. huxleyi* and *Gephyrocapsa*, and the peripheral endoplasmic reticulum playing a role in coccolithogenesis in *Algirosphaera robusta*.

Very few holococcolithophore cultures have been maintained and only three species have been studied in TEM sections; *Coccolithus braarudi* (as *Crystallolithus braarudi)* was studied by Manton and Leedale (1963, 1969) and by Rowson et al. (1986). *Calyptrosphaera sphaeroidea* was studied by Klaveness (1973) and Sym and Kawachi (2000) studied *Calyptrosphaera radiata*. As with heterococcoliths, holococcoliths are underlain by an organic baseplate scale and develop in Golgi vesicles. Individual rhombohedral calcite crystals have been observed within Golgi cisternae in *C. radiata* (Sym and Kawachi 2000), but despite numerous observations, fully formed holococcoliths have not been observed inside cells. It has therefore been inferred that the assemblage of crystals to form holococcoliths occurs outside the cell membrane, after exocytosis of the baseplate scale. This poses obvious problems for understanding how calcification is regulated. A potential explanation is provided by observations that a delicate hyaline "skin" envelopes the coccosphere of holococcolithophores, meaning that even if holococcoliths are assembled outside the cell membrane, this is likely to occur in a regulated environment. Alternatively, it is possible that holococcoliths are assembled just below the cell membrane but that it is a rapid process immediately preceding exocytosis and so has not been captured in TEM preparations.

Life Cycles

Organisms with heteromorphic phases in their life histories are common in the Haptophyta (Fig. 9a–c). An alternation of a haploid stage with a diploid stage has been documented in all orders and many families within the Coccolithophyceae. So

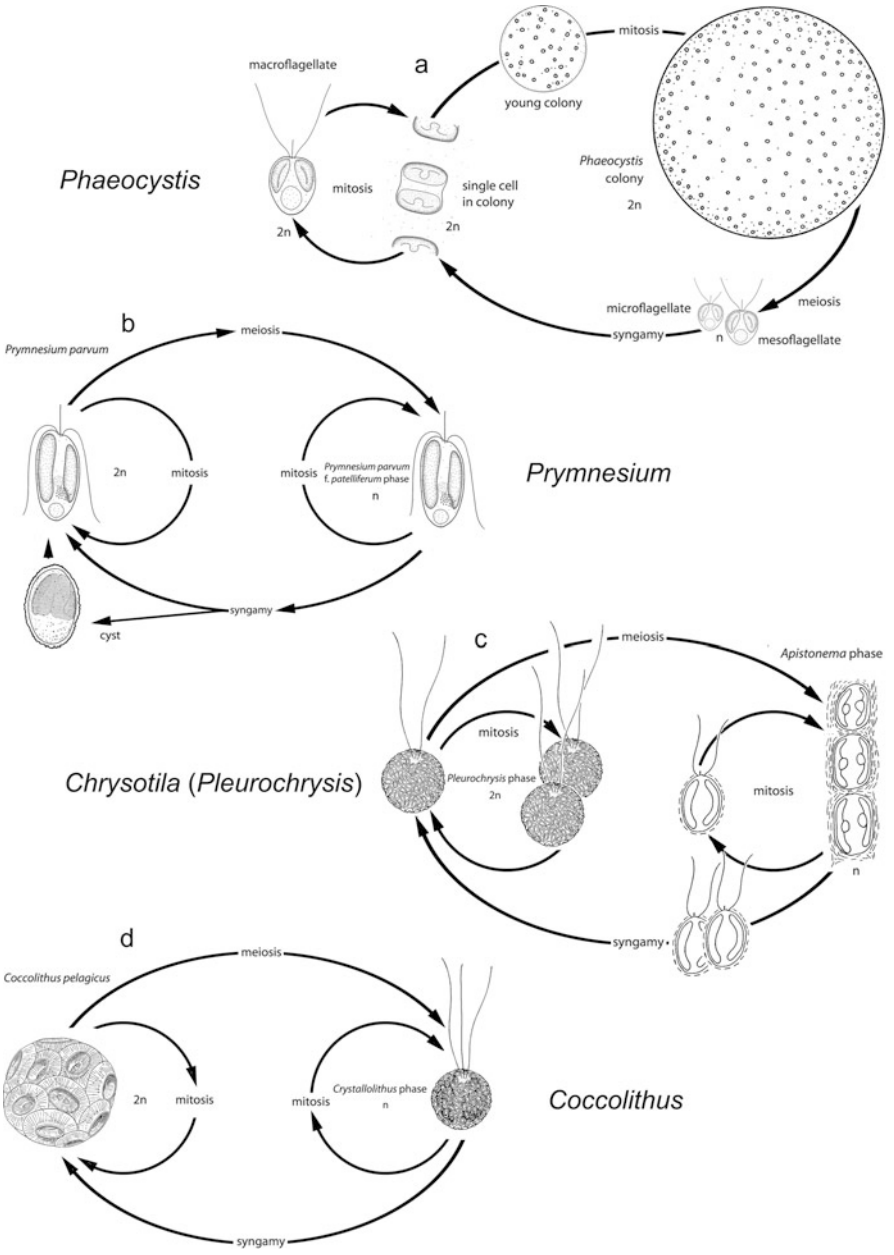

Fig. 9 Schematic life cycles in Coccolithophyceae: (**a**) *Phaeocystis globosa*, (**b**) *Prymnesium parvum*, (**c**) *Chrysotila carterae*, (**d**) *Coccolithus pelagicus*

far alternation of generations has not been demonstrated in members of the Pavlovophyceae, although transitions between motile and nonmotile forms occur in many species (Billard 1994; Bendif et al. 2011).

Alternation between a nonmotile planktonic palmelloid phase (Figs. 1c, g and 9a) and motile swarmers occurs in *Phaeocystis pouchetii*, *P. antarctica*, *P. jahnii*, and *P. globosa.* In *P. globosa*, four different cell types have been distinguished; two of them are haploid: the microflagellate and the slightly larger mesoflagellate. The largest flagellate type, the macroflagellate, and the palmelloid phases are diploid. The function of these cells and their order of appearance in the life cycle are still unknown, but the short-lived macroflagellate is believed to develop into a colony. Micro- and mesoflagellates (meiosis) are produced within the colony and are eventually released and multiply vegetatively. The life cycle is completed by syngamy between a micro- and mesoflagellate that develops into a macroflagellate that forms a new colony (Peperzak et al. (2000) and references therein). A nonmotile zygote linking the haploid unicellular stages and the diploid colonial stages has been documented in *P. antarctica* (Gaebler-Schwarz et al. 2010). The zygote can divide vegetatively as a benthic palmelloid stage and not revert to the colonial stage at least in culture conditions. The characteristic α-chitin containing pentagonal stars (Chrétiennot-Dinet et al. 1997) are produced by the mesoflagellate in *P. globosa* (Peperzak et al. 2000). These star-shaped structures are also produced by *P. antarctica*, *P. pouchetii*, and *P. cordata* but have not been observed in *P. jahnii.* In *P. scrobiculata*, a nine-ray star has been reported. *Phaeocystis scrobiculata* and *P. cordata* are believed to occur as flagellates only. *Phaeocystis* flagellates may be covered by minute scales (Fig. 6d) and the ornamentation of the scales may vary slightly between species (Rousseau et al. 2007). A plausible life cycle for *Phaeocystis* is illustrated in Fig. 9a.

In *Prymnesium*, two distinct cell types with differing scale morphology and cell size may occur within their haplodiploid life cycle as is seen in *Prymnesium polylepis* (Edvardsen and Vaulot 1996; Edvardsen and Medlin 1998) (Fig. 6d, j, k) and *P. parvum* (Larsen and Medlin 1997; Larsen and Edvardsen 1998). Their life cycle may also contain nonmotile cells (Parke et al. 1955) and even a silicified cyst in the case of *P. parvum* (Pienaar 1980) (Fig. 9b).

Life cycles of coccolithophores provide an excellent documentation of the alternation between haploid and diploid generations. Each generation is characterized by a specific cell covering and is capable of vegetative multiplication and dispersal. Diploid generations bear heterococcoliths, whereas haploid generations, depending on the family/genera they represent, are either covered by holococcoliths (Coccolithaceae, Helicosphaeraceae, Figs. 7i and 9d) or, nannoliths (Ceratolithaceae, Figs. 6s and 7e), or are a non-calcifying benthic stage (Pleurochrysidaceae, Hymenomonadaceae, Fig. 9c), or a non-calcifying motile stage (Noëlhaerhabdaceae) (Billard and Inouye 2004).

Parke and Adams (1960) showed that the heterococcolithophore *Coccolithus pelagicus* ssp. *braarudii* (Geisen et al. 2002) phase alternated with a motile

holococcolithophore (*Crystallolithus braarudii*) phase. The haploid and diploid state of the holococcolith and heteroccoccolith stage, respectively, has been more recently demonstrated by flow cytometric DNA analysis (Houdan et al. 2004a). Reports of combination cells with the heterococcolithophore placed inside the gametangium (zygote stage) bearing holococcoliths are increasing in occurrence since their first reports in the early 1900s (Kamptner 1941; Thomsen et al. 1991; Kleijne 1993; Cros et al. 2000).

Alternation of a nonmotile stage ("Apistonema stage") with one or more motile forms (Fig. 9c) has been observed in *Chrysotila* (Leadbeater 1970; Gayral and Fresnel 1983) and *Ochrosphaera* Schussnig (Schwarz 1932; Lefort 1975). In *Chrysotila pseudoroscoffensis*, the diploid coccolith-bearing phase produces motile spores without coccoliths after meiosis. These give rise to a haploid benthic filamentous phase that eventually releases isogametes with flagella and a haptonema. Fusion takes place and a zygote is formed that releases diploid, coccolith-bearing motile cells within 24 h. In *Ochrosphaera neapolitana* meiosis, isogamete formation and syngamy were reported already by Schwarz (1932).

In *Ruttnera* species, the benthic, nonmotile, mucilage ensheathed cells form the dominant stage; the nonmotile cell may divide within the mucilage sheath to form a variable number of offspring cells (usually 8 or 16) that are released as swarmers. They settle quickly and secrete a new mucilage sheath after which they divide vegetatively (Green and Parke 1975).

A complex life cycle involving naked, scale-bearing, and coccolith-bearing stages was described in *Emiliania huxleyi* by Klaveness (1972). Flow cytometric analysis has shown that its life cycle includes a diploid and a haploid phase (Green et al. 1996) where the motile scale-covered flagellate is haploid and the coccolithophore may be diploid; however, haploid coccolithophores were reported by Medlin et al. (1996). This is a type of life cycle (Fig. 9d) that is considered typical of the Noëlhaerhabdaceae (Billard and Inouye 2004).

So far alternation of generations has not been demonstrated in members of the Pavlovophyceae, although transitions between motile and nonmotile forms occur in many species (Billard 1994; Bendif et al. 2011).

There are few reports of cysts in the Haptophyta. Cysts of *Prymnesium* were described by Carter (1937) and have been investigated by Pienaar (1980) who has shown that the walls of *Prymnesium parvum* cysts are composed of layers of scales with siliceous material on the distal surfaces. There is a simple sub-anterior pore. Cysts have also been reported in *Isochrysis galbana* (Parke 1949).

Differences in gene expression between the haploid flagellate and the diploid coccolith-bearing stage have been demonstrated (Von Dassow et al. 2009; Rokitta et al. 2011), but the ecological role of the different life cycle stages, their occurrence, and distribution are poorly understood. It has been shown that the colonial diploid stage of *Phaeocystis* may be resistant to viral attacks, whereas single cells are more susceptible to viral infection (Jacobsen et al. 2007). In *Emiliania huxleyi*, the haploid flagellate has been reported to escape viral infection (Frada et al. 2008).

Maintenance and Cultivation

Some haptophytes are easily cultivated, but many are more demanding to isolate and keep in culture. Of those in culture, most are euryhaline with wide nutritional tolerance. *Isochrysis* and *Pavlova* are easily cultivated and extensively used as feed in the aquaculture industry. *Prymnesium parvum* is extremely euryhaline and eurytherm and thrives in eutrophic waters (Edvardsen and Paasche 1998) and is also easily cultivated. About half of the described Prymnesiales species in all genera have at some point been cultured and most *Phaeocystis* species are presently kept in culture. Of the coccolithophore species, approximately half of those currently accepted are in culture, but most culture-based studies on coccolithophore physiology, genetics, and biochemistry use the cosmopolitan *Emiliania huxleyi*. Most cultured coccolithophores are from coastal waters of the families Pleurochrysidaceae, Hymenomonadaceae, Noëlaerhabdaceae, and Coccolithaceae. Few oceanic oligotrophic species have been cultured likely because they are sensitive to high nutrient concentrations (Probert and Houdan (2004) and references therein). The few oceanic picoplanktonic haptophyte cultures partly reflect our lack of information on nutritional requirements and physiology of haptophytes in oligotrophic oceanic waters but also the logistical problems of isolating rare and small species in remote places.

Many isolates were made of single cells by micropipette, either from the original water sample or from an enriched culture (10:1 or 20:1). Motile haptophytes, such as *Chrysochromulina*, tend to swim toward the light and can be concentrated at the surface prior to isolation. It is advisable to have a selection of media and dilutions available. The serial-dilution method can yield small and abundant haptophytes, such as *Dicrateria* and *Chrysochromulina* (Edvardsen et al. 2000). These and other isolation techniques are described in Andersen and Kawachi (2005).

Many marine media (f/2, IMR1/2, ErdSchriber, for a review, see Andersen (2005) are based on natural seawater with added nutrients, vitamins, and trace elements and have proved useful in the maintenance of many haptophytes. IMR1/2 and f/2 have the advantage that no soil extract is used in the enrichment, thus eliminating a variable component. Artificial media, such as ASP- and S-media (Provasoli et al. 1957) and their modifications made by Paasche (1964) have been useful in the culture of some coccolithophores. Paasche's medium is broadly similar to ASP2 but includes a number of minor elements (Br, Sr, Al, Rb, Li, I) and the only vitamins are B_{12}, thiamine, and biotin. A thorough review on culturing coccolithophores was published by Probert and Houdan (2004). Light intensity and quality, day length, temperature, mixing, mode of sterilization of media, and growth containers are other factors that must be taken into consideration for the cultivation of haptophytes, similar to other sensitive microalgae (Probert and Houdan 2004; Andersen 2005).

Axenic cultures of haptophytes have been obtained (Guillard 2005), although not all species readily lend themselves to bacteria-free culturing; presumably the bacteria are providing, or making available, micronutrients or organic compounds not accessible to the algae in the standard media used. Several haptophytes have been shown

to be mixotrophic and can feed on bacteria (Jones et al. 1994) or algae (Tillmann 1998). The method generally used to remove bacteria is treatment with a range of concentrations of antibiotics (penicillin, streptomycin, gentamicin, etc.) followed by subculturing into fresh, antibiotic-free medium.

Some haptophytes produce nonmotile cells surrounded by masses of mucilage, and these may be difficult to obtain bacteria-free, seemingly because bacteria are embedded in the mucilage and are protected from the antibiotics. Green and Course (1983) found that *Chrysotila lamellosa* could be obtained apparently bacteria-free by first inducing the formation of motile swarmers, which do not carry a mucilage investment, and exposing these to antibiotics before subculturing them into new medium.

Several haptophyte species are cultivated as feed for both experimental and commercial shellfish farming, particularly for oysters (Jeffrey et al. 1994; Moestrup 1994). *Isochrysis galbana* has proved to be particularly valuable and has been in use since its isolation into culture by Parke (Flagellate "I" in Bruce et al. (1940)) more than 70 years ago. *Tisochrysis lutea* (as *Isochrysis* affinis *galbana* or T-Iso, a culture isolated from the sea off Tahiti) is also widely used as feedstock in bivalve aquaculture (Jeffrey et al. 1994; Brown et al. 1997; Bougaran et al. 2003). The pavlovophytes *Diacronema lutheri* (as *Monochrysis lutheri*, then *Pavlova lutheri*) and *Pavlova gyrans* are also being extensively used as feed in the aquaculture of bivalves, crustaceans, and fish (Green 1975; Meireles et al. 2003; Ponis et al. 2006). Haptophytes are rich in fatty acids, a large proportion of which may be of the valuable, long-chain polyunsaturated omega-3 fatty acids EPA (eicosapentaenoic acid, C20:5n-3) and DHA (docosahexaenoic acid, C22:6n-3) (Meireles et al. 2003; Guschina and Harwood 2006). Algae are the only producers of EPA and DHA in marine food webs, and cultivation of microalgae, including haptophytes, as supplement in fish feed is rapidly increasing.

Evolutionary History

Fossil Record

Fossil coccoliths and other fossil remains of calcifying nanoplankton (calcareous nannofossils) first appear ca. 225 Ma. Older reports are disputed and represent forms that are not ancestral to the latest Triassic and Early Jurassic coccoliths (Bown 1998).

Pioneering studies of calcareous nannofossils date back to the nineteenth century (Ehrenberg 1836; Huxley 1858; Siesser 1994). Species-level taxonomy is based on nannofossil morphology (Perch-Nielsen 1985a; Perch-Nielsen 1985b; Bown 1998; Jordan et al. 2004). Not all extant coccolithophores are well represented in the fossil record. Selective dissolution of rare and fragile taxa arguably impedes the reconstruction of "true" species richness through time (Young et al. 2005). The fossil record is largely composed of dissolution-resistant heterococcoliths, recording the diploid life stage of coccolithophores and rarely the haploid holococcoliths (Dunkley Jones et al. (2008)). Bown et al. (2004) compiled an overview of calcareous nannofossil morphospecies richness and evolutionary rates over the last 225 Ma.

Mesozoic Era

The earliest known coccoliths, of Late Triassic (Norian and Rhaetian) age, are very small (~2 μm) and possess very simple "murolith" morphologies. The Triassic/Jurassic boundary is characterized by significant extinctions, and only one species (*Crucirhabdus primulus*) survived (Bown 1998). Following this event, calcareous nanoplankton abundance and diversity steadily increased during the Jurassic and Cretaceous periods, with relatively low background extinction and speciation. Maximum morphospecies diversity was reached during the Late Cretaceous (Campanian-Maastrichtian), with large coccoliths and nannofossils with sophisticated architecture (Perch-Nielsen 1985a; Bown et al. 2004). Provincialism in calcareous nannofossil assemblages, between low (tethyan realm) and high (boreal and austral realms) paleolatitudes, is well documented during the Cretaceous (Roth and Bowdler 1981).

The Cretaceous-Paleogene boundary (K-T boundary; 65.5 Ma) is marked by a catastrophic event in which 93% of all species or 83% of all genera went extinct leaving only 10 survivors (Perch-Nielsen et al. 1982; Bown et al. 2004; Bown 2005). Survivor species included both common, opportunistic taxa as well as those typically rare in Late Cretaceous assemblages.

Cenozoic Era

After the K-T event, calcareous nanoplankton was characterized by the dominance of few survivor species and the appearance of small-sized (~2 μm) Cenozoic newcomers. Paleocene species diversity rapidly increased, with new coccolith morphologies and novel nannolith groups (*Sphenolithus*, *Fasciculithus*, *Heliolithus*, *Discoaster*) that differed significantly from the Mesozoic architectures (Perch-Nielsen 1985a; Bown et al. 2004). The Paleocene-Eocene Thermal Maximum (PETM; ca. 55 Ma) represents a relatively short-lived but rapid climatic perturbation of global warming, elevated atmospheric CO_2, and lowered ocean pH that drove significant evolutionary turnover affecting both fragile and robust taxa across broad ecological preferences (Gibbs et al. 2006).

The transition from the Eocene "greenhouse" into the Oligocene "icehouse" (ca. 34 Ma) was characterized by a rapid decline in diversity and global prominence (Aubry 1992; Hannisdal et al. 2012) and a macroevolutionary decrease in coccolith size (Aubry 1992; Henderiks and Pagani 2008). Nannofossil diversity recovered and increased again in the Middle and Early Late Miocene but decreased sharply during the remainder of the Miocene into the Pliocene and Pleistocene, marked by the loss of discoasters, sphenoliths, and large coccolith morphospecies (*Coccolithus miopelagicus*, *Reticulofenestra pseudoumbilicus*, *Calcidiscus macintyrei*). The latter part of the Neogene is characterized by the dominance of small placoliths and rapid evolution within the *Reticulofenestra*, which gave rise to the modern genera *Gephyrocapsa* and *Emiliania* (Thierstein et al. 1977; Takayama 1993; Bollmann et al. 1998; Okada 2000). Modern coccolithophores are extremely small in comparison to most of their Mesozoic and Cenozoic ancestors (Henderiks et al. 2004; Aubry 2007).

Nannofossil Evolution and Climate Change

In the short term, biocalcification releases CO_2 to the environment. On geological timescales, the burial of calcium carbonate into deep-sea sediments represents a long-term removal ("sink") of carbon from the surface oceans and the atmosphere. Calcareous nannofossils have been a major component of pelagic carbonates since the Early Jurassic, ca. 180 Ma (Bramlette 1958; Mattioli and Pittet 2002). The widespread Late Cretaceous chalk deposits (the white cliffs of Dover) are prime examples of calcifying nanoplankton as rock-forming organisms. Quantitative estimates of calcareous nannofossils and their corresponding calcite mass, as determined by morphometry (Young and Ziveri 2000) and polarized light microscopy (Beaufort 2005; Beaufort et al. 2014), provide important insights into the process of deep-sea carbonate burial and its climatic feedbacks, from the Jurassic to Recent times (Bornemann et al. 2003; Ziveri et al. 2007). Advances in the geochemical analysis of nannofossils (Stoll and Ziveri 2004; Rickaby et al. 2007), automated microscopy techniques (Beaufort 2005), and the quantification of fossil coccosphere and cell geometries (Henderiks 2008; Gibbs et al. 2013) have expanded investigations of ancient coccolithophore productivity, calcification, and their phenotypic evolution.

It remains a challenge to understand the mechanisms and rates of climatic adaptation by coccolithophores, on both ecological and evolutionary timescales. Scenarios for the future ecological success of coccolithophores are largely informed by short-term experiments on few extant species and field studies. Extrapolation of current physiological knowledge to studies of nanoplankton evolution should be treated with caution because of the very different temporal scales involved. Nevertheless, a range of hypotheses exist linking nanoplankton evolution and ecological prominence to climate variability and ocean chemistry in the past (Aubry 1992; Bown et al. 2004; Erba 2006; Aubry 2007; Henderiks and Rickaby 2007; Henderiks and Pagani 2008).

Not all modern coccolithophore lineages are represented in the fossil record, but the evolution of some morphospecies (*Coccolithus pelagicus*, *Helicosphaera carteri*, and *Braarudosphaera bigelowii*) can be traced back to the Paleocene or even the Cretaceous. Detailed coccolith morphometric analyses can address the evolutionary significance of phenotypic variation in coccolithophores (Knappertsbusch 2000; Reitan et al. 2012).

The first sedimentary evidence of alkenones in Cretaceous black shales, ca. 120 Ma (Farrimond et al. 1986; Brassell and Dumitrescu 2004) post-dates the molecular divergence between the Isochrysidales and other coccolithophore clades (Fig. 10), ca. 195 Ma (Medlin et al. 2008). This supports the notion that the Isochrysidales clade is distinct from all other clades and that modern *Gephyrocapsa* and *Emiliania* may have some unique strategies in adapting to current climatic change (Henderiks and Rickaby 2007).

Molecular Clock Reconstructions

A haptophyte molecular clock with selected extant genera is presented in Fig. 10 and calibrated with the fossil coccolith record. Clocks that average the rate of evolution across all lineages (Takezaki et al. 1995; Medlin and Zingone 2007) and that allowed

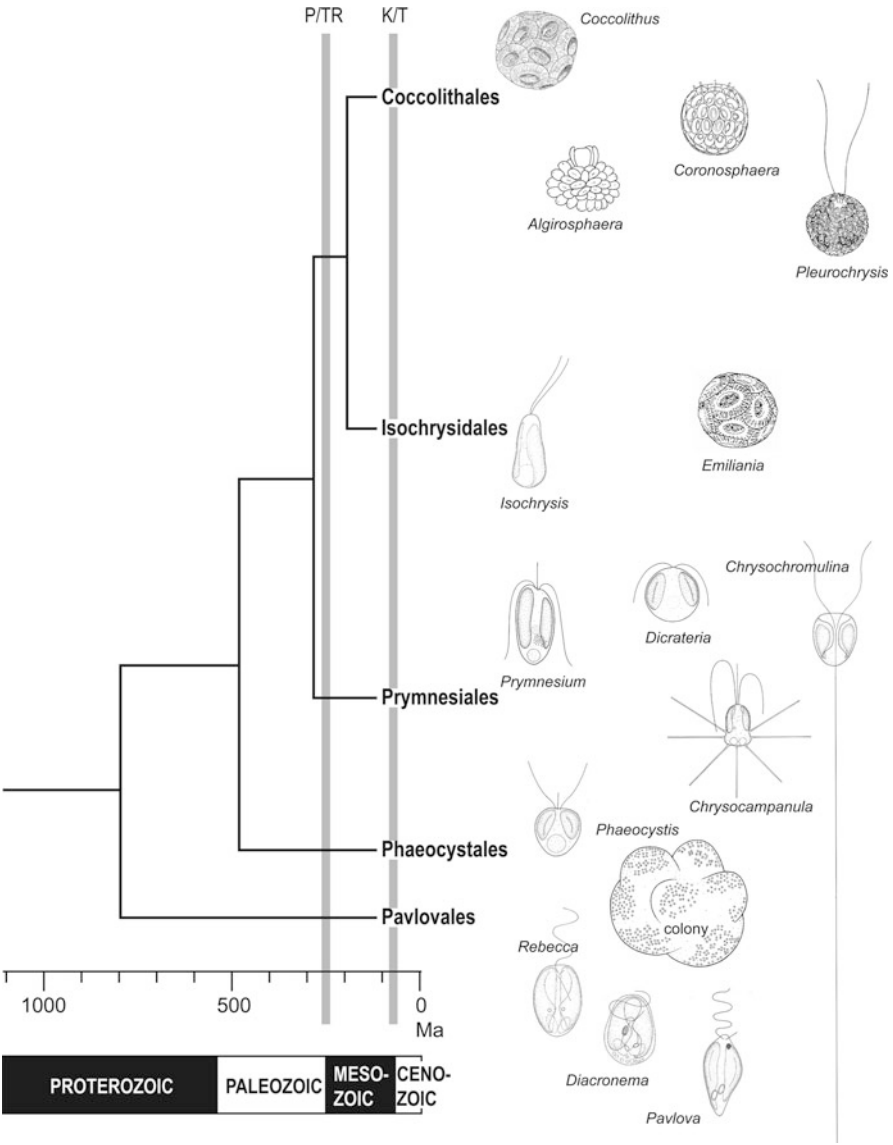

Fig. 10 Haptophyte molecular clock, with selected extant genera, illustrated to represent five orders. Coccolithophyceae, Coccolithales: *Coccolithus*; *Algirosphaera*; *Coronosphaera*; *Chrysotila*. Isochrysidales: *Isochrysis*; *Emiliania*. Prymnesiales: *Prymnesium*; *Dicrateria*; *Chrysochromulina*; *Chrysocampanula*. Phaeocystales: *Phaeocystis*, Pavlovophyceae. Pavlovales: *Rebecca*, *Diacronema*, *Pavlova*. Timescale million years ago (Ma), geologic eras indicated

the evolution to vary across the lineages (Sanderson 2006; Medlin et al. 2007) have been made and calibrated using characters and divergence points, such as the character-based constraint of 195 Ma for the emergence of all coccolithophores

and the divergence-based constraints of 64 Ma for the divergence of *Coccolithus* from *Cruciplacolithus* and 50 Ma for the divergence of Helicosphaeraceae from Pontosphaeraceae. The molecular clock extrapolates to dates of origin for some of the undated nodes. Another molecular clock has been made using the SSU and LSU rRNA genes (de Vargas et al. 2007). Divergence dates in that study are slightly older than those found by Medlin and coworkers who used a relaxed molecular clock.

The Haptophyta as a group diverged from other eukaryotes deep in the Proterozoic, >1200 Ma, in the crown group radiation (Medlin et al. 1997). Their true sister group has never been confirmed, and various trees place them in different positions in the crown group radiation. The long time period between the origin of haptophytes and the initial divergence (~800 Ma) of the two classes, Pavlovophyceae and Coccolithophyceae (Fig. 10), indicates that many of the early evolutionary branches in this group are extinct or that they have not yet been sampled (Edvardsen et al. 2000). A new group of picoplankton (Cuvelier et al. 2010) breaks up this long branch substantiating the hypothesis that the latter reason was the cause of this long branch. The order Phaeocystales diverged from all other Coccolithophyceae at ~480 Ma and then the Prymnesiales diverged from the Coccolithales plus Isochrysidales at ~280 Ma, making this a Late Paleozoic-Early Mesozoic event that may be associated with Permian-Triassic boundary (250 Ma). Modern diversifications in these lineages occurred some time after the lineage origin so many taxa were presumably lost during this time.

Within the order Phaeocystales, the divergence of the warm water *Phaeocystis* species from the cold water species occurs at 30 Ma when the Drake Passage opened to isolate the Antarctic Continental waters and dispersal to the Arctic occurred across the equator during a cooling trend at 15 Ma, and then the two polar populations were separated by a warming trend that then isolated the two polar species (Medlin and Zingone 2007).

Molecular diversification occurred earlier within the Prymnesiales than within the Coccolithales plus Isochrysidales where most of these latter divergences occurred fairly late in the haptophyte timetree (Fig. 10). The diversification within the Coccolithales plus Isochrysidales occurred predominantly after the Mesozoic-Cenozoic boundary (66 Ma), as predicted by the fossil record. Mesozoic coccolithophores have been intensively studied, and at the Mesozoic-Cenozoic boundary, an abrupt extinction is documented in the fossil record with ~90% of end-Cretaceous species disappearing (MacLeod et al. 1997; Bown 2005). After that, there is a major radiation in the Early Cenozoic with new clades rapidly diversifying and forming the origins of the modern coccolithophore biota (Bown et al. 2004).

One significant insight learned from the haptophyte molecular tree is that the Mesozoic-Cenozoic boundary extinction does not seem to have affected the Prymnesiales, Phaeocystales, or Pavlovales to the same degree as the Coccolithales, which is assessed by comparing the depth of clade diversification. In non-calcifying groups, there are 25 clades/lineages that cross the Mesozoic-Cenozoic boundary (Medlin et al. 2007) as compared to 11 coccolithophore clades. This type of branching pattern is suggestive of a major extinction (Medlin et al. 2008). This interpretation of tree branching pattern has been confirmed in other groups

(Vergroeben et al. 2014). One possible explanation for this difference in their survival may be the mode of nutrition in the haptophyte lineages. Those that are mixotrophic (Jones et al. 1994) or who could produce resting stages did not go extinct, whereas those that were obligate phototrophs without resting stages did. There appears to be a selective extinction of the order Coccolithales at the Mesozoic-Cenozoic boundary where calcified organisms were affected by ocean chemistry, and the uncalcified lineages likely switched to mixotrophy to take advantage of the poor light conditions at this extinction event. Modern coccolithophores represent terminal points of a number of evolutionary lines, some extending back to the Triassic and before. These lines have developed to some extent in parallel with each other and with those represented by the Prymnesiales, Phaeocystales, and the Pavlovophyceae.

Taxonomy

A haptophyte taxonomy (Table 1) has been constructed from Silva et al. (2007), de Vargas et al. (2007), and Edvardsen et al. (2011). Two classes are included in the phylum Haptophyta, Pavlovophyceae, and Coccolithophyceae (Prymnesiophyceae) and are separated by features of cell shape, flagellar insertion, and type and location

Table 1 The major taxonomic groups within the Haptophyta, their main characteristics, and examples of the genera of living algae included in them[a]

Phylum Haptophyta	Cells with haptonema
Class: Pavlovophyceae	Cells with knob scales
Order: Pavlovales	Motile cells with two unequal flagella, the longer with an investment of small knob scales and fine hairs and the shorter sometimes vestigial. Haptonema present but reduced (e.g., *Diacronema*, *Pavlova*)
Class: Coccolithophyceae (Prymnesiophyceae)	Cells with organic scales, with or without coccoliths
Order: Isochrysidales	Motile cells with two equal or subequal flagella, haptonema reduced or absent (e.g., *Ruttnera*, *Emiliania*, *Isochrysis*)
Order: Coccolithales	Cells coccolith bearing many genera with a polymorphic life cycle. Haptonemata recorded in several genera (e.g., *Acanthoica*, *Braarudosphaera*, *Calyptrosphaera*, *Chrysotila*, *Coccolithus*, *Corisphaera*, *Crenalithus*, *Cyclolithella*, *Discosphaera*, *Helicosphaera*, *Laminolithus*, *Rhabdosphaera*, *Syracosphaera*, *Umbellosphaera*, *Umbilicosphaera*)
Order: Prymnesiales	Motile cells with two (rarely four) equal or subequal flagella and usually an obvious haptonema which may be very long and coiling (e.g., *Chrysochromulina*, *Haptolina*, *Prymnesium*)
Order: Phaeocystales	Motile cells with two flagella and short haptonema. Flagellate alternating with colonial stage (*Phaeocystis*)

[a]Based on Parke and Adams (1960), Parke and Dixon (1976), Edvardsen et al. (2000), de Vargas et al. (2007), Silva et al. (2007), and Edvardsen et al. (2011) (note that these are not formal taxonomic descriptions)

of scales (Edvardsen et al. 2000). Pavlovophyceae has one order, the Pavlovales, and the class Coccolithophyceae (Prymnesiophyceae) may be divided into four orders: the Phaeocystales, Prymnesiales, Isochrysidales, and Coccolithales. Of these, the Phaeocystales with the genus *Phaeocystis* is the most basal and clearly defined in SSU rDNA phylogenetic trees. The Prymnesiales consists of two well-supported molecular clades, which have been separated at the family level (Prymnesiaceae and Chrysochromulinaceae) based on the shape of cells (saddle shaped or rounded, respectively) and the length and nature of the haptonema (Edvardsen et al. 2011). The coccolithophores have traditionally been classified on the basis of coccolith morphology, and the Coccolithales is the order within the Haptophyta that includes the highest number of described genera and species and is almost certainly a heterogeneous assemblage. de Vargas et al. (2007) erected the subclass Calcihaptophycidae and recognized the orders Isochrysidales, Syracosphaerales, Zygodiscales, and Coccolithales (see also Jordan et al. 2004). Molecular data show Isochrysidales to be the sister of Coccolithales. The Isochrysidales includes two families, the Noëlhaerhabdaceae with the coccolith-bearing genera *Emiliania*, *Gephyrocapsa*, and *Reticulofenestra* and the Isochrysidaeae with the non coccolith-bearing genera *Isochrysis*, *Chrysotila*, and *Tisochrysis* (Bendif et al. 2013).

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Centrohelida and Other Heliozoan-Like Protists

26

Rebecca J. Gast

Abstract

The Centrohelida has arisen through the dissolution of the Heliozoa and the gradual removal of morphologically similar, but ultrastructurally and genetically distinct taxa from the group. The taxonomy of these other heliozoan-like protists is still largely in flux, as are the groups within the Centrohelida. Centrohelida and heliozoan-like protists are heterotrophic, free-living species that are found in most aquatic benthic environments where they feed on bacteria and other protists, including algae. Morphologically the cells are conspicuous, generally round in shape with eye-catching raylike axopodia. They can be found in habitats that represent a wide range of temperatures and salinities, including extreme environments. Most are free floating, but some attach to substrates by a stalk. Interest in the heliozoan-like protists ("sun animalcules") is largely in regard to cell biology. Their size (some can be 500 μm in diameter) and axopodial structure have made them useful subjects for biochemical and ultrastructural studies of microtubules.

Keywords

Axoplast • Axopodia • Centrohelids • Centroplast • Heliozoa

Contents

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Summary Classification

●**Centrohelida**
●●**Acanthocystidae** (*Acanthocystis*, *Choanocystis*, *Pseudoraphidiocystis*, *Echinocystis*, *Pseudoraphidiphrys*, *Pterocystis*)
●●**Heterophryidae** (*Sphaerastrum*, *Heterophrys*, *Oxnerella*, *Chlamydaster*)
●●**Raphidiophryidae** (*Parasphaerastrum*, *Polyplacocystis*, *Raphidiophrys*, *Raphidiocystis*)

●**Retaria**
●●**Acantharia**
●●●**Taxopodida** (*Sticholonche*)

●**Stramenopiles**
●●**Actinophryida** (*Actinophrys*, *Actinosphaerium*)

●**Rhizaria**
●●**Cercozoa**
●●●**Granofilosea**
●●●●**Clathrulinidae** (*Clathrulina*, *Cienkowskya*, *Hedriocystis*, *Penardiophrys*)

●**Rhizaria incertae sedis**
●●**Gymnosphaerida** (*Hedraiophrys*, *Actinocoryne*, *Gymnosphaera*)

[Eukaryota incertae sedis: Heliomonadida/Dimorphida (*Heliomorpha*, *Tetradimorpha*, *Acinetactis*)]
[Other incertae sedis heliozoan-type genera: *Wagnerella*, *Actinolophus*, *Lithocolla*, *Actinosphaeridium*]

Introduction

General Characteristics and Occurrence

The phagotrophic spherical amoebae with microtubule-supported axopodia once called "sun animalcules" used to be grouped together into a formal class called Heliozoa. The cells range from 10 to 500 μm in size, either naked or coated with organic or siliceous scales, and radiating long axopodia (Fig. 1). Centrohelida and other heliozoan-like protists have been isolated from fresh and marine water; from polar, temperate, and subtropical regions; as well as from some extreme environments. They are generally found just above the sediment-water interface, but can be isolated from the pelagic environment as well.

Literature and History of Knowledge

The body of literature on heliozoan-like protists includes works from the end of the nineteenth century and the beginning of the twentieth century devoted to light microscopy and systematics and more recently papers focusing on ultrastructure (electron microscopy), life cycles and cell physiology (including motility and feeding processes), molecular systematics, and biogeochemistry. Articles that comprise the basis of this work include Penard's monograph (Penard 1904); the descriptions of Valkanov (1940), Rainer (1968), and Tregouboff (1953); studies by Febvre-Chevalier (Febvre-Chevalier 1982; Febvre-Chevalier and Febvre 1984); and work by Mikrjukov, Patterson, and Cavalier-Smith (Mikrjukov 1998, 2000a, b; Mikrjukov and Patterson 2001; Cavalier-Smith and von der Heyden 2007).

Haeckel first described spherical protists with raylike pseudopodia as heliozoan in 1866 Haeckel 1866), and the name was eventually applied to many organisms with similar morphologies. Penard (1904) proposed the first classification of the group based largely upon morphological observations of their skeleton, resulting in the separation of the Heliozoa from the radiolarians. The Heliozoa included the groups Centrohelida, Actinophryida, Clathrulinidae, Dimorphida (or Heliomonadida), and Gymnosphaerida. *Sticholonche*, belonging to the Taxopodida, was also placed within the Heliozoa. Light and electron microscopy studies were next used to propose more comprehensive systematics (Hartmann 1913; Kühn 1926; Valkanov 1940; Rainer 1968; Febvre-Chevalier and Febvre 1984; Smith and Patterson 1986). Most recently, molecular phylogenetic methods have been used to help understand the evolutionary relationships between groups and species (Nikolaev et al. 2004; Cavalier-Smith and von der Heyden 2007; Bass et al. 2009; Yabuki et al. 2012). It is now generally recognized that the class Heliozoa was established based upon convergent morphological characteristics, and it has been dissolved in recent taxonomic revisions (Mikrjukov 1998, Mikrjukov and Patterson 2001; Adl et al. 2005, 2012). Currently many of the heliozoan-like protists are placed within the Centrohelida (see section "Centrohelida and Heliozoan-Like Taxonomy" below).

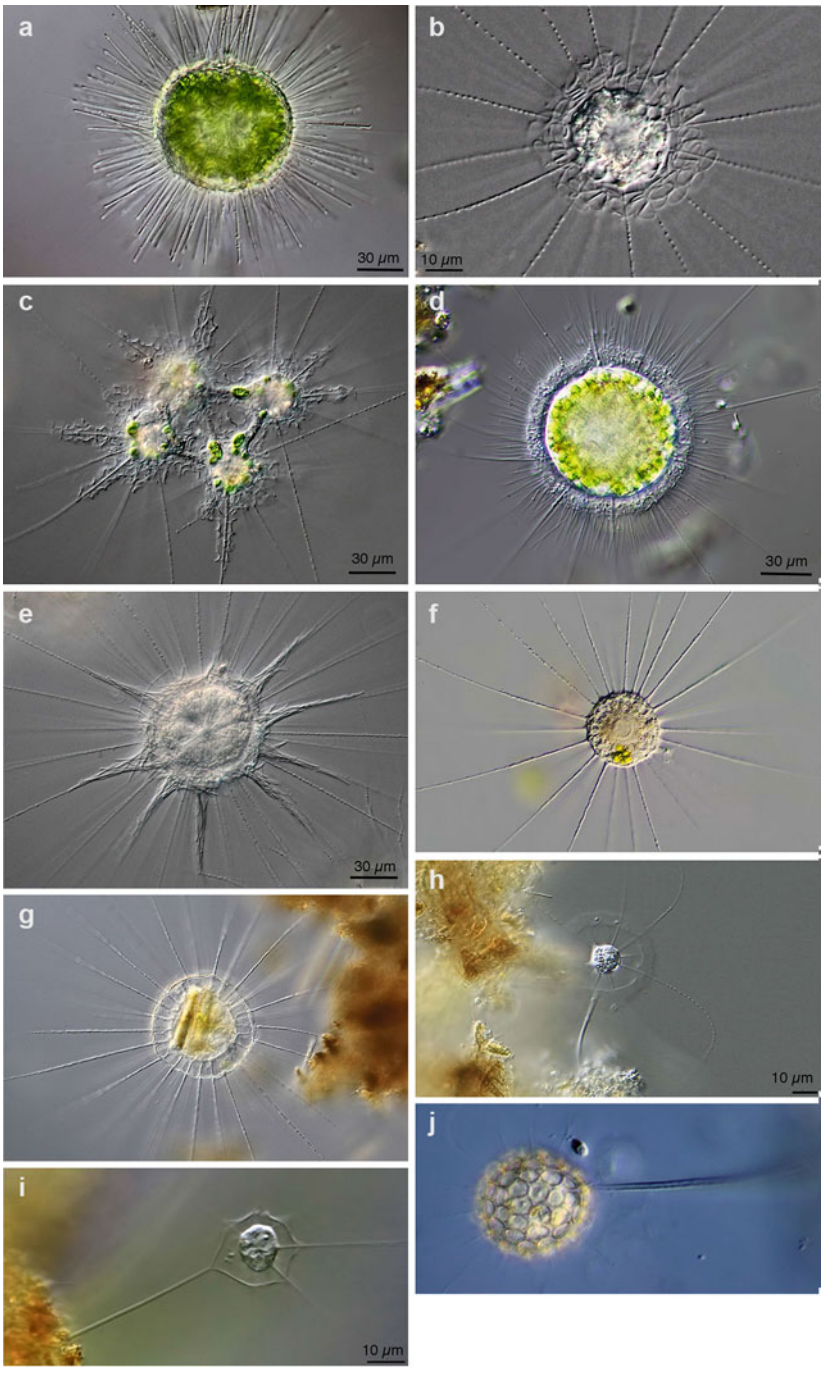

Fig. 1 Light microscope images of centrohelid and heliozoan-like protists. (**a**) *Acanthocystis turfacea*, (**b**) *Raphidophrys intermedia*, (**c**) *Raphidiophrys elegans*, (**d**) *Heterophrys myriopoda*,

Practical Importance

The Centrohelida and heliozoan-like protists have been useful for investigating aspects of cell biology. Studies of fibrillar proteins and microtubules involved in cell shape and movement (Cachon et al. 1977; Cachon and Cachon 1984; Febvre-Chevalier and Febvre 1980), the role of the cell membrane in detection and response to stimuli and control of contraction (Febvre-Chevalier et al. 1983, 1986), and the influence of the environment on the cell (Febvre-Chevalier 1981) were conducted using *Sticholonche* and *Actinocoryne*. Morphogenesis (Tilney and Byers 1969; Roth and Shigenaka 1970; Edds 1975), feeding (Suzaki et al. 1980; Patterson and Hausmann 1981; Hausmann and Patterson 1982; Linnenbach et al. 1983), and the biochemistry of tubulin and associated proteins within the axoneme (Little et al. 1983) were all studied using isolates of *Actinosphaerium*. $Ca2^{+}$-dependent axopodial contraction (Arikawa et al. 2006; Kakuta and Suzaki 2008) and feeding behavior (Pierce and Coats 1999; Sakaguchi et al. 1998) have been studied using *Actinophrys sol*.

Habitats and Ecology

Centrohelida and other heliozoan-like protists are widely distributed in aquatic environments, and while they have been isolated primarily from freshwater, they have also been observed in brackish or marine water in the euphotic zone. Primarily sub-benthic or benthic, they can be sampled with plankton net tows and can be abundant when conditions are favorable. The only exclusively pelagic marine form is *Sticholonche zanclea*. Freshwater centrohelid-like organisms have been collected from diverse sources of relatively still water, including lakes, regions of rivers, stagnant water, artificial ponds, marshes, and temporary pools (Penard 1904; Rainer 1968). Marine and brackish species are found in the coastal zone, again in waters that are not energetic, like harbors, coves, and brackish channels. Most species are free living and float or roll in the water, but *Sticholonche* is unique and moves by using an axopodial rowing motion (Cachon and Cachon 1978). Some species may secrete a long proteinaceous peduncle or stalk that temporarily attaches them to surfaces in the environment (e.g., *Clathrulina*, *Wagnerella*, *Actinocoryne*).

Centrohelida and heliozoan-like cells seem to prefer oxygenated water with plenty of organic matter to support the growth of other protists that serve as prey organisms. They also appear to be able to tolerate a wide range of temperatures and salinities. For example, *Cienkowskya mereschkovskyi* was found by Villeneuve (1937) in salt-marsh channels near Sete, France, that experience highly variable salinities, and

Fig. 1 (continued) (**e**) *Polyplacocystis pallida*, (**f**) *Actinophrys sol* (cell body is approximately 43 μm in diameter), (**g**) *Actinosphaerium eichornii*, (**h**) *Actinosphaeridium* sp., (**i**) *Hedriocystis pellucida*, and (**j**) *Clathrulina elegans* (the shell is 35 μm and the stalk 112 μm) (All images are courtesy of Ferry Siemensma. Additional images of amoeboid protists may be viewed at Microworld (http://www.arcella.nl))

Febvre-Chevalier reported collecting it in Villefranche in water of salinities between $37^0/_{00}$ and $38^0/_{00}$ (Febvre-Chevalier 1990). *Cienkowskya* also illustrates the wide temperature range that some isolates can tolerate; it has been isolated from the White Sea (mean temperature of 2 °C), from Villefranche (temperatures between 13 °C and 27 °C), and from salt-marshes near Sete (temperatures up to 30 °C).

pH also likely plays a role in the distribution of these species with different habitats varying from pH 4.6–8.5, (Rainer 1968). *Actinophrys sol* and *A. eichorni* tolerate very wide ranges in pH (4.6–8.5), while species like *Raphidiophrys elegans* and *Acanthocystis echinata* are observed at more limited ranges (between 6.0 and 8.2 for the former and 4.5–5.4 for the latter). *Polyplacocystis symmetrica*, *Raphidophrys intermedia*, *R. ovalis*, *R. echinata*, *Clathrulina elegans*, and *Pompholyxophrys punicea* were isolated from acidic bogs (pH 4.3–5.1) in Russia (Leonov 2010). Recently, the acidic limits of pH tolerance have been lowered further with reports of *Actinophrys* species in Spain's Rio Tinto (pH approximately 2.0; Amaral Zettler et al. 2000) and of *Actinophrys sol* in lower Lusatia, Germany, at pH 2.3 and 2.6 (Packroff 2000).

Centrohelida and heliozoan-like protists feed by phagocytosis of bacteria, other protists (including algae), and larvae of invertebrates. They are generally considered passive predators that capture prey as it comes along. Despite the occurrence of free-floating forms, their ecological niche is considered to be the benthic environment where they inhabit the superficial layer of detritus and interstitial spaces. Swimming prey is thought to impact and stick to the mucous coat of the axopodia, stimulating contraction and movement of the prey toward the cell where a food vacuole is formed (Febvre-Chevalier and Febvre 1980; Patterson and Hausmann 1981; Suzaki et al. 1980).

Characterization and Recognition

General Appearance and Ultrastructure

Centrohelida and heliozoan-like protists are spherical, 10–500 μm in diameter, with long slender axopodia and short pseudopods, or branched filopods (Fig. 1). The genera *Heliomorpha*, *Tetradimorpha*, and *Acinetactis* have one or more flagella in addition to axopodia. In general, Centrohelida lack a central capsule and are either naked or covered with a mucoid cell coat. Some members of the heliozoan-like protists belonging to the Clathrulinidae have latticed organic capsules. The mucous coat, ranging in thickness from 0.05 to 5 μm depending upon the species and physiology, is secreted at the cell surface. Most cell surfaces also contain external skeletal spicules, scales, or small spheres. These can be composed of silica or organic material, and the morphology of the spheres, spicules (spatula, needles, tubes, cups, funnels, clubs), and scales (elliptic, lens shaped) is a key element of species identification and systematics.

Various kinds of extrusomes (organelles involved in prey capture) are scattered in the axopodial and cortical cytoplasm. Their contents are ejected after excitation by an outside stimulus by rupture of the cell membrane. The different types of extrusomes that have been described include dense and mottled granules (Actinophryidae), mucocysts, and kinetocysts (Centrohelida and Clathrulinidae) (Febvre-Chevalier 1985; Mikrjukov 1998; Davidson 1976).

Vegetative cells can be either mono- or multinucleated, and some genera alternate between spherical free-living and stalked sessile forms (members of the Gymnosphaerida and Clathrulinidae). Some stalks are inert, while others are cytoplasmic and may be capable of contraction (*Actinocoryne* and *Wagnerella*). The formation of resting cysts when growth conditions become unfavorable has been observed. Some heliozoan-like protists have also been reported to contain symbiotic algae (*Hedraiophrys* Febvre-Chevalier 1973a), or to retain functional chloroplasts from their algal food (*Acanthocystis*, *Raphidiocystis*, and *Chlamydaster*; Patterson and Dürrschmidt 1987).

A key feature of heliozoan-like protists is the axopodia, although this is now considered to be a trait acquired independently in the different lineages rather than an indication of shared evolutionary history. These are long, thin projections supported by arrays of microtubules called axonemes. Axopodia are able to contract rapidly, at a velocity of 50–300 lengths of the cell per second (Davidson 1975; Febvre-Chevalier and Febvre 1980). Filopods, long supple projections of the cell body, and pseudopods, temporary extensions of the cell surface, lack microtubular structure. All three structures are involved in feeding.

The axopodia are made up of bundles of microtubules (Little et al. 1983) connected to one another by cross-bridges resulting in distinct patterns (see Dustin 1978). There are five basic patterns that are generally recognized. There are the slightly irregular hexagons and equilateral triangles found in the Centrohelida (Fig. 2, panel 1b and 2b, e.g., *Heterophrys*, *Raphidiophrys*, *Acanthocystis*; Tilney 1971; Bardele 1975). Two interlocking coils in a spiral pattern are present in the Actinophryidae (Tilney and Porter 1965; Roth et al. 1970; Ockleford and Tucker 1973). Irregular adjacent hexagons that form a "parquet" pattern are present in the Gymnosphaerida (Febvre-Chevalier 1973a, 1975, 1982). A square microtubule arrangement is found in the Heliomonadida (Fig. 2, panel 3b; Brugerolle and Mignot 1983, 1984), while the Clathrulinidae exhibit an irregular pattern (Fig. 2, panel 4b; Bardele 1972).

Microtubules are generated by microtubule-organizing centers or microtubule-nucleating centers (MTOC or MNC: Pickett-Heaps 1969). These are located in the center of the cell or on the outer nuclear membrane (Actinophryidae and Clathrulinidae). The central MTOC, called a centroplast, may possess a central disc sandwiched between two dense caps about 0.1–1.5 μm in diameter (Centrohelida; Fig. 2, panel 1a, 2a). In other instances it may lack this inner differentiation and is then sometimes called an axoplast (Anderson 1988; Febvre-Chevalier 1973b; Gymnosphaerida and Heliomonadida; Fig. 2, panel 3a, 4a).

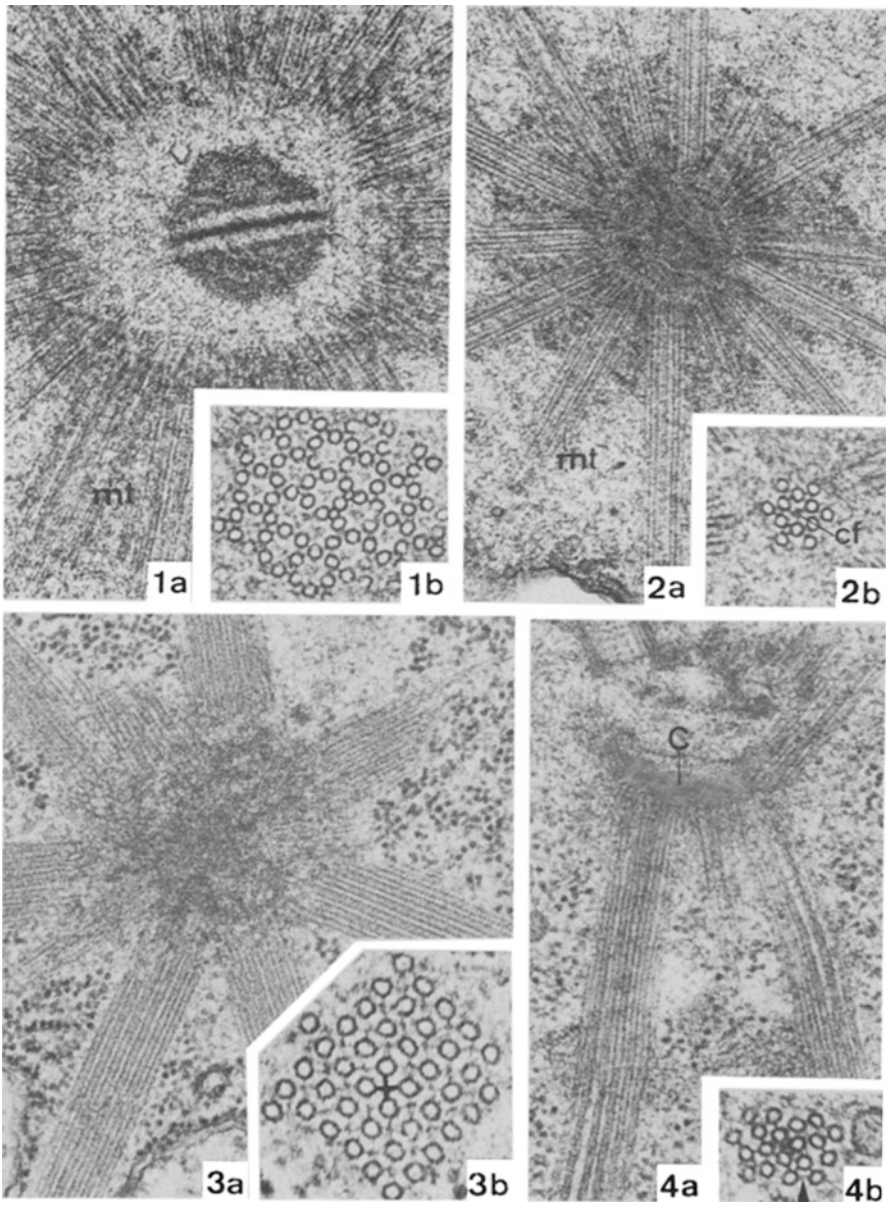

Fig. 2 Transmission electron micrographs of *Raphidiophrys elegans*, *Acanthocystis turfacea*, *Dimorpha mutans*, and *Tetradimorpha radiata*. Panel **1a** The centroplast of *Raphidiophrys elegans* has a tripartite central disc with an electron dense equatorial plate. Axopodial MTs arise from a shell around the centroplast. ×60,000. **1b** Axopodial MTs are triangles grouped in x-shapes, resulting in hexagonal or irregular hexagon patterns. ×90,000. Panel **2a** In the centroplast of *Acanthocystis turfacea*, the dense plate at the equator of the central disc is less obvious, and the axopodial MTs originate directly from the dense material around the disc. ×60,000. **2b** Axopodial MTs are

Life Cycle

Reproduction is usually asexual, with binary cell division most commonly observed. Division can give rise to equal- or unequal-sized offspring cells, with the smaller cell in the unequal division called a bud. Multiple fission, where multiplication of nuclei is followed by rapid synchronous division, can result in a large number of offspring cells produced at the same time. Division in stalked, sessile species takes place in the head, or in the base after withdrawal of both stalk and head (Zuelzer 1909; Febvre-Chevalier 1982). The offspring cells become free and fall onto the substratum where they undergo morphogenesis to give rise to a stalked cell.

Sexual reproduction occurs through autogamy in the cysts of *Actinophrys* and *Actinosphaerium* (Mignot 1979; Bělař 1923). First, the parent cell encysts and forms the gamontocyst, followed by progamic fission, resulting in two gamonts. Each gamont goes through meiotic division, after which one offspring nucleus degenerates. The remaining two cells differentiate into male and female gametes, and they fuse to form a zygote.

Centrohelida and Heliozoan-Like Taxonomy

The revised taxonomy of the Centrohelida and other heliozoan-like protists presented here is based upon Adl et al. (2005, 2012). The taxonomy of these protists is actively under revision, and other versions can be found in Cavalier-Smith and von der Heyden 2007; Mikrjukov et al. 2000; Mikrjukov 2000a, b; Mikrjukov and Patterson 2001; Yabuki et al. 2012; and on the web at *Microworld, world of amoeboid organisms* Siemensma, F. J. 2015 http://www.arcella.nl.

Eukaryota; Centrohelida (Kühn 1926)

Members of the Centrohelida have axonemes arising from a centroplast that has a tripartite disc flanked by two regions of electron-dense material. Axonemes have

Fig. 2 (continued) arranged in a single hexagon containing a central filament (cf) which is linked to the six neighboring MTs. ×90,000. Panel **3a** The centroplast of *Dimorpha mutans* is composed of microfibrillar material from which the axopodial axonemes arise. ×54,000. **3b** Axopodial MTs are arranged in a "quincunx" pattern that makes a squared packed array. ×150,000. Panel **4a** In *Tetradimorpha radiata* the lens-shaped centroplast (C) is composed of unstructured dense material, with the axopodial MTs arising from its periphery. ×93,000. **4b** The MTs are arranged irregularly with some having more than four links and others having triangular figures (*arrow*). ×102,000 (Used with kind permission from Springer Science+Business Media: Origins of Life, *The cell characters of two Helioflagellates related to the Centrohelidian lineage: Dimorpha and Tetradimorpha*, volume 13, 1984, 305–314, Guy Brugerolle and Jean-Pierre Mignot, Figs. 1, 2, 3, 4; original (first) copyright by D. Reidel Publishing Company)

hexagonal and triangular patterns of microtubules, and the mitochondrial cristae are lamellate (flat) in shape. The kinetocysts are complex ball-and-cone-shaped structures. Some members have mucous stalks, and cell body coverings include naked, mucous, organic spicules, and siliceous rods, platelike scales, and spicules (tubelike, trumpetlike, and spine-like).

Heterophryidae (Poche 1913). Members of this group are naked or with a mucous coat. Some have tangential or radial organic spicules (revised in Mikrjukov 1996a).

Genera *Sphaerastrum*, *Heterophrys*, *Oxnerella*, *Chlamydaster.*

Acanthocystidae (Claus 1874). The surface of these protists is composed of two to three types of siliceous scales. The basal layer is usually composed of oval scales, the outer layer is composed of funnel-like structures or radial spicules (may have branched tips and/or flat, centrally attached basal disc).

Genera *Acanthocystis*, *Choanocystis*, *Pseudoraphidiocystis*, *Echinocystis*, *Pseudoraphidiphrys*, *Pterocystis.*

Raphidiophyridae (Mikrjukov 1996b). This group of centrohelid protists has siliceous scales or spicules (trumpetlike, tubelike, or funnel-like) (revised in Mikrjukov 1996b).

Genera *Parasphaerastrum*, *Polyplacocystis*, *Raphidiophrys*, *Raphidiocystis.*

Rhizaria; Cercozoa; Granofilosea; Clathrulinidae (Claus 1874; Desmothoracida Hertwig and Lesser 1874)

The most distinctive characteristic of this group is the presence of a perforated or latticed capsule. Some have a non-cytoplasmic stalk, while others do not. There is a single, central nucleus, and the mitochondrial cristae are tubular. Axopodia tend to be long and are sometimes branched or forked, with the axonemes terminating on the nuclear envelope. The axonemes have unorganized microtubular arrays. Extrusomes are present, but resemble ones of cercomonads more than those of centrohelids. Reproduction occurs by binary fission, with one of the daughter cells forming a uni- or biflagellated cell that transforms into an amoeba after settling. The stalk and capsule are then secreted. Most isolates are freshwater organisms.

Genera *Clathrulina*, *Cienkowskya*, *Hedriocystis*, *Penardiophrys.*

Incertae sedis Clathrulinidae *Servetia* – A marine genus with a naked spherical head and hollow, non-cytoplasmic stalk with a broad base.

SAR; Stramenopiles; Actinophryidae (Claus 1874; Emend Hartmann 1926)

Members of the actinophryid group are round bodied with stiff axopodia that taper from the base out to the tip. Microtubule organization within the axopodia is a striking double hexagonal spiral array, and the MTOCs are present on electron-dense material at the surface of the nucleus or near a nucleus. Cells have either a single

central nucleus or multiple nuclei located centrally in the cell, and mitochondrial cristae are tubular. The extrusomes are of two types – large and osmiophilic and small and granular. The cell surface is naked and cysts with multiple walls can form. Binary fission is the primary mode of reproduction, but autogamy within the cyst occurs through the formation and fusion of amoeboid gametes. Actinophryids are the heliozoan-type most commonly recovered from freshwater, but are also found in marine and soil environments. The flagellated genus *Ciliophrys* (Cienkowsky 1876) was originally included among the actinophryids, but is now considered a member of the pedinellids.

Genera *Actinophrys*, *Actinosphaerium.*

Retaria; Acantharia; Taxopodida

The single member of this pelagic marine group is *Sticholonche zanclea* Hertwig, 1877. It is about 200 μm in size and has a bilateral symmetry rather than the radial symmetry seen in other members of the heliozoan-like protists. Its oar-shaped axopodia are arranged in 50–60 rows that terminate on the surface of the large central nucleus. Axopodia are used for buoyancy and movement. The microtubules are arranged in irregular hexagonal arrays. There has been debate regarding the taxonomic placement of *Sticholonche*, but molecular evidence indicates this organism is related to the Polycystinea and Acantharea (Nikolaev et al. 2004).

Incertae Sedis Rhizaria; Gymnosphaerida (Poche 1913; Emend Mikrjukov 2000b)

Most of the gymnosphaerid protists are found in marine environments. Cells can be uni- or multinucleate, and the nuclei can be present in the amoeboid base of the cytoplasmic stalk. The cell body with radiating axopodia is present at the top of the stalk, and the surface of the cell may be naked or covered by mucous or siliceous spicules. Mitochondria have tubular cristae. The life cycles appear to be complex and are not fully resolved. The original description of *Hedraiophrys hovassei* reported the presence of algal and bacterial symbionts (Febvre-Chevalier 1973a).

Genera *Hedraiophrys*, *Actinocoryne*, *Gymnosphaera.*

Incertae Sedis Eukaryota; Heliomonadida (Formerly Dimorphida); Heliomorphidae/Acinetactidae/Tetradimorphidae (Helioflagellates or Heliomonads; Siemensma 1991)

Axopodial microtubules arise from MTOCs near the flagellar bases. The cells are mononuclear and have tubular mitochondrial cristae, and kinetocysts are present.

Members of the genus *Heliomorpha (Dimorpha)* and *Acinetactis* have two flagella, while members of the genus *Tetradimorpha* have four. Molecular studies suggest placement of these organisms as relatives of the Cercozoa, and *Acinetactis* was added to this group by Bass et al. (2009).

Genera *Heliomorpha (Dimorpha)*, *Tetradimorpha*, *Acinetactis.*

"Other" Heliozoan-Type Genera

Wagnerella incertae sedis Rhizaria – This marine genus has a noncontractile cytoplasmic stalk with an enlarged base and a spherical head. The axoplast is located centrally within the head, and the head is covered by mucilaginous material and siliceous spicules. Amoeboid cells are produced during reproduction, from both the head and from the base.

Actinolophus incertae sedis Rhizaria – A marine genus with a noncontractile cytoplasmic stalk and a pyriform head that is covered by a gelatinous layer. The single nucleus is located eccentrically within the head, and the axoplast is pear shaped.

Actinosphaeridium incertae sedis Granofilosea – A space is present between the mucous layer and the cell body of this organism, and the stalk ends at the mucous coat rather than on the cell body. The species was previously called *Nuclearia caulescens*.

Lithocolla incertae sedis Eukaryota – Found in both marine and freshwater, this organism is covered in a dense coating of sand grains. Movement is accomplished by rodlike filopods, and the nucleus is located centrally. The sand coat has made ultrastructure studies difficult, and its taxonomic position remains largely uncertain.

Maintenance and Cultivation

Benthic cells are collected by direct sampling of the sediment surface at the water-sediment interface. *Sticholonche zanclea* and other pelagic forms can be collected using a plankton net (mesh size of 40 μm). The organisms can be cultivated from collected sediments using serial dilution into a culture medium (e.g., SES medium for freshwater or Plymouth Erdschreiber medium for marine, Catalogue of the UK National Culture Collection). They can be enriched from water samples by adding Cerophyll or a grain of rice or barley to the collected sample. Algae, such as *Chlorogonium elongatum* (freshwater) or *Dunaliella* (marine; Davidson 1975), can be added as a food source, but enrichments generally support the growth of bacteria and small flagellated protists that serve as prey. Although enrichment cultures are usually successful in initially recovering heliozoan-like cells, it can be difficult to maintain them for long periods and to isolate the organisms into clonal culture. Some species may be available from culture collections like the Culture Collection of Algae and Protozoa, but this is rare.

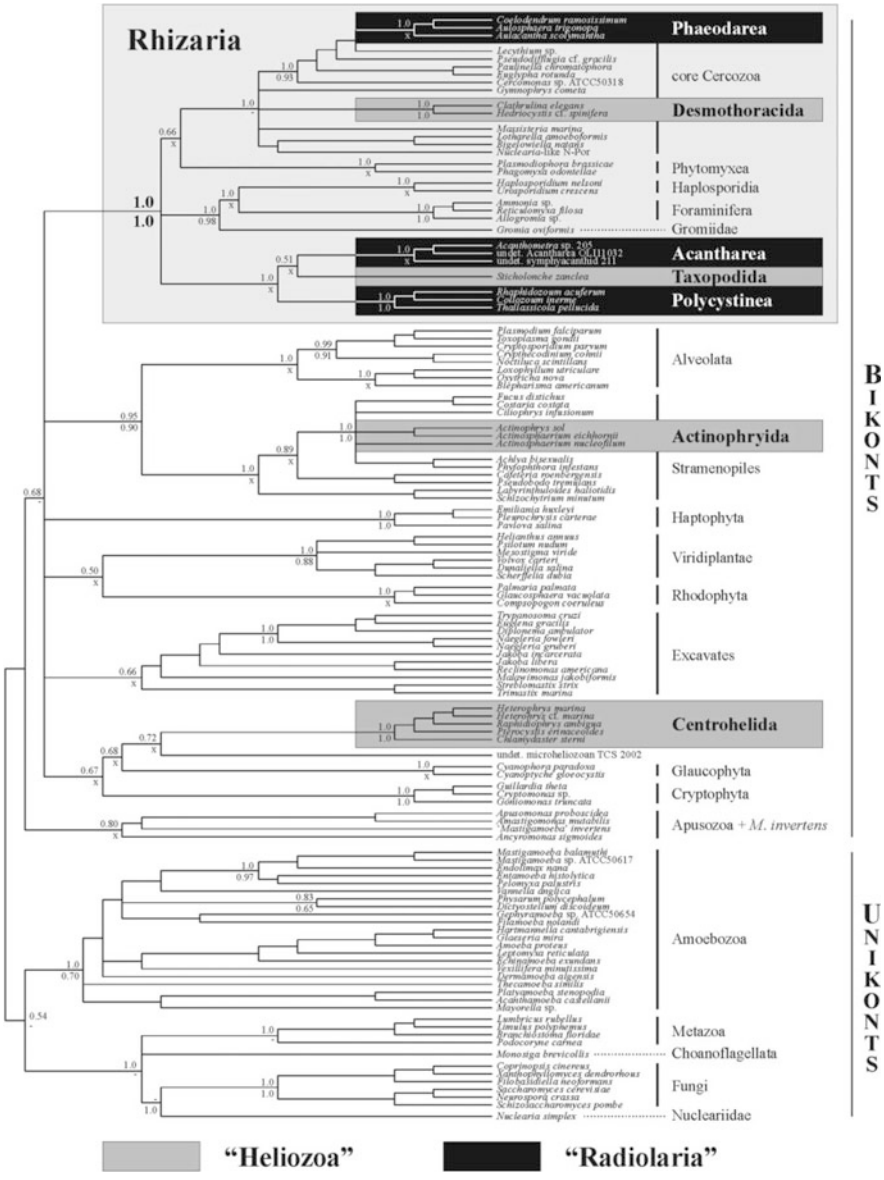

Fig. 3 Molecular phylogeny of Centrohelida and heliozoan-like protists (Used with kind permission from National Academy of Sciences. *The twilight of Heliozoa and rise of Rhizaria, an emerging supergroup of amoeboid eukaryotes*, volume 101, issue 21, 2004, 8066-8071, Sergey I. Nikolaev, Cedric Berney, Jose F. Fahrni, Ignacio Bolivar, Stephane Polet, Alexander P. Mylnikov, Vladimir V. Aleshin, Nikilai B. Petrov and Jan Pawlowski, Fig. 1. Copyright (2004) National Academy of Sciences, U.S.A.)

Evolutionary History

Over the past 15 years, it has become accepted that the heliozoan-like protists are a polyphyletic group based upon both morphological (Smith and Patterson 1986; Mikrjukov 1998, 1999, 2000a, b; Mikrjukov et al. 2000) and largely 18S rDNA-based molecular studies (e.g., Nikolaev et al. 2004; Cavalier-Smith and von der Heyden 2007; Cavalier-Smith and Chao 2003). The centrohelids are proposed to share a molecular evolutionary history with the haptophytes and cryptomonads and more broadly with the stramenopile/alveolate/Rhizaria (SAR; Cavalier-Smith and von der Heyden 2007; Burki et al. 2009). The Heliomorphids (Dimorphids) and Clathrulinids (Desmothracids) are considered to share an evolutionary history with the Cercozoa, and actinophryids are proposed to share common ancestry with the Stramenopiles (Fig. 3; Nikolaev et al. 2004; Bass et al. 2009). Due to a lack of molecular data, the history of the gymnosphaerids remains unresolved, with their placement limited to incertae sedis within the Rhizaria.

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Jakobida

27

Alastair G. B. Simpson

Abstract

Jakobida is a small group (<20 described species) that is related to Heterolobosea and Euglenozoa. Jakobids are free-living heterotrophs with two flagella. They primarily eat prokaryotes that are captured by suspension feeding, using a current produced by the posterior flagellum (which has a dorsal vane), and an "excavate"-type feeding groove. Most are marine or freshwater aerobes, although the Stygiellidae (*Stygiella*, *Velundella*) are marine and brackish water anaerobes. Most jakobids are free-swimming cells, some of which temporarily attach to surfaces, while Histionidae (e.g., *Histiona*, *Reclinomonas*) are freshwater sessile forms that sit within conical or wineglass-shaped organic loricas. Jakobids have rarely been identified as major components of microbial ecosystems, except in some anoxic marine waters. They are of special evolutionary importance, however, because their mitochondrial genomes retain more ancestral bacterial-like features than those of other eukaryotes. The mitochondrial genomes of aerobic jakobids encode more genes than those of any other eukaryote group; around 100 genes in total, including up to 69 protein-coding genes, ~10 of which occur in no other mitochondrial genome examined to date. In particular, they encode (subunits of) a bacterial-type RNA polymerase, while the mitochondrial RNA polymerase in other eukaryotes is a nucleus-encoded single-subunit enzyme with viral affinities. This retention by jakobids of the inferred-to-be-original mitochondrial RNA polymerase is an important datum for inferring the evolutionary history of eukaryotic cells, including the mitochondrial symbiosis. Malawimonads are a small group of heterotrophic flagellates that superficially resemble jakobids, but are of uncertain evolutionary position within eukaryotes and thus also of particular evolutionary importance.

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Contents

Summary Classification

●**Jakobida**
●●**Histionina**
●●●**Jakobidae** (*Jakoba*)
●●●**Histionidae** (*Histiona*, *Reclinomonas*, *Stomatochone*?, *Stenocodon*?)
●●●**Moramonadidae** (*Moramonas*, *'Seculamonas' nomen nudum*)
●●**Andalucina**
●●●**Andaluciidae** (*Andalucia*)
●●●**Stygiellidae** (*Stygiella*, *Velundella*)

Introduction

General Characteristics

Jakobids (Jakobida) are small free-living heterotrophic flagellates. All well-studied species have two flagella. Jakobids are one group of the "typical excavates," which signifies that they have a conspicuous feeding groove supported by a particular set of

cytoskeletal elements that originate in association with the basal body of the posterior flagellum (Simpson 2003). As in other typical excavates, the posterior flagellum bears vanes and beats within the groove to generate the feeding current. There are <20 formally described jakobids, including *incertae sedis* species. Species of *Jakoba*, *Moramonas*, *Andalucia*, *Stygiella*, and *Velundella* are free swimming (as are some undescribed forms), while the Histionidae ("histionids") are sessile and usually reside within a lorica.

Jakobids are of interest primarily because of their ancestral-like mitochondrial genomes. Jakobid mitochondrial genomes are uniquely gene rich, as first demonstrated by Lang et al.'s (1997) seminal study of *Reclinomonas americana*. In addition to a large total number of genes (up to 100), jakobid mitochondrial genomes typically contain about nine genes that have not been found in the mitochondrial genomes of any other eukaryote (Burger et al. 2013; Gray et al. 2004). Most remarkably, this includes genes encoding subunits of a bacterial-type RNA polymerase, whereas other eukaryotes use a completely different "phage-type" RNA polymerase in their mitochondria.

Occurrence

Jakobids have been observed or detected in many habitats, including water column and sediments from both freshwater and saline/marine habitats (e.g., Behnke et al. 2006; Flavin and Nerad 1993; Pascher 1942, 1943; Patterson 1990). They have also been isolated from soil and observed in hypersaline samples (e.g., Lara et al. 2006, 2007; Ruinen 1938; Strassert et al. 2016). Jakobids do not appear to be particularly abundant in most habitats. One exception is seen in anoxic marine waters, where sequences from the presumably anaerobic taxon Stygiellidae can be abundant in environmental SSU rDNA/rRNA surveys or even dominant (Stock et al. 2009; Weber et al. 2014). Some accounts describe histionids as rare (Nicholls 1984).

Literature and History of Knowledge

Jakobids have been recognized as a group for a little over two decades, yet organisms now classified as jakobids were first described more than 100 years ago. Voigt (1901) described the loricate species now known as *Histiona velifera* and in 1902 introduced the genus name *Histiona* itself (Voigt 1902), while Penard (1921) and Pascher (1943) described additional *Histiona* species. These early accounts were based primarily on light microscopy of living cells. In the systematics of the time, *Histiona* was generally appended to other groups of loricate flagellates, namely, bicosoecids or chrysophyceans, or to the prymnesiophytes/haptophytes (e.g., Bourrelly 1968; Pascher 1942, 1943).

Petersen and Hansen (1961) and Nicholls (1984) subsequently combined detailed light microscopy studies with transmission electron microscopy (TEM) of whole

mounts of *Histiona* spp. This showed better the organization of the lorica and the flagella – the "posterior" flagellum had been overlooked in most previous studies. Further observations of *Histiona aroides* by Mylnikov (1984, 1989) included some ultrathin-section TEM data. These data led to skepticism about the previous systematic assignments for *Histiona* (e.g., Petersen and Hansen 1961), but did not connect *Histiona* positively to any other group.

Ruinen (1938) had described a free-swimming flagellate as *Cryptobia libera*. True *Cryptobia* species are parasites or commensals and are now recognized to be kinetoplastids (see ▶ Kinetoplastea). Patterson (1990) rediscovered this organism in marine samples. Light microscopy and TEM observations showed that it lacked the diagnostic features of kinetoplastids and did not closely resemble any other well-studied group. Patterson (1990) therefore proposed a new genus, *Jakoba*, and new family, Jakobidae, and renamed the species *Jakoba libera*. The genus name was chosen in honor of Ruinen – "Jakoba" was her given name.

Soon afterward Flavin and Nerad (1993) described a new loricate flagellate, *Reclinomonas americana*, using TEM and scanning electron microscopy (SEM). They realized that *Reclinomonas* was similar to both *Histiona* and *Jakoba*. They nonetheless placed *Reclinomonas* and *Histiona* in a separate new family, Histionidae. O'Kelly (1993) then compared *Reclinomonas*, *Histiona*, and *Jakoba* in detail and referred to them collectively as the "jakobids." O'Kelly (1993) also discussed an "undescribed jakobid" that was later formally described as *Malawimonas jakobiformis* (O'Kelly and Nerad 1999). Malawimonads, it turns out, are not specifically related to jakobids, despite their similar appearance by light microscopy (see Coda).

Bernard et al. (2000) described a new species from oxygen-poor intertidal sediments under the name *Jakoba incarcerata*. TEM studies demonstrated a strong similarity with other jakobids (Bernard et al. 2000; Simpson and Patterson 2001), but subsequent molecular phylogenies did not group *Jakoba incarcerata* with *Jakoba libera* (Edgcomb et al. 2001; Simpson et al. 2002). Lara et al. (2006) then reported a new soil flagellate that was specifically related to *Jakoba incarcerata* in small subunit ribosomal DNA (SSU rDNA) phylogenies. A new genus, *Andalucia*, was introduced, with the soil species described as *Andalucia godoyi* and with *Jakoba incarcerata* renamed *Andalucia incarcerata* (Lara et al. 2006). Recently, an extensive cultivation effort focused on anaerobic jakobids was reported by Pánek et al. (2015), who also proposed splitting *Andalucia* into multiple genera. *Jakoba*/*Andalucia incarcerata* was transferred to the new genus *Stygiella*, as *Stygiella incarcerata*, along with three new species, *Stygiella adherens*, *Stygiella agilis*, and *Stygiella cryptica.* Two further new species of anaerobic jakobids were assigned to a second new genus, *Velundella*, as *Velundella nauta* and *Velundella trypanoides* (Pánek et al. 2015). *Stygiella* and *Velundella* are closely related (Pánek et al. 2015).

The genera *Stenocodon* and *Stomatochone* were introduced by Pascher (1942) for several small, sessile flagellates. *Stenocodon epiplankton*, currently considered the only species in its genus, is a *Histiona*-like loricate organism (Pascher 1942; Flavin and Nerad 1993). The several nominal species of *Stomatochone* are similar to *Stenocodon*, except that they have no lorica, and most reportedly have a single

flagellum (Pascher 1942). There are no molecular or TEM data for *Stenocodon* or *Stomatochone*, and both are considered Histionidae *incertae sedis*. As a matter of purely historical interest, one of Pascher's *Stomatochone* species, *Stomatochone excavata*, was originally described in the late nineteenth century (as *Oikomonas excavata;* see Pascher 1942). This species might be the first-described jakobid, if it is indeed correctly assigned.

Two other jakobids are currently under study and are referred to in the literature as *Seculamonas ecuadoriensis* and *Jakoba bahamensis* or *J. bahamiensis* (e.g., Burger et al. 2003, 2013; Gray et al. 2004; Marx et al. 2003; Rodriguez-Ezpeleta et al. 2007; Shutt and Gray 2006a, b). As of yet, there are no published morphological data or a formal description of either species. Very recently, however, a new soil isolate that is specifically related to *Seculamonas ecuadoriensis* was formally described as *Moramonas marocensis* (Strassert et al. 2016). It is possible that *Moramonas* will be a suitable generic vehicle for the *Seculamonas ecuadoriensis* organism, if and when it is formally described.

Much of the work on jakobids over the last two decades has been in the fields of biochemistry, molecular biology, and evolutionary genomics. The mitochondrial genome of *Reclinomonas americana* strain ATCC 50394 was published in 1997 (Lang et al. 1997; see also Burger et al. 1996; Lang et al. 1996). Mitochondrial genome sequences have since been completed for three other strains of *Reclinomonas americana* and for *Histiona aroides*, *Jakoba libera*, *Jakoba bahamensis*, *Seculamonas ecuadoriensis*, and *Andalucia godoyi* (Burger et al. 2013; see also Gray 1999; Gray et al. 1998, 1999, 2004; Lang et al. 1999a, b). In addition, extensive mitochondrial genome sequence data, including most or all of the coding regions, was recently reported for *Moramonas marocensis* (Strassert et al. 2016). The uniquely bacterial-like nature of jakobid mitochondrial genomes makes them important for understanding the evolution and full capabilities of mitochondria. This has inspired several studies of mitochondrial genes, proteins and RNAs in jakobids, including the positive identification of a mitochondrial gene of previously unknown function (Burger et al. 2003), characterization of respiratory complex organization (Marx et al. 2003), examination of the catalytic properties of the RNA component of mitochondrial RNase P (Seif et al. 2006), identification and/or characterization of additional (nearly) "jakobid-specific" mitochondrial genes (Jacob et al. 2004; Keiler et al. 2000; Tong et al. 2011), and studies of mitochondrial tRNA processing (Leigh and Lang 2004). Recently it was established that at least some jakobids belong to the selection of protists with nuclear genes that encode a bacterial-like FtsZ plus MinC-E protein system, which is inferred to form part of the ancestral mitochondrial division mechanism in eukaryotes (Leger et al. 2015). In fact, mitochondrial localization of the Min proteins was demonstrated using heterologous expression of *Stygiella incarcerata* proteins in yeast (in parallel with studies of *Dictyostelium* sequences).

Examination of nuclear genes from jakobids began primarily for phylogenetic purposes (Archibald et al. 2002; Cavalier-Smith 2000; Edgcomb et al. 2001; Simpson and Roger 2004; Simpson et al. 2002, 2006). *Jakoba libera*, *Reclinomonas americana*, *Histiona aroides*, *Andalucia godoyi*, *Stygiella incarcerata*, *Jakoba bahamensis*, and *Seculamonas ecuadoriensis* have all been the subjects of expressed

sequence tag (EST) projects or more extensive transcriptomic surveys (e.g., Hampl et al. 2009; Leger et al. 2016; O'Brien et al. 2007; Lang, personal communication), while whole genome sequencing projects are underway for *Andalucia godoyi* and *Reclinomonas americana* (Lang, personal communication; see Burger et al. 2013). Some phylogenetic studies focusing on jakobids have relied heavily on these resources (e.g., Hampl et al. 2009; Rodríguez-Ezpeleta et al. 2007; Simpson et al. 2008). These databases have also been used for gene discovery, for example, for finding nucleus-encoded mitochondrial or hydrogenosomal proteins (Shutt and Gray 2006a, b; Tong et al. 2011; Leger et al. 2015, 2016), for intron confirmation (Russell et al. 2005), and to establish that jakobids have "TTAGGG" telomeres like those of vertebrates (actually inferred to be the ancestral type for eukaryotes; Fulnečkova et al. 2013).

Cultures identified as *Jakoba libera* have been used to examine several aspects of predation by nanoflagellates. These include comparative studies of feeding and growth kinetics with different species of predators and/or prey (Christaki et al. 2005; Eccleston-Parry and Leadbeater 1994; Mohapatra and Fukami 2004a, 2005), production of hydrolytic enzymes during predation (Mohapatra and Fukami 2004b), chemosensory attraction of predators to different bacterial prey (Mohapatra and Fukami 2007), and the influence of different predators on the composition of experimental prokaryote communities (Vázquez-Domínguez et al. 2005).

Practical Importance

At present jakobids are studied primarily because of their evolutionary importance rather than any direct impacts on humans. They are all free living, and there are no indications of pathogenicity or toxicity. There is no known commercial exploitation of jakobids.

Habitats and Ecology

Free-swimming jakobids have been reported from diverse habitats, including marine water column, marine sediments, freshwater, and soil (Lara et al. 2007; Patterson 1990; Patterson et al. 1993; Vørs et al. 1995; Strassert et al. 2016). The morphospecies *Jakoba libera* was first observed in hypersaline samples, apparently including saturated brine (Ruinen 1938). All described species of *Stygiella* and *Velundella* were isolated from anoxic marine or saline material (Bernard et al. 2000; Pánek et al. 2015; Simpson et al. 2008), and related SSU rDNA/rRNA sequences have been detected from different oxygen-poor, sulfide-rich water samples, including some associated with deep hypersaline anoxic basins (Alexander et al. 2009; Behnke et al. 2006; Luo et al. 2005; Pánek et al. 2015; Stock et al. 2009, 2012; Weber et al. 2014). By contrast, histionids seem to be restricted to freshwater. They attach to surfaces, including larger algae, or are neustonic (Penard 1921; Petersen and Hansen 1961).

As with other "typical excavates," jakobids are suspension feeders. The beating of the posterior flagellum creates a current through the feeding groove. Individual suspended particles are phagocytosed within the groove, usually at its posterior end. Jakobids are generally considered to be bacterivores (e.g., O'Kelly 1997; Patterson et al. 1993), although *Jakoba libera* can also consume the extremely small (1 μm) eukaryote *Ostreococcus tauri* (Christaki et al. 2005). Unsurprisingly, very different growth rates have been recorded for *Jakoba libera* when fed different prey species (Mohapatra and Fukami 2004a, 2005).

Jakobids are fairly slow swimmers (e.g., Eccleston-Parry and Leadbeater 1994) and generate a modest feeding current if tethered. This suggests that their clearance rate (the volume from which prey is extracted per unit time) may be low compared with many suspension-feeding flagellates. Single-bacterium growth experiments on *Jakoba libera* yielded clearance rates of <5 nl cell^{-1} h^{-1}, at the low end of those reported for nanoflagellates (Eccleston-Parry and Leadbeater 1994; Mohapatra and Fukami 2004a, 2005). In one trial *Jakoba libera* had by far the lowest volume-specific clearance rate (clearance rate/predator cell volume) of six flagellates tested under common conditions (Eccleston-Parry and Leadbeater 1994). It is possible that jakobids are adapted to relatively high concentrations of prokaryotes, such as those associated with surfaces in sediments and on detritus particles. The loricate histionids are normally attached to surfaces, while *Jakoba libera*, *Stygiella incarcerata*, and *Velundella nauta* often attach temporarily to surfaces by the distal portion of the anterior flagellum (Bernard et al. 2000; Patterson 1990; Pánek et al. 2015). Meanwhile, cells of *Stygiella adherens* and *Stygiella cryptica* adhere with either flagellum, while those of *Stygiella agilis* and *Velundella trypanoides* reportedly adhere by the cell body (Pánek et al. 2015). These attachment strategies may also improve clearance, relative to feeding while swimming (see Cristensen-Dalsgaard and Fenchel 2003).

Characterization and Recognition

General Appearance

Free-swimming jakobids are usually <12 μm long (though *Velundella trypanoides* can reach 15 μm; Pánek et al. 2015) and roughly pyriform in shape (Figs. 1a, b and 2a–f). The "ventral" side of the cell is formed into a broad groove. The right margin of the groove is more conspicuous than the left margin, which becomes reduced about halfway down the cell (Fig. 1b). The two flagella insert near the anterior end of the cell and are generally 1–2 times the length of the cell body (Figs. 1 and 2). Cultures of stygiellids often contain swimming forms with reduced grooves and sometimes different flagellum lengths (Bernard et al. 2000: Pánek et al. 2015). For example, cultures of *Stygiella incarcerata* contain both grooved cells and cells with reduced or absent grooves, with many of the latter having a markedly lengthened anterior flagellum (Fig. 2c, d). The posterior flagellum, also called flagellum 1, is directed posteriorly and usually beats within the groove (Figs. 1 and 2c). The anterior

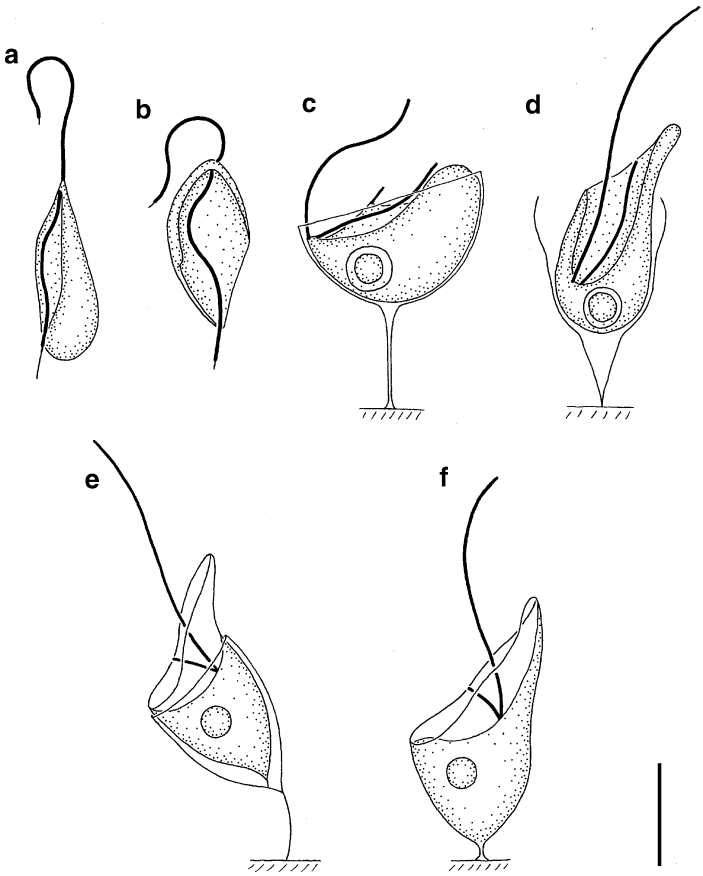

Fig. 1 Appearance of representative jakobids, viewed by light microscopy. (**a**) *Jakoba libera* (Jakobidae), lateral view; (**b**) *Stygiella incarcerata* (Stygiellidae), ventral view; (**c**) *Reclinomonas americana* (Histionidae), lateral view; (**d**) *Histiona aroides* (Histionidae); (**e**) *Stenocodon epiplankton* (Histionidae *incertae sedis*); (**f**) *Stomatochone infundibuliformis* (Histionidae *incertae sedis*). Scale bar represents 5 μm for all images (Drawings by AGBS, after drawings and images in Patterson (1990), Micro*scope (http://starcentral.mbl.edu/microscope), Simpson and Patterson (2001), O'Kelly (1997), Nicholls (1984) and Pascher (1942))

flagellum (or flagellum 2) emerges anteriorly or laterally and sometimes curves to be directed posteriorly (Figs. 1 and 2). In *Jakoba libera* and *Stygiella incarcerata*, the anterior flagellum can assume a shepherd's crook shape, and the curved portion adheres temporarily to the substrate (Figs. 1a, b and 2a, c). Most species locomote by swimming with a spiraling motion.

Loricate jakobids (i.e., almost all Histionidae) are typically around 10–15 μm. To facilitate comparisons, the site of flagellar insertion can be taken to define the anterior end. The lorica is delicate and is organic rather than mineralized. *Reclinomonas* resembles a free-swimming jakobid that is reclining, groove-side

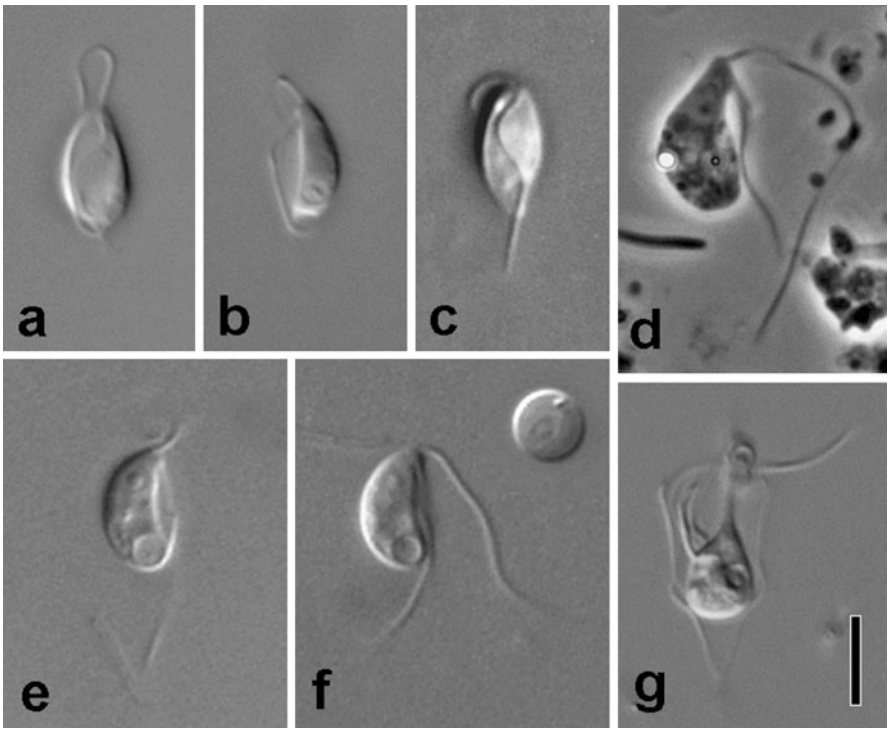

Fig. 2 Light micrographs of living jakobids. All micrographs except **d** are differential interference contrast: (**a**) *Jakoba libera*, ventral view, with hooked anterior flagellum; (**b**) *Jakoba libera*, lateral view of attached cell; (**c**) *Stygiella incarcerata*, morph with short anterior flagellum (only proximal portion visible), ventral view; (**d**) *Stygiella incarcerata* lateral view of morph with long anterior flagellum, cell is slightly compressed, phase contrast; (**e**) *Andalucia godoyi*, lateral view, showing groove; (**f**) *Andalucia godoyi*, showing flagellum lengths. *Inset at top right* shows a cyst; (**g**) *Histiona* sp. Scale bar represents 5 μm for all micrographs (**a**, **b** images by J.S. Park and AGBS. **c** image by AGBS, reproduced from Simpson and Patterson (2001), with permission. **d** image by AGBS. **e**, **f** images by E. Lara. **g** image by Y. Eglit)

uppermost, within a close-fitting wineglass-shaped lorica (Flavin and Nerad 1993; O'Kelly 1997; see Fig. 1c). The "stem" of the wineglass, or pedicel, attaches the organism to the substrate. *Histiona* cells have rounded anterior ends and normally reside anterior-end-downwards within the lorica (Figs. 1d and 2g). The groove margins, especially the right margin, are very broad. This gives the posterior portion of the cell a sail-like appearance (it is sometimes referred to as the "velum"), and it usually projects from the lorica (Figs. 1d and 2g). The lorica is more conical than in *Reclinomonas* and includes a shelflike structure upon which the cell itself lies (Petersen and Hansen 1962). In both taxa the anterior flagellum usually curves over to be directed posteriorly (Fig. 1c, d). The posterior flagellum lies within the groove and is difficult to see by light microscopy. In both *Reclinomonas* and *Histiona*, the left margin of the groove detaches about halfway down the cell,

forming a projection called the epipodium (Flavin and Nerad 1993; Penard 1921; O'Kelly 1997; see Fig. 1c). The epipodium can be mistaken for a short flagellum by light microscopy, but is actually supported by microtubules from the left "R1" flagellar root (see below). Some accounts suggest that the epipodium attaches the cell to its lorica (Flavin and Nerad 1993; Penard 1921), but this is disputed by O'Kelly (1997). Cells of *Stenocodon* and *Stomatochone* (both Histionidae *incertae sedis*) are more conical, and one of their two flagella is very short (Fig. 1e, f) or absent altogether (three nominal species of *Stomatochone*; Pascher 1942). *Stenocodon* has a conical lorica, while *Stomatochone* is similar to *Stenocodon* but lacks the lorica and attaches to surfaces by a pointed part of the cell (Pascher 1942).

Ultrastructure

The nucleus has a central nucleolus and is positioned in the anterior part of the cell, closely associated with the flagellar basal bodies (Fig. 3a). The mitochondria and single dictyosomal Golgi body are also located anteriorly. There is a single mitochondrion in most cases (Flavin and Nerad 1993; Lara et al. 2006; O'Kelly 1993; but see Strassert et al. 2016). The mitochondrial cristae are tubular/ampuliform in *Reclinomonas*, *Histiona*, *Moramonas*, and *Andalucia* (Lara et al. 2006; Mylnikov 1989; O'Kelly 1993, 1997; Strassert et al. 2016; see Fig. 3b), but flattened in *Jakoba libera* and absent in *Stygiella* and *Velundella* (Pánek et al. 2015; Patterson 1990; Simpson and Patterson 2001). The endoplasmic reticulum is observed throughout the cell, while food vacuoles are mostly in the posterior two-thirds (Fig. 3a). A microbody has been documented in *Andalucia* and *Moramonas* (Lara et al. 2006; Strassert et al. 2016). *Jakoba libera* and histionids possess small, round extrusomes that lie under the dorsal cell membrane (Mylnikov 1989; O'Kelly 1993, 1997). Their positioning suggests a defensive function. The lorica of *Reclinomonas americana* is decorated with fine scales that resemble carpentry nails (Flavin and Nerad 1993; O'Kelly 1997). These are produced within the endomembrane system prior to cell division, and zoospores contain a vesicle that holds numerous scales (O'Kelly 1997; see below).

The anterior flagellum (F2) is unremarkable, but the posterior flagellum (F1) of trophic cells has a vane on its "dorsal" side (i.e., the side closest to the cell body – Fig. 3d–f). The vane is not normally seen by light microscopy, but can be observed in fixed, stained material (Petersen and Hansen 1962). It is as broad as the axoneme in *Jakoba libera* and *Andalucia godoyi* (Lara et al. 2006; Patterson 1990), but 2–4 times as broad in other well-studied species (Flavin and Nerad 1993; Mylnikov 1989; Simpson and Patterson 2001; see Fig. 1d). The vane is supported by a fine paraxonemal leaf of an unknown protein composition, which can appear striated in grazing sections (Lara et al. 2006; Mylnikov 1989; Simpson and Patterson 2001). Presumably the vane plays a role in generating the feeding current (or maintaining the current close to the cell surface) or in the mechanics of food capture. The vane is absent in *Reclinomonas* zoospores (O'Kelly 1997), consistent with a role in feeding rather than locomotion.

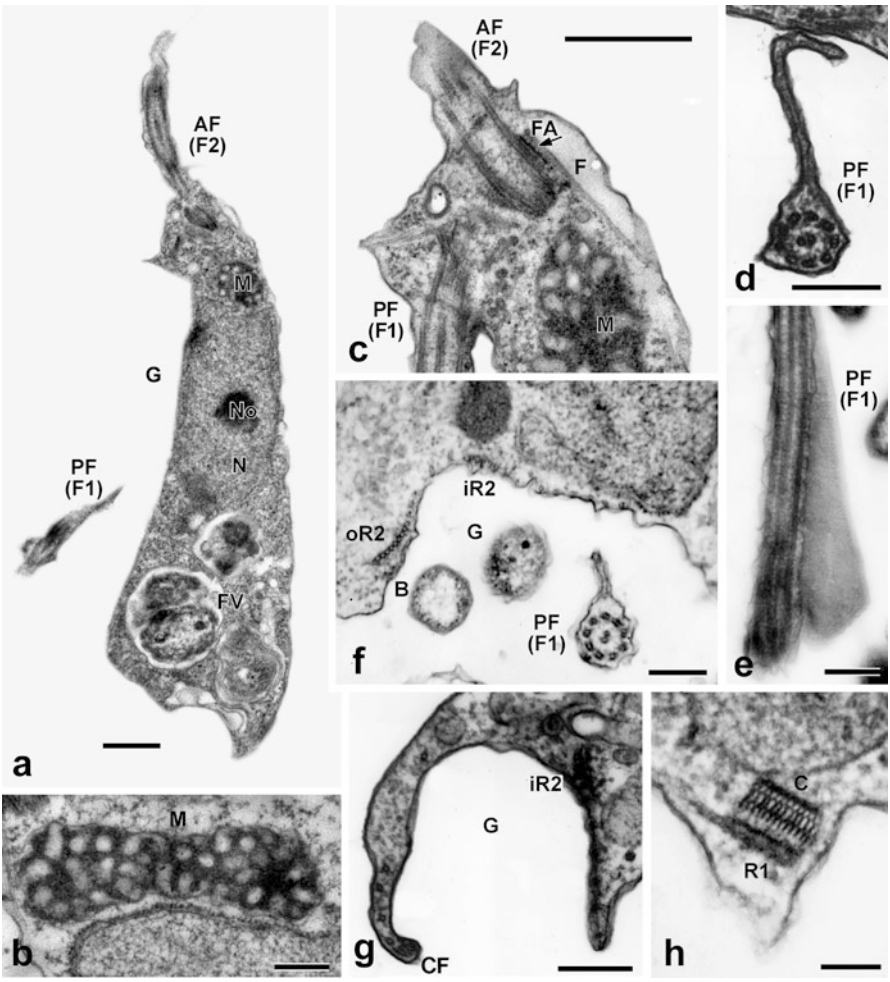

Fig. 3 Transmission electron micrographs of *Andalucia godoyi* (**a**–**c**) and *Stygiella incarcerata* (**d**–**h**): (**a**) Whole cell in longitudinal section; (**b**) mitochondrion, with tubular cristae; (**c**) flagellar apparatus, showing the dorsal fan originating close to the basal body of the anterior flagellum (flagellum 2); (**d**) transverse section of the posterior flagellum (flagellum 1) showing the single broad "dorsal" vane; (**e**) longitudinal section of the posterior flagellum and vane; (**f**) transverse section through the groove, showing the outer portion of the R2 (right) root (labeled "*oR2*") supporting the right wall of the groove together with the B fiber (here shown shortly before its end), while the inner portion of the R2 root (labeled "*iR2*") and, especially, microtubules derived from the R1 (left) root, support much of the floor of the groove. Note the dorsal position of the flagellar vane relative to the axoneme of the posterior flagellum; (**g**) the groove near the posterior end of the cell. Note the thin right wall of the groove, supported by the composite fiber (*CF*), and microtubules derived from R2; (**h**) transverse section of the C fiber, attached to the dorsal side of the R1 (left) root, at the anterior end of the groove. The R1 microtubules are sectioned obliquely here and cannot be distinguished individually. *AF(F2)* anterior flagellum/flagellum 2, *B* B fiber, *C* C fiber, *CF* composite fiber, *F* dorsal fan, *FA* fan-associated sheet, *FV* food vacuole, *G* groove, *iR2* inner portion of the R2 root (right root), *M* mitochondrion, *N* nucleus, *No* nucleolus, *oR2* outer

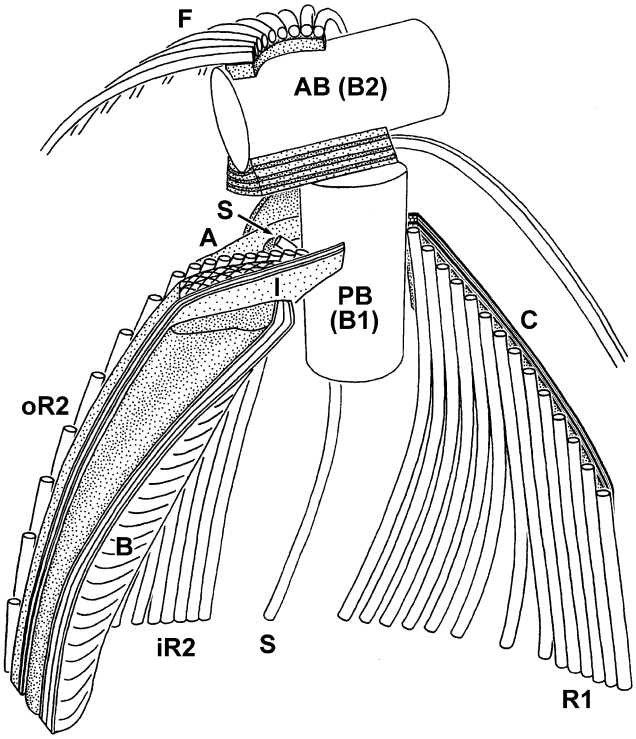

Fig. 4 Reconstruction of the proximal flagellar apparatus of *Stygiella incarcerata*, seen from the ventral side. *A* A fiber, *AB(B2)* anterior basal body/basal body 2, *B* B fiber, *C* C fiber, *F* dorsal fan, *I* I fiber, *iR2* inner portion of the R2 (right) root, *R1* left "R1" microtubular root, *PB(B1)* posterior basal body/basal body 1, *R2* right "R2" microtubular root, *S* singlet microtubular root (Modified from Simpson and Patterson (2001), reproduced with permission)

The flagellar apparatus is depicted in Fig. 4. The basal bodies lie at a wide angle to one another (see also Fig. 3c). There are two main flagellar microtubular roots: the left root "R1" and the right root or "R2" (see Yubuki et al. 2013 for corrected universal numbering for the flagellar microtubular roots of "typical excavates"). These originate in association with the posterior basal body (basal body 1). There is also a single microtubule – the "singlet root" – that originates close to basal body 1, near the dorsal side of the R2 (right) root. The proximal portion of the R2 root associates with three non-microtubular fibers, "A," "B," and "I" (O'Kelly 1997;

Fig. 3 (continued) portion of the R2 root (right root), *PF(F1)* posterior flagellum/flagellum 1, *R1* left 'R1' root. Scale bars in **a** and **c** represent 500 nm. Bars in **b** and **d**–**g** represent 250 nm. Scale bar in h represents 100 nm (**a** and **c** Images by E. Lara, reproduced from Lara et al. (2006), with permission. **b** image by E. Lara. **d**–**h** Images by AGBS, reproduced from Simpson and Patterson (2001), with permission)

Patterson 1990). The "A" fiber lies on the dorsal side of the R2 root, the "I" fiber on the ventral side, and the "B" fiber is initially aligned at an angle to the ventral side of the R2 root (Fig. 3f). The non-microtubular "C" fiber is attached to the dorsal side of the R1 (left) root and has a conspicuous multilayered appearance (Fig. 3h). It supports the origins of individual microtubules that are gradually added to the left side of R1 (see Fig. 4).

The main role of the cytoskeleton associated with basal body 1 is supporting the feeding groove. Shortly after its origin, the R2 (right) root divides into an inner portion with 4–8 microtubules (Lara et al. 2006; O'Kelly 1997; Simpson and Patterson 2001) and an outer portion with the remainder. The outer portion and the associated B fiber support the right margin of the groove (Fig. 3f). Microtubules from the R1 (left) root support the left margin. Most of the groove floor is supported by microtubules that diverge from the R1 root, while the rightmost portion only is supported by the singlet root, the inner portion of the R2 root, and a few individual microtubules that emerge between the two portions of the R2 root (Figs. 3f and 4). Progressing down the groove, the "I" fiber and "B" fiber gradually disappear, while the left margin abruptly reduces, and the microtubules supporting the left margin terminate. In histionids, the left margin edge detaches and continues to be supported by R1 microtubules and the C fiber for some distance, forming the epipodium (see above, and O'Kelly 1997; Mylnikov and Mylnikov 2014). Further down, many of the microtubules supporting the right margin terminate. The remaining microtubules are supplemented by a (partly) striated "composite fiber," which supports the right margin of the groove to the posterior end of the cell (Simpson and Patterson 2001; Fig. 3g). The groove wall at this point can become very tall and extremely thin, especially in histionids (Flavin and Nerad 1993), resulting in the sail-like appearance ("velum") seen in light microscopy (see above). The histionid velum margin is supported by more microtubules than the posterior right margin of free-swimming jakobids, and the composite fiber is more extensive as well (Mylnikov and Mylnikov 2014).

The anterior basal body (basal body 2) is associated with a fan of individual microtubules that spread out to support the dorsal side of the cell (Fig. 3c). There is a small plaque or sheet of dense material sandwiched between the originating microtubules and the basal body ("FA" in Fig. 3c; Lara et al. 2006; O'Kelly 1997). There are probably no true microtubular roots associated with basal body 2 in jakobids. A two-microtubule "root" was originally reported in *Stygiella incarcerata* (Simpson and Patterson 2001), but is not seen in the related *Andalucia godoyi*, and likely represents two individual microtubules (Lara et al. 2006).

Life Cycle

Jakobids reproduce by binary fission. Two new basal bodies form prior to mitosis and serve as the organizing centers for the mitotic spindle, which forms externally to the nucleus (O'Kelly 1993). O'Kelly (1993) reported that the nuclear envelope breaks down after spindle formation, but that the nucleolus persists through at

least the early stages of mitosis. Following cytokinesis in *Reclinomonas*, one daughter cell inherits the parental lorica while the other swims away as a zoospore, settles elsewhere, and constructs a new lorica (O'Kelly 1997). Similar swimming cells have been reported in other histionids (Mylnikov 1984; Penard 1921). There are no reports of sexuality in jakobids.

Some species form cysts with relatively delicate, unmineralized cyst walls. The soil-isolated species *Andalucia godoyi* and *Moramonas marocensis* both have spherical cysts (Lara et al. 2006; Strassert et al. 2016; Fig. 2f inset). The cysts of histionids generally remain within the lorica and have a small button-like projection (Mylnikov 1984; O'Kelly 1997; Petersen and Hansen 1961). The presence of cysts in *Jakoba libera* is uncertain (see O'Kelly 1997). The cysts of jakobids retain some vestiges of the flagellar apparatus, i.e., basal bodies and parts of the flagellar microtubular root system (O'Kelly 1997; Strassert et al. 2016).

Mitochondrial Genomes

The mitochondrion was originally an independent bacterium, specifically an alpha-proteobacterium, that was incorporated as an endosymbiont in some common ancestor of all extant eukaryotes. The mitochondrial genome is the reduced and highly modified remnant of this bacterium's genome (see Gray et al. 1999). Of all mitochondrial genomes studied to date, those of jakobids most closely resemble the ancestral mitochondrial genome, that is, they have lost the fewest bacterial features.

The exemplar jakobid *Reclinomonas americana* strain ATCC 50394 has a circular-mapping mitochondrial genome that is ~90% coding and contains nearly 100 genes, including 67 identified protein-coding genes and large ORFs (Fig. 5; Burger et al. 2013; Lang et al. 1997). This is about five times as many protein-coding genes as in animal mitochondria, for example. The several other jakobid mitochondrial genomes sequenced subsequently are generally very similar to that of *Reclinomonas americana* ATCC 50394. The biggest exceptions are in *Jakoba libera*, in which the mitochondrial genome is linear and has slightly fewer (89) putative genes (Burger et al. 2013; Gray et al. 2004), and *Moramonas marocensis*, whose mtDNA apparently includes a very large amount of noncoding sequence and is of unknown configuration (Strassert et al. 2016). Notably, the mitochondrial genome of *Andalucia godoyi* proved to have even more coding potential than *Reclinomonas*, with 100 genes, 69 of which are protein-coding genes or ORFs (Burger et al. 2013). These include *rpl35* (encoding a ribosomal protein) and *cox15* (encoding a cytochrome oxidase assembly protein), neither of which is mitochondrion encoded in any other eukaryote examined to date. Interestingly, the *Andalucia cox15* is not closely related to the nucleus-encoded *cox15* sequences from other eukaryotes and those from the great majority of alpha-proteobacteria (He et al. 2016). It most likely represents a recent gene transfer into the *Andalucia* mitochondrion from a prokaryotic source (and not an ancestral feature of the eukaryotic mitochondrial genome that has been uniquely retained in *Andalucia*).

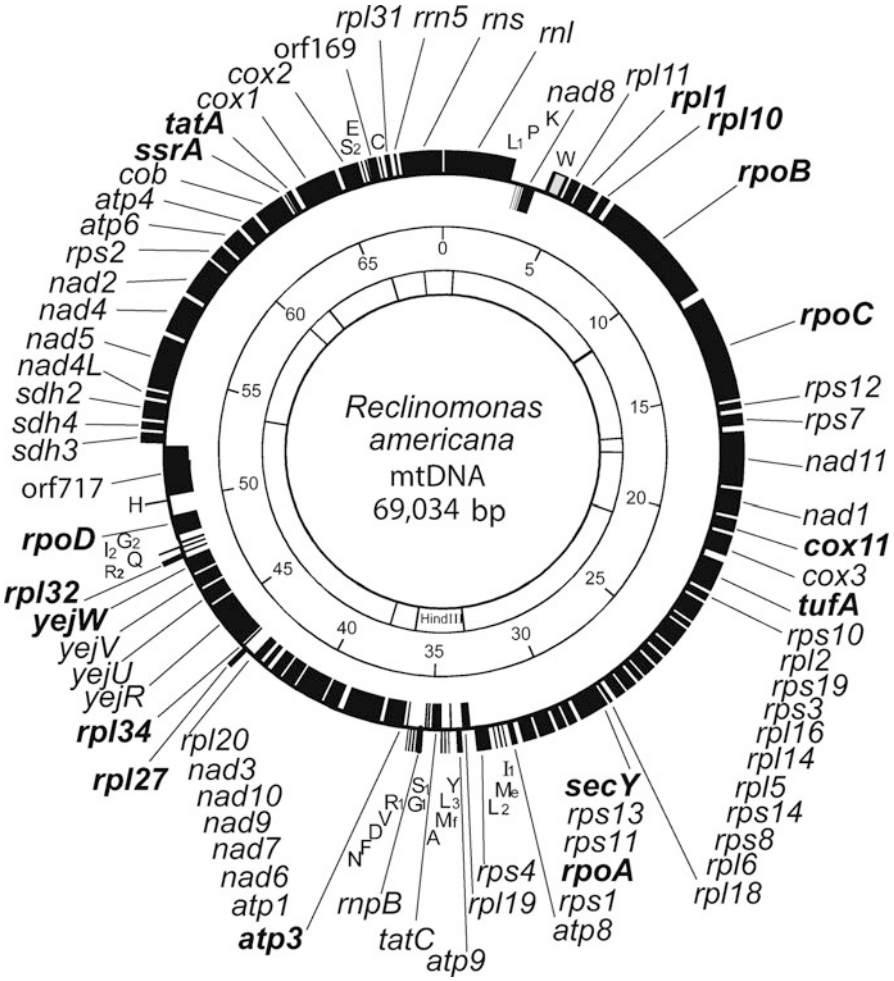

Fig. 5 Map of the mitochondrial genome of *Reclinomonas americana* (strain ATCC 50394). tRNA genes are denoted by individual capital letters according to the corresponding amino acid. Genes that appear to be unique to jakobid mitochondria, or nearly so, are denoted in bold – among these are *rpoA-D*, which encode subunits of a bacterial-type $\alpha_2\beta\beta'\sigma$ RNA polymerase; *tufA*, which encodes a translation elongation factor (also found in *Hartmannella* mtDNA – see Burger et al. 2013); *secY*, which encodes a SecY-type transporter; and *ssrA*, which encodes a transfer-messenger RNA (tmRNA) molecule (also found in oomycete mtDNA – see Burger et al. 2013) (Modified and updated from an original kindly provided by B. Franz Lang (Université de Montréal; Organelle Genome Megasequencing Program))

There are about nine protein-coding genes that are present in most or all jakobid mitochondrial genomes but are absent from all other sequenced mitochondrial genomes (Fig. 5; Burger et al. 2013). Products encoded by "jakobid-specific" genes include several ribosomal proteins, and a protein transporter of the bacterial

SecY type that is probably involved in the insertion of mitochondrion-encoded integral inner membrane proteins, though the latter is not encoded on *Andalucia godoyi* mtDNA (Burger et al. 2013; Tong et al. 2011). Most jakobid mitochondrial genomes also encode a transfer-messenger RNA (tmRNA), involved in clearing stalled ribosomes, otherwise only known from unpublished oomycete mitochondrial genome data (Burger et al. 2013; Jacob et al. 2004; Keiler et al. 2000). Most dramatically however, jakobid mitochondrial genomes encode subunits of a bacterial-type $\alpha_2\beta\beta'\sigma$ RNA polymerase. Genes for four subunits are present in *Reclinomonas americana* and most other jakobids, while *Jakoba libera* mtDNA encodes only two subunits (Burger et al. 2013; Gray et al. 2004; Lang et al. 1999a; Strassert et al. 2016). This seems to be a unique retention of a bacterial-like feature, since the mitochondrial RNA polymerases of other eukaryotes are nucleus-encoded single-subunit enzymes with no affinity to bacterial-type RNA polymerases. These have a completely distinct, though still obscure, evolutionary origin, but are ultimately related to the RNA polymerases of T7/T3 bacteriophage (Cermakian et al. 1996; Shutt and Gray 2006a).

Jakobid mitochondrial genomes also include several features that are found in only a few other eukaryotes. This includes the genes for the 5S rRNA and the RNA component of RNase P (*rrn5*, *rnpB*) and genes for some proteins required for maturation of electron transport chain components (Gray et al. 2004; Lang et al. 1996, 1997, 1999a). Like some other gene-rich mitochondrial genomes, gene order in jakobid mitochondrial genomes preserves vestiges of proteobacterial operons (Gray 1999; Lang et al. 1999a). It is especially noticeable in jakobids because of the larger number of genes. For example, *secY*, which is unique to jakobids, is positioned adjacent to the remaining ribosomal protein genes from the proteobacterial *spc* operon, and the gene order is the same as in proteobacteria (Burger et al. 2013; Lang et al. 1999a). Similarly, the RNA polymerase subunit gene *rpoA* is located "correctly" within a reduced proteobacterial "alpha" operon in all jakobids (Burger et al. 2013; Lang et al. 1999a, b). Finally, all jakobid mitochondrial genomes, except that of *Jakoba libera*, retain conserved Shine-Dalgarno-like sequences upstream of coding regions (Burger et al. 2013; Lang et al. 1997; Strassert et al. 2016).

The Nuclear Genome

As of early 2016, there are nuclear genome sequencing and annotation projects underway for at least two jakobids: *Andalucia godoyi* and *Reclinomonas americana* (Lang, personal communication; see Burger et al. 2013), but these have yet to be fully published. It appears that nuclear genes in *Jakoba*, *Reclinomonas*, and *Moramonas* (all members of Histionina) usually do include spliceosomal introns (e.g., Edgcomb et al. 2001; Archibald et al. 2002; Russell et al. 2005; Strassert, personal communication). By contrast, introns are lacking from most (but not all) of the protein-coding gene sequences reported from *Andalucia*, *Stygiella*, and *Velundella* species (Edgcomb et al. 2001; Leger et al. 2015; Simpson et al. 2008),

hinting that Andalucina as a group (see below) might have relatively intron-poor genomes. Curiously, the alpha tubulin genes from Andalucina are not closely related to those of other jakobids and instead show a strong phylogenetic affinity with alpha tubulin genes from Obazoa (e.g., opisthokonts), fornicates (e.g., diplomonads), and parabasalids (Edgcomb et al. 2001; Pánek et al. 2015; Simpson et al. 2008). This is most likely due to eukaryote-to-eukaryote gene transfer (Simpson et al. 2008) or perhaps an ancient paralogy and differential paralog retention (Eliáš, personal communication).

Systematics

The formal taxon for all jakobids is Jakobida Cavalier-Smith 1993, emended by Adl et al. (2005). Described jakobids are divided into two subtaxa: Histionina Cavalier-Smith 2013 and Andalucina Cavalier-Smith 2013. The original morphological diagnoses of these taxa do not distinguish them because the diagnosis for Andalucina is inaccurate; however, each corresponds to a well-supported clade in molecular phylogenies (see below and Pánek et al. 2015). Histionina includes Jakobidae Patterson 1990, Moramonadidae Strassert et al. 2016, and Histionidae Flavin and Nerad 1993. Jakobidae and Moramonadidae each only contains a single genus (*Jakoba* and *Moramonas*, respectively), while Histionidae includes *Histiona* and *Reclinomonas*, with *Stomatochone* and *Stenocodon* currently considered as Histionidae *incertae sedis* (Adl et al. 2005, 2012; Patterson et al. 2002). *Jakoba bahamensis*/*Jakoba bahamiensis* and *Seculamonas ecuadoriensis* are *nomina nuda* that refer to organisms that branch within Histionina, with the latter assignable specifically to Moramonadidae (Strassert et al. 2016). Andalucina is subdivided into Andaluciidae Cavalier-Smith 2013 (which includes only *Andalucia*) and Stygiellidae Pánek et al. 2015, which contains the recently introduced genera *Stygiella* and *Velundella*. A list of species (including well-studied *nomina nuda*) is given in Table 1.

Maintenance and Cultivation

Many jakobids, especially free-living forms, have been observed, encountered, and isolated from crude cultures, rather than directly from environmental samples (e.g., Bernard et al. 2000; Patterson et al. 1993). One jakobid was encountered as a contaminant in an algal culture (O'Kelly 1993). Histionids may be collected as epibionts on larger algae (Bourrelly 1953; Penard 1921).

Many of the described species are maintained in monoprotistan culture. Jakobids have been isolated by serial dilution (Flavin and Nerad 1993; Lara et al. 2006; pers. obs) or even simple serial transfer (pers. obs). Existing cultures are not axenic – they include prey bacteria. Most cultures have been isolated and maintained in standard liquid media of appropriate salinity and mineral composition (e.g., Page's amoeba saline or "WCL" for freshwater and soil organisms; f/2, sterile seawater or diluted

Table 1 Described and studied species of jakobids, and availability of various data

Higher taxon	Species	Authority	Habitat[a]	TEM	SSU rDNA[b]	mtDNA	Comments
Jakobidae	*Jakoba libera*	(Ruinen 1938) Patterson 1990	Marine[c]	*	**	*	
	Jakoba bahamensis/ Jakoba bahamiensis	*nomina nuda*	Marine (?)			*	Specific relationship with *Jakoba libera* unclear
Histionidae	*Histiona aroides*	Pascher 1943	Freshwater	*		*	
	Histiona campanula	Penard 1921	Freshwater				Sometimes assigned to *Reclinomonas* or *Stenocodon*
	Histiona velifera	(Voigt 1901) Pascher 1943	Freshwater				*Histiona zachariasi* is an objective junior synonym
	Reclinomonas americana	Flavin and Nerad 1993	Freshwater	*	**	****[d]	First jakobid mtDNA fully sequenced (ATCC 50394)
	Stenocodon epiplankton[e]	Pascher 1942	Freshwater				Not to be confused with *Stomatochone epiplankton*
	Stomatochone cochlear[e]	Pascher 1942	Freshwater				
	Stomatochone epiplankton[e]	Pascher 1942	Freshwater				Not to be confused with *Stenocodon epiplankton*
	Stomatochone excavata[e]	Pascher 1942	Freshwater				Assignment to *Stomatochone* tentative
	Stomatochone infundibuliformis[e]	Pascher 1942	Freshwater				

Moramonadidae	*Moramonas marocensis*	Strassert et al. 2016	Soil	*	*	*[f]	mtDNA with large amounts of noncoding sequence
	Seculamonas ecuadoriensis	*nomen nudum*	Freshwater/ soil		*	*	
Andaluciidae	*Andalucia godoyi*	Lara et al. 2006	Soil	*	**	*	Most gene-rich mtDNA known
Stygiellidae	*Stygiella adherens*	Pánek et al. 2015	Marine		***		Anaerobe
	Stygiella agilis	Pánek et al. 2015	Marine		***		Anaerobe
	Stygiella cryptica	Pánek et al. 2015	Marine		*		Anaerobe
	Stygiella incarcerata	(Bernard et al. 2000) Pánek et al. 2015	Marine/ Saline	*	**********		Anaerobe; formerly assigned to *Jakoba* and *Andalucia*
	Velundella nauta	Pánek et al. 2015	Marine		*		Anaerobe
	Velundella trypanoides	Pánek et al. 2015	Marine	*[g]	*****		Anaerobe

[a]"Marine" includes brackish and modestly hypersaline marine habitats
[b]Asterisks represent the number of near-full-length SSU rDNA sequences obtained from distinct cultures as of early 2016; does not include closely related environmental sequences
[c]Possibly also (markedly) hypersaline habitats
[d]Mitochondrial genomes completed for four distinct strains identified as *Reclinomonas americana*
[e]Histionidae *incertae sedis*
[f]Mitochondrial genome map incomplete
[g]Limited TEM data published

sterile seawater for marine/brackish organisms), supplemented with an organic carbon source to promote bacterial growth, for example, dilute tryptone soy broth (0.3%), dilute LB media (0.3%), or a sterile barley grain (Lara et al. 2006; Patterson 1990). In some cases live prey bacteria (e.g., *Klebsiella* or *Enterobacter*) have been added instead of the carbon source or in addition to it (Burger et al. 2013; Flavin and Nerad 1993). *Reclinomonas americana* grows in ATCC 802 medium (i.e., Sonneborn's *Paramecium* medium), a simple cereal grass infusion (Flavin and Nerad 1993). *Stygiella incarcerata*, which is anaerobic, but not strictly so, grows well on fairly rich media that promotes dense bacterial growth and thus reduces free oxygen, e.g., 50% seawater supplemented with 3% LB (Simpson et al. 2008). However, this species also grows in seawater variants of ATCC 802 medium (ATCC 1525 medium) and in 50% seawater supplemented with a barley grain or with 802 media (Bernard et al. 2000; Pánek et al. 2015; Simpson et al. 2008). Other *Stygiella* and *Velundella* species were also originally cultivated on ATCC 1525 medium (Pánek et al. 2015). *Jakoba libera* has been grown on several different bacteria during experimental ecology studies (Eccleston-Parry and Leadbeater 1994; Mohapatra and Fukami 2004a). Most cultured jakobids grow well at 20–25 °C (e.g., Burger et al. 2013; Mohapatra and Fukami 2004a; pers. obs). Strains of the marine/brackish species *Stygiella incarcerata* and *Velundella trypanoides* will grow at salinities 1.5 times that of seawater (56 ppt), but show poor-to-no growth at double marine salinity (Pánek et al. 2015). At least one *Jakoba c.f. libera* strain grows well at 100 ppt salinity (i.e., almost three times marine salinity; pers. obs.).

Evolutionary History

Overall Phylogenetic Position

Jakobids have been considered as eukaryotes of particular evolutionary importance for more than two decades. O'Kelly (1993) noted that jakobid flagellates were morphologically similar to some "amitochondriate" flagellates, especially retortamonads. Retortamonads, in turn, were usually considered to be related to diplomonads. At the time certain amitochondriate flagellates, including retortamonads and diplomonads, were widely thought to be the earliest-branching extant eukaryotes (Cavalier-Smith 1987; Patterson and Sogin 1992). O'Kelly (1993) proposed that jakobids represented a series of the earliest branches among the mitochondrion-bearing eukaryotes, in other words, that all amitochondriate eukaryotes were descended from jakobid or jakobid-like ancestors. The idea of jakobids being a paraphyletic group that was especially deeply branching among mitochondriate eukaryotes was later refuted by molecular phylogenetics (see below). Nonetheless, this concept was a primary motivation for Lang and colleagues to sequence mitochondrial genomes from jakobids, leading to the discovery that these genomes were uniquely bacterial-like (Gray et al. 2004; Lang et al. 1997 – see "Characteristics", above).

Early phylogenies based on one or two nuclear genes did not strongly resolve the position of jakobids within eukaryotes (Archibald et al. 2002; Cavalier-Smith 2000; Edgcomb et al. 2001; Simpson et al. 2002), although some studies hinted at a relationship with Euglenozoa and/or Heterolobosea (Archibald et al. 2002; Cavalier-Smith 2004; Edgcomb et al. 2001). Later molecular phylogenies based on multiple (4-140) nucleus-encoded proteins demonstrated with increasing conviction that jakobids are closely related to Euglenozoa and Heterolobosea, collectively known as Discicristata (Hampl et al. 2009; Rodríguez-Ezpeleta et al. 2007; Simpson and Roger 2004; Simpson et al. 2006, 2008). This clade containing Jakobida, Euglenozoa, and Heterolobosea is now referred to as Discoba (Hampl et al. 2009). Phylogenomic analyses indicate that the recently discovered heterotrophic flagellate *Tsukubamonas* also belongs to Discoba, but is probably more closely related to Euglenozoa and Heterolobosea than to Jakobida (Kamikawa et al. 2014).

Phylogenetic Relationships Within Jakobida

The phylogenetic relationships among jakobids, as currently understood, are summarized in Fig. 6a. Until recently, the principal problem was establishing the relationship between the first-described jakobids (the clade now known as Histionina) and the group now known as Andalucina. Analyses of morphological data supported the monophyly of all jakobids (Simpson 2003; Lara et al. 2006); however, phylogenetic trees of SSU rDNA sequences or tubulin genes never place Andalucina in a clade with other jakobids (e.g., Berney et al. 2004; Cavalier-Smith 2004; Edgcomb et al. 2001; Lara et al. 2006; Pánek et al. 2015; Simpson et al. 2002; Strassert et al. 2016), and phylogenies based on 6–7 nucleus-encoded proteins have given extremely weak support for a jakobid clade, at best (Pánek et al. 2015; Simpson et al. 2008). Nonetheless, phylogenomic analyses based on datasets of >100 proteins, or mitochondrion-encoded proteins, recover a jakobid clade with strong statistical support, with Andalucina as the sister group to other jakobids (Burger et al. 2013; Hampl et al. 2009; Kamikawa et al. 2014). Jakobid monophyly, and the deep branching of Andalucina (represented by *Andalucia*), is also recovered in analyses of genes of probable mitochondrial origin in eukaryotes (Derelle et al. 2015).

The relationships within Andalucina are currently inferred from recent analyses of SSU rDNA, which demonstrate the monophyly of Stygiellidae to the exclusion of Andalucidae (i.e., *Andalucia*) and the reciprocal monophyly of *Stygiella* and *Velundella* (Pánek et al. 2015; Fig. 6a). The relationships within Histionina are inferred from an assortment of partial, overlapping datasets. Morphological analyses and phylogenetic/phylogenomic analyses of nucleus-encoded proteins, as well as mitochondrial proteins, all support the monophyly of *Histiona* and *Reclinomonas*, suggesting strongly that Histionidae is indeed monophyletic (Burger et al. 2013; Hampl et al. 2009; Lara et al. 2006; Rodríguez-Ezpeleta et al. 2007; Pánek et al. 2015; Simpson 2003; Strassert et al. 2016). The relationships between Histionidae, Moramonadidae, *Jakoba libera*, and *Jakoba bahamensis* remain

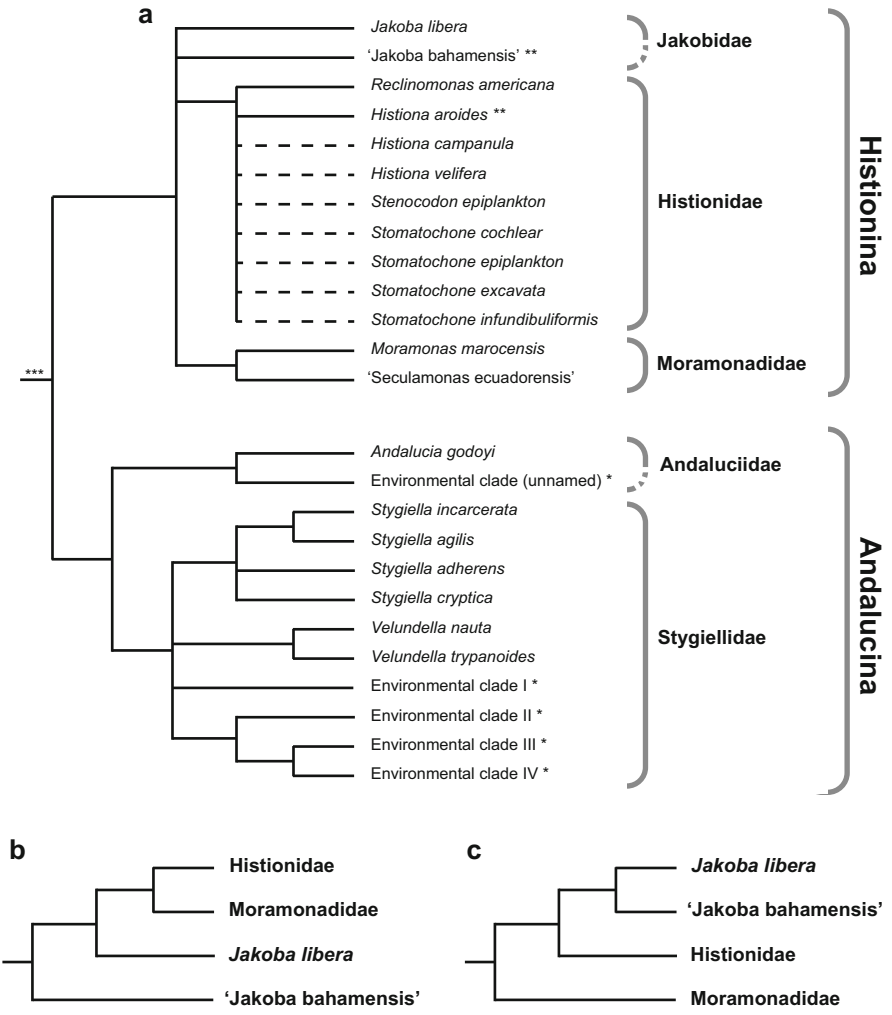

Fig. 6 Inferred phylogenetic relationships within Jakobida. (**a**) Summary phylogeny, based mainly on analyses of SSU rDNA by Pánek et al. (2015) and Strassert et al. (2016). Poorly supported branches (<65% bootstrap support in one or both studies) have been collapsed. Single asterisks represent clades containing multiple environmental sequences identified by Pánek et al. (2015); isolated environmental sequences have been excluded. Double asterisks represent species for which there are no published SSU rDNA sequences, which have been placed on the basis of multiprotein phylogenies inferred from nucleus- or mitochondrion-encoded sequences (see text). The monophyly of Jakobida (basal branch with triple asterisk) is not recovered in SSU rDNA phylogenies, but is well supported by phylogenomic analyses (see text). Dashed lines represent species for which no molecular data are available, whose positions are inferred from morphological considerations; (**b**) phylogenetic relationships within Histionina as recovered by most analyses of nuclear genes (see text); (**c**) relationships within Histionina inferred from mitochondrial genome phylogenies (e.g., Burger et al. 2013; Strassert et al. 2016)

unclear. Molecular phylogenies of nucleus-encoded proteins typically place *Jakoba bahamensis* as the deepest branch within Histionina and not specifically related to *Jakoba libera* (Hampl et al. 2009; He et al. 2014; Pánek et al. 2015; Rodríguez-Ezpeleta et al. 2007; Simpson et al. 2008; see Fig. 6b). By contrast, phylogenies of mitochondrion-encoded proteins recover *Jakoba* sensu lato as a clade, albeit with a very deep divergence between *Jakoba libera* and *Jakoba bahamensis*, and place Moramonadidae (e.g., *Seculamonas ecuadoriensis*) in the more deeply diverging position (Burger et al. 2013; Strassert et al. 2016; see Fig. 6c).

Importance for Mitochondrial Evolution

As discussed above, jakobid mitochondrial genomes encode subunits of a bacterial-type $\alpha_2\beta\beta'\sigma$ RNA polymerase, whereas all other studied eukaryotes have a single-subunit phage-type enzyme of separate evolutionary origin as their only known mitochondrial RNA polymerase. It appears that jakobids have retained this ancestral feature of the prokaryotic symbiont that became the mitochondrion, while it was replaced in all other eukaryotes. Several scenarios might explain this remarkable phylogenetic distribution (Simpson and Roger 2004; Simpson et al. 2006).

One possible scenario is that jakobids are a very early-diverging eukaryote lineage. After the divergence of jakobids, a common ancestor of all other living eukaryotes then acquired the phage-type RNA polymerase by lateral gene transfer (LGT) and lost their now-redundant bacterial-type RNA polymerase. This hypothesis is intuitively appealing, but is difficult to reconcile with the probable close phylogenetic relationship of jakobids with Heterolobosea and Euglenozoa (see above), since these latter two groups are known to have the phage-type RNA polymerase (Clement and Koslowsky 2001; Cermakian et al. 1996). This scenario would be tenable only if the eukaryotic tree should be rooted on jakobids. This would mean that the apparent Jakobida-Heterolobosea-Euglenozoa-*Tsukubamonas* clade recovered in phylogenomic analyses (Discoba; see above) is actually the basal grade for all living eukaryotes. There is no independent evidence positively supporting a jakobid rooting of the eukaryote tree (and see Derelle et al. 2015, for example, for phylogenetic evidence that the root of the eukaryote tree is more likely to lie elsewhere).

A second scenario is that the phage-type RNA polymerase has moved between eukaryote lineages through multiple events of LGT, each time replacing the bacterial-type RNA polymerase. This is less parsimonious (how much less depends on the position of the root of the eukaryote tree), but does not require that jakobids are a uniquely early-diverging lineage. For example, the scenario could be reconciled with jakobids being the sister group to Heterolobosea and Euglenozoa if it is supposed that the common ancestor of the latter two lineages was the recipient of one of these LGT events.

A third scenario supposes that it is actually the phage-type RNA polymerase that is ancestral for living eukaryotes and that the bacterial-like RNA polymerase was

more recently transferred from a bacterium into the mitochondrial genome of an ancestor of jakobids. The transfer of protein-coding genes into mitochondrial genomes is uncommon, but documented (e.g., Bergthorsson et al. 2003; Pont-Kingdon et al. 1998), including in the jakobid *Andalucia* (He et al. 2016; see above). A considerable difficulty with this scenario is that the bacterial-type RNA polymerase is a multi-subunit enzyme, and several genes would need to be transferred at once. In fact, *rpoA* in jakobids (where present) is located in its expected position within an apparent vestige of the proteobacterial alpha operon (Burger et al. 2013; Lang et al. 1999a, b), which does not include the other *rpo* genes. This supports an ancestral origin of *rpoA* rather than a recent acquisition of a set of *rpo* genes through LGT.

A fourth possibility, and perhaps the most plausible at present, is that the last common ancestor of eukaryotes had both bacterial-type and phage-type mitochondrial RNA polymerases (Stechmann and Cavalier-Smith 2002). Under this scenario, the bacterial RNA polymerase was subsequently lost in most eukaryotic lineages, but not in jakobids, which may or may not have lost the phage-type polymerase instead. This scenario does not require that the jakobids be uniquely deep-branching eukaryotes (assuming that multiple independent losses of bacterial RNA polymerase are plausible), but does imply an extended period where both polymerases are maintained together by eukaryotes.

Indirect evidence supporting this fourth scenario was presented by Shutt and Gray (2006a, b). They noted that mitochondrial RNA polymerase is only one of several nucleus-encoded mitochondrial replication/transcription proteins of possible phage origin. For example, the mitochondrial replicative helicase "Twinkle" is related to gp4 from T7 phage. Shutt and Gray (2006a) proposed that all of the phage-related proteins were acquired together, perhaps from an integrated prophage in the proteobacterial symbiont that became the mitochondrion. This model implies that jakobids would have originally had all the phage-type proteins and likely still have some or all of them. Indeed, transcripts encoding Twinkle are produced by *Seculamonas ecuadoriensis* and *Jakoba bahamensis* (Shutt and Gray 2006b). Shutt and Gray (2006a) suggest that the original function of the phage-type RNA polymerase in eukaryotes was not conventional transcription, but instead generating primers for mitochondrial DNA replication. Subsequently this enzyme was co-opted to also act as the transcriptional mitochondrial RNA polymerase, replacing the bacterial-type enzyme that originally performed this function. This co-option presumably happened several times in eukaryote evolution, and jakobids represent the only known lineage in which this did *not* occur. Appealingly, this model provides a plausible explanation as to why both polymerases could have been maintained simultaneously by early eukaryotes – they originally had different functions. Furthermore, an analogous situation occurs in the plastids of land plants, in which two different RNA polymerases transcribe different sets of plastid-encoded protein-coding genes (Gray and Lang 1998). One polymerase is of the bacterial type, while the other is of the phage type, and was apparently derived from mitochondrial RNA polymerase by gene duplication during the history of land plants.

Coda: Malawimonads

The first synthetic account of jakobid flagellates by O'Kelly (1993) included an "undescribed jakobid" from freshwater that differed from *Jakoba*, *Reclinomonas*, and *Histiona* in having discoidal mitochondrial cristae and an anterior microtubular root. This organism was later described formally as *Malawimonas jakobiformis* (O'Kelly and Nerad 1999). A related organism has been studied for more than a decade under the names *Malawimonas californiana*, *M. californiensis*, and *M. californianus* (e.g., Gray et al. 2004; Rodriguez-Ezpeleta et al. 2007; Russell et al. 2005), but still awaits formal description. An additional species of malawimonad has recently been characterized (Heiss et al. unpublished).

Like jakobids, malawimonads are small flagellates with two flagella (Fig. 7a, b) and a feeding groove supported by the "typical excavate" architecture (O'Kelly and Nerad 1999; Simpson 2003). Malawimonads, however, do not show the multilayered structure to the "C" fiber that is characteristic of jakobids, and the "dorsal fan" of microtubules mostly originates in association with the anterior "R3" microtubular root (which is absent in jakobids – see above). Malawimonads either have a pair of vanes on the posterior flagellum, as in most other "typical excavates" (Heiss et al. unpublished), or have a single vane on the ventral side of the axoneme, not

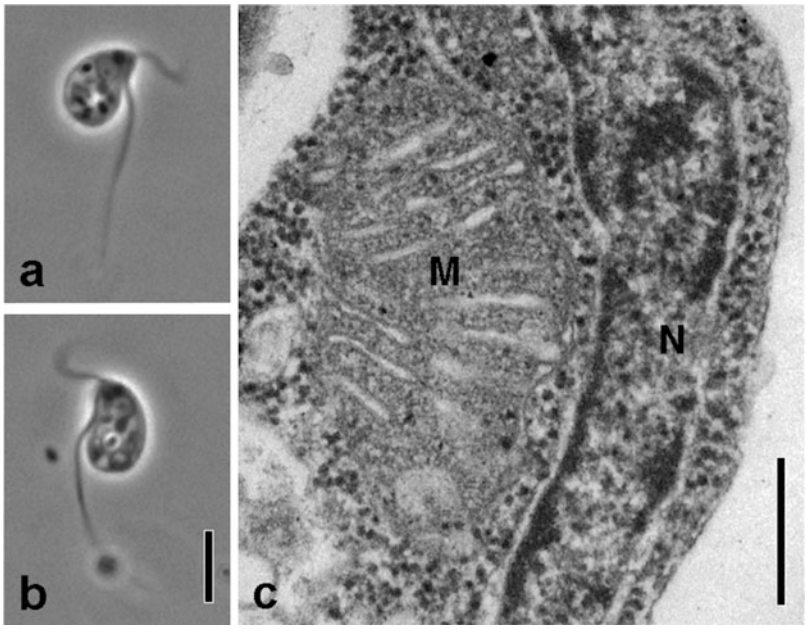

Fig. 7 Undescribed malawimonad "isolate 249": (**a**, **b**) Phase contrast light micrographs of living cells; (**c**) transmission electron micrograph showing mitochondrion with discoidal cristae and a narrow profile through the nucleus. *M* mitochondrion, *N* nucleus; scale bar in **b** represents 5 μm for **a** and **b**. Bar in **c** represents 250 nm (**a**, **b** Images by AGBS, **c** Image by A.A. Heiss)

the dorsal side as in jakobids (O'Kelly and Nerad 1999). Overall, the cell cytoskeleton of malawimonads more closely resembles that of *Carpediemonas* and other *Carpediemonas*-like organisms (CLOs), which are typical excavates that belong to the anaerobic Metamonada clade and are especially closely related to diplomonads and retortamonads (Simpson and Patterson 1999; Simpson et al. 2002, 2006; Yubuki et al. 2007; see ▶ Retortamonadida (with notes on *Carpediemonas*-Like-Organisms and Caviomonadidae)). The discoidal mitochondrial cristae noted by O'Kelly (1993) are indeed a distinctive characteristic of malawimonads as a whole (Fig. 7c). The mitochondrial genomes of *Malawimonas jakobiformis* and *Malawimonas californiana* have been sequenced (Gray et al. 2004). They are relatively gene rich, but contain many fewer genes than the mitochondrial genomes of jakobids, and in particular they lack genes encoding subunits of bacterial-type RNA polymerase (Gray et al. 2004).

Molecular phylogenies have not resolved the position of malawimonads within eukaryotes. Analyses of small numbers of nucleus-encoded genes typically (but not always) place malawimonads as close relatives of metamonads, either as the sister group to Metamonada as a whole or as sister to Preaxostyla (oxymonads and trimastigids; Simpson et al. 2006, 2008). As of early 2016, most phylogenomic analyses place malawimonads separately from other excavates, sometimes as sister to other obscure groups without close affinities to well-studied taxa, for example, collodictyonids (Derelle and Lang 2012; Derelle et al. 2015; Hampl et al. 2009; Rodríguez-Ezpeleta et al. 2007; Zhao et al. 2012). Nonetheless a close relationship with metamonads and/or other excavates has been recovered under certain specific conditions, in particular the exclusion of many rapidly evolving taxa (Burki et al. 2009; Hampl et al. 2009; Rodríguez-Ezpeleta et al. 2007). Irrespective, phylogenies of SSU rDNA, tubulins, 5–7 nucleus-encoded proteins, and phylogenomic analyses all concur that malawimonads are not specifically related to jakobids (Edgcomb et al. 2001; Hampl et al. 2009; Rodríguez-Ezpeleta et al. 2007; Simpson et al. 2002; 2006, 2008). The similarities between jakobids and malawimonads apparently reflect the ancestral morphology of "excavate" protists, or perhaps convergence, and not a close phylogenetic relationship.

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Heterolobosea

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Abstract

Heterolobosea is a group of ~150 described species of heterotrophs, almost all free living. Many are "amoeboflagellates" with a three-phase asexual life cycle, centered on trophic amoebae that can reversibly transform into flagellates (some of which undertake phagocytosis and/or division) and cysts. The amoebae are usually lobose, with "eruptive" pseudopodia. Flagellates typically have two or four near-parallel flagella and, if phagocytic, a feeding groove and/or elongate cytostome. Some taxa have simpler lifecycles, for example, *Vahlkampfia* spp. apparently lack flagellates, while *Percolomonas* and *Stephanopogon* lack amoebae. *Stephanopogon*, uniquely, has numerous flagella in rows like the kineties of ciliates. Acrasids, meanwhile, are terrestrial "slime molds" in which amoebae aggregate to form stalked fruiting bodies. The mitochondria are often enveloped in endoplasmic reticulum and usually have discoidal cristae, while the Golgi apparatus lacks dictyosomal stacking. Most flagellates have a "doubled" flagellar apparatus with two sub-identical halves. The flagellar apparatus typically includes a large "R2" microtubular root (or two) and a striated rhizoplast. Most heteroloboseans are marine, freshwater, or terrestrial aerobes, but the group shows considerable ecological breadth, for example,

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Psalteriomonadidae and *Creneis* are anaerobes, three groups are (mostly) obligate halophiles, and many species are thermophiles. The best-known genus is *Naegleria*. *Naegleria gruberi* is a cell biology model (e.g., for flagellar apparatus development). *Naegleria fowleri* is a facultative human pathogen responsible for primary amoebic meningoencephalitis (PAM). This infection is usually acquired from warm water via the nasal passages; it is extremely rare but almost always fatal.

Keywords

Amoeba • Amoeboflagellate • Cyst • Discoba • Excavata • Extremophile • Flagellate • Heterolobosea • *Naegleria* • Pathogen • Protozoa • Slime mold

Contents

Summary Classification

●**Heterolobosea**
●●**Pharyngomonada** (*Pharyngomonas*)
●●**Tetramitia**
●●●**Acrasidae** (e.g., *Acrasis*, *Allovahlkampfia*, *Pocheina*)
●●●**Creneidae** (*Creneis*)
●●●**Gruberellidae** (*Gruberella*, *Stachyamoeba*)

●●●**Percolatea**
●●●●**Percolomonadidae** (*Percolomonas*)
●●●●**Stephanopogonidae** (*Stephanopogon*)
●●●**Psalteriomonadidae** (e.g., *Harpagon*, *Psalteriomonas*, *Sawyeria*)
●●●**Tulamoebidae** (*Pleurostomum*, *Tulamoeba*)
●●●**Vahlkampfidae*** (e.g., *Heteramoeba*, *Naegleria*, *Tetramitus*, *Vahlkampfia*)

*Vahlkampfidae is known to be highly paraphyletic.

Introduction

General Characteristics

The taxon Heterolobosea, Page and Blanton, 1985 (Excavata: Discoba), unites a diverse array of heterotrophic amoeboflagellates, amoebae, flagellates, and certain "slime molds" (the acrasids) that had previously been studied in relative isolation from one another (Page and Blanton 1985; Roger et al. 1996; Yubuki and Leander 2008; Brown et al. 2012a; Harding et al. 2013). Typical heteroloboseans are amoeboflagellates that have distinct amoeba and flagellate stages. In most species the amoeba stage can also transform into a cyst, resulting in a characteristic three-phase life cycle, with amoebae as the central phase (Fig. 1). This life cycle is likely an ancestral feature of Heterolobosea (Harding et al. 2013); however, many lineages seem to have lost the ability to form flagellates, or, conversely, do not form amoebae in culture (see Page 1988; Patterson et al. 2000; Pánek and Čepička 2012). Heterolobosean amoebae are typically limax, with eruptive pseudopodial formation, characterized by sudden outwards (and lateral) bulging during locomotion (Page 1978; Page and Blanton 1985). Flagellates usually possess two or four flagella. They often have a feeding groove and a generally reduced version of the flagellar apparatus cytoskeleton of "typical excavates" (Simpson 2003; Brugerolle and Simpson 2004; Park and Simpson 2011; Pánek et al. 2014b), but there are several exceptions, with the multiflagellated "pseudociliate" *Stephanopogon* being particularly notable (Yubuki and Leander 2008). The acrasids differ from other heteroloboseans in that they form multicellular sorocarps through an aggregative process (Fig. 2), which is

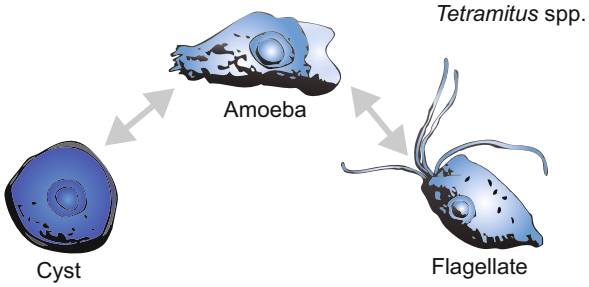

Fig. 1 Typical three-phase life cycle of Heterolobosea, as seen in the genus *Tetramitus*, with amoebae as the central phase

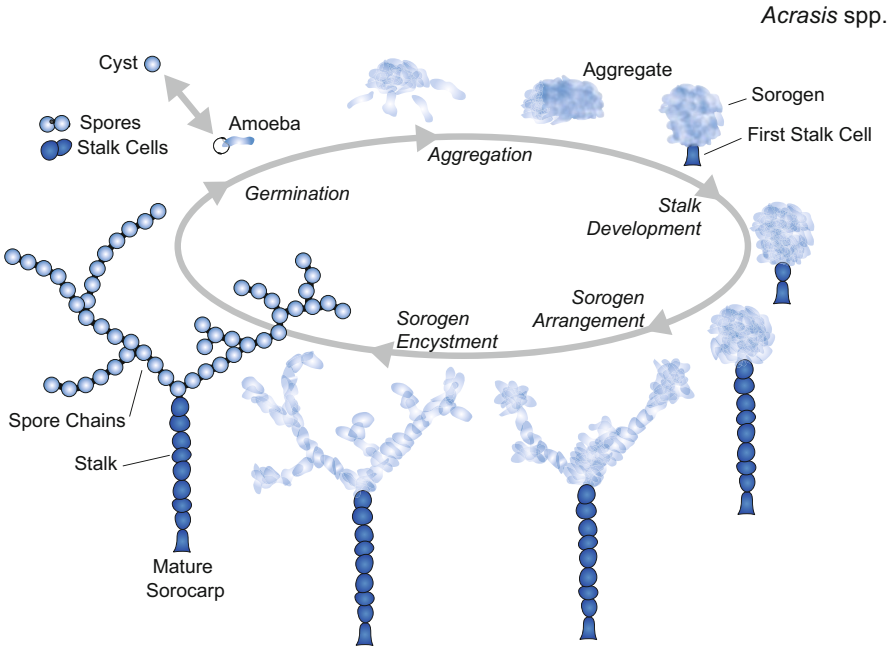

Fig. 2 Life cycle of acrasids, as seen in the genus *Acrasis*, with acrasoid sorogenesis. Mature sorocarp arranged as a branched structure with several chains of spores. Sorocarp displays basal stalk cells (*dark blue*) with distal spore cells (*light blue*). Spores germinate as limax amoebae. Amoebae may form cysts, which can germinate as amoebae. Amoebae aggregate to form small mounds. When aggregation ceases, cells in aggregate encyst to become the stalk, with a mass of amoebae (sorogen) remaining on top. Cells of the sorogen align into chains and then encyst to become spores. Developmental process descriptions are italicized. Names of structures are in regular font

somewhat similar to that seen in dictyostelid slime molds (Brown et al. 2012a; Brown and Silberman 2013; see ▶ Dictyostelia).

Almost all known heteroloboseans are free-living microbivores. *Naegleria fowleri* is a facultative parasite of humans that causes fatal meningoencephalitis (Carter 1970). Despite advances in drug treatment and supportive care, the mortality rate associated with the disease has remained over 95% (http://www.cdc.gov/parasites/naegleria/general.html; Siddiqui and Khan 2014). A close relative, *Naegleria gruberi*, is a useful cell biology model for examining development of the flagellar apparatus (Lee 2010; Fritz-Laylin and Fulton 2016). Heterolobosea have also attracted attention because of their ecological diversity, as the group includes many thermophiles, several clades of halophiles, and a considerable diversity of anaerobes, in addition to mesophilic soil-dwelling, freshwater, and marine forms (e.g., Reeder et al. 2015; Park and Simpson 2011; Pánek et al. 2012). Currently, the group comprises ~150 described species and 35 genera assigned to nine families and two main clades, Pharyngomonada and Tetramitia. Pharyngomonada is a

plesiomorphic group that contains a single described genus, *Pharyngomonas*, most isolates of which are halophilic amoeboflagellates. Tetramitia is morphologically and ecologically much more diverse and includes almost all described heterolobosean species.

Occurrence

Heteroloboseans have been reported from all continents, including Antarctica, and contribute to microbial communities from tropical to polar regions. Most (~86 species including all 47 *Naegleria* spp.) have been reported from soil and freshwater. Other species inhabit a wide range of habitats, including thermal springs, hypersaline brines, anoxic sediments, intestinal tracts of animals, bat guano, acidic rivers, and marine and brackish sediments. The human pathogen *Naegleria fowleri* is amphizoic; its primary habitat is warm bodies of freshwater (Visvesvara et al. 2007). Some other species are suspected to be opportunistic pathogens of animals (see De Jonckheere 2011). Culture-based approaches continue to reveal new heterolobosean species and genera (e.g., Brown et al. 2012a; Pánek et al. 2012, 2014a, b; Geisen et al. 2015; Park and Simpson 2016; Tyml et al. 2016). By contrast, environmental PCR-based methodologies have not yet contributed significantly to our knowledge of the occurrence and diversity of Heterolobosea in nature. The limited detection of Heterolobosea in such studies is almost certainly due to primer bias (Murase et al. 2014).

Literature and History of Knowledge

Although Heterolobosea has been formally recognized as a group for only three decades (Page and Blanton 1985; Roger et al. 1996), some organisms now classified as Heterolobosea were first described more than 150 years ago. Perty (1852) introduced the genus *Tetramitus* and described the well-known amoeboflagellate *Tetramitus rostratus*. A few decades later, descriptions were published of the acrasids now known as *Pocheina rosea* (Cienkowski 1873) and *Acrasis granulata* (Van Tieghem 1880), as well as an account of *Stephanopogon colpoda* (Entz 1884). These descriptions were based only on light microscopy; hence, their close relationships to each other remained hidden. In fact, they tended to be studied by different researchers; acrasids were examined primarily by mycologists, while amoebae and amoeboflagellates such as *Tetramitus* and *Naegleria* were studied by protozoologists, especially those who focused on amoebae. Meanwhile, the multiflagellated *Stephanopogon* was generally considered to be an unusual ciliate until the 1980s (Lipscomb and Corliss 1982; Patterson and Brugerolle 1988). It was transferred to Heterolobosea only recently, based on molecular phylogenies (Cavalier-Smith and Nikolaev 2008; Yubuki and Leander 2008).

Vahlkampfiid amoebae and amoeboflagellates have been investigated by many authors before the 1970s, with studies of *Naegleria* and *Tetramitus* being quite

extensive (e.g., Bunting and Wenrich 1929; Dingle and Fulton 1966; Droop 1962). Page (1976) placed them into a separate order, Schizopyrenida, within the subclass Gymnamoebia. Two years later, Page (1978) further suggested that Schizopyrenida are closely related to Acrasida because their forms of amoeboid locomotion are similar. This grouping was formalized by Page and Blanton (1985) when they established the class Heterolobosea. This action was bolstered by ultrastructural characteristics shared by acrasids and schizopyrenids, in particular both usually have discoidal mitochondrial cristae and a characteristic mitochondrion/ER complex (Page and Blanton 1985; see below).

Prior to the 1950s, acrasids were associated with the other sorocarpic amoebae that were assigned to Mycetozoa *sensu lato* and were little studied. In 1960, Olive and Stoianovitch described a new species of the genus *Acrasis*, *Acrasis rosea*, established stable culture of this species, and documented its life cycle in detail (Olive and Stoianovitch 1960). On the basis of studies of *A. rosea*, Olive (1975) formally separated class Acrasea from other sorocarpic amoebae. Since then, several species of the genera *Acrasis* and *Pocheina* have been described and studied in detail (Olive et al. 1983; Brown et al. 2010, 2012a).

Until recently, taxonomists studying heteroloboseans have focused primarily on the amoeba and cyst stages. However, there has been an increased emphasis on flagellates over the last couple of decades (e.g., Fenchel and Patterson 1986; Brugerolle and Simpson 2004; Park et al. 2007; Park and Simpson 2011; Pánek et al. 2012). This is in line with a greater scientific interest in heterotrophic flagellates in general and with the realization that Heterolobosea is closely related to certain flagellate groups, especially Euglenozoa (see below).

The establishment of Heterolobosea as a coherent group, and an accurate understanding of its diversity and phylogenetic placement, required the use of electron microscopy and molecular sequencing techniques. The taxon Heterolobosea was primarily based on ultrastructural similarities, such as the discoidal shape of the mitochondrial cristae, the absence of dictyosomes, and the rough endoplasmic reticulum elements that surround the mitochondria (Page and Blanton 1985). Later molecular phylogenetic analyses confirmed the monophyly of the grouping (Roger et al. 1996). Page and Blanton (1985) had primarily distinguished Heterolobosea from other lobose amoebae; however, it was soon suggested that they had a closer evolutionary connection to Euglenozoa on the basis of cristae structure, as well as similarities of mitosis (Patterson 1988). A specific relationship between Heterolobosea and Euglenozoa was subsequently confirmed by molecular phylogenetics and phylogenomic analyses (e.g., Baldauf et al. 2000; Rodríguez-Ezpeleta et al. 2007; Hampl et al. 2009). Molecular studies, primarily SSU rDNA phylogenies, have also helped to reveal the relationships between particular heterolobosean lineages and to place within Heterolobosea some taxa of previously uncertain affinity (*Pleurostomum*, *Stephanopogon*; Park et al. 2007; Yubuki and Leander 2008), or newly discovered protists with bizarre morphology, specifically the amoeboid flagellate *Creneis carolina* (Pánek et al. 2014b).

Conversely, ultrastructural studies and/or phylogenetic analyses also clearly showed that some traditional heterolobosean genera are paraphyletic or polyphyletic, e.g., some nominal species originally assigned to *Tetramitus* (Vahlkampfiidae) have been transferred to *Trimastix*, then *Paratrimastix*, which are genera of Preaxostyla (Brugerolle and Patterson 1997; Bernard et al. 2000; Zhang et al. 2015; see ▶ Preaxostyla), while others have ultimately been transferred to other genera in different families within Heterolobosea, such as *Percolomonas* (Percolomonadidae; Fenchel and Patterson 1986) and *Harpagon* (Psalteriomonadidae; Pánek et al. 2012). Many purely amoeboid species were originally assigned to a single genus, *Vahlkampfia*, but some of these are now classified in *Paravahlkampfia*, or *Neovahlkampfia* on the basis of molecular phylogenetic information (Brown and De Jonckheere 1999), or, in the case of *Vahlkampfia anaerobica* (Smirnov and Fenchel 1996), transferred to *Monopylocystis* (Psalteriomonadidae; Pánek et al. 2012). One special case is *Pharyngomonas salina*, which was first described by Entz (1904) as *Trichomastix salina*. Kirby (1932) transferred this species to the *Tetramitus*, then Larsen and Patterson (1990) transferred it to the genus *Percolomonas*, and, finally, Cavalier-Smith and Nikolaev (2008) accommodated it in a newly established genus, *Pharyngomonas*. Recent molecular phylogenetic studies also revealed that some presumed acrasids in fact belong to different eukaryotic supergroups; *Fonticula* is an opisthokont (Brown et al. 2009), *Copromyxa* belongs to Amoebozoa (Brown et al. 2011), and *Guttulinopsis* has now been placed in Rhizaria, along with the peculiar amoeba *Rosculus*, previously also considered to be a heterolobosean (Brown et al. 2012b; Bass et al. 2016).

Naegleria is the best known and most studied genus within Heterolobosea. In 1899, Schardinger discovered an amoeba that was able to transform into a flagellate and called it *Amoeba gruberi* (Schardinger 1899; Alexeieff 1912) then suggested a new genus name for this organism – *Naegleria*. Before 1970, *Naegleria gruberi* was studied mainly as a model organism for amoeba-to-flagellate transformation (e.g., Willmer 1956; Dingle and Fulton 1966; Fulton and Dingle 1967). The genus attracted much more attention when Rodney Carter described *Naegleria fowleri* as an agent causing primary amebic meningoencephalitis (PAM) in humans and mice (Carter 1970). Since then, another 45 nominal species of *Naegleria* have been described and isolated from different freshwater habitats from all over the world (see De Jonckheere 2002, 2014); currently, ~30% of all described heterolobosean species belong to the genus *Naegleria*. *Naegleria fowleri* is the only species known to cause classic PAM in humans, although *Naegleria australiensis* and *N. italica* produce disease in experimental animals (see De Jonckheere 2011). Visvesvara et al. (2009) proposed that some cases of nonfatal PAM in humans could be caused by *Paravahlkampfia francinae*, another heterolobosean species.

The nuclear and mitochondrial genomes of both *N. gruberi* and *N. fowleri* have recently been published (Fritz-Laylin et al. 2010; Herman et al. 2013; Zysset-Burri et al. 2014; GenBank AF288092). The metabolism of *N. gruberi* has been inferred *in silico* using the genome data and seems to be extremely versatile. Although

considered to be fully aerobic, some important anaerobic genes have been identified as well (Fritz-Laylin et al. 2010; Opperdoes et al. 2011). Potential pathogenicity factors in *N. fowleri* have also been noted (Herman et al. 2013; Zysset-Burri et al. 2014). Some transcriptomic data are available from several other species of Heterolobosea (*Percolomonas cosmopolitus*, *Pharyngomonas kirbyi*, *Psalteriomonas lanterna*, *Stachyamoeba* sp. ATCC 50324, *Sawyeria marylandensis*), and the mitochondrial genomes of *Acrasis kona* ATCC MYA3509 and *Stachyamoeba* sp. ATCC 50324 have also been studied (Barberà et al. 2010; De Graaf et al. 2009; Fu et al. 2014; Harding et al. 2016; Rodríguez-Ezpeleta et al. 2007; Valach et al. 2014; http://data.imicrobe.us/sample/view/2025; http://data.imicrobe.us/sample/view/2029).

Practical Importance

As noted above *Naegleria fowleri* causes a disease in humans (Carter 1970) that is usually known as primary amoebic meningoencephalitis, or naegleriasis (see Visvesvara et al. 2007). This is a rare, but extremely acute, fulminant, necrotizing, and hemorrhagic meningoencephalitis that leads to death in most cases (>95%). Amoebae invade the body via intact or disrupted nasal mucosa, migrate across the cribriform plate to the brain via the olfactory nerves, and incite meningoencephalitis with rapid cerebral edema, resulting in cerebellar herniation (see Visvesvara 2010; Siddiqui and Khan 2014). Initial symptoms start about 5 days after infection (range 1–7 days) and may include headache, fever, nausea, or vomiting. Later symptoms can include stiff neck, confusion, loss of balance, seizures, and hallucinations. The disease progresses rapidly following the onset of symptoms and usually causes death within about five days (http://www.cdc.gov/parasites/naegleria/general.html). Although *N. fowleri* has been isolated in all continents except Antarctica (De Jonckheere 2011, 2014), most of the ~250 confirmed cases have been reported in developed regions (Southern USA, Europe, Australia), which is likely due to underreporting in developing countries. *Naegleria fowleri* typically infects people when they swim in warm freshwater lakes, or hot springs, or when inadequately chlorinated swimming pool water or contaminated tap water enters their nose, e.g., due to religious and cultural practices such as use of unboiled tap water in nasal cleansing equipment (Bartrand et al. 2014; De Jonckheere 2014; Siddiqui and Khan 2014).

Two other species, *Naegleria italica* and *Naegleria australiensis*, are pathogenic for experimental animals, but no human infections are recorded (see De Jonckheere 2014). Visvesvara et al. (2009) isolated a distantly related heterolobosean, *Paravahlkampfia franciae*, from the cerebrospinal fluid of a patient with typical symptoms of naegleriasis. No other protists, viruses, or bacteria were detected. This amoeba destroyed mammalian cell cultures, but did not kill experimental animals after intranasal inoculation. Some other heterolobosean are occasionally reported in studies of amoebic infections associated with keratitis; however, pathogenicity has not been clearly demonstrated (Aitken et al. 1996; Alexandrakis et al. 1998; Dua et al. 1998; Ozkoc et al. 2008).

Habitats and Ecology

Feeding and Locomotion

Most heteroloboseans feed primarily on bacteria, though in practice, amoebae are likely to consume diverse prey. For example, *Naegleria fowleri* has the ability to engulf yeasts and even human cells (John et al. 1985; Visvesvara et al. 2007). Cannibalism has been reported in some species, including *Pseudovahlkampfia emersoni* (Sawyer 1980) and *Acrasis rosea* (Olive and Stoianovitch 1960). Acrasids may feed on yeasts and conidia of ascomycetes (Olive and Stoianovitch 1960; Olive et al. 1983). Members of the genus *Stephanopogon* feed primarily on surface-associated protists, including pennate diatoms, and a variety of small flagellates (e.g., *Rhynchomonas*, *Ancyromonas*), but will also eat yeast in culture as well as bacteria (Lipscomb and Corliss 1982; Patterson and Brugerolle 1988; Yubuki and Leander 2008; Lee et al. 2014).

Heterolobosean amoebae are raptorial feeders that move relatively rapidly when compared to amoebae of some other eukaryotic groups. They are probably firmly associated with substrates most of the time, rather than being unattached and free floating.

Flagellates usually feed, although in some amoeboflagellates, notably *Naegleria*, this form instead serves as a transient motile stage (see below). Trophic flagellates usually possess a conspicuous feeding groove, and synchronized flagellar beating creates a feeding current that carries suspended particles to it. Flagellates can capture prey when swimming or can temporarily adhere to the substrate. *Harpagon descissus*, for example, attaches to surfaces by the posterior end of the cell (Pánek et al. 2012), while in *Percolomonas cosmopolitus* one of the four flagella is much longer than the cell and anchors to the substrate (Fenchel and Patterson 1986). The two anterior flagella of *Pharyngomonas kirbyi* often mediate attachment, while the two posterior flagella generate the feeding current (Park and Simpson 2011). *Percolomonas cosmopolitus* ingests food particles at the posterior end of the groove (Fenchel and Patterson 1986), while *Pharyngomonas* possesses a cytopharynx that opens into the groove's anterior portion (Kirby 1932; Park and Simpson 2011). Several genera (e.g., *Tetramitus*, *Heteramoeba*, *Pleurostomum*, and *Tulamoeba)* have retained just a short groove-like region in the anterior of the cell, or lack a feeding groove altogether, but feed using a long tubular cytostome (Droop 1962; Darbyshire et al. 1976; Kirby et al. 2015; Park et al. 2007). Some heterolobosean flagellates swim relatively rapidly, especially those with many flagella (*Creneis carolina*; *Psalteriomonas lanterna*).

Stephanopogon species differ from other heterolobosean flagellates in their basic feeding and locomotion. Although capable of swimming, they usually crawl rapidly along surfaces using their many ventral flagella. They use a well-defined anterior feeding apparatus to feed raptorially on surface-associated microbes (Yubuki and Leander 2008; Lee et al. 2014).

Acrasid amoebae have the ability to aggregate to make sorocarps, with chains of spores or a globular spore mass perched atop a stalk composed of cells

(Brown et al. 2012a). It is not known how often acrasids form sorocarps in the natural environment. Sorocarps presumably develop in response to the exhaustion of food or some other environmental cue and provide a means of dispersal by air or animals. Starvation may be induced on a Petri dish, which is where the most reliable observations of acrasids have actually been made (Olive 1975). It seems likely from such observations that sorocarps form in the morning, perhaps after a period of amoebal growth in the morning dew. Individual amoebae of acrasids are also able to form cysts, termed "microcysts," that are similar to the cysts of other heterolobosean. Microcyst formation, rather than sorocarp development, may predominate when moisture is insufficient to allow continued growth.

Habitats

Most heterolobosean species and genera have been reported from freshwater habitats or soils (~86 described species, 17 genera). However, many heterolobosean live in marine or brackish sediments (~30 species, 15 genera). Marine/freshwater transitions have been relatively common at the suprageneric level, but are rarely apparent within a single genus – in most possible exceptions, sequence data are not available, and the current generic assignments may be spurious (*Stachyamoeba lipophora*, *Percolomonas sulcatus*, *Vahlkampfia dumnonica*). One clear exception is *Harpagon salinus*, which actually branches as sister to two other *Harpagon* spp. living in freshwater habitats (Pánek et al. 2014a). Studies of anaerobic heterolobosean (Psalteriomonadidae) have shown that there are no marine/freshwater transitions reported within individual species, while at least two such transitions took place over the evolutionary history of the family Psalteriomonadidae (Pánek et al. 2012, 2014a).

Acrasids live in terrestrial environments. *Acrasis* spp. have been cultured from a variety of dead plant parts (seeds, pods, berries, inflorescences) and occasionally from the bark of living trees (see Brown et al. 2012a). *Acrasis granulata* was isolated from a beer yeast culture (van Tieghem 1880).

Heterolobosea make up a large proportion of the heterotrophic protist species recorded from extremely hypersaline habitats, including (near-) saturated brines (see Hauer and Rogerson 2005; Park et al. 2009). At least three heterolobosean lineages (*Pharyngomonas*, Tulamoebidae, and *Euplaesiobystra*) appear to be largely or entirely composed of halophiles (Park et al. 2009; Park and Simpson 2011, 2015). Strains of *Pharyngomonas salina*, *Pharyngomonas kirbyi*, *Pleurostomum flabellatum*, *Tulamoeba peranophora*, *Tulamoeba bucina*, and *Euplaesiobystra hypersalinica* have been shown experimentally to grow in media of at least 20% salinity (Park et al. 2007, 2009; Harding et al. 2013; Park and Simpson 2011; Kirby et al. 2015).

At least two groups, Psalteriomonadidae and Creneidae, adapted to life in anoxic and microoxic habitats. Psalteriomonadidae is quite a common group in anoxic sediments, both marine and freshwater; 16 species have been described (Pánek et al. 2012, 2014a; Murase et al. 2014). The taxon Creneidae currently contains a

single species, the recently described *Creneis carolina* (Pánek et al. 2014b). The mitochondrial derivatives of these anaerobic Heterolobosea do not possess cristae, and they function as hydrogenosomes in three studied psalteriomonads (Broers et al. 1993; De Graaf et al. 2009; Barberà et al. 2010).

Naegleria sp. and *Paravahlkampfia* sp. have been detected in habitats with pH <3 using environmental PCR approaches (Sheehan et al. 2003; Amaral Zettler et al. 2002), while *Tetramitus thermacidophilus* was actually isolated from an acidic hot spring. This species grows at pH from 1.2 to 6.0, with an optimal pH of 3.0 (Baumgartner et al. 2009). Recently, Reeder et al. (2015) showed that *T. thermoacidophilus* can be a dominant bacterivore in this type of environment.

Several heteroloboseans, including the three pathogenic *Naegleria* species (see above), survive and divide in temperatures around 40–45 °C (De Jonckheere 2014). *Marinamoeba thermophila*, *Fumarolamoeba ceborucoi*, *Euplaesiobystra hypersalinica*, and the undescribed species "BB2" have been shown to grow at 50 °C (De Jonckheere et al. 2009, 2011b; Park et al. 2009; Harding et al. 2013), while *T. thermacidophilus* and *Oramoeba fumarolia* will grow at temperatures up to 54 °C (Baumgartner et al. 2009; De Jonckheere et al. 2011a). There are also a few reports on psychrophilic species adapted to cold environments. The growth optimum of *Vahlkampfia signyensis* is 10 °C, and the cells die when the temperature exceeds 20 °C (Garstecki et al. 2005). *Tetramitus vestfoldii* was isolated from the microbial mat of a brackish Antarctic lake and grows at 5 °C (Murtagh et al. 2002).

Some heteroloboseans have been found in animal intestinal tracts or associated with microbial infections, including *Allovahlkampfia* sp. (Ozkoc et al. 2008), *Naegleria* spp. (De Jonckheere 2014; Dyková et al. 2006), *Paravahlkampfia* spp. (Schuster et al. 2003; Visvesvara et al. 2009), *Percolomonas sulcatus* (Brugerolle and Simpson 2004), *Pseudovahlkampfia emersoni* (Sawyer 1980), and *Tetramitus ovis* (De Jonckheere and Brown 2005a). So far, parasitologists have paid almost no attention to endobiotic heteroloboseans other than *Naegleria*. Recently, a new species, *Neovahlkampfia nana*, was isolated from degraded gill tissue from rainbow trout (Tyml et al. 2016), but it is unclear whether this organism is a true ecto- or endobiont.

Characterization and Recognition

General Characteristics

Amoebae

Almost all heterolobosean amoebae are cylindrical and more-or-less monopodial (i.e., limax), and usually move relatively rapidly via broad hyaline bulges (Fig. 3a–g, j). The sudden "eruptive" formation of these hyaline bulges distinguishes them from most lobopodial limax amoebae from the taxon Amoebozoa. They are able to feed by phagocytosis using their pseudopodia. Some *Naegleria* spp., including *N. fowleri*, are known to produce sucker-like "amebostomes" as special structures

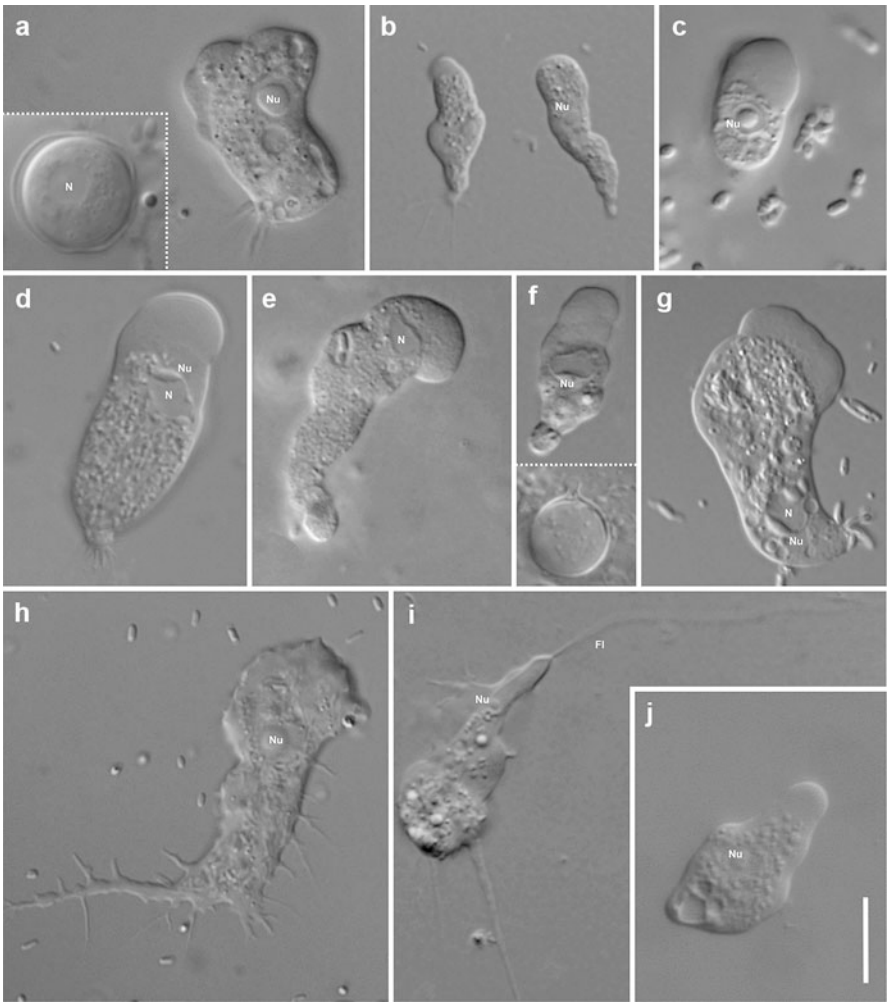

Fig. 3 Light micrographs of living heteroloboseans in amoeba and cyst stages. All micrographs are differential interference contrast: (**a**) *Tetramitus* sp. cyst and amoeba stage; amoeba with clearly visible central nucleolus and long filiform pseudopodia in the cell's posterior. (**b**) *Neovahlkampfia damariscottae* strain CCAP 1588/7. (**c**) *Naegleria* sp. strain H1N; amoeba with central nucleolus. (**d**) *Selenaion koniopes*; amoeba with parietal nucleoli. (**e**) *Pseudoharpagon longus* strain EVROS1; border between hyaloplasm and granuloplasm is less apparent than in other heterolobosean amoebae. (**f**) *Monopylocystis* spp. cyst and amoeba stage; amoeba of *M. elegans* strain EVROS1M with parietal nucleoli and cyst of *M. visvesvarai* strain TSUKIMV with single conspicuous pore with a plug. (**g**) *Psalteriomonas lanterna* strain BUKITLA; amoeba with parietal nucleoli. (**h**) *Pharyngomonas turkanaensis* strain LO; amoeba stage. (**i**) *Creneis carolina* strain PC4AM; amoeboid cell with single flagellum. (**j**) *Acrasis kona*. *Abbreviations: N* nucleus, *Nu* nucleolus. Scale bar represents 10 μm (**a** image by Yana Eglit; **c** by Petr Táborský; **f** (cyst) by Ivan Čepička; **h** by Tommy Harding)

for phagocytosis (John et al. 1984). Amebostomes vary in size depending on prey (John et al. 1985; Visvesvara et al. 2007).

Amoebae of the deep-branching taxon *Pharyngomonas* differ from typical heterolobosean amoebae because they are flattened, with an undulate or crenulated anterior edge. Some of them trail long filiform pseudopodia from the posterior end, and they generally move slowly (Fig. 3h). More typical eruptive pseudopodial formation has been observed in some *Pharyngomonas* strains, however, particularly under conditions of stress (Harding et al. 2013; Plotnikov et al. 2015). *Stachyamoeba lipophora* is also able to form two distinct morphotypes of amoebae. One is a typical limax form with eruptive lobopodia, while the other is rather flattened, with spine-shaped pseudopodia produced from an anterior hyaline zone (Page 1975, 1987). The amoeboid stage of *Creneis carolina* produces an extremely flattened, hyaloplasmic pseudopodium at the anterior end; no eruptive bulges have been observed (Fig. 3i). In addition, *C. carolina* is the only known heterolobosean that maintains flagella in the amoeboid stage (Pánek et al. 2014b).

Flagellates

Heterolobosean flagellates typically possess either two flagella (e.g., *Heteramoeba*, *Euplaesiobystra*, *Pleurostomum*, most *Naegleria* spp.) or four flagella (e.g., *Pharyngomonas*, *Percolomonas*, *Harpagon*, *Pseudoharpagon*, most *Tetramitus* spp.). These usually arise apically or subapically at the anterior end of the feeding apparatus, where present (Fig. 4a–d). Only a few heterolobosean species have a different number of flagella in a single mastigont. *Creneis carolina* possesses just a single flagellum in its amoeboid stage, but more than ten flagella in a distinct multiflagellate stage (Figs. 3i and 4h). *Tetramitus jugosus* and *Oramoeba fumarolia* flagellates possess two flagella, but cells with more flagella (up to ten in *O. fumarolia*) were also reported in these species (Darbyshire et al. 1976; De Jonckheere et al. 2011a). Most flagellates of *Pseudoharpagon longus* possess five flagella, while 20% of cells have more than five flagella (typically ten in two clusters, see Fig. 4e). *Psalteriomonas lanterna* has four nuclei and four mastigonts, each with four flagella, and four feeding grooves (Broers et al. 1990; Fig. 4f, i). *Trimastigamoeba philippiensis* is unusual because its four flagella arise from the bottom of a gullet-like tube (Bovee 1959).

The feeding groove arises subapically in most heteroloboseans (e.g., *Percolomonas*, *Harpagon*, *Pseudoharpagon*, *Psalteriomonas*, *Pharyngomonas*). *Tetramitus* and *Heteramoeba* instead possess a groove-like region that opens anteriorly, while *Pleurostomum* and *Tulamoeba* have a long tubular cytostome only. Most *Naegleria* spp. have no groove-like structure remaining.

Representatives of the genus *Stephanopogon* have little in common with other heterolobosean flagellates in terms of general morphology. *Stephanopogon* cells are vase shaped with a flattened "neck" region and dorsoventrally flattened (Fig. 4g). The ventral side of the cell bears numerous flagella (several dozen to more than 100),

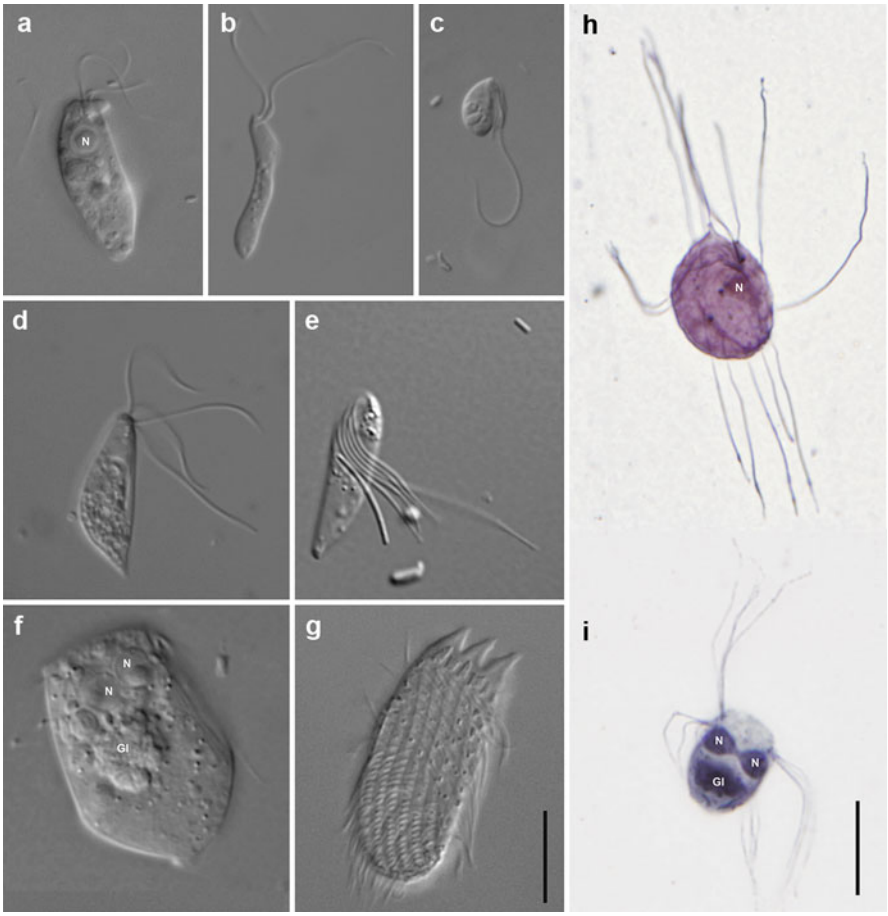

Fig. 4 Light micrographs of heteroloboseans in the flagellate stage. (**a**–**g**) Differential interference contrast images of live cells, (**h**–**i**) protargol-stained cells: (**a**) *Tetramitus* sp. (**b**) *Tulamoeba peronaphora*. (**c**) *Percolomonas cosmopolitus*. (**d**) *Pharyngomonas kirbyi*. (**e**) *Pseudoharpagon longus* strain EVROS1. (**f**) *Psalteriomonas lanterna* strain BUKITLA; (**g**) *Stephanopogon pattersoni*. (**h**) *Creneis carolina* strain PC4AM; multiflagellate. (**i**) *Psalteriomonas lanterna* strain KORTUN. *Abbreviations*: *Gl* globule of aggregated hydrogenosomes, *N* nucleus, *Nu* nucleolus. Scale bars represent 10 μm (**a** image by Yana Eglit; **d** by Jong Soo Park; **g** by Won Je Lee)

while just a few flagella arise from the dorsal side, mainly near the base of the "neck" (Yubuki and Leander 2008). The cytostome is slit shaped, with a bulging dorsal lip, and is accompanied by large ventral "barbs" in most species (Yubuki and Leander 2008; Patterson and Brugerolle 1988; Lipscomb and Corliss 1982; Lee et al. 2014; see Fig. 4g).

Cysts

Cyst stages are spherical or subspherical and usually have distinct outer and inner layers (i.e., an ectocyst and endocyst, see Fig. 3a, f). Some heterolobosean cysts have no pores and presumably excyst by wall rupture (e.g., *Paravahlkampfia* spp., some *Tetramitus* spp.); others have one or more pores that penetrate either both layers (e.g., *Naegleria*, *Pernina*) or just the endocyst (*Euplaesiobystra hypersalinica*). Mature cysts of *Selenaion koniopes* are covered by numerous granules about 100 nm across, which are visible by electron microscopy (Park et al. 2012).

Acrasids form two types of cysts – individual microcysts and spore cells of the fruiting bodies (Fig. 5a, b). Spores are distinguished by the presence of "hilae," disc-shaped structures that connect adjacent spore cells in the sorocarp (see below). Hilae are absent in microcysts, which are rounded.

Acrasid Sorocarps

Members of the Acrasidae may develop a simple multicellular fruiting body (sorocarp, Fig. 5c–e). The cells in the mature sorocarp are differentiated into two types: basal stalk cells and distal spore cells. They do not undergo programmed cell death, however, and stalk cells retain viability. The complexity of the sorocarp varies among species, from elaborate arborescent sorocarps to simple uniseriate chains of spores, to globose sori. The sorocarp structure can be used to identify the species within the group (Brown et al. 2010, 2012a).

Life Cycle

Typical Amoeboflagellates

The classic heterolobosean life cycle consists of three distinct forms: amoeba, flagellate, and cyst (Fig. 1). The amoeba stage plays a central role since it is able to encyst and transform (reversibly) to the flagellate form. The flagellate may be purely a dispersal stage, incapable of feeding. However, flagellates of many amoeboflagellates are capable of feeding and division, and a clone can often exist as flagellates for numerous generations (e.g., *Tetramitus*, *Heteramoeba*, *Monopylocystis*, *Pseudoharpagon*, *Pharyngomonas* – Bunting and Wenrich 1929; Droop 1962; Harding et al. 2013; Pánek et al. 2012; Plotnikov et al. 2015). This three-phase life cycle, with the central role of the amoeba stage, is probably ancestral for Heterolobosea since it has been reported from both major clades, Pharyngomonada and Tetramitia (Harding et al. 2013).

The life cycle of *Naegleria gruberi* has been studied in detail. The main active stage is the amoeba, which has no flagella and no basal bodies or cytoplasmic microtubules (Fulton and Dingle 1971). It is capable of both feeding and division. Under stress conditions (e.g., changes in temperature or osmolarity, or starvation), the amoebae rapidly transform into cysts, or flagellates. The highly resistant cysts are

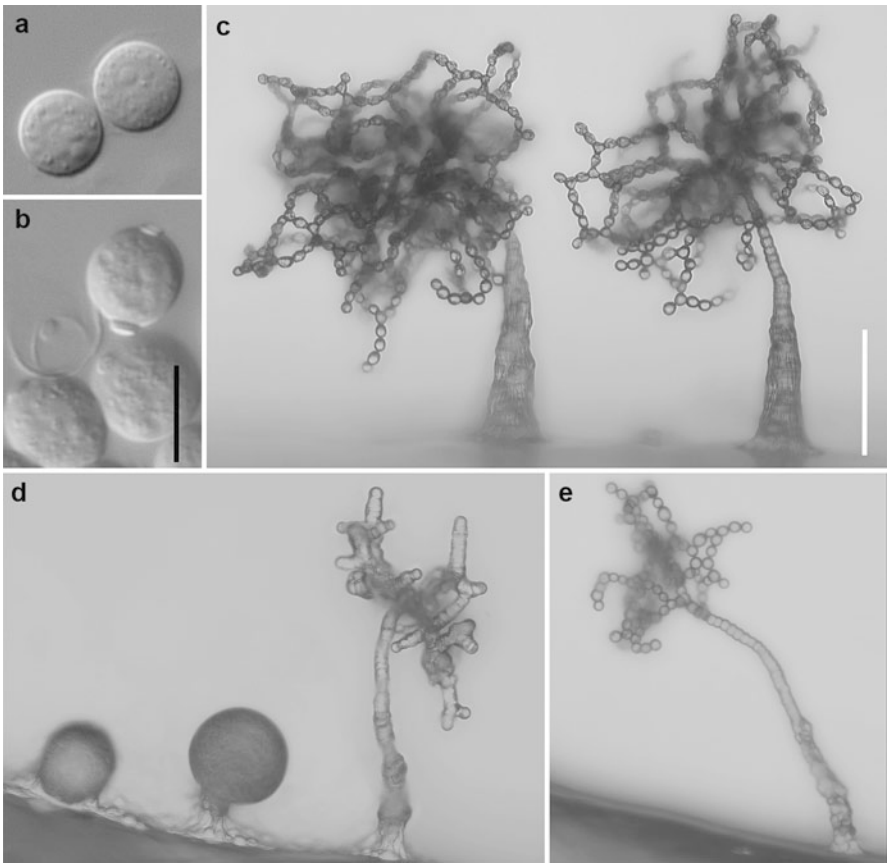

Fig. 5 Light micrographs of *Acrasis kona* in different life stages. (**a**, **b**) Differential interference contrast, (**c**–**e**) bright-field micrographs. (**a**) Round cysts. (**b**) Spores with prominent hilae. (**c**) Sorocarps with a thick-based stalk that is tapered to a uniseriate row of stalk cells; highly complex branching of the sorocarp. (**d**) Developing sorocarps in various stages. From *left* to *right*: early sorogen before stalk formation, sorogen atop a developed short stalk, and a sorocarp about to sporulate. (**e**) Mature sorocarp. Scale bars represent 10 μm (**a**, **b**) 100 μm (**c**–**e**), respectively

double walled, with three or more plugged pores. Excystation is achieved by dissolving the plugs. The cells excyst in the amoeba form.

The flagellate of *N. gruberi* is a temporary stage that persists only for a few hours and has no ability to divide or feed; it instead plays a role in dispersal (Preston and King 2003). During amoeba-to-flagellate transformation, the entire microtubular cytoskeleton of the flagellate is formed *de novo*. Fulton and Dingle (1971) examined 250 sections through a number of *N. gruberi* cells during various phases of this transformation, and only observed basal bodies in cells fixed 55 min or more after initialization. Similarly, fluorescence microscopy examinations by Walsh (2007) identified the first putative basal bodies 50–55 min after the onset of transformation,

although some other microtubules were detected earlier. Recently, Fritz-Laylin et al. (2016) showed that basal body assembly is actually a two-step process. The first basal body is assembled *de novo* within 40 min from the initiation of differentiation. The second basal body is then assembled adjacent to the elongating first basal body ("mentored" or "templated" assembly) over the next 10 min. Some authors argue for the existence of a transient duplicating complex that provides a site for the *de novo* assembly of the next generation of basal bodies (Lee et al. 2015). Transformation also involves the formation of the microtubular roots and non-microtubular elements of the flagellar apparatus, such as the rhizoplast (see below). The whole transformation process takes ~120 min.

Flagellates in nutrient-rich medium revert to amoebae by kinetid dedifferentiation; they lose their fixed shape and resorb their flagellar apparatus completely (Fulton 1977, 1983). In some other *Naegleria* spp. (*N. minor* and *N. robinsoni*), the juvenile flagellates possess four flagella and divide once to form biflagellate cells (De Jonckheere 2002).

Acrasids

The life cycle of acrasids includes the distinctive process of aggregative sorocarp formation (sorogenesis). The limax-type amoebae resemble other heterolobosean amoebae; they live solitarily, feed, multiply, and can encyst. During sorogenesis, they begin to aggregate and form a simple multicellular stage; the signalling mechanism is unknown. During this process, cells retain their individuality (i.e., no plasmogamy occurs), although they are covered by a common extracellular slime sheath. Aggregated amoebae form a "sorogen" – a mass of undifferentiated amoeboid cells that are in process of fruiting. Eventually, sorogens begin to rise from the substratum as amoeboid cells, then encyst to form the cuboidal cells of the stalk. Generally, acrasid sorogenesis follows one of two patterns – acrasoid and pocheinoid (Brown et al. 2012a). In acrasoid sorogenesis (Fig. 2), the sorogen usually begins to invaginate and lobe to form finger-like projections (though lobing does not occur in small sorogens; the sorogen simply elongates). These projections elongate and branch as the amoeboid cells maneuver themselves into a single line while still remaining within the slime sheath. Once the cells forming these branches are in single rows, they round up and form walls to become spores. The acrasoid fruiting structure ranges from a single chain of spores supported by a uniseriate stalk (a single row of stalk cells) to a large sorus of branching chains of spores perched on a stalk composed of several rows of stalk cells. In the pocheinoid type of sorogenesis, the spore mass instead remains globose; the sorocarp develops directly into this rounded mass of spores without forming chains or branches.

The thin slime sheath is often missing from around the spore chains, but generally remains over the stalk cells. Most isolates will not form sorocarps in either constant light or darkness; they require instead a dark period with a strain-specific minimal length of about 8 h (Reinhardt 1968).

The acrasid *Pocheina flagellata* also forms flagellates, but only upon germination of the stalk cells and spores and then only if the germinating cell has first undergone

mitosis. If the cell fails to divide, germination yields a single amoeboid cell (Olive et al. 1983). The flagellate cells become amoebae within 24 h. Amoebae are not known to convert directly to flagellates, but given the paucity of data, this transformation cannot be discounted. Flagellates have two flagella that appear to be apical, but their exact point of insertion and orientation during swimming has been difficult to observe. The elongated flagellates bear pellicular surface striations and a longitudinal groove (Olive et al. 1983).

Exceptions to the Typical Life Cycles

Differences in the presence of certain stages in the life cycle have been reported for different species within a single genus and even for different strains of a single species. For example, although most *Naegleria* and many *Tetramitus* species are known to produce flagellates, attempts to induce amoeba-to-flagellate transformation *in vitro* have been unsuccessful for others (e.g., Darbyshire et al. 1976; De Jonckheere et al. 2001; De Jonckheere 2007). Strains of particular species known to produce flagellates (*Psalteriomonas lanterna*, *Heteramoeba clara*, *Willaertia magna*, some *Tetramitus* spp.) or cysts (*Percolomonas cosmopolitus*) were observed to lose these abilities after prolonged cultivation (Broers et al. 1990; Droop 1962; Fenchel and Patterson 1986; Page 1988). In addition, the abundance of transformed flagellates depends on the type of bacterial prey (shown in *Oramoeba fumarolia*; De Jonckheere et al. 2011a). Recently, it has been found that members of some genera previously considered to be purely amoebae or flagellates are, in fact, able to form all three life stages, e.g., *Pharyngomonas* (Harding et al. 2013), *Pseudoharpagon* (Pánek et al. 2014a), *Tulamoeba* (Kirby et al. 2015), and *Monopylocystis* (Pánek et al. 2014a). Based on these results, one could speculate that most Heterolobosea currently known as strict flagellates are capable of forming amoebae under certain conditions. However, the closely related genera *Percolomonas* and *Stephanopogon* probably truly lost the amoeba stage, at least as a central stage in the life cycle; interestingly, members of both genera are able to encyst from the flagellate stage, which is atypical among Heterolobosea (Fenchel and Patterson 1986; Lwoff 1936; Raikov 1969).

The life cycle of *Stephanopogon* is very unusual since the number of nuclei within a flagellate cell increases during its life and only "mature" cells with several to many nuclei will encyst. Before germination from the cyst, the cell undergoes cytokinesis, which results in the formation of several daughter cells, each possessing a single nucleus that is subsequently duplicated prior to excystation (Lwoff 1936; Raikov 1969). The life cycle of *Creneis carolina* is even more atypical (Pánek et al. 2014b). It includes an amoeboid flagellate stage with 1–2 long anterior flagella and a flagellate with more than 10 flagella. The amoeboid flagellate is the main trophic stage in culture. The multiflagellate stage possesses two anterior flagella (which are probably homologous to the flagella of the amoeboid cell) and multiple lateral ones. No true cysts or aflagellated amoebae have been observed. This means that *Creneis carolina* apparently has multiple distinct life history stages, but none of them resembles those of typical heteroloboseans.

Nuclei, Mitosis, and Sex

Most heteroloboseans possess one nucleus, with a central or subcentral nucleolus (e.g., Balamuth et al. 1983; Page 1984; Park and Simpson 2011; Park et al. 2009; Schuster 1975; Tyml et al. 2016). By contrast, peripheral nucleolar material or parietal nucleoli are seen in *Selenaion*, *Heteramoeba*, *Stachyamoeba lipophora*, and Psalteriomonadidae, as shown by both light and electron microscopy (Carey and Page 1985; Droop 1962; Pánek et al. 2012, 2014a; Page 1975; Park et al. 2012; see Fig. 3d, f, g). In addition, a few multinucleate species have been described (*Fumarolamoeba ceborucoi*, *Gruberella flavescens*, *Pseudovahlkampfia emersoni*, *Psalteriomonas lanterna*, *Stephanopogon* spp.; Broers et al. 1990; De Jonckheere et al. 2011b; Lee et al. 2014; Page 1984; Sawyer 1980).

All examined heterolobosean species have closed intranuclear orthomitosis, in which the nuclear membrane remains intact until separation of the daughter nuclei and the intranuclear mitotic spindle displays axial symmetry (see Page and Blanton 1985). On the other hand, the fate of the nucleolus during mitosis differs among lineages. The nucleolus disintegrates during mitosis in *Acrasis rosea*, *Gruberella flavescens*, *Stachyamoeba lipophora*, and probably also *Pocheina flagellata* (Olive et al. 1983; Page 1978). The nucleolus persists through mitosis in other studied heteroloboseans (including *Naegleria*, *Tetramitus*, *Pernina*, and *Pocheina rosea*) and divides into two polar masses (Balamuth et al. 1983; El Kadiri et al. 1992; Olive et al. 1983; Schuster 1975). The nucleolus of *Naegleria gruberi* contains 3,000–5,000 copies of a 14 kbp circular plasmid that carries the 18S, 28S, and 5.8S rRNA genes (Clark and Cross 1987; Maruyama and Nozaki 2007). Because the nuclear chromosomes do not contain copies of these genes, it is essential that each daughter nucleus obtains a suitable portion of these plasmids during nucleolar division. The fate of the nucleolus and the structure and formation of mitotic spindle during mitosis have been studied in detail in *Naegleria pringsheimi* by confocal microscopy (Walsh 2012). This study suggests that specific nucleolar binding sites for microtubules allow mitotic spindle formation and attachment, and that spindle elongation drives nucleolar division.

The question of the sexuality of Heterolobosea has not been fully resolved yet. An isoenzyme study encompassing several dozen strains of *Naegleria lovaniensis* uncovered strong evidence of genetic recombination (Pernin et al. 1992). The presence of meiosis-associated genes in the genome of *Naegleria gruberi* was documented by Fritz-Laylin et al. (2010). Later, two key genes encoding proteins involved in gamete- and nucleus fusion (HAP2 and GEX1) were also identified in this genome (Speijer et al. 2015). A few studies have described cell fusion between heterolobosean flagellates and amoebae. Bunting (1926) documented adherence and fusion of *Tetramitus rostratus* flagellates by light microscopy, but did not report nucleus fusion. Frequently, a pair of fusing *Tetramitus* flagellates consisted of one very small and one normal-sized individual. Similarly, amoebae of *Acrasis rosea* were seen to produce anastomoses, or to fuse (Olive et al. 1961; Olive 1963). Droop (1962) studied sexual processes in the amoeboflagellate *Heteramoeba clara*. He performed crossing experiments with clonal cultures of flagellates that

all derived from a culture established from a single amoeba cell. He established six cultures that only produced flagellates (no amoebae observed over 4 months). These cultures were crossed with each other, and amoebae reappeared in certain combinations of cultures. This pattern was consistent with the existence of two mating types, with amoebae being produced only in crosses of flagellates of opposite mating types.

Ultrastructure

The Golgi apparatus does not show a classic stacked dictyosomal form. There are multiple mitochondria, which (in aerobic forms) have flattened cristae that are generally reported as discoidal and in some cases clearly take the form of rigid, pedicellate discs (Fig. 6a; Page and Blanton 1985; Fenchel and Patterson 1986; Lee et al. 2014). Anaerobic forms have mitochondrion-related organelles that lack cristae, although single crista-like structures have been reported in *Pseudoharpagon pertyi* (Fig. 7a; Pánek et al. 2014a). Many heteroloboseans show a close association of mitochondria and endoplasmic reticulum (Fig. 6b), and this was originally considered characteristic of Heterolobosea (Page and Blanton 1985). This arrangement is not seen in a number of unrelated species and genera, however (e.g., *Pharyngomonas* spp., *Stephanopogon* spp., *Percolomonas cosmopolitus*, and *Pleurostomum flabellatum;* Fenchel and Patterson 1986; Patterson and Brugerolle 1988; Park et al. 2007; Park and Simpson 2011; Lee et al. 2014; see Fig. 6b). Putative peroxisomes with a single membrane and a paracrystalline inclusion have been reported from *Pharyngomonas kirbyi* (Park and Simpson 2011) and *Selenaion koniopes* (Park et al. 2012). Various electron-dense organelles bounded by a single membrane have been observed, for example, the "black bodies" of *Naegleria* spp. and *Selenaion koniopes*, which are of unclear function (Stevens et al. 1978; Park et al. 2012), and the "dense bodies" of *Pseudoharpagon pertyi* (Pánek et al. 2014a).

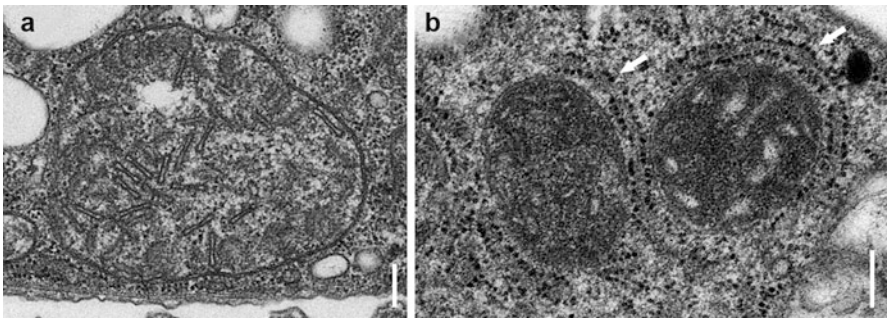

Fig. 6 Electron micrographs of mitochondria of heteroloboseans. (**a**) *Stephanopogon pattersoni* (Stephanopogonidae), showing rigidly discoidal cristae, but an absence of endoplasmic reticulum envelopment of the mitochondria. (**b**) *Selenaion koniopes* (*incertae sedis*; clade VII), showing rough endoplasmic reticulum envelopment of the mitochondria (*arrows*), but note the less defined form of the cristae. Scale bars represent 200 nm (**a** image by Won Je Lee; **b** by Jong Soo Park)

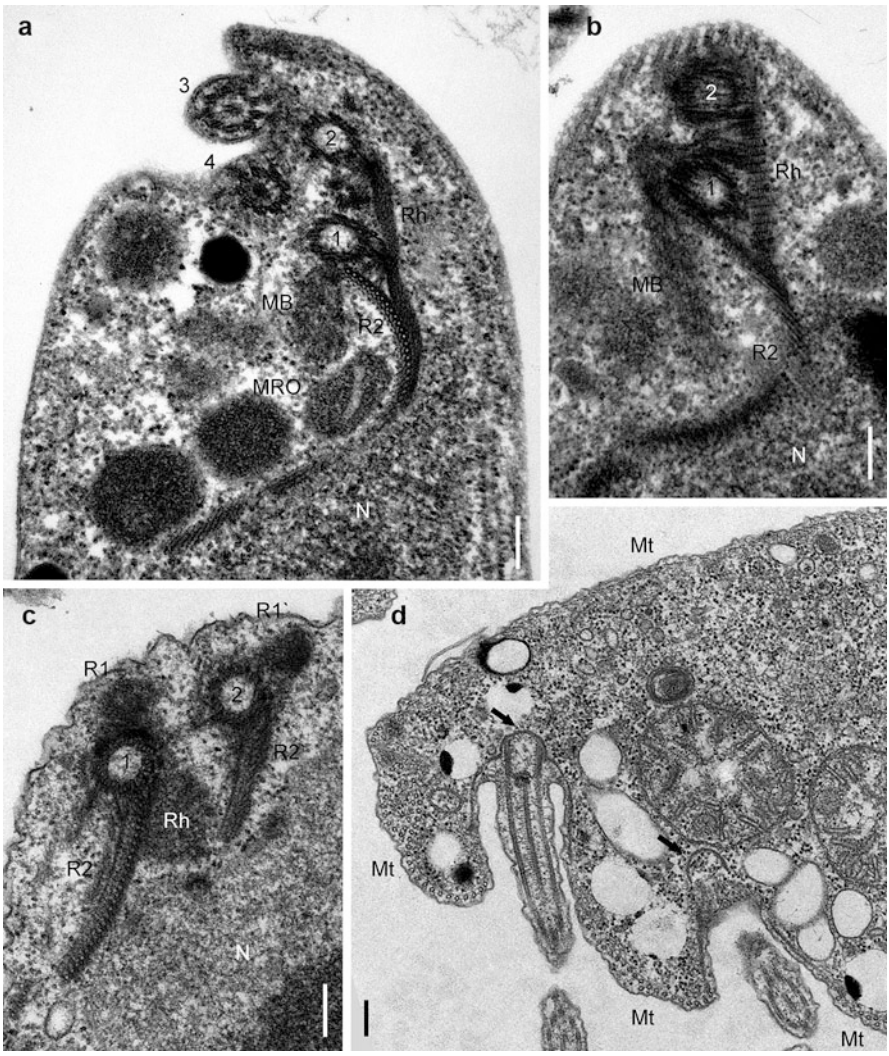

Fig. 7 Electron micrographs of the flagellar apparatus of heterolobosean. (**a**) *Pseudoharpagon pertyi* (Psalteriomonadidae) showing the R2 microtubular root supported by the rhizoplast (Rh) on one face, and the I fiber (not labelled) on the other. Note the mitochondrion-related organelles (MRO) of this anaerobe. (**b**) *Pseudoharpagon pertyi*, showing substructure of the rhizoplast and position of the microfibrillar bundle (MB). (**c**) *Pharyngomonas kirbyi* (Pharyngomonada) showing a well-developed instance of "doubled flagellar apparatus" organization, with similar microtubular root systems associated with basal body 1 (R1, R2) and basal body 2 (R1′, R2′). Note the well-developed R1 (and R1′) and diffuse rhizoplast homolog (Rh), which are particular to Pharyngomonada among Heterolobosea. The R1 and R1′ roots are sectioned obliquely here and individual microtubules are not visible. (**d**) *Stephanopogon pattersoni* (Stephanopogonidae), showing sections through two rows of flagella, with *arrows* indicating the dense material that underlies each individual basal body. Note the abundant microtubules (Mt) supporting the cell membrane, especially between the flagellar rows. *Abbreviations: 1* Basal body 1; *2* Basal body 2; *3* Basal body 3; *4* Basal body 4; *MB* Microfibrillar bundle, *MRO* Mitochondrion-related organelles, *Mt* microtubules, *N* nucleus, *R1* Root 1; *R1′* Root 1′; *R2* Root 2, *R2′* Root 2′, *Rh* Rhizoplast. Scale bars represent 200 nm (**c** image by Jong Soo Park)

Endoplasmic reticulum vesicles containing aggregations of dense material were reported in *Naegleria* spp. and *Selenaion koniopes* cells. These structures probably contain cyst wall material that is secreted from the cell during encystation (Chávez-Munguía et al. 2009; Park et al. 2012). The cytoplasm of many species contains rounded lipid droplets.

Most heteroloboseans have either two or four near-parallel basal bodies lying close together in a single mastigont (Fig. 7a–c). Heterolobosean flagellates have a peculiar "doubled" flagellar apparatus; in cells with two basal bodies, each is associated with a broadly similar set of microtubular and non-microtubular elements (Brugerolle and Simpson 2004; Park et al. 2007). In cells with four basal bodies, these form two parallel pairs, with each pair then supporting a similar set of associated cytoskeletal elements (Fig. 7c; Brugerolle and Simpson 2004; Park and Simpson 2011). In almost all cases, one of these two sets (the more posterior) is more extensive than the other; they are inferred to be developmentally linked (at least in forms with dividing flagellates) with the more anterior set transforming into the fully developed posterior set during the next round of cell division. This general organization was probably ancestral for Heterolobosea and is found today both in Pharyngomonada and many Tetramitia (except that the flagella within each pair are not parallel in *Pharyngomonas*; see below and Park and Simpson (2011)). The more developed unit of the flagellar apparatus usually includes just a single large flagellar microtubular root, now understood to represent "R2," which is associated with the posterior-most basal body (BB1, inferred to be the "eldest"; see Fig. 7a–c). The immature unit includes a (usually) reduced version of R2 (called R2` here), which is associated with basal body 2 (BB2; see Fig. 7c).

The R2 root is typically curved in cross section. It is supported on its concave face by a lattice-work "I" fiber and on the convex face by a rhizoplast system that takes the place of the "A" fiber seen in other excavates (Fig. 7a, c; Brugerolle and Simpson 2004). The rhizoplast is an elongated paracrystalline structure with regular striations (Fig. 7b). There is generally a single rhizoplast system that branches proximally to connect basal bodies BB1 and BB2, as seen in the biflagellated *Naegleria* or tetraflagellated *Harpagon* (Brugerolle and Simpson 2004); however, there can be two separate rhizoplasts, each for one pair of flagella and R2 (*Percolomonas sulcatus*; Brugerolle and Simpson 2004), or even a branching complex of rhizoplasts that extend in different directions through the cytoplasm, as reported in *Tetramitus rostratus* (Balamuth et al. 1983; Brugerolle and Simpson 2004). Purification and indirect immunofluorescence staining of the *Naegleria gruberi* rhizoplast suggested that it contains proteins homologous to UNC-119 proteins, which are best known for their expression in metazoan neural tissues, including photoreceptor cells (Chung et al. 2007). A microfibrillar bundle is "strung" across the concave face of the R2 in some taxa, notably psalteriomonads and *Percolomonas* (Fig. 7a, b; Broers et al. 1990; Fenchel and Patterson 1986; Brugerolle and Simpson 2004). The R2 root often splits into two portions to support the margins of the feeding groove, where present (Broers et al. 1990; Brugerolle and Simpson 2004; Park and Simpson 2011). The R2′ typically has an I fiber but is much shorter and typically does not split into

inner and outer portions (though *Percolomonas sulcatus* is an exception; Brugerolle and Simpson 2004).

Flagellates of the deep-branching taxon Pharyngomonada have some unusual features in their flagellar apparatus that may represent retentions of plesiomorphic features. The arrangement of basal bodies in a pair is orthogonal, not (near-) parallel as in other heteroloboseans. Pharyngomonads have retained a long R1 microtubular root with attached multilaminate C fiber originating from BB1, as seen in "typical excavates" such as the closely related jakobids (Park and Simpson 2011); because of the doubled flagellar apparatus, there is also an R1` with C fiber originating from BB2, which is actually somewhat larger than R1 (Fig. 7c). These R1 and R1′ elements join the left portion of R2 to support the left margin area of the feeding groove. Their rhizoplast homologue is not striated and not elongated (Fig. 7c). More uncertainly, the R2/I system of *Pharyngomonas* also possesses a spur-like element possibly homologous to the B fiber of other excavates (Park and Simpson 2011), although a similar structure is also seen in *Creneis* (Pánek et al. 2014b).

The cytoskeleton of *Stephanopogon* is very different from that of other heterolobosean flagellates (Fig. 7d). The ventral flagella are arranged in more-or-less longitudinal rows superficially similar to the kineties typical of ciliates. Each flagellum lies in a small depression that is supported by a ring of short, spaced microtubules. These originate from a cone-like complex of electron-dense material that surrounds the (very short) basal body. A sheetlike extension of the same or similar material passes underneath the basal body and extends to connect to the next basal body in the row (Patterson and Brugerolle 1988; Yubuki and Leander 2008; Lee et al. 2014). The few dorsal flagella are mostly arranged in pairs, though one of the flagella in a pair can be a stub (Yubuki and Leander 2008). The regions between the flagellar rows are supported by longitudinal ribbons of microtubules (Fig. 7d). The cytostome is lined by regularly spaced longitudinal microtubules. The cytoplasm surrounding the cytostome contains an array of rods, each made up of a block of cross-linked microtubules. Very large numbers of small electron-dense secretory vesicles (often referred to as extrusomes) are present in the cytostomes lips and between the longitudinal microtubules of the cytostome (Patterson and Brugerolle 1988; Yubuki and Leander 2008; Lee et al. 2014).

Hohl and Hamamoto (1968, 1969) described the ultrastructure of the sorogenic cells of *Acrasis rosea*. Numerous spherical-to-oblong granules, perhaps representing pigment granules, were present. Bundles of microfilaments in pseudopod-like extensions were found in the sorogenic cells. The nucleoli of sorogenic cells appeared to have three components: (i) dispersed, round masses of granular material; (ii) intensely staining homogeneous bodies found within the dispersed masses; and (iii) a large round mass of finely granular material. The dispersed mass contained parallel stacks of lamellar elements. This complex nucleolar structure has not been observed in either *Acrasis* trophic amoebae (Hohl and Hamamoto 1968) or those of other acrasids. Three-colored carotenoid pigments were detected in *A. rosea* (Fuller and Rakatansky 1966). One of these was identified as the xanthophyll torulene. The unidentified pigments were (i) a yellow-green carotenoid and (ii) an orange

carotenoid that had some characteristics of a xanthophyll, with the latter being the major pigment.

Taxonomy and Systematics

Page and Blanton (1985) proposed the taxon Heterolobosea to unite schizopyrenid amoebae/amoeboflagellates and acrasids, based on the presence of eruptive lobopodia, discoidal mitochondrial cristae, and the absence of stacked Golgi bodies. Fenchel and Patterson (1986) then included in Heterolobosea the newly described genus *Percolomonas*. *Percolomonas* initially housed flagellates with no known amoeba stage that had previously been assigned to the genus *Tetramitus* (whose type species is a well-known schizopyrenid amoeboflagellate). Most authors now use the name Heterolobosea for the entire clade containing all descendants of the last common ancestor of the genus *Naegleria* and *Pharyngomonas*, i.e., including both Pharyngomonadea and Tetramitia (e.g., Hampl et al. 2009; Park and Simpson 2011; Adl et al. 2012; Brown et al. 2012a; Pánek et al. 2012; Harding et al. 2013; Plotnikov et al. 2015). Contrarily, Cavalier-Smith and co-workers have used the name Percolozoa for the same clade (Cavalier-Smith 1993; Cavalier-Smith and Nikolaev 2008). They divide the phylum Percolozoa into four classes: Pharyngomonadea (*Pharyngomonas*), Percolatea (*Percolomonas*, *Stephanopogon*), Lyromonadea (*Psalteriomonadidae*), and Heterolobosea *sensu* Cavalier-Smith (1993). The latter three classes are united within the subphylum Tetramitia. In this chapter, we prefer the former "broad" concept of Heterolobosea (Heterolobosea *sensu lato*): Firstly, the restricted concept of Heterolobosea (i.e., *sensu* Cavalier-Smith 1993) refers explicitly to a paraphyletic group (Cavalier-Smith and Nikolaev 2008). Secondly, there is now good evidence that the common ancestor of all Heterolobosea *sensu lato* was an amoeboflagellate with the ability to form eruptive pseudopodia, which had discoidal mitochondrial cristae, but lacked discrete dictyosomes. This is mostly consistent with the original concept of Heterolobosea (see Page and Blanton 1985; Harding et al. 2013).

The internal classification of Heterolobosea has changed dramatically since 1985, with many new lineages having been described or transferred to the group. Cavalier-Smith and Nikolaev (2008) proposed the division of Heterolobosea *sensu lato* into two subphyla, Pharyngomonada and Tetramitia, and this is now widely accepted (e.g., Pánek and Čepička 2012). Pharyngomonada and Tetramitia both represent clades in SSU rDNA phylogenies (Cavalier-Smith and Nikolaev 2008; Harding et al. 2013). Synapomorphies of Tetramitia include a specific 17-1 helix in the secondary structure of the SSU rRNA molecule (Cavalier-Smith and Nikolaev 2008; Nikolaev et al. 2004) and, possibly, (near-)parallel basal bodies (Cavalier-Smith and Nikolaev 2008; Park and Simpson 2011). Isolate "BB2" (ATCC strain PRA-19) is an undescribed thermophilic amoeba with typical heterolobosean characteristics that remains *incertae sedis* within Heterolobosea. Phylogenetic analyses of SSU rDNA sequences indicate that isolate "BB2" might represent the sister lineage to all other Heterolobosea, or be specifically related to *Pharyngomonas*, but statistical support

either way is very low (Harding et al. 2013). An amoeba referred to as *Soginia* (a *nomen nudum*) was inferred to be a possible deep branch within Heterolobosea based on multigene phylogenies (Parfrey et al. 2010), but this is almost certainly because the SSU rDNA attributed to this organism actually derives from a gregarine (Cavalier-Smith 2015).

Pharyngomonada currently contains a single genus, *Pharyngomonas*, in its own family, Pharyngomonadidae, and class, Pharyngomonadea; these taxa currently refer to an identical set of species as Pharyngomonada and are sometimes used instead of Pharyngomonada to denote the whole group (Adl et al. 2012; Park and Simpson 2016).

Based on phylogenetic analyses of SSU rDNA sequences, Tetramitia can be divided into seven well-supported major clades that have been defined by Pánek et al. (2012, 2014a). Unfortunately, relationships between these clades remain unresolved and thus, no phylogenetic classification of subphylum Tetramitia into monophyletic classes has been proposed. Eight families of Tetramitia are currently recognized: Acrasidae van Tieghem 1880, Creneidae Pánek et al. 2014, Gruberellidae Page and Blanton 1985, Percolomonadidae Cavalier-Smith 2008 (in Cavalier-Smith and Nikolaev 2008), Psalteriomonadidae Cavalier-Smith 1993, Stephanopogonidae Corliss 1961, Tulamoebidae Kirby et al. 2015, and the paraphyletic Vahlkampfiidae Jollos 1917. Future work will hopefully allow resolution of Vahlkampfiidae into a series of monophyletic taxa. A full list of genera, divided by higher taxa, is given in Table 1. More details of the correspondence between this taxonomy and the phylogenetic structure within Heterolobosea are given in section "Evolutionary History" (see below).

Maintenance and Cultivation

Most heterolobosean amoebae and flagellates have been cultivated on bacterial prey or on nutrient-enriched media that fosters the growth of bacteria. *Naegleria gruberi* is the only heterolobosean amoeba that has been grown on a chemically defined medium (Fulton et al. 1984). Amoebae are frequently grown on agar plates (though many can also be cultivated in liquid media), while flagellates are typically maintained in liquid media. Smirnov and Brown (2004) give detailed methods for cultivation of soil amoebae in general (not just heteroloboseans), including recipes for various media and recommendations for inoculation. *Protocols in Protozoology* (Lee and Soldo 1992) is also a good source for detailed information on the cultivation of freshwater and marine amoebae.

Techniques for examining amoeba-to-flagellate transformation have been reviewed by Page (1976) and Fulton (1977). However, the precise conditions for transformation have been determined for very few species, the best known being *Naegleria gruberi*. In general, high-nutrient conditions are optimal for the feeding and dividing amoeba stage, and drastic dilution of nutrients induces the flagellate stage (e.g., by suspension in distilled water, sometimes for extended periods).

Table 1 Full list of heterolobosean genera, divided by higher taxon. Number of described species, typical habitats, and availability of SSU rDNA sequences are indicated

Family	Genus	spp.	Habitats	SSU rDNA[a]
Pharyngomonadidae	*Pharyngomonas*	3	Hypersaline	***
Acrasidae	*Acrasis*	5	Aerial	******
	Allovahlkampfia	1	Soil, aerial	******
	Pocheina	3	Aerial	*
	Solumitrus	1	Soil	*
Creneidae	*Creneis*	1	Marine	*
Gruberellidae	*Gruberella*	1	Marine	–
	Stachyamoeba	1	Marine, fresh water	*
Percolomonadidae	*Percolomonas*	4?	Marine, fresh water	**
Stephanopogonidae	*Stephanopogon*	7	Marine	***
Psalteriomonadidae	*Harpagon*	3	Fresh water, saline	******
	Monopylocystis	6	Marine, brackish, saline	******
	Psalteriomonas	3	Fresh water	******
	Pseudoharpagon	3	Marine, brackish	*****
	Sawyeria	1	Fresh water	***
Tulamoebidae	*Pleurostomum*	6	Hypersaline	*****
	Tulamoeba	2	Hypersaline	**
Vahlkampfiidae[b]	*Fumarolamoeba*	1	Soil	*
	Heteramoeba	1	Marine	*
	Marianamoeba	1	Marine	*
	Naegleria	47	Fresh water, some spp. facultative endobionts	******
	Neovahlkampfia	2	Marine, fresh water	**
	Parafumarolamoeba	1	Soil	*
	Paravahlkampfia	3	Soil, some spp. endobiotic	****
	Pseudovahlkampfia	1	Marine, endobiotic	–
	Tetramastigamoeba	1	fresh water	–
	Tetramitus	15	Soil, fresh water, acidic hot springs	******
	Vahlkampfia	6?	Fresh water, marine	**
	Willaertia	1	Fresh water	***
No family affiliation	*Euplaesiobystra*	1	Hypersaline	*
	Oramoeba	1	Marine	*
	Pernina	1	Marine	–
	Selenaion	1	Hypersaline	*
	Trimastigamoeba	1	Fresh water	–
	Vrihiamoeba	1	Soil	*

[a]Six asterisks indicate that 6+ sequences are available
[b]Vahlkampfiidae is currently a nonmonophyletic and essentially artificial assemblage

Anaerobic Heteroloboseans

Anaerobic amoebae and flagellates can be isolated from marine, brackish, or freshwater sediments. A typical strategy is to initially inoculate approximately 2 ml of sample into 9 ml of relatively rich sterile medium in 15 ml culture tubes (Pánek et al. 2012). Freshwater strains are isolated in Sonneborn's *Paramecium* medium (ATCC medium 802, solution 1) or 3% LB medium. Marine and brackish strains are isolated in seawater 802 medium (ATCC medium 1525) or 5% TYGM-9 medium (ATCC medium 1171) prepared with sterilized seawater. The strains are maintained in xenic agnothobiotic cultures at room temperature and subcultured once a week.

Halophilic Heteroloboseans

Halophilic and halotolerant heteroloboseans have usually been isolated in, and grown on, liquid medium consisting of 'Medium V' (Park 2012; previously known as 'AS medium'), typically at 10–25% final salinity (Park et al. 2007, 2009, 2012; Park and Simpson 2011, 2015; Harding et al. 2013). Halotolerant forms such as *Selenaion koniopes, Tulamoeba bucina* and *Pharyngomonas* strain RL have also been grown in seawater-strength media (e.g., f/2, Smaltz-Pratt medium or sterilised seawater), or in Page's Amoeba Saline (PAS, Page 1988) supplemented with double the NaCl concentration of seawater (2X SPAS; ~7% salinity; Park et al. 2012; Kirby et al. 2015; Plotnikov et al. 2015). Media are enriched with a carbon source to support growth of prokaryote food (often sterile barley grains and/or 0.05–1% v/v LB medium; 1.5 g/L yeast extract also used). In the case of some lower salinity media, separately grown *Pseudomonas fluorescens* or *Escherichia coli* have been added directly, usually instead of the organic enrichment. Some strains have been isolated or cultivated as amoebae on 1–2% agar plates made with Medium V at 10–25% final salinity, 2X SPAS medium, or f/2, and supplemented with separately grown bacteria (*E. coli or Salinivibrio* sp.) as a food source (Park et al. 2012; Harding et al. 2013). Subculturing is typically performed every 2–4 weeks, or longer.

Stephanopogon

Only a few *Stephanopogon* strains have been cultivated. Lipscomb (see Lipscomb and Corliss 1982) maintained a strain of *Stephanopogon apogon* for a few months (albeit with a low growth rate) using filtered seawater, split peas, and unidentified bacteria. Nerad established *Stephanopogon apogon* in monoxenic culture in ATCC medium 1405, using the kinetoplastid nanoflagellate *Rhynchomonas nasuta* as a food source; *S. apogon* has subsequently been cryopreserved (Culture: ATCC

50096). Yubuki and Leander (2008) temporarily maintained a low-abundance culture of *Stephanopogon minuta* with a standard f/2 seawater medium and a small pennate diatom (*Nitzschia* sp.) as a food source, while Lee et al. (2014) cultivated the similar *Stephanopogon pattersoni* indefinitely in sterile seawater enriched with 1% LB media together with the nanoflagellate *Ancyromonas* (and prokaryotes).

Acrasids

Acrasis spp. are most commonly encountered on dead attached plant parts and *Pocheina rosea* on the bark of living trees. For *Acrasis*, dead and decaying plant parts (inflorescences, berries, pods, fruits, etc.) still attached to the plant should be collected in paper sacks. The material is torn into small fragments and placed on Petri plates prepared with weak malt-yeast agar ('wMY agar'; 0.002 g malt extract, 0.002 g yeast extract, 0.75 g K_2HPO_4, 15 g bacteriological agar, 1.0 L deionized water; see Brown et al. 2010). A small drop of water is then placed on each piece of collected plant substrate and left to air-dry with the Petri dish lid on. The plant materials and surrounding agar surfaces should be examined microscopically after 3–5 days. If the sorocarps of acrasids are observed, the end of an insect minuten needle can be used to collect spore chains, best done under a dissecting microscope. The spores should then be placed on wMY agar with a streak of the yeast *Rhodotorula mucilaginosa* added for food (in the case of *Acrasis rosea*). Other yeasts, some bacteria, and conidial fungi have also been used as food sources for *Acrasis* spp. (Olive and Stoianovitch 1960; Olive et al. 1961; Reinhardt 1968). The orange growth of the *Acrasis* amoebae, cysts, and sorocarps will become apparent among the pink yeast colonies within a few days. *Allovahlkampfia* spp. can be grown on bacteria; however, sorocarps may be induced through the addition of sterilized bark pieces soaked in a slurry of *R. mucilaginosa* and water (Brown et al. 2012a). Cultures may be transferred by cutting out a block of agar containing amoebae, cysts, or fruiting bodies and placing it in a streak of *R. mucilaginosa* on a plate of wMY (for more abundant growth, the inoculum block can be pushed along the yeast streak to spread the amoebae, cysts, and spores). Isolation plates and cultures should be kept in either natural or artificial day-night light conditions for sorocarp formation.

Species of *Pocheina* are cultured with great difficulty (Olive et al. 1983). *Pocheina* is frequently encountered on pine bark in moist chambers. Pieces of bark from living trees should be placed on a wMY agar Petri dish and wetted with sterile distilled water. The bark should be examined with a dissecting microscope for the minute pink or orange sorocarps that appear after 1–4 days. The sorocarps are removed with a needle; the sori alone can be removed by touching them with a block of agar on the end of a needle. It is difficult to germinate stalk cells and spores, but germination may occur if entire sorocarps are placed on a malt extract-yeast extract medium at pH 5.5 with 0.75% agar. Fungi, yeast, and bacteria must also be isolated from the bark. Sorocarp formation may occur on the wMY agar, or on sterilized

pieces of bark on agar. *Pocheina* is usually found repeatedly on bark collections from the same tree, allowing continued collections for trials with various food organisms.

Evolutionary History

Evolutionary Position and Significance; History and Present Understanding

Early SSU rDNA phylogenies of eukaryotes had indicated that heterolobosean were amongst the deepest-branching lineages within eukaryotes, especially mitochondrion-bearing lineages (Hinkle and Sogin 1993). Cavalier-Smith (1993) suggested that the heterolobosean (under the name Percolozoa; see "Taxonomy and Systematics") were especially important to understanding the evolutionary transition between primitive eukaryotic cells and modern cells with classical mitochondria, peroxisomes, and a stacked Golgi apparatus. He proposed that heterolobosean were a group at the base of mitochondrion-bearing eukaryotes that primitively lacked dictyosomes and stemmed from, or included, the lineage in which mitochondria were acquired. Notably, Cavalier-Smith emphasized that *Psalteriomonas vulgaris* (syn. *Lyromonas vulgaris* – see Pánek et al. 2012) differed from other Heterolobosea (and resembled primitive eukaryotes) in lacking classical mitochondria (Broers et al. 1993), while *Psalteriomonas lanterna* was originally thought to possess both hydrogenosomes (associated with methanogenic prokaryotes) and "modified mitochondria" located throughout the cytoplasm and surrounded by rough endoplasmic reticulum (Broers et al. 1989, 1990).

The hypothesis that certain anaerobic eukaryotes (e.g., diplomonads, archamoebae, and microsporidia) represent amitochondrial, deep-branching lineages has now been abandoned; further research confirmed that the ancestor of eukaryotes possessed mitochondria (Roger et al. 1998; Embley and Hirt 1998; Dolezal et al. 2006) as well as a stacked Golgi apparatus (Mowbrey and Dacks 2009; Klute et al. 2011). Also, subsequent phylogenetic analyses showed clearly that psalteriomonads are a clade that branches well within Heterolobosea and must descend from heterolobosean with aerobic mitochondria (Park and Simpson 2011; Harding et al. 2013; Pánek et al. 2012, 2014a, b). Further, De Graaf et al. (2009) demonstrated that the organelles of *Psalteriomonas* are two different morphs or developmental stages of hydrogenosomes, rather than two distinct types of organelles.

Further, it is now clear that the deepest-branching eukaryote lineages cannot be reliably identified based on rooted SSU rDNA phylogenies (Philippe et al. 2000). Instead, phylogenetic analyses over the last 15 years, especially of multiprotein and phylogenomic datasets, have shown that Heterolobosea is closely related to Euglenozoa (Baldauf et al. 2000; Rodríguez-Ezpeleta et al. 2007; Hampl et al. 2009; Kamikawa et al. 2014). In analysis with broad taxon sampling of other lineages, Heterolobosea and Euglenozoa emerge as sister taxa within the Discoba

clade (Heterolobosea, Euglenozoa, Jakobida, and *Tsukubamonas*; Rodríguez-Ezpeleta et al. 2007; Hampl et al. 2009; Kamikawa et al. 2014), albeit the precise position of *Tsukubamonas* is not settled.

Internal Phylogeny

The internal phylogeny of Heterolobosea, as currently understood, is based largely on SSU rDNA data (plus ITS1, ITS2, and 5.8S rDNA in case of genus *Naegleria* – De Jonckheere 2004). Two major lineages of Heterolobosea have been described: Pharyngomonada, which contains just a single genus, and Tetramitia, which contains most heteroloboseans. Based on SSU rDNA analyses, Pánek et al. (2012, 2014b) divided Tetramitia into seven well-supported major clades (I-VII). No morphological synapomorphies are described for most of these clades, and the relationships between them remain largely unclear (see Fig. 8). Furthermore, the positions of the "BB2" lineage and of *Creneis carolina* remain unresolved in SSU rDNA phylogenies (Harding et al. 2013; Pánek et al. 2014b) and including the divergent *Creneis carolina* sequence in SSU rDNA analyses decreases statistical support for Tetramitia clades VI and IV. Molecular data from some other taxa with peculiar morphology are not available (e.g., *Trimastigamoeba*).

Tetramitian clades I and VII are each represented by just one or two described species: *Neovahlkampfia damariscottae* and *N. nana* (Vahlkampfiidae *pro parte*) for clade I (Tyml et al. 2016) and *Selenaion koniopes* (Heterolobosea *incertae sedis*) for clade VII. SSU rDNA analyses indicate that these two clades might represent deep-branching lineages within Tetramitia. These are also the only two major clades of Tetramitia for which no flagellates have been reported.

Tetramitian clade II unites acrasid slime molds, which produce sorocarps and allovahlkampfiid amoebae (*Allovahlkampfia*, *Solumitrus palustris*). The evolution of sorocarpic multicellularity appears to precede the last common ancestor of tetramitian clade II (Brown et al. 2012a). Thus the name Acrasidae or Acrasida may be used for this clade. There appears to be some potential for the loss of sorocarpy, as only one isolate of *Allovahlkampfia* has been observed to produce sorocarps (*Allovahlkampfia spelaea* isolate BA; Brown et al. 2012a). However the lack of records of sorocarps in other allovahlkampfiids may simply be because the culture conditions were not conducive to fruiting. The monophyly of the genus *Pocheina* is currently uncertain, because *Pocheina rosea* has been tentatively synonymized with *Acrasis rosea* (Brown et al. 2012a) and *Pocheina flagellata* was reported to transform into flagellates, unlike other described acrasids (Olive et al. 1983). This opens a question about the phylogenetic position of *Pocheina flagellata*, but no molecular data are currently available.

Tetramitian clade III includes *Naegleria*, *Willaertia*, *Marinamoeba* (Vahlkampfiidae *pro parte*), and Tulamoebidae (*Pleurostomum*, *Tulamoeba*). While *Naegleria* is a freshwater lineage, members of Tulamoebidae are halophilic or extremely halotolerant (Kirby et al. 2015), and *Marinamoeba thermophila* is a

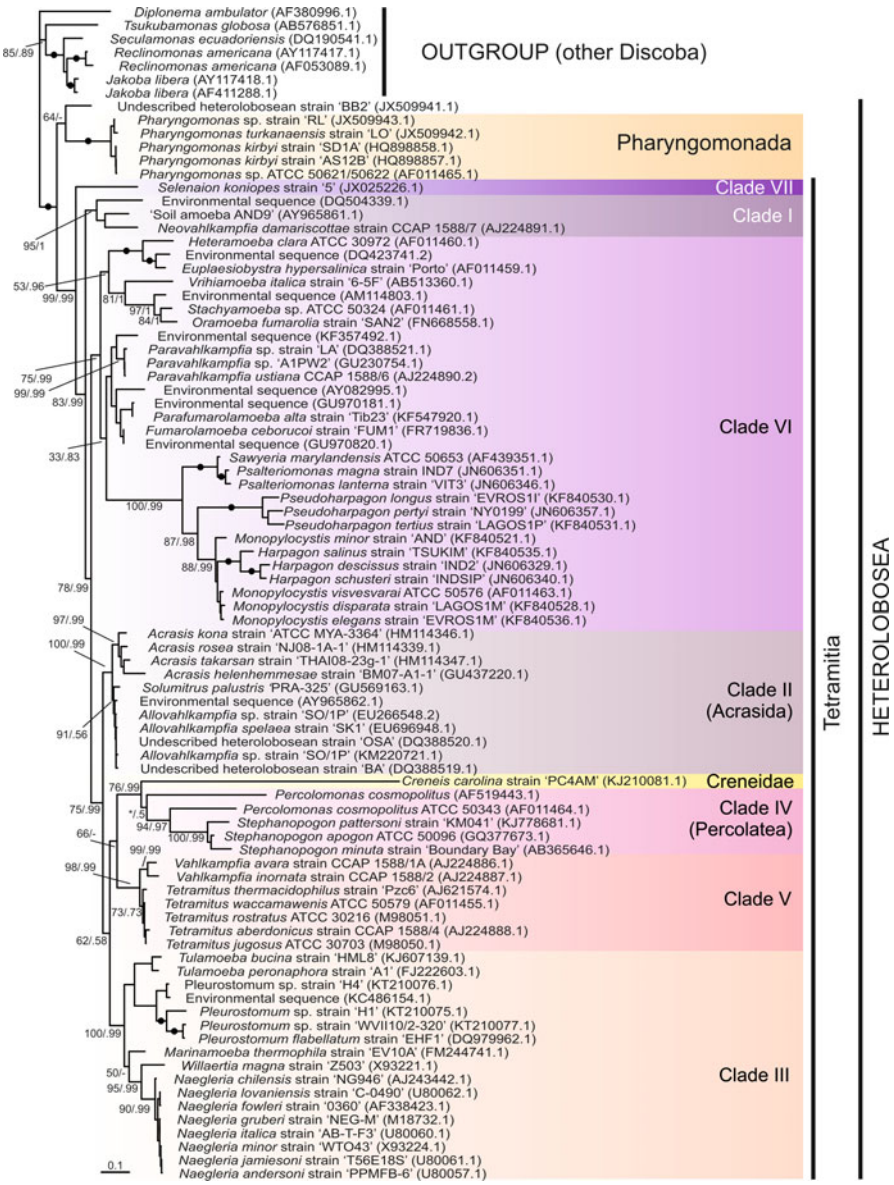

Fig. 8 SSU rDNA phylogeny of Heterolobosea. The tree is based on 1,283 well-aligned positions. The topology was inferred in RAxML version 8.2.8 using maximum likelihood with the GTRGAMMA model and 500 rapid bootstraps. The values represent RAxML bootstrap percentages followed by PhyloBayes posterior probabilities. Absolute statistical support (100/1) is indicated by *black dots*. Values lower than 50% or 0.5 are marked by "–." Bayesian analysis was performed using PhyloBayes MPI version 1.5a with the GTR CAT model (maxdiff 0.94, minimum effective size 100, number of cycles excluded from convergence checks 1,000)

marine amoeba that does not grow in high salinities, but is thermophilic (De Jonckheere et al. 2009).

Tetramitian clade VI, or Percolatea *sensu* Cavalier-Smith and Nikolaev (2008), is represented by two genera of flagellates, *Percolomonas* (Percolomonadidae) and *Stephanopogon* (Stephanopogonidae). Members of these two genera are the only heteroloboseans able to encyst as flagellates. They probably descend from a common ancestor that lost the amoeba stage. Yubuki and Leander (2008) identified two possible morphological synapomophies of Percolatea, but reexamination in light of new data indicates that neither feature is likely to define this clade (Lee et al. 2014).

Tetramitian clade V currently includes the genera *Vahlkampfia* and *Tetramitus* (both Vahlkampfiidae *pro parte*). Genus *Vahlkampfia* comprises several species with no known flagellate stage. The close relationship of some species has been confirmed by molecular methods, but some others are classified as *Vahlkampfia* based only on the absence of a flagellate stage (e.g., the marine *Vahlkampfia dumnonica*); their real phylogenetic position is uncertain. Species of the genus *Tetramitus* are extremely diverse in terms of morphology and ecology. Thus, they were assigned to multiple different genera in the past (e.g., *Adelphamoeba*, *Didascalus*, *Learamoeba*, *Paratetramitus*, *Singhamoeba*). Currently, these names are considered to be junior synonyms for *Tetramitus*, based mainly on molecular sequence comparisons (Brown and De Jonckheere 1999; De Jonckheere et al. 1997; De Jonckheere and Brown 2005b).

Tetramitian clade VI is a diverse lineage that includes some endobiotic taxa (*Paravahlkampfia* spp., representing Vahlkampfiidae *pro parte*), obligate anaerobes (Psalteriomonadidae), and halophiles/thermophiles (*Euplaesiobystra hypersalinica*), as well as non-thermophilic aerobes that inhabit freshwater/soil (*Vrihiamoeba*, *Parafumarolamoeba*) or marine/brackish habitats (*Heteramoeba*).

Fossil Record

The size and lack of mineralization of heterolobosean cells makes them poor candidates for fossilization. Nonetheless, cysts that have been identified as similar to those of *Naegleria* have been reported in mid-Cretaceous amber (Waggoner 1993).

The Evolutionary Importance of Heteroloboseans; Selected Cases

The phylogenetic position of Heterolobosea within Discoba makes it an important comparison group for examining the evolution of mitochondrial genomes and information systems. This is because jakobids have the most bacterial-like mitochondrial genomes known (Burger et al. 2013; see ▶ Jakobida), while Euglenozoa have aberrant mitochondrial genetic information systems, with different kinds of extensive gene fragmentation or extensive insertion/deletion RNA editing

(Flegontov et al. 2011). The mitochondrial genomes of *Naegleria* spp. are not particularly unusual, with a moderately large gene complement, while *Acrasis rosea* has a much smaller number of genes. There is no widespread genome fragmentation, and RNA editing in these two genera seems to be restricted to substitution-type editing at six or fewer positions (Rüdinger et al. 2011; Herman et al. 2013; Fu et al. 2014).

Flagella of many eukaryotic lineages undergo a fundamental morphogenetic process called ciliary transformation. During this process, each basal body/flagellum unit passes through a developmental program that requires more than one cell cycle to complete, such that their functional roles can change across generations (e.g., the anterior flagellum in a parent cell becomes the posterior flagellum of one of its daughters; Beech et al. 1991; Heimann et al. 1995; Melkonian et al. 1987; Nohýnková et al. 2006; Perasso et al. 1992; Yubuki and Leander 2012; Yubuki et al. 2013). With this in mind, *Naegleria*'s amoeba-to-flagellate transformation is atypical, because the whole flagellar apparatus, with two different basal bodies, is assembled *de novo* within a single generation (see Fulton and Dingle 1971; Fulton 1993). *De novo* assembly of the whole flagellar apparatus (or equivalent) is not unique for Heterolobosea; it has been described in several other eukaryotic lineages including mammals (see Fritz-Laylin et al. 2016). Nevertheless, *Naegleria* offers a particularly promising system to study *de novo* basal body assembly, as well as the mechanisms regulating the number of centrioles assembled per cell. This process has been studied for more than 45 years in *Naegleria* and protocols for straightforward control of amoeba-to-flagellate transformation have been developed. Furthermore, a genome sequence is available, as well as axenic cultures and well-characterized antibodies to orthologs of basal body components (see Fulton 1970; Fritz-Laylin and Fulton 2016).

Generally, the architecture of the eukaryotic flagellar apparatus is robust and conservative; the main components can be identified and homologized with elements in related taxa based on similar morphology as well as relative position (Yubuki and Leander 2013). For example, the R2 microtubular root has been conserved in many taxa across the tree of extant eukaryotes and has usually retained a similar structure and function (Cavalier-Smith 2013; Heiss et al. 2013; Yubuki and Leander 2013). The recently described *Creneis carolina* appears to be an exception to this rule. Ultrastructural study showed that the R2, or even the entire flagellar root system, has most probably undergone a reversal of chirality relative to the flagellum (Pánek et al. 2014b).

The term "acrasid" has historically included all cellular slime molds (see a timeline of "acrasid" research in Fig. 2 in Brown and Silberman (2013)); however, it is now known that protistan organisms have converged upon an aggregative behavior that ends in a sorocarp structure at least seven times in the evolutionary history of eukaryotes (Brown et al. 2009, 2010, 2011, 2012a, b; Brown and Silberman 2013; Tice et al. 2016). In addition to Acrasidae (which is the only example within excavates), this capability has evolved twice in Amoebozoa (dictyostelids and copromyxids) and once in each of Alveolata (the ciliate genus *Sorogena*), Opisthokonta (*Fonticula*), Stramenopiles (*Sorodiplophrys*), and

Rhizaria (*Guttulinopsis*). Little is currently known about whether there are molecular similarities among these organisms with similar life cycles. In the future, comparative genomics and transcriptomics will help to shed a light on this topic.

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Euglenida

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Brian S. Leander, Gordon Lax, Anna Karnkowska, and Alastair G. B. Simpson

Abstract

Euglenids are a group of >1500 described species of single-celled flagellates with diverse modes of nutrition, including phagotrophy and photoautotrophy. The group also encompasses a clade of specialist "primary" osmotrophs (Aphagea) and, very likely, one group of phagotrophs that are ectosymbiont-supporting anaerobes (Symbiontida). Almost all euglenids are free-living. The (usually) one or two emergent flagella have thick paraxonemal (paraxial) rods and originate in a deep pocket/reservoir, while the cell surface is almost always supported by a pellicle of parallel proteinaceous strips underlain by microtubules. Cells with 4–12 strips are rigid; most of those with more strips (typically ~20–40) have them arranged helically and exhibit active cell deformation called "euglenid motion" or "metaboly." Most phagotrophic euglenids are surface-associated bacterivores or eukaryovores that employ a flagellar gliding motility; they are abundant in marine and freshwater sediments. Photoautotrophic species (Euglenophyceae) constitute a single subclade within euglenids and have a plastid (chloroplast) of secondary endosymbiotic origin, with three bounding membranes. The plastid is typically green, with chlorophylls *a* + *b*, and was derived from a chloroplastidan alga related to the Pyramimonadales. Photoautotrophic euglenids move primarily by swimming, and most (members of the taxon Euglenales, e.g., *Euglena*) have a single emergent flagellum and are generally restricted to fresh and brackish waters.

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Contents

Summary Classification

●**Euglenida**
●●**Petalomonadida** (e.g., *Petalomonas, Notosolenus, Scytomonas, Sphenomonas*)
●●**"Ploeotiids"*** (e.g., *Ploeotia*, *Entosiphon*, *Keelungia*)
●●**Symbiontida** (*Bihospites, Calkinsia,* and *Postgaardi*)
●●**Spirocuta** (formerly "H clade" or "HP clade")
●●●**Aphagea** (e.g., *Rhabdomonas, Menoidium, Distigma, Astasia*)
●●●***Neometanema***
●●●**"Anisonemids"** (*Anisonema*, *Dinema*)
●●●**"Peranemids"*** (e.g., *Peranema, Jenningsia, Heteronema, Urceolus*)
●●●**Euglenophyceae**
●●●●***Rapaza***
●●●●**Eutreptiales** (e.g., *Eutreptia*, *Eutreptiella*)
●●●●**Euglenales** (= Euglenea)
●●●●●**Phacaceae** (*Lepocinclis, Phacus,* and *Discoplastis*)
●●●●●**Euglenaceae** (e.g., *Euglena*, *Colacium, Trachelomonas*)

* Both "ploeotiids" and "peranemids" are paraphyletic assemblages.

Introduction

Euglenids (sometimes referred to as "euglenoids") are a prominent group of free-living, aquatic flagellates, usually with one or two active flagella. Most of the >1500 descibed species are unicells that are 5–50 μm in length; a few are larger. Almost all are motile, either by swimming or by surface-associated gliding on the flagella or cell body.

Euglenida represents one of three major subgroups within the Euglenozoa, along with ▶ Kinetoplastea and Diplonemea, which they resemble in several conspicuous ways. For example, as in kinetoplastids, the flagella are inserted at the base of a deep pocket (also known as the reservoir), and active flagella are conspicuously thickenend due to the presence of paraxonemal (paraxial) rods. The mitochondrial cristae are also discoidal. However, euglenids are readily distinguishable by their cell surface architecture, which almost always is supported by a pellicle of abutting parallel strips of protein that lie directly under the cell membrane (Fig. 1). Cells with many helically arranged strips (>20) are often capable of a characteristic squirming or pulsing form of active cell deformation called "euglenid motion" or "metaboly," which is effected by sliding of adjacent strips.

Euglenids are notable for their diverse modes of nutrition, including phagotrophy (consumption of particles, especially other cells), osmotrophy (absorbtion of organic molecules), and photoautotrophy (photosynthesis) (Figs. 2, 3, 4, and 5). Among the phagotrophs, there is a convenient, if imperfect, distinction drawn between predominantly "bacterivorous" taxa, which have rigid pellicles with 12 or fewer strips and tend to be smaller in size, and predominantly "eukaryovorous" taxa that have pellicles with many strips, are usually flexible, and tend to be larger. The latter typically consume microbial eukaryotes, including unicellular algae. Meanwhile, some phototrophic forms are apparently also capable of pinocytosis, or even

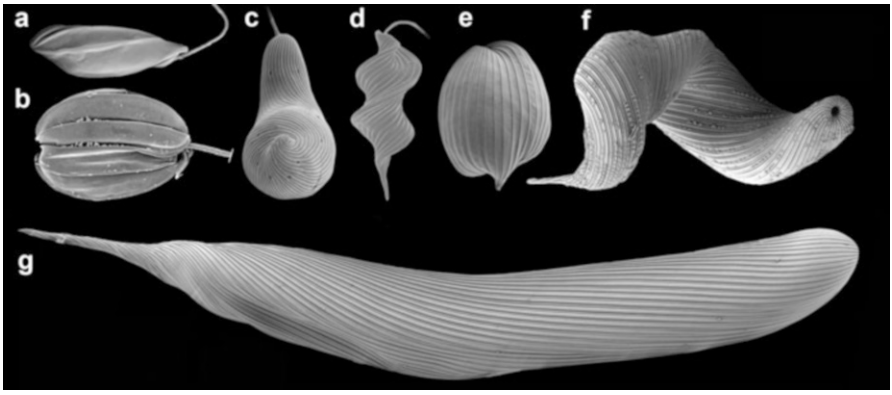

Fig. 1 Scanning electron micrographs showing the diversity of euglenids. (**a**) Petalomonad (phagotroph), (**b**) Ploeotiid (phagotroph), (**c**) *Euglena* (phototroph), (**d**) *Monomorphina* (phototroph), (**e**) *Phacus* (phototroph). (**f**–**g**) *Lepocinclis* (phototroph). Images not to scale; all cells between 10 and 100 μm

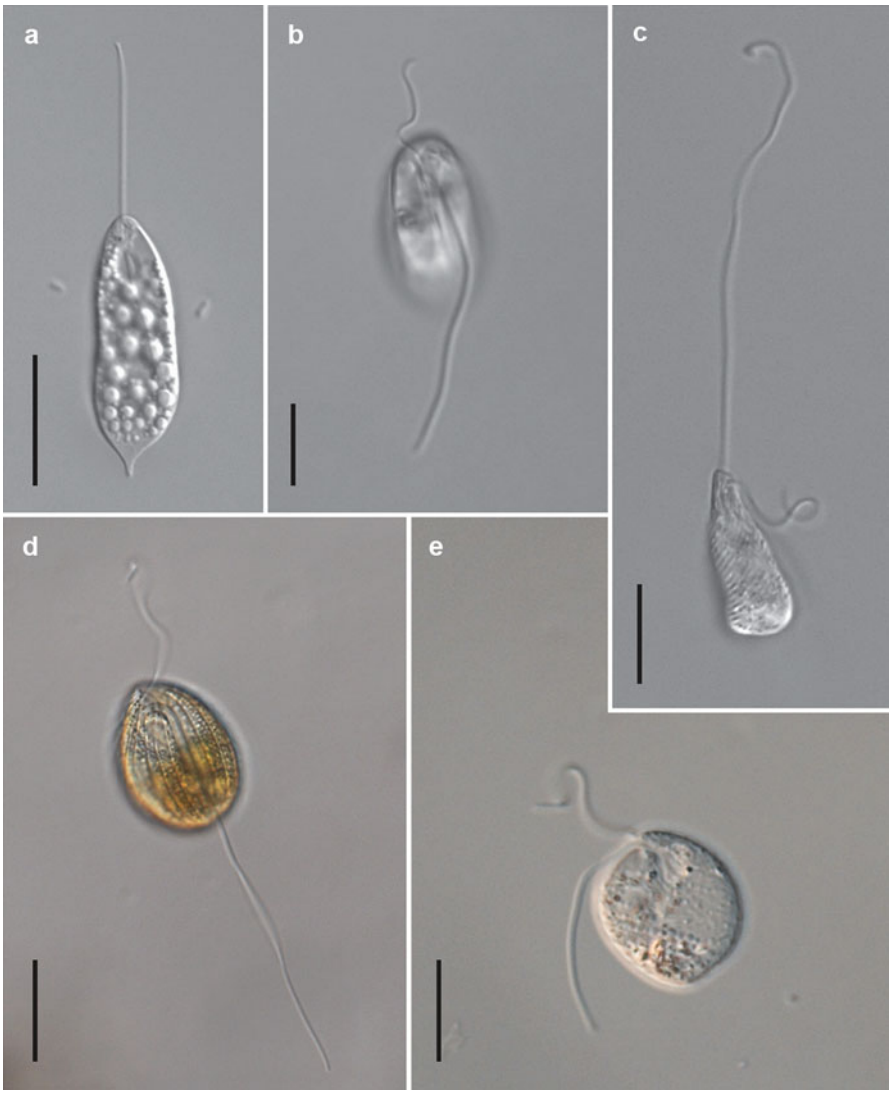

Fig. 2 Light micrographs (DIC) of phagotrophic euglenids, demonstrating various orientations of flagella and modes of locomotion. (**a**) *Petalomonas planus*, a rigid petalomonad. This species has only one flagellum, which is directed anteriorly. (**b**) *Ploeotia vitrea*, a "ploeotiid." While gliding on the posterior flagellum, the cell body is above the substrate, while the anterior flagellum beats from side to side. (**c**) *Heteronema globuliferum*, a flexible "peranemid" that glides on the anterior flagellum, with the posterior flagellum trailing under the cell during actual locomotion. (**d**) *Anisonema acinus*, an "anisonemid" gliding on its posterior flagellum, while the anterior flagellum beats anteriorly. (**e**) *Neometanema parovale* "skids" along surfaces, "skidding" being a form of swimming where the posterior flagellum is in loose contact with the substrate. The anterior flagellum beats freely. Scale bars are 20 μm for **a** and **d** and 10 μm for **b**, **c**, and **e**. Credit: **e**: Won Je Lee

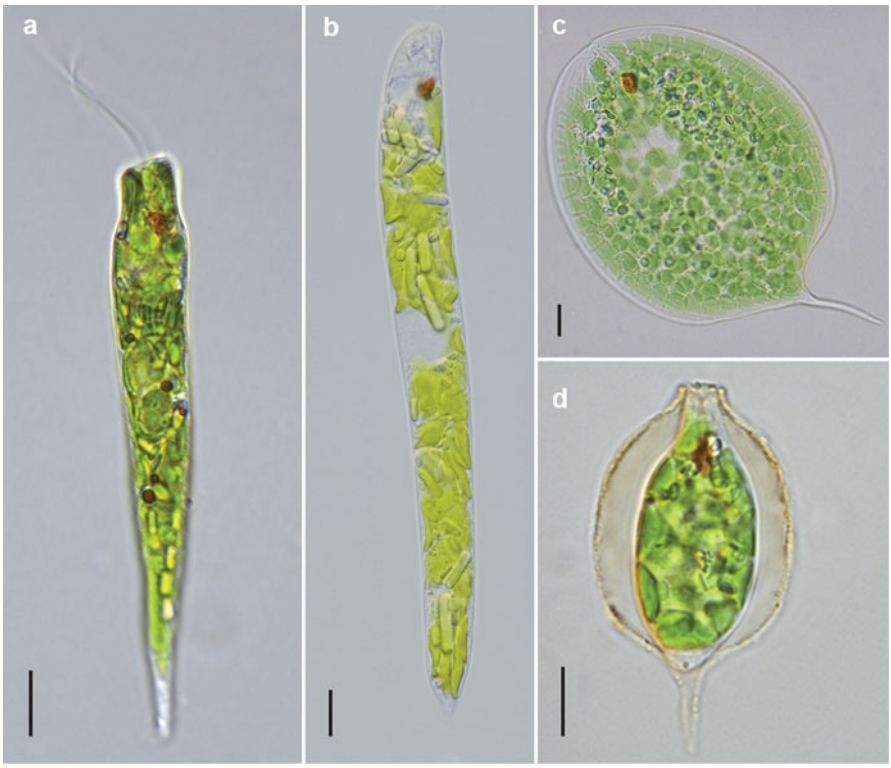

Fig. 3 Light micrographs (bright field) showing the diversity of photoautotrophic euglenids. (**a**) *Eutreptiella*, a marine cell showing two emergent flagella. (**b**) *Euglena*, a cell with shield-shaped plastids. (**c**) *Phacus*, rigid cell with small discoidal plastids. (**d**) *Strombomonas*, a cell enveloped by an organic lorica. Scale bars 10 μm. Credit: Bożena Zakryś

phagotrophy of eukaryotic algae in the case of the deep-branching phototroph *Rapaza* (Yamaguchi et al. 2012).

The "bacterivores" include the petalomonads (Petalomonadida), which glide with a forward-directed flagellum (e.g., *Petalomonas*, *Notosolenus*), and "ploeotiids," which glide on the posterior/ventral flagellum (e.g., *Ploeotia*, *Keelungia, Entosiphon*) (Fig. 2a, b). The eukaryovores include some taxa that glide primarily on a forward-directed anterior flagellum (i.e., similarly to petalomonads; Fig. 2c). An example is the well-known genus *Peranema*, and these organisms are referred to here as "peranemids." Other eukaryovores resemble ploeotiids in gliding on the posterior flagellum; the best known example is *Anisonema,* and these are referred to here as "anisonemids" (Fig. 2d). The unusual phagotroph *Neometanema* normally "skids" along surfaces rather than gliding (Fig. 2e). Ploeotiids and peranemids appear to be paraphyletic groups, the anisonemids may be as well.

Photoautotrophic euglenids are phylogenetically less diverse than phagotrophs, although more species have been described. Most are elongate, flexible cells that

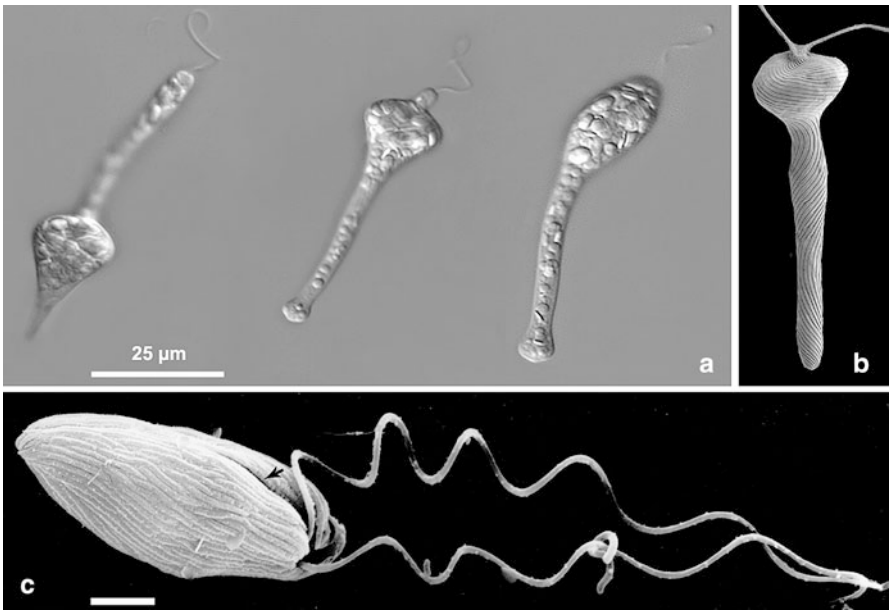

Fig. 4 Micrographs showing primary osmotrophs and symbiontids. (**a**) Three light micrographs (DIC) of a cell of *Astasia* sp. (primary osmotroph). This series also illustrates the process of metaboly ("euglenoid movement") in this particularly flexible euglenid. (**b**) Scanning electron micrograph of *Distigma* sp. (primary osmotroph), showing multiple distortions of the helical organization of the pellicle due to sliding of adjacent strips. (**c**) Scanning electron micrograph of *Postgaardi mariagerensis* (symbiontid) showing the epibiotic bacteria enveloping the cell. The arrow indicates a subtle ventral groove. Scale bars: **a**, 25 µm; **c**, 2 µm. Credit: **a**: William Bourland, **c**: modified from Simpson et al. 1997, reproduced with permission

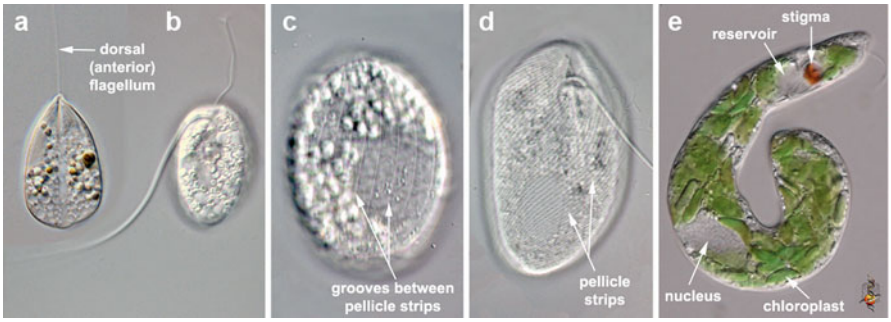

Fig. 5 Labeled light micrographs (DIC) showing several conspicuous traits in euglenids. (**a**) Petalomonad (phagotroph), (**b**) ploeotiid (phagotroph; note thickness of the ventral/posterior flagellum), (**c**, **d**) anisonemids (phagotrophs), (**e**) *Euglena* (phototroph). All cells between 20 and 50 µm. Credit: Linda Amaral Zettler and David Patterson

swim using one or (more rarely) two emergent flagella (e.g., *Eutreptia*, *Euglena*; Fig. 3a, b). Other commonly encountered forms are rigid cells with various cell shapes (e.g., *Phacus*; Fig. 3c) and cells that are enclosed in an extracellular lorica but are nonetheless capable of swimming (*Trachelomonas* and *Strombomonas*; Fig. 3d).

Among the osmotrophs, there are "primary osmotrophs" (the Aphagea, e.g., *Rhabdomonas*, *Distigma*, and *Astasia*; Fig. 4a, b), which descended from within eukaryovorous lineages, and "secondary osmotrophs," which are a polyphyletic collection of species and strains that descended from various photoautotrophs. Secondary osmotrophs now tend to be assigned to predominantly photoautotrophic genera, reflective of their evolutionary histories (see "Taxonomy").

The existence of both phagotrophic and photoautotrophic species led to euglenids being examined both as plant-like and animal-like life-forms. Among other things this resulted in competing classification schemes under the International Code of Botanical Nomenclature and the International Code of Zoological Nomenclature (i.e., they are "ambiregnal taxa" – see Patterson and Larsen 1992). Of course euglenids are neither plant nor animal, so the group does not fall neatly within the archaic plant-animal dichotomy. Photoautotrophic euglenids in fact acquired photosynthesis via a secondary endosymbiosis involving a chloroplastidan green alga (see below). The morphological and behavioral diversity of the group is also exceptional and provides compelling illustrations of major events in evolution, such as the punctuated effects of secondary endosymbiosis and changes in underlying developmental mechanisms (Leander et al. 2007; see "Evolutionary History").

Several photoautotrophic and osmotrophic species are bloom-formers in nutrient-rich conditions and are useful indicators of environmental pollution. Phagotrophic species are ubiquitous primary consumers and are likely to be important components of microbial food webs, especially in sediments. A few euglenids have been used as model systems for addressing a wide variety of questions in basic cell biology and physiology and as teaching aids. *Euglena gracilis*, for instance, is familiar to nearly every student who has taken a general biology course in high school, college, or university.

Literature and History of Knowledge

Knowledge of euglenids extends back to the late 1600s and involves several of the pioneers of eukaryotic microbiology. Photoautotrophic euglenids were among the organisms documented by Leeuwenhoek. In the eighteenth century, O.F. Mueller described some species, though he assigned them to non-euglenid genera. The first genera of photoautotrophs were introduced in the early nineteenth century by Ehrenberg, notably *Euglena* (1830), *Cryptoglena* (1832), *Colacium* (1835), and *Trachelomonas* (1835). By the mid-twentieth century, the number of described species had increased markedly, and several other important freshwater genera were introduced (*Phacus* Dujardin 1841, *Lepocinclis* Perty 1849, *Monomorphina* Mereschkowski 1877, *Strombomonas* Deflandre 1930) as well as two marine genera (*Eutreptia* Perty 1852 and *Eutreptiella* Da Cunha 1913). Accounts of osmotrophic

and phagotrophic euglenids accumulated in the nineteenth century, with genera introduced or regularized by Ehrenberg, Dujardin (1841; e.g., *Anisonema*, *Ploeotia*), Perty (1852), and Stein (1878), among others. In the mid-late twentieth century, monographic accounts of freshwater species, based on light microscopy, were produced by Gojdics (1953), Huber-Pestalozzi (1955), Pringsheim (1956), Popova (1966), Popova and Safonova (1976), Starmach (1983), and Tell and Conforti (1986). Those monographic studies were mainly focused on photoautotrophic species. Leedale's 1967 book "*Euglenoid flagellates*" summarized the ultrastructural and biochemical/cell physiological information available at the time for the group, and Buetov (1968) summarized research on *Euglena*.

Despite this long history, a considerable number of species and several genera have been described since the original publication of the *Handbook of Protoctista* (e.g., Larsen and Patterson 1990; Lee and Patterson 2000; Triemer et al. 2006; Linton et al. 2010; Bennett et al. 2014). Among the most important advances was the definition of the anaerobic Symbiontida, including the first descriptions of *Postgaardi* and *Bihospites* (Fenchel et al. 1995; Yubuki et al. 2009; Breglia et al. 2010; Fig. 4c), though as discussed below, the case that symbiontids are euglenids is not fully settled and some authors currently treat them as a separate group within the Euglenozoa (Cavalier-Smith 2016). As with most other groups of protists, the advent of molecular phylogenetics has resulted in considerable taxonomic and systematic changes, especially in the last ~15 years. Important syntheses and revisions of photoautotrophic euglenids include Marin et al. (2003), Linton et al. (2010), Kim et al. (2010), Karnkowska et al. (2015) and Kim et al. (2015), and Preisfeld et al. (2001) for osmotrophic euglenids. The phylogenetic relationships and systematics of phagotrophic euglenids remain much more poorly understood and are currently in a state of flux (Lax and Simpson 2013; Cavalier-Smith 2016; Cavalier-Smith et al. 2016).

Practical Importance

Euglenids are not known to cause disease in humans or livestock; rare possible cases of parasitism by euglenids involve noneconomic organisms such as tadpoles and gastrotrichs (Wenrich 1924; Brumpt and Lavier 1924; Kisielewska et al. 2015). However, some bloom-forming photoautotrophic euglenids have been shown to produce neurotoxins that can cause widespread fish die-offs in freshwater aquaculture facilities (Zimba et al. 2004, 2010).

A couple of species of euglenids have been exploited as model systems for biological research. For example, *Euglena gracilis* has been investigated for the production of important compounds (Krajčovič et al. 2015) such as vitamins A, C, and E (e.g., Takeyama et al. 1997; Fujita et al. 2008); polyunsaturated fatty acids (e.g., Korn 1964; Wallis and Browse 1999; Meyer et al. 2003); the carbohydrate paramylon (e.g., Santek et al. 2009; Rodríguez-Zavala et al. 2010; Shibakami et al. 2012); and wax esters (e.g., Inui et al. 1982; Teerawanichpan and Qiu 2010; Tucci et al. 2010; Dasgupta et al. 2012). *Euglena gracilis* can be grown in a wide range of

conditions: autotrophically or heterotrophically on various carbon sources (or both), under a broad range of pH values, and in high concentrations of cadmium, chromium, lead, mercury, and zinc. Therefore, it can be used for bioremediation of polluted waters (Krajčovič et al. 2015).

Habitats and Ecology

Phagotrophic euglenids are widespread in marine, brackish, and freshwater sediments. These cells glide within the spaces between sand grains and within the narrow interface between mud and the water column. They can compose up to 85% of the biomass of bacterivorous flagellates in certain aerobic freshwater, marine, and brackish sediments and are presumably important predators in these ecosystems (Boenigk and Arndt 2002; Dietrich and Arndt 2000). Despite clear microscopical evidence of their presence, phagotrophic euglenids are suspiciously rare in many environmental sequencing datasets from sediments (e.g., Forster et al. 2016). Similarly phototrophic euglenids are poorly represented in freshwater environmental surveys (e.g., Simon et al. 2015). A possible reason for this contradiction might be that euglenids often have divergent and expanded SSU rRNA gene sequences, including the V4 region that is routinely used in environmental surveys. Divergence can result in "universal" primers not binding efficiently. Additionally, many euglenids exhibit such enlarged V4 regions that they cannot be fully sequenced using current high-throughput sequencing technology (Busse and Preisfeld 2002; Karnkowska-Ishikawa et al. 2013). To address these problems (in photoautotrophic euglenids at least), careful investigation of possible DNA barcodes was recently performed, and specific primers were proposed for the V2–V3 and V4 regions of the SSU rDNA (Łukomska-Kowalczyk et al. 2016).

Phagotrophic euglenids are mostly raptorial feeders on other microbial cells, although it is documented that some act as detritivores (e.g., consume cytoplasm and organelles from large ruptured cells), and at least one species, *Dolium sedentarium*, is a sessile "ambush" predator (Larsen and Patterson 1990). As discussed above, it has become common to divide phagotrophic euglenid taxa into "bacterivores" and "eukaryovores," based on morphological correlates of food preference and phylogenetic position. The bacterivores (petalomonads and ploeotiids) are rigid cells with few pellicular strips and tend to be relatively small (most are <25 μm long). The rigidity of the pellicle constrains them by gape limitation; thus they feed on small prey, primarily prokaryotes. The eukaryvores (e.g., "peranemids" and "anisonemids") are mostly slightly-to-highly flexible cells with unfused and more numerous pellicular strips, and they also tend to be larger (most are >20 μm long). As a consequence, they are typically capable of consuming larger prey items in both absolute and relative terms, such as large eukaryotic cells. For example, Chen (1950) documented that *Peranema trichophorum* can engulf whole *Euglena gracilis* cells, which are almost as large as themselves. Many eukaryovorous euglenids specialize in consuming benthic microalgae, especially pennate diatoms (e.g., Lee and Patterson 2000).

Despite the usefulness of this phylogenetic bacterivore/eukaryovore dichotomy, it is not a clear-cut autecological distinction. For example, it is documented that many rigid species that are phylogenetically grouped with "bacterivores" are quite capable of consuming eukaryotic cells; *Ploeotia*/*Serpenomonas costata* is known to eat yeast in culture (Linton and Triemer 1999), and large petalomonads and ploeotiids are not infrequently observed with food vacuoles containing the remains of algae (e.g., Larsen and Patterson 1990; Lax and Simpson 2013; see Fig. 5a). In fact, *Dolium*, a rigid cell with six pellicular strips, ingests whole pennate diatoms (Larsen and Patterson 1990).

Phototrophic lineages mainly inhabit the water column of freshwater environments. Extremely large and vermiform species have reduced flagella and often inhabit the interface between the sediment and water column (Leander and Farmer 2000b; Esson and Leander 2008) (Fig. 1g). Only a few phototrophs inhabit the marine plankton (e.g., the Eutreptiales), however, several species are found in brackish water and estuaries, either in sediments or in the water column. Some species migrate vertically in marine sand, in coordination with tidal and diurnal cycles (e.g., *Euglena rustica*). These species are usually found in high abundance and form easily visible green patches in marine sand during low tides (Brown et al. 2002).

The deep-branching euglenophycaean *Rapaza viridis* has an interesting, if little-studied autecology. The sole known isolate is a mixotroph that houses an apparently functional euglenid plastid (see below), but also feeds on cells of a particular strain of the chloroplastidan alga *Tetraselmis* (Yamaguchi et al. 2012). This feeding is extremely selective (other algae are rejected as prey, including other strains of *Tetraselmis*), but obligate; *R. viridis* could not survive in culture more than ~1 month without prey (Yamaguchi et al. 2012).

Characterization and Recognition

The following is a summary of characteristic morphological features of euglenids. Important systems are covered in more detail in later subsections.

- The best synapomorphy for the group is a pellicle consisting of proteinaceous strips beneath the plasma membrane, associated with microtubules. The pellicle strips are oriented longitudinally in bacterivorous euglenids and usually helically in eukaryovorous, photoautotrophic, and osmotrophic euglenids. The strips are secondarily longitudinal in some rigid photoautotrophs (e.g., *Phacus*) and primary osmotrophs (e.g., *Menoidium*).
- Cells usually have two heterodynamic flagella that originate within an anteriorly-directed flagellar pocket. One flagellum extends anteriorly or anterio-laterally, but historically has been called the "dorsal" flagellum; the other, the "ventral flagellum," bends to run posteriorly. In most photoautotrophs, most osmotrophs, and a

few phagotrophs, only the dorsal flagellum is emergent, while the ventral flagellum is reduced in length and confined to the flagellar pocket (or is absent altogether).

- The flagellar pocket in photoautotrophic species is modified into a "reservoir" (equivalent to the flagellar pocket sensu stricto) and a narrower cylindrical-shaped "canal" leading to the exterior of the cell.
- The flagella are thickened, sometimes extremely so, due to the presence of paraxonemal (paraxial) rods: As in other Euglenozoa, the rod in the dorsal flagellum has a tubular appearence when viewed in transverse section using TEM, while the rod in the ventral flagellum is a 3-dimensional lattice.
- The flagellar apparatus consists of two basal bodies (ventral and dorsal, representing basal bodies 1 and 2, respectively) and three microtubular roots: the dorsal root (R3), the ventral root (R2), and the intermediate root (R1), as in most other Euglenozoa (numbering for basal bodies and roots after Yubuki and Leander 2013).
- Freshwater lineages have contractile vacuoles associated with the reservoir.
- Photoautotrophic species have green plastids (chloroplasts) containing chlorophylls *a* and *b*. The plastids are surrounded by three membranes and have thylakoids in stacks of three. Pyrenoids are absent in the Phacaceae (*Discoplastis*, *Phacus,* and *Lepocinclis*) and *Euglenaformis*.
- Photoautotrophic species respond to the direction and intensity of light using a shading stigma ("eyespot") and a photosensory swelling at the base of the emergent flagellum.
- Cells have a feeding apparatus consisting of a tube or pocket reinforced longitudinally by microtubules. These originate ultimately from the ventral root, where traced (e.g., Surek and Melkonian 1986; Willey and Wibel 1987). The feeding apparatus in many phagotrophs is further elaborated by four or five electron-dense "vanes" and reinforced by two robust rods partly composed of microtubules. The feeding apparatus in photoautotrophic and osmotrophic species is highly reduced.
- Diverse and dynamic modes of motility are seen, including metaboly, substrate-mediated gliding, and swimming.
- Mitochondria have discoidal (paddle-shaped) cristae (as in other euglenozoans).
- The nucleus has permanently condensed chromosomes and a conspicuous nucleolus.
- The main storage polymer of most euglenids (perhaps all) is paramylon, a distinctive beta-1,3-glucan. Cytoplasmic paramylon granules may be small or extremely large (especially in some photoautotrophic species).
- Extrusomes (ejectile organelles) are common, almost always in the form of "typical" tubular extrusomes, mucocysts, or muciferous bodies.
- The Golgi bodies are usually elaborate, with a large number of cisternae (see Fig. 11a).
- Cytokinesis involves a longitudinal cleavage furrow.

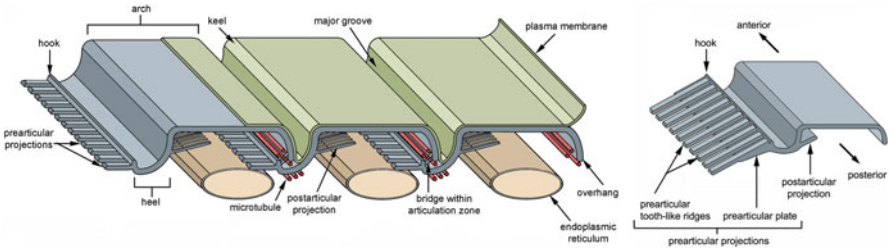

Fig. 6 Labeled illustrations showing the general organization of pellicle ultrastructure in flexible photoautotrophic euglenids. (*Left*) The configuration of three articulating strips and associated microtubules positioned beneath the plasma membrane and subtended by tubular cisternae of endoplasmic reticulum. (*Right*) A pellicle strip with robust toothlike prearticular projections and robust postarticular projections (e.g., some *Lepocinclis*)

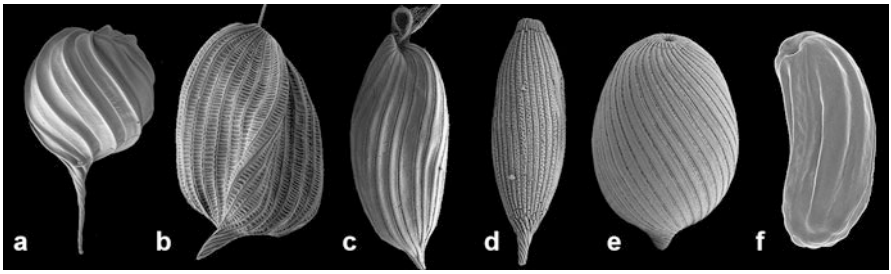

Fig. 7 Scanning electron micrographs showing the diversity of pellicle structure in rigid photoautotrophic euglenids (**a-e**) and primary osmotrophs (**f**). (**a**) *Monomorphina*. (**b**) *Phacus*. (**c**) *Phacus*. (**d**) *Lepocinclis*. (**e**) *Phacus*. (**f**) *Rhabdomonas*. All cells between 20 and 60 μm

Pellicle and Metaboly

The best synapomorphy for the Euglenida is a novel cytoskeleton comprised of parallel proteinaceous strips, underlain by microtubules, that run along the length of the cell (Leander 2004; Leander and Farmer 2000a, 2001a; Leander et al. 2001a, b) (Figs. 1, 5c, d, 6, 7). These elements are positioned immediately beneath the plasma membrane and are closely associated with cisternae of the endoplasmic reticulum. Collectively, this ultrastructural system is referred to as the euglenid pellicle.

The number of individual strips varies from 4 or 5 in some petalomonads to 120 in some very large euglenophytes (Esson and Leander 2008). Bacterivores (petalomonads and ploeotiids) have 12 or fewer longitudinal strips (often 8 or 10) that are fused to form a rigid pellicle. Eukaryovores (e.g., peranemids and anisonemids) have 20 to about 60 strips that are usually helically arranged and slide to allow metaboly; photoautotrophic (and secondarily osmotrophic) euglenids have 16–120 helically arranged strips, and most are capable of metaboly (Leander et al. 2007). However, some photoautotrophs (and secondary osmotrophs) have

secondarily become rigid and have longitudinally arranged strips. The earliest diverging primary osmotrophs (e.g., *Distigma*) have about 20 helically arranged strips and are metabolic; however, some lineages became rigid and now have fewer strips (e.g., 14) that run more longitudinally and that are often fused into a continuous proteinacous layer around the cell (e.g., *Menoidium* and *Rhabdomonas*; Leander et al. 2001b; Fig. 7f).

The strips are composed mostly of a family of proteins called "articulins" (Marrs and Bouck 1992). In general, the main frame of each pellicle strip is "S-shaped" in cross section and consists of an arch region and a heel region that defines a groove (Leander et al. 2007; Leander and Farmer 2001a) (Fig. 6). Adjacent strips articulate along their lateral margins; the strip arch overlaps with the heel of a neighboring strip, giving the surface of euglenid cells a striated appearance (Fig. 6).

The articulation zones between adjacent strips allow the dynamic changes in cell shape called "metaboly," "euglenoid motion," or "euglenoid movement" (Fig. 4a, b). They also facilitate cytoskeletal replication prior to cell division (i.e., cytokinesis). Metaboly is observed in most cells that have a large number of pellicle strips (16 or more). As well as serving a secondary locomotory role, metaboly is thought to facilitate the ingestion of large food particles, such as other eukaryotic cells, in eukaryovorous phagotrophs (Leander 2004; Leander et al. 2001, 2007; Yamaguchi et al. 2012).

In photoautotrophic and secondary osmotrophic lineages, the frame of each strip contains periodic arrays of projections that branch laterally from the heel (Leander et al. 2001b, 2007; Leander and Farmer 2001a). The projections of one strip articulate with the projections of an adjacent strip beneath the arches (Fig. 6). The projections that branch beneath the arch of an adjacent strip, so-called prearticular projections, and those that branch beneath the arch of the same strip, so-called postarticular projections, can vary considerably in robustness: some lineages possess delicate threadlike projections; some species possess more robust toothlike projections; and some species possess projections that form robust continuous plates (Fig. 6). Euglenid cells with more delicate strips tend to demonstrate more dramatic degrees of metaboly (Fig. 5); euglenid cells with robust strips tend to be rigid, or nearly so (Angeler et al. 1999; Leander 2004; Leander et al. 2001b, 2007; Leander and Farmer 2001a) (Fig. 7). Phagotrophic and primary osmotrophic euglenids lack strip projections altogether.

The euglenid pellicle is multigenerational; each strip or cohort of strips represents a different cytokinetic event in the history of any particular cell (Esson and Leander 2006, 2008; Leander et al. 2007; Yubuki and Leander 2012). Prior to cytokinesis, the number of pellicle strips around the cell periphery doubles. Each daughter cell (usually) inherits the same number of pellicle strips as the parent cell in a semiconservative manner. During strip doubling, new strips emerge within the articulation zones between mature strips. In the photoautotrophic euglenids, the newly produced pellicle strips do not extend to the posterior tip of the cell and consequently form whorled surface patterns of strip termination (Esson and Leander 2006, 2008; Leander and Farmer 2000a; Leander et al. 2001b). Strips that terminate before reaching the posterior tip of the cell occupy a relative position along the length of

the cell called a "whorl." The number of posterior whorls varies between different species, ranging from one to four. In some species, the whorls themselves can be dissociated into one or more subwhorls (Esson and Leander 2008; Leander and Farmer 2000a; Leander et al. 2001b; Yubuki and Leander 2012). Comparative analyses of the strip termination patterns in several different species have provided important insights into the developmental processes associated with the control and evolutionary diversification of the euglenid pellicle (Esson and Leander 2006, 2008; Leander and Farmer 2000a; Leander et al. 2001b; Yubuki and Leander 2012).

Variation in the number of strips within many species (though usually with a strong mode) indicates that strips are not necessarily distributed evenly during cell division. For instance, a parent cell with 40 strips doubles the number of strips to 80 prior to cell division. In most cases, the two daughter cells will each receive 40 strips and recover the number that was present in the parent cell. In other cases, the daughter cells might receive some other proportion, such as 38 and 42 or 36 and 44. It is also possible that strip duplication is not always faithful; for instance, a parent cell with 40 strips might only produce 39 new strips, in which case the daughter cells will receive 39 and 40 strips, respectively.

Permanent strip duplication events refer to cases where a cell duplicates its strips but fails to divide. Permanent strip halving events refer to cases where a cell divides without duplicating its strips. The distribution of strip numbers found in euglenids suggests that these events happened several times during the evolution of the group (see "Evolutionary History").

Flagella and Locomotion

Most euglenids possess two heterodynamic flagella that emerge from the flagellar pocket. A few lineages have more than two (e.g., some Eutreptiales have four flagella; McLachlan et al. 1994), and some have highly reduced flagella, giving the appearance of one or none when viewed with the light microscope (Figs. 2, 3, 4, and 5). Euglenids possess paraxonemal (paraxial) rods within the flagella that run alongside the 9 + 2 microtubular axoneme (Fig. 8a, b). The paraxonemal rods make euglenid flagella conspicuously thick when viewed under the light microscope (e.g., Fig. 5b); the thickest flagellum can approach or exceed 1 μm width in many larger cells, especially in large phagotrophic euglenids (e.g., Larsen and Patterson 1990). The paraxonemal rod in each flagellum has a different structure: the rod in the ventral flagellum has a latticelike structure, and the rod in the dorsal flagellum has, at core, a whorled structure that appears tubular in transverse sections (Fig. 8a, b). A major component of both structures are the paraxonemal rod proteins PAR1 and PAR2, whose genes arose through duplication prior to the divergence of euglenids and kinetoplastids (Talke and Preisfeld 2002).

Euglenid flagella characteristically have very thick investments of fine hairs, which generally emerge in horizontal (or shallowly helical) rows of tufts associated with the flagellar axoneme and/or paraxonemal rod (Bouck et al. 1978; Dawson and Walne 1991; Hilenski and Walne 1985; Mignot 1965, 1966) (Fig. 8). These hairs

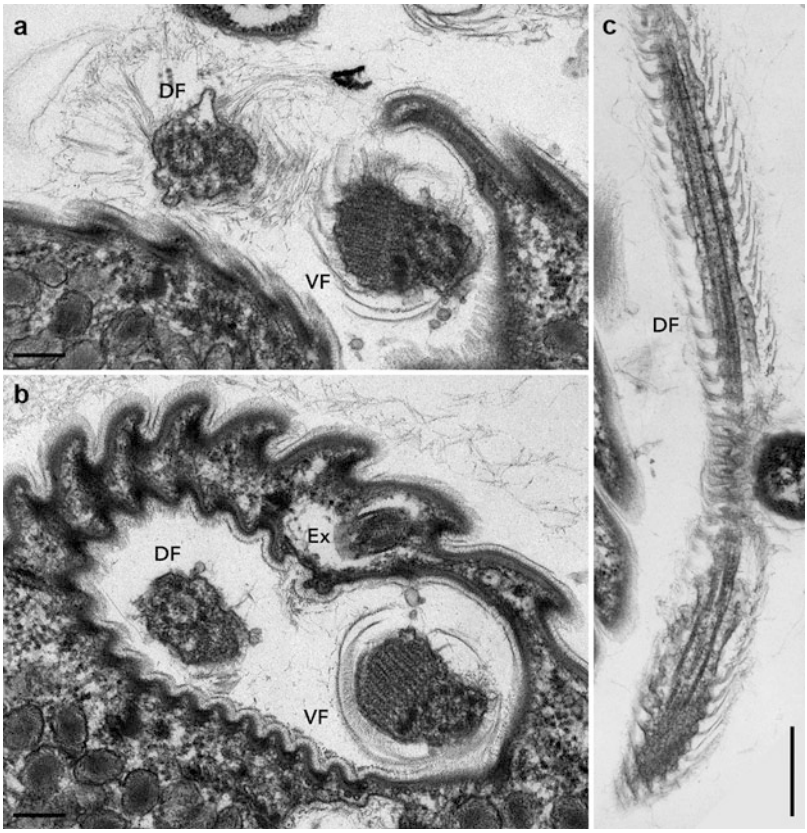

Fig. 8 Transmission electron micrographs of flagella. (**a**, **b**) Near-transverse sections of the proximal portions of the dorsal/anterior flagellum (*DF*) and ventral/posterior flagellum (*VF*), showing the paraxonemal rods (tubular in *DF*, latticed in *VF*) and the flagellar hairs. Note also the oblique section of an undischarged tubular extrusome (*Ex*); *Neometanema parovale*. (**c**) Longitudinal view of dorsal/anterior flagellum (distal end to top of page), showing flagellar hairs; *N. parovale*. Scale bars: **a**, 200 nm; **b**, 200 nm; **c**, 500 nm. Credit: **a**: courtesy of Won Je Lee, **b**, **c**: image by Won Je Lee, slightly modified from Lee and Simpson 2014a, reproduced with permission

typically lie oriented with their distal ends pointing toward the distal end of the flagellum (Fig. 8c). In addition, emergent flagella may have a single longitudinal row of bundles of larger hairs, which can be several micrometers long; these hairs are best studied in the sole emergent flagellum of Euglenean photoautotrophs such as *Euglena* (e.g., Leedale 1967; Bouck et al. 1978; Melkonian et al. 1982). The phototroph *Eutreptia*, which has two emergent flagella, has these rows of bundles of long hairs on both flagella (Dawson and Walne 1991), as, apparently, does the biflagellated primary osmotroph *Distigma proteus* (Leedale 1967), while the

peranemid eukaryotroph *Peranema* has them on the anterior flagellum only (Hilenski and Walne 1985). Petalomonads have sparser arrangements of flagellar hairs than other euglenids and/or finer hairs (though data is limited; Cann and Pennick 1986; Lee and Simpson 2014b), while flagellar hairs have not been reported at all in symbiontids (Yubuki et al. 2009).

In phagotrophic lineages, the flagella are heterodynamic, with one flagellum (i.e., the dorsal/anterior flagellum) held ahead of the cell, while the other flagellum (i.e., the ventral, recurrent, or posterior flagellum) bends backward and extends posteriorly from the cell, often within a ventral groove or sulcus (Figs. 2b–e, 5b). The hairs and paraxonemal rods of these flagella facilitate gliding motility across substrates (Saito et al. 2003). In petalomonads and peranemids, the dorsal/anterior flagellum is involved in gliding. During this gliding most of the anterior flagellum is held stiffly against the substrate, but the tip is in constant motion and functions as a sensory apparatus (Figs. 2a, c and 5a). In these cells the posterior/ventral flagellum is shorter and thinner than the anterior flagellum; in some cases it lacks a paraxonemal rod, does not emerge from the reservoir, or is completely absent (e.g., Cann and Pennick 1986; Lee and Simpson 2014b). In ploeotiids and anisonemids, only the posterior flagellum is involved in gliding, and the whole anterior flagellum sweeps from side to side; in these cells the anterior flagellum is almost always thinner and usually shorter than the posterior flagellum (Figs. 2b, d, e and 5b). Some phagotrophic euglenids also use the anterior flagellum like a hook to shovel prey cells into the feeding apparatus (Breglia et al. 2013).

Most osmotrophic and photoautotrophic euglenids primarily move using swimming motility (Leander 2004). They usually possess an emergent dorsal flagellum that extends from the canal and is highly dynamic, while the reduced ventral flagellum does not emerge from the canal and is inactive. The emergent flagellum beats in an organized and consistent pattern that takes the form of a "figure-eight" or a lasso. This beat pattern pulls the euglenid cell through the water column (Leander 2004). By contrast, eutreptialean photoautotrophs possess two emergent flagella that both beat during swimming (some primary osmotrophs also have two emergent flagella).

Although phagotrophic euglenids are usually poor swimmers, the symbiontid *Postgaardi* swims with a spiralling motion (Simpson et al. 1997), while the anisonemid-like *Neometanema* normally moves by rapidly "skidding" (i.e., swimming while maintaining physical contact with the substrate), powered by beating of the anterior/dorsal flagellum (Lee and Simpson 2014a; see Larsen and Patterson 1990, 2000). Conversely, gliding is seen in some photoautotrophic euglenids (Euglenophyceae), but gliding cells typically hold the cell body against the substrate, not the flagellum (which is often greatly shortened).

Flagellar Apparatus

The flagellar axonemes are anchored by basal bodies that are situated at the base of the flagellar pocket: the ventral flagellum originates from the ventral basal body, and

the dorsal flagellum originates from the dorsal basal body. A striated fiber connects both basal bodies. Three microtubular roots extend from the basal bodies: the dorsal root extends from the lateral side of the dorsal basal body, the ventral root extends from the lateral side of the ventral basal body, and an intermediate root extends from the medial side of the ventral basal body and thus lies initially between the basal bodies (Yubuki and Leander 2012). In the universal numbering system for the eukaryotic flagellar apparatus, the ventral basal body represents basal body 1, and the dorsal basal body is basal body 2 (i.e., the dorsal basal body is predicted to transform into the ventral basal body during cell division; Moestrup 2000). Meanwhile the dorsal root represents R3, the ventral root R2, and the intermediate root R1 (Yubuki and Leander 2013, noting that the identification of the roots in Moestrup 2000 was inaccurate). This flagellar apparatus constitutes the organizing center from which several other cytoskeletal elements arise – such as the microtubules associated with the cell surface (or pellicle), which originate in association with the dorsal root, and the central microtubules of the feeding apparatus, which, when traced, prove to originate from the ventral root (Belhadri et al. 1992; Belhadri and Brugerolle 1992; Farmer and Triemer 1988; Hilenski and Walne 1985; Leander 2004; Shin et al. 2001, 2002; Simpson 1997; Solomon et al. 1987; Surek and Melkonian 1986; Willey and Wibel 1985; Yubuki and Leander 2012).

Feeding Apparatus

Phagotrophic euglenids have feeding apparatuses that range from relatively simple microtubule-reinforced pockets or tubes (MtR pockets) to highly complex systems of rods and vanes (Leander et al. 2007; Triemer and Farmer 1991a, b). One major group of bacterivorous euglenids, petalomonads, have MtR pockets, with some of the reinforcing microtubules likely derived from the ventral root of the flagellar apparatus via the MtR structure (although this connection has not been proved yet in any species; Lee and Simpson 2014b). Ploeotiids (e.g., *Ploeotia* and *Entosiphon)* and eukaryovorus euglenids possess feeding apparatuses that are much more complex. These include two robust rods composed of ordered arrays of microtubules embedded within an amorphous matrix (Triemer and Farmer 1991a; Linton and Triemer 1999). In *Entosiphon*, one of the rods bifurcates near the anterior end of the cell and gives the impression of three feeding rods in transverse view (Triemer and Fritz 1987; Leander et al. 2007). The feeding rods in ploeotiids typically extend the entire length of the cell, as do those of some eukaryovorous euglenids, like *Dinema*. By contrast, the feeding rods are confined to the anterior third of the cell in eukaryovorous euglenids that are capable of extreme metaboly, such as *Peranema*, *Urceolus*, and *Jenningsia*. A smaller "accessory rod" is sometimes positioned along the lateral margin of each feeding rod in both bacterivorous and eukaryovorous euglenids (Nisbet 1974; Breglia et al. 2013). Between the two feeding rods are four to five plicate or lamellar vanes, depending on the species.

The action of the feeding apparatus has been studied in some detail in *Entosiphon*; when a prey cell is about to be ingested, the rods of the feeding

apparatus protrude from the anterior end of the cell, and the vanes twist open like the blades of a pinwheel (Triemer and Fritz 1987). When the feeding apparatus retracts, the vanes twist back into their original position, gripping and internalizing the prey in the process. Although most phagotrophic euglenids ingest their prey whole, some euglenids (e.g., *Peranema*) can also feed by myzocytosis (Triemer 1997). This mode of feeding is vampire-like, in that the feeding rods pierce the prey cell, allowing the cell contents to be sucked into a phagosomal vacuole within the euglenid. The feeding apparatuses present in photoautotrophic and osmotrophic euglenids are highly reduced, corresponding to the switch from predominantly phagotrophic modes of nutrition to photoautotrophy and surface absorption, respectively (Leander et al. 2001a; Shin et al. 2002; Surek and Melkonian 1986; Willey and Wibel 1985).

Plastids (Chloroplasts)

Photoautotrophic euglenids (Euglenophyceae) evolved once from eukaryovorous euglenid ancestors that established a secondary endosymbiosis with green algal prey cells (Gibbs 1978; Leander 2004). These algae were related to the prasinophyte *Pyramimonas* (Turmel et al. 2009; Hrdá et al. 2012). The chloroplasts of euglenophytes are themselves green, are surrounded by three membranes, and possess thylakoids in stacks of three (Fig. 9). Most euglenid plastids contain a conspicuous pyrenoid (a region containing RuBisCO protein), although the small disc-shaped plastids of *Discoplastis*, *Lepocinclis*, *Phacus*, and *Euglenaformis* lack pyrenoids altogether (Figs. 3, 9a, c). Carbohydrate storage in the form of paramylon granules is also often associated with the pyrenoids, but is also distributed throughout the cytoplasm (Fig. 9a). Plastids with conspicuous paramylon caps on both sides

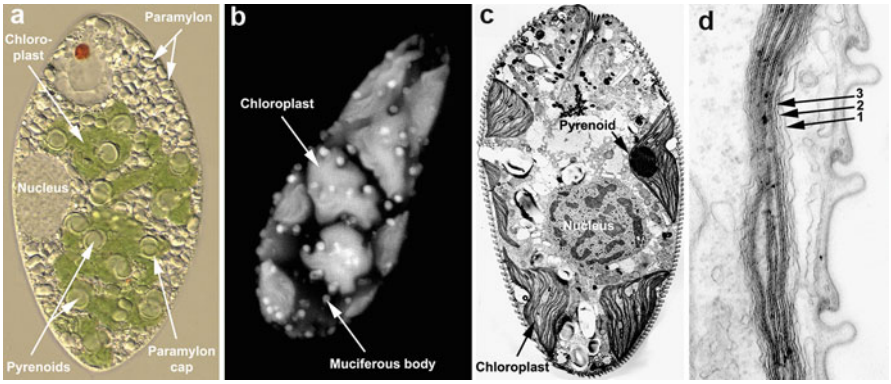

Fig. 9 Light and electron micrographs showing the general ultrastructure of chloroplasts (i.e., plastids) in *Euglena*. (**a**) Light micrograph showing paramylon, pyrenoids, chloroplasts, and the nucleus. (**b**) Confocal micrograph showing autofluorescence and the spatial distribution of chloroplasts and muciferous bodies. (**c**) Low magnification transmission electron micrograph showing the nucleus, pyrenoid, and chloroplasts. (**d**) High magnification transmission electron micrograph showing three membranes surrounding the chloroplast

of the pyrenoid are referred to as being "diplopyrenoidal" and on the one side as "haplopyrenoidal" (Brown et al. 2003; Monfils et al. 2011).

The number and morphology of euglenid plastids are very diverse (e.g., shield-shaped, disc-shaped, and star-shaped) and reflect evolutionary relationships, different stages in cell development and environmental conditions. Some photoautotrophic euglenids are known to switch nutritional modes and survive in the dark, whereby the plastids become "bleached" over time. Several different groups of photoautotrophic euglenids include species that have independently lost photosynthesis (e.g., *Euglena quartana*, *Euglena longa*, and *Lepocinclis cyclidiopsis;* Triemer and Farmer 2007; Bennett and Triemer 2014). Plastids with reduced genomes still exist in at least some of these secondary osmotrophs (e.g., *Euglena longa*, Hachtel 1998; see below).

The plastid genome of the model species *Euglena gracilis* was sequenced more than 20 years ago (Hallick et al. 1993). The genome is surprisingly large (~143 kb) but not because of the gene repertoire, which is relatively small (97 predicted genes), but due to extensive noncoding DNA sequence, including an enormous number of introns (~150). For comparison, the representative prasinophyte *Pyramimonas parkeae* has only one intron (Turmel et al. 2009). The next sequenced plastid genome was that of the secondarily non-photosynthetic species *Euglena longa* (Gockel and Hachtel 2000). This is about half the size of the *E. gracilis* plastid genome due to the loss of all genes encoding photosynthesis-related proteins, except for the *rbc*L gene encoding the large subunit of RuBisCO. The *E. longa* plastid genome is required for cell growth and viability (Gockel et al. 1994; Gockel and Hachtel 2000; Hadariová et al. 2016).

Fifteen more plastid genomes have been sequenced since 2010, covering most of the genera of Euglenophyceae (Bennett et al. 2012, 2014; Hrdá et al. 2012; Wiegert et al. 2012, 2013; Pombert et al. 2012; Bennett and Triemer 2015; Dabbagh and Preisfeld 2017; Kasiborski et al. 2016). Comparative studies revealed that they have very similar complements of protein-coding genes; however, there have been major changes in gene arrangement. The most striking differences are the numbers of introns. Two early-diverging Eutreptiales have few introns (7–23; Hrdá et al. 2012; Pombert et al. 2012; Wiegert et al. 2012), the only sequenced representative of Phacaceae (*Phacus orbicularis*) has 67, and representatives of Euglenaceae have 53–150. The pattern of intron proliferation observed in the Euglenophyceae corresponds with the number of identified maturases (Eutreptiales, 1; Phacaceae, 2; Euglenaceae, 3), which are possibly involved in intron mobility (Kasiborski et al. 2016).

Photoreception

Euglenophytes (and most secondary osmotrophs) can respond to the intensity and direction of light and orient themselves in the water column accordingly (Kuznicki et al. 1990). Photoreception is accomplished by an apparatus consisting of a photosensory swelling at the base of the emergent dorsal flagellum and a closely

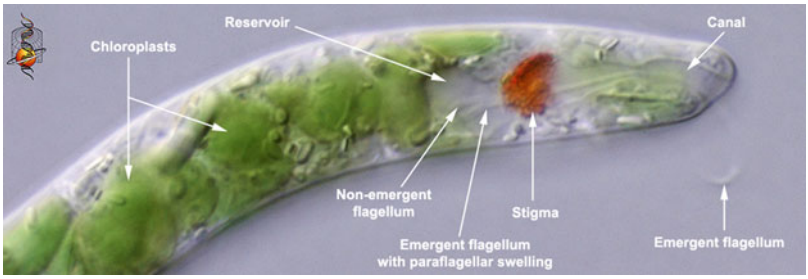

Fig. 10 Labeled light micrograph showing the photoreceptor apparatus in a photoautotrophic euglenid, consisting of an expanded reservoir, a canal, a paraflagellar swelling near the base of the emergent flagellum, and a shading stigma (i.e., eyespot). Credit: Linda Amaral Zettler and David Patterson

associated shading structure composed of orange or red carotenoids, called the "stigma" or "eyespot" (Fig. 10). The stigma of euglenids is positioned near the base of the flagellar pocket/reservoir. Interestingly, it lies in the cytoplasm, instead of being embedded within the plastid as in most other photosensory algae (e.g., within the green algae, dinoflagellates, and chrysophyceans). The stigma shades one side of the flagellar swelling; as the cell rotates through the water, the swelling can detect the direction of the most intense light source. The behavior of the swimming flagellum will then respond in a way that allows the cell to maintain a position in the water column that is optimal for photosynthesis.

There is evidence of a photosensory swelling and stigma in the phagotrophic eukaryovorous euglenid *Urceolus* (Leander et al. 2001a). This putative photoreception apparatus in *Urceolus* might enable it to maintain a position in light regimes favoured by its algal prey. There is no evidence that *Urceolus* has or had plastids, but it was inferred to be a close relative of Euglenophyceae in analyses of morphological data (Leander et al. 2001a), and thus it is possible that its putative photoreception apparatus is homologous to that of photoautotrophic euglenids.

Mitochondria

The mitochondria are distinctive in having stalked, paddle-shaped cristae, usually referred to as "discoidal" cristae (Fig. 11b). They are homologous to the discoidal cristae of ▶ Kinetoplastea and probably those of ▶ Heterolobosea. The mitochondrion of *Euglena gracilis* forms a large reticulated network (Pellegrini 1980). This conformation may be widespread among euglenids, although numerous separate elongated mitochondria are reported in some taxa (e.g., *Peranema*; Roy et al. 2007; Leedale 1967). The anaerobic symbiontids retain conspicuous mitochondria-related organelles; these have a homogeneous matrix, and profiles through them generally lack cristae altogether (Simpson et al. 1997; Yubuki et al. 2009; Fig. 11g). Nonetheless, a few flattened crista-like structures have been seen in *Bihospites bacati* (Breglia et al. 2010).

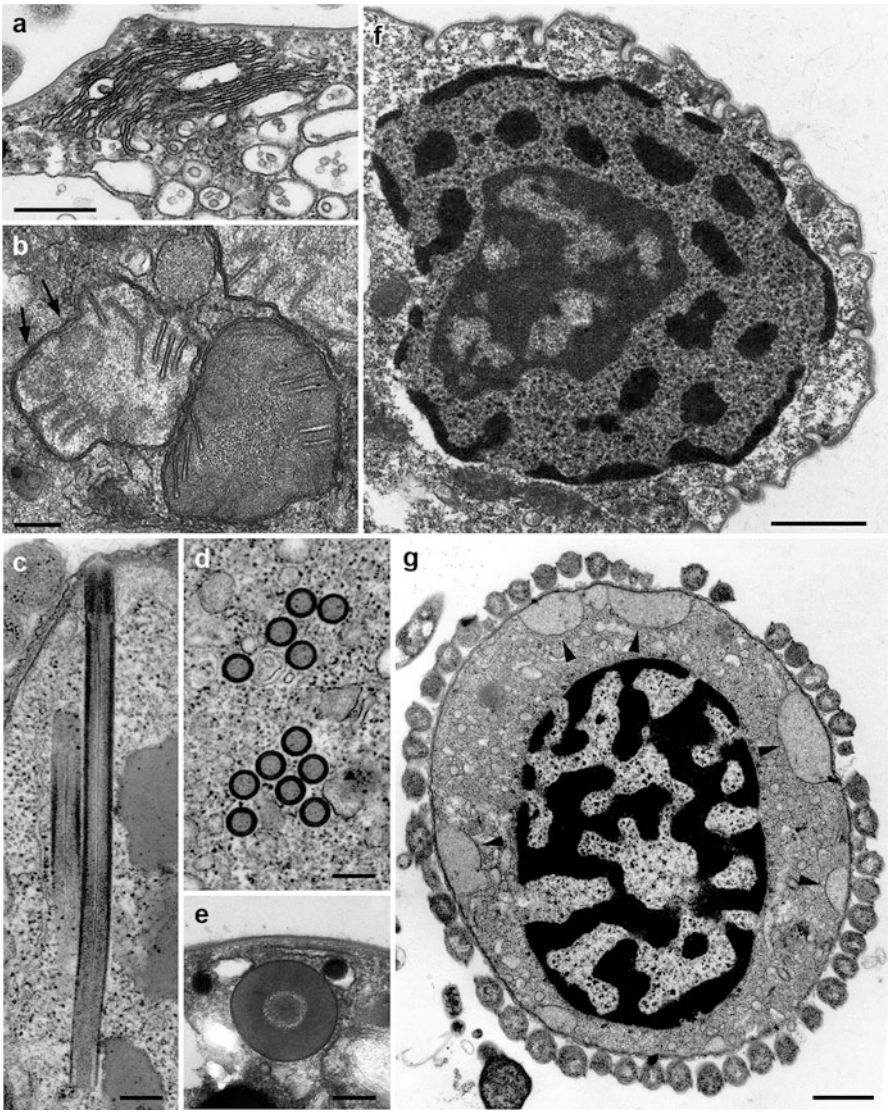

Fig. 11 Transmission electron micrographs of major organelles. (**a**) Golgi apparatus; *Notosolenus urceolatus*. (**b**) Profiles of mitochondria with rigid, discoidal mitochondrial cristae. Note two cristae lying parallel to the plane of section, thus showing discoidal profile (*arrows*); *N. urceolatus.* (**c**) Two tubular extrusomes, one viewed in longitudinal section; *Postgaardi mariagerensis*. (**d**) Transverse sections of a dozen tubular extrusomes; *P. mariagerensis*. (**e**) Globular presumptive extrusome of a petalomonad, shown at the same scale as the tubular extrusosomes in d and e; *N. urceolatus.* (**f**) Nucleus, showing extensive permanently condensed chromatin; *Neometanema parovale*. (**g**) Transverse section through a symbiontid (*P. mariagerensis*). Note the large nucleus with extensive condensed chromatin, the mitochondrion-related organelles that lack cristae (*arrowheads*), and the epibiotic bacteria (>40 cut transversally in this section). Scale bars: **a**, 500 nm; **b**–**e**, 200 nm; **f**, **g**, 1 μm. Credit: **a**, **b**, **e**: courtesy of Won Je Lee. **c**, **d**, **e**; modified from Simpson et al. 1997, reproduced with permission. **f**: image by Won Je Lee, modified from Lee and Simpson 2014a, reproduced with permission

The mitochondrial genome of euglenids is not well understood. For example, it is only recently that the mitochondrial genome of *E. gracilis* was fully sequenced (Dobakova et al. 2015). This consists of a heterogeneous population of DNA molecules roughly 1–10 kb long (Spencer and Gray 2010; Dobakova et al. 2015) and houses just seven protein-coding genes, while the mitochondrial ribosomal RNAs are encoded as multiple fragments (Spencer and Gray 2010; Dobakova et al. 2015). No evidence of kinetoplastid-type RNA editing or RNA-editing machinery was found in *Euglena* (Dobakova et al. 2015). Nonetheless, transmission electron micrographs of the petalomonads *Petalomonas cantuscygni* and *Notosolenus urceolatus* show fibrous compacted inclusions within the mitochondria that are similar in appearance to the kDNA inclusions present in kinetoplastids (Leander et al. 2001a; Lee and Simpson 2014b). This fibrous nature was not seen in a subsequent study of *P. cantuscygni* (perhaps due to fixation differences); however, some small circular DNA molecules were observed in electron micrographs of mtDNA preparations, along with many larger linear molecules (Roy et al. 2007). Therefore, it is currently unclear whether the mitochondrial genomes of some deep-branching euglenids might contain "minicircles" (encoding "guide rRNA" genes) like those found in ▶ Kinetoplastea.

Extrusomes

Some typical bacterivorous euglenids display thick-walled "tubular extrusomes," often with cruciate central filaments, that are similar to those present in a few diplonemids and free-living kinetoplastids (Brugerolle 1985; Schuster et al. 1968). These extrusomes were studied in detail in the ploeotiid *Entosiphon sulcatum* (Mignot 1966; Mignot and Hovasse 1973). They are also present in all described symbiontids; *Bihospites,* Postgaardi, and *Calkinsia* (Breglia et al. 2010; Simpson 1997; Yubuki et al. 2009; Fig. 11c, d). Where well documented, these extrusomes are highly elongated in the undischarged state (>2 μm; see Fig. 11c) and expand in length during discharge into an open lattice structure (Breglia et al. 2010; Mignot 1966; Simpson et al. 1997). Their function has not been studied directly, but presumably they operate in predation or in protection from predation. A homologous but modified form is seen in some eukaryovorous euglenids. These are usually shorter, have a dense central region when in the undischarged state (Lee and Simpson 2014a; see Fig. 8b), and exhibit less length expansion upon discharge, where known (Hilenski and Walne 1983). They have been found in *Teloprocta*/*Heteronema scaphurum*, in *Neometanema parovale*, and in *Peranema trichophorum*, where they are called mucocysts (Breglia et al. 2013; Hilenski and Walne 1983; Lee and Simpson 2014a; Mignot 1966).

Mucilaginous bodies called "mucocysts" are present in two subclades within *Euglena* and might be homologous to the extrusomes of phagotrophic euglenids. Mucocysts of photoautotrophic euglenids sit beneath pores positioned in rows within the articulation zones between the pellicle strips (Leander et al. 2001b; Esson and Leander 2008). The number of strips between the rows of mucosyst pores is variable,

which makes them suitable diagnostic characters at the species level (Leander et al. 2001b; Kosmala et al. 2009).

Tubular extrusomes have not been observed in petalomonads, instead various globular membrane-bounded bodies have been imaged or illustrated in ultrastructural reports (see Lee and Simpson 2014b). The only detailed study is in *Notosolenus urceolatus*, where the bodies are pill-shaped or rounded, about 0.5 μm in diameter and have a dense axial core (Fig. 11e). Typically, several are present in the anterior portion of the cell. It was proposed that these organelles represent a class of extrusome that is not homologous to the tubular extrusomes of other euglenozoans, but discharge has not been observed (Lee and Simpson 2014b).

The symbiontid *Bihospites* possesses ejectile ectosymbionts, known as epixenosomes, in addition to tubular extrusomes (Breglia et al. 2010). These epixenosomes are verrucomicrobial bacteria and are closely related to similar defensive symbionts reported earlier in certain ciliates (Petroni et al. 2000). In *Bihospites* the epixenosomes lie in rows between the rod-shaped epibiotic bacteria (see "Habitats and Ecology") and discharge by rapidly unwinding a central filament structure (Breglia et al. 2010).

Extracellular Structures

Conspicuous extracellular structures enclosing the main cell body are rare in euglenids. A strikingly thickened glycocalyx is present in several taxa, including *Neometanema* and several osmotrophs (Lee and Simpson 2014a). Most spectacularly, a group of photoautotrophic euglenids comprising *Trachelomonas* and *Strombomonas* produce a globular organic lorica that may be smooth or decorated with spines. The lorica has a single opening for the flagellum, and the cells locomote by swimming. The primary component of the lorica is mucus (Hilenski and Walne 1983; Mignot 1966), and during its development, the lorica slowly becomes thicker and ornamented. Iron and manganese are the main nutrients necessary for the lorica formation (e.g., Pringsheim 1953; Singh 1956). Differences in lorica formation between *Trachelomonas* and *Strombomonas* (Brosnan et al. 2005) are concordant with molecular phylogenetic data showing two distinct genera of loricate euglenids (e.g., Brosnan et al. 2005; Ciugulea et al. 2008). The sister group to the loricates is *Colacium*, which also has the ability to produce copious amounts of mucus, but instead forms mucilaginous stalks and dendroid colonies (Leedale 1967).

The Nucleus, Reproduction, and Cytokinesis

Euglenids cells usually have a single, large nucleus during interphase. The nucleus typically has a conspicuous subcentral nucleolus and large amounts of permanently condensed chromatin. This chromatin may give the nuclear material a lumpy appearance when viewed by light microscopy (Fig. 5e) and appears electron dense in transmission electron micrographs (Fig. 11f, g; see also Fig. 9c).

The nuclear genome organization of euglenids exhibits some bizarre features; rRNA genes are extrachromosomal, circular molecules, with thousands of copies per cell (Cook and Roxby 1985; Ravel-Chapuis 1988). Moreover, three types of introns are present in euglenid genomes; in addition to conventional spliceosomal introns, both noncanonical introns (for which a splicing mechanism is unknown) and so-called intermediate introns have been documented (Canaday et al. 2001; Milanowski et al. 2014). All euglenid species studied so far add a noncoding capped spliced-leader (SL) RNA to nucleus-encoded mRNAs via spliceosome-dependent *trans*-splicing (Frantz et al. 2000; Kuo et al. 2013), a process also reported in the other groups of Euglenozoa: kinetoplastids (Walder et al. 1986) and diplonemids (Sturm et al. 2001, Gawryluk et al. 2016). Full sequencing of the nuclear genome of *Euglena gracilis* is in progress but has been hindered by the genome size (approximately 2 Gb) and the high percentage of repetitive regions (O'Neill et al. 2015; see also EuglenaDB https://sites.dundee.ac.uk/euglenadb/). Furthermore, the nuclear DNA contains the unusual base "J," which makes up approximately 0.2% of all the bases (Dooijes et al. 2000) and hampers sequencing.

Asexual reproduction in euglenids occurs by mitosis followed by cytokinesis. The basal bodies and associated flagellar root system replicate first, followed by the feeding apparatus (if present) and then the pellicle. In many species the probasal bodies form early in interphase, such that they are present alongside the flagellated basal bodies in most cells within a population (e.g., *Entosiphon*; Solomon et al. 1987; *Peranema*; Hilenski and Walne 1985).

The mechanics of mitosis in euglenids was summarized at the level of light microscopy by Leedale (1967) and at the ultrastructural level by Triemer and Farmer (1991a). As with many protists, the nuclear envelope persists throughout mitosis, and the nucleolus does not break down but elongates and divides (in a few species, there are multiple nucleoli that divide separately; Leedale 1967; Zakryś 1986). The chromosomes are usually reported as permanently condensed (see above) but attached to the nuclear envelope prior to mitosis; they detach to assemble loosely at the division plane during metaphase (though spindle microtubules connect to the chromosomes before detachment in *Anisonema*; Triemer 1985). The relative timing of this assembly on one hand, and the process of chromosome replication through the separation of sister chromatids on the other, reportedly varies from species to species (Leedale 1967). The mitotic spindle system is intranuclear, with microtubules originating against the nuclear envelope. Almost all accounts indicate the presence of multiple subspindles originating from different foci around each pole of the dividing nucleus (Triemer and Farmer 1991a). Separation of the chromosomes is initially due to elongation of the nucleus rather than shortening of the spindle microtubules, which only happens near the end of anaphase; Triemer and Farmer (1991a) refer to this pattern as a "reversed anaphase A/B sequence." There is normally an association of the poles of the dividing nucleus and the replicated flagellar apparatus, but not always; in *Anisonema* the flagellar apparatus completes replication and begins segregation only after mitosis is well advanced (Triemer 1985).

After the nucleus and cytoskeleon have duplicated, a cleavage furrow forms at the base of the flagellar pocket near the basal bodies and migrates toward the anterior opening, forming two flagellar pockets within the cell. The cleavage furrow subsequently migrates posteriorly down the longitudinal axis of the cell; the posterior tip of the cell is the last part to become cleaved. The cleavage furrow forms between a (mature) parent strip and a newly generated (nascent) strip on two sides of the cell (Esson and Leander 2006). Each daughter cell (usually) contains the same number of pellicle strips as the parent cell (Yubuki and Leander 2012); however, an unequal distribution of strips can also occur during cytokinesis (see above).

Sexuality is almost unknown in euglenids, but Mignot (1962) gave a light microscopy account of a small petalomonad, *Scytomonas pusilla*, that included normal-looking cells behaving as isogametes and undergoing syngamy (i.e., pairs of cells fused, and then their nuclei fused).

Taxonomy

About 1500 species of euglenids are recognized, with the majority being photoautotrophs. The taxonomy of the photoautotrophs was extensively scrutinized over the last 20 years based on molecular and morphological data, and the current assignment of species to genera largely follows phylogeny. Notably, a large number of species that were formerly placed within *Euglena*, but are not closely related, have been given new generic assignments. Conversely, certain genera of secondary osmotrophs have been suppressed on phylogenetic grounds (e.g., *Hyalophacus* – Marin et al. 2003; *Cyclidiopsis* – Bennett and Triemer 2014). The traditional genus *Astasia* turned out to include species of both primary and secondary osmotrophs, but now includes only primary osmotrophs.

Photoautotrophic euglenids, or Euglenophyceae, are a monophyletic group (Marin et al. 2003; Karnkowska et al. 2015; Kim et al. 2015; Cavalier-Smith 2016) comprising the basal monotypic genus *Rapaza* (Yamaguchi et al. 2012), Eutreptiales and Euglenales (here and elsewhere we use the dominant botanical-tradition naming for higher taxa of Euglenophyceae; see Cavalier-Smith 2016 for a recent but particular "zoological" taxonomy for photoautotrophic euglenids). Eutreptiales comprise two predominantly marine genera – *Eutreptia* and *Eutreptiella* – while a third genus, *Tetraeutreptia,* was subsumed within *Eutreptiella* (Marin et al. 2003; Cavalier-Smith et al. 2016). Due to the limited number of taxa and genes used for phylogenetic reconstructions, the genus-level taxonomy is not well resolved within the Eutreptiales and *Eutreptiella* might be paraphyletic (Marin et al. 2003; Cavalier-Smith 2016). The Euglenales is much more diverse and is subdivided into two clades: Phacaceae (with three genera) and Euglenaceae (with eight genera).

The Phacaceae contains *Discoplastis*, *Lepocinclis*, and *Phacus* (Kim et al. 2010; Linton et al. 2010; Karnkowska et al. 2015; Kim et al. 2015). *Discoplastis* was erected to accomodate two species previously classified in the genus *Euglena* (Triemer et al. 2006). *Phacus* and *Lepocinclis* are closely related (Kim et al. 2010;

Linton et al. 2010; Karnkowska et al. 2015; Kim et al. 2015). Both genera have been intensively studied over the last 15 years, with several species transferred from other genera to either *Phacus* (Linton et al. 2010) or *Lepocinclis* (Marin et al. 2003; Kosmala et al. 2005; Bennett and Triemer 2012; Bennett and Triemer 2014). Those taxonomic changes resulted in the loss of morphological characters distinguishing those two genera (Linton et al. 2010). Some phylogenetic analyses have indicated paraphyly of the genus *Phacus* (Kim and Shin 2014; Karnkowska et al. 2015); however, the most comprehensive study (Kim et al. 2015) supported the division into two genera.

The Euglenaceae contains seven monophyletic genera (*Euglenaria*, *Euglenaformis*, *Colacium*, *Cryptoglena*, *Monomorphina*, *Strombomonas*, and *Trachelomonas*) and the paraphyletic *Euglena* (Kim et al. 2010; Linton et al. 2010; Karnkowska et al. 2015; Kim et al. 2015). The earliest branching lineage is the recently established genus *Euglenaformis* (Bennett et al. 2014) with one species, *Euglenaformis proxima* (formerly *Euglena proxima*). The remaining genera form two sister clades. One clade includes *Colacium* and the closely related loricate genera *Trachelomonas* and *Strombomonas*. Marin et al. (2003) proposed merging *Trachelomonas* and *Strombomonas*, but all recent phylogenetic analyses have supported their phylogenetic distinction (Brosnan et al. 2005; Triemer et al. 2006; Ciugulea et al. 2008; Kim and Shin 2008; Kim et al. 2010; Linton et al. 2010; Karnkowska et al. 2015; Kim et al. 2015). The second clade includes the closely related rigid genera *Monomorphina* and *Cryptoglena,* together with *Euglena* and *Euglenaria* (Karnkowska et al. 2015; Kim et al. 2010, 2015; Linton et al. 2010). *Euglenaria* was erected to accommodate three *Euglena* species placed outside the main clade of *Euglena* (Linton et al. 2010). *Euglenaria* is sister to *Monomorphina* and *Cryptoglena* in most phylogenetic analyses (Linton et al. 2010; Kim et al. 2010; Karnkowska et al. 2015), but branched as sister to *Euglena* in one recent study (Kim et al. 2015). The taxonomy of the genus *Euglena* is the most problematic because species which did not fit morphologically into other genera were assigned to it, resulting in an amalgam of species. Currently, two species of *Euglena* fall outside the main well supported *Euglena* clade: *E. archaeoplastidiata* (Kim and Shin 2008; Kim et al. 2010; Karnkowska et al. 2015; Kim et al. 2015) and *E. velata* (Karnkowska-Ishikawa et al. 2012; Kim et al. 2015).

The genera *Euglenamorpha* and *Hegneria*, which were originally observed in tadpole guts, are not represented in any molecular phylogenetic trees. Therefore, their validity and phylogenetic positions are questionable.

Most of the species of phototrophic euglenids were described in the nineteenth and twentieth centuries based solely on morphology. Thousands of taxa have been described (~3000 including forms and varieties according to AlgaeBase: http://www.algaebase.org) because of the great morphological diversity of euglenid cells. The species-level taxonomy of the group is riddled with duplications and re-descriptions, as well as formulations of artificial classification schemes. The advent of DNA sequencing combined with careful morphological investigation allowed some of the taxonomic confusions to be resolved. Many species have been verified, and new taxa have been described to accommodate the observed

molecular and morphological diversity (Bennett and Triemer 2012; Kosmala et al. 2005, 2007a, 2007b, 2009; Karnkowska-Ishikawa et al. 2010, 2011, 2012, 2013, 2014; Kim et al. 2013a, b, 2014; Kim et al. 2016; Linton et al. 2010; Łukomska-Kowalczyk et al. 2015; Shin and Triemer 2004; Zakryś 1997; Zakryś et al. 2002, 2004, 2013).

The primary osmotrophs (Aphagea) are a phlyogenetically cohesive group that includes the Rhabdomonadales (*Menoidium*, *Rhabdomonas*, *Gyropaigne*, *Parmidium*, and *Rhabdospira*) plus *Distigma* and *Astasia* (see above). Both *Distigma* and *Astasia* appear to be paraphyletic at present (Preisfeld et al. 2001; Muellner et al. 2001; Cavalier-Smith 2016).

The taxonomy of phagotrophs is far less well organized than that of photoautotrophic euglenids, partly because sequence information is sparse. Current genus-level taxonomy is a mix of traditional systems that emphasize a few conspicuous morphological characters on one hand (e.g., flagellar number and lengths, degree of flexibility, visibility of the feeding apparatus) and molecular phylogenetic information derived from very few species on the other (plus a small amount of ultrastructural data). The genus-level taxonomy is covered here using the four informal assemblages introduced earlier. Throughout we will use the the predominant "zoological" genus names (but see below).

Petalomonads (Petalomonadida) are probably monophyletic, and this group contains several dozen species assigned to the genera *Petalomonas*, *Notosolenus*, *Calycimonas*, *Sphenomonas*, *Scytomonas*, *Tropidoscyphus*, *Atraktomonas*, the recently created *Biundula*, and perhaps *Dolium* and *Dylakosoma* (Lee and Simpson 2014b; Cavalier-Smith 2016). The boundaries among many of these genera are highly uncertain; the morphological differences between them are often subtle, and some are known to currently represent non-monophyletic groupings (e.g., *Notosolenus*; Lee and Simpson 2014b). Much better DNA sequence coverage of genera and species (including type species) is needed, and it is likely to precipitate considerable changes to the genus-level taxonomy.

Most ploeotiid species, with the exception of *Entosiphon* spp., were descibed within the last 30 years, and most have been included at some point in the genus *Ploeotia* (Larsen and Patterson 1990). However recent phylogenies inferred from SSU rDNA sequences indicate that ploeotiids are genetically diverse and not monophyletic (Lax and Simpson 2013; Chan et al. 2013; Cavalier-Smith 2016; Cavalier-Smith et al. 2016), and the current trend is to recognize several genera in addition to *Ploeotia* (and *Entosiphon*), namely, *Decastava, Keelungia*, *Lentomonas*, and *Serpenomonas* (see Chan et al. 2013; Cavalier-Smith 2016). These are a mix of new taxa and genera that were previously considered as synonyms of *Ploeotia*. The rational distribution of most ploeotiid species to genera awaits further molecular sequence data (e.g., from the type species of *Ploeotia, P. vitrea*).

Peranemids include several genera, namely *Peranema*, *Chasmostoma*, *Urceolus*, and *Jenningsia* (and *Peranemopsis*, a synonym of *Jenningsia* according to Lee et al. 1999), as well as most but not all of the organisms that have typically been assigned to *Heteronema* (see below), including that assigned to the newly proposed *Teloprocta* (Cavalier-Smith et al. 2016). Peranemids in this broad sense

are probably not monophyletic (note that the taxa Peranemia, Peranemida, and Peranemidae have all recently been used to encompass just *Peranema*, *Urceolus, Jenningsia*, and *Peranemopsis*; Cavalier-Smith 2016; but this more restricted "peranemid" assemblage is likely not monophyletic either). As of late 2016, there are sequences available from just two species from this entire assemblage, so the phylogenetic appropriateness of the genus-level taxonomy is difficult to evaluate at present.

The anisonemid assemblage includes *Dinema* and *Anisonema* and, almost certainly, some species currently assigned to *Heteronema* (see below). It is unclear at present whether anisonemids are monophyletic (compare Lax and Simpson 2013; Lee and Simpson 2014a; Cavalier-Smith 2016). The assignment of species to *Anisonema* and *Dinema* is problematic; molecular phylogenies usually recover *Dinema* as non-monophyletic (Lee and Simpson 2014a; Cavalier-Smith 2016), while *Anisonema* has a very diffuse circumscription, to the extent that some species are probably actually ploeotiids.

Neometanema and the taxonomic entity *Semihia* are related to (and possibly derived from) anisonemids, from which they differ by having a distinctive "skidding" motility, although they also retain a supplementary ability to glide (Lee and Simpson 2014a). *Neometanema* and *Semihia* collectively absorb all the euglenid species previously assigned to *Metanema* (e.g., Larsen 1987), which has a zoological homonym, as well as a couple of species of *Heteronema* (see below; Lee and Simpson 2014a). Molecular phylogenetic analysis shows with moderate support that *Neometanema* is closely related to Aphagea (Lax and Simpson 2013; Lee and Simpson 2014a; Cavalier-Smith 2016), and the taxon name Natomonadida has recently been proposed for this grouping, based on the frequent use of swimming locomotion (Cavalier-Smith 2016).

The genus *Heteronema* is particularly problematic. At present it mainly includes "peranemids," but also includes a small number of "anisonemids" (see Larsen and Patterson 1990) even after the recent transfer of species to *Neometanema* (Lee and Simpson 2014a). Although the first described *Heteronema, H. marina* (Dujardin 1841), was some kind of anisonemid (as defined here), the modern concept of the genus comes from Stein (1878) and is based on peranemid species. This switch has long been recognized and tolerated (Larsen and Patterson 1990). Cavalier-Smith (2016) recently proposed returning to Dujardin's earlier concept, but we advocate overlooking this proposal, which is potentially destabilizing for no real gain (and if carried to a logical conclusion, could dramatically affect the application of the genera *Dinema* and/or *Anisonema* as well as *Heteronema)*.

The Symbiontida (synonym Postgaardea – see Cavalier-Smith et al. 2016) encompasses the three genera *Calkinsia*, *Postgaardi*, and *Bihospites*. Each includes a single described species at present (Yubuki et al. 2009; Breglia et al. 2010).

It is important to note that several genera of phagotrophic euglenids have homonyms in botanical taxonomy, and alternative botanical names have been

proposed: *Dinema* = *Dinematomonas*; *Entosiphon* = *Entosiphonomonas*; and *Peranema* = *Pseudoperanema*.

Evolutionary History

The phylogeny of euglenids has been addressed most extensively using nucleotide sequences amplified from ribosomal genes (i.e., small and large subunit rRNA genes) (Brosnan et al. 2003, 2005; Busse et al. 2003; Ciugulea et al. 2008; Karnkowska et al. 2015; Kim and Shin 2008; Kim et al. 2015; Linton et al. 1999, 2000, 2010; Marin et al. 2003; Milanowski et al. 2001, 2006; Montegut-Felkner and Triemer 1997; Müllner et al. 2001; Nudelman et al. 2003; Preisfeld et al. 2001; Triemer et al. 2006; von der Heyden et al. 2004; Zakryś et al. 2002). Although these genes have been helpful in resolving the phylogeny of photoautotrophic euglenids, they do not provide satisfactory phylogenetic signal at deeper levels in the phylogeny (e.g., among the bacterivorous euglenids). Additional evidence for deep-level phylogenetic relationships of euglenids comes from comparative analyses of morphological data and some nucleus-encoded protein genes (e.g., heat shock protein 90), although the latter are still very sparse (Breglia et al. 2007; Leander et al. 2001a, b; Karnkowska et al. 2015; Montegut-Felkner and Triemer 1997; Simpson et al. 2002; Simpson and Roger 2004; Talke and Preisfeld 2002; Cavalier-Smith et al. 2016). These data also confirm the placement of euglenids within the Euglenozoa, as sister to kinetoplastids and diplonemids. Multigene molecular phylogenetic analyses also strongly support the placement of the Euglenozoa as a whole within a clade, the Discoba, that also includes Heterolobosea, Jakobida, and *Tsukubamonas* (e.g., Hampl et al. 2009; Kamikawa et al. 2014).

The following summarizes the current state of knowledge about phylogenetic relationships among euglenids (see also Fig. 12).

- Photoautotrophic euglenids (Euglenophyceae or euglenophytes) are a monophyletic subgroup nested within a paraphyletic assemblage of phagotrophic lineages.
- Euglenophytes with one emergent flagellum (Euglenales) are monophyletic; the Eutreptiales, with two emergent flagella (or rarely more), are their closest relatives. The recently described mixotroph *Rapaza* (also with two emergent flagella) is the deepest branch within euglenophytes.
- *Phacus* and *Lepocinclis* are each probably monophyletic and together form a more inclusive monophyletic group within the euglenophytes; these lineages tend to have 32 pellicle strips, are rigid, show great diversity in cell shape, and possess many small disc-shaped chloroplasts without pyrenoids and dimorphic paramylon grains.

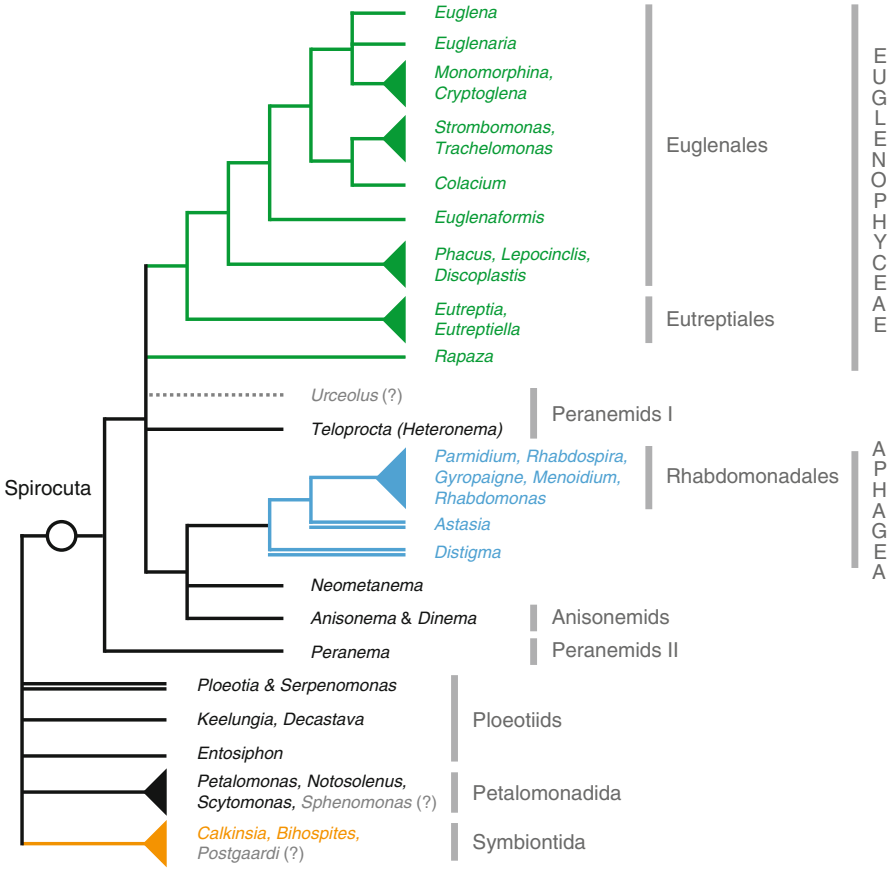

Fig. 12 Current knowledge of the evolutionary tree of euglenids, based primarily on SSU rRNA gene phylogenies. Photoautotrophic taxa are shown in green, primary osmotrophs in blue, "typical" phagotrophic taxa in black, and symbiontids in orange. Selected higher taxa are depicted to the right, though Spirocuta (= "H" or "HP" clade) is marked with a circle on its basal branch. Polytomies indicate regions of the tree that are poorly supported and/or resolved differently in various recent analyses. Genera shown in gray are important taxa whose positions are inferred from morphological information alone, since no molecular data are currently available (genera for which there are relatively limited data are not shown). Double lines on a branch denote paraphyletic groups (note also that both "peranemids" and "ploeotiids" appear to be paraphyletic). *Ploeotia* is probably paraphyletic at present, but it is also unclear whether *Serpenomonas* and *Ploeotia* are phylogenetically distinct (sequence information is awaited from *Ploeotia vitrea*, the type species of *Ploeotia*). The clade containing *Phacus*, *Lepocinclis*, and *Discoplastis* represents the taxon Phacaceae; the clade containing all other genera within Euglenales corresponds to the taxon Euglenaceae (see text)

- *Discoplastis* is the monophyletic sister group to *Phacus* and *Lepocinclis* and shares several morphological features with them (e.g., disc-shaped plastids without pyrenoids and 32 pellicle strips); however, these cells undergo dynamic metaboly.

- The monotypic genus *Euglenaformis* branches at the base of the assemblage of Euglenaceae.
- The loricate taxa *Trachelomonas* and *Strombomonas* are each monophyletic and together form a monophyletic group.
- The nearest sister group to the loricates is *Colacium*, which forms mucilaginous stalks.
- The rigid euglenophytes *Monomorphina* and *Cryptoglena* form a monophyletic group; these lineages have only one plastid and a relatively small number of broad pellicle strips (around 16–20).
- *Euglenaria* is monophyletic and possess morphological features similar to those of some representatives of *Euglena* (lobate plastids with diplopyrenoids), but distinct molecular signatures in nuclear SSU rDNA sequences. The molecular phylogenetic position of that lineage is not well resolved.
- The modern (revised) version of the genus *Euglena* is monophyletic with two known exceptions (see above); *Euglena* species often have 40 pellicle strips, undergo metaboly, and show great diversity in cell shape and plastid morphology (e.g., shield-shaped, stellate, lobed, spherical).
- Photosynthesis was lost several times independently within the euglenophytes (e.g., *Euglena longa* and *Euglena quartana* – previously assigned to "*Astasia*" and "*Khawkinea,*" respectively).
- The nearest sister lineages to euglenophytes are certain eukaryovorous euglenids, possibly *Teloprocta* (formerly *Heteronema*) and/or *Urceolus*.
- Primary osmotrophic euglenids (Aphagea, e.g., *Distigma*, *Rhabdomonas, Astasia*) are monophyletic and diverged from eukaryovorous ancestors independently from euglenophytes.
- Euglenophytes, primary osmotrophs, and eukaryovorous euglenids form a monophyletic group (Spirocuta; formerly the "H" or "HP" clade).
- Eukaryovorous euglenids are paraphyletic because they gave rise, independently, to both primary osmotrophs and euglenophytes – see above.
- Bacterivorous euglenids are probably paraphyletic.
- One clade of bacterivorous euglenids, petalomonads (Petalomonadida), has retained several possibly ancestral characters, such as few pellicle strips (10 or fewer), an MtR pocket, kDNA-like mitochondrial inclusions, and bacterivorous modes of nutrition; however, phylogenetic evidence that petalomonads are a particularly deep branch within euglenids is equivocal at best.
- Other bacterivorous euglenids ("ploeotiids," including *Entosiphon*) have unclear molecular phylogenetic positions vis-à-vis each other and petalomonads and symbiontids. These lineages have rigid pellicles with 12 or fewer strips (usually 10), somewhat similar to petalomonads, but have complex feeding apparatuses, including rods and vanes, similar to eukaryovorous euglenids.
- Symbiontids are a monophyletic group of anaerobes that lack pellicular strips, but usually branch among bacterivorous euglenids in molecular phylogenies, albeit with weak statistical support. They likely descended from "classical" bacterivorous euglenids, and secondarily lost pellicular strips, perhaps as a consequence of entering into symbioses with epibiotic bacteria. However,

transverse sections through the cell surface of *Bihospites* show many S-shaped profiles that are reminsicent of pellicle strips.
- Knowledge about the overall diversity and phylogenetic relationships of bacterivorous and eukaryovorous euglenids is still very poor.

Morphological Evolution, Especially the Pellicle

The euglenid pellicle is very diverse, and comparative analyses have demonstrated a great array of intermediate states for several cytoskeletal characters. This diversity placed in a molecular phylogenetic context demonstrates many large-scale evolutionary trends within the group (Leander et al. 2007).

The evolution of strip number involved at least three mechanisms associated with cytoskeletal replication and cell division: (1) asymmetrical segregation of strips to daughter cells, (2) permanent strip doubling events, and (3) permanent strip halving events (Esson and Leander 2006; Leander 2004; Leander et al. 2001a, b, 2007; Yubuki and Leander 2012). Permanent strip duplication events refer to a cell that duplicates its strips but fails to divide. Permanent strip halving events refer to a cell that divides without first duplicating its strips. The distribution of strip numbers found in euglenids suggests that these mechanisms collectively happened several times during the evolution of the group; there is evidence for the following events: four strips to eight strips (or vice versa) in petalomonads, 10 strips to 20 strips coincident the emergence of Spirocuta (the HP clade), and 20 strips to 40 strips near the origin of the Euglenales (Esson and Leander 2006; Leander et al. 2001a, b, 2007; Leander 2004).

The ancestral state for the number of strips in phototrophic euglenids is between 40 and 50; strip numbers that are significantly higher or lower than 40–50 are inferred to represent derived states. For instance, some relatively enormous species have either doubled or tripled this number of strips (e.g., 80 strips in *Lepocinclis helicoideus* and 120 strips in *Euglena obtusa*) (Esson and Leander 2008; Leander and Farmer 2000b). The phototrophic lineages that have lost metaboly, such as *Phacus* and *Lepocinclis*, tend to have 32 strips, which is the inferred ancestral state for the more inclusive clade consisting of these two genera plus *Discoplastis*. A subgroup of *Phacus* reduced the number of strips even further to about 20; these cells are among the smallest of all known photoautotrophic euglenids (Fig. 6). The strip-halving process (see above) helps explain the reduction of strips during the evolution of the rigid photoautotrophic lineage *Monomorphina* (32 strips to 16 strips; Leander and Farmer 2001b) (Figs. 1 and 6).

Fossil Record

Euglenid fossils are sparse. Aside from the loricas of *Trachelomonas* and *Strombomonas*, euglenids do not secrete hard parts that would promote fossilization.

However, some photoautotrophic euglenids have exceedingly thick proteinaceous strips, which could presumably fossilize. *Moyeria* is an enigmatic fossil with euglenid-like features (e.g., strips and a canal opening) that was discovered in Silurian deposits (Gray and Boucot 1989). The size, shape, and surface morphology of these fossils are reminiscent of some phototrophic euglenids in the genus *Monomorphina*.

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Kinetoplastea

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Wendy Gibson

Abstract

The class Kinetoplastea Cavalier-Smith 1981 (previously known as the order Kinetoplastida Honigberg 1963) constitutes an important group of free-living and parasitic flagellates. The group is named after the kinetoplast, a unique cell organelle consisting of the tightly packaged mitochondrial DNA, which forms a stainable structure within the single mitochondrion. The Kinetoplastea includes several important human pathogens that are carried by bloodsucking insect vectors, e.g., *Trypanosoma brucei*, *T. cruzi*, *Leishmania donovani*, *L. major*, and *L. tropica*, as well as vector-borne animal pathogens such as the African tsetse-transmitted trypanosomes that cause nagana. Some kinetoplastids are plant parasites, e.g., *Phytomonas*, transmitted by phytophagous bugs. While these pathogenic kinetoplastids are of major medical, veterinary, and economic importance, many other kinetoplastid species also have a parasitic lifestyle, either in a single host species or alternating between two different hosts. The ubiquitous free-living kinetoplastids such as *Bodo* are of major ecological importance as heterotrophs in marine and freshwater environments. Some kinetoplastid species are popular and significant laboratory model species for biochemical and molecular biology investigations. In particular, *Trypanosoma brucei* is notorious for its ability to undergo antigenic variation, and *Leishmania* infection is a paradigm for T-helper cell type I and type II immune responses.

Revised from the original chapter of Keith Vickerman

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Introduction

General Characteristics

The class Kinetoplastea Cavalier-Smith 1981 constitutes a group of small colorless flagellates with one or two flagella and massed mitochondrial DNA that forms a stainable structure – the kinetoplast – within the single mitochondrion. The size, shape, and position in the cell of the kinetoplast are of taxonomic and ontogenetic significance. In many genera the kinetoplast is found close to the kinetosomes (basal bodies) of the flagella and consists of a network of interlocked circular DNA

molecules, tightly packaged into a disc-shaped bundle. The kinetoplast DNA may also be dispersed and occupy all or a large part of the mitochondrion. In a few mutants stainable kinetoplast DNA may be absent (dyskinetoplasty).

The kinetoplastids are classified together with the euglenids and diplonemids in the phylum Euglenozoa Cavalier-Smith 1981, which is recovered as a monophyletic group in molecular phylogenetic trees based on ribosomal RNA and protein-coding genes (Keeling et al. 2005). Kinetoplastids can be divided by morphology into two groups: one that is uniflagellate and the other with two heterodynamic flagella – one directed anteriorly and locomotory, the other directed posteriorly and recurrent or trailing (Figs. 1 and 2). These groups were traditionally classified as the suborders Trypanosomatina (family Trypanosomatidae) and Bodonina (families Bodonidae, Cryptobiidae), respectively. However, molecular phylogenetic evidence indicates that while the Trypanosomatina and Trypanosomatidae are both monophyletic, the suborder Bodonina and its constituent families are not (Callahan et al. 2002; Dolezel et al. 2000; Wright et al. 1999). With increasing diversity of kinetoplastids represented in phylogenetic analyses, a more complete picture is now beginning to emerge (Moreira et al. 2004; von der Heyden et al. 2004). There is a core group of

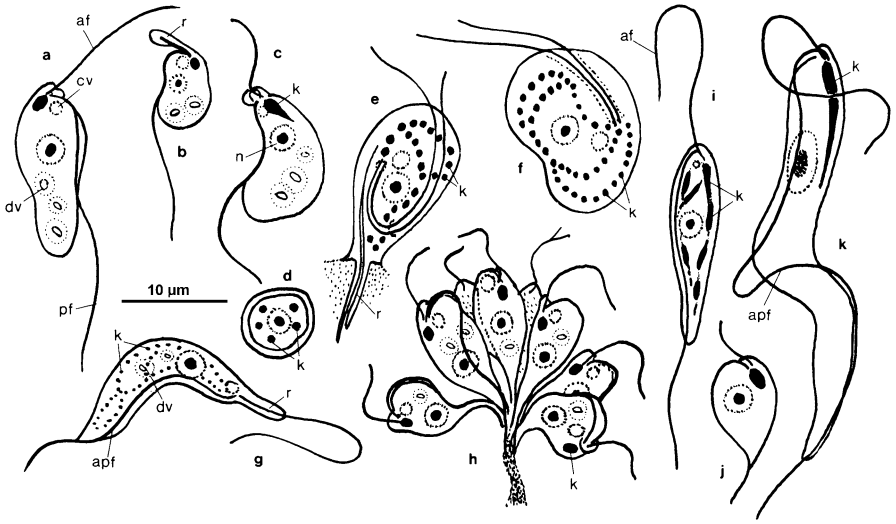

Fig. 1 Morphology of bodonid flagellates: *k* kinetoplast, *n* nucleus, *cv* contractile vacuole, *dv* digestive vacuole containing bacteria, *af* anterior flagellum, *pf* posterior flagellum, *apf* attached pf, *r* rostrum, *EUK* eukinetoplastic, *PLK* polykinetoplastic, *PNK* pankinetoplastic. (**a**) *Parabodo caudatus* (EUK); (**b**) *Rhynchomonas nasuta* (EUK); (**c**) *Procryptobia glutinosa* (EUK); (**d**) *P. glutinosa* cyst (PLK); (**e**) *Ichthyobodo necator* (PLK) attached phase on fish skin; (**f**) *I. necator* (PLK) migratory phase; (**g**) *Dimastigella trypaniformis* (PLK); (**h**) *Cephalothamnium cyclopum* (EUK) colony with secreted stalk; (**i**) *Cryptobia vaginalis* from leech (PNK phase); (**j**) *Cryptobia vaginalis* (EUK phase); (**k**) *Trypanoplasma keysselitzi* (EUK) from tench (After Vickerman and Preston 1976)

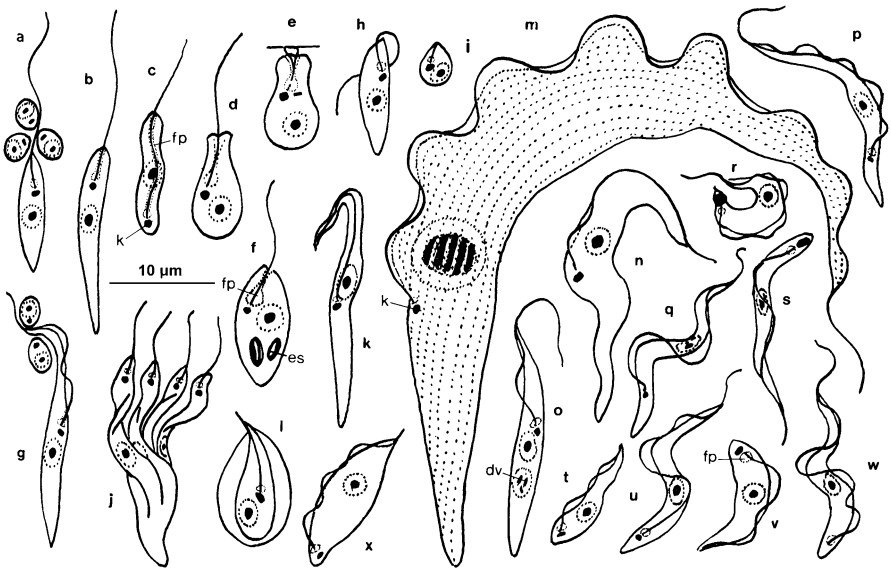

Fig. 2 Morphology of trypanosomatid flagellates: *fp* flagellar pocket, *PM* promastigote, *AM* amastigote, *OPM* opisthomastigote, *CHM* choanomastigote, *EPM* epimastigote, *TPM* trypomastigote, other abbreviations as in Fig. 1. (**a**) *Leptomonas oncopelti* (PM) with "straphanger" cysts; (**b**) *Herpetomonas muscarum* (PM); (**c**) *H. muscarum* (OPM); (**d**) *Crithidia fasciculata* (CHM, nectomonad); (**e**) *C. fasciculata* (CHM, haptomonad); (**f**) *C. oncopelti* (CHM) with endosymbionts (es); (**g**) *Blastocrithidia familiaris* (EPM) with cysts; (**h**) *Leishmania major* (PM); (**i**) *L. major* (AM); (**j**) *Phytomonas elmassiani* (PM), multiple fission stage in plant latex; (**k**) *Rhynchoidomonas drosophilae* (TPM); (**l**) *Endotrypanum schaudinni* (EPM) in sloth red cell; (**m**), *Trypanosoma grayi* (TPM) from crocodile blood; (**n**) *T. (Megatrypanum) cyclops* (TPM) from blood of Macaque; (**o**) *T. cyclops* (EPM, with pigment in digestive vacuole) from culture; (**p**) *T. (Herpetosoma) musculi* (TPM) from mouse blood; (**q**) *T. (Tejeraia) rangeli* (TPM) from human blood; (**r**) *T. (Schizotrypanum) dionisii* (TPM) from pipistrelle bat; (**s**) *T. (Duttonella) vivax* and (**t**) *T. (Nannomonas) congolense* (TPM), both from cattle blood; (**u**) *T. brucei* (TPM, slender bloodstream form); (**v**) *T. brucei* (TPM, short stumpy form); (**w**) *T. evansi* (TPM, dyskinetoplastic) from camel; (**x**) *T. (Pycnomonas) suis* (TPM) from pig blood (After Vickerman and Preston 1976)

kinetoplastids, subclass Metakinetoplastina (Moreira et al. 2004) that comprises the trypanosomatids and bodonids, with a highly divergent sister group, and subclass Prokinetoplastina (Moreira et al. 2004) that includes the fish ectoparasite *Ichthyobodo necator*, a related kinetoplastid symbiont of amoebae and organismal DNA recovered from environmental samples (Callahan et al. 2002; Moreira et al. 2004; von der Heyden et al. 2004).

Molecular phylogenetic studies are adding to the number of kinetoplastid genera through the formal recognition of polyphyletic genera, e.g., the revision of genus *Bodo* (Moreira et al. 2004), and the discovery of new genera, particularly among the trypanosomatids from arthropod hosts (Maslov et al. 2013). Methods of obtaining molecular phylogenetic information from previously unculturable organisms, living, for example, in marine sediments and soil, also add to the number of bodonid genera

and species (von der Heyden and Cavalier-Smith 2005). A general problem for kinetoplastid taxonomy is the lack of discernable morphological differences between sometimes quite different species. Molecular approaches have been widely used to discriminate species and strains of clinically important genera such as *Trypanosoma* and *Leishmania* and are now being employed more widely for species identification.

Many bodonids are free living and as heterotrophs constitute an important part of the community in marine and freshwater environments. The group also includes parasitic forms traditionally grouped into the Cryptobiidae, now subsumed into the parabodonid group (Moreira et al. 2004). Parasitic bodonids occur in fish, either in the bloodstream or gut, and also in other aquatic or terrestrial organisms. The ectoparasite *Ichthyobodo necator* lives on the gills and skins of fish and a few other organisms (see Table 1). Endosymbiotic kinetoplastids have been described in other protists. Bodonids are usually phagotrophic, ingesting food through a cytopharynx with a cytostome bordered by prominent lips often drawn out into a projecting rostrum (Fig. lb, e, g). The bodonid kinetoplast is highly variable in morphology and structure, generally being larger and often more diffuse or dispersed that in the trypanosomatids. The fundamental difference between the kDNAs of bodonids and trypanosomatids appears to be that the kDNA does not form a network in any of the representative bodonid species studied thus far, in contrast to the trypanosomatids where kDNA mini- and maxicircles are interlinked and densely packaged (Lukeš et al. 2002). The diagnostic characteristics of some representative bodonid genera are summarized in Table 1.

Trypanosomatids are invariably parasitic and their host ranges are indicated in Table 2. They have a small, strongly staining kinetoplast – referred to as the eukinetoplastic condition (Vickerman 1990). During their life cycles, the position of the kinetoplast-basal body-flagellar pocket complex may shift in relation to the nucleus and body extremities, and the flagellum may change in length. Thus, the following forms are recognized (Fig. 2): *amastigote*, round to oval body, flagellum short, not emerging from pocket; *promastigote*, kinetoplast close to anterior end of elongate body, flagellum emerging anteriorly and unattached; *opisthomastigote*, similar, but kinetoplast is postnuclear and flagellar pocket forms long canal to anterior end of body; *choanomastigote*, body pyriform, kinetoplast just in front of nucleus, flagellum emerging anteriorly; *epimastigote*, prenuclear kinetoplast, flagellum emerging from pocket along the body and attached to the body along its anterior portion; *trypomastigote*, similar, but kinetoplast and flagellar pocket are postnuclear. The term spheromastigote is sometimes used for a rounded body with an emerging flagellum attached to it. *Endomastigote* has been coined for the condition described in *Wallaceina* where the flagellum is enclosed within the cell. The different genera are characterized according to which of these morphological forms are present in the life cycle and whether the cycle is monogenetic (monoxenous) or digenetic (heteroxenous) (Table 2), i.e., involves one host or alternates between two different animal hosts, usually a vertebrate and an invertebrate. The clinically important genera, *Trypanosoma* and *Leishmania*, are divided into sections and subgenera, summarized in Table 3.

Table 1 Principal bodonid genera of Kinetoplastea

Order	Genus	Diagnostic characters[a]	Feeding	Habitat/life cycle
Eubodonida Vickerman 2004	*Bodo* Ehrenberg 1830 emend. Vickerman 2004	Eukinetoplastic; AF non-tubular hairs; PF free from body; lateral cytostome-cytopharynx lacks PR	Phagotrophic, bacterivorous	Solitary, free living (fresh- or seawater, soil); can colonize fleeting habitats (dew, spittle bug exudate)
Parabodonida Vickerman 2004	*Parabodo* Skuja 1939 emend. Vickerman 2004	Eukinetoplastic in trophozoite; pankinetoplastic in cyst; body flattened; PF free/mainly free from body and used as skid; cytostome-cytopharynx lacks PR	Phagotrophic, bacterivorous	Solitary, free living (commonly coprozoic)
	Procryptobia Vickerman 1979	Eukinetoplastic trophozoite; PF attached to body over greater length, acts as "skid." Cysts sometimes polykinetoplastic	Phagotrophic, bacterivorous	Free living, commonly coprozoic
	Cryptobia[b] Leidy 1846	Eukinetoplastic or pankinetoplastic trophozoites; PF attached along the length of body; cysts unknown	Osmotrophic	Parasitic or endocommensal in gut or reproductive system of invertebrates
	Trypanoplasma[b] Laveran and Mesnil 1901	Eukinetoplastic; PF attached along length of body and forms "undulating membrane"; cysts unknown	Osmotrophic	Parasitic in blood (and gut?) of fish; cyclical transmission of blood trypanoplasms primarily by leeches. Occasionally pathogenic

Neobodonida	*Neobodo* Vickerman 2004	Eukinetoplastic; PF free/mainly free from body and used as skid; apical cytostome-cytopharynx supported by PR	Phagotrophic, bacterivorous	Solitary, free living (fresh- or seawater, soil)
	Rhynchomonas Klebs 1893	Eukinetoplastic trophozoite; PF mostly free from body, acts as "skid"; short AF attached to long proboscis formed from rostrum. Cysts undescribed	Phagotrophic, bacterivorous	Free living (fresh water, marine planktonic, soil); coprozoic
	Dimastigella Sandon 1928	Polykinetoplastic trophozoite and cyst; PF attached along elongate body acts as "skid." Long rostrum alongside AF	Phagotrophic, bacterivorous	Free living in soil and coprozoic
Prokinetoplastida Vickerman 2004	*Ichthyobodo* Pinto 1928 (*Costia* Leclerque 1890)	Polykinetoplastic; trophozoite attached to host by elongate rostrum; free-swimming dispersive stage lacks rostrum; PF free from body. No cysts	Phagotrophic ingesting cytoplasm of host cell (histophagic)	Biphasic life cycle; trophozoite ectoparasitic on fish and urodeles; alternates with free-swimming dispersive phase. Pathogenic in young fish

Notes: [a]Abbreviations *AF* anterior flagellum, *PF* posterior flagellum, *PR* prismatic rod of microtubules
[b]*Trypanoplasma* and *Cryptobia* are treated as separate genera here, although the taxonomy of the fish gut and invertebrate cryptobias is still under debate (Moreira et al. 2004; Woo 1994)

Table 2 Principal trypanosomatid genera of Kinetoplastea

Genus	Diagnostic characters[a]	Hosts and practical significance
Leptomonas[b] Kent 1880	Monogenetic; *promastigotes* and cysts only in life cycle	Mainly insects (Hemiptera, Diptera, Hymenoptera, Blattoidea, Lepidoptera, Siphonaptera, Anoplura), rarely other invertebrates and ciliates. Nonpathogenic
Herpetomonas[b] Kent 1880	Monogenetic; promastigotes and *opisthomastigotes* (not all species) in life cycle	Diptera, Heteroptera, Siphonaptera. Nonpathogenic
Crithidia[b] Léger 1902	Monogenetic; *choanomastigotes* only	Diptera, Hemiptera, Trichoptera; Hymenoptera. Nonpathogenic
Blastocrithidia[b] Laird 1959	Monogenetic; *epimastigotes* and cysts only	Diptera, Hemiptera, Siphonaptera, and ixodid ticks. Possibly pathogenic in some species
Rhynchoidomonas Patton 1910	Monogenetic; *trypomastigote* stage only (but genus poorly known)	Diptera. Nonpathogenic
Wallaceina Podlipaev, Frolov, and Kolesnikov 1999 (*Proteomonas* Podlipaev, Frolov & Kolesnikov 1990)	Monogenetic; *endomastigotes* and promastigotes	Hemiptera
Angomonas Souza and Corte-Real 1991	Monogenetic; defined phylogenetically; endosymbiont bearing	Diptera, Heteroptera
Strigomonas Lwoff and Lwoff 1931	Monogenetic; defined phylogenetically; endosymbiont bearing	Diptera, Heteroptera
Sergeia Svobodová and Votýpka 2007	Monogenetic; defined phylogenetically	Diptera
Phytomonas Donovan 1909	Digenetic; *promastigotes*	Plants (Euphorbiaceae, Asclepiadaceae, Moraceae, Palmae, mainly) *and* phytophagous Hemiptera. Pathogenic species cause hartrot in oil and coconut palms and wilt disease in coffee plant
Leishmania Ross 1903	Digenetic; intracellular *amastigotes* (mammal) and promastigotes (vector)	Mammals (Primates, Rodentia, Edentata, Hyracoidea, Carnivora, Marsupialia) and Diptera (Phlebotominae). Pathogenic species in humans cause dermal, mucocutaneous, and visceral leishmaniasis (see Table 5)
Sauroleishmania Ranque 1973	Digenetic; intracellular *amastigotes* (reptiles) and promastigotes (vector)	Reptiles (lizards and snakes) and Diptera (Phlebotominae). Nonpathogenic

(*continued*)

Table 2 (continued)

Genus	Diagnostic characters[a]	Hosts and practical significance
Endotrypanum Mesnil and Brimont 1908	Digenetic; intra-erythrocytic trypomastigotes and epimastigotes (in mammal); promastigotes and amastigotes (in vector)	Edentata (sloths) and Diptera (Phlebotominae, genus *Lutzomyia*). Nonpathogenic
Trypanosoma Gruby 1843	Digenetic; *trypomastigotes* (and more rarely epimastigotes or intracellular amastigotes) in vertebrate; trypomastigotes, epimastigotes (rarely promastigotes, amastigotes) in vector	Vertebrates (all classes) and Hirudinea or Arthopoda (insects, mites). Pathogenic species cause sleeping sickness and Chagas disease in humans and nagana and related diseases in domestic animals (see Tables 3 and 4)

References: (Maslov et al. 2013; Merzlyak et al. 2001; Wallace 1966, 1979)
Notes: [a]The morphological type characteristic of each genus is given in italics. In *Endotrypanum* the type found in the mammal depends on the species
[b]Genera known to be paraphyletic and currently under revision. The endosymbiont-bearing genera *Strigomonas* and *Angomonas* have been revised, *Leptomonas* split into two clades and *Herpetomonas* redefined; see (Maslov et al. 2013) and references therein

Literature

Accounts of the pathogenic Trypanosomatidae loom large in texts on tropical medicine and veterinary medicine as well as in frequent papers in journals dealing with these subjects. Research into the biochemistry, immunology, and molecular genetics of the pathogenic trypanosomatids, including *T. cruzi*, has been fueled by the need for new drugs and vaccines. As 2005 saw the publication of genome sequences for *T. b. brucei*, *T. cruzi*, and *L. major* – the so-called Tritryps (El-Sayed et al. 2005b) – research on these organisms has now entered the post-genomic era. *Trypanosoma brucei* and *Leishmania* species have proved to be tractable laboratory models, fostering a huge literature of recent work on molecular biology and immunology. Trypanosome antigenic variation is now a standard textbook example for gene rearrangements, while *Leishmania* infection is a paradigm for T-helper cell type I and type II immune responses in the mouse. Molecular phylogenetic studies have refocused attention on some of the more obscure kinetoplastids, as these have proved important in constructing accurate evolutionary trees. The great diversity of insect trypanosomatids has also come under scrutiny, following the introduction of molecular taxonomic methods (Maslov et al. 2013; Podlipaev 2001).

Practical Importance

Several kinetoplastids are of medical, veterinary, or agricultural importance on account of their pathogenicity to humans, livestock, and even crop plants.

Table 3 Subgenera of *Trypanosoma* from mammals: distinguishing features and representative species

Subgenus	Mammalian trypomastigote distinguishing features	Representative species	Behavior in mammal
Section: *Stercoraria*			
Megatrypanum[a] Hoare 1964	Large TPM(40–100 μm); long pointed PE; medium, nonterminal kinetoplast; long FF	*T. (M.) theileri*	Division in BS as EPM; ND TPM
Herpetosoma[a] Doflein 1901	Medium TPM (21–36 μm); long pointed PE; large rodlike nonterminal kinetoplast; long FF	*T. (H.) lewisi*	Multiple fission in visceral capillaries as EPM; ND TPM
Schizotrypanum Chagas 1909	Small TPM(15–24 μm); short pointed PE; large subterminal kinetoplast; long FF	*T. (S.) cruzi*	Division as intracellular AM in muscle or MNP; ND TPM in blood
Tejeraia[a,b] Anez 1982	Medium TPM (25–35 μm); long pointed PE; medium nonterminal kinetoplast; long FF	*T. (T.) rangeli*	ND TPM only, known in mammal
Section: *Salivaria*			
Duttonella Chalmers 1908	Medium TPM (21–26 μm); blunt, small (14–17 μm) to rounded PE; large terminal kinetoplast; long FF	*T. (D.) vivax*	Division in BS as medium TPM; wholly intravascular
Nannomonas Hoare 1964	Small TPM (12–18 μm); blunt PE; medium, subterminal marginal kinetoplast; no or short FF	*T. (N.) congolense* Savannah, forest, and Kilifi (Kenya coast) subgroups	Division in BS as TPM attached to endothelia
		T. (N.) simiae	As *T. congolense*, but long and short forms occur (pleiomorphic)
		T. (N.) godfreyi	As *T. congolense*
Pycnomonas[c] Hoare 1964	Small TPM (8.5–19 μm); very short pointed PE; small subterminal kinetoplast; short FF	*T. (P.) suis*	Division in BS as TPM
Trypanozoon Lühe 1906	Pleomorphic TPM; long slender forms (mean 30 μm) with long FF and short stumpy forms (mean 18 μm) with no FF; small subterminal kinetoplast	*T. (T.) brucei* Subspecies *T. (T.) b. brucei*, *T. (T.) b. gambiense*, *T. (T.) b. rhodesiense*	Dividing slender TPM in blood, lymph, CT; nondividing stumpy TPM in blood and lymph

(*continued*)

Table 3 (continued)

Subgenus	Mammalian trypomastigote distinguishing features	Representative species	Behavior in mammal
		T. (T.) evansi	As *T. brucei* but monomorphic
		T. (T.) equiperdum	

References: (Hoare 1972; McNamara et al. 1994)
Abbreviations: AM amastigote, *BS* bloodstream, *CT* connective tissues, *EPM* epimastigote, *FF* free flagellum, *ND* nondividing, *MNP* mononuclear phagocyte, *PE* posterior extremity, *TPM* trypomastigote
Notes: [a]There is no phylogenetic support for subgenera *Megatrypanum*, *Herpetosoma*, or *Tejeraia* (Hamilton et al. 2004; Stevens et al. 1999b)
[b]Originally included by (Hoare 1972) in subgenus *Herpetosoma*, removed to subgenus *Tejeraia* by (Anez 1982) on account of anomalous life cycle
[c]Rarely reported. New subgenus created to reflect unique developmental cycle in tsetse: development in midgut, salivary glands, and proboscis (Hoare 1972; Peel and Chardome 1954)

Kinetoplastids were first recognized as pathogens when Evans in 1880 discovered the trypanosome (*Trypanosoma evansi*) that causes the disease surra of horses and camels in India. In Africa, the threat of trypanosomiasis (nagana) to livestock has had a profound effect on the agricultural economies of the tsetse-infested countries south of the Sahara, as this disease has prevented the exploitation of livestock for transport and traction as well as for human food production. Three approaches are used in the control of diseases caused by the kinetoplastids: chemotherapy, vaccination, and vector control, as these diseases are usually insect-borne. The lack of safe and effective drugs for chemotherapy, investment in development of new drugs, and the withdrawal of some existing drugs have kindled interest in the indirect control of disease by tackling the insect vectors. This strategy has proved very successful in halting transmission of Chagas disease in the so-called Southern Cone countries of South America. While vaccination against leishmaniasis is a realistic possibility (Kedzierski et al. 2006), this is ruled out for the African trypanosomes by their renowned ability for antigenic variation and for *T. cruzi* by strain variation.

The trypanosomes responsible for human and animal trypanosomiasis in Africa (see Table 4) are transmitted by tsetse flies (*Glossina* spp.) in which the parasites undergo a complex cycle of development before they reach the infective metacyclic stage in the fly's mouthparts (cyclical transmission, Fig. 5, Table 4, and *Life Cycles*). In tsetse-infected areas, susceptible animals die from severe anemia and other pathogenic consequences of trypanosome infection, while general production losses (decreased rate of weight gain, sterility, abortion) debilitate infected livestock. Human African trypanosomiasis (HAT), colloquially known as sleeping sickness, is a severe and fatal disease if untreated and is caused by two subspecies of *T. brucei*. The chronic form of HAT in West and Central Africa is caused by *T. b. gambiense* and is typically transmitted by riverine tsetse such as *Glossina palpalis*, while *T. b. rhodesiense* causes the acute East African form of the disease and is transmitted by a number of different tsetse species, e.g., *G. morsitans*, *G. pallidipes*, and *G. fuscipes*. Both forms of the disease are zoonoses, with known reservoirs in domestic pigs for

Table 4 Trypanosomes of mammals: hosts, transmission, and relation to disease of man and domestic animals

Species	Main hosts/ reservoirs	Vectors/ transmission	Disease	Geographical distribution
T. (Megatrypanum) theileri	Cattle	Tabanid flies (C; PS)	Nonpathogenic	Cosmopolitan
T. (Herpetosoma) lewisi	Rats	Rat fleas (C; PS)	Nonpathogenic	Cosmopolitan
T. (Schizotrypanum) cruzi[a]	Humans, opossums, armadillos, raccoons, cats, dogs, rodents	Triatomine bugs (C; PS)	Chagas disease	C. and S. America
T. (Tejeraia) rangeli	Humans, dogs, cats, opossums, monkeys	Triatomine bugs (C; AS; SG)	None (pathogenic in vector only)	S. America
T. (Duttonella) vivax	Ruminants	Tsetse flies (C; AS; P); outside Africa tabanid flies (M)	Nagana	Sub-Saharan Africa, S. America, W. Indies, Mauritius
T. (Nannomonas) congolense	Ruminants, equines, pigs, carnivores, rarely camels	Tsetse flies (C; AS; P)	Nagana	Sub-Saharan Africa
T. (N.) simiae	Pigs, camels, monkeys	Tsetse flies (C; AS; P)	Acute in pigs	Sub-Saharan Africa
T. (N.) godfreyi	Pigs	Tsetse flies (C; AS; P)	Mild disease in pigs	Sub-Saharan Africa
T. (Pycnomonas) suis[b]	Pigs, warthog	Tsetse flies (C; AS; P)	Acute in piglets	Sub-Saharan Africa
T. (Trypanozoon) brucei brucei	Ruminants, equines, camels, pigs, carnivores	Tsetse flies (C; AS; SG)	Nagana	Sub-Saharan Africa
T. (T.) brucei rhodesiense	Humans, ruminants, antelopes, carnivores	Tsetse flies (C; AS; SG)	Acute sleeping sickness	E. Africa
T. (T.) brucei gambiense	Humans, domestic pig, dog	Tsetse flies (C; AS; SG)	Chronic sleeping sickness	W. and C. Africa
T. (T.) evansi	Camels, equines, ruminants, Indian elephant, carnivores, vampire bat	Tabanid flies (M); vampire bats	Surra, Mal de caderas	N. Africa, Middle East, S. Asia, E. Indies, Mauritius, C. and S. America

(*continued*)

Table 4 (continued)

Species	Main hosts/ reservoirs	Vectors/ transmission	Disease	Geographical distribution
T. (T.) equiperdum	Equines	Venereal contact	Dourine	Cosmopolitan

References: (Hoare 1972); (McNamara et al. 1994)
Abbreviations: Transmission *C* cyclical, *M* mechanical. Metacyclics formed in *AS* anterior station (mouth parts of vector, for injection with saliva); *P* in proboscis, *SG* salivary glands, *PS* posterior station (hindgut of vector for deposit with feces)
Notes: [a]A consensus was reached among researchers on Chagas disease that *T. cruzi* strains should be referred to by six discrete typing units (*T. cruzi* I–VI) characterized by multilocus genotype (Zingales et al. 2009, 2012). *T. cruzi* I–VI vary in geographical distribution, ecotope, reservoir hosts and vectors, and the severity of human disease. *T. cruzi* V and VI are believed to be natural hybrids of *T. cruzi* II and III
[b]Rarely reported. Metacyclics in tsetse proboscis, but trypanosomes also develop in salivary glands

the Gambian disease and in cattle or wild animals such as bushbuck for the rhodesian parasite; however, the animal reservoir assumes greater importance in the epidemiology of the rhodesian disease. According to the World Health Organization, annual incidence has fallen over the past decade, with numbers of reported cases falling below 10,000 in 2009; the true incidence is likely to be higher, as many cases go unreported. Thirty-six African countries are currently listed as at risk from HAT, although many of these reported few or no cases during the decade 2000–2009 (WHO 2013b). Gambian HAT is more prevalent and widespread than rhodesian HAT, which is a more sporadic disease. In the recent past, epidemic resurgence of HAT has been blamed on the breakdown of disease surveillance and control measures by civil disturbance and diversion of resources to more pressing health priorities such as malaria and AIDS.

Outside the tsetse belt of Africa, *Trypanosoma evansi*, a monomorphic descendant of *T. brucei* (see Tables 3 and 4 and Evolutionary History), and *T. vivax* may be spread by biting flies (especially tabanids) without a cycle of parasite development in the vector. The principal victims of such mechanical transmission are the draft animals of the unmechanized farming world, horses and camels in particular (Table 4). Vampire bats (*Desmodus* spp.) may act as mechanical vectors of *T. evansi*, with the trypanosomes moving from blood of the ungulate to that of the bat and vice versa via the bat's saliva. The now rare equine disease dourine is due to *T. equiperdum*, which, like *T. evansi*, is another monomorphic descendant of *T. brucei* now transmitted venereally between horses. Carnivores (e.g., lions, leopards, dogs) can acquire infection with the salivarian trypanosomes by eating the carcasses of infected ungulates (Hoare 1972), and this has been demonstrated experimentally by feeding infected goat meat to cats and dogs (Moloo et al. 1973).

Chagas disease caused by *T. cruzi* currently affects an estimated 7–8 million people worldwide, predominantly in South and Central America, where it is an important etiological agent of cardiac disease (WHO 2013a). The trypanosome multiplies intracellularly in the heart and other muscles, emerging into the blood as a trypomastigote to invade other cells or to be taken up by the bloodsucking triatomine

bug vector for cyclical transmission. The disease is transmitted through the bug's feces, which are contaminated with metacyclic trypomastigotes and ejected by the bug after feeding. The metacyclics penetrate the vertebrate host through skin lesions or normal eye mucous membranes. In rural areas these bugs readily invade dilapidated housing where they hide in crevices in wall and thatch, emerging at night to feed. Besides transmission by live bugs, outbreaks of infection have also occurred as a consequence of the contamination of food or drink by dead bugs or their feces. Blood transfusion and organ transplants account for direct transmission in urban areas, and vertical transmission is also possible via perinatal infection or breast milk. The disease is a zoonosis with a reservoir in several mammals (Table 4). It has an acute phase (1–2 months) during which trypanosomes are found in the blood, followed by the apparent disappearance of the parasite during the chronic phase that may last for years. Most people do not show further symptoms, but a minority (estimated 30%) progress to chronic inflammatory disease of the heart or digestive tract – the so-called mega-syndromes. An autoimmune reaction is suspected in pathogenesis of Chagas disease, but this is controversial (Tarleton and Zhang 1999; Urbina 2010). Chagas disease is increasingly reported far beyond its traditional area of distribution in Latin America, as a consequence of the increased mobility of the human population.

The leishmaniases in humans are caused by a complex of 11 principal species of *Leishmania* (Ashford and Crewe 2003; Bates 2007) and have a wide distribution including Southern Europe, Africa, the Middle East, the Indian subcontinent, Asia, and Central and South America (Piscopo and Mallia 2006; WHO 2013c). All leishmanias are transmitted by sand flies (Phlebotominae), the ingested amastigotes transforming to promastigotes in the insect gut (Table 5). Infective forms develop in a plug of proteinaceous material secreted by the parasite, which blocks the pharynx; in order to feed the fly regurgitates this plug of infectious material into the bite wound and substances within the plug exacerbate infection. As the morphology of these parasites in their macrophage host cell is similar, species recognition depends on the clinical features of the disease caused and on molecular identification of the parasites in the laboratory (see *Classification Schemes*). Clinical manifestations of leishmaniasis include both cutaneous and visceral forms of the disease as summarized in Table 5. The most serious disease, kala-azar (visceral), involves the macrophages of the liver, spleen, and bone marrow, causing a fatal anemia that, left untreated, has a high mortality rate. The danger these diseases represent for the health of children should be especially emphasized, as infants are more vulnerable and risk of failure of diagnosis is high. Leishmanial infections in humans induce both humoral and cellular immune responses, but the balance of their expression varies with the type of disease. In visceral leishmaniasis, the cell-mediated immune response is deficient, and although antibody levels are high, infected macrophages disseminate widely, producing disease in various organs. In cutaneous leishmaniasis, a cutaneous delayed hypersensitivity reaction develops early, controlling proliferation and spread of the parasite; however, in patients lacking immune responsiveness to the parasite, diffuse cutaneous leishmaniasis can develop (Evering and Weiss 2006).

Other kinetoplastids of practical importance are the fish-parasitizing bodonids (Woo 1994) and species of *Phytomonas* that parasitize palm trees and coffee plants

Table 5 Principal species of *Leishmania* causing human disease

Species	Disease/lesion	Distribution	Reservoir hosts
Old World species[a]			
L. tropica[b]	Dry cutaneous; chronic; urban OS; (LR)	Central and W. Asia, N. Africa	Rock hyrax, dog (rare)
L. major	Wet cutaneous; acute; rural OS	Asia, Africa	Rodents
L. aethiopica	Dry cutaneous rural OS; (mucocutaneous, DCL)	Ethiopia, Kenya	Hyrax
L. donovani[b]	Visceral kala-azar (PKDL)	Africa, Asia	Rodents in Africa
L. infantum	Infantile visceral	Mediterranean, Central and W. Asia	Dogs
New World species[c]			
L. mexicana	Cutaneous (Chiclero's ulcer); sylvatic	Central America	Forest rodents
L. amazonensis	Cutaneous (DCL); sylvatic	S. America	Forest rodents
L. braziliensis	Mucocutaneous (espundia); metastasizing; sylvatic	Central and S. America (exc. Argentina, Chile)	Forest rodents
L. guyanensis	Cutaneous (pian bois); metastasizing; sylvatic	Guyana, N. Brazil	Sloth, anteater
L. panamensis	Cutaneous; metastasizing; sylvatic	Panama	Sloth
L. peruviana	Cutaneous (uta)	Peruvian Andes	Dogs
L. infantum	Infantile visceral	Central and South America	Dogs, foxes

References: (Ashford and Crewe 2003; Bates 2007)
Abbreviations: *OS* Oriental sore, *(DCL)* disseminative cutaneous leishmaniasis in some individuals in the absence of cell-mediated immunity, *(LR)* Leishmaniasis recidivans, chronic, nonhealing lesion not responding to treatment, *(PKDL)* post-kala-azar dermal leishmaniasis, skin lesions occurring after apparent cure of visceral leishmaniasis. Abbreviations in brackets represent occasional complications
Notes: [a]Vectors, species of *Phlebotomus*
[b]Humans and other hosts required (anthroponotic). Humans are considered incidental hosts for the other species
[c]Vectors, species of *Lutzomyia*

(Dollet 1984; Wallace et al. 1992) (see Tables 1 and 2 and "Habitats and Ecology"). The kinetoplastids have assumed importance in molecular biology by virtue of the unusual structure of kinetoplast DNA and the phenomenon of RNA editing (kDNA, see "The Kinetoplast") and as a result of detailed studies on the genetic basis of antigenic variation – the process whereby trypanosomes evade their host's immune response (see "The Surface Membrane") – and of flagellar structure and function (see "Cytoskeleton and Motility").

Habitats and Ecology

Completely free-living kinetoplastids are all phagotrophic bodonids (Table 1), and many are common in infusions. Most feed on bacteria while creeping along surfaces, using the anterior flagellum for locomotion and for propelling food particles toward the cytostome, while the posterior flagellum functions as a "skid" that permits contact of the kinetoplastid with the substratum. These organisms are most abundant in organic-rich environments, but many species have strict oxygen requirements and tend to accumulate at a characteristic distance from the air/water interface. Common as coprozoic organisms, they have often been mistaken for parasites in stools or urine samples from a variety of patients (Vickerman 1978). Since bodonids do not survive body temperature, these instances must result from contamination of the samples with free-living organisms. Most free-living bodonids can form thin-walled cysts (Fig. 1d) and thus can pass through the gut of vertebrates to hatch out in the voided feces. One of the most ubiquitous bodonids, *Bodo saltans*, however, appears to be unable to form a cyst.

Epizoic bodonids include *Cephalothamnium cyclopum* (Fig. 1h), which forms colonies attached to its copepod host. Many bodonids live on the gills and skin of fish where they adopt a variety of lifestyles. *Cryptobia branchialis* and *C.* (*Bodomonas*) *concava*, which attach by their recurrent flagella, are claimed to feed on bacteria and dead cells in the gill mucus and so should be transferred to the genus *Procryptobia. Ichthyobodo necator* (*Costia necatrix*), an important parasite of freshwater fish and especially of alevins in fish hatcheries (Woo 1994), attaches to epithelial cells by an anterior rostrum and ingests cell cytoplasm (Fig. 1f). A dispersive free-living phase (Fig. 1g) lacks the rostrum and probably does not feed.

The bloodstream trypanoplasms of fish are transmitted primarily by aquatic bloodsucking leeches but can also be transmitted directly between fish (Woo 1994). Several species of *Cryptobia* have been described from the foregut of marine teleosts, the esophagus or stomach sometimes being thickly carpeted with these bodonids (Becker 1977; Woo 1994). Gut cryptobias are common, too, in freshwater planarians (e.g., *C. dendrocoeli* in *Dendrocoelum lacteum*). *C. iubilans* of the gut of cichlid fishes (Nohýnkovà 1984) can spread to other organs (the gall bladder, spleen, ovary, liver) where it appears to multiply inside macrophages within a parasitophorous vacuole (cf. *Leishmania* spp., see below). Transmission of these gut cryptobias is probably via a free-swimming aquatic phase (Woo 1994), but the cryptobias reported from the gut of terrestrial gecko lizards (Bovee and Telford 1962) may have an encysted stage that ensures transmission. Copulation seems the most likely means of transmission of the cryptobias (e.g., *C. vaginalis*: Fig. 1i, j) found in the female reproductive tract of invertebrates. *Cryptobia helicis* of terrestrial and aquatic gastropods attaches to the microvillar surface of its host organ by tentacle-like outgrowths of its anterior flagellum (Current 1980). All true cryptobias feed by pinocytosis through a cytostome-cytopharynx. A trypanoplasm-like organism, *Jarrellia atramenti*, was described from mucus recovered from the blow hole of a stranded pygmy whale, an unusual report of a bodonid from a warm-blooded vertebrate (Poynton et al. 2001).

While some bodonids have blatantly parasitic lifestyles, the nature of the relationship between other endo- or ectosymbiotic bodonids and their hosts is doubtful. Perhaps the most curious endosymbiotic kinetoplastids are those living in other protists. Kinetoplastids related to *Ichthyobodo necator* (*Perkinsela*, previously referred to as *Perkinsiella* and "*Perkinsela*-related" forms) appear to be obligate symbionts of paramoebid amoebae, such as *Paramoeba*/*Neoparamoeba pemaquidensis*, which causes amoebic gill disease in sea-farmed salmonids (Caraguel et al. 2007). The trypanosomatids, *Leptomonas karyophilus* and *L. ciliatorum*, were described from the macronuclei of ciliates (Gillies and Hanson 1963; Gortz and Dieckmann 1987).

Most of the monogenetic trypanosomatids of arthropods (Wallace 1979) are strictly localized in the guts of their larval or adult hosts. *Crithidia* species tend to be found in the hindgut, where they attach to the chitinized lining by their shortened flagella (Fig. 2e). Such attached or "haptomonad" stages (Molyneux 1983) are also found in *Leptomonas*, *Herpetomonas*, and *Blastocrithidia.* More often species of these genera are found as free-swimming "nectomonads" in the gut lumen, either within or outside the peritrophic membrane. Occasionally the Malpighian tubules are invaded (e.g., *Rhynchoidomonas* spp.) or the hemocoel and salivary glands. The developmental stages of digenetic trypanosomatids occupy similar sites. Those trypanosomes (Stercoraria, see Table 3) that produce the infective metacyclic stage in the hindgut have no reason to leave the alimentary tract. Although *Phytomonas* spp. (Table 2) and *Trypanosoma rangeli* (Table 4) invade the hemipteran's salivary glands from the gut via the hemocoel, the evidence that *Trypanosoma brucei* follows the same route in *Glossina* is poor, and it is generally accepted that migration to the glands is via the foregut and proboscis (Fig. 5) (Lewis and Langridge 1947; Van den Abbeele et al. 1999; Vickerman 1985). Attached stages in the trypanosomatid life cycle may densely carpet the body surface to which they attach. Although the environment of these organisms is aerobic, anaerobic conditions may exist locally, especially in the parasite-packed insect hindgut.

A remarkable interaction of *Leishmania* with the sand fly vector facilitates transmission of the parasite to the vertebrate host. Parasites in the anterior gut secrete a proteinaceous gel, which blocks the pharynx causing flies to regurgitate the plug of flagellates and gel in order to feed, thereby depositing it in the skin of the host. Components of the gel exacerbate disease in the host (Bates 2007; Rogers et al. 2004). Sand fly saliva itself has notable pharmacological effects in the host, such as vasodilation, and also plays a role in disease exacerbation (Bates 2007; Titus and Ribeiro 1988).

Transmission of the monogenetic trypanosomatids is apparently through the nectomonad form in the aqueous environment and more rarely via an encysted stage (see *Life Cycles*) contaminating food. These trypanosomatids survive the diapause in some endopterygote insects, but their location in the pupa is uncertain. Transovarian transmission to offspring of *Leptomonas jaculum* and *Blastocrithidia euryophthalmi* has been reported (McGhee and Cosgrove 1980). Predatory Hemiptera may "adopt" the trypanosomatids of their prey insects, and the so-called *Leishmania* spp. found as promastigotes in the hindgut and rectum of chameleons and other lizards may be similarly acquired (Telford 1995).

Phytomonas spp. inhabit the latex or phloem vessels of their plant hosts, and *P. serpens* is also found in fruit (e.g., tomatoes), but not in the leaves, stems, or unripe fruit of the plant (Dollet 1984; Wallace et al. 1992). Phytomonads are transmitted by various bugs that suck plant juices, but must be distinguished from other trypanosomatids carried by these insects. The nonpathogenic species from lactiferous plants are probably transmitted cyclically by phytophagous hemiptera of the families Lygaeidae and Coreidae (Wallace et al. 1992), but details of the transmission of the pathogenic phytomonads found in the phloem of coffee bushes and palms are limited; the phytomonad of palm trees is transmitted by genus *Lincus* (Wallace et al. 1992).

The digenetic trypanosomes and trypanoplasms of the blood of aquatic poikilotherms share transmission by marine or freshwater leeches. After ingestion in the blood meal of the annelid, trypanosomes undergo a cycle of morphological changes culminating in the production of infective metacyclics in the proboscis sheath (Lom 1979), ready to infect the vertebrate host; multiplication without a developmental cycle has been described for trypanoplasms in the leech crop and proboscis (Woo 1994).

The trypanosomes are best known as free-swimming flagellates in vertebrate blood. However, they may become sequestered in the capillaries of certain organs, especially during the multiplicative phase (e.g., *T. lewisi*), attach to peripheral capillary endothelia (e.g., *T. congolense*), or leave the vascular system and invade the lymphatics and connective tissue fluid (e.g., *T. brucei*, *T. evansi*; see Table 3) (Losos and Ikede 1972). *Trypanosoma cruzi* invades and multiplies as amastigotes inside many different host cell types including muscle cells, macrophages, and fibroblasts (Andrade and Andrews 2005). Like *Leishmania* parasites in macrophages, *T. cruzi* must avoid killing and digestion by the host cell's lysosomal enzymes. *T. cruzi* surmounts this problem by escaping from the parasitophorous vacuole in which it was engulfed into the cytosol, using a hemolysin related to the C9 component of complement to punch holes in the lysosomal membrane (Andrade and Andrews 2005). In contrast, *Leishmania* parasites survive and proliferate as amastigotes inside the phagolysosome of the host macrophage, somehow resisting destruction while benefiting from the nutritionally rich environment (Naderer and McConville 2008). *Endotrypanum* species, which like leishmanias are also transmitted by phlebotomine sand flies, avoid the lysosome threat in the mammal by inhabiting erythrocytes (Table 2).

Characterization and Recognition

Summary Description of Class Kinetoplastea

Flagellates with one or two flagella, each typically possessing a paraflagellar rod in addition to the axoneme and arising from a flagellar pocket or pit. They contain a single mitochondrion, typically extending the length of the body – linear, branched, or reticulate – that contains a prominent DNA kinetoplast usually

located close to the flagellar kinetosomes that insert on (or close to) the mitochondrial outer membrane. Mitochondrial cristae are discoid or tubular. Primary enzymes of the glycolytic pathway are housed in glycosomes – microbody-like organelles characteristic of the taxon. The cytoskeleton is composed of supporting pellicular microtubules (microtubular arrays associated with the plasma membrane). A microtubule-surrounded cytopharynx is present in many species, presumably secondarily lost by many osmotrophic forms. Pseudopodia are absent. There is a single vesicular nucleus with a prominent nucleolus. Nuclear division with intranuclear spindle, lacking polar structures. Condensed chromosomes are not visible. No plastids or storage carbohydrate bodies are present, but lipid globules commonly are present. The Golgi apparatus is typically in the region of the flagellar pocket, but is not connected to kinetosomes. Contractile vacuoles, if present, empty into the flagellar pocket. Reproduction typically is by binary fission; genetic exchange has been described in a handful of species, but likely occurs widely throughout the group, since genes for meiosis-specific proteins have been identified in the genome sequences obtained thus far – see "Sex and Genetics". Kinetoplastids are free living or parasitic. Encystation is common among free-living forms, rare in parasitic forms.

The ultrastructure of representative bodonids and trypanosomatids is depicted in Figs. 3 and 4.

The Kinetoplast and RNA Editing

The kinetoplast stains with basic dyes, the Feulgen reaction, and other DNA-specific stains. The kinetoplast divides before the nucleus, although the DNA replication periods (S phase) of the two organelles partially overlap (Matthews and Gull 1994). In transmission electron micrographs of sections, kDNA is seen as a disc, sphere, or rod composed of 2.5–3.0 nm thick fibrils. In trypanosomatids these are oriented anisotropically and are orthogonal to the face of the disc. In bodonids the structure of the kDNA is more variable: kDNA may be arranged in bundles of anisotropic fibrils as several distinct kinetoplasts (polykinetoplasty; Fig. 1e, f, g) or as bundles isotropically distributed through part or all of the mitochondrial lumen (pankinetoplasty; Fig. 1i) (Lukeš et al. 2002; Vickerman and Preston 1976). The terms eu-, poly-, and pankinetoplasty were coined by Vickerman (1990) to describe the appearance of the kinetoplast, with pro-kDNA and mega-kDNA added more recently (Lukeš et al. 2002). These various structures can also be understood according to the arrangement of the minicircle component of the kDNA and have been interpreted as an evolutionary progression from free minicircles to either an intercalated network (in the trypanosomatids) or giant circles (mega-kDNA found in *Trypanoplasma borreli*) composed of many minicircles joined together in tandem (Lukeš et al. 2002). The key difference between the kDNA of trypanosomatids and bodonids lies in the presence or absence, respectively, of a network structure, rather than in the degree of compaction and mitochondrial distribution of the kDNA as seen by microscopy.

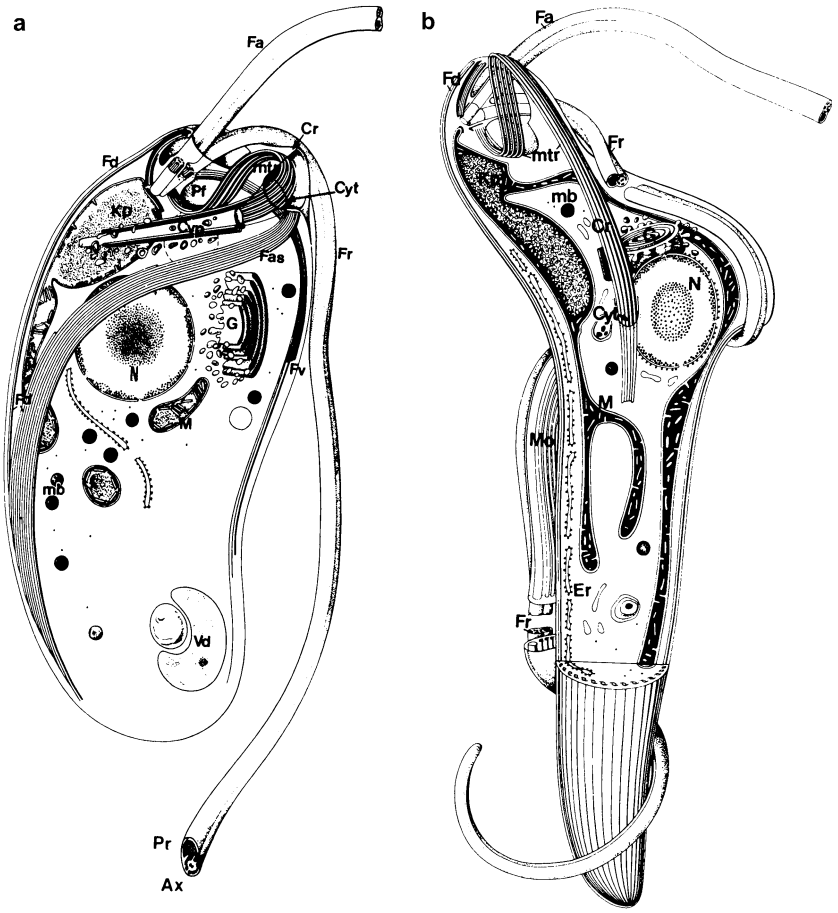

Fig. 3 Ultrastructure of bodonid flagellates. (**a**) Schematic dissection of trophozoite of *Parabodo caudatus* seen in right lateral view. The two flagella (*Fa*, anterior flagellum; *Fr*, posterior (recurrent) flagellum) arise from a flagellar pocket (*Pf*); each has a paraflagellar rod (*Pr*) in addition to an axoneme (*Ax*) in its shaft. Their kinetosomes are separated from the kinetoplast (*Kp*) region of the mitochondrion by a dense plate; the single mitochondrion (seen as several profiles *M*) forms a figure eight in the cell. The cytoskeleton consists of three microtubular bands: *Fd*, the dorsal fiber arising from the dorsal *Pf* wall and connected to the kinetosome of *Fa* by three microtubules; *Fv*, the ventral fiber, connected to the kinetosome of *Fr* and passing along the left side of the *Pf* wall; *mtr*, a band of microtubules from the same kinetosome reinforcing the preoral crest (*Cr*) and deflected inward at the cytostome (*Cyt*) to support the cytopharynx (*Cyp*). A band of microtubules *Fas*, associated with the cytostome, passes along the right side to become incorporated into a sheet of microtubules along with the dorsal fiber. The contractile vacuole (which empties into *Pf*) is seen behind *Cyp* and *Fas*. *N* nucleus, *Er* endoplasmic reticulum, *G* Golgi apparatus, *Vd* digestive vacuole, *mb* microbody (probably a glycosome) (From Brugerolle et al. 1979). (**b**) Schematic dissection of *Trypanoplasma borreli* from blood of goldfish *(Carassius auratus).* Although similar in structure to *Parabodo,* the posterior (recurrent) flagellum (*Fr*) adheres to the body and in beating draws up its surface into an "undulating membrane" (*Mo*). The body is enclosed in an incomplete corset of microtubules corresponding to the *Fd* and *Fv* of *Bodo.* The preoral crest (*Cr*) is extremely long and supported by a microtubule band (*Mtr*) which plunges in the cytostome/cytopharynx about one third of the way along the body. Label abbreviations as in a (From Brugerolle et al. 1979)

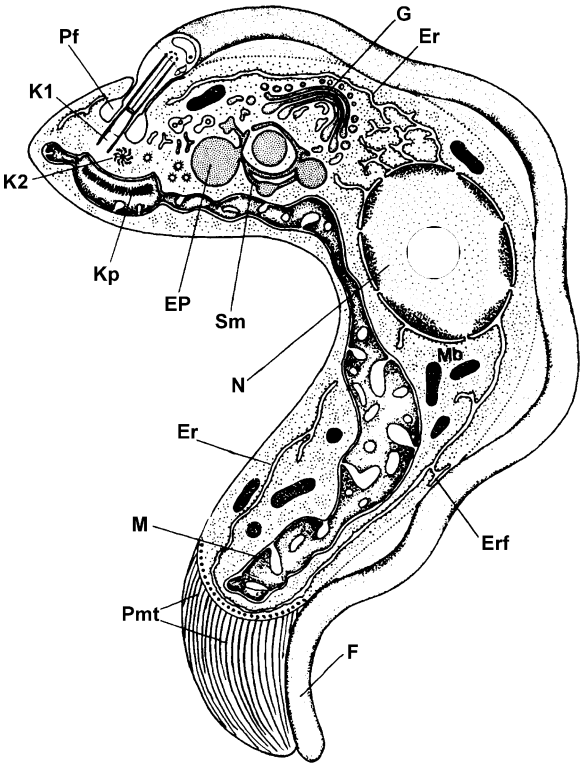

Fig. 4 Ultrastructure of a trypanosomatid, *Trypanosoma congolense*, from the blood of its mammalian host. The single flagellum (*F*) arises from a flagellar pocket (*Pf*) and corresponds to the posterior/recurrent flagellum of bodonids; in *Trypanosoma* it adheres to the body to form an undulating membrane. Close to its kinetosome (*Kl*) lies a barren kinetosome (*K2*), which is all that remains of the anterior flagellum. The kinetoplast (*Kp*) lies in an expansion of the single mitochondrion (*M*), which has tubular cristae. Pellicular microtubules (*Pmt*) form a corset encasing the entire body. Granular endoplasmic reticulum (*Er*) is abundant in the cytoplasm, as are glycosomes (microbodies, *Mb*); a specialized cisterna of ER (*Erf*) runs along the entire length of the undulating membrane. Between the nucleus (*N*) and flagellar pocket, the cytoplasm contains a smooth-membraned reticulum (*Sm*) with saclike dilations; this probably forms a sequestering and digestive system for protein (*EP*) endocytosed from the flagellar pocket. *G*, Golgi apparatus (After Vickerman 1969)

The kDNA of trypanosomatids is the only DNA known which is in the form of a network consisting of thousands of interlocked circles (Shapiro and Englund 1995). The network structure causes the DNA to band rapidly in CsCl density gradients when deproteinized cell lysates are centrifuged. Spread networks are seen by EM to consist usually of two classes of circle, with, depending on species, 25–50 maxicircles each 20–40 kb and 5,000–10,000 minicircles each 0.5–10 kb. Maxicircles, which are equivalent to the mitochondrial DNAs of other eukaryotes, contain genes essential for mitochondrial biogenesis and code for mitochondrial ribosomal RNA and subunits of some proteins involved in electron transport and

ATP synthesis. Transcripts from some of these genes require extensive editing by insertion or deletion of uridine residues before they can be translated correctly; the precise position of each RNA edit is directed by a guide RNA molecule, typically transcribed from one of the minicircles but also encoded by maxicircles in some species (Benne et al. 1986; Shaw et al. 1988; Stuart et al. 2005). Phylogenomic evidence supports the view that RNA editing evolved early in kinetoplastids and is an ancient feature of the group (Deschamps et al. 2011). The whole sequence of the maxicircle genome is known for *T. brucei*, *T. cruzi*, and *Leishmania tarentolae* (Sloof et al. 1992; Westenberger et al. 2006) and also for the partially deleted maxicircle of *T. equiperdum* (Lai et al. 2008). Maxicircles can be removed from the network by restriction enzyme digestion without affecting network integrity, as it is fundamentally a structure of intercalated minicircles. Indeed, this structural role was the only function assumed for minicircles until their key role in RNA editing was discovered. Minicircles are heterogeneous in nucleotide sequence, except in mutants, such as *T. evansi* and *T. equiperdum*, in which they have become homogeneous, presumably as a result of an active mitochondrial system no longer being required.

Why trypanosomatids alone among eukaryotes should have their mitochondrial DNA organized in a network is not known, neither is the purpose of RNA editing, which has been found to varying extent in all kinetoplastids studied and thus is presumed to have evolved before the divergence of the group (Deschamps et al. 2011; Maslov et al. 1994; Simpson et al. 2000). The gene transcripts requiring editing and the extent of edits vary between species, and it has been postulated that retroposition events have gradually corrected the genomic sequences. Transfer RNAs have to be imported into the kinetoplastid mitochondrion, as there are no tRNA genes in the maxicircle, and a second RNA editing process targeting the tRNA anticodon converts the tryptophan tRNA to read the stop codon UGA (Simpson et al. 2000).

The structure of kDNA has been studied in relatively few bodonids. In *Parabodo caudatus* (formerly *Bodo caudatus*), the kinetoplast appears as a single structure at the base of the flagellum – eukinetoplasty (Vickerman 1990) – but becomes fragmented and dispersed during encystment; the kDNA fraction from gradients contained free circular DNAs identified as minicircles of 10 and 12 kb, and 19 kb maxicircles (Hajduk et al. 1986). A similar nonnetwork organization of the kDNA was found in *Bodo saltans* with 70 kb maxicircles and 1.4 kb minicircles, abundant and free circles for the most part with a few catanenes (Blom et al. 1998); in the EM image shown, the kinetoplast sits at the base of the flagellum as a disc-shaped mass and resembles that of trypanosomatids, i.e., eukinetoplastic condition, although subsequently described as pro-kDNA (Lukeš et al. 2002). In *Cryptobia helicis*, a parasite of snails, the kDNA is seen as multiple foci within the mitochondrion (pankinetoplasty) and consists of ~43 kb maxicircles and 4.2 kb minicircles, which, unusually for minicircles, are supercoiled; again there is no network structure (Lukeš et al. 1998). In *Trypanoplasma borreli* the minicircles are joined in tandem to make 180 kb circular DNAs (mega-kDNA), with 80 kb maxicircles (Maslov and Simpson 1994). Despite the fact that the kDNA of genus *Dimastigella* appears as

multiple distinct nucleoids in the mitochondrial lumen – polykinetoplasty (Vickerman 1990) – no network structure was revealed in the analysis of kDNA and only free minicircles recovered (Stolba et al. 2001). Thus, albeit on a limited sample of bodonids, kDNA network structure is absent and bodonid mini- and maxicircles are more variable in size than those of trypanosomatids.

Replication of the kinetoplast in trypanosomatids raises problems not found with any other DNAs, owing to the network structure (Liu et al. 2005). This complex and intricate process has been intensively studied in *Crithidia fasciculata* and *Trypanosoma brucei* (Jensen and Englund 2012). Minicircles are released from the kDNA network by a topoisomerase and replicated daughter minicircles then reattach to the periphery of the network at opposite sides. At the end of replication, the network has doubled in size and is composed entirely of replicated minicircles, each of which has a nick in one DNA strand; the network then divides in two. In *C. fasciculata* the kDNA network rotates so that as replicated minicircles are attached at the two poles, they become evenly distributed on the periphery of the network; in *T. brucei* the same problem is solved in a different way – the network oscillates from side to side (Liu and Englund 2007). Less is known about the replication of maxicircles: like minicircles, they replicate only once per generation, but do not detach from the network; replication is via a theta intermediate structure and initiates in the variable region of the maxicircle (Carpenter and Englund 1995; Liu et al. 2005). The kinetoplast replicates during the division cycle after kinetosome (basal body) reproduction is complete. The kinetoplast is connected to the kinetosome by filaments – the tripartite attachment complex (TAC) – ensuring linkage between the replication of both structures (Jensen and Englund 2012).

The maxicircle gene products are essential for activation of the mitochondrion. Some mutants of *T. brucei* that cannot transform to the procyclic stage (i.e., undergo development in the insect) have maxicircle deletions or even complete absence of maxicircles. Some such mutants – the so-called dyskinetoplastic trypanosome lines – arise through disruption of network structure and dispersion of kDNA throughout the mitochondrion (Schnaufer et al. 2002). They lack a stainable kinetoplast and typical maxicircles and minicircles cannot be isolated from them (Cuthbertson 1981). Dyskinetoplasty can be induced by certain trypanocidal drugs (acridines, phenanthridines, diamidines). Dyskinetoplastic lines of *T. brucei* and its evolutionary derivatives, *T. evansi* and *T. equiperdum* (see Evolutionary History), can live indefinitely in the mammalian host – presumably because the mitochondrion is repressed in that phase of the life cycle and maxicircle gene products are not required. Other dyskinetoplastic kinetoplastids have not been described in nature (Schnaufer et al. 2002).

The Glycosome and Basic Metabolism

All kinetoplastids contain glycosomes – microbody (peroxisome)-like organelles – in their cytoplasm, diameter 0.02–1.0 μm, with a finely granular matrix and a bounding membrane similar in thickness to that of the endoplasmic reticulum

(Fig. 3). In some cases crystalline bodies are present in the matrix. Glycosomes were thought to be unique to kinetoplastids, being absent in *Euglena* (Michels and Hannaert 1994; Opperdoes et al. 1988), but evidence of glycosomes in *Diplonema papillatum* now suggests that these organelles evolved in the common ancestor of diplonemids and kinetoplastids (Gualdron-Lopez et al. 2012). Glycosomes play a key role in energy metabolism in that they harbor seven primary enzymes of the glycolytic pathway as well as two enzymes involved in the metabolism of glycerol-glycerol kinase and NAD^+-linked glycerol-3-phosphate dehydrogenase. Together, these enzymes account for the conversion of glucose and glycerol to 3-phosphoglycerate (Hannaert et al. 2003; Opperdoes 1985). A major peculiarity of *T. brucei* and related species is that reoxidation of NADH generated in glycolysis is via a dihydroxyacetone phosphate/glycerol-3-phosphate shunt operating in conjunction with a terminal glycerophosphate oxidase. The oxidase is cyanide/azide insensitive and is located in the mitochondrial membrane. It reacts with oxygen directly and lacks coupling to ATP synthesis. The high activity of this glycerophosphate oxidase accounts for the high oxygen demands of bloodstream trypanosomes; the enzyme is inhibited by aromatic hydroxamic acids. A similar oxidase is found in many other organisms but not mammals (Chaudhuri et al. 2006).

Glycosomes also contain adenylate kinase, two enzymes of de novo pyrimidine synthesis, and, in insect stages of the life cycle, malate dehydrogenase and phosphoenolpyruvate carboxykinase, which participate in the glycolytic reoxidation of NADH and CO_2 fixation, respectively. Glycosomes also have an important role in the synthesis of ether lipids as do mammalian peroxisomes (Hannaert et al. 2003; Opperdoes 1985). Microbodies are believed to be derived from the ER (endoplasmic reticulum) rather than having an endosymbiotic origin, like mitochondria and chloroplasts; indeed, phylogenetic analysis of glycolytic enzymes indicates a eukaryote rather than prokaryote origin (Michels and Hannaert 1994). The compartmentalization of glycolysis in the glycosome concentrates enzymes and substrates, making glycolysis more efficient; however, among extant kinetoplastids, only some trypanosomes and phytomonads benefit from the ability to maintain a high glycolytic flux, and therefore the evolutionary advantage of the glycosome requires alternative explanations (Michels and Hannaert 1994).

Glycosomes undergo a morphological change in the life cycle of *T. brucei*; the spherical structures of the metacyclic and bloodstream forms become bacilliform and more electron dense in the uncoated vector stages (Fig. 5), paralleling the repression of glycolysis in the insect host (Vickerman 1985). The glycosome, being essential to kinetoplastids, may be an appropriate target for rational chemotherapy of diseases caused by these organisms.

Trypanosomatids show flexibility in their basic metabolism, which may end in oxidative phosphorylation or proceed only as far as aerobic fermentation, according to the extent of mitochondrial suppression and corresponding dependence on glycolysis for energy production (Bringaud et al. 2006; Chaumont et al. 1994). As parasites, they rely on available carbon sources in their hosts; for example, glucose is the preferred carbon source for mammal stages of *Trypanosoma brucei* and *T. cruzi*, which dwell in the bloodstream or cytoplasm of the host, whereas for insect stages

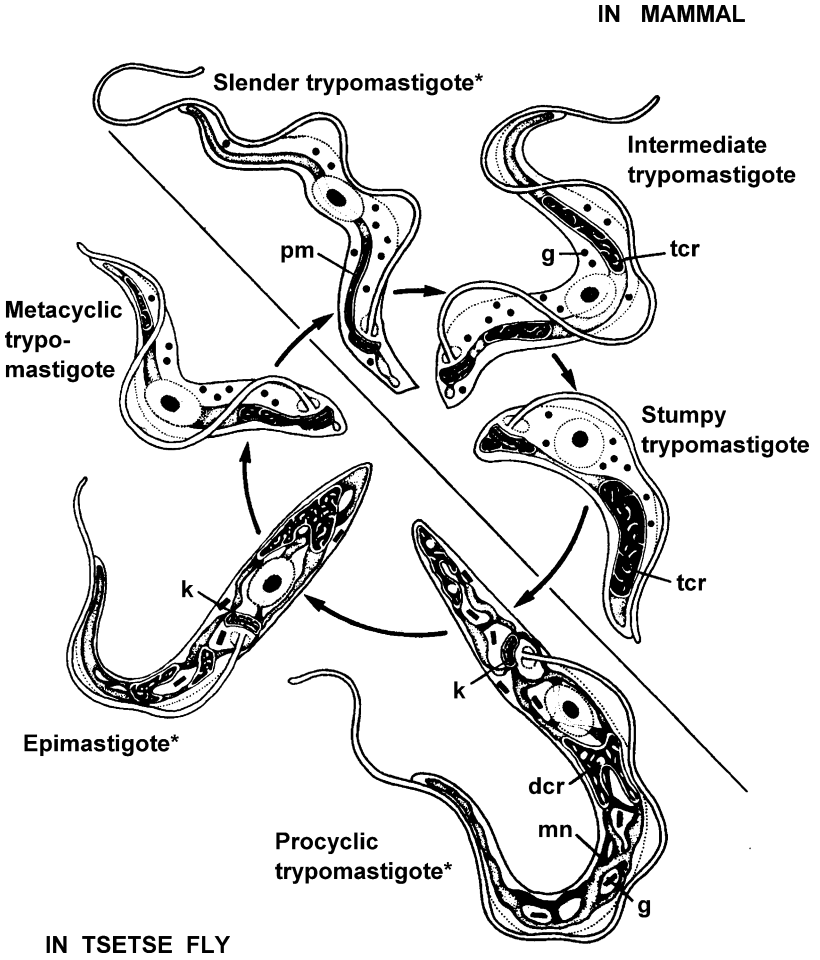

Fig. 5 Life cycle of *Trypanosoma brucei* showing changes in position of kinetoplast and in the form of the mitochondrion and glycosomes (*g*). The mitochondrion is shown cut open to display its interior. Stages in which division occurs are marked with an asterisk. *In mammalian host:* Long slender trypomastigotes divide in blood and tissue fluids giving rise to nondividing intermediate and short stumpy trypomastigotes. Slender forms have a simple promitochondrion (*pm*) with few or no internal cristae; a functional respiratory chain is missing and the parasite depends entirely on glycolysis for energy production. Glycolytically reduced NADH is reoxidized by a dihydroxyacetone phosphate/glycerol 3-phosphate oxidase shuttle in combination with a cyanide- and azide-insensitive glycerophosphate oxidase, located in the mitochondrial membrane, which reacts with O_2 without the intervention of cytochromes and without coupling to ATP synthesis. Transformation to the stumpy form is accompanied by acquisition of proline and α-ketoglutarate oxidase activities as the mitochondrion swells and develops tubular cristae (*tcr*). *In the insect host:* On entering the tsetse midgut, the mitochondrion expands into a network (*mn*) as the post-kinetoplast and prenuclear regions of the trypomastigote increase in size. Succinoxidase activity appears and proline and α-ketoglutarate oxidases are augmented as the trypanosome switches from a glucose-based energy metabolism to one based on proline. Later, in the midgut, discoid cristae (*dcr*) replace the tubular type and a complete cytochrome chain appears; the chain may be branched at cytochrome

only amino acids are readily available; similarly *Leishmania* promastigotes in the insect use amino acids, whereas amastigotes in the phagolysosome of the mammalian macrophage use fatty acids as their energy source (Bringaud et al. 2006). *Phytomonas* spp. from fruit or the phloem or latex of plants use glucose and the other simple sugars present in plant sap or produced by enzymatic digestion of complex carbohydrates to fuel glycolysis, meanwhile suppressing mitochondrial activity (Fernandez Becerra et al. 1997; Sanchez-Moreno et al. 1992).

Many aspects of basic metabolism in the so-called Tritryps (*Trypanosoma brucei*, *T. cruzi*, and *Leishmania major*) are now plain to see from the genome data, if the constituent enzymes of a particular biochemical pathway are present (Berriman et al. 2005). In addition, analysis of all the small metabolites present in the cell (metabolomics) provides a global picture of cell metabolism (Creek et al. 2012). In contrast, little is yet known about the metabolism of bodonids due to the difficulties of growing these organisms in axenic culture, but the availability of a draft genome sequence of *Bodo saltans* will speed up further investigation (Jackson et al. 2008). Presumably the free-living bodonids such as *Bodo* are able to obtain and utilize the wide variety of organic compounds from their prey, while parasitic bodonids such as *Trypanoplasma* and *Cryptobia* are dependent on available carbon sources in their hosts. Glycosomes, glycosomal enzymes, and genes have been studied in *Trypanoplasma borreli* (Adje et al. 1998; Opperdoes et al. 1988; Wiemer et al. 1995). The free-living bodonids tolerate a wide range of conditions, including extremes of salinity or temperature and anoxic environments, and have even been found in deep-sea vent communities (Atkins et al. 2000). This indicates great adaptability in their metabolic and physiological requirements, but to what extent individual species are capable of these feats of adaptation is open to debate. For example, superimposing the salt tolerance of various isolates of *Neobodo (=Bodo) designis* onto their phylogenetic tree has revealed a high degree of cryptic speciation (Koch and Ekelund 2005).

Trypanosomatids such as *Trypanosoma brucei*, *T. cruzi*, and *Leishmania* spp. lack catalase, although this enzyme has been detected in the glycosomes of *Crithidia luciliae*, *Leptomonas samueli*, and possibly *Phytomonas* spp., as well as the bodonid, *Trypanoplasma borreli* (Fernandez Becerra et al. 1997; Opperdoes et al. 1988; Sanchez-Moreno et al. 1992). The antioxidant defense system of

Fig. 5 (continued) b allowing flow of electrons to oxygen either via cytochromes a-a_3 (cytochrome oxidase). Procyclics migrate anteriorly to the proventriculus (cardia) of the fly, where the nucleus elongates and the cell undergoes an asymmetric division resulting in one short and one long epimastigote (not shown). All these stages are found among the migratory stages in the foregut, but it is believed to be the short epimastigote that becomes an attached, multiplicative epimastigote, with prenuclear kinetoplast (*k*) and tubular mitochondrial cristae, on reaching the salivary glands. On transformation to the metacyclic trypomastigote, the mitochondrion becomes a linear structure again; as yet nothing is known about the respiration of the salivary gland stages in the life cycle. The surface of the mammal stages and metacyclics is covered with variant surface glycoprotein; note change in form of glycosomes (*g*) from spherical to bacilliform structures and vice versa (Based on Lewis and Langridge 1947; Opperdoes 1985; Vickerman 1985)

Trypanosoma and *Leishmania* species instead relies on a unique dithiol, trypanothione (Fairlamb et al. 1985); trypanothione reductase and other enzymes involved in the biosynthesis and metabolism of trypanothione are prime candidates for the development of new drugs against trypanosomiasis (Fairlamb and Cerami 1992). Trypanothione metabolism has also been studied in *Crithidia luciliae*, but whether this is a system unique to trypanosomatids, or kinetoplastids in general, is unknown.

Before leaving metabolic pathways, the distinctive nucleotide biosynthesis of kinetoplastids deserves mention. While kinetoplastids have to scavenge purines, they have both synthesis and salvage pathways for pyrimidines. In *Trypanosoma cruzi* one of the key enzymes in this biosynthetic pathway, dihydroorotate dehydrogenase (DHOD), is cytosolic rather than being located in the mitochondrial membrane and uses fumarate as electron acceptor rather than ubiquinone; the same type of DHOD was also found in two free-living bodonids – *Neobodo saliens* and *Parabodo caudatus* (formerly *Bodo saliens* and *Bodo caudatus*) – but not in *Euglena gracilis* and is hypothesized to have entered the kinetoplastid lineage by horizontal gene transfer from prokaryotes (Annoura et al. 2005).

The Surface Membrane and Antigenic Variation

Most probably all kinetoplastids have a plasma membrane that is divided into several functional domains – minimally, main cell body, flagellum, flagellar pocket, cytostome – but, as ever, there is little information on bodonids, the bulk of research being on the pathogenic trypanosomatids. Each stage in the kinetoplastid life cycle may have a variety of glycoproteins and glycoconjugates exposed on its surface, some of which are stage specific and may also show inter- or intrastrain variation. Much current research focuses on the structure and function of exposed macromolecules as ligands binding to host cells during invasion, as antigens playing a part in the induction of immunity and evasion of the host's immune response or as receptors for the uptake of molecules from the environment. In addition, small glycolipid molecules – glycosylinositolphospholipids or GIPLs – cover the surface of *T. cruzi*, *Leishmania*, and other trypanosomatids, providing a protective physical barrier between the parasite and the harsh environment of the host.

The chronic infection and fluctuating parasitemia of African trypanosomiasis are due to evasion of the host's immune response through antigenic variation on the part of the parasite. As trypanosomes multiply by fission in the rising parasitemia, a small number of variable antigen types (VATs) are present in the trypanosome population and these are the focus of the host antibody (IgM) response. The parasitemia goes into remission as trypanosomes of these VATs are killed off, leaving trypanosomes that have switched to other VATs to form the next wave of parasitemia. VAT switching does not require host antibody to induce it and is a stochastic mutational process (Borst and Cross 1982). The molecular basis for antigenic variation in the African tsetse-transmitted trypanosomes has attracted much attention and is now a textbook example of developmental gene rearrangements in eukaryotes (reviewed

by Borst and Cross 1982; Pays et al. 2004; Taylor and Rudenko 2006). Most studied is *Trypanosoma brucei*, with relatively little known about antigenic variation in the other salivarian trypanosomes such as *T. congolense* or *T. vivax.*

The variant surface glycoprotein (VSG) can be seen as a 12–15 nm thick coat covering the entire trypanosome plasma membrane, except the flagellar pocket where it is recycled (Engstler et al. 2004); the coat is composed of a monomolecular layer of VSG dimers. The various VSGs differ in the amino acid sequence of the N-terminus and hence in their antigenic specificity; the N-terminal portion of the VSG is exposed, while the C-terminus is conserved (Blum et al. 1993) and attached to the membrane proper (lipid bilayer) via a glycosyl phosphate inositol (GPI) anchor (Ferguson 1994). Each VSG is the product of a single *VSG* gene, which is expressed in a telomeric expression site. Antigenic variation comes about by replacement of the expressed *VSG* gene with the whole or part of another *VSG* gene. Only one *VSG* gene is expressed at a time, which was thought to result from there being only a single expression site; however, this hypothesis fell by the wayside when it was realized that a single trypanosome had about 20 different expression sites and could switch between them. Instead it turns out that *VSG* is transcribed in a special compartment in the nucleus – called the expression site body or ESB (Navarro and Gull 2001) – which is unique to trypanosomes.

The number of *VSG* genes in the antigenic repertoire of a single trypanosome was estimated at 1000 (Borst and Cross 1982), a ballpark figure confirmed by the results of the *T. brucei* genome sequencing project (Berriman et al. 2005); silent VSG genes were found clustered in subtelomeric arrays on the 11 pairs of large chromosomes, but surprisingly most were found to be either pseudogenes or part genes, which would require recombination with another VSG gene to produce a functional protein. Besides devoting an estimated 10% of its genes to encode VSG (Berriman et al. 2005), *T. brucei* also transcribes these genes at high rate using RNA polymerase I, making VSG mRNA one of the most abundant mRNAs in a bloodstream form cell. *VSG* gene transcription appears to be tightly bound to cell division, as cells undergo pre-cytokinesis cell cycle arrest when *VSG* gene transcription is disrupted (Sheader et al. 2005). The VSG protein is continuously recycled via the flagellar pocket, the whole coat being turned over in an astonishing 12 min (Engstler et al. 2004). While antigenic variation is clearly a successful immune evasion strategy, it comes at a huge metabolic cost to the trypanosome.

When the trypanosome embarks on cyclical development in the tsetse fly, the VSG coat is discarded and replaced by a less dense covering of other stage-specific, GPI-anchored glycoproteins, such as the acidic procyclins of *T. brucei* and *T. congolense*, which are characterized by repetitive amino acid sequences (Roditi et al. 1987; Utz et al. 2006). The VSG coat is reacquired by the metacyclic trypanosomes in the vector's salivary glands (*T. brucei*) or hypopharynx (*T. vivax*, *T. congolense*). The metacyclic VAT repertoire is limited and these *VSG* genes are expressed in a special set of expression sites (Barry et al. 1998). The metacyclic VATs are the first to appear in the mammal following a bite by an infected tsetse fly

and are succeeded by bloodstream form VATs expressed from the usual telomeric expression sites. The seemingly inexhaustible series of antigenic variants that a single trypanosome can produce makes vaccination a hopeless proposition.

The cell surface of *Trypanosoma cruzi* is covered with a dense layer of mucins, protruding above a GIPL layer (Guha-Niyogi et al. 2001). Mucins serve key roles in host cell invasion and protection of the parasite surface and show stage-specific expression, with several different mucin gene families recognized. Many hundreds of mucin genes occur in the *T. cruzi* genome and they are found clustered with genes for the aptly named mucin-associated surface proteins (MASPs, function unknown), proteases (GP63), and trans-sialidases (El-Sayed et al. 2005a). Why so many genes are required, how they are regulated during development, and what role they play in evasion of host immune responses are major unanswered questions (Buscaglia et al. 2006). Trypanosomes cannot synthesize sialic acid de novo, so trans-sialidases serve to transfer sialic acid residues from host to parasite molecules, including mucins; such modifications can be crucial for host cell recognition and invasion (Schenkman et al. 1991). *Trypanosoma cruzi* epimastigotes are characterized by abundant lipopeptidophosphoglycan (LPPG) on their surface (Singh et al. 1994).

Cell-surface molecules of *Leishmania* also show stage-specific expression and have key roles in host cell invasion and protection of the parasite against host defenses (Olivier et al. 2005). Promastigotes in the sand fly vector are protected by a thick glycocalyx consisting of a heterogeneous mix of GPI-anchored glycoproteins, GIPLs, and lipophosphoglycans (LPGs). Some *Leishmania* species use LPG to attach to the gut epithelium of their particular sand fly vector (Bates 2007), while the glycocalyx on the surface of the mammal-infective metacyclics includes branched LPG molecules, capable of resisting complement attack on transfer to the mammalian host (Olivier et al. 2005). Invasion of host macrophages involves a complex interplay of parasite surface molecules and macrophage receptors, facilitating attachment and uptake of the parasite, while deactivating and delaying macrophage defenses; once inside, the parasite continues to subvert its host cell by manipulation of signaling pathways, thereby modulating the host immune response – of key relevance to the very different clinical pictures produced by different *Leishmania* species (Olivier et al. 2005).

A thick, protective glycocalyx seems to be a feature of the trypanosomatid cell surface, whether produced by GIPLs and LPG in *Leishmania* or the bulky carbohydrate side chains of the GPI anchors for surface proteins in trypanosomes (Ferguson 1994). GIPLs, LPG, and LPPG share a core structural motif with GPI anchors – the mannose-glucosamine-phosphatidylinositol linkage to the phospholipid bilayer; further carbohydrate and lipid groups are then linked to this "anchor" group (Ferguson 1994). GIPLs have been found in *Leishmania* spp. and *T. cruzi* as well as some insect trypanosomatids (Guha-Niyogi et al. 2001). Mucins may also be a general feature of the trypanosome surface, having been found in both *T. cruzi* and in the fish trypanosome, *T. carassii*, although lost in the Salivaria (Overath et al. 2001).

Nutrition and Symbionts

Knowledge of nutritional requirements is fragmentary, with the focus of research as always on the pathogenic trypanosomatids, and feeding mechanisms remain a mystery in the majority of kinetoplastid species. In bodonids, a cytostome-cytopharynx is invariably present, and through it bacteria and macromolecules are phagocytosed for digestion in food vacuoles, which subsequently empty into the flagellar pocket (Vickerman and Preston 1976). The preoral crest (Fig. 3a) and wall of the flagellar pocket form seizing lips for prey and these lips may be drawn out to form a prehensile rostrum (Fig. 1). In trypanosomatids, a cytostome-cytopharynx has been reported in *Crithidia* and certain invertebrate stages of the life cycle of some trypanosome species (e.g., *T. cruzi*, *T. cyclops*). The cytostome has been shown to be active in protein uptake in *T. cruzi* epimastigotes and empties into the reservosome – see below (Porto-Carreiro et al. 2000). However, the flagellar pocket seems to be the main site for endo- and exocytosis in most trypanosomatids (Field and Carrington 2009). The cytostome and flagellar pocket are the only two regions without pellicular microtubules and a protective glycocalyx or protein coat and are therefore available for vesicular traffic. Endocytic pathways and cellular machinery have been well characterized in *T. brucei* and other pathogenic trypanosomatids (Morgan et al. 2002a, b).

Few trypanosomatids have been grown in defined media but these include representatives of the major genera; actual nutritional requirements, however, have been determined for very few. Metabolomic analysis is now being used to provide a global view of metabolic flux, facilitating the rational design of new culture media, as well as improving understanding of cellular metabolism (Creek et al. 2013). Trypanosomatids studied to date require minimally the following nutrients: at least ten but often more amino acids, depending on other constituents of the medium; hemin; purine (hemomastigotes can synthesize their own pyrimidines); thiamine, riboflavin, pantothenic acid, nicotinamide pyridoxamine, biotin, folic acid, and biopterin; and a mixture of inorganic nutrients and possibly specific lipids (Trager 1974). Since they have no storage carbohydrates, kinetoplastids are primarily dependent upon exogenous substrates, such as carbohydrates or amino acids, for their energy supplies. However, *T. cruzi* epimastigotes store proteins and lipids in reservosomes – large membrane-bound organelles found at the posterior end of the cell (Figueiredo et al. 2004) – these in association with the cytostome may be adaptations for survival in a vector prone to prolonged bouts of starvation, interrupted by periods of plenty after a blood meal. Storage lipid occurs in several kinetoplastids and fatty acids are used as an energy source by *Leishmania* amastigotes (Bringaud et al. 2006).

Parasitic kinetoplastids exploit the nutrients available in their particular host environment, so their biosynthetic capabilities vary according to life cycle stage. Blood-dwelling parasites such as *Trypanosoma brucei* are bathed in nutrient-rich serum, allowing uptake to replace de novo synthesis. Thus, in the vertebrate host, the trypanosome's requirement for sterols is met by extraction of cholesterol from low-density lipoprotein (LDL) particles taken up from serum by receptor-mediated

endocytosis; in addition, procyclic insect forms maintain the capacity to synthesize ergosterol from scratch (Coppens and Courtoy 2000). Trypanosomatids require exogenous heme and, indeed, the first two enzymes of the heme biosynthetic pathway are missing in the genome sequences of *T. brucei*, *T. cruzi*, and *Leishmania major* (Berriman et al. 2005). In *T. brucei*, heme is obtained by uptake of hemoglobin coupled to haptoglobin by a specific receptor in the flagellar pocket; this seems to be an Achilles heel of the parasite as the same receptor also internalizes apolipoprotein L1, which is the trypanolytic factor in human serum (Vanhollebeke et al. 2008). Apolipoprotein L1 ends up in the lysosomes, where it creates holes in the membrane, leading to swelling and eventual cell lysis (Perez-Morga et al. 2005). The human pathogenic subspecies, *T. b. rhodesiense*, uses its own serum resistance-associated (SRA) protein to deactivate apolipoprotein L1, thereby escaping lysis (Vanhamme et al. 2003). The essential nutrient iron is bound to transferrin in host serum and taken up by receptor-mediated endocytosis; in bloodstream form *T. brucei*, the transferrin receptors are located in the flagellar pocket, while in *T. cruzi* epimastigotes, transferrin enters via the cytostome and is routed to the reservosome; the location of *Leishmania* amastigotes within the lysosome of the host macrophage has the interesting consequence that nutrients are delivered via the endocytic system of the host cell (Morgan et al. 2002a).

Symbiotic prokaryotes are common in the cytoplasm of bodonids and occur in some insect trypanosomatids; those of *Crithidia oncopelti*, *C. deanei*, *C. desouzai*, *Blastocrithidia culicis*, and *Herpetomonas roitmani* have been studied in some detail. Antibiotic treatment has produced aposymbiotic (symbiont-free) strains of most of these species, enabling detailed examination of the role of the endosymbiont in supplying essential nutrients (de Souza and Motta 1999). Symbionts relieve *C. deanei* and *C. oncopelti* of their heme requirements and also synthesize several metabolites essential for growth. Cells generally host one or two endosymbionts, but their large size (1.3–2.3 μm in length and 0.3–1.0 μm in width) leads to some distortion of cell structure (de Souza and Motta 1999). A phylogenetic comparison of the three trypanosomatid genera with endosymbionts demonstrated that the endosymbiont-containing trypanosomatids are a monophyletic group, separate from naturally occurring, symbiont-free species of the same genera (Hollar et al. 1998). This implies that a single endosymbiosis event occurred in this lineage, and in agreement with this, phylogenetic analyses show that the bacterial symbionts of *Crithidia* and *Blastocrithidia* are very closely related within the β division of the Proteobacteria (de Souza and Motta 1999). The endosymbionts of bodonids appear to be intact encapsulated gram-negative bacteria (Eyden 1977; Vickerman 1977).

Cytoskeleton and Motility

Microtubules are the only known element of the kinetoplastid cytoskeleton. Axonemal microtubules are involved in locomotion, pellicular microtubules (PMT) in the maintenance of cell shape, and nuclear spindle microtubules in cell division. In the bodonids the PMT array is usually incomplete (Brugerolle et al. 1979) and composed

of a series of microtubule bands ("fibers") as depicted for *Parabodo caudatus* in Fig. 3a. In trypanosomatids, evenly spaced PMT form a corset enveloping the entire body (Fig. 4), the spindle shape of the cell being accommodated by variation in the length of individual microtubules; only in certain aposymbiotic insect trypanosomatids is the corset interrupted by PMT-aligned branches of the mitochondrion. The PMT are cross-linked to each other and to the plasma membrane, thus holding the trypanosomatid cell in shape; during cell division, the old PMT array is maintained and used as the framework for addition of the new microtubules of the daughter cell (Gull 1999).

Microtubule assembly and disassembly play an important role in kinetoplastid morphogenesis and differentiation. The major structural units of trypanosomatid microtubules, α- and β-tubulin, are subject to a range of protein modifications, although of uncertain significance to the living cell, some of these modifications have proved to be valuable markers for the analysis of microtubule assembly, e.g., the removal and addition of tyrosine (Gull 1999). Other minor tubulin types have been detected and have discrete roles: as well as being associated with the microtubule-organizing centers (MTOCs) of *Trypanosoma brucei* (Scott et al. 1997), γ-tubulin is essential for building a normal flagellar axoneme, since the central two microtubules were missing in cells lacking this protein (McKean et al. 2003).

The axoneme of kinetoplastids has a characteristic "9 + 0" transition zone between it and the kinetosome, the two central axonemal microtubules arising beyond the second of two transverse plates delimiting the zone (Fig. 4). No arms are present on the doublets of the transition zone, and a fine "collarette" of unknown composition surrounds the zone outside its bounding membrane. A latticelike chord, the paraflagellar rod (PFR), running alongside and linked to the axoneme is a second characteristic of the kinetoplastid (and euglenid) flagellum. Other names for the PFR include "paraxonemal rod" and "paraxial rod." The PFR is composed largely of two similar proteins; loss of either has been shown to severely reduce motility in *Trypanosoma brucei* and *Leishmania* (Bastin et al. 1998; Santrich et al. 1997). Striated rootlets are undeveloped in the kinetoplastids; the kinetosomes, which serve as microtubule-organizing centers (Vickerman 1976), are associated with the kinetoplast (mitochondrial) capsule membrane. Nontubular mastigonemes are found on the anterior flagellum of certain bodonids (e.g., *Bodo saltans*, *Cephalothamnium cyclopum)*.

All kinetoplastids have two kinetosomes. Both bear flagella in the anisokont bodonids (Fig. 3). In the trypanosomatids the single axoneme-bearing kinetosome (Fig. 4) corresponds to the posterior flagellum of the bodonids. Attachment of the recurrent flagellum to the body occurs in several bodonids (Table 1) and in some trypanosomatid genera epimastigote and trypomastigote stages (Table 2). A linear series of desmosome-like attachments binds the flagellum to the body in trypanosomatids, but similar structures are rare in bodonids. Detailed analysis of the flagellar attachment zone (FAZ) in *Trypanosoma brucei* reveals a complex structure consisting of a longitudinal bundle of cytoplasmic filaments attached to four specialized PMTs, which arise near the kinetosomes (Gull 1999). Molecular

dissection of flagellar attachment has demonstrated essential roles for the surface membrane glycoprotein, FLA1 (Cooper et al. 1993; LaCount et al. 2002), and the FAZ protein, FAZ1 (Vaughan et al. 2008), as mutant trypanosomes lacking expression of these genes had defects in attachment and cytokinesis. In dividing trypanosomes, the new flagellum arises alongside the old flagellum and, as it lengthens, remains attached by its tip to the old flagellum by a structure called the flagellar connector (Moreira-Leite et al. 2001); the flagellar connector thus guides the growth of the new flagellum and thereby directs the correct configuration of other associated cell structures. The flagellum is therefore central to cell pattern control, and it is therefore no surprise that disruption of flagellar structure impacts on cell division as well as motility (Broadhead et al. 2006).

No detailed studies of movement in bodonids are available, but trypanosomatids have found a use in studies on hydrodynamic aspects of flagellar movement. A notable feature of these flagella is that they propagate waves from tip to base as well as from base to tip (Holwill 1980). Indeed, bends can be initiated at any point along the flagellum, whereas in most other flagella, bending is dependent upon activation in an adjacent region. The ability to dissect flagellar function at the molecular level has renewed interest in the control of flagellum beating (Branche et al. 2006).

A final peculiarity of the kinetoplastid flagellum is its ability to act as an attachment organelle in parasitic species, securing the "haptomonad" to its substratum or to the flagellum of an adjacent kinetoplastid. The flagellum may develop extensive cortical outgrowths to aid attachment (Molyneux 1983; Tetley and Vickerman 1985). Such attached stages play an important part in the life cycles of trypanosomatids. The physical nature of the attachment is unknown, but hemidesmosome-like plaques are present on the axonemal membrane in attachments to a foreign substratum and desmosomes are present in attachments to other flagella (Molyneux 1983; Vickerman 1973).

Nucleus and Transcription: Sex and Genetics

All kinetoplastids have a vesicular nucleus with large central nucleolus. Perinuclear chromatin is visible in electron micrographs, but no chromosomal condensation cycle is apparent. Nuclear division takes place without dissolution of the nuclear envelope; an intranuclear microtubular spindle structure is present but lacks polar structures; a few kinetochore-like plaques which split into hemiplaques that migrate to the poles have been demonstrated (Solari 1983).

There is detailed knowledge about the chromosomal complement and genetics of the pathogenic trypanosomatids. All are fundamentally diploid, although aneuploidy has been reported in *Leishmania* and *T. cruzi*, and cloned isolates of *T. cruzi* were shown to have a wide range of DNA contents (Dvorak et al. 1982). *Trypanosoma brucei* has 11 pairs of large chromosomes, a few small chromosomes, and an estimated 100 minichromosomes (Melville et al. 1998). In contrast, *T. cruzi* and *Leishmania* spp. have large numbers of chromosomes – the genome strains of *T. cruzi* and *L. major* have ~28 and 36 pairs of chromosomes, respectively

(El-Sayed et al. 2005b; Ivens et al. 2005). Notwithstanding these differences in chromosome number, the Tritryp genome sequences reveal large-scale conservation of gene order (synteny) when chromosomes are aligned (El-Sayed et al. 2005b; Ivens et al. 2005).

Present in the Tritryp genome sequences are six meiosis-specific genes, indicating that these kinetoplastids have the machinery for a meiotic division and production of haploid gametes (El-Sayed et al. 2005a). Genetic exchange has now been demonstrated in laboratory crosses of *T. brucei* (Jenni et al. 1986), *T. cruzi* (Gaunt et al. 2003), and *Leishmania major* (Akopyants et al. 2009), as well as in the bumblebee parasite *Crithidia bombi* (Schmid-Hempel et al. 2011). In *T. brucei* genetic exchange occurs during co-transmission of different strains via the tsetse fly and takes place in the insect's salivary glands (Gibson et al. 2008). Analysis of allelic inheritance indicates that the mechanism includes conventional meiosis (MacLeod et al. 2005), meiosis-specific proteins are expressed by epimastigotes in the salivary glands (Peacock et al. 2011), and haploid gametes have been demonstrated (Peacock et al. 2014). As in *T. brucei*, the process of genetic exchange in *Leishmania* occurs in the insect vector and appears to involve meiosis (Akopyants et al. 2009). In contrast, genetic exchange in *T. cruzi* was demonstrated in mammalian cell culture and the process appears to be parasexual; the parental trypanosome genomes fuse with subsequent chromosome loss (Gaunt et al. 2003). Naturally occurring hybrids have been described in *T. cruzi* and *Leishmania* spp. (Kelly et al. 1991; Miles et al. 2009; Ravel et al. 2006), and there is evidence that *T. b. brucei* and *T. b. rhodesiense* are genetically intermixed in East Africa (Balmer et al. 2011).

Studies of gene expression in trypanosomes and *Leishmania* have revealed several features in these trypanosomatids that are unusual in eukaryotes. Transcription of protein-coding genes is polycistronic as in prokaryotes, with posttranscriptional regulation of individual messenger RNAs (mRNAs); the genes lack introns. During processing of the transcript, the usual 3′ poly A tail is added, but in addition a conserved 39-nucleotide leader sequence or mini-exon is spliced onto the 5′ end of each mRNA; as the spliced leader is transcribed from a separate array of tandemly linked repeats, transcription is referred to as discontinuous (Borst 1986). Spliced leader genes have been widely found in kinetoplastids and also in *Diplonema* and *Euglena gracilis* (Sturm et al. 2001). The presence of the molecular machinery for RNA interference (RNAi) in some trypanosomatids (*Trypanosoma brucei*, *Leishmania braziliensis*) has enabled targeted knockdown of particular genes, facilitating investigation of gene expression (Ngo et al. 1998; Subramaniam et al. 2006). An unusual base – β-D-glucosyl-hydroxymethyluracil or J – initially discovered in *T. brucei* appears to be characteristic of the Euglenozoa in general (Dooijes et al. 2000). Base J was thought to be involved in DNA silencing or suppression of recombination, but recent results from *Leishmania* show that it controls the correct termination of transcription (van Luenen et al. 2012).

The development of methods for genetic transformation of trypanosomatids (Beverley and Clayton 1993) was a major advance and opened up the many avenues of investigation predicated on genetic manipulation. Coupled with the

publication of several genome sequences (Berriman et al. 2005; El-Sayed et al. 2005b; Ivens et al. 2005; Jackson et al. 2010, 2012; Peacock et al. 2007), with more to follow, the pathogenic trypanosomatids are one of the best-studied groups of protists.

The thorniest taxonomic problems lie in species recognition, especially among the trypanosomatids of practical importance. Here, exact identification is necessary for epidemiological studies, but as morphology is of no help, there is increasing reliance on molecular approaches. For population genetics analysis, initial characterization studies based on isoenzyme variation have been largely superseded by DNA analyses, such as microsatellite genotyping. Identification of individual species, subspecies, or strains was accomplished initially using DNA probes and subsequently by PCR. A range of different target genes have been chosen for PCR: kDNA minicircles offer ease of purification and limited complexity and have proved useful for identification of *T. evansi* (Masiga and Gibson 1990) and *Leishmania* spp. (Lambson et al. 2000); the spliced leader gene is easily amplified from a wide variety of trypanosomatid species by virtue of the conserved 39-nucleotide spliced leader sequence (Maslov et al. 2007); single-gene PCRs have been developed for specific identification of the human pathogens, *T. b. gambiense* and *T. b. rhodesiense* (Radwanska et al. 2002a, b). Similarly, application of DNA-based approaches should prove very helpful for bodonid identification.

Maintenance and Cultivation

The introduction of cryopreservation to maintain stocks of kinetoplastid organisms in the 1960s, using glycerol or dimethyl sulfoxide (DMSO) as cryoprotectants, removed the need for routine maintenance of cell lines and greatly facilitated research. Similarly, the development of semi-defined media for in vitro cultivation of parasitic trypanosomatids in the 1970s reduced reliance on animals to maintain cell lines; recipes for media in routine laboratory use and detailed protocols may be found in Taylor and Baker (1978). Complex biphasic blood agar media such as NNN or the simpler "Sloppy Evans," which is a mixture of blood and agar, are useful for initial isolation into culture, but thereafter organisms are more easily cultivated by adapting to monophasic media. Widely used complex monophasic media are FYTS (Roitman et al. 1972) for *Leishmania* spp., LIT medium (Camargo 1964) for *T. cruzi*, and SDM (Brun and Schonenberger 1979) or Cunningham's medium (Cunningham 1977) for *T. brucei* and related trypanosomes. Bacterial contamination may be controlled with broad-spectrum antibiotics such as gentamycin (10–100 μg ml^{-1}). Yeast or other fungal contamination may be controlled by 5-fluorocytosine in primary cultures (Maser et al. 2002). Insect hosts in particular are liable to be infected with several species, or even genera, of trypanosomatids, so once a culture is isolated and growing vigorously, cloning should be attempted. Cloning can be achieved by plating on culture medium solidified with agar or agarose or by serial dilution in microtiter plates.

The salivarian trypanosomes (except *T. vivax*, which lacks a vector midgut stage in the life cycle) are readily grown in culture as the vector midgut procyclic form. Other insect developmental stages are more difficult to culture in vitro, but a recent breakthrough was the discovery that overexpression of the RNA-binding protein RPB6 triggers mass metacyclogenesis of *T. brucei* procyclics in culture (Kolev et al. 2012). The blood and extravascular tissue forms of *T. brucei* and related species were first cultivated by Hirumi and co-workers using bovine fibroblast cultures as feeder layers, but semi-defined liquid media containing various additives such as bathocuproine, cysteine, and mercaptoethanol are now used routinely in many labs to support continuous growth of bloodstream forms (Hirumi et al. 1997). Metabolomic analysis holds potential for further refinement of culture methods (Creek et al. 2013).

Bloodstream forms of the salivarian trypanosome species *T. brucei*, *T. evansi*, *T. equiperdum*, and *T. congolense* can be readily grown in laboratory rodents and rabbits, but bloodstream forms of other species usually fail to grow in these small laboratory animals and require experimental calves, goats, or pigs. Intracellular stages of *Leishmania* species and *T. cruzi* may be grown in mammalian tissue culture cells. Various cell types can be used to grow the intracellular trophic amastigote stage, e.g., chick embryo, bovine embryo muscle, HeLa, Vero, and myocardial cells; nondividing trypomastigotes are released into the medium and can be collected to infect fresh cultures.

The phagotrophic free-living bodonids may be isolated in monoprotist culture from rich infusions of soil, feces, or vegetable matter by serial dilution with soil extract (SE) diluted 1:10 with distilled water (or seawater in the case of marine species). Monoxenic cultures can sometimes be obtained by migration: packed, washed cells are layered on the surface of diluted SE medium in a long Pasteur pipette, with the sealed fine end repeatedly bent to give a zigzag path; flagellates migrate toward the sealed tip leaving behind their accompanying bacteria, so that axenic specimens can be recovered from the tip and placed in 0.05% liver infusion with a suitable food bacterium (e.g., *Enterobacter aerogenes)*; however, axenic cultures of these organisms have not been achieved. Media formulae for cultures provided by the American Type Culture Collection (ATCC) are in (Nerad 1991).

Of the parasitic bodonids, only trypanoplasms of freshwater fish have been cultivated to date, with various blood agar media, such as NNN and SNB9, giving good results for both bloodstream and vector stages (Woo 1994). Blood forms of *Trypanoplasma salmositica* (previously *Cryptobia salmositica*) and *T. bullocki* were successfully cultivated in MEM liquid medium supplemented with 20–25% inactivated fetal calf serum, subculturing every 15 days; culture forms were infective to fish (Woo 1994). Other parasitic bodonids have been obtained in bulk from their hosts, e.g., large numbers of *Ichthyobodo necator* were obtained directly from parasitized fish (Callahan et al. 2002) and *Cryptobia helicis* was recovered from dissected snails (Lukeš et al. 1998).

Population doubling times vary from 6 to 18 h in trypanosomatid cultures, and yields of up to 10^7 organisms per ml can be obtained under optimum growth conditions.

Evolutionary History

There are few fossil kinetoplastids, although trypanosomatids, including putative digenetic parasites, have been recovered from insects preserved in amber (Poinar and Poinar 2004). Therefore, the evolutionary history of the group must largely be reconstructed from comparative morphology, cell biology, and gene sequences. The euglenids, which have more morphological features in common with kinetoplastids than any other major extant group (basically two flagella with paraxonemal rods and flagellar pocket, peripheral microtubular cytoskeleton, mitochondrial network with discoid cristae, nuclear division with persistent nucleolus), and the diplonemids are grouped together with kinetoplastids in the Euglenozoa Cavalier-Smith 1981. Molecular phylogenies indicate that diplonemids are more closely related to kinetoplastids than euglenids are (Kamikawa et al. 2014; Simpson and Roger 2004), and consistent with this they have similar spliced leader RNA sequences (Sturm et al. 2001) and appear to have glycosomes (Makiuchi et al. 2011). Massive development of the mitochondrial DNA to form the kinetoplast sets kinetoplastids apart, although it is noteworthy that the diplonemid *Hemistasia* has a kinetoplast-like structure identified by EM (see Elbrachter et al. 1996; Simpson and Roger 2004; Yabuki and Tame 2015).

Molecular phylogenies have confirmed the long-held assumption that the mainly free-living and biflagellate bodonids evolved earlier than the exclusively parasitic and uniflagellate trypanosomatids (e.g., Callahan et al. 2002; Deschamps et al. 2011; Moreira et al. 2004; Simpson et al. 2004). Most bodonids fall into three distinct clades: Neobodonida, Parabodonida, and Eubodonida (Moreira et al. 2004; Simpson et al. 2002; von der Heyden et al. 2004). Neobodonida mostly includes free-living taxa with moderately to highly elongate rostra that extend beyond the flagellar pocket and contain the ingestion apparatus, e.g., *Neobodo*, *Dimastigella*, and *Rhynchobodo*. In the most extreme case, the rostrum of *Rhynchomonas* has evolved as a proportionately huge bulbous-ended structure that is attached to the short anterior flagellum (invisible by light microscopy) and sweeps from side to side as the flagellum beats. Parabodonida includes the free-living *Procryptobia* and *Parabodo* (such as the well-known *Parabodo caudatus*, formerly known as *Bodo caudatus*) but also the parasitic/commensal *Cryptobia* spp. and the digenetic trypanoplasms (*Trypanoplasma*), which are therefore very likely to have evolved from free-living ancestors independently of trypanosomatids. Eubodonida currently contains the free-living taxon *Bodo* only, but is of great evolutionary importance as the sister group to the trypanosomatids (Deschamps et al. 2011). Curiously, the deepest confirmed branch within the kinetoplastid phylogenetic tree, Prokinetoplastina, contains no known free-living forms, just the ectoparasitic *Ichthyobodo* and the *Perkinsela*(-like) permanent symbionts of paramoebid amoebae (Callahan et al. 2002; Moreira et al. 2004; von der Heyden et al. 2004).

Loss of the anterior flagellum, leaving only the posterior (recurrent) as the organ of propulsion, and the adoption of obligate parasitism occurred early in the history of the trypanosomatid lineage. Catenation of the circular kDNA molecules to form a network may have occurred at the same time. A widely favored hypothesis has been

that trypanosomatids were ancestrally monogenetic parasites of insects, and this hypothesis received additional support with the recent discovery that a presumably monogenetic parasite of mosquitos, *Paratrypanosoma confusum*, represents the deepest branch within trypanosomatids (Flegentov et al. 2013). Among the other monogenetic trypanosomatids, the promastigote *Leptomonas* genus may represent the simplest form, with *Crithidia* and *Herpetomonas* possibly representing later developments in one-host parasitism (although note that these genera are not monophyletic). *Phytomonas* and *Leishmania* retain the simple promastigote form while becoming two-host parasites. The genus *Trypanosoma* is a separate, monophyletic lineage of two-host parasites. The hematozoic habit (living in animal blood) thus evolved in at least two separate lineages in kinetoplastids, in the trypanoplasms when the blood-gills-skin contact route of transmission was improved by leech transmission and in the trypanosomatids when the insect host became hematophagous. In this scenario, the leech-transmitted trypanosomes of fish arose secondarily from insect-transmitted forms, with amphibia forming the bridge between terrestrial and aquatic transmission cycles (Hamilton et al. 2004; Maslov et al. 1996).

In trypanosome phylogenies, the African tsetse-transmitted trypanosomes – the Salivaria – form a divergent group, with large evolutionary distances separating this clade from the rest of the genus (Hamilton et al. 2004; Stevens et al. 1999a). The two human pathogens, *T. brucei* and *T. cruzi*, thus had separate origins, borne out by their distinctive life cycles in their respective vertebrate and invertebrate hosts. The date of divergence of the *T. brucei* and *T. cruzi* clades has been inferred to coincide with the separation of South America and Africa 100 mya (Stevens et al. 1999a), but *T. cruzi* may have evolved more recently from bat trypanosomes, considering the potential for long-range dispersal offered to parasites of these highly mobile, flying mammals (Hamilton et al. 2012).

In historical time, *T. evansi* is believed to have evolved from the cyclically transmitted *T. brucei* of the African tsetse belt by transportation across the Sahara in camel trains (Hoare 1972). Tabanid flies served to transmit the blood infection mechanically with concomitant loss of trypanosome pleomorphism, kinetoplast DNA maxicircles, and mitochondrial function. From North Africa, *T. evansi* spread in horses east across Southern Asia to the East Indies and Taiwan and West to South America. In the latter case, vampire bats as well as biting flies have become responsible for transmission. Similarly, *T. vivax* was imported into the New World in infected cattle from West Africa in the seventeenth century, and *Leishmania infantum* (known as *L. chagasi* in South America) was carried by people and dogs from the Mediterranean region. The origin of the venereally transmitted, equine parasite *T. equiperdum* is unclear, although it resembles *T. evansi* in loss of pleomorphism and in the homogeneity of the kinetoplast DNA minicircles; however, few bona fide lab isolates remain, and these have either lost maxicircles completely like *T. evansi* or have a deleted maxicircle (Lai et al. 2008). The distinctive pathology and mode of transmission of *T. equiperdum*, clearly described in ancient texts and evident to modern scholars when dourine was a common equine disease (Hoare 1972), mark it out as a distinct species. Although both *T. evansi* and *T. equiperdum* are now viewed as mutants of *T. brucei* and, from an evolutionary perspective,

should be renamed as subspecies of *T. brucei*, the rules of taxonomic precedence do not allow this, because *T. evansi* was described first. The pragmatic solution is for use of the species names to prevail, in recognition of the distinct pathology of these two parasites and the fact that they are now genetically isolated from *T. brucei*, because they cannot develop in tsetse flies where sexual reproduction occurs.

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Preaxostyla

31

Vladimir Hampl

Abstract

Preaxostyla comprises Oxymonadida, containing 14 genera of gut endosymbionts plus two genera of free-living bacterivorous flagellates from low oxygen sediments (*Trimastix* and *Paratrimastix*). The group was recognized on the basis of 18S rRNA phylogenies, and ultrastructural investigations have revealed a synapomorphy in the organization of the "I" fiber that supports microtubular root R2. *Trimastix* and *Paratrimastix* are typical excavates with three anterior/lateral flagella and the recurrent flagellum passing through a conspicuous ventral feeding groove. The cellular structure of oxymonads is more derived, and a particularly striking diversity of large cellular forms is observed in genera inhabiting guts of lower termites and wood-eating cockroaches. Here the large oxymonad species and their bacterial ecto- and endosymbionts are probably involved in the cellulose digestion, similarly to the large species of parabasalids. All Preaxostyla live in low oxygen environments, and this has affected their metabolism and organelle complement. Glycolysis is apparently the main source of cellular ATP and mitochondria are either reduced to hydrogenosome-like compartments (in *Trimastix* and *Paratrimastix*) or lost completely (in oxymonads). Peroxisomes are absent in the whole group. Stacked Golgi bodies are unknown in oxymonads; however, genes encoding proteins functional in Golgi are present, indicating the existence of a cryptic Golgi. Phylogenomic analyses have shown that Preaxostyla represent one of the three main lineages of Metamonada (within Excavata). Because oxymonads are the only known eukaryotes that have completely lost the mitochondrial organelle, they may serve as models for studies of amitochondriality and mitochondrial evolution.

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Bacterivore • Endosymbionts • Termites • Excavata • *Trimastix* • *Paratrimastix* • Oxymonads • Amitochondriate

Contents

Summary Classification

●**Preaxostyla**
●●**Trimastigidae** (*Trimastix)*
●●**Paratrimastigidae** (*Paratrimastix*)
●●**Oxymonadida**
●●●**Polymastigidae** (*Monocercomonoides*, *Polymastix*, *Tubulimonoides*, *Paranotila*)
●●●**Streblomastigidae** (*Streblomastix*)
●●●**Pyrsonymphidae** (*Pyrsonympha*, *Dinenympha*, *Pyrsonymphites*†, *Dinenymphites*†)
●●●**Saccinobaculidae** (*Saccinobaculus*, *Notila*)
●●●**Oxymonadidae** (*Oxymonas*, *Microrhopalodina*, *Barroella*, *Sauromonas*, *Oxymonites*†, *Microrhopalodites*†, *Sauromonites*†)
●●●***Opisthomitus***

Introduction

General Characteristics

Preaxostyla are heterotrophic protists, typically bearing four flagella. *Trimastix* and *Paratrimastix* have a typical excavate morphology, with a hunched appearance and conspicuous excavate ventral groove. Flagella originate subapically, and the posterior flagellum trails through the cytostome and bears two vanes. Oxymonadida Grassé 1952 are morphologically diverse group, and they never form cytostomes. In oxymonads, flagella are arranged in two separated pairs, and their number can multiply to eight in *Pyrsonympha* and to eight or 12 in *Saccinobaculus* or increase to many in, e.g., *Microrhopalodina* and *Sauromonas*. Nuclei or whole karyomastigonts (nucleus, flagella, basal bodies, preaxostyle, axostyle, and microtubular roots) are multiplied in the oxymonad genera *Microrhopalodina* and *Barroella*. Cells of *Trimastix* and *Paratrimastix* contain hydrogenosome-like derivates of mitochondria and, usually, stacked Golgi bodies. Neither mitochondria nor peroxisomes nor Golgi bodies were reported in oxymonads with the potential exception of *Saccinobaculus doroaxostylus*. Several oxymonad species have developed a microfibrillar organelle for attachment to the intestinal wall (holdfast) often situated on an anterior extension of the cell (rostellum). Preaxostyla divide by binary fission and have either open mitosis (*Paratrimastix*) or mitosis of a closed type with an intranuclear spindle (Oxymonadida). Trophozoites are the dominant life stages of the cell cycle; formation of gametes and cysts has been demonstrated in only a few species.

Occurrence

Trimastix and *Paratrimastix* are free-living inhabitants of hypoxic sediments in marine or freshwater habitats, respectively. The typical habitat of oxymonads is the gut of insects; the exceptions are several species of *Monocercomonoides* that inhabit intestines of vertebrates. The largest diversity of oxymonads, in terms of both species count and morphology, is found in the hindgut of lower termites and the cockroach genus *Cryptocercus*.

Literature and History of Knowledge

Light microscopy of *Trimastix* and *Paratrimastix* was studied by Saville Kent (1880), Grasse (1952), and Bernard et al. (2000). Light microscopy and ultrastructure was studied by Brugerolle and Patterson (1997), O'Kelly et al. (1999), Simpson et al. (2000), and, most recently, Zhang et al. (2015), who also revised the taxonomy and created genus *Paratrimastix* for two species originally classified as *Trimastix*.

Transcriptomic and cell biological studies, all on *Paratrimastix pyriformis*, were performed by Stechmann et al. (2006), and Zubáčová et al. (2013). The evolutionary history of the group has been investigated by Dacks et al. (2001), Hampl et al. (2009), and Zhang et al. (2015).

Oxymonads (*Pyrsonympha vertens* and *Dinenympha gracilis*) were first observed by Leidy (1877). During the first half of the twentieth century, all genera and most species were described using light microscopy. Between 1960 and 1990, the ultrastructure of the most important genera was reconstructed using electron microscopy, with a particular focus on the structure of the axostyle and the mechanism of its movement (e.g., McIntosh et al. 1973; Brugerolle and Joyon 1973; Bloodgood et al. 1974). The first papers that employed molecular methods to study the diversity and evolutionary history of oxymonads and their bacterial symbionts were published at the very end of the twentieth century (Moriya et al. 1998; Iida et al. 2000; Tokura et al. 2000; Dacks et al. 2001). Fragmentary information on oxymonad biochemistry, molecular genetics, and cellular biology became available from 2003 onward (e.g., Keeling and Leander 2003; Liapounova et al. 2006). The genome project of *Monocercomonoides* sp. was finished in 2016 (Karnkowska et al. 2016).

The earliest light microscopic observations of oxymonads were performed by Porter (1897), Kofoid and Swezy (1919, 1926), Kidder (1929), Powell (1928), Kirby (1928), Jírovec (1929), Georgevitch (1932), Cleveland et al. (1934), Cross (1939, 1946), Kirby and Honigberg (1949), Nie (1950), Cleveland (1950a, b, c, 1966), Moskowitz (1951), Gabel (1954), and Jensen and Hammond (1964). The most comprehensive light microscopic tract is in Grassé (1952). The fossils of oxymonads have been studied by Poinar (2009a, b). Oxymonad ultrastructure was studied with electron microscopy by Grimstone and Cleveland (1965), Hollande and Carruette-Valentin (1970a, b), Brugerolle (1970), Smith and Arnott (1973a), Mcintosh et al. (1973), Lavette (1973), Brugerolle and Joyon (1973), Bloodgood et al. (1974), Kulda and Nohýnková (1978), Cochrane et al. (1979), Brugerolle (1981), Radek (1994), Brugerolle and König (1997), Rother et al. (1999), Simpson et al. (2002), Brugerolle et al. (2003), Leander and Keeling (2004), Maass and Radek (2006), Carpenter et al. (2008), and Tamschick and Radek (2013). Physiological and electron microscopic studies regarding axostyle motility were performed by Mcintosh et al. (1973), Mcintosh (1973, 1974), Bloodgood and Fitzharris (1978), Heuser (1986), and Jensen and Smaill (1986). The symbiotic bacteria of oxymonads were studied by Smith and Arnott (1974b), Iida et al. (2000), Tokura et al. (2000), Noda et al. (2003, 2006), Stingl et al. (2005), Yang et al. (2005), and Hongoh et al. (2007). The cell biology and biochemistry of oxymonads have been studied by Keeling and Leander (2003), Slamovits and Keeling (2006a, b), Liapounova et al. (2006), de Koning et al. (2008), and Dacks et al. (2008). The first genomic project was carried out by Karnkowska et al. (2016). The evolutionary history of oxymonads has been studied by Moriya et al. (1998, 2001, 2003), Dacks et al. (2001), Hampl et al. (2005, 2009), Heiss and Keeling (2006), Carpenter et al. (2008), and Radek et al. (2014).

Practical Importance

Oxymonads are of indirect practical importance because of their obligate association with their wood-destroying hosts, the dry wood and subterranean lower termites, and the closely related wood-feeding cockroach *Cryptocercus* (Lo et al. 2000; Inward et al. 2007). Because of their large microtubular axostyles, pyrsonymphids, and saccinobaculids have been useful subjects for research into microtubule function. Oxymonads represent the only known group of eukaryotes containing amitochondriate representatives (Karnkowska et al. 2016).

Habitats and Ecology

Trimastix and *Paratrimastix* are small free-living bacterivorous flagellates inhabiting marine and freshwater sediments that are low in oxygen, where they presumably play a role in grazing bacteria, creating a food-web link between the bacterial biomass and larger organisms.

All oxymonads are endobiotic, and most representatives inhabit the hindgut of lower termites and the intestine of the wood-feeding cockroaches. Several species live in the intestine of larvae of the crane fly and Scarabaeoidea beetles, myriapods, and the intestine of vertebrates. The list of oxymonad species and their hosts is given in Table 1. There are no known pathogenic species.

Oxymonads are often involved in symbiotic relationships. Oxymonads of termites and wood-feeding cockroaches are members of large communities of bacteria, archaea, and anaerobic protists (especially parabasalids) in the hindgut of the host (Brune and Ohkuma 2011; Ohkuma and Brune 2011). The community is essential for cellulose digestion, and if the microorganisms are killed, the insect dies within a few weeks (Cleveland 1924). The exact role of the flagellates (oxymonads and parabasalids) in cellulose digestion is not clear (for review, see Radek (1999), Li et al. (2006), Brugerolle and Radek (2006), Brune and Ohkuma (2011)). Microscopic observations clearly show that large oxymonads (*Pyrsonympha*, *Oxymonas*, *Microrhopalodina*), similarly to large parabasalids (e.g., *Trichonympha*), internalize and digest large pieces of wood. High-resolution imaging mass spectrometry (NanoSIMS) gave direct evidence for the flow of organic carbon from ^{13}C-enriched cellulose to the cell interior of *Oxymonas dimorpha* (Carpenter et al. 2013). The smaller species are probably not involved in cellulose digestion (Cleveland 1925; Radek 1999). Production of cellulolytic enzymes has been reported in several parabasalid species (Yamin 1981; Nakashima et al. 2002; Zhou et al. 2007) but not, so far, in any oxymonad. The association of oxymonads with termites and roaches was observed in 97–110 mya old Cretaceous fossils (Poinar 2009a, b).

The surface and cytoplasm of most oxymonads are colonized by prokaryotic symbionts. The surface bacteria belong to the groups *Spirochaetes* (Iida et al. 2000; Noda et al. 2003) and *Bacteroidales* (Noda et al. 2006; Hongoh et al. 2007). Protists and/or bacteria often form special attachment structures (Bloodgood et al. 1974; Smith

Table 1 List of species of Preaxostyla and their hosts. The older synonyms are given in brackets. † Extinct species

Species	Host
Barroella coronaria Cross 1946	*Postelectrotermes [Neotermes] howa*
Barroella zeteki (Zeliff 1930)	*Calcaritermes brevicollis*
Dinenympha aculeata Georgevitch 1951	*Reticulitermes lucifugus*
Dinenympha aviformis Georgevitch 1951	*Reticulitermes lucifugus*
Dinenympha exilis Koidzumi 1921	*Reticulitermes [Frontotermes] speratus*
Dinenympha fimbriata Kirby 1924	*Reticulitermes lucifugus*, *Reticulitermes flavipes. Reticulitermes hageni*, *Reticulitermes hesperus*, *Reticulitermes virginicus*
Dinenympha gracilis Leidy 1877	*Reticulitermes lucifugus*, *Reticulitermes flavipes*, *Reticulitermes hesperus*, *Reticulitermes tibialis*
Dinenympha leidyi Koidzumi 1921	*Reticulitermes speratus*
Dinenympha mukundia Mukherjee and Maiti 1989	*Reticulitermes tirapi*
Dinenympha nobilis Koidzumi 1921	*Reticulitermes speratus*
Dinenympha parva Koidzumi 1921	*Reticulitermes speratus*
Dinenympha porteri Koidzumi 1921	*Reticulitermes speratus*
Dinenympha rayi Mukherjee and Maiti 1989	*Reticulitermes tirapi*
Dinenympha rugosa Koidzumi 1921	*Reticulitermes speratus*
Dinenymphites spiris Poinar 2009a †	*Kalotermes burmensis*
Microrhopalodina hofmanni (De Mello and De Mello 1944)	"Indian *Cryptotermes*"
Microrhopalodina inflata (Grassi and Foà 1911)	*Kalotermes flavicollis*
Microrhopalodina multinucleata (Kofoid and Swezy 1926)	*Cryptotermes dudleyi*
Microrhopalodina occidentis (Lewis 1933)	*Pterotermes* [*Kalotermes*] *occidentis*
Microrhopalodites polynucleatis Poinar 2009a †	*Kalotermes burmensis*
Monocercomonoides adarshii Mali et al. 2001	*Oryctes rhinoceros*
Monocercomonoides aurangabadae Mali and Patil 2003	*Blattella germanica*
Monocercomonoides blattae	*Blatta* sp.
Monocercomonoides bovis Jensen and Hammond 1964	*Bos taurus*
Monocercomonoides caprae (Das Gupta 1935) [*Monocercomonoides sayeedi* Abraham 1961]	*Capra hircus*
Monocercomonoides caviae daCunha and Muniz 1921 [*Monocercomonoides hassalli* daCunha and Muniz 1927]	*Cavia aperea var. porcellus*
Monocercomonoides chakravartii Krishnamurthy and Sultana 1976	*Polyphaga indica*
Monocercomonoides cunhai (daFonseca 1939)	*Cuniculus paca*
Monocercomonoides digranula (Crouch 1933)	*Marmota monax*

(*continued*)

Table 1 (continued)

Species	Host
Monocercomonoides dobelli Krishnamurthy and Madre 1979	Amphibians (Bufo melanostictus)
Monocercomonoides exilis Nie 1950	*Cavia aperea var. porcellus*
Monocercomonoides filamentum Janakidevi 1961 maybe identical with *Monocercomonoides lacertae* (Tanabe 1933)	*Testudo elegans*
Monocercomonoides ganapatii Rao 1969	*Gryllotalpa africana*
Monocercomonoides garnhami Rao 1969	*Periplaneta americana*
Monocercomonoides globus Cleveland et al. 1934	*Cryptocercus punctulatus*
Monocercomonoides gryllusae Sultana and Krishnamurthy 1978	*Gryllus bimaculatus*
Monocercomonoides hausmanni Radek 1996/1997	*Kalotermes sinaicus*
Monocercomonoides indica Navarathnam 1970	*Tatera indica*
Monocercomonoides khultabadae Mali and Mali 2004	*Pycnoscelus surinamensis*
Monocercomonoides krishnamurthii Sultana 1976	*Pycnoscelus surinamensis*
Monocercomonoides lacertae (Tanabe 1933) [? *Monocercomonoides filamentum* Janakidevi 1961, *Monocercomonoides mehdii* Krishnamurthy 1967, *Monocercomonoides singhi* Krishnamurthy 1967]	Lizards, snakes, tortoises (*Erimias argus*)
Monocercomonoides lepusi Todd 1963	*Lepus nigricollis*
Monocercomonoides marathwadensis Krishnamurthy and Sultana 1976	*Periplaneta americana*
Monocercomonoides mehdii Krishnamurthy 1967 maybe identical with *Monocercomonoides lacertae (Tanabe* 1933)	*Calotes versicolor*
Monocercomonoides melolonthae Grassi 1879 [*Monocercomonoides cetoniae* (Jollos) Travis 1932, *Monocercomonoides ligrodis* Travis 1932]	*Coleoptera* larvae, *Tipula* larvae (*Tipula* sp.)
Monocercomonoides nimiei Ray 1949	*Cavia cutleri*
Monocercomonoides omergae Mali et al. 2001	*Oryctes rhinoceros*
Monocercomonoides orthopterorum Parisi 1910	*Ectobius lapponicus*, *Periplaneta orientalis*, *Periplaneta americana*, *Tipula abdominalis* larvae
Monocercomonoides oryctesae Krishnamurthy and Sultana 1977	*Oryctes rhinoceros*
Monocercomonoides panesthiae Kidder 1937	*Panesthia* sp.
Monocercomonoides pileata Kirby and Honigberg 1949	*Citellus beecheyi*, *Citellus beldingi*, *Citellus lateralis chrysoideus*, *Citellus leucurus*, *Citellus tridecemlineatus*, *Peromyscus maniculatus*

(*continued*)

Table 1 (continued)

Species	Host
Monocercomonoides polyphagae Krishnamurthy and Sultana 1976	*Polyphaga indica*
Monocercomonoides qadrii Rao 1969	*Oryctes rhinoceros*
Monocercomonoides quadrifunilis Nie 1950	*Cavia aperea var. porcellus*
Monocercomonoides robustus Gabel 1954	*Marmota monax*
Monocercomonoides rotunda (Bishop 1932)	Anuran amphibians
Monocercomonoides sayeedi Abraham 1961	*Capra aegagrus hircus*
Monocercomonoides segoviae Perez Reyes 1966	?
Monocercomonoides shortii Navarathnam 1970	*Rattus rattus frugivorus*
Monocercomonoides singhi Krishnamurthy 1967 maybe identical with *Monocercomonoides lacertae* (Tanabe 1933)	*Chameleon zeylanicus*
Monocercomonoides spirostreptae Krishnamurthy and Sultana 1980	*Spirostreptus* sp.
Monocercomonoides viperae Mandrae and Krishnamurthy 1976	*Vipera russelli*
Monocercomonoides termitis Krishnamurthy and Sultana 1977	"Indian termite"
Monocercomonoides tipulae Grassé 1926	*Tipula l*arvae
Monocercomonoides wenrichi Nie 1950	*Cavia aperea var. porcellus*
Notila proteus Cleveland 1950c	*Cryptocercus punctulatus*
Notila proteus ussuriensis Bobyleva 1973	*Cryptocercus relictus*
Opisthomitus avicularis Duboscq and Grasse 1934	*Kalotermes flavicollis*
Opisthomitus longiflagellatus Radek et al. 2014	*Neotermes jouteli*
Opisthomitus flagellae Hollande and Carruette-Valentin 1970b	*Kalotermes dispar*
Opisthomitus brasiliensis De Mello 1953	*Cryptotermes brevis*
Oxymonas barbouri Zeliff 1930	*Glyptotermes angustus [barbouri]*
Oxymonas bastiensis Tiwari 2005	*Neotermes bosei*
Oxymonas bengalensis Das, 1974	*Cryptotermes havilandi*
Oxymonas bosei Das 1974	*Neotermes bosei*
Oxymonas brevis Zeliff 1930	*Cryptotermes brevis*
Oxymonas caudata Cross 1946 maybe identical with *Oxymonas panamae* Zeliff 1930	*Proneotermes* [*Calotermes*] *perezi*
Oxymonas chilensis Guzman 1961	*Calotermes chilensis*
Oxymonas clevelandi Zeliff 1930	*Incisitermes immigrans* [*Kalotermes clevelandi*], *Incisitermes* [*Kalotermes*] *tabogae*, *Incisitermes fruticavus*
Oxymonas dimorpha Connell 1930	*Paraneotermes simplicicornis*
Oxymonas diundulata Nurse 1945	*Kalotermes brouni*
Oxymonas gigantea Poinar 2009b †	Blattellidae
Oxymonas gracilis Kofoid and Swezy 1926	*Rugitermes [Kalotermes] magninotus*

(*continued*)

Table 1 (continued)

Species	Host
Oxymonas grandis Cleveland 1935	*Neotermes dalbergiae*, *Neotermes tectonae*, *Neotermes bosei*
Oxymonas granulosa Janicki 1915	*Incisitermes marginipennis*, *Neotermes connexus*
Oxymonas hirtelli Mello 1954	*Neotermes hirtellus*
Oxymonas hubbardi Zeliff, 1930	*Incisitermes marginipennis*, *Marginitermes [Kalotermes] hubbardi*
Oxymonas janicki Zeliff 1930	Kalotermitidae
Oxymonas jouteli Zeliff 1930	*Neotermes [Kalotermes] jouteli*
Oxymonas kirbyi Zeliff 1930	*Rugitermes kirbyi*
Oxymonas megakaryosoma Cross 1946	*Glyptotermes* sp.
Oxymonas megarostelata Bala and Bhagat 1993	*Odontotermes obesus*
Oxymonas minor Zeliff 1930	*Incisitermes [Kalotermes] minor*
Oxymonas notabilis Cross 1946	*Postelectrotermes [Neotermes] howa*
Oxymonas ovata Zeliff 1930	*Calcaritermes brevicollis*
Oxymonas panamae Zeliff 1930 maybe identical with *Oxymonas caudata* Cross 1946	*Rugitermes panamae*
Oxymonas parvula Kirby 1926	*Cryptotermes domesticus [hermsi]*
Oxymonas pediculosa Kofoid and Swezy 1926	*Calcaritermes [Kalotermes] nigriceps*, *Rugitermes panamae*
Oxymonas projector Kofoid and Swezy 1926	*Incisitermes seeversi [Kalotermes perparvus]*
Oxymonas protus Poinar 2009a †	*Kalotermes burmensis*
Oxymonas rotunda Cross 1946 [*Oxymonas ovata* Zeliff 1930]	*Calcaritermes emarginicollis*, *Incisitermes marginipennis*
Oxymonas synderi Zeliff 1930	*Cryptotermes breviarticulatus*
Oxymonas tenuicollis Grassé and Hollande	*Neotermes aburiensis*
Oxymonites gerus Poinar 2009a †	*Kalotermes burmensis*
Paranotila lata Cleveland 1966	*Cryptocercus punctulatus*
Paratrimastix eleionoma Zhang et al. 2015	Free-living, freshwater
Paratrimastix pyriformis (convexa) (Zhang et al. 2015)	Free-living, freshwater
Polymastix ganapatii Sultana 1976	Scarabeid larvae
Polymastix hystrix Grassé 1952	*Neotermes aburiensis*
Polymastix indica Krishnamurthy and Sultana 1978	*Polyphaga indica*
Polymastix jadhavii Mali 1993	*Periplaneta americana*
Polymastix legeri Grassé 1926	*Glomeris*
Polymastix melolonthae Grassi 1879 maybe identical with *Polymastix wenrichi* Geiman 1933	Coleoptera larvae, *Tipula* larvae
Polymastix nitidus Hasselmann 1928	*Rhizocrinus*
Polymastix periplanetae Qadri and Rao 1963	*Periplaneta americana*

(*continued*)

Table 1 (continued)

Species	Host
Polymastix phyllophagae Travis and Becker 1931	Larvae of *Phyllophaga*
Polymastix rayi Sultana 1976	*Periplaneta americana*
Polymastix wenrichi Geiman 1933 maybe identical with *Polymastix melolonthae* Grassi 1879	*Tipula abdominalis*
Pyrsonympha affinis Fedorowa 1923	*Coptotermes* sp.
Pyrsonympha elongata Georgevitch 1932	*Reticulitermes lucifugus*
Pyrsonympha flagellata Grassi and Sandias 1893	*Reticulitermes lucifugus*
Pyrsonympha grandis Koidzumi 1921	*Reticulitermes speratus*
Pyrsonympha granulata Powell 1928	*Reticulitermes lucifugus*, *Reticulitermes hesperus*
Pyrsonympha havilandi Das 1972	*Cryptotermes havilandi*
Pyrsonympha major Powell 1928	*Reticulitermes flavipes*, *Reticulitermes lucifugus*, *Reticulitermes tibialis*, *Reticulitermes hesperus*
Pyrsonympha minor Powell 1928	*Reticulitermes lucifugus*, *Reticulitermes hageni*, *Reticulitermes tibialis*, *Reticulitermes hesperus*
Pyrsonympha modesta Koidzumi 1921	*Reticulitermes speratus*
Pyrsonympha omblensis Georgevitch 1951	*Reticulitermes lucifugus*
Pyrsonympha rostrata Mukherjee and Maiti 1988	*Reticulitermes tirapi*
Pyrsonympha tirapi Mukherjee and Maiti 1988	*Reticulitermes tirapi*
Pyrsonympha vertens Leidy 1877	*Reticulitermes flavipes*
Pyrsonymphites cordylinus Poinar 2009a †	Kalotermitidae
Saccinobaculus ambloaxostylus Cleveland et al. 1934	*Cryptocercus punctulatus*
Saccinobaculus doroaxostylus Cleveland et al. 1934 [*Oxymonas doroaxostylus* emend. Cleveland 1950a]	*Cryptocercus punctulatus*
Saccinobaculus gloriosus Bobyleva 1973	*Cryptocercus relictus*
Saccinobaculus minor Cleveland et al. 1934 [*Oxymonas nana* emend. Cleveland 1950a]	*Cryptocercus punctulatus*
Saccinobaculus lata Cleveland 1950b	*Cryptocercus punctulatus*
Saccinobaculus scabiosus Bobyleva 1973	*Cryptocercus relictus*
Saccinobaculus spatiatus Bobyleva 1973	*Cryptocercus relictus*
Sauromonas m'baikiensis Grassé and Hollande 1952	*Glyptotermes boukoko*
Sauromonites katatonis Poinar 2009a	Kalotermitidae
Streblomastix strix Kofoid and Swezy 1919	*Zootermopsis angusticollis*, *Zootermopsis nevadensis*
Trimastix elaverinus Dumas 1930	Free-living, freshwater

(*continued*)

Table 1 (continued)

Species	Host
Trimastix inaequalis, Bernard et al. 2000	Free-living, marine
Trimastix marina Saville Kent 1880–1882	Free-living, marine
Tubulimonoides aurangabadae Mali et al. 2003	*Oryctes rhinoceros*
Tubulimonoides gryllotalpae Krishnamurthy and Sultana 1976	*Gryllotalpa africana*
Tubulimonoides shivamurthi Mal and Sultana 1993	*Oryctes rhinoceros*

and Arnott 1974b; Rother et al. 1999; Brugerolle 1981; Leander and Keeling 2004). Ectobiotic bacteria are occasionally phagocytosed by the host (Brugerolle 1981; Leander and Keeling 2004; Noda et al. 2006). The prokaryotes in the cytoplasm of oxymonads belong to the groups *Endomicrobia* (TG-1), which are specific to this environment (Stingl et al. 2005; Yang et al. 2005), methanogens (Tokura et al. 2000), and mycoplasmas (Yang et al. 2005). Verrucomicrobial symbionts have been reported from the nuclei (Sato et al. 2014). The essence of the oxymonad-bacterial relationship is unclear, although some metabolite transfers have been proposed between parabasalid protists and their endosymbionts living in the same environment (Hongoh 2010). The association of protists with prokaryotes is not strictly one-to-one specific, i.e., unrelated protists are associated with closely related bacteria and several types of bacteria are associated with a single oxymonad.

Characterization and Recognition

Organization of Cytoskeleton

The organization of the *Trimastix* and *Paratrimastix* cytoskeleton closely follows the basic scheme known from other typical excavates (Simpson 2003; Yubuki et al. 2013), and it likely represents the ancestral organization of the group. Four basal bodies are arranged in a cruciate pattern. Left (R1) and right (R2) microtubular roots are connected to a recurrent basal body B1 and support the margins of the cytostome. The right root (R2) is associated with a thick I fiber with a lattice-work substructure (see below). From the anterior basal body B2 originates the anterior root (R3), which is associated with the dorsal fan of microtubules (F) supporting the dorsal side of the cell. Differences between *Trimastix* and *Paratrimastix* are subtle. Common features of both, which distinguish them from other typical excavates, can be found in the organization of the supportive fibers B, C, and I (Zhang et al. 2015). In particular, the I fiber forms one thin sheet connected to R2 by lattice-like structure, which resembles the structure of the paracrystalline part of the preaxostyle in oxymonads. These two cytoskeletal components are regarded as homologous, and similarity of their fine structure is the defining synapomorphy of Preaxostyla (Simpson 2003).

The structure of the oxymonad cytoskeleton has diverged from the canonical excavate form. Here it will be described using the genus *Monocercomonoides*, which probably resembles the ancestral state in oxymonads, employing terminology according to Radek (1994) (Figs. 1 and 2). Each oxymonad cell contains one karyomastigont (as in the case of *Monocercomonoides*) or sometimes more than one. Each karyomastigont consists of a nucleus, four basal bodies with flagella that are organized in two pairs, and a preaxostyle that connects the pairs of basal bodies. The preaxostyle (="primary row" in older works) is made of two layers. The layer facing the nucleus consists of a single row of microtubules (homologous to R2 in excavates), and this attaches to a second layer made of non-microtubular material (homologous to the I fiber in excavates). The preaxostylar region is rich in polysaccharide granules. The cell's anterior-posterior axis is formed by an axostyle that consists of parallel rows of microtubules that are interconnected by bridges. In the nuclear region, the axostyle is associated with the preaxostyle by the single row of microtubules that is continuous between both structures. The axostyle is contractile in Pyrsonymphidae, Saccinobaculidae, and Oxymonadidae, where it serves as the organelle for locomotion. Microtubular root R1 or funis (fully developed in *Monocercomonoides*) is connected to the basal body of the recurrent flagellum (basal body 1) and underlies this flagellum. In *Monocercomonoides*, the most

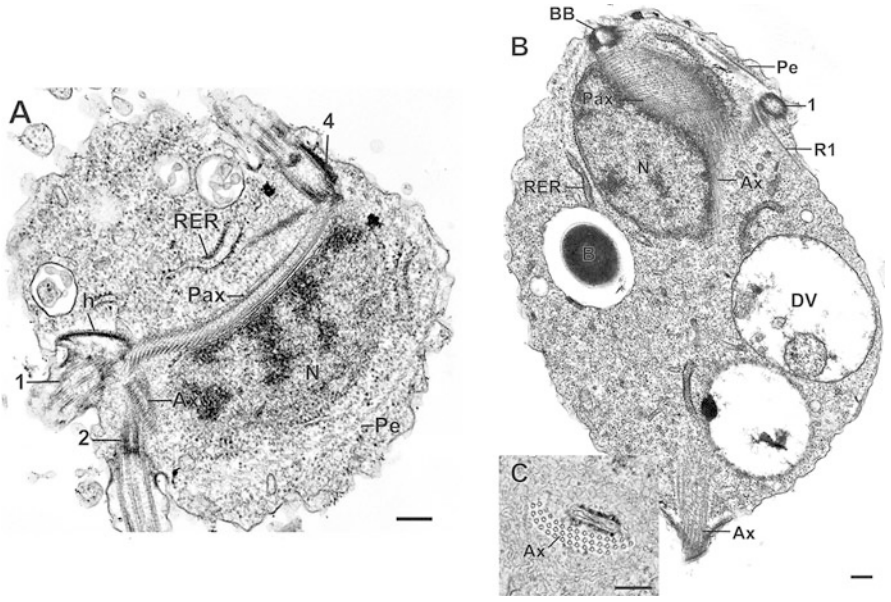

Fig. 1 Transmission electron micrographs of *Monocercomonoides* sp. from *Parasphaeria boleiriana*. (**a**) Transverse section of the nuclear region, (**b**) longitudinal section, and (**c**) transverse section of the axostyle composed of microtubular rows connected by bridges. *1, 2, 4* basal bodies 1, 2, 4, *Ax* axostyle, *B* bacterium, *BB* basal body, *DV* digestive vacuole, *h* hook-like fiber, *N* nucleus, *Pax* preaxostyle, *Pe* pelta, *RER* rough endoplasmic reticulum; bars 200 nm. Terminology according to Radek (1994) (Courtesy of Guy Brugerolle)

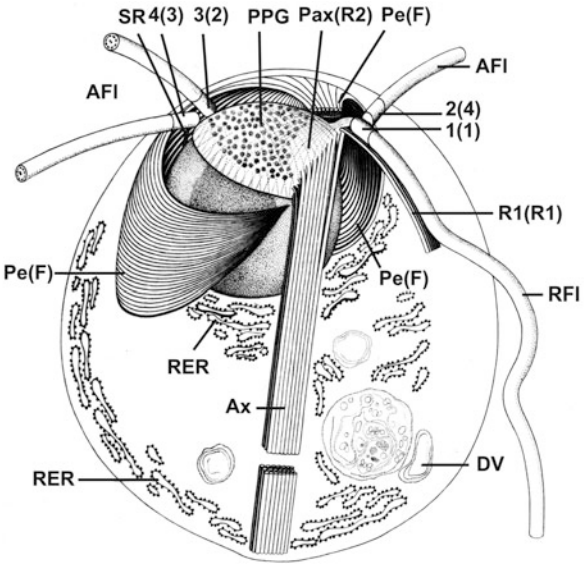

Fig. 2 Ultrastructure of *Monocercomonoides*. Terminology follows Radek (1994); terminology according to Yubuki and Leander (2013) is given in brackets. *1, 2, 3, 4* basal bodies 1–4, *AFl* anterior flagella, *Ax* axostyle, *DV* digestive vacuole, *F* fan, *Pax* preaxostyle, *Pe* pelta, *PPG* perinuclear polysaccharide granules, *R1* microtubular root R1, *R2* microtubular root R2, *RFl* recurrent flagellum, *RER* rough endoplasmic reticulum, *SR* striated root. The axostyle in its distal part is artificially interrupted to show the organization of microtubules (Courtesy of Eva Nohýnková, adapted)

anterior basal body (4) is associated with a microtubular root (not shown in Figures), which underlies the pelta. This pelta is a microtubular sheet that covers the nucleus and that is homologous to the dorsal fan of typical excavates. Simpson et al. (2002) suggested homologies between the oxymonad cytoskeleton and cytoskeleton of typical excavates; the excavate terminology for cytoskeletal structures according to Yubuki and Leander (2013) is given in Fig. 2 in brackets.

Sex and Reproduction

Preaxostyla reproduce by binary fission. *Paratrimastix* use an open mitosis, while mitosis is of a closed type in oxymonads. A characteristic migration of nuclei through the cell is typical for family Oxymonadidae and will be described in more detail below. Sexual processes comprising gametogenesis, fertilization, and meiosis were reported in the oxymonads of the wood-feeding cockroach *Cryptocercus*, namely, *Notila*, *Saccinobaculus*, and *Paranotila* (Cleveland 1950b, c; Cleveland 1966). Cleveland also described sexual processes in two *Oxymonas* species from *Cryptocercus* (*O. doroaxostylus* and *O. nana*) (Cleveland 1950a); however, these species are currently regarded as members of the genus *Saccinobaculus*

(*S. doroaxostylus*, *S. minor*; Heiss and Keeling 2006). Synaptonemal complexes characteristic for meiosis were reported from *Pyrsonympha flagellata* (Hollande and Carruette-Valentin 1970a). Encystation has been reported in *Monocercomonoides*, *Saccinobaculus*, and *Sauromonas*, as well as in *Paratrimastix* (Cleveland 1950a; Grassé 1952; O'Kelly et al. 1999). The developmental cycles of flagellates and the sexual cycles (where present) are synchronized with the molting cycle of the insect host and are governed by the molting hormone ecdysone (May 1941; Grassé 1952; Cleveland 1956; Cleveland et al. 1960). Termites lose all intestinal protozoa during nymphal molt; both young termites, and post-molt adult termites must establish their protozoan biota by proctodeal feeding from adults (Brugerolle and Radek 2006; Brune and Ohkuma 2011).

Molecular Genetics and Biochemistry

Due to the impossibility of axenic cultivation, our knowledge on molecular genetics and biochemistry is very fragmentary, and the studies are restricted to transcriptomic and genomic surveys and gene fishing from genomic DNA and cDNA. The only sequenced genome of the group (*Monocercomonoides* sp.) is ~75 MB in size and 36.8 GC and contains 16,629 predicted protein coding genes (Karnkowska et al. 2016).

The cytoplasm of *Trimastix* and *Paratrimastix* contains electron-dense mitochondrion-like organelles with poorly known biochemistry. In *Paratrimastix pyriformis*, the only protein experimentally localized into these organelles is an enzyme of the glycine cleavage system, part of amino acid metabolism. Transcriptome studies in *P. pyriformis* indicate the presence of pyruvate:ferredoxin oxidoreductase and [FeFe]hydrogenase, suggesting an extended glycolysis in this organism (Zubacova et al. 2013). Peroxisomes have not been reported in *Trimastix* and *Paratrimastix*.

Energetic metabolism of oxymonads seems to be broadly similar to other studied anaerobes such as *Trichomonas*, *Giardia*, or *Entamoeba* (Reeves et al. 1977; Upcroft and Upcroft 1998; Müller 1992). Among the glycolytic enzymes of *Monocercomonoides*, several were acquired by lateral gene transfer from prokaryotes, including the ATP-efficient alternatives pyrophosphate fructose-6-phosphate phosphotransferase and pyruvate orthophosphate dikinase (Liapounova et al. 2006; Slamovits and Keeling 2006b). Pyruvate is probably oxidatively decarboxylated by pyruvate:ferredoxin oxidoreductase (PFO) in the cytosol, and the resulting acetyl-CoA is further fermented to ethanol (Karnkowska et al. 2016). Transcripts of [FeFe]hydrogenase are abundant (Karnkowska et al. 2016), but the production of hydrogen has not been established. Dacks et al. (2008) found that the expression of cathepsin B cysteine proteases in *Monocercomonoides* is relatively high and comparable to housekeeping genes. Unlike other metamonads (*Giardia*, *Trichomonas*), the oxymonad genome is relatively intron rich (1.1 and 1.9 introns per gene in *Streblomastix* and *Monocercomonoides*, respectively) (Slamovits and Keeling 2006a; Karnkowska et al. 2016). Some oxymonads (*Streblomastix*, some *Monocercomonoides*) use a noncanonical genetic code, in which the codons TAA and TAG encode the amino acid glutamine (Keeling and Leander 2003; de Koning et al. 2008).

Mitochondria, stacked Golgi, and peroxisomes have not been clearly demonstrated in oxymonads. Electron-dense organelles of uncertain nature, but resembling mitochondria, were reported from *Saccinobaculus doroaxostylus* however (Carpenter et al. 2008). In the case of *Monocercomonoides* sp. strain from chinchilla, no genes for mitochondrion-specific proteins have been detected in the fully sequenced genome, confirming the absence of any mitochondrion suggested by electron microscopy (Karnkowska et al. 2016). The same applies to peroxisomes, but in the case of the Golgi apparatus, a full set of genes coding for "Golgi-associated" proteins was found. The cellular localization of their protein products is unknown.

Taxonomy

Preaxostyla are classified within the phylum Metamonada (Cavalier-Smith 2003), a subgroup of the taxon Excavata (Cavalier-Smith 2002; Simpson 2003; Adl et al. 2005; Hampl et al. 2009; Adl et al. 2012). Preaxostyla contains three described species of Trimastigidae, two of Paratrimastigidae (Zhang et al. 2015), and approximately 140 described species of oxymonads, divided into five families – Polymastigidae, Saccinobaculidae, Pyrsonymphidae, Streblomastigidae, and Oxymonadidae (Brugerolle and Lee 2000), plus the isolated genus *Opisthomitus* (Fig. 3). List of described species is given in Table 1.

Trimastigidae

The family contains a single genus *Trimastix* Saville Kent. Cells bear four flagella stretched roughly in the anterior, right, left, and posterior directions. The posterior flagellum passes through a suspension-feeding groove and bears two vanes. Vane margins are not thickened. The genus contains two marine species *T. marina* and *T. inaequalis* and one freshwater species *T. elaverinus* with uncertain status. Light microscopy of *Trimastix* was studied by Saville Kent (1880), Dumas (1930), Grasse (1952), and Bernard et al. (2000). Light microscopy and ultrastructural observations were reported by Zhang et al. (2015).

Paratrimastigidae

The family contains a single genus *Paratrimastix* Zhang, Taborsky, Silberman, Panek, Čepička, and Simpson. Cells bear four flagella directed anteriorly, to the right and left, and posteriorly. The posterior flagellum passes through a suspension-feeding groove and bears two vanes with thickened margins. The genus contains two species *P. pyriformis* (syn. *convexa*) and *P. eleionoma* from freshwater habitats around the globe (Fig. 3). Light microscopy and ultrastructure of *Paratrimastix* was studied by Brugerolle and Patterson (1997), O'Kelly et al. (1999), and Simpson

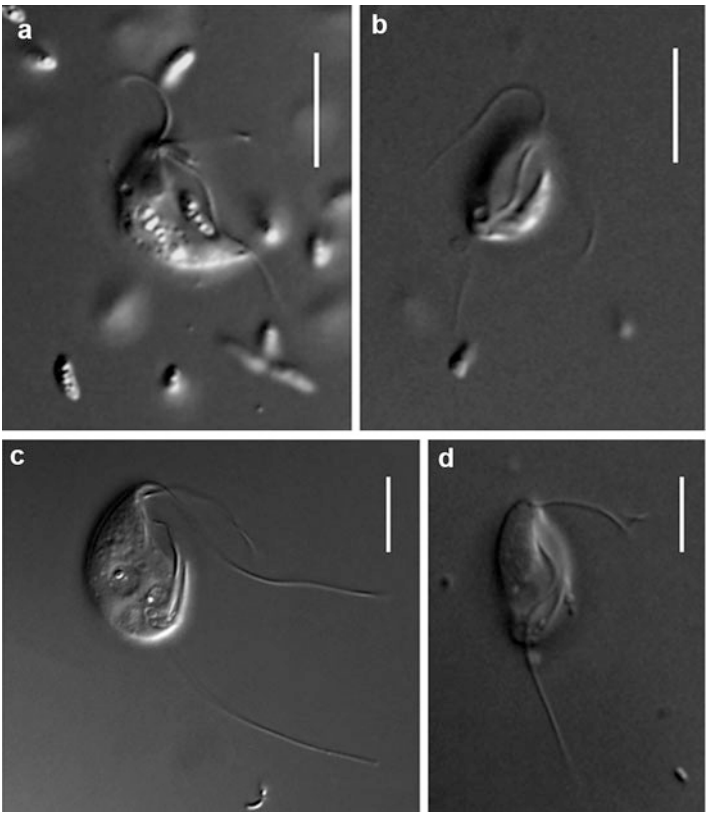

Fig. 3 **DIC images of *Paratrimastix.*** *P. pyriformis* (**a**, **b**) and *P. eleionoma* (**c**, **d**); bars 10 μm

et al. (2000). In the literature between years 1997 and 2013, these two species are referred to as *Trimastix pyriformis* and *Trimastix marina*, respectively.

Oxymonadida

More than 140 described species of oxymonads (Table 1 and Fig. 4) are all gut endobionts. They are classified into five families (Polymastigidae, Streblomastigidae, Pyrsonymphidae, Saccinobaculidae, and Oxymonadidae) and a genus *Opisthomitus*.

Polymastigidae

There are four described genera of small tetraflagellates with pelta and slender noncontractile axostyle and without attachment organelles.

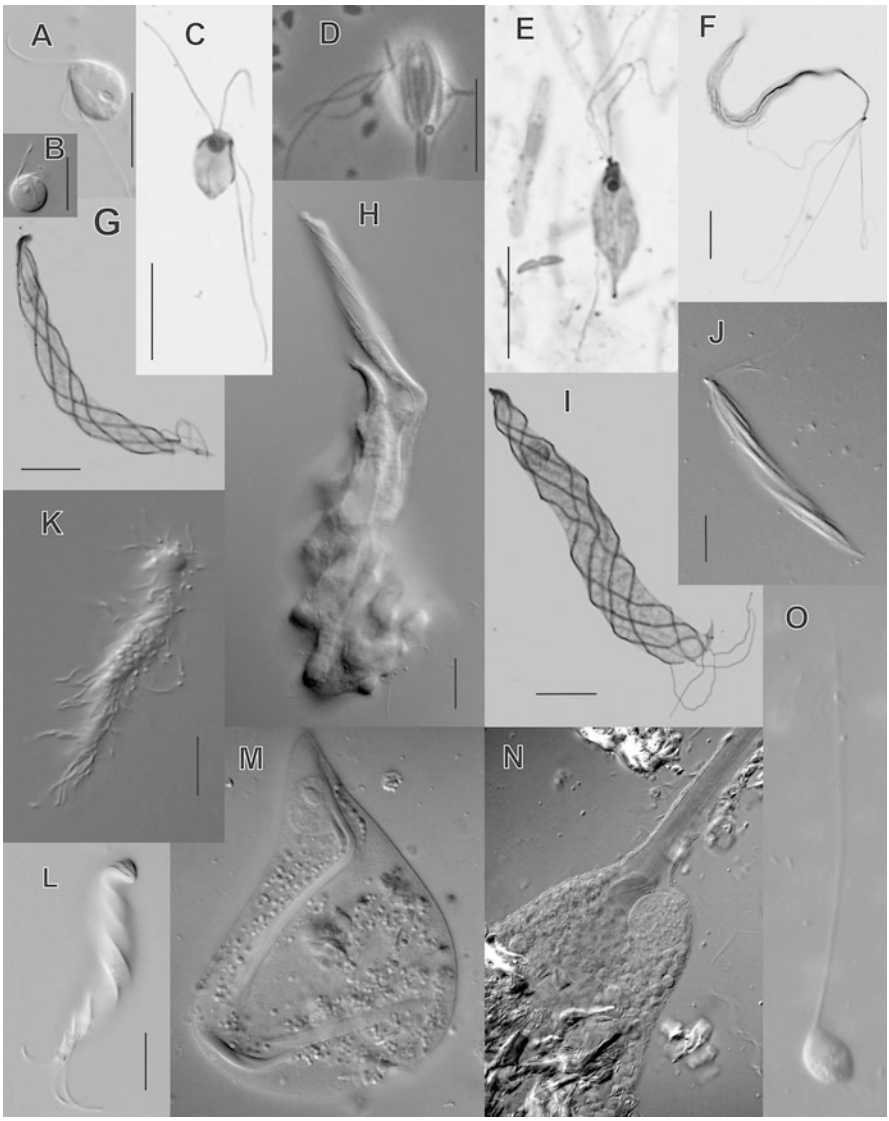

Fig. 4 DIC images and protargol preparations of oxymonads. (**a**, **b**) DIC images and (**c**) protargol preparations of *Monocercomonoides* sp. from *Chinchilla*, (**d**) DIC image of *Polymastix* sp. from *Parasphaeria boleiriana*, (**e**) protargol preparation of *Polymastix melolonthae* from crane fly larva, (**f**) protargol preparation and (**j**) DIC image of *Streblomastix strix* from *Zootermopsis angusticollis*, (**g**) protargol preparation of *Dinenympha gracilis* from *Reticulitermes lucifugus*, (**h**) DIC image of *Pyrsonympha vertens* from *Reticulitermes flavipes*, (**i**) protargol preparation of *Pyrsonympha* sp. from *Reticulitermes lucifugus*, (**k**) DIC image of *Dinenympha fimbriata* from *Reticulitermes lucifugus*, (**l**) DIC image of *Dinenympha* sp. from *Reticulitermes lucifugus*, (**m**) DIC image of *Saccinobaculus ambloaxostylus* from *Cryptocercus punctulatus*, (**n**) DIC image of nuclear region, and (**o**) whole cell of *Oxymonas* sp. from *Cryptocercus punctulatus*; bars 10 μm. (**d**) was kindly provided by Guy Brugerolle; (**m**–**o**) were kindly provided by Patrick Keeling and Kevin Carpenter

Monocercomonoides Travis

Monocercomonoides Travis has a small oval to pyriform body (5–15 μm in length) and four flagella arranged in two pairs, with one which is recurrent and attached to the body (Fig. 4a–c). The organization of the *Monocercomonoides* cytoskeleton was described above and is depicted in Fig. 2. Over 40 species have been described (Table 1), but the validity of some of them is uncertain. About half of the species inhabit the posterior part of the digestive tract of wood-eating insect imagoes (the cockroaches *Cryptocercus* and *Parasphaeria* and lower termites), insect larvae (*Tipula*, Coleoptera), or millipedes, while the rest live in the gut of vertebrates (rodents, bovids, reptiles, and amphibians). The ultrastructure was studied by Brugerolle and Joyon (1973), Kulda and Nohýnková (1978), Radek (1994), Simpson et al. (2002), and Brugerolle et al. (2003).

Tubulimonoides Krishnamurthy and Sultana

The genus *Tubulimonoides* described from the gut of *Gryllotalpa africana* (African mole cricket) is very similar to *Monocercomonoides* but differs from it by its tubular axostyle. In the type species (*Tubulimonoides gryllotalpae*) the flagella are reportedly organized into groups of three and one, unlike all other oxymonads. The other two species have the flagella organized in a typical 2:2 fashion. Because of these discrepancies and in the absence of electron microscopic study, the validity of this genus is questionable. Light microscopy was carried out by Krishnamurthy and Sultana (1976), Mali and Sultana (1993), and Mali et al. (2003).

Polymastix Bütschli

Spindle-shaped tetramastigotes (5–22 μm in length) differ from *Monocercomonoides* by the absence of a recurrent (cell-adhering) flagellum, very short or no fiber R1, the presence of a microfibrillar bundle connecting the nucleus to the first pair of basal bodies, a narrow and grooved preaxostyle, a slender axostyle composed of about 10 microtubules, a small pelta, and, most strikingly, the presence of long symbiotic *Fusiformis* bacteria on the surface (Fig. 4d, e). Up to 11 species are currently recognized (Table 1); another as-yet undescribed species has been observed in the cockroach *Parasphaeria* (Brugerolle et al. 2003). *Polymastix* was found in the gut of larvae of Scarabaeoidea beetles and crane flies, myriapods (*Glomeris* and *Rhizocrinus*), cockroaches, and termites. EM studies were conducted by Brugerolle (1981) and Brugerolle et al. (2003).

Paranotila Cleveland

A single species *P. lata* was described from the gut of *Cryptocercus punctulatus*. On the basis of morphology, Brugerolle and Lee (2000) classified *Paranotila* among polymastigids. The uninuclear cell is larger than *Monocercomonoides* (15–25 μm) and has four flagella only slightly adhering to the cell and directed laterally and a single axostyle that does not protrude from the cell. Under the influence of molting hormone ecdysone, *Paranotila* undergoes a sexual cycle that involves automixis. During the nuclear division without cytokinesis, the cell transforms to a gametocyte containing eight male and eight female gametic nuclei.

The nuclei fuse to form an eight-nuclear zygote that breaks gradually into eight uninuclear cells. A single morphological study was conducted by Cleveland (1966); no EM study has been published.

Streblomastigidae

The family contains a single genus with one described species, *Streblomastix strix* Kofoid and Swezy 1919, that inhabits the hindgut of termopsid termites, e.g., *Zootermopsis angusticollis* (Fig. 4f, j). Noda et al. (2006) report undescribed *Streblomastix* sp. from *Archotermopsis* sp. The relatively rigid spindle-shaped cells of *S. strix* are typically 15–50 μm long, but rare giant forms can be as long as 300 μm. Four flagella are inserted subapically and do not adhere to the cell. The anterior tip of the cell forms a thin rostellum with a cup-like holdfast. This structure can be lengthened and retracted, and it serves for attachment to the gut epithelium. In many individuals (probably recently divided cells), the rostellum is small or absent. The surface of the cell (besides the very anterior tip) is covered by 100–200 long rod-shaped epibiotic bacteria of at least three morphotypes, and the sequencing of 16S rRNA revealed three closely related phylotypes related to *Bacteroides* (Leander and Keeling 2004; Noda et al. 2006). In transverse section, *Streblomastix* shows a stellate organization with the cytoplasm reduced to a dense central core from which radiate 6–7 thin vanes. The ridges between vanes are apparent in the light microscope and typically show torsion from left to right starting at the anterior end. The ragged cell shape is probably an adaptation to accommodate bacterial epibionts and naked cells, produced by antibiotic treatment, shift to a teardrop shape (Leander and Keeling 2004). The nucleus is a dense thin spiral rod. The microtubular cytoskeleton consists of axostyle, pelta, and preaxostyle. In the prenuclear region, the microtubules of the axostyle are organized in several parallel rows (syn. "rhizoplast" in Kidder (1929)); in the nuclear region, microtubules form a single row that envelops the nucleus, and in the post-nuclear region, the axostyle consists of a loose bundle of microtubules. The pelta helically encircles the prenuclear axostyle and covers the anterior part of the nucleus. The cell divides by binary fission, and the cell cycle is probably affected by the molting cycle of the termite. No cysts have been reported. Morphology was studied by Kofoid and Swezy (1919) and Kidder (1929). Electron microscopy was conducted by Hollande and Carruette-Valentin (1970b) and Leander and Keeling (2004).

Pyrsonymphidae

All 25 described species in two genera are hindgut symbionts of the lower termite genus *Reticulitermes* (Table 1). The nucleus is situated anteriorly. Four or eight flagella are organized in two or four pairs separated by preaxostyle(s). Flagella emerge at the anterior end of the cell, bend posteriorly, insert into grooves on the cell surface, wind around the cell in left-handed spirals, and trail posteriorly. The

contractile axostyle is the main motile organelle. It extends the entire length of the organism and consists of thousands of microtubules arranged in many parallel rows connected by bridges. Most, if not all, pyrsonymphids contain endobiotic bacteria in the cytosol, and many species also harbor epibiotic bacteria on the surface (Smith and Arnott 1974b; Iida et al. 2000; Tokura et al. 2000; Stingl et al. 2005; Yang et al. 2005; Hongoh et al. 2007). The bacteria are often attached to the cell by specialized structures developed by both bacteria and protists (Smith and Arnott 1974b). Two extant and two fossil genera are currently recognized (Poinar 2009a, b).

Pyrsonympha Leidy

Representatives of this genus (13 described species; Table 1) are relatively large cells (up to 150 μm) and show a pyriform, sack-like appearance (Fig. 4h, i). The broader posterior end of the cell is filled with phagocytic vesicles containing wood pieces. *Pyrsonympha* often develops an attachment organelle (holdfast) at the anterior pole of the cell (Cochrane et al. 1979). Many individuals of *Pyrsonympha vertens* have eight flagella and two preaxostylar fibers (Bloodgood et al. 1974). These individuals likely represent a prolonged stage in the life cycle prior to cell division. The axostyle of *Pyrsonympha* can be isolated and movement reactivated in vitro (Bloodgood et al. 1974). In *P. vertens*, a loose bundle of microtubules (paraxostyle) runs parallel to the axostyle from the basal bodies region (Brugerolle 1970). The pelta is reduced to several microtubules (= solénolemme in Hollande and Carruette-Valentin (1970b)). The surface of *Pyrsonympha* is covered by fine scales of unknown function and composition (Smith and Arnott 1973). Ring-like structures were reported on the surface of an undetermined pyrsonymphid from the gut of *Neotermes cubanus* (Maass and Radek 2006). Hollande and Carruette-Valentin (1970a) reported synaptonemal complexes in *P. flagellata*, suggesting the existence of meiosis.

Dinenympha Leidy

Dinenympha Leidy are smaller (tens of μm) freely motile cells with four flagella, which are characterized by a screw-like shape. If not associated with epibiotic bacteria like *D. fimbriata*, the cells exhibit distinctive wiggly movement (*D. gracilis*). Twelve species have been described (Fig. 4g, k, l and Table 1).

The long-lasting debate as to whether *Pyrsonympha* and *Dinenympha* represent separate genera or life-cycle stages of the same genus was apparently resolved by molecular studies (Moriya et al. 2003; Stingl and Brune 2003) showing that the sequences of *Dinenympha* and *Pyrsonympha* form separate groups and, importantly, that specific DNA probes hybridize exclusively to one genus but not the other and vice versa (see Fig. 1 in Moriya et al. (2003)). Light microscopic observations of pyrsonymphids were carried out by Porter (1897), Powell (1928), Jírovec (1929), Georgevitch (1932), and Grassé (1952) and electron microscopy by Brugerolle (1970), Hollande and Carruette-Valentin (1970a, b), Smith and Arnott (1973, 1974a, b), Smith et al. (1975), Bloodgood et al. (1974), Cochrane et al. (1979), and Maass and Radek (2006). Two fossil species of Pyrsonymphidae – *Dinenymphites spiris* and *Pyrsonymphites cordylinis* – have been described from

Cretaceous amber from Burma. The age of the amber was dated between 97 and 110 mya (Poinar 2009a, b). The protists were found in association with a fossil termite, *Kalotermes burmensis*.

Saccinobaculidae

Saccinobaculidae are hindgut symbionts of the wood-feeding cockroaches *Cryptocercus punctulatus* and *C. relictus*. The four, eight, or 12 flagella do not adhere to the body except, in some cases, in the proximal part (Fig. 4m). No attachment organelle has been observed. The large axostyle is contractile and is responsible for cell locomotion. It undulates vigorously inside the cell, like "a snake in a bag," causing rapid and dramatic changes in the cell shape (Cleveland et al. 1934). The waves originate at the anterior end and propagate posteriorly in a single plane – they are sinusoidal rather than helical (Mcintosh 1973; Mcintosh et al. 1973). As in *Pyrsonympha*, the movement of the isolated axostyle can be reactivated in vitro (Mooseker and Tilney, 1973). A sexual process was reported in this family (Cleveland 1950a, b, c). The family contains two morphologically very similar genera.

Saccinobaculus Cleveland

As in other oxymonads, the basic unit of the mastigont consists of two pairs of basal bodies associated with a preaxostyle, and multiplication of flagella is accompanied by the multiplication of preaxostyles. The microtubules of the preaxostyle continue to form the first row of axostylar microtubules facing away from the nucleus. In the prenuclear region, similar but shorter rows of microtubules gradually attach to this primary row, forming the axostyle that contains more than 8000 microtubules in the largest sections. The axostyle forms an arch anteriorly to the nucleus and then runs posteriorly, twisting and forming a crescent that almost closes to a circle or spiral to the distal end, where it protrudes from the cell. The number of microtubules decreases significantly toward the posterior end. The nucleus is tightly associated with the axostyle by its dorsal side. The region of the nucleus and preaxostyle is wrapped from the posterior and ventral side in a thin single layer of microtubular sheet, the pelta (= thin lamina in Mcintosh et al. (1973)). Conspicuous electron-dense granules were reported from the cytoplasm of *Saccinobaculus* (Mcintosh et al. 1973; Carpenter et al. 2008), which may represent peroxisomes or a modified mitochondrion (Carpenter et al. 2008). The surface of the cell is covered by circular concavities that sometimes show circular pits in the center (Carpenter et al. 2008). These are similar to those reported from pyrsonymphids (Maass and Radek 2006). Their function is unknown, but the presence of what appears to be clathrin coating in these pits suggests they may play a role in endocytosis. Epibiotic bacteria are present only rarely. Seven species of *Saccinobaculus* are currently recognized (Table 1). They differ in size and presence of granules in the axostyle or cytoplasm (Cleveland et al. 1934; Heiss and Keeling 2006). Cleveland (1950b) transferred *S. doroaxostylus* and *S. minor* into the genus *Oxymonas* as *O. doroaxostylus* and *O. nana*, but the

molecular phylogenetic study by Heiss and Keeling (2006) showed that they should be classified as *Saccinobaculus*. Light microscopy studies were performed by Cleveland et al. (1934), Cleveland (1950b, c), and Heiss and Keeling (2006), with electron microscopy by Grimstone and Cleveland (1965), Mcintosh et al. (1973), Mcintosh (1973), and Carpenter et al. (2008).

Notila Cleveland

Cleveland (1950c) distinguished *Notila* from *Saccinobaculus* on the basis of differences in their sexual cycles. The major difference is that both trophozoites and "gametes" of *Notila* are diploid. As late as after fusion of two diploid "gametic" cells, their nuclei undergo single-step meiosis to form four haploid gametic nuclei. The gametic nuclei fuse to form a double zygote that soon undergoes cytokinesis. Morphologically, *Notila* differs from *Saccinobaculus* by its axostyle that does not protrude, has no terminal sheath, and contains granules. The validity of the genus has yet to be confirmed. A single species, *Notila proteus*, was studied using light microscopy by Cleveland (1950c), Grassé (1952), and Bobyleva (1973); no EM study has been done.

Oxymonadidae

All described species are hindgut symbionts of termites, specifically Kalotermitidae. They can either take the form of free-swimming flagellates or attach to the intestinal wall by a microfibrillar holdfast situated at the tip of a cellular extension – the rostellum. In some cases the rostellum may be several times longer than the cell (Fig. 4o). It is probably able to contract or extend by a slow passive movement. The stout axostyle is contractile, but does not undulate as violently as in *Saccinobaculus*. Locomotion probably results from the combined activity of the axostyle and flagella. Oxymonadidae may have single or multiple nuclei. Nuclei migrate posteriorly during mitosis and travel back after telophase. The surface of the cell (including the rostellum of most species) is densely covered by epibiotic rod-shaped bacteria, oriented perpendicularly to the cell. Four extant and three fossil genera of Oxymonadidae are currently recognized (Table 1).

Oxymonas Janicki

Oxymonas Janicki are club-shaped cells, usually containing a single nucleus, two pairs of flagella, and a single axostyle (Fig. 4n, o). Amoeboid forms have been also reported (Tamschick and Radek 2013). Over 30 species have been described (Table 1), including two fossil species. The length of the reported species varies between 5 and 240 μm and the width between 4 and 165 μm. The rostellum of *Oxymonas* is supported by a paraxostyle (homology to the paraxostyle of *Pyrsonympha* is unclear) and a bundle of free microtubules. The paraxostyle originates at the dense microtubule-organizing center at the tip of the rostellum and extends posteriorly to the cell body. It consists of microtubules organized in convoluted ribbons. Free microtubules originate at various positions in the trunk of the

rostellum, extend posteriorly, and continue to the axostyle. The stout axostyle consists of parallel, stacked rows containing thousands of microtubules. It originates at the base of the rostellum by inserting new microtubules among the free microtubules continuing from the rostellum. The microtubules in the axostyle are interconnected by cross-bridges. The axostyle is tightly adpressed to the nucleus, continues posteriorly, splits into smaller bundles, and often enrolls at the posterior end. In some cells, the axostyle protrudes posteriorly. The preaxostyle that connects the pairs of basal bodies is situated close to the origin of the axostyle, but studies do not show any connection between the two structures. A dense plate adjacent to the preaxostyle underlies a region in the flagellar area where long spirochetes attach (Cross 1946; Brugerolle and König 1997; Rother et al. 1999).

The surface of *Oxymonas*, under the epibiotic bacteria, is densely covered by external surface structures that form a honeycomb-like pattern. They are formed by a cylindrical base and are covered by a lid. Pits with a coat resembling clathrin are formed from the bottom. The surface structures are composed of carbohydrates and likely function in pinocytosis. The lid also serves as an attachment place for bacteria (Rother et al. 1999). Light microscopic observations of *Oxymonas* were conducted by Kofoid and Swezy (1926) and Cross (1939, 1946). Fossils were studied by Poinar (2009a, b). Studies using EM were conducted by Brugerolle and König (1997), Rother et al. (1999), Tamschick and Radek (2013), and Radek et al. (2014).

Microrhopalodina (syn. *Proboscidiella*) Grassi and Foa

Four species are described (Table 1). Cell dimensions range from 23 to 165 μm in length and 11–113 μm in width and contain multiple karyomastigonts. The number of karyomastigonts varies from four to 50, but the common numbers are four, eight, and 12. The karyomastigonts are arranged in a collar at the base of the rostellum. Every karyomastigont associates to its own axostyle. Posterior to the nuclei, axostyles extend independently as bands composed of parallel microtubular rows connected by electron-dense bridges. The bands are strongly curved at the posterior end. In the region of the nucleus, at least one row of axostylar microtubules splits from the band and laterally encircles the nucleus, forming a calyx. In the anterior direction, the microtubules lose the periodic organization, and microtubules from all axostyles join into a single loose bundle that extends into the rostellum. One lamella of microtubules encircles this loose bundle. Similarly to *Oxymonas*, the rostellum contains microtubules of the paraxostyle that originate in the holdfast and extend into the cell as convoluted ribbons. These ribbons are less developed than in *Oxymonas*. The cell body contains numerous vesicles filled with digested material. The surface of the cell is covered by external surface structures and bacteria, as in *Oxymonas* (Rother et al. 1999). Light microscopy was carried out by Kofoid and Swezy (1926), Kirby (1928), Cross (1946), and Rother et al. (1999) and EM by Lavette (1973) and Rother et al. (1999).

Barroella (syn. *Kirbyella*) Zeliff

Only two species are described (Table 1), with cell dimensions ranging between 27 and 224 μm in length and 11–80 μm in width (Cross 1946). The mature cell has a

club-like shape, no flagella, and multiple nuclei (2–114), which are scattered throughout the body. Slender axostyles are tortuously curved and much longer than the body. Axostyles and nuclei are rarely equal in number. Immature cells are similar to *Microrhopalodina*, with a collar of flagella and shorter axostyles. They originate by budding from larger cells that are distinguished by formation of multiple karyomastigont coronas (Cross 1946). No EM study has been done.

Sauromonas Grassé and Hollande

The single species, *Sauromonas m'baikiensis*, is a symbiont of the termite *Glyptotermes boukoko*. In the attached form, the cell is organized like *Oxymonas* and possesses a single nucleus, four flagella, and a single axostyle. The rostellum of *Sauromonas* contains a recurvent fibrillar bundle, which may in fact correspond to the paraxostyle of *Oxymonas* and *Microrhopalodina*. When the termite molts, the organism detaches from the intestinal wall and undergoes series of transformations resulting in a polyflagellated cell, which then loses the flagella and encysts. Light microscopy was carried out by Grassé (1952). No EM study has been done. Three fossil species of Oxymonadidae – *Oxymonites gerus*, *Microrhopalodites polynucleatis*, and *Sauromonites katatonis* – have been described in association with a fossil termite species *Kalotermes burmensis* from Cretaceous amber from Burma, 97–110 mya (Poinar 2009a, b).

Opisthomitus Duboscq & Grassé

Opisthomitus Duboscq and Grassé 1934 are small oxymonads bearing four flagella. The anterior end of the cell is pointed and forms a conspicuous lappet that may be homologous to a rostellum; however, the attachment of the cells to the gut wall has never been observed. The organization of the cytoskeleton resembles *Monocercomonoides*, including the presence of a pelta supported by a microtubular root associated with anterior basal body 4. The surface of the body is covered by numerous ring-like bulges resembling the concavities in *Saccinobacullus*. Light microscopy was studied by Duboscq and Grassé (1934), De Mello (1953), Hollande and Carruette-Valentin (1970b), and Radek et al. (2014). An EM study was performed by Radek et al. (2014). Two valid species, *Opisthomitus avicularis* and *O. longiflagellatus*, and two species with uncertain status, *O. brasiliensis* and *O. flagellae*, have been described. The genus is not classified into any oxymonad family, and the phylogeny based on 18S rRNA suggests its affiliation to Pyrsonymphidae (Radek et al. 2014).

Maintenance and Cultivation

Stable cultures have been established so far only for representatives of *Trimastix*, *Paratrimastix*, and *Monocercomonoides*. The cultures are monoeukaryotic but polyxenic (with admixed bacteria). *Trimastix* grows on ATCC 1525 medium that

should, for some strains, be supplemented by 1 ml of simplified ATCC 1034 medium (without folic acid and yeast nucleic acid added; Zhang et al. 2015). *Paratrimastix* grows well on bacterized ATCC 802 (Sonneborn's *Paramecium* medium). *Monocercomonoides* grows on TYSGM (Diamond 1982) or Dobell-Laidlaw two-phase medium (Dobell and Laidlaw 1926; Hampl et al. 2005). Cultures are maintained in 22 °C, or 37 °C if from a mammalian host, and are transferred every 4–7 days. Insect oxymonads can be maintained in the lab in their hosts.

Phylogeny and Evolution

The close relationship between endobiotic oxymonads and free-living *Paratrimastix* was first realized through phylogenetic analyses of 18S rRNA genes (Dacks et al. 2001). Based on this finding and on ultrastructural comparisons, the taxon Preaxostyla was established and defined by ultrastructural synapomorphy – a characteristic appearance of the I fiber in *Paratrimastix* and its homologue, the paracrystalline part of preaxostyle, in oxymonads (Simpson 2003). The name Anaeromonada has also been used for this grouping (Cavalier-Smith 2003). The "typical excavate" morphology of *Paratrimastix* justified inclusion of Preaxostyla into the supergroup Excavata (Cavalier-Smith 2002; Simpson 2003). Within Excavata, Preaxostyla are regarded as members of Metamonada – a commonly recognized group containing most of the other anaerobic Excavata (i.e., parabasalids and fornicates) (Cavalier-Smith 2003; Hampl et al. 2009). Both Metamonada and Excavata represent reasonable taxonomic hypotheses based on data available today, but the statistical support specifically for Excavata is never strong in molecular phylogenetic/phylogenomic analyses (Hampl et al. 2005, 2009; Simpson et al. 2006; Rodriguez-Ezpeleta et al. 2007a, b; Parfrey et al. 2010; Grant and Katz 2014; Kamikawa et al. 2014). The validity of all Excavata as a clade has been strongly challenged by a potential rooting of eukaryotes "within" Excavata, with *Malawimonas* on one side of the root and other examined Excavata on the other (Derelle et al. 2015); however, these analyses have not included Metamonada. The position of Metamonada relative to this proposed root therefore remains to be established.

The internal phylogeny of Preaxostyla recovered using 18S rRNA genes by Zhang et al. (2015) is schematically depicted in Fig. 5. It suggests that the common ancestor of Preaxostyla was a typical excavate with four flagella resembling the extant genera *Trimastix* and *Paratrimastix*. The morphology of oxymonads is derived and probably affected by their endobiotic way of life. A striking evolutionary explosion of morphological diversity is apparent in oxymonads from cockroach and termite guts. Fossils resembling some current genera of oxymonads have been reported in association with *Kalotermes burmensis* and a blattellid cockroach found in early Cretaceous amber (97–110 mya) from a mine in the Hukawng Valley, southwest of Maingkhwan, Burma (Table 1, Poinar 2009a, b). Sequence data have been obtained from the oxymonad genera *Pyrsonympha*, *Dinenympha*, *Oxymonas*, *Streblomastix*, *Monocercomonoides*, *Saccinobaculus*, and *Opisthomitus*

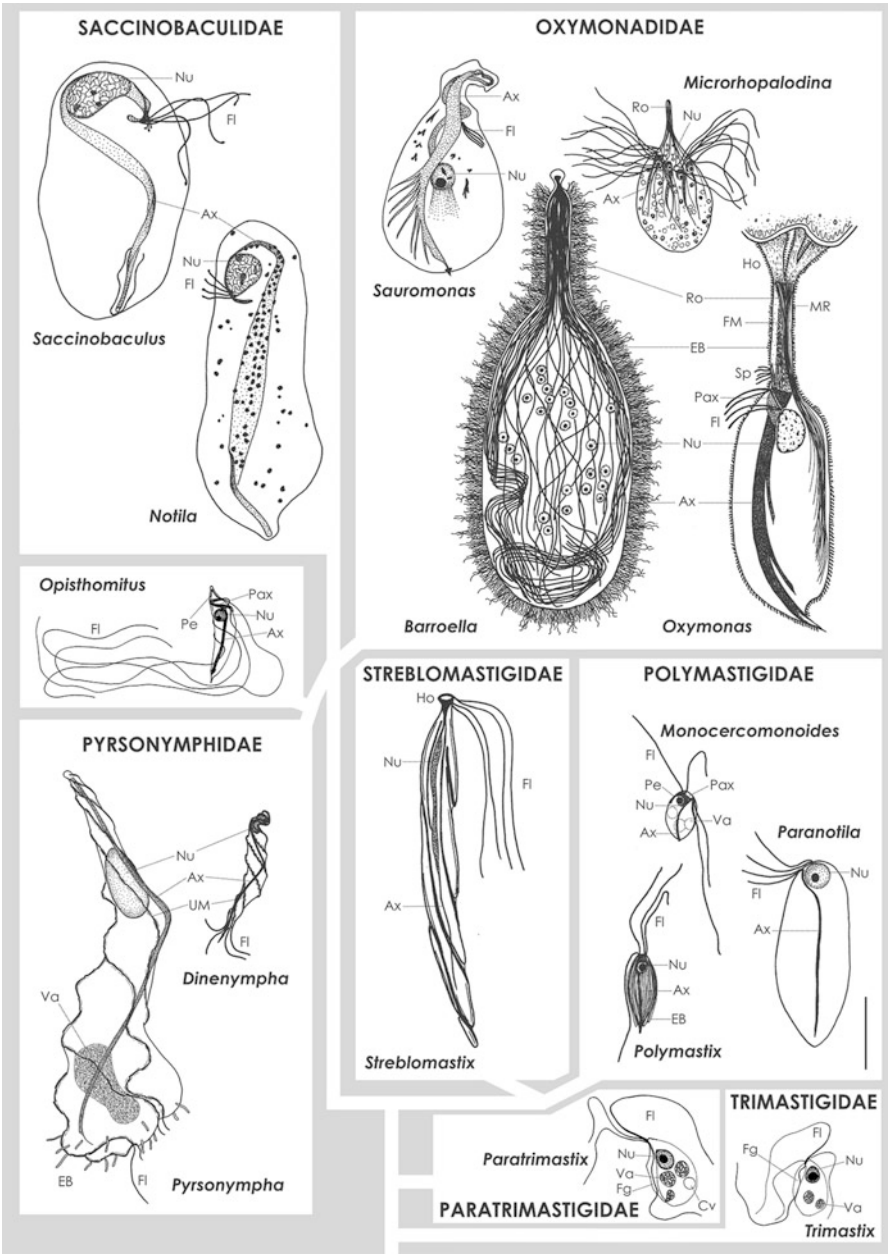

Fig. 5 Genera of oxymonads, their division into families, and probable relationships between the families. *Ax* axostyle, *Cv* contractile vacuole, *EB* ectosymbiotic bacteria, *Fg* feeding groove, *Fl* flagellum, *FM* free microtubules, *Ho* holdfast, *Nu* nucleus, *MR* microtubular ribbons, *Pe* pelta, *Pax* preaxostyle, *Ro* rostellum, *Sp* spirochaetes, *UM* undulating membrane, *Va* vacuole. bar: 10 μm for Polymastigidae and Streblomastigidae; 20 μm for Pyrsonymphidae,

representing all five families. The relationships within oxymonads are not well resolved, but all recent analyses generally agree on the relatively robust clade of Polymastigidae + Streblomastigidae and a weakly supported clade of the remaining oxymonads (Hampl et al. 2005; Heiss and Keeling 2006; de Koning et al. 2008; Radek et al. 2014).

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Fig. 5 (continued) Saccinobaculidae, and *Oxymonas*; and 40 μm for *Sauromonas*, *Microrhopalodina*, and *Barroella*. Drawing of *Barroella* after Cross (1946), *Oxymonas* after Brugerolle and König (1997), *Microrhopalodina* after Kirby (1928), *Sauromonas* after Grassé (1952), *Saccinobaculus* after Cleveland (1950b), *Notila* after Cleveland (1950c), and *Paranotila* after Cleveland (1966); remaining figures are original

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Parabasalia

32

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Abstract

The Parabasalia are a clade of single-celled, anaerobic flagellates that are mainly obligate symbionts or parasites of insects and vertebrates. The group includes the common and widespread human sexually transmitted species *Trichomonas vaginalis*. Many species are found exclusively in the guts of termites and the wood-feeding roach *Cryptocercus*, where they contribute to wood digestion as part of a complex microbial community that sustains the insects. These insect symbionts often harbor an extensive and diverse assortment of ecto- and endosymbionts. The Parabasalia are characterized by a parabasal body (Golgi complex supported by a parabasal fiber), which is associated with the flagellar apparatus. Their mitochondria have evolved into hydrogenosomes, double-membrane-bounded organelles that derive energy from the breakdown of pyruvate to acetate, CO_2, and H_2. They vary in size from the minute *Tricercomitus*, which is only a few microns long, to the half-a-millimeter-long *Mastotermes* gut symbiont *Mixotricha paradoxa*. Historically, the Parabasalia have been treated as two groups: the smaller, simpler "trichomonads" which bear up to six flagella and the typically much larger, multiflagellate "hypermastigotes." Ultrastructural and molecular evidence have shown that together these groups form a monophyletic Parabasalia, and though neither "trichomonads" nor "hypermastigotes" are monophyletic, they continue to be useful as descriptive terms.

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Introduction

General Characteristics

Parabasalia is a clade of anaerobic protists, almost all of which are flagellates. Parabasalians are characterized by the presence of a parabasal body (a densely packed Golgi complex that is associated with striated fibers connected to the basal bodies), hydrogenosomes (anaerobic derivatives of mitochondria), closed pleuromitosis with an extranuclear mitotic spindle, and a particular arrangement of microtubular and non-microtubular elements of the mastigont, notably the axostyle and pelta (see below). Although belonging to the Excavata, parabasalians have lost the typical excavate features, particularly the ventral feeding groove and cytostome. Many species possess a characteristic undulating membrane formed by a recurrent flagellum and cytoplasmic projection. Most parabasalians are uninucleate, but multinucleate cells have evolved in some lineages. Although the parabasalians are not particularly species rich, including approximately 450 described species in 100 genera, they display immense variability in cell complexity and in the number of flagella, which ranges from zero to several thousands. Accordingly, Parabasalia has been historically divided into two assemblages, the trichomonads with up to six flagella per mastigont and usually simpler cells, and the hypermastigotes, which can possess thousands of flagella and extraordinarily complex cells. However, it has been shown that an increase in the number of flagella has evolved several times independently from the trichomonad-like cells. The current taxonomy of the Parabasalia consists of six classes that better reflect the evolution of this group. Nonetheless, "trichomonad" and "hypermastigote" are still routinely used as terms of convenience to differentiate simpler cell types from multiflagellate forms.

Occurrence

Almost all parabasalians are symbionts of the digestive tracts of animals, both invertebrates and vertebrates, including humans. Much of the known diversity of Parabasalia occurs in the guts of termites and their sister lineage, the wood-feeding

roach *Cryptocercus*. Parabasalians belonging to the orders Trichonymphida, Spirotrichonymphida, and Cristamonadida are found nowhere else. These parabasalians form obligate, vertically inherited symbioses with the insect families Archotermopsidae, Hodotermitidae, Kalotermitidae, Mastotermitidae, Rhinotermitidae, Serritermitidae, Stolotermitidae, and Stylotermitidae of the infraorder Isoptera, collectively referred to as the lower termites, and *Cryptocercus*, the sole extant genus of infraorder Cryptocercoidea (Krishna et al. 2013). By contrast, termites from the most speciose family, Termitidae, only sporadically harbor small parabasalians such as *Trichomonas* and *Monocercomonas* and never hypermastigotes (Kirby 1937). While the lower termites are found on all continents except Antarctica, *Cryptocercus* has only been reported from the northern hemisphere, in certain mountainous regions of China, Korea, Russia, and the USA.

Besides termites, a number of trichomonad species have been described from other insects, such as cockroaches, crane flies, beetles, and true bugs, and some other invertebrates such as horse leeches and snails (Alexeieff 1911; Mackinnon 1913; Grassé 1926; Bishop 1932; Kozloff 1945; Brugerolle et al. 2003; Zhang 2003; Smejkalová et al. 2014), though nothing is known about their importance for their hosts. Trichomonads of vertebrates are mostly harmless intestinal commensals and can be found in diverse birds, fish, amphibians, reptiles, and mammals (e.g., Alexeieff 1910; Honigberg 1978; Čepička et al. 2005, 2006; Smejkalová et al. 2012). The best-known parabasalians are the few human and livestock parasites that have escaped the lower intestinal tract and live in the genitourinary, upper digestive, or respiratory tracts: *Trichomonas vaginalis*, *Trichomonas gallinae*, *Tritrichomonas foetus*, and *Histomonas meleagridis*.

Although most parabasalian species are host-associated, a few free-living ones have been described as well, for example, *Monotrichomonas carabina*, *Ditrichomonas honigbergii*, *Pseudotrichomonas keilini*, and *Lacusteria cypriaca* (Bishop 1935, 1939; Farmer 1993; Bernard et al. 2000; Yubuki et al. 2010). They inhabit freshwater, brackish, and marine anoxic/microoxic sediments and have been found worldwide.

Literature and History of Knowledge

The first parabasalians to be described were trichomonads associated with humans and animals. The first was *Trichomonas vaginalis* from a human vaginal tract (Donné 1836), followed by three more species of *Trichomonas*, each now known by different names: *Tetratrichomonas limacis* from slugs (Dujardin 1841), *Tritrichomonas suis* from pig intestines (Gruby and Delafond 1843), and *Trichomitus batrachorum* from frogs (Perty 1852). Another early description was of *Pentatrichomonas hominis* from human intestines, originally named *Cercomonas hominis* (Davaine 1854, 1860). The first multiflagellated species (hypermastigotes) were described not long after: *Lophomonas blattarum* (Stein 1860), from the hindgut of a common cockroach, and *Trichonympha agilis* (Leidy 1877) from the hindgut of a termite. Leidy wrote that the multiflagellate swimming cell he called *Trichonympha*

reminded him of "nymphs in a recent spectacular drama, in which they appeared with their nakedness barely concealed by long cords suspended from the shoulders" (Leidy 1877), hence the origin of the *-nympha* suffix that proliferated through the nomenclature of hypermastigotes. Toward the end of the nineteenth century, more researchers began to study the protist hindgut community of termites, using only light microscopy and relatively unsophisticated staining techniques. The parabasalians, like the ciliates, proved accessible to iron hematoxylin and then protargol staining, however, revealing a wealth of taxonomically useful morphological characters. Many new genera of parabasalians from various hosts, both trichomonads and hypermastigotes, were described, and a classification system with elements recognizable in our current classification was in place by the early twentieth century (Grassi and Foà 1911).

The order Trichomonadida (corresponding roughly to the "trichomonad" assemblage) was created by Kirby (1947). His system of families and genera was revised by Honigberg (1963). Honigberg's view on the evolution of the trichomonads was largely supported by electron microscopy (Brugerolle 1976), and his version of the trichomonad system survived to the beginning of the twenty-first century with some minor modifications. Then, it was gradually replaced by the contemporary system present in this chapter (see below), which is based both on morphology, including ultrastructure, and results of molecular phylogenetic studies.

The concept of hypermastigotes is even older than that of trichomonads. An affinity between the multiflagellate *Lophomonas* and *Trichonympha* was first proposed after the discovery of *Joenia*, another termite hindgut protozoan that appeared morphologically intermediate between *Lophomonas* and *Trichonympha* (Grassi 1885). This led to the creation of the order Hypermastigida for multiflagellate forms (Grassi and Foà 1911). Although much of the diversity of both trichomonads and hypermastigotes was described early in the twentieth century, it was still many years before their relatedness was understood. Hypermastigotes were initially thought to be ciliates, or intermediate between ciliates and gregarines (Leidy 1881; Kent 1882). Similarities between hypermastigotes and flagellates were soon recognized, however, and support for this view grew (Stein 1878; Kofoid and Swezy 1919; Cleveland 1923; Kirby 1947; Grassé 1952). With the advent of electron microscopy, ultrastructural studies began to reveal strong evidence that trichomonads and hypermastigotes were specifically related (Hollande and Valentin 1969a; Hollande and Carruette-Valentin 1971; Hollande and Carruette-Valentin 1972; Tamm and Tamm 1973). The superorder Parabasalia was proposed in 1973 to formally unite these two groups (Honigberg 1973). Molecular phylogenetic studies have confirmed the monophyly of Parabasalia but have also shown that neither trichomonads nor hypermastigotes are monophyletic, and at times their results have conflicted directly with morphology-based scenarios of parabasalian evolution (see "Evolutionary History").

No treatise of the Parabasalia exists, and even the economically important species have not been reviewed in depth for nearly 20 years. The most recent books reviewing these species are *Trichomonads Parasitic in Humans* (Honigberg 1990) and *Parasitic Protozoa* (Kreier 1991). An excellent genus-level descriptive key of

the group by Brugerolle and Lee (2000) can be found in *An Illustrated Guide to the Protozoa* (Lee et al. 2000). Several chapters describing the biology and evolution of termite hindgut parabasalians can be found in the *Intestinal Microorganisms of Termites and Other Invertebrates* (König and Varma 2006) and *Biology of Termites: A Modern Synthesis* (Bignell et al. 2011). The American Museum of Natural History houses the extensive microscope slide collections of Harold Kirby and Lemuel Roscoe Cleveland, two of the most prolific investigators of termite and *Cryptocercus* hindgut parabasalians. A set of 35 mm films made by Cleveland is also housed there.

Practical Importance

Parabasalians have evolved as symbionts of the animal digestive tract. Intestinal parabasalids generally cause little or no harm to their hosts (BonDurant and Honigberg 1994), with some pertinent possible exceptions (see examples below). A few species have moved to other areas of the body, where they are parasites. Humans are infected by several species, for example, *Trichomonas vaginalis* in the urogenital tract, *Trichomonas tenax* in the oral cavity, and *Pentatrichomonas hominis* and *Dientamoeba fragilis* in the large intestine (Honigberg 1978; McDougald and Reid 1978). *Trichomonas vaginalis* is the most important by far, infecting 180 million people worldwide annually. It is the most common of the sexually transmitted urogenital infections in humans. The pathogenicity of *Dientamoeba fragilis* for humans is not well understood, but it seems that certain bowel disorders can be attributed to this species (Barratt et al. 2011). A single report of *Dientamoeba* being pathogenic for gorillas was published (Lankester et al. 2010). *Pentatrichomonas hominis* is considered nonpathogenic (Honigberg and Burgess 1994). Several parabasalian species have been found in the respiratory tract of humans, for example, *Trichomonas tenax*, *Trichomonas vaginalis*, *Tritrichomonas foetus*, *Pentatrichomonas hominis*, *Tetratrichomonas gallinarum*, and *Tetratrichomonas empyemagena* (Jongwutiwes et al. 2000; Čepička et al. 2005; Kutisova et al. 2005; Duboucher et al. 2006, 2007; Carter and Whithaus 2008; Leterrier et al. 2012); their pathogenic potential is usually unclear. Although *Lophomonas blattarum*, a hypermastigote from cockroaches, has been repeatedly reported from the respiratory tract of humans, it was possibly confused with epithelial cells (see Li and Gao 2016).

Histomonas meleagridis causes histomoniasis or "blackhead" disease that affects the ceca and liver of turkeys, chicken, quail, and peafowl. It has been effectively treated with dimetridazole and nifursol in the feed, but these drugs are now banned in the European Union. Symptoms in turkeys are listlessness, anorexia, droopy wings, and yellow, sulfur-colored feces. *Histomonas* interacts with cecal worms, earthworms, coccidia, and other intestinal microbiota (AbdulRahman and Hafez 2009). It can be transmitted between birds by the nematode *Heterakis gallinarum*, and earthworms may serve as paratenic hosts (McDougald and Reid 1978).

Another parabasalian affecting birds is *Trichomonas gallinae*, which lives in the upper digestive tract of birds where it can cause disease. It infects turkeys, raptors,

and gulls, but its primary host is the domestic pigeon. In pigeons it is transferred in the "milk" from the crop of an affected bird to the offspring. Virulent strains cause ulcers of the mouth, pharynx, esophagus, and crop, from which the organism enters the blood stream and passes to the liver. From this site it can kill a bird in two weeks (BonDurant and Honigberg 1994). A large outbreak of *T. gallinae* recently led to population declines in two finch species in the UK (Lawson et al. 2011).

Cattle are the primary hosts of *Tritrichomonas foetus*, which is transmitted exclusively as a venereal infection. In infected females, fertilization of the ovum occurs but the embryo may die and be expelled or absorbed. Besides cattle, *T. foetus* is found also in the large intestine and nasal cavity of pigs, where it is considered a harmless commensal, and in the intestine of cats, where it causes diarrhea (Yao and Köster 2015). For a detailed account of trichomoniasis, see BonDurant and Honigberg (1994).

Aside from causing disease in humans and animals, the main impact of parabasalians on society is their role in the destruction of buildings by wood-eating termites. While *Cryptocercus* is found only within decaying tree trunks (Nalepa 1984), at least 370 of the 3000 living species of termites are considered pests. The most damaging of the termite pests are *Cryptotermes brevis*, *Cryptotermes domesticus*, *Cryptotermes dudleyi*, *Coptotermes formosanus*, *Coptotermes gestroi*, *Reticulitermes flavipes*, and *Reticulitermes lucifugus*; these all harbor symbiotic hindgut parabasalians (Krishna et al. 2013).

Habitats and Ecology

Parabasalians are all anaerobes, and most are intestinal symbionts or parasites. The majority of described species are obligate symbionts of wood-eating insects (the so-called "lower" termites and *Cryptocercus* wood-feeding roaches), and these associations in particular have received sustained attention in terms of evolutionary history and functional ecology. Each termite or wood roach usually harbors several species of parabasalians. These species help their host to digest cellulose, in cooperation with the other microorganisms present in the intestine. Their evolution as gut symbionts has led to many morphological adaptations including cell enlargement and multiplication of flagella in some lineages (see below). It has been shown that the symbiosis between termites and their parabasalians is obligate and that the insect host will starve to death despite continued feeding if the symbionts are removed (Cleveland 1925). The termite parabasalians are considered highly host specific and coevolving with their hosts, with few host switches (Kirby 1947; Kitade 2004; Noda et al. 2007; Tai et al. 2015).

Many parabasalian species found in vertebrates are understudied and have not been reported since the original description. The species living in the intestine are usually commensals, though a possible pathogenicity for the host is a consideration in some cases. The host specificity differs from species to species (e.g., Čepička et al. 2006). Some species seem to be restricted to a few closely related hosts or a single host lineage such *Tetratrichomonas limacis* from gastropods or several trichomonad species from guinea pigs (Nie 1950; Čepička et al. 2006). Others can infect many

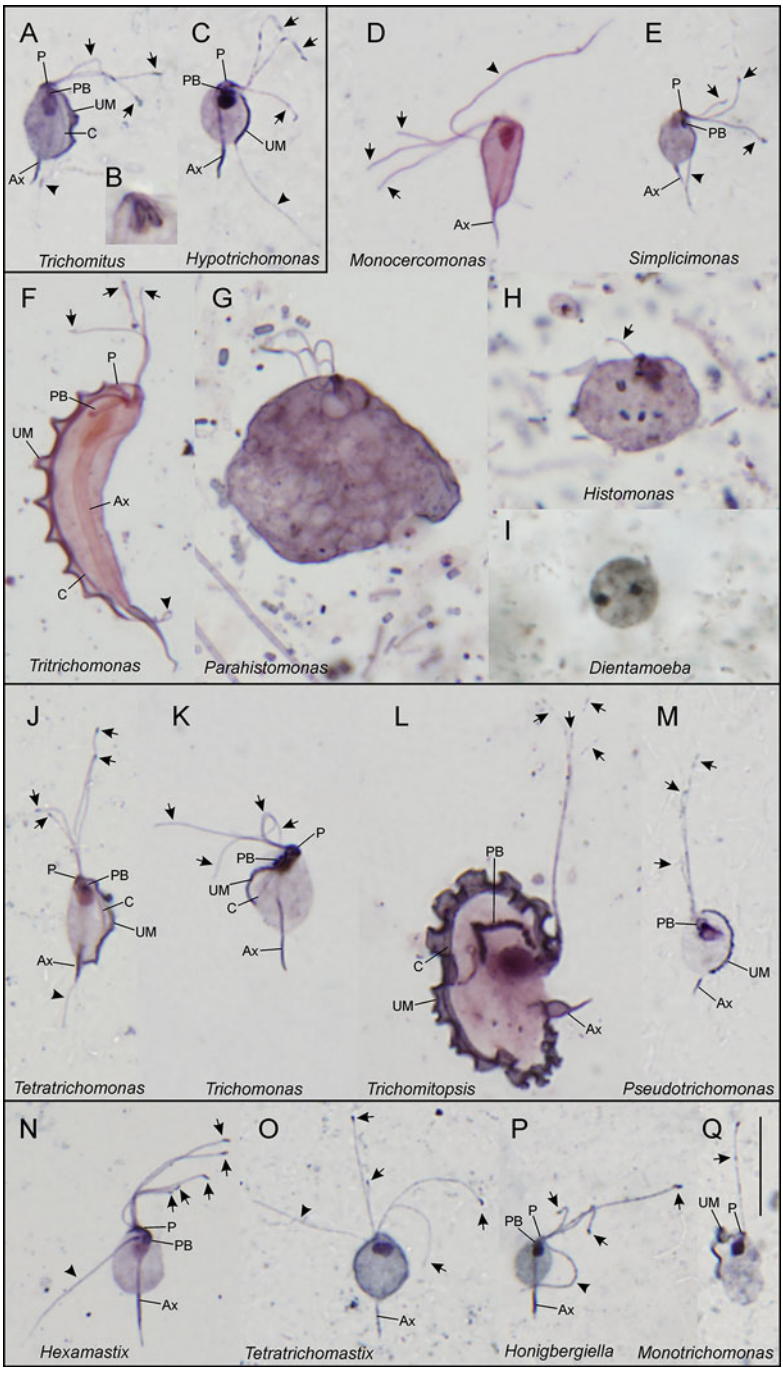

Fig. 1 (continued)

species representing one or even more vertebrate classes, e.g., *Trichomitus batrachorum* from a wide diversity of amphibians and reptiles, *Pentatrichomonas hominis* from many mammalian orders, *Tetratrichomonas gallinarum* from birds and primates, and *Tetratrichomonas* sp. "lineage 10" from tortoises, cattle, and primates (Honigberg 1953; Honigberg and Burgess 1994; Čepička et al. 2005; Smejkalová et al. 2012).

Several species of parabasalians, notably *Trichomonas* spp. and *Tritrichomonas foetus*, have colonized other internal organs, such as the oral cavity (e.g., *Trichomonas tenax* from humans and *Trichomonas gallinae* from birds) and genitourinary tract (*Trichomonas vaginalis* from humans and *Tritrichomonas foetus* from cattle). These species are often pathogenic for their hosts, causing various diseases. The trichomonads from extra-intestinal locations were generally believed to be highly host specific, with the exception of *Tritrichomonas foetus* that infects the intestine and nasal cavity of pigs as well as the genitourinary tract of cattle. Nonetheless, recently published studies have shown that the true host range may be wider in several cases (Šlapeta et al. 2012; Morin-Adeline et al. 2015).

Characterization and Recognition

Light Microscopy

Parabasalia is a morphologically diverse lineage and can be divided into two assemblages according to the cell complexity: trichomonads (relatively simple cells with up to six flagella per mastigont) and hypermastigotes (complex, often very large cells, with many flagella per mastigont). These two groups more or less correspond with the traditional orders Trichomonadida and Hypermastigida, but it has been shown that neither is monophyletic (see below). The trichomonads represent morphologically plesiomorphic forms of the Parabasalia, whereas the hypermastigotes are a polyphyletic assemblage of highly derived forms that are adapted to

Fig. 1 Light-microscopic morphology of Hypotrichomonadida (a–c), Tritrichomonadida (d–i), Trichomonadida (j–m), and Honigbergiellida (n–q). Protargol-stained cells, bright field. **(a)** *Trichomitus batrachorum* from *Bufo bufo*. **(b)** Parabasal body of *Trichomitus batrachorum* from *Testudo radiata*. **(c)** *Hypotrichomonas acosta* from *Leptopelis* sp. **(d)** *Monocercomonas colubrorum* from *Tropidophis melanurus*. **(e)** *Simplicimonas similis* from *Melamphaus faber*. **(f)** *Tritrichomonas augusta* from *Lacerta vivipara*. **(g)** *Parahistomonas wenrichi* from *Meleagris gallopavo*. **(h)** *Histomonas meleagridis* from *Meleagris gallopavo*. **(i)** *Dientamoeba fragilis* from *Homo sapiens*. **(j)** *Tetratrichomonas* sp. from *Macaca silenus*. **(k)** *Trichomonas tenax* from *Homo sapiens*. **(l)** *Trichomitopsis termopsidis* from *Zootermopsis angusticollis*. **(m)** Free-living *Pseudotrichomonas keilini*. **(n)** *Hexamastix coercens* from *Acomys* sp. **(o)** *Tetratrichomastix* sp., origin uncertain. **(p)** *Honigbergiella ruminantium* from *Bos taurus*. **(q)** Free-living *Monotrichomonas* sp. **Scale bar in Q** = 10 μm; it applies for the whole plate. **Labels**: *arrows* anterior flagella, *arrowhead* recurrent flagellum, *Ax* axostyle, *C* costa, *P* pelta, *PB* parabasal body, *UM* undulating membrane

phagocytosis of relatively large wood particles in the intestines of termites. Trichomonads are now classified among Trichomonadida, Honigbergiellida, Tritrichomonadida, Hypotrichomonadida, and Cristamonadida, while hypermastigotes belong to Trichonymphida, Lophomonadida, Spirotrichonymphida, and Cristamonadida, and one species is classified within Honigbergiellida (Čepička et al. 2010; Gile and Slamovits 2012; James et al. 2013).

Morphology of Trichomonad Cells

Trichomonad cells are usually spindle-shaped or pyriform (Figs. 1, 2). They do not possess any cytostome. Instead, phagocytosis generally occurs anywhere on the cell surface. Certain taxa tend to be amoeboid, for example, *Histomonas meleagridis* and *Parahistomonas wenrichi* (Fig. 1g, h). *Trichomonas vaginalis* also becomes amoeboid when attached to the vaginal epithelium, though it retains its flagella, as does the cristamonad *Gigantomonas herculea*, which forms gigantic plasmodia as part of its life cycle. *Dientamoeba fragilis* has completely lost its flagella and is the only true amoeba within Parabasalia (Fig. 1i). Cells of trichomonads from vertebrates measure about ten micrometers; trichomonads from termites may have much larger cells with diameters of tens or even hundreds of micrometers, for example, cells of *Mixotricha paradoxa* measure up to half a millimeter in length (Cleveland and Grimstone 1964; Brugerolle 2004).

Trichomonads are predominantly uninucleate. Many cells of *Dientamoeba fragilis* are binucleate, because they are arrested in the telophase stage of the cell cycle (Camp et al. 1974) (see Fig. 1i). The nucleus of a trichomonad is typically in close association with the flagellar basal bodies (which in simple forms are grouped together into a single "mastigont") and associated cytoskeletal fibers; in other words most trichomonads have a "karyomastigont." The number of flagella in a mastigont varies in trichomonads from zero in the amoeboid *Dientamoeba fragilis* (Fig. 1i) to six in the genera *Hexamastix*, *Pentatrichomonas*, *Pentatrichomonoides*, *Cochlosoma*, and *Cthylla* (Fig. 1m). The ancestral number is four (e.g., *Trichomitus*, *Tritrichomonas*, *Parahistomonas*, *Monocercomonas*, *Simplicimonas*, *Honigbergiella*, *Devescovina*; Figs. 1a–g, m, p and 2d, g), but five flagella are common (e.g., *Trichomonas*, *Tetratrichomonas*, *Pseudotrypanosoma*, *Trichomitopsis*, *Tetratrichomastix*; Fig. 1j–l, n), and trichomonads with three flagella (*Ditrichomonas*), two flagella (*Monotrichomonas*, Fig. 1q), or a single flagellum (*Histomonas*, Fig. 1h) are known as well.

Two independent lineages of Cristamonadida, the first one being represented by the genera *Calonympha*, *Stephanonympha*, and *Snyderella* and the second one by the genus *Coronympha*, have multiplied their nuclei and possess eight (*Coronympha* young forms) to dozens or even hundreds (e.g., *Snyderella*) of nuclei per cell (see Figs. 2h, j and 6c) (Harper et al. 2009; Gile et al. 2011). Such "polymonad" trichomonads are collectively called the calonymphs (Čepička et al. 2010). As in simpler trichomonads, the nuclei of calonymphs are each associated with flagellar basal bodies in a mastigont, forming an organelle system called the karyomastigont

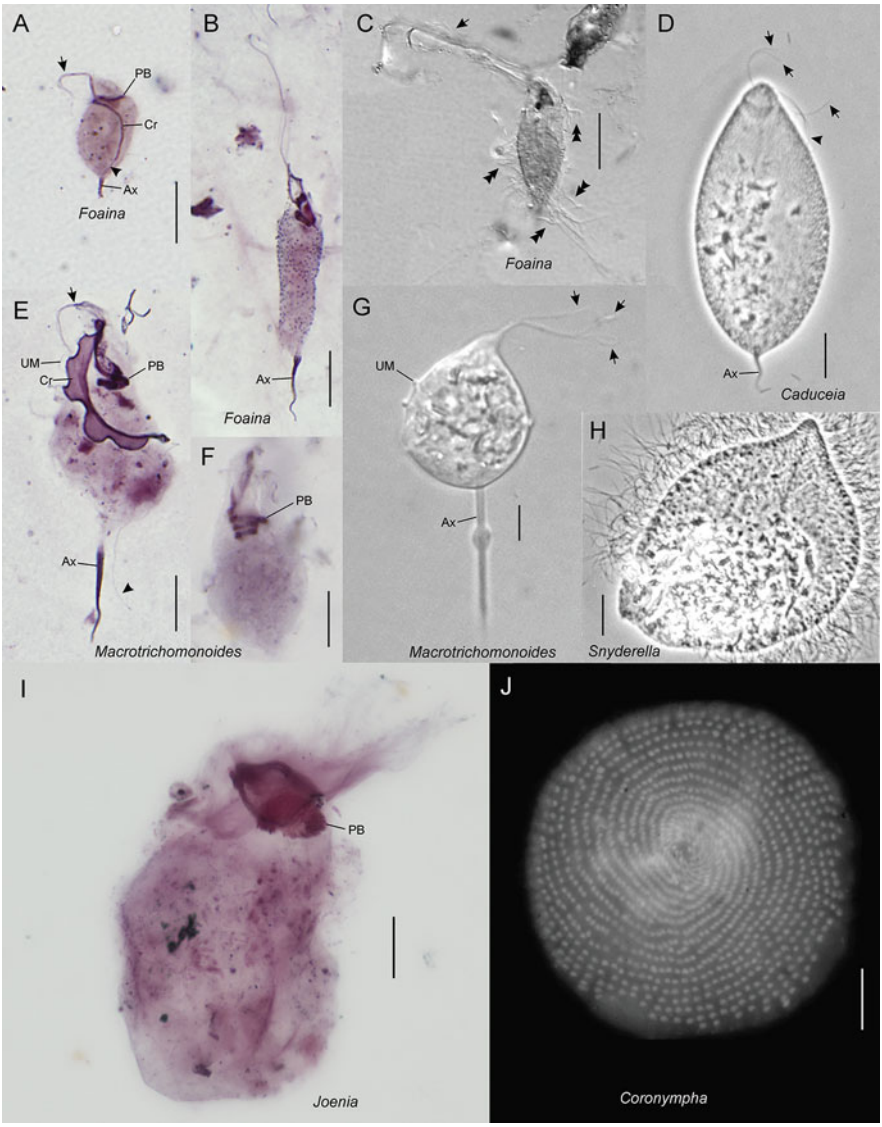

Fig. 2 Light-microscopic morphology of Cristamonadida. (a) Protargol-stained *Foaina dogieli* from *Kalotermes flavicollis*. (**b)** Protargol-stained *Foaina* sp. from *Neotermes cubanus*. The dots on the surface are the sites of attachment of epibiotic bacteria. (**c)** Protargol-stained *Foaina* sp. from *Neotermes cubanus* showing epibiotic bacteria, observed under DIC. The partial cell in the picture belongs to a polymastigid oxymonad. (**d)** Living *Caduceia versatilis* from *Cryptotermes cavifrons* observed under phase contrast. (**e)** Protargol-stained *Macrotrichomonas* sp. from *Neotermes cubanus*. (**f)** Protargol-stained *Macrotrichomonoides* sp. from *Neotermes cubanus*, detail of the parabasal body coiling around the axostyle. (**g)** Living *Macrotrichomonoides restis* from *Neotermes jouteli* observed under DIC. (**h)** Living *Snyderella* sp. from *Cryptotermes cavifrons* observed under phase contrast. (**i)** Protargol-stained *Joenia annectens* from *Kalotermes flavicollis*. (**j)** Top view of

(as above). In the genera *Calonympha* and *Prosnyderella*, there is a proliferation of mastigonts that lack an associated nucleus, the akaryomastigonts. Only in the genus *Snyderella* are the nuclei disassociated from the mastigonts and suspended in the cytoplasm. In this case, all flagella are found in akaryomastigonts across the surface of the cell. Each karyo- or akaryomastigont has four flagella. Although the calonymphs are multiflagellate, their mastigont organization is trichomonad-like and clearly derives from a multiplication of nucleomotor systems, rather than the proliferation of individual flagella that has occurred multiple times in hypermastigotes. Accordingly, two to four flagella are present in individual karyo- or akaryomastigonts.

Flagella of trichomonads insert apically or subapically. One flagellum is usually recurrent and runs posteriorly along the cell body, while the other flagella are directed anterolaterally (Figs. 1 and 2a, e, g). The posterior flagellum is acronematic, while the anterior flagella usually end with structures called "knobs" when stained (Fig. 1a, c, e, j, n–q), though the knobs may be artifacts due to the cell shrinkage during the fixation (Céza et al. 2015). The recurrent flagellum of some trichomonads is associated with the cell body, forming an undulating membrane. The undulating membrane may reach the posterior end of the cell (e.g., *Tetratrichomonas*, *Pentatrichomonas*, *Tritrichomonas*, *Trichomitus*; Fig. 1a, c, f, j, l), or it can be shorter (e.g., *Trichomonas*, *Ditrichomonas*, *Monotrichomonas*; Fig. 1k, q). In most cases, the recurrent flagellum extends beyond the undulating membrane (e.g., *Tetratrichomonas*, *Tritrichomonas*, *Trichomitus*), but in *Trichomonas* and *Pseudotrichomonas*, the recurrent flagellum is associated with the cell body along its whole length, and no free portion is developed (Fig. 1k, m). The undulating membrane is usually underlain by a fiber of varying thickness called a costa (Fig. 1a, f, j–l). Some genera, e.g., *Hypotrichomonas*, *Pseudotrichomonas*, *Ditrichomonas*, and *Monotrichomonas*, possess an undulating membrane but no costa (Fig. 1c, m, q). The undulating membrane of some members of Cristamonadida is instead underlain by a fibrous cresta (Fig. 2a, e) that is not homologous to the costa (Kirby 1942; Hollande and Valentin 1969b; Brugerolle 1976; Brugerolle and Lee 2000). The presence/absence of costa and cresta was historically suggested to be an important taxonomic feature (Kirby 1947; Honigberg 1963).

The karyomastigont of trichomonads is associated with characteristic cytoskeletal elements. Four of these are visible under the light microscope: costa/cresta, pelta, axostyle, and, with appropriate staining, parabasal fibers (Fig. 1). The axostyle is a hyaline rod and is differentiated into the proximal, spatulate capitulum, which laterally covers the nucleus, and a distal trunk, which usually protrudes from the posterior end of the cell. Trichomonad taxa differ in the shape of the capitulum,

Fig. 2 (continued) cell apex of *Coronympha* (*Metacoronympha*) sp. from *Incisitermes snyderi* showing nuclei stained with DAPI. **Scale bars** = 10 μm for **a**–**c**, **e**, **f**, **h**, and **j** and 20 μm for **d**, **g**, and **i**. **Labels**: *arrows* anterior flagella, *arrowhead* recurrent flagellum, *Ax* axostyle, *Cr* cresta, *double arrowhead* epibiotic bacteria, *PB* parabasal body, *UM* undulating membrane

thickness of the trunk, and shape of its ending. In general, two types of axostyles are recognized (Čepička et al. 2010): *Trichomonas* type, which tapers gradually (e.g., *Trichomonas*, *Trichomitus*, *Monocercomonas*, and many others; Fig. 1a, c, d, j, k, m–o), and *Tritrichomonas* type, which tapers abruptly (e.g., *Tritrichomonas*, *Simplicimonas*, and many cristamonad genera; Fig. 1e, f, l). The pelta is a crescent-shaped structure that curves over the anterior side of the nucleus. In the bird parasite genus *Cochlosoma*, the pelta has been modified to support an adhesive disc superficially resembling that of the diplomonad *Giardia* (Pecka et al. 1996). Each mastigont of the calonymphs includes an individual axostyle and pelta. Trunks of the axostyles are either separated from each other (e.g., in *Coronympha*) or they form a bundle along the cell's axis (e.g., *Calonympha* and *Stephanonympha*) (Kirby 1929; Rösel et al. 1996). Both axostyle and pelta are absent in the amoeboid *Dientamoeba fragilis* (Camp et al. 1974).

Usually one or two parabasal fibers run from the basal bodies into the cell. These are associated with the Golgi apparatus and together with the Golgi form the so-called parabasal body, which is the apomorphy for which Parabasalia is named. The parabasal body may be V-shaped (e.g., *Trichomonas*, *Trichomitus*, *Hypotrichomonas*; Fig. 1b), sausage-shaped (e.g., *Tritrichomonas*, *Monocercomonas*; Fig. 1f), discoid (e.g., *Tetratrichomonas* and *Pseudotrichomonas*; Fig. 1j, m), drop-shaped (e.g., *Pentatrichomonas*, *Simplicimonas*, *Hexamastix*; Fig. 1e, n), elongate (e.g., *Trichomitopsis*; Fig. 1l), or branched (*Pseudotrypanosoma*).

The parabasal body shape is particularly striking in the devescovinids, a grouping of large trichomonads from termite hindguts. In genera such as *Devescovina*, *Metadevescovina*, *Caduceia*, and *Macrotrichomonas*, the parabasal body winds around the axostyle, with the number of turns being used as a species-level taxonomic feature (Fig. 2f). The coiling of the parabasal body reminded Kirby of the snakes winding around Hermes' staff, the caduceus, and prompted him to name a new genus *Caduceia* (Kirby 1942). Apart from being larger than other trichomonads, the devescovinids have a similar overall morphology, with three anterior and one recurrent flagellum. The latter sometimes adheres to the cell, forming an undulating membrane. The recurrent flagellum is typically thickened to form a cord or a ribbon-like band (Foà 1905; Janicki 1915; Brugerolle and Lee 2000).

Morphology of Hypermastigote Cells

Hypermastigotes measure from several to several hundred micrometers in length and bear more flagella than trichomonads, ranging from several tens to several thousands. Almost all hypermastigote cells possess a single nucleus. Most structures typical for trichomonads, i.e., pelta, axostyle, and parabasal body, are also present in hypermastigotes but usually have been expanded or transformed. Three broad morphological categories of hypermastigotes can be recognized. Trichonymphida (e.g., *Trichonympha*, *Staurojoenina*; Fig. 3a–h) have many flagella arranged along and around a bilaterally symmetrical rostrum. Spirotrichonymphida (e.g., *Spirotrichonympha*, *Holomastigotes*; Fig. 4) lack a true rostrum, and the complex

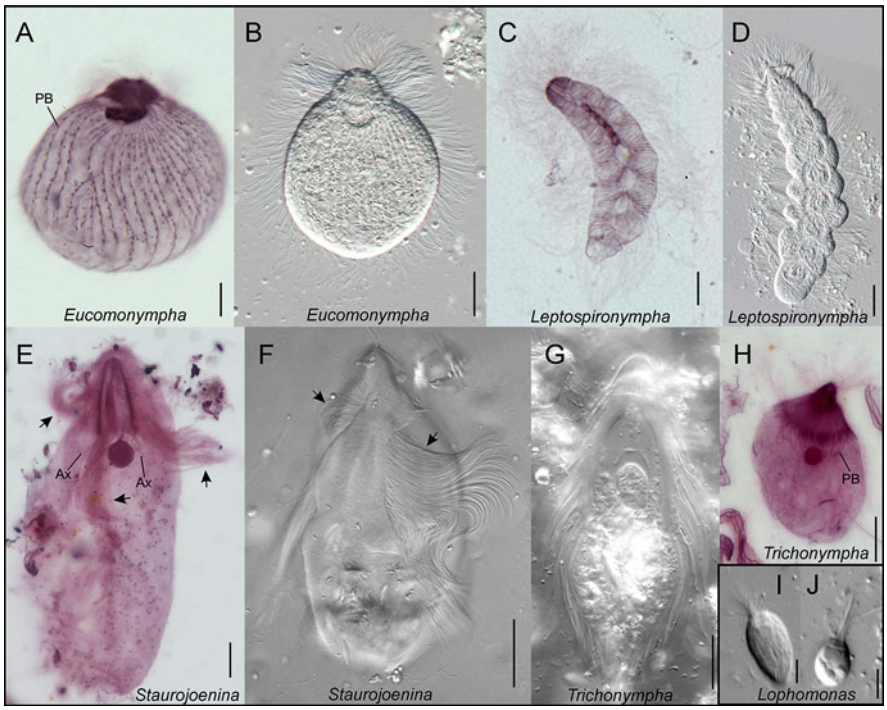

Fig. 3 **Light-microscopic morphology of Trichonymphida (a–h) and Lophomonadida (i, j).** **(a)** Protargol-stained *Eucomonympha* sp. from *Cryptocercus primarius*. **(b)** Living *Eucomonympha* sp. from *Cryptocercus primarius* observed under DIC. **(c)** Protargol-stained *Leptospironympha* sp. from *Cryptocercus primarius*. (**d)** Living *Leptospironympha* sp. from *Cryptocercus primarius* observed under DIC. (**e)** Protargol-stained *Staurojoenina* sp. from *Neotermes cubanus*. (**f)** Living *Staurojoenina mulleri* from *Neotermes jouteli* observed under DIC. (**g)** Living *Trichonympha* sp. from *Cryptocercus punctulatus* observed under DIC. (**h)** Protargol-stained *Trichonympha* sp. from *Reticulitermes flaviceps*. (**i)** Living *Lophomonas striata* from *Periplaneta americana* observed under DIC. (**j)** Living *Lophomonas blattarum* from *Periplaneta americana* observed under DIC. **Scale bars** = 10 μm for **a**, **c**, **i**, and **j**; 20 μm for **b**, **d**, **e**, **g**, and **h**; and 50 μm for F. **Labels**: *arrows* flagellar bundles of *Staurojoenina, Ax* axostyle/axostylar filaments, *PB* parabasal body

cytoskeletal structure supporting the flagella is arranged in two counterclockwise spirals (Brugerolle and Lee 2000) (see below for more details). The third group, collectively and informally called the lophomonads, were placed together in early taxonomies because of their shared anterior tuft of many flagella but are now known to be polyphyletic. All lophomonads, with the exception of *Lophomonas* itself, belong to the Cristamonadida, though they do not branch together within that order. Another shared feature of lophomonads is the resorption of parabasal bodies, axostyle, flagella, and basal bodies during cell division (Brugerolle and Patterson 2001). Only the four privileged basal bodies (homologous to the ancestral four basal bodies, see below) are not resorbed. These are duplicated and then separated, and the

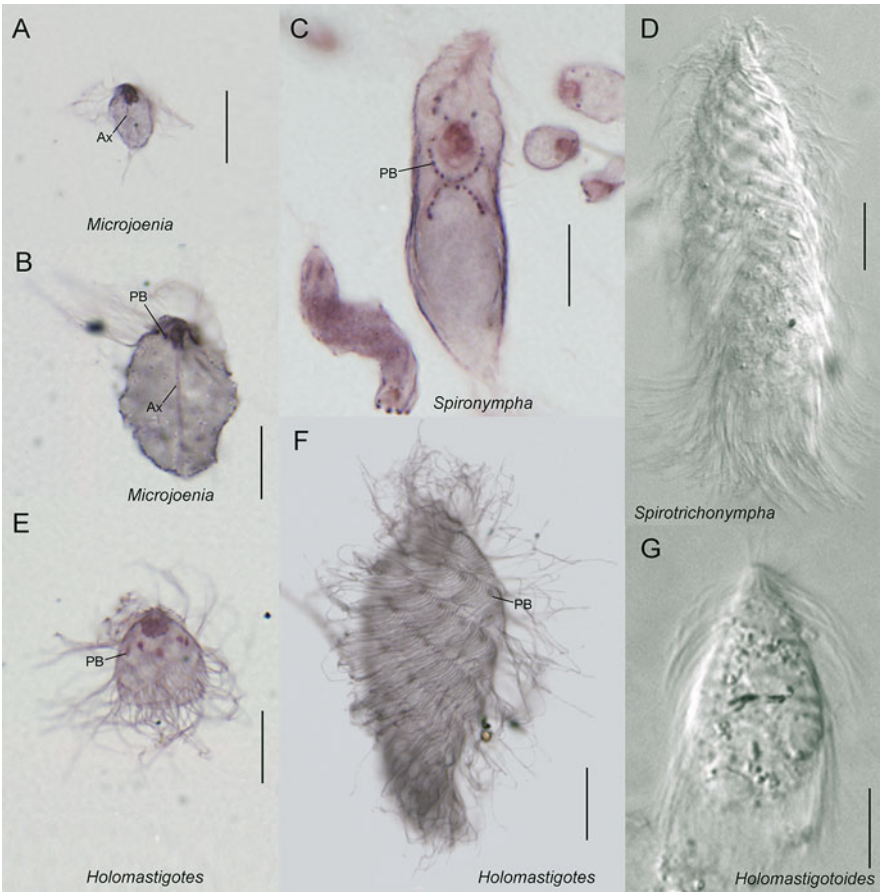

Fig. 4 Light-microscopic morphology of Spirotrichonymphida. (a) Protargol-stained juvenile *Microjoenia* sp. from *Reticulitermes lucifugus*. **(b)** Protargol-stained adult *Microjoenia* sp. from *Reticulitermes lucifugus*. (**c)** Protargol-stained *Spironympha* sp. from *Reticulitermes flaviceps*. (**d)** Living *Spirotrichonympha flagellata* from *Reticulitermes hesperus* observed under DIC. (**e)** Protargol-stained juvenile *Holomastigotes elongatum* from *Reticulitermes lucifugus*. **(f)** Protargol-stained adult *Holomastigotes elongatum* from *Reticulitermes lucifugus*. (**g)** Living *Holomastigotoides* sp. from *Coptotermes testaceus* observed under DIC. **Scale bars** = 10 μm for **a**–**c**, **e**, and **f**; 20 μm for **d**; and 50 μm for **g**. **Labels**: *Ax* axostyle/axostylar filaments, *PB* parabasal body

additional flagella and associated structures are rebuilt in each daughter cell (Hollande and Carruette-Valentin 1971).

Projoenia sawayai (Cristamonadida) displays the most plesiomorphic morphology among cristamonad hypermastigotes in general (Lavette 1970). Its cells are 45–100 μm long and strongly resemble cells of devescovinids by possessing a recurrent flagellum proximally supported by a cresta, a stout axostyle, and a single, spiral parabasal body, yet its mastigont is an apical flagellar area with as many as 500 flagella. The morphology of other cristamonad hypermastigotes (informally

referred to as "joeniids," e.g., *Joenia*, *Joenoides*, *Placojoenia*, *Joenina*, and *Joenopsis*) is similar to that of *Projoenia*, though the cells are more complex, bear more flagella, and lack the cresta (Brugerolle and Lee 2000) (Fig. 2i). Joeniid genera differ mainly in the shape of the flagellar area. *Rhizonympha jahieri* is a peculiar lophomonad whose cells are plasmodia with several hundred karyomastigonts, each containing multiple flagella (Grassé and Hollande 1951). *Kofoidia loriculata* is another unusual lophomonad with several bundles of flagella on the apex that are arranged in an open circle (Light 1927). Genera *Deltotrichonympha* and *Koruga* (which is likely a synonym of *Deltotrichonympha*) have rows of flagella extending down the cell body in addition to the apical flagellar area.

Lophomonas cells are 20–60 μm long and contain a single karyomastigont (Fig. 3i, j). The many flagella are arranged in an ear-shaped row partially encircling the nucleus. The axostyle is thin and can protrude through the cell body (Kudo 1926a, b; Hollande and Carruette-Valentin 1972).

In the monophyletic hypermastigote order Trichonymphida, cells are divided into a rostrum and postrostral area (Fig. 3a–h). The postrostral area contains the nucleus, which usually lies close to the boundary of the two areas. The rostrum is bilaterally or tetraradially symmetrical and bears two (most trichonymphids) or four (Staurojoeninidae; Fig. 3e) flagellar areas, each composed of longitudinal rows of flagella. The areas separate from each other during cell division and are distributed into the daughter cells (Hollande and Carruette-Valentin 1971). Some trichonymphids bear flagella also on the postrostral area: in Spirotrichosomidae (e.g., *Spirotrichosoma*, *Leptospironympha*, *Apospironympha*; Fig. 3c, d), the postrostral basal bodies are arranged in spiral rows, which makes them superficially similar to Spirotrichonymphida (see below), while in Trichonymphidae (Fig. 3g, h), the rows are longitudinal. In Teranymphidae, the postrostral flagella are either arranged in longitudinal rows as well (*Eucomonympha* and *Pseudotrichonympha*; Fig. 3a, b) or form multiple circular transverse rows (*Teranympha*). In Hoplonymphidae (e.g., *Hoplonympha*, *Barbulanympha*) and Staurojoeninidae (e.g., *Staurojoenina*), the postrostral area is devoid of flagella (Fig. 3e, f). The parabasal complex of trichonymphids is divided into numerous branches around the nucleus (*Trichonympha*) or consists of numerous bodies within the cell (Brugerolle and Lee 2000).

The flagella of Spirotrichonymphida are arranged in two or more counterclockwise spiral rows that are distributed into daughter cells during the division (Fig. 4). The number of flagellar lines can vary among cells of a single species (Brugerolle 2006a). Some genera (*Spirotrichonympha*, *Spironympha*, *Spirotrichonymphella*) possess an apical structure ("pseudorostrum") that resembles the rostrum of Trichonymphida, while the others (e.g., *Holomastigotes*, *Holomastigotoides*) do not. The axostyle is either absent (*Spirotrichonymphella*), split into individual filaments (*Spirotrichonympha* and *Holomastigotoides*), or resembles the axostyle of trichomonads (*Microjoenia*, *Micromastigotes*). Parabasal fibers and multiple, small parabasal bodies are associated with the flagellar rows or are scattered in the cytoplasm (Brugerolle and Lee 2000; Brugerolle 2001).

Cthulhu macrofasciculumque is the only hypermastigote member of Honigbergiellida (James et al. 2013). Its cells are only about 20 μm long and bear about 20 flagella. Otherwise, their morphology is largely trichomonad-like.

Structure of the Cytoskeleton

Although Parabasalia belongs to the supergroup Excavata, they do not possess the ventral feeding groove supported by cytoskeleton, which is typical for plesiomorphic excavates such as *Carpediemonas*, *Trimastix*, and *Jakoba*. Instead, they have developed a characteristic system of microtubular and non-microtubular cytoskeletal elements, for which homology with elements of the flagellar apparatus of typical excavates usually cannot be determined (Simpson 2003). The mastigont system that is most similar to the hypothetical last common ancestor of Parabasalia is found in some trichomonads (Fig. 5b, c) and comprises four basal bodies, three of which (here referred to as B2, B3, and B4) are parallel, are directed anteriorly, and bear the three anterior flagella. The remaining basal body (B1) lies in proximity to B2–B4 but is perpendicular to them and bears the recurrent flagellum. Note that in much of the Parabasalia literature, the basal body of the recurrent flagellum is instead designated R, and the anterior flagellar basal bodies are B1–B3. The B1–B4 scheme adopted here allows microtubular roots to be designated and compared across eukaryotic lineages (Moestrup 2000; Yubuki and Leander 2013; Yubuki et al. 2016). In genera with four anterior flagella, such as *Trichomonas*, *Tetratrichomonas*, and *Pseudotrypanosoma* (Trichomonadida), the basal body bearing the additional flagellum (B5) lies in the same plane as B2–B4 and is parallel to them, making a four-sided bundle (Fig. 5a). A fifth anterior flagellum has been added into the mastigont of Parabasalia in two different ways. In *Pentatrichomonoides* (Trichomonadida) and *Hexamastix* (Honigbergiellida), the basal body of the fifth anterior flagellum (B6) is adjacent and parallel to B2–B5 (Hampl et al. 2007; Fig. 5e, f). In *Pentatrichomonas* and *Cochlosoma* (Trichomonadida), on the other hand, B6 is separate and not parallel to the others (Honigberg et al. 1968; Pecka et al. 1996). In species with fewer than four flagella, the four basal bodies remain but one or more of them are barren. For example, *Ditrichomonas*, which has two anterior flagella, has one barren basal body, while *Histomonas*, with only one flagellum, has three barren basal bodies (Schuster 1968; Farmer 1993).

The basal bodies of the anterior flagella, B2–B4, bear striated rootlets running posteriorly into the cytoplasm. The rootlets of B3 and B4 are short and single, while B2 bears multiple, long fibers, called sigmoid fibers or preaxostylar filaments, that are curved toward the dorsal side of the cell and run toward the pelta-axostyle junction (Fig. 5a–c, f). In addition to striated rootlets, B3 and B4 each bear a short, hooked lamina along their length (Brugerolle 1991). Another typical cytoskeletal structure that originates from the area of the basal bodies (specifically, between B1 and B4) is the striated marginal lamella. It underlies the proximal part of the recurrent flagellum and participates in the undulating membrane structure (Fig. 5a, b, g).

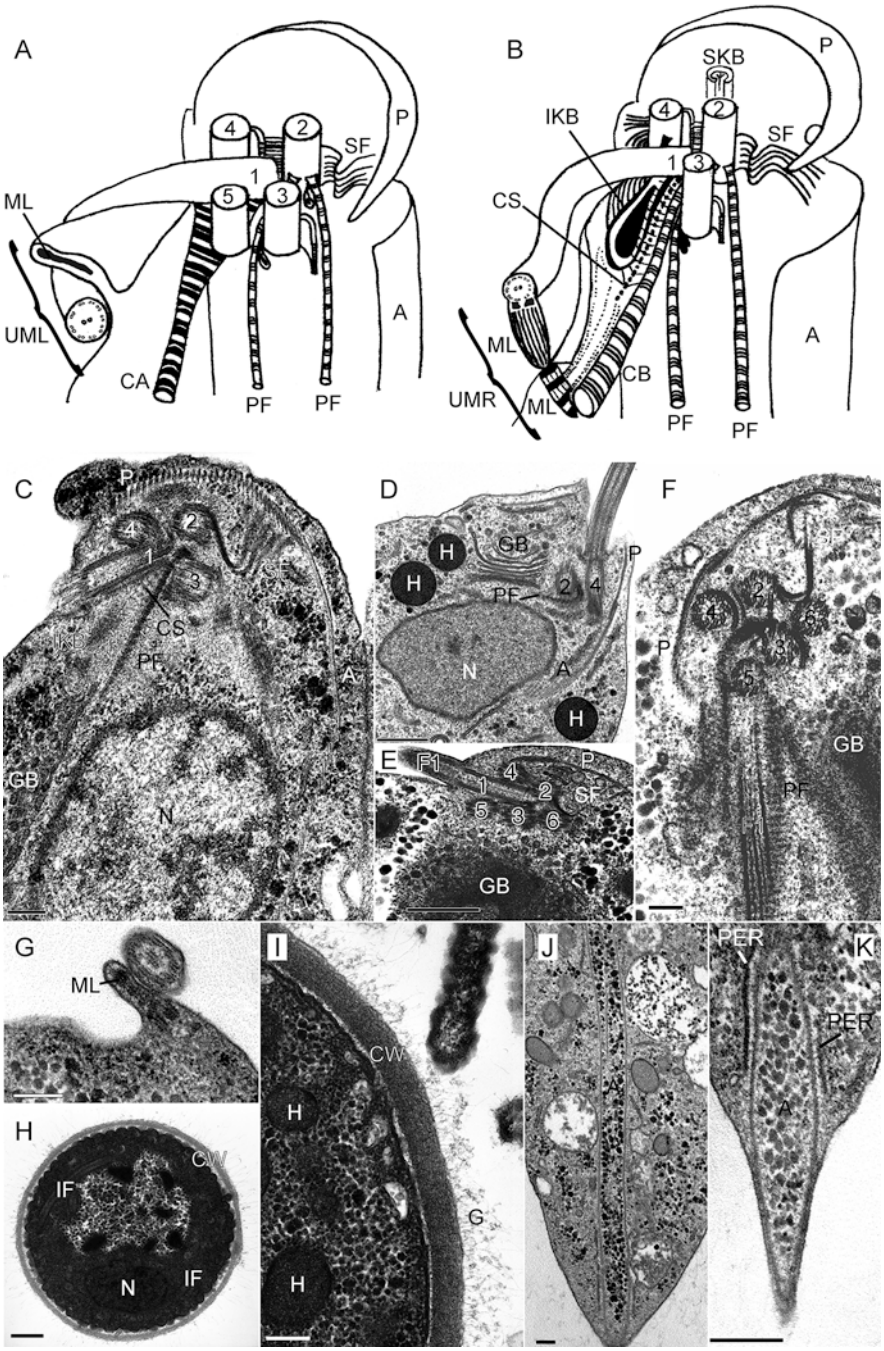

Fig. 5 Ultrastructure of trichomonads. **(a)** Organization of the cytoskeleton of *Tetratrichomonas* sp. (Trichomonadida). **(b)** Organization of the cytoskeleton of *Tritrichomonas muris*

The recurrent flagellum of some species is attached to the ventral cell surface, and an undulating membrane develops (Fig. 5a, b, g). The side of the cell where the recurrent flagellum runs is referred to as "dorsal" in the older literature, but here we consider it "ventral," because the ventral feeding groove had been located here before it was lost. There are two basic types of undulating membrane in trichomonads. The first one, called a lamelliform undulating membrane, is found in Trichomonadida, Honigbergiellida, and Hypotrichomonadida, and a very simple version is also seen in *Simplicimonas* (Tritrichomonadida) (Fig. 5a, g). It is formed by a thin cytoplasmic projection that is laterally supported by the recurrent flagellum. The recurrent flagellum usually has a typical structure with no modifications, but in some genera from termites, such as *Trichomitopsis*, *Pseudotrypanosoma*, *Trichomonoides*, and *Pentatrichomonoides*, it is enlarged and contains paraxonemal fibers (Hollande and Valentin 1968; Brugerolle et al. 1994; Brugerolle 1999). The distal part of the cytoplasmic projection of the cell body contains the marginal lamella. The second type of undulating membrane is found in the genus *Tritrichomonas* (Tritrichomonadida) and is called a "rail"-type undulating membrane (Brugerolle 1976) (Fig. 5b). Here, the recurrent flagellum is applied directly to the distal part of the cytoplasmic projection, which is much thicker than in the lamelliform undulating membrane. Both the cytoplasmic projection and recurrent flagellum contain electron-dense material derived from the marginal lamella. *Tritrichomonas* species differ considerably in the fine structure of the rail-type undulating membrane (Joyon et al. 1969) (see Fig. 5b–d in Brugerolle & Lee 2000). In some members of Cristamonadida, the recurrent flagellum adheres to the cell body as well, and a homolog of the rail-type undulating membrane is developed. In this case, however, there is no cytoplasmic projection, though a sharp transition can be seen where one side of the undulating membrane meets the cell membrane, and the enlarged recurrent flagellum applies directly to the cell surface and is subtended by an electron-dense fiber, the cresta (Fig. 6b). The cresta is thus not homologous to the costa that underlies the undulating membrane of many trichomonads, but instead

Fig. 5 (continued) (Tritrichomonadida). (**c–k)** Transmission electron micrographs. (**c)** Mastigont of *Monocercomonas colubrorum* (Tritrichomonadida). (**d)** Apical portion of *Honigbergiella ruminantium* (Honigbergiellida). (**e, f)** Mastigont of *Hexamastix kirbyi* (Honigbergiellida). (**g)** Simple lamelliform undulating membrane of *Simplicimonas similis* (Tritrichomonadida). (**h, i)** Cyst of *Honigbergiella ruminantium* (Honigbergiellida). (**j, k)** Trunk of the axostyle of *Simplicimonas moskowitzi* (Tritrichomonadida). **Scale bars** = 200 nm for **c**, **f**, **g**, and **i** and 500 nm for **d**, **e**, **h**, **j**, and **k**. **Labels**: *1*, *2*, *3*, *4*, *5*, *6* basal bodies 1–6, *A* axostyle, *CA* costa (A-type), *CB* costa (B-type), *CS* comb-like structure, *CW* cyst wall, *F1* recurrent flagellum (flagellum 1), *G* glycocalyx, *GB* Golgi body (parabasal body), *H* hydrogenosome, *IF* internalized flagellum, *IKB* infrakinetosomal body, *ML* marginal lamella, *N* nucleus, *P* pelta, *PER* periaxostylar ring, *PF* parabasal fiber, *SF* sigmoid fibers, *SKB* suprakinetosomal body, *UML* undulating membrane (lamelliform), *UMR* undulating membrane (rail type). (**a**) After Brugerolle (1976), (**b)** after Brugerolle (1991), and (**c**–**i**) after Hampl et al. (2007), with permission from Elsevier, modified, and (**j** and **k)** after Čepička et al. (2010), with permission from Elsevier, modified

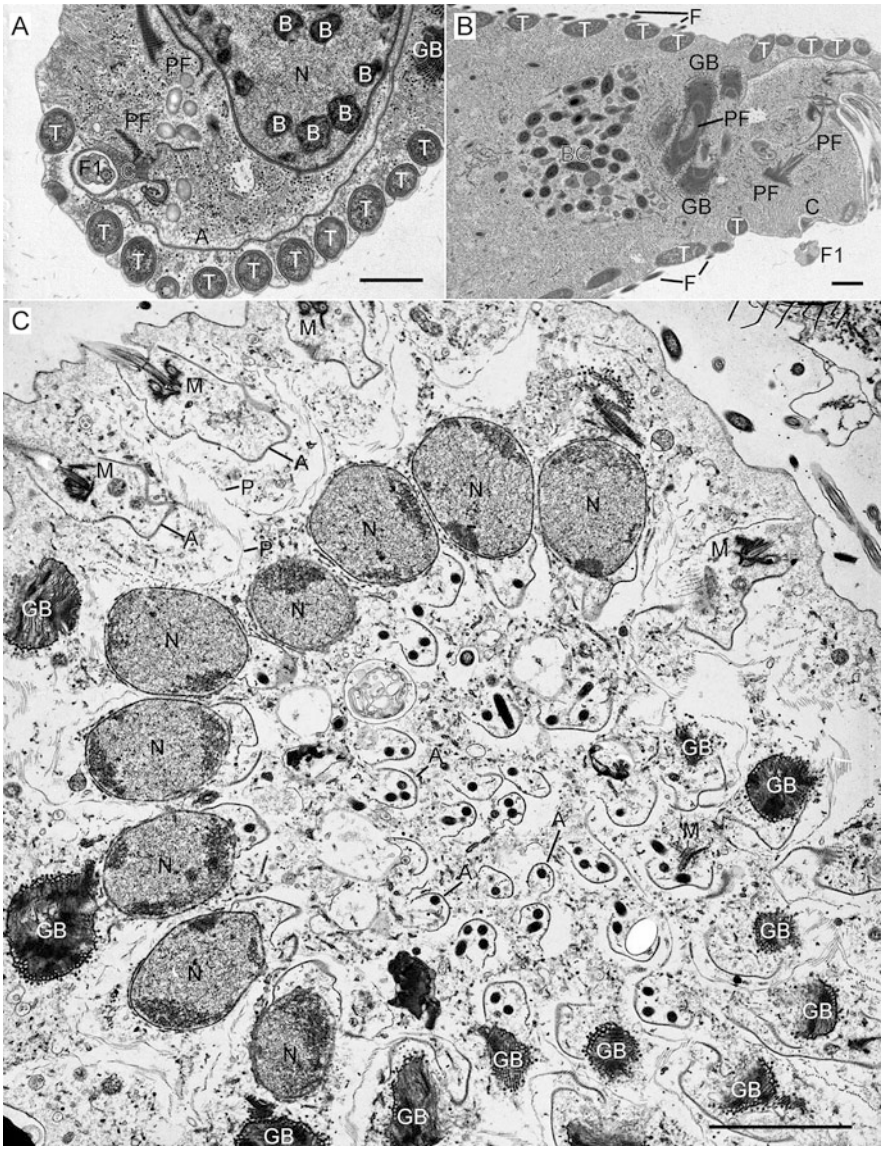

Fig. 6 **Ultrastructure of Cristamonadida. (a, b)** Electron micrographs of *Caduceia versatilis*. **(c)** Electron micrograph of *Calonympha grassii*. **Scale bars** = 1000 nm for **a** and **b** and approximately 3000 nm for **c**. **Labels**: *A* axostyle, *B* unidentified bacteria in the nucleus of *Caduceia*, *BC* "bacterial cup," a group of undescribed bacteria surrounding the axostyle directly posterior to the parabasal body, *C* cresta, *F* symbiotic fusiform bacteria of *Caduceia*, *F1* recurrent flagellum (flagellum 1), *GB* Golgi body (parabasal body), *M* mastigont of *Calonympha* with four basal bodies, *PA* pelta-axostyle complex, *PF* parabasal fiber, *N* nucleus, *T Candidatus* Tammella caduceiae ectosymbiotic bacteria

may be homologous to the proximal part of the rail-type undulating membrane of *Tritrichomonas* (Hollande and Valentin 1969b; Gile et al. 2015).

The undulating membrane of *Trichomitus* (Hypotrichomonadida), *Tritrichomonas* (Tritrichomonadida), and most members of Trichomonadida is underlain along its length by a thick striated fiber, the costa (Fig. 5a, b). Although costae are relatively uniform when observed under the light microscope, their structure and the exact site of their origin in the area of the basal bodies differ among lineages. Generally, two types are distinguished: A-type and B-type (historically, they had been also referred to as C_1- and C-type, respectively). The A-type costa is found in *Tritrichomonas* and *Trichomitus*. Its striations have a periodicity of about 40 nm and consist of repetitions of four transverse bands. In contrast, the B-type costa of Trichomonadidae, although with similar periodicity of 40 nm, consists of the repetition of a single basic line of dense filaments in cross section (Honigberg et al. 1972). The costa of most trichomonads is a rigid structure; in the closely related genera *Trichomitopsis* and *Pseudotrypanosoma*, it is contractile, and its movement contributes to the movement of the entire cell (Mattern and Honigberg 1971; Amos et al. 1979).

The parabasal apparatus is a defining feature of parabasalians. In trichomonads it includes two striated filaments (PF1 and PF2) with periodicity (ca. 40 nm) and structure very similar to that of the A-type costa (Mattern et al. 1967; Honigberg et al. 1971; Honigberg et al. 1972; Brugerolle 1976). They emerge from the basal bodies, run posteriorly into the cytoplasm, and are associated with the cisternae of stacked Golgi apparatus, which is extensively developed (Fig. 5d, f).

The axostyle and pelta are conspicuous structures of trichomonad cells under the light microscope (see above). Transmission electron microscopy revealed that each consists of a single, broad sheet of cross-linked microtubules (Brugerolle 1986) (Fig. 5c, f). Although pelta and axostyle are two separate structures, they meet in the area called the pelta-axostylar junction, where their microtubules overlap for some length. The inner side of the pelta-axostylar junction is associated with the sigmoid fibers that descend from B2. The pelta curves to the right and encircles the area where the basal bodies are located and supports the periflagellar canal, an external pit or chamber that houses the proximal portion of the flagella (Honigberg and Brugerolle 1990). The axostyle is divided into an anterior, spoon-shaped capitulum that curves over the nucleus (Fig. 5a–c) and a posterior, tubular trunk that extends axially to the posterior taillike tip of the cell (Fig. 5j, k). The axostylar trunk is formed from a sheet of microtubules, either with edges joined to form a hollow tube, as in *Simplicimonas* (Čepička et al. 2010), or rolled into a spiral, as in *Tritrichomonas* (Brugerolle and Lee 2000). In *Pentatrichomonoides* (Trichomonadida), the trunk has been modified into a microtubular corset that underlies most of the cell surface (Brugerolle et al. 1994).

There are several non-microtubular structures in the mastigont of trichomonads that are lineage specific. Some of these are various kinds of striated fibers. Although their structure has been well documented by TEM studies, their compositions and

functions are unknown. For example, the so-called infrakinetosomal body is typical for some Tritrichomonadida and Cristamonadida, and the comb-like structure has been documented from the mastigont of Tritrichomonadida, Cristamonadida, and Hypotrichomonadida (Fig. 5b, c; see Čepička et al. 2010).

Hypermastigotes have many unique ultrastructural features, though certain aspects of their cytoskeletons are directly comparable to those of simpler trichomonads. In particular, the "privileged" basal bodies, those that are homologous to the ancestral B1–B4, can be found among the many additional flagella (Hollande and Carruette-Valentin 1971). These are most clearly apparent in the lophomonads, where they are at the heart of the apical flagellar bundle and polarize the parabasal bodies and pelta-axostyle complex just as in trichomonads (Hollande and Carruette-Valentin 1972; Brugerolle 1991). Upon cell division, all flagella are resorbed and only the privileged basal bodies remain. The privileged basal bodies are arranged in the three anterior, one recurrent arrangement typical of trichomonads, with a hooked lamina on B2 and B4 (Honigberg and Brugerolle 1990). One exception to this is found in *Lophomonas*, where the direction of B1 has changed such that all four privileged basal bodies are parallel (Hollande and Carruette-Valentin 1972; Brugerolle 1991). This significant ultrastructural difference is consistent with the distant relationship between *Lophomonas*, and the cristamonad hypermastigotes according to molecular phylogenies (Gile and Slamovits 2012). Furthermore, the basal bodies of the proliferated flagella in *Joenia* and *Deltotrichonympha* (Cristamonadida) each bear a hooked lamina, suggesting they arose by multiplication of B2 or B4, while the proliferated flagella in *Lophomonas* have unadorned basal bodies, suggesting that they derive from B1 (Brugerolle 1991).

In Trichonymphida (Fig. 7), the proliferated flagella are organized into two regions, with the parabasal fibers forming a base plate for each. These plates meet along their longitudinal edges to form the distinctive rostral tube characteristic of *Trichonympha*, *Pseudotrichonympha*, and *Teranympha*, or they are separated by lobes of ectoplasm, as in, e.g., *Hoplonympha* and *Barbulanympha*. In *Staurojoenina*, there are four such rostral plates separated by four ectoplasmic lobes. At the apex of each rostral plate can be found one (*Staurojoenina*), two (*Trichonympha*), or three (*Urinympha*) privileged basal bodies (Hollande and Carruette-Valentin 1971; Brugerolle and Lee 2000).

In Spirotrichonymphida, the proliferated flagella are organized into two to six helical rows in which the basal bodies are connected by short connecting fibers and longer fibrous bands (Lingle and Salisbury 1995). Depending on the genera, these bands might reach the cell's posterior (e.g., *Spirotrichonympha*) or traverse most of the cell (e.g., *Holomastigotoides*) or remain confined to the cell's apex (*Microjoenia*) (Brugerolle 2001, 2005, 2006b; Brugerolle and Bordereau 2004). Parabasal bodies may be interspersed regularly or irregularly between the rows (Brugerolle and Lee 2000). Each flagellar line has a set of one to three basal bodies at its apex, one of which bears the hooked lamina characteristic of B2 and B4 of trichomonads, while homologs of the recurrent basal body (B1) have not been identified (Brugerolle 2001).

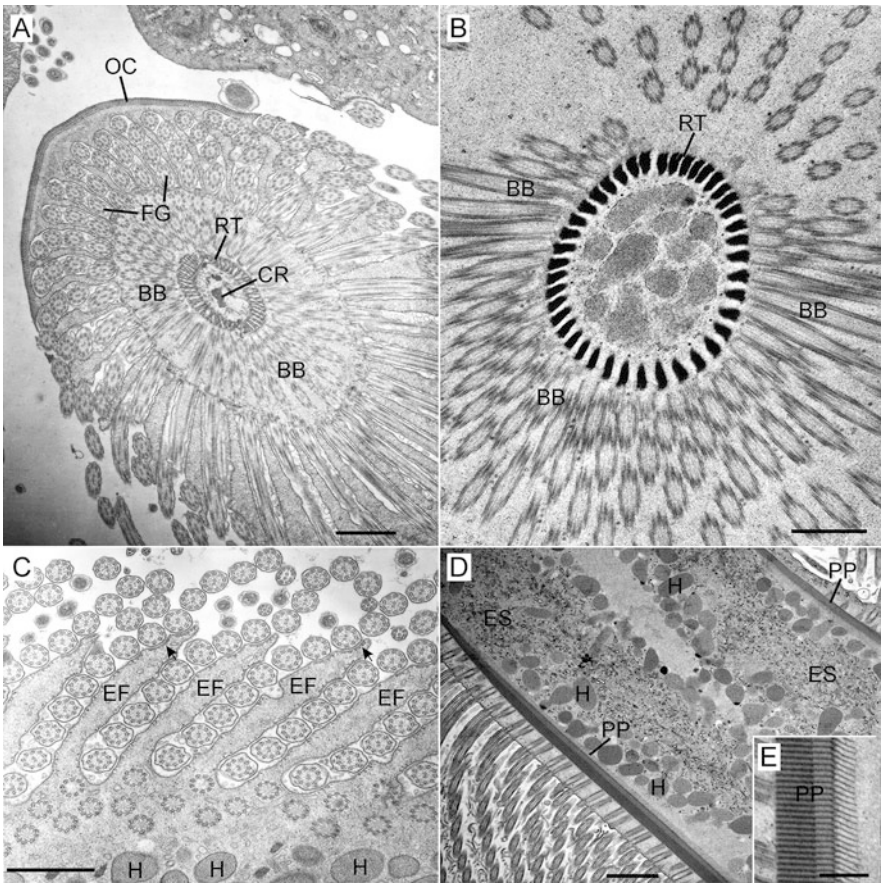

Fig. 7 Ultrastructure of *Trichonympha* spp. (a–c) and *Staurojoenina* sp. (d, e). (**a)** A lower-resolution, tangential section through the *Trichonympha* rostrum showing the microtubule-containing outer cap covering the flagellar grooves and the rostral tube with the centriolar rod. (**b)** The anterior tip of the *Trichonympha* rostrum with the electron-dense rostral tube, to which long basal bodies are attached. (**c)** A higher-resolution view of the postrostral region of *Trichonympha* in which ectoplasmic flanges separate flagellar grooves. (**d)** The rostral lobes of *Staurojoenina* are underlain by parabasal plates, in which basal bodies are embedded. **(e)** Detail of the striated parabasal plate of *Staurojoenina*. **Scale bars** = 1000 nm for **a**–**c**, 2000 nm for **d**, and 500 nm for **e**. **Labels**: *arrows* electron-dense material bound to the cytoplasmic side of the cell membrane that attaches axonemes to the flanges, *BB* basal bodies, *CR* central rod, *EF* ectoplasmic flanges separating flagellar grooves, *ES* endomembrane system, *FG* flagellar grooves, *H* hydrogenosome, *OC* outer cap, *RT* rostral tube

Hydrogenosomes

Parabasalians are characterized by a distinctive double-membrane-bounded organelle called the hydrogenosome. The hydrogenosome's basic biochemistry was first defined by Lindmark and Müller in *Tritrichomonas foetus* (1973). These organelles

function in anaerobic ATP generation via the partial oxidation of pyruvate to acetate, carbon dioxide, and molecular hydrogen (Tachezy 2008). They are homologous to mitochondria (although the precise evolutionary history vis-à-vis obligately aerobic mitochondria has been extensively debated; Martin and Müller 2007), a relationship that was demonstrated through molecular/biochemical evidence such as the presence within hydrogenosomes of mitochondrial-type chaperones (Germot et al. 1996) and of the NADH dehydrogenase module of the mitochondrial respiratory chain (Hrdý et al. 2004). Like most hydrogenosomes, those from parabasalians lack a genome (Turner and Müller 1983; Clemens and Johnson 2000; Van Der Giezen et al. 2005). The anti-trichomoniasis drug metronidazole (Flagyl) receives an electron in the hydrogenosome, making it cytotoxic (Benchimol 2009).

Using TEM, the hydrogenosome is seen as an organelle delimited by two closely appressed membranes, with a homogenous, granular matrix (Figs. 5d, g, 7c). It lacks cristae. In *Trichomonas vaginalis* the hydrogenosomes occupy about 6% of the cell volume (Nielsen and Diemer 1976). Hydrogenosomes proliferate in the cell by a fission process similar to that described for peroxisomes and mitochondria (Wexler-Cohen et al. 2014).

Mitosis and Reproduction

The mitotic process used by parabasalians in cell division is a form of "cryptopleuromitosis." In this case the nuclear envelope remains intact, while the chromosomes' kinetochores are embedded in the envelope. The mitotic spindle, also called a paradesmose, remains outside the nucleus and consists of pole-to-pole microtubules and pole-to-kinetochore microtubules. The spindle pole bodies, called atractophores, are associated with the mastigont structures, such that the spindle segregates the two groups of kinetosomes as it separates the two sets of chromosomes in the nucleus. This is best seen in Fig. 3.7 in Honigberg and Brugerolle (1990). The atractophore is a somewhat amorphous granular material in the trichomonads but has a distinctive bell-clapper appearance in the trichonymphid and spirotrichonymphid hypermastigotes (Hollande and Carruette-Valentin 1972; Ritter et al. 1978). Kubai (1973) gives a very detailed ultrastructural study of the kinetochores and their movement within the nuclear envelope prior to attachment to the spindle tubules in *Trichonympha*.

Mitosis of the multiple nuclei in calonymphs occurs simultaneously, whether in the nonmastigont-associated nuclei of *Snyderella* or in the karyomastigonts of all other genera, but the nuclei can then be segregated asymmetrically, so that the two offspring cells do not have the same number of nuclei. For example, a cell with 100 nuclei can divide into two cells of 70 and 30 nuclei (Dolan et al. 2000a, b). It is often difficult to resolve the chromosomes in these mitoses. Among all the trichomonad species described, only a few karyotypes have been reported (Zubáčová et al. 2008).

Most parabasalian species are assumed to reproduce only asexually. The sexual cycles of many *Cryptocercus* parabasalians have been studied extensively by Cleveland (1947) and involve whole cell fusion of haploid gametes. In *Trichonympha* the male gamete enters the female gamete from the posterior end and is fully absorbed. The male's organelles disintegrate, and the two nuclei fuse. The cell then undergoes meiosis. This sex cycle is triggered by ecdysone and the molting of the insect and does not occur otherwise. For a critical view of Cleveland's work, see Raikov (1995). Though not observed by other workers or in parabasalians outside the *Cryptocercus* hindgut, the presence of a sexual cycle in parabasalians is supported by genes for meiotic machinery in the genome of *Trichomonas vaginalis* (Malik et al. 2008).

Cysts

While many intestinal symbiotic protists are propagated between hosts by a cyst stage, few of the parabasalian gut flagellates do this. Certain hypermastigotes are the main exceptions to this rule, for example, *Staurojoenina* from *Neotermes* and *Macrospironympha* from the wood roach *Cryptocercus* (Cleveland et al. 1934; Dolan et al. 2004). *Trichonympha* from *Cryptocercus* encyst when their host molts. While encysted, the cells divide such that two daughter cells are released upon excystment (Cleveland et al. 1934). The cockroach symbiont *Lophomonas* also forms cysts in which one or more nuclear divisions take place (Kudo 1926a, b). Among non-termite gut parabasalians, true cysts have been observed from *Honigbergiella ruminantium* (Fig. 5h, i), *Trichomitus batrachorum*, *Trichomitus sanguisugae*, *Monocercomonas tipulae*, *Ditrichomonas honigbergii*, and possibly *Dientamoeba fragilis* (Brugerolle 1973; Farmer 1993; Hampl et al. 2007; Clark et al. 2014).

In other parabasalians, the rounded, resistant, resting form is called a pseudocyst because it lacks a cell wall (Pereira-Neves et al. 2003). Pseudocysts are particularly well characterized in *Trichomonas vaginalis* and *Tritrichomonas foetus* (Pereira-Neves et al. 2003; Pereira-Neves and Benchimol 2009). *Barbulanympha* forms pseudocysts upon molting of its host, *Cryptocercus* (Cleveland et al. 1934).

Prokaryotic Symbionts

Many of the parabasalian symbionts in the guts of termites and *Cryptocercus* are themselves host to a wide diversity of ecto- and endosymbiotic bacteria (Fig. 6a, b). A single host can harbor multiple types of bacterial symbionts that occupy distinct regions of the host cell (Sato et al. 2009; Strassert et al. 2010). While this has been known since early descriptions of the flagellates and was often incorporated into their name, e.g., *Devescovina striata* (Dolan 2001), only the development of molecular phylogenetic techniques has allowed researchers to place them into new and

existing bacterial phyla and study their metabolic interactions. Many of these bacterial symbionts are from the *Bacteroidales*, the *Elusimicrobia* (formerly Termite Group 1), and the *Spirochaetales*. They have been found to fix nitrogen, produce acetate, and serve as motility symbionts (Tamm 1982; Ohkuma et al. 2015). Just as many of the termite gut symbiotic parabasalians remain to be described, so are there many cases of bacterial symbioses of flagellates that need investigation. There is considerable evidence that these bacterial symbionts are specific to their host flagellates and that the two groups of organisms have coevolved within the termite's gut (Noda et al. 2007; Desai et al. 2010; Strassert et al. 2010).

Many of the cases of ectosymbiotic bacteria are from the order *Bacteroidales*. These include the ectosymbionts of the cristamonads *Joenia annectens* and *Devescovina* spp. and the *Cryptocercus* trichonymphids *Barbulanympha* and *Urinympha*, which are all likely nitrogen fixers (Noda et al. 2006; Strassert et al. 2010; Desai and Brune 2012; Tai et al. 2016). The order *Bacteroidales* is well represented on the termite's gut wall (Nakajima et al. 2006). It has been hypothesized that these ectosymbionts may consume small amounts of oxygen, in effect protecting the anaerobic host (Noda et al. 2006). A nitrogen-fixing *Bacteroidales* endosymbiont has been found in *Pseudotrichonympha grassii* from *Coptotermes formosanus* (Hongoh et al. 2008).

Spirochetes can be either ecto- or endosymbionts of parabasalians in the termite hindgut, or they may be free in the gut fluid (Ohkuma 2008). The cristamonad *Mixotricha paradoxa* from *Mastotermes darwiniensis* is an example of motility symbiosis with hundreds of spirochetes arrayed across the parabasalian cell surface, propelling the huge 500 micron-long flagellate through the gut (Cleveland and Grimstone 1964). Molecular phylogenetic work has found three species of *Treponema* spirochetes occupying distinct regions of the *M. paradoxa* surface, each associated with a *Bacteroides*-related rod-shaped bacterium (Wenzel et al. 2003; König et al. 2005). Three distinct spirochetes were also described from *Spirotrichonympha leidyi* in *Coptotermes formosanus* (Inoue et al. 2008). Acetogenesis and nitrogen fixation were confirmed from the complete genome of an unusual spirochete endosymbiont of *Eucomonympha* from the termite *Hodotermopsis sjoestedti*. In this case the spirochete is a short rod, devoid of its normal periplasmic flagella (Ohkuma et al. 2015).

Recent studies have found distinct termite gut lineages of several bacterial phyla associated with parabasalian flagellates, including the *Synergistes*, *Verrucomicrobia*, and *Elusimicrobia*. The motility symbionts on *Caduceia versatilis*, first reported by Tamm (1982), have been identified as affiliated with the *Synergistes* and named "*Candidatus* Tammella caduceiae" (Hongoh et al. 2007). The endonuclear organisms seen in *Trichonympha agilis* have been found to be *Verrucomicrobia* and named "*Candidatus* Nucleococcus spp." (Sato et al. 2014). The symbionts originally affiliated with Termite Group 1 have been named the *Elusimicrobia* and include the group Endomicrobia, which have been found widely in *Trichonympha* both from termites and *Cryptocercus* (Geissinger et al. 2009; Ikeda-Ohtsubo and Brune 2009).

Taxonomy

The taxonomic system of Parabasalia adopted here (outlined at the end of this section) consists of six classes, eight orders, and 17 families and corresponds as closely as possible to the current consensus of molecular phylogenetic analyses while remaining consistent with morphological data. However, molecular phylogenies are not resolved in all areas of the tree, and some parabasalians have not yet been included. Names may yet change as new data become available, as they have many times in the last century. In order to allow readers to understand the meaning of taxon names at different time points in the literature on parabasalians, a brief historical account of parabasalian taxonomy follows.

Traditionally, the Parabasalia was divided into two orders, Trichomonadida and Hypermastigida, according to the number of flagella per mastigont and cell complexity (e.g., Levine et al. 1980). Trichomonadida included the simpler forms along with polymonad ones (those whose nuclei and flagella were multiplied together) (Honigberg 1963; Pecka et al. 1996). Hypermastigida was divided into three suborders according to the arrangement of flagella. Lophomonadina had flagella arranged in a tuft at the cell apex, Trichonymphina had many flagella arranged along a bilaterally symmetrical rostrum, and Spirotrichonymphina had flagella arranged in spiral rows (Grassi and Foà 1911; Hollande and Carruette-Valentin 1971). Although early phylogenetic trees already showed that this classification system did not correspond to the actual phylogeny of Parabasalia because of a paraphyletic Trichomonadida and extensively polyphyletic Hypermastigida (see above), this taxonomy remained unrevised until the twenty-first century. Brugerolle and Patterson (2001) were the first to formally recognize the polyphyletic nature of hypermastigids in a taxonomic system and united certain genera of trichomonads and the whole Lophomonadina within a new order, Cristamonadida (Brugerolle and Patterson 2001).

Čepička et al. (2010) revised the higher taxonomy of Parabasalia in order to bring it in line with the contemporary results of molecular phylogenetic studies. They divided Parabasalia into six classes, Trichomonadea, Tritrichomonadea, Hypotrichomonadea, Cristamonadea, Trichonymphea, and Spirotrichonymphea. Most classes include a single order, but Trichomonadea was further divided into two orders, Trichomonadida and Honigbergiellida. The first three classes contain only trichomonads, whereas all members of Trichonymphea and Spirotrichonymphea are hypermastigotes; Cristamonadea and Honigbergiellida contain both trichomonads and hypermastigotes (though only a single hypermastigid species, *Cthulhu macrofasciculumque*, belongs to the latter). The system of six classes was adopted in the recently revised classification of eukaryotes (Adl et al. 2012).

Establishing an internal taxonomy for Cristamonadea has proven problematic. Molecular phylogenies have been unable to resolve the internal relationships. Similarly, while morphological and ultrastructural features are able to circumscribe

individual genera, few characters have been identified to group genera into families. Finally, some of the traditional families have proven non-monophyletic. For these reasons, all genera of Cristamonadea were lumped into a single family, Lophomonadidae (Čepička et al. 2010). However, it was later shown that the genus *Lophomonas* is not related to the rest of Cristamonadea, but instead forms a sister lineage to Trichonymphea (Gile and Slamovits 2012), making Lophomonadidae and Cristamonadea sensu Čepička et al. (2010) polyphyletic.

The problem of the polyphyly of Cristamonadea was addressed in a recent system by Cavalier-Smith (2013). He divided Parabasalia (which he treated as a superclass) into two classes, Trichomonadea and Trichonymphea. Trichomonadea was further divided into subclasses Eotrichomonadea (order Trichomonadida with suborders Trichomonadina and Honigbergiellina and order Tritrichomonadina) and Cristamonadea (orders Cristamonadida and Spirotrichonymphida). Trichonymphea was divided into orders Trichonymphida and Lophomonadida; the latter consisted of the genus *Lophomonas*. However, according to the current understating of the evolution of the phylogeny of Parabasalia, several taxa of this system are paraphyletic or polyphyletic (Cavalier-Smith 2013).

Here, we mostly follow the system of Čepička et al. (2010) and divide Parabasalia into six classes: Trichomonadea, Tritrichomonadea, Hypotrichomonadea, Cristamonadidea, Spirotrichonymphea, and Trichonymphea. We also recognize the order Lophomonadida sensu Cavalier-Smith (2013) (within Trichonymphea) as well as the family Lophomonadidae containing *Lophomonas* and Joeniidae as the only family of Cristamonadida comprising all genera contained in Lophomonadidae sensu Čepička et al. 2010 except *Lophomonas* itself. The detailed taxonomy used here is as follows:

Class Trichomonadea
- Order Trichomonadida
  - Family Trichomonadidae (*Cochlosoma*, *Lacusteria*, *Pentatrichomonas*, *Pentatrichomonoides*, *Pseudotrichomonas*, *Pseudotrypanosoma*, *Tetratrichomonas*, *Trichomitopsis*, *Trichomonas*, *Trichomonoides*)
- Order Honigbergiellida
  - Family Honigbergiellidae (*Ditrichomonas*, *Honigbergiella*, *Monotrichomonas*)
  - Family Hexamastigidae (*Hexamastix*, *Tetratrichomastix*)
  - Family Tricercomitidae (*Tricercomitus*)
  - Unplaced genera *Cthulhu* and *Cthylla*

Class Tritrichomonadea
- Order Tritrichomonadidae
  - Family Tritrichomonadidae (*Tritrichomonas*)
  - Family Dientamoebidae (*Dientamoeba*, *Histomonas*, *Parahistomonas*, *Protrichomonas*)
  - Family Monocercomonadidae (*Monocercomonas*)
  - Family Simplicimonadidae (*Simplicimonas*)

Class Hypotrichomonadea
  Order Hypotrichomonadida
    Family Hypotrichomonadidae (*Hypotrichomonas*, *Trichomitus*)

Class Cristamonadea
  Order Cristamonadida
    Family Joeniidae (*Achemon*, *Astronympha*, *Bullanympha*, *Caduceia*, *Calonympha*, *Coronympha*, *Criconympha*, *Cyclojoenia*, *Deltotrichonympha*, *Devescovina*, *Diplonympha*, *Evemonia*, *Foaina*, *Gigantomonas*, *Gyronympha*, *Hyperdevescovina*, *Joenia*, *Joenina*, *Joenoides*, *Joenopsis*, *Kirbyina*, *Kofoidia*, *Koruga*, *Macrotrichomonas*, *Macrotrichomonoides*, *Metadevescovina*, *Mixotricha*, *Pachyjoenia*, *Parajoenia*, *Parajoenopsis*, *Placojoenia*, *Polymastigotoides*, *Projoenia*, *Prosnyderella*, *Pseudodevescovina*, *Rhizonympha*, *Snyderella*, *Stephanonympha*)

Class Spirotrichonymphea
  Order Spirotrichonymphida
    Family Holomastigotoididae (*Holomastigotes*, *Holomastigotoides*, *Microjoenia*, *Micromastigotes*, *Rostronympha*, *Spiromastigotes*, *Spironympha*, *Spirotrichonympha*, *Spirotrichonymphella*, *Uteronympha*)

Class Trichonymphea
  Order Trichonymphida
    Family Trichonymphidae (*Trichonympha*)
    Family Hoplonymphidae (*Barbulanympha*, *Hoplonympha*, *Rhynchonympha*, *Urinympha*)
    Family Staurojoeninidae (*Idionympha*, *Staurojoenina*)
    Family Teranymphidae (*Eucomonympha*, *Pseudotrichonympha*, *Teranympha*)
    Family Spirotrichosomidae (*Apospironympha*, *Bispironympha*, *Colospironympha*, *Leptospironympha*, *Macrospironympha*, *Spirotrichosoma*)

Order Lophomonadida
  Family Lophomonadidae (*Lophomonas*)

Parabasalian genera *Incertae sedis*
  Trichocovina (Trichomonadida or Tritrichomonadida or Cristamonadida)
  Prolophomonas (Lophomonadida or Cristamonadida)
  Eulophomonas (Lophomonadida or Cristamonadida)
  Chilomitus (formerly Monocercomonadidae)

Maintenance and Cultivation

Many trichomonad species from vertebrates (including most species from humans) and some species from non-termite insects can be maintained relatively easily in polyxenic cultures with bacteria, using various media such as Dobell and Laidlaw's biphasic medium (Dobell and Laidlaw 1926) or liquid medium TYSGM-9 (Diamond 1982). Trichomonads from mammals and birds are maintained at 42 °C and are subcultured approximately every third day; those isolated from poikilotherms and insects are maintained at room temperature and are subcultured approximately once a week (Čepička et al. 2006). Free-living trichomonads such as *Pseudotrichomonas keilini*, *Tetratrichomonas undula*, or *Ditrichomonas honigbergii* were also cultured in Dobell and Laidlaw's biphasic medium or TYSGM-9 (Farmer 1993; Čepička et al. 2006; Yubuki et al. 2010), though various media used for free-living protists such as Sonneborn's *Paramecium* medium (ATCC medium 802), its 9:1 mixture with TYSGM-9, or 5% PYNFH medium (ATCC medium 1034) have been used as well (Yubuki et al. 2010).

Several species from vertebrates, such as *Trichomonas vaginalis*, *Pentatrichomonas hominis*, *Tritrichomonas foetus*, *Trichomitus batrachorum*, *Hypotrichomonas acosta*, *Monocercomonas colubrorum*, and *Simplicimonas moskowitzi* can be cultured axenically in the TYM medium. The pH of the medium is usually adjusted to 7.2; for *Trichomonas vaginalis*, the optimum pH is 6.2.

Most cultured species can be easily cryopreserved. Cells at early-stationary or late-log growth phase are supplemented by DMSO to a final concentration of 5%. The suspension is then cooled at 6 to 8 °C per minute to the point of release of the latent heat of fusion. Then, the suspension is subjected to rapid cooling to take the organisms past the latent heat of fusion zone within 1.5 minutes. Then, the suspension is cooled at the rate of 1 to 2 °C per minute to −60 °C, and then it is immersed in liquid nitrogen (Honigberg and Burgess 1994).

As obligate anaerobic symbionts, often with bacterial symbionts of their own, the parabasalians of the termite gut have proven difficult to culture. Only a few termite gut parabasalians, such as *Trichomitopsis termopsidis* (Cleveland) from *Zootermopsis angusticollis*, have been brought into axenic culture (Yamin 1978; Odelson and Breznak 1985). None has been cultured on a defined medium. In brief, a buffered salt solution (pH 6.9) is used: K_2HPO_4, 10.8 mM; KH_2PO_4, 6.9 mM; KCl, 21.5 mM; NaCl, 24.5 mM; $MgSO_4$, 5.2 mM; and $CaCl_2$, 0.53 mM. To this solution is added 0.1% (w/v) cellulose particles small enough for the cells to ingest. This salt solution supplemented with cellulose is boiled and then cooled, while bubbling with O_2-free N_2. The solution is poured into tubes and sealed under N_2 with rubber stoppers and autoclaved. N_2-flushed plastic syringes are used to add the following after autoclaving: $NaHCO_3$ to 10 mM and heat-inactivated fetal calf serum to 2.5% (v/v). After surface-sterilizing the termite with 70% ethanol, the hindgut is removed by forceps and broken open with a syringe plunger tip. The plunger is inserted in the syringe, which is flushed with N_2. The syringe is used to draw up medium from the tube and then to plunge the medium, with protist cells, back into the tube. Cultures are incubated at 27 °C with subcultures made every 2–4 weeks (Yamin 1978).

Evolutionary History

External Relationships

Parabasalia belongs to the Metamonada clade within the Excavata supergroup, a supergroup whose members are characterized in part by a feeding groove (Simpson 2003). Parabasalia is one of the three major subclades of metamonads, along with Preaxostyla (comprising Oxymonada and trimastigids) and Fornicata (comprising Diplomonadida, Retortamonadida, and *Carpediemonas*-like organisms) (Simpson 2003; Adl et al. 2012; Zhang et al. 2015). Though the Parabasalia and Oxymonada have lost the ancestral excavate-type feeding groove, molecular phylogenetic evidence links them to their more plesiomorphic relatives in Fornicata and the trimastigids. Multigene phylogenies suggest that Fornicata is the sister group of Parabasalia, while Preaxostyla is the deepest branch in the clade (Hampl et al. 2005, 2009; Katz and Grant 2015).

Metamonads are mostly anaerobic gut commensals. The most recent ancestor of Parabasalia was probably a gut commensal, and the free-living species are secondarily adapted to life outside an animal host. However, the free-living species have not yet been included in rooted, multigene analyses, so the possibility that they might form the deepest branches cannot be completely excluded (Hampl et al. 2007; Noda et al. 2012). The other two metamonad lineages have deep-branching, free-living representatives, e.g., *Trimastix* in Preaxostyla and *Carpediemonas* in Fornicata (Kolisko et al. 2008; Zhang et al. 2015), so it is likely that Metamonada as a whole is ancestrally free-living.

Parabasalia is likely a relatively young phylum. Parabasalia certainly predates the origin of termites, which has been dated back to the Jurassic/Cretaceous boundary, roughly 150 million years ago (Misof et al. 2014; Bourguignon et al. 2015). This provides a minimum age for the group. There are no sound estimates to provide a maximum age: molecular clock age estimates have not yet been applied to the Parabasalia specifically, and such age estimates for Excavata are highly method sensitive and range between 900 million and 1.8 billion years (Parfrey et al. 2011; Eme et al. 2014). Parabasalians attributable to the orders Trichonymphida, Cristamonadida, and Spirotrichonymphida have been described from 100-million-year-old early Cretaceous amber (Poinar 2009).

Internal Relationships

Inferences of character evolution in Parabasalia depend largely on the position of the root for the clade, an inference that eluded molecular phylogenetic analyses for years (Hampl et al. 2004). Outgroup rooting with SSU rDNA or protein sequences failed to find a supported position for the root but tended to place the root near or within the Trichonymphida (Keeling et al. 1998; Ohkuma et al. 2000, 2007a). This position is clearly artifactual, deriving from the attraction of the long stem branch of Parabasalia to the long branches of the Trichonymphida (Keeling and Palmer 2000; Hampl et al.

2004). Also, the Trichonymphida rooting contradicts morphology-based scenarios in which the simpler, smaller cells are considered most similar to the ancestral form (Kirby 1947; Honigberg 1963; Brugerolle 1976). With the addition of elongation factor 1-alpha sequences to multi-protein analyses, a different root position was inferred, between the clade of Trichomonadida and Trichonymphida on one hand and Hypotrichomonadida, Spirotrichomonadida, Tritrichomonadida, and Cristamonadida on the other (see Fig. 8). Honigbergiellida, Lophomonadida, and free-living members of Trichomonadida were not included in the analysis (Noda et al. 2012). This root position results in simpler parabasalians forming the deeper branches and the complex hypermastigotes arising later, a more intuitively plausible scenario (Fig. 8).

The ancestral morphology of parabasalians under this rooting was therefore likely similar to *Trichomitus* and *Hypotrichomonas* (Hypotrichomonadida): small cells with four flagella, a costa, and a lamelliform undulating membrane (Čepička et al. 2010). Variations on this body plan have taken different directions among the simpler parabasalians. The undulating membrane has been lost several times, for example, in *Honigbergiella*, *Simplicimonas*, *Monocercomonas*, and Dientamoebidae, and altered to a rail type in Tritrichomonadida (Brugerolle 1976; Čepička et al. 2010). Flagellar number is particularly changeable among trichomonads and has increased to five or six in the Trichomonadida and Honigbergiellida and reduced to three or two in certain Honigbergiellida and to zero in *Dientamoeba*, with an anomalous increase to at least 20 flagella in *Cthulhu* (James et al. 2013).

It is in the hypermastigote taxa that the most impressive morphologies have evolved. Though traditionally united on the basis of many flagella but just one nucleus, molecular phylogenetic analyses have demonstrated the polyphyly of hypermastigotes. While trichonymphids and spirotrichonymphids are each monophyletic groups, their complex multiflagellate morphologies evolved independently of each other. Lophomonads, on the other hand, are actually polyphyletic. *Lophomonas* forms the sister lineage to trichonymphids (Gile and Slamovits 2012); *Kofoidia* is closely related to the cristamonad genera *Devescovina* and *Metadevescovina*, which have trichomonad cell organization (Tai et al. 2014); and the rest, genera such as *Joenia*, *Joenina*, *Joenoides*, and *Deltotrichonympha*, branch separately near the base of the Cristamonadida (Ikeda-Ohtsubo et al. 2007; Noda et al. 2009).

The evolutionary tendency to multiply flagella is restricted to parabasalians that live in the termite or roach hindgut. It is difficult to count the number of distinct flagellar multiplication events in Parabasalia because relationships among cristamonads are not resolved, but within the boundaries of this uncertainty, there must have been at least five and possibly more than seven distinct instances (not including cases of nuclear multiplication). This number includes the recently described genus *Cthulhu*, which bears at least 20 flagella and branches with *Hexamastix* and *Cthylla* in the Honigbergiellida (James et al. 2013). The termite/roach hindgut environment also appears to favor evolutionary increases in cell size, as, for

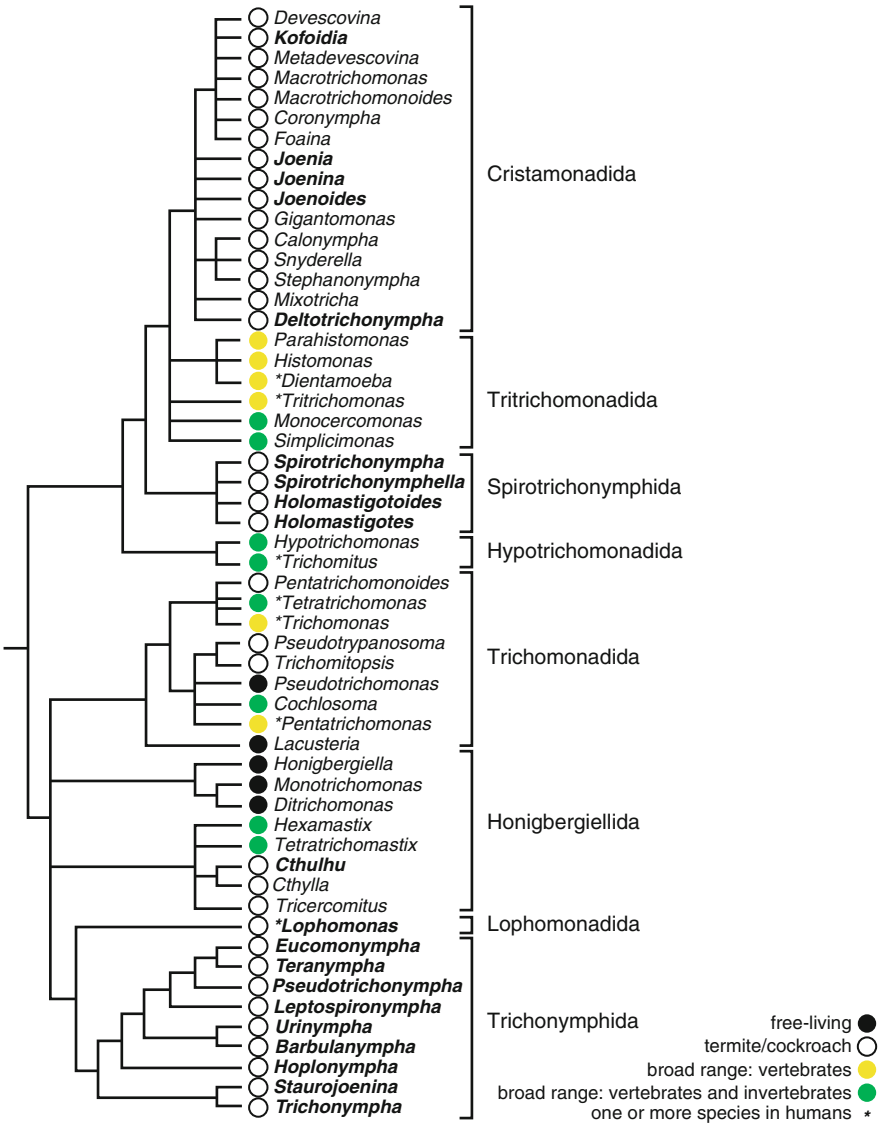

Fig. 8 Schematic phylogenetic tree of Parabasalia based on multiple molecular phylogenetic analyses (see text for details). The eight orders according to this scheme, based on Čepička et al. (2010) and Cavalier-Smith (2013), are indicated to the *right*. *Circles* at tips indicate habitat: genera with *open circles* are exclusive to termite and/or cockroach hindguts. *Black circles* indicate genera with free-living species. *Colors* indicate a broad host range of described species, *yellow* for vertebrates and *green* for vertebrates and invertebrates. *Asterisks* indicate genera in which one or more species has been found in humans. *Bold type* indicates "hypermastigote" genera, i.e., parabasalians with many flagella

example, in the large (50–100 μm long) trichomonad *Trichomitopsis termopsidis* (Keeling 2002) and in cristamonads such as *Devescovina* and *Macrotrichomonas* that have only four flagella despite reaching lengths of 80–90 μm (Brugerolle and Lee 2000; Gile et al. 2015). Meanwhile, parabasalians that are not restricted to the roach or termite hindgut tend to remain small (under 30 μm, usually under 20 μm) and retain six or fewer flagella (Brugerolle and Lee 2000).

Intergeneric relationships in the Parabasalia are resolved to differing degrees in different parts of the tree. The schematic representation of these relationships (Fig. 8) is a synthesis of results from multiple phylogenetic analyses: some using protein-coding sequences (Gerbod et al. 2004; Ohkuma et al. 2007; Čepička et al. 2010; Noda et al. 2012), but most using SSU rDNA (Gerbod et al. 2002; Hampl et al. 2004; Hampl et al. 2006; Noël et al. 2007; Noda et al. 2009; Carpenter et al. 2010; Čepička et al. 2010; Yubuki et al. 2010; Gile et al. 2011; Gile and Slamovits 2012; Tai et al. 2014; Gile et al. 2015). Most genera with at least some molecular data are included in the figure, but many important genera have yet to be included in molecular phylogenetic analyses and are not represented. Some evolutionary trends in Parabasalia are also indicated in Fig. 8. Multiplications of flagella (hypermastigote genera) are indicated by bold text. Termite and cockroach gut residents are indicated by open circles. Note that the orders Cristamonadida, Spirotrichonymphida, and Trichonymphida have radiated entirely within this habitat (Lophomonadida are from cockroaches but not termites). Many trichomonad genera have broad host ranges, with species found across vertebrates (yellow circles) or across vertebrates and invertebrates, in some cases including the termite/roach hindgut (green circles). The few free-living species belong to genera indicated by black filled circles.

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Diplomonadida

33

Rodney D. Adam

Abstract

The diplomonads ("two units") are characterized by their possession of two nuclei that are similar in appearance, replication, and function. Together with the *Carpediemonas*-like organisms and retortamonads, the diplomonads are classified within Fornicata. Each "unit" of the diplomonad cell includes a karyomastigont that has one nucleus and (usually) four flagella, which are used for locomotion. Thus, most diplomonads have two karyomastigonts. However, the "enteromonads" present an exception in that they have a single karyomastigont per cell. The diplomonads have anaerobic metabolism and lack conventional mitochondria, so they were thought to be pre-mitochondriate organisms. However, they have subsequently been shown to have highly reduced mitochondria called mitochondrion-related organelles (MRO) that perform some of the functions of conventional mitochondria. The most studied diplomonads are the *Giardia* species, which are intestinal pathogens or commensals for a variety of vertebrates from amphibians to mammals and include pathogens of humans. Like *Giardia* spp., the *Spironucleus* species also replicate in the host intestine, in this case in vertebrates or invertebrates and include notable fish pathogens. In contrast, *Hexamita* and *Trepomonas* species can be either free-living or parasitic.

Keywords

Mastigont • Binucleate • Mitosome • Mitochondrion-like organelle (MRO) • Anaerobic • *Hexamita* • *Spironucleus* • *Trepomonas* • *Enteromonas* • *Trimitus* • *Trigonomonas* • *Gyromonas* • *Giardia* • *Octomitus* • *Brugerolleia*

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Introduction

The diplomonads are small anaerobic flagellates originally defined by the remarkable binary axial symmetry of the cell body, with each of its two "karyomastigont" systems facing outward from the central axis. Each karyomastigont system has a single nucleus with four basal bodies (kinetosomes); typically, a flagellum emerges from each, so there are four flagella per karyomastigont. One flagellum is recurrent and associated with the cytostome or forms the intracellular axis of the cell. In addition to the genera with two karyomastigonts, some unizoic forms (*Enteromonas* and *Trimitus* spp.) have been identified as part of the diplomonad assemblage by morphological and molecular criteria (Kolisko et al. 2008). These unizoic organisms lack the twofold symmetry, having only a single karyomastigont.

The first published description of a diplomonad was by Antony van Leeuwenhoek in 1681, when he gave a recognizable description of *Giardia lamblia* (syn. *Giardia duodenalis*, *Giardia intestinalis*) from his own diarrheic stools, making it one of the first protists on record (Dobell 1920). Lambl (1859) gave a formal

description of the human *Giardia*'s size, shape, and sucking disk; (Grassi 1881) added an account of the flagella and nuclei in the trophozoite and described the cyst. The most commonly observed genera of free-living diplomonads, *Trepomonas* and *Hexamita*, were described by Dujardin in 1841 (Dujardin 1841).

Diplomonads are now placed phylogenetically within a large clade of anaerobes called Metamonada and specifically within the subgroup Fornicata (Cavalier-Smith 2013; Simpson 2003). A recent classification system considered Fornicata as a superclass comprising the classes Carpediemonadida and Eopharyngia. In turn, the Eopharyngia are made up of the orders Diplomonadida and Retortamonadida (Cavalier-Smith 2013). Alternatively, a molecular evaluation of multiple *Carpediemonas*-like organisms (CLOs) placed the CLOs into several clades within Fornicata and did not use Eopharyngia as part of the classification system (Kolisko et al. 2010). The evolutionary relationships within Fornicata are a complex topic and are discussed in depth in another chapter (▶ Retortamonads).

The diplomonads provide a remarkable model system for testing some of the current ideas regarding parasite biology and evolution, since the taxon includes free-living, commensal, and pathogenic organisms. Those that are parasitic infect a wide variety of invertebrates and vertebrates. The obligate parasite *Giardia* has relatively little biosynthetic function. Which of those synthetic functions are maintained by free-living or other parasitic diplomonads?

Habitats and Ecology

Most diplomonads are parasitic, surviving in the alimentary canals of their hosts, and some cause diseases in their host organisms. However, *Trepomonas* species are free-living (Xu et al. 2016) and *Hexamita* species may be free-living or parasitic (Xu et al. 2016). For example, *Hexamita inflata* is a free-living organism, but other *Hexamit*a species are parasitic to a variety of invertebrate and vertebrate hosts (Kulda and Nohynkova 1978). *Hexamita parva* may cause extensive intestinal disease in infected turtles and tortoises and may invade the kidney and bladder as well as other organs (Zwart and Truyens 1975). *Hexamita nelsoni* infects oysters and has been found in the stomach and pericardium, but not necessarily associated with a high mortality (Scheltema 1962).

Spironucleus species are primarily intestinal organisms and are parasitic in a wide variety of vertebrates, from fish to birds and mammals. They are recognized as significant pathogens for salmon and other fish. *Spironucleus salmonicida* has also been isolated from muscle abscesses of salmon from fish farms in Norway and from "systemically" infected fish, suggesting the ability of these parasites to cause invasive infection (Fard et al. 2007; Jorgensen and Sterud 2006; Sterud 1998). Increased mortality has been associated with heavy infection of trout by *Spironucleus salmonis*, accompanied by hepatocellular necrosis and abdominal ascites (Poynton et al. 2004). *Enteromonas* species are intestinal parasites and

Enteromonas hominis infects humans, possibly causing disease on occasion (Spriegel et al. 1989). *Brugerolleia algonquinensis* has been identified in the blood of a frog (Desser et al. 1993).

Giardia lamblia, the most common human parasite, causes infection of the small intestine that may be symptomatic or asymptomatic. When symptomatic, people have subacute diarrhea that is commonly accompanied by nutrient malabsorption and weight loss (Ortega and Adam 1997). Infection results when the environmentally stable cyst is ingested and, after passing through the stomach, excysts as the vegetative trophozoite in the proximal small intestine. Some of the organisms then encyst in the small intestine and are passed in the feces to continue the cycle of transmission. Trophozoites attach to the lumen of the small intestine via their ventral disks (see Fig. 4 for EM of the ventral disk) by a mechanism that is probably mechanical (Elmendorf et al. 2003). There is no known receptor-mediated attachment and no intracellular or intraluminal invasion. There is some villous disruption during infection as well as observable lymphocyte infiltration in the *lamina propria*, but whether these or other phenomena are responsible for the diarrhea is not yet known. The secretory IgA response has been proposed as the major means of eradicating the infection and is supported by the observation of refractory diarrhea due to *Giardia* in patients with X-linked hypogammaglobulinemia. However, more recent studies using animal models suggest that that cell-mediated immunity may also be important in eradication of infection (Singer and Nash 2000). Diagnosis of human infection is documented by the identification of cysts or trophozoites in fecal specimens, trophozoites in small intestinal contents, or *Giardia* antigen (by ELISA or DFA) in fecal specimens. Zoonotic transmission to humans has been controversial in part because of the confusing results obtained with cross-transmission studies. However, "human" genotypes have been found in beavers, and beavers have been implicated as the source for a number of human infections. In contrast, dogs, cats, and livestock generally have different genotypes and are unlikely to be important sources of human infection.

Characterization and Recognition

General Morphology and Classification

The diplomonads typically have bilateral axial symmetry, with each half of the trophozoite having a karyomastigont consisting of a nucleus and four basal bodies. Typically, a flagellum emerges from each basal body (exceptions to this general rule are described below). One flagellum is recurrent and associated with the cytostome (in the Hexamitidae) or forms the intracellular axis of the cell. The unizoic forms (*Enteromonas* and *Trimitus* spp.) lack the twofold symmetry, having only a single

karyomastigont. At an ultrastructural level, the diplomonads lack some of the canonical eukaryotic organelles, including classical mitochondria (though highly modified forms remain – see below), perixosomes, and a well-organized Golgi (Desser et al. 1993; Poynton and Sterud 2002).

The diplomonads have been classified by using a combination of morphological and molecular characteristics. Although the names of the genera have been constant, the classification systems have evolved far more quickly than the organisms being described, including names and rankings of the groups. However, all recent classification systems place the diplomonads into two monophyletic groups; Hexamitinae (or Distomatina (Cavalier-Smith 2013)) and Giardiinae. The genera can be distinguished by the pattern of flagellar organization and/or the shape of the nuclei and by electron microscopic features (Table 1).

Among the Heximatidae, the *Hexamita* species have round nuclei with the basal bodies located on the external surface of the nucleus (Fig. 1). Each of the flagella in a karyomastigont has a different orientation and role, so the flagella in a cell can be considered to form four functional "pairs," with each pair consisting of one flagellum from each karyomastigont. Three pairs of flagella emerge anteriorly, while the other pair is recurrent, running lateral to the nucleus. The recurrent flagella each run through a cytostomal canal or flagellar pocket. These pockets allow nutrients to pass for internalization. The nuclei of the *Spironucleus* species are spiral-shaped and subapical in location (Fig. 2). The recurrent flagella run medial to the nucleus along a flagellar pocket. Despite this distinctive architecture, phylogenies of the *Spironucleus* species based on SSU rDNA sequences place them into three different clades that differ as much from each other as from *Hexamita* spp. (Jorgensen and Sterud 2007). Even two *Spironucleus* species (*Spironucleus barkhanus* and *S. salmonicida*) that are in the same clade and are morphologically identical nonetheless demonstrate substantial differences at a genomic level (Andersson et al. 2007; Roxstrom-Lindquist et al. 2010).

The cell body of *Trepomonas agilis* (the most commonly reported member of the genus by far) is oval- to pear-shaped and has two oral grooves, one on each side (Eyden and Vickerman 1975). The cells have elongated nuclei that each has an apex posteriorly where the basal bodies are located. The organisms are notable for food vacuoles that rapidly move phagocytosed bacteria and other nutrients within the trophozoite. *Trigonomonas* cells are triangular- or spindle-shaped and 8–30 μm long, with the posterior end of the cell flattened. Only three flagella are inserted at the base of each nucleus; one flagellum is longer than others and used for locomotion. Cytostomal grooves extend from the flagella emergence to the posterior of the body. The organism rotates around its axis while swimming (as in *Trepomonas*). The cells of the rarely recorded genus *Gyromonas* are smaller, with subtle depressions on each side of the cell, rather than well-defined grooves. *Gyromonas* cells have just two flagella per karyomastigont.

The *Enteromonas* and *Trimitus* species differ from other diplomonads by their possession of only one karyomastigont and thus, one nucleus and up to four flagella (four in *Enteromonas*, three in *Trimitus*).

Table 1 Genera of Diplomonadida. Diplomonadida and Hexamitinae classified according to Brugerolle et al. (Brugerolle 1975), Giardinae according to Kulda and Nohynkova (1978); given suborder ranking in Cavalier-Smith (2013)

Suborder	Genus	Morphologic features	Ecology
Hexamitinae (~ Distomatida) (phagotrophs with two cytostomes, alternate genetic code)	*Enteromonas* (da Fonseca 1915)	Three free, one recurrent F, one karyomastigont, and one N	Entozoic (mammals including man), nonpathogenic
	Trimitus (Alexeieff 1910)	One karyomastigont with one N, two free, one recurrent F	Entozoic (poikilotherms)
	Trepomonas (Dujardin 1841)	Two large ant. pyriform N; two free locomotory F, three in each large lateral cytostome	Free-living or entozoic (poikilotherms), nonpathogenic
	Hexamita (Dujardin 1838)	Two spherical ant. N; six free locomotory F; recurrent F initially in intracellular channels opening at broad caudal cytostomes	Free-living or entozoic (invertebrates and vertebrates), some species pathogenic in fish, oysters, tortoises
	Gyromonas (Seligo 1886)	Small (6–10 μm) with two N and two pairs of F. Each side mostly occupied by two posterior and largely opened grooves	Free-living
	Trigonomonas (Klebs 1892)	Two N; three F at the base of each nucleus; one flagellum is longer than others and used for locomotion; cytostomal grooves extend from the flagella emergence to the posterior of the cell; moving contractile vacuole	Free living in freshwater
	Spironucleus (Lavier 1936)	Two "S"-shaped ant. N; six free locomotory F; recurrent F in narrow channels opening at small posterolateral cytostomes	Entozoic (vertebrates), frequently pathogenic (fish poultry, laboratory rodents)

(*continued*)

Table 1 (continued)

Suborder	Genus	Morphologic features	Ecology
Giardiinae (osmotrophs without cytostomes; ciliary roots between nuclei)	*Octomitus* (Prowazek 1964)	Two bean-shaped ant. N; six free locomotory F recurrent F initially intracytoplasmic; cytostomes absent	Entozoic (vertebrates), nonpathogenic
	Brugerolleia (Desser 1993)	Two spherical ant. N; three pairs of F emerging on side of N, one pair of F run parallel through cytoplasm to emerge posteriorly; rudimentary cytostome	Entozoic (frogs)
	Giardia (Kunstler 1882)	Two oval sub-ant. N; attachment to the host intestine by the ventral sucking disk; anterolateral F, posterolateral F, and caudal F with initially intracytoplasmic axonemes; ventral flagella beat in ventrocaudal groove; cytostomes absent	Entozoic (tetrapods including man), may be pathogenic

There remains good consensus regarding the placement of these nine genera within the Diplomonadida. There is also good agreement regarding the placement of *Giardia*, *Octomitus*, and *Brugerolleia* within a single clade and *Trepomonas*, *Hexamita*, and *Spironucleus* within another clade. The remainder of relationships and hierarchy within the diplomonads is less clear. Earlier classification systems place *Caviomonas* spp. within the diplomonads, but more recent data suggests that they are more accurately placed with the *Carpediemonas*-like organisms (Yubuki et al. 2016)
ant anterior, *F* flagellum, flagella, *N* nucleus, nuclei

The Giardiinae are characterized by the lack of a cytostome or cytostomal canal. The recurrent flagella run through the cytoplasm rather than through a flagellar pocket. Among the three genera, the ventral disk is unique to *Giardia* species. *Giardia* and *Octomitus* species lack a cytostome altogether, while *Brugerolleia algonquinensis* has rudimentary cytostomal homologues (Desser et al. 1993). The *Giardia* nuclei are round, while the *Octomitus* and *Brugerolleia* nuclei are kidney-shaped.

Giardia species were initially named on the basis of host of origin, but in subsequent seminal work by Filice (1952), three major morphological types were proposed: (i) *Giardia lamblia* (syn. *Giardia intestinalis* and *Giardia duodenalis*, the latter being the name used by Filice; of mammals and birds), (ii) *Giardia muris* (rodents), and (iii) *Giardia agilis* (amphibians). These are distinguishable from one another by light microscopy by the shape of the cell body, the relative length of the adhesive disk, and the shape and position of the median bodies. *Giardia agilis* has a

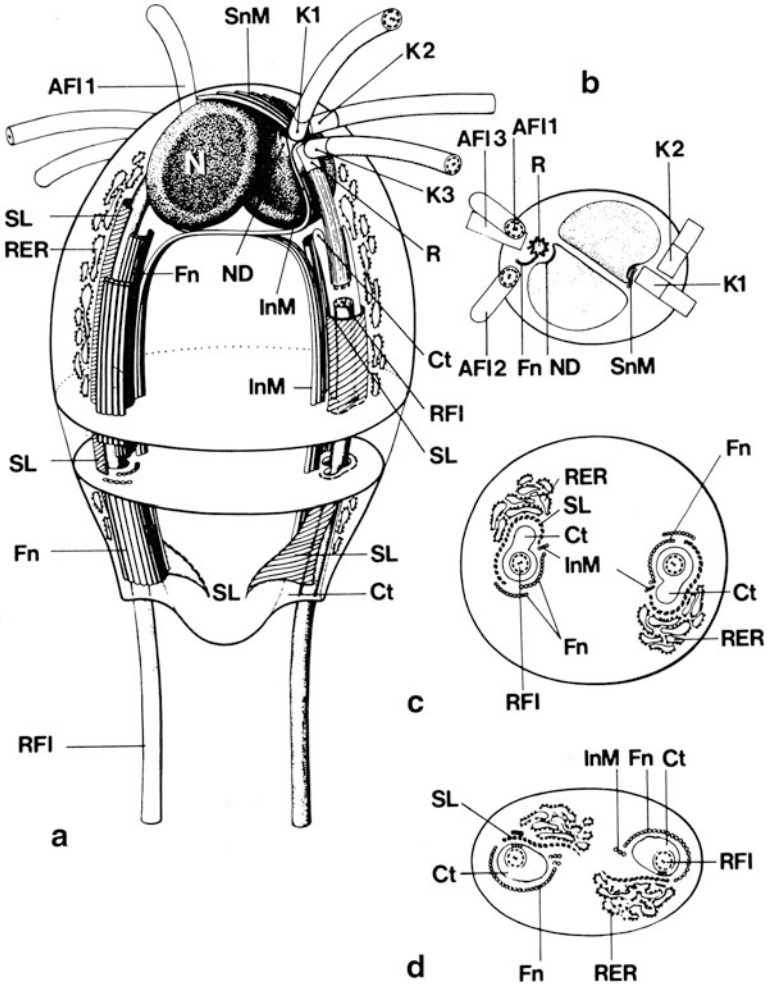

Fig. 1 Ultrastructure of *Hexamita.* (**a**) Diagrammatic reconstruction of trophozoite. (**b**) Slightly oblique, (**c**, **d**) transverse sections of anterior **b**, central **c**, and posterior **d** regions of the body to show axial binary symmetry. K1, K2, K3, and R – kinetosomes; AFl1, AFl2, and AFl3 – anterior flagella emerging anterolaterally. Recurrent flagella (*RFl*) protrude through cytostomal tubes (*Ct*) which are each surrounded by a supporting striated lamella (*SL*), the funis microtubule ribbon (*Fn*), the infranuclear microtubule ribbon (*InM*, originating at K1 on opposite side), and rough endoplasmic reticulum (*RER*). *SnM* supranuclear microtubule ribbon, *N* nucleus, *ND* nuclear depression housing kinetosomes (basal bodies). (**a**, Kulda and Nohynkova 1978; **b**–**d**, After Brugerolle 1974)

long narrow cell body, sucking disk approximately one-fifth of the body length, and a median body that is single, club-shaped, and parallel to the longitudinal axis of the cell. *Giardia muris* has a short broad pyriform cell body, sucking disk over half the cell length, and two small round median bodies. *Giardia lamblia* has a pyriform cell

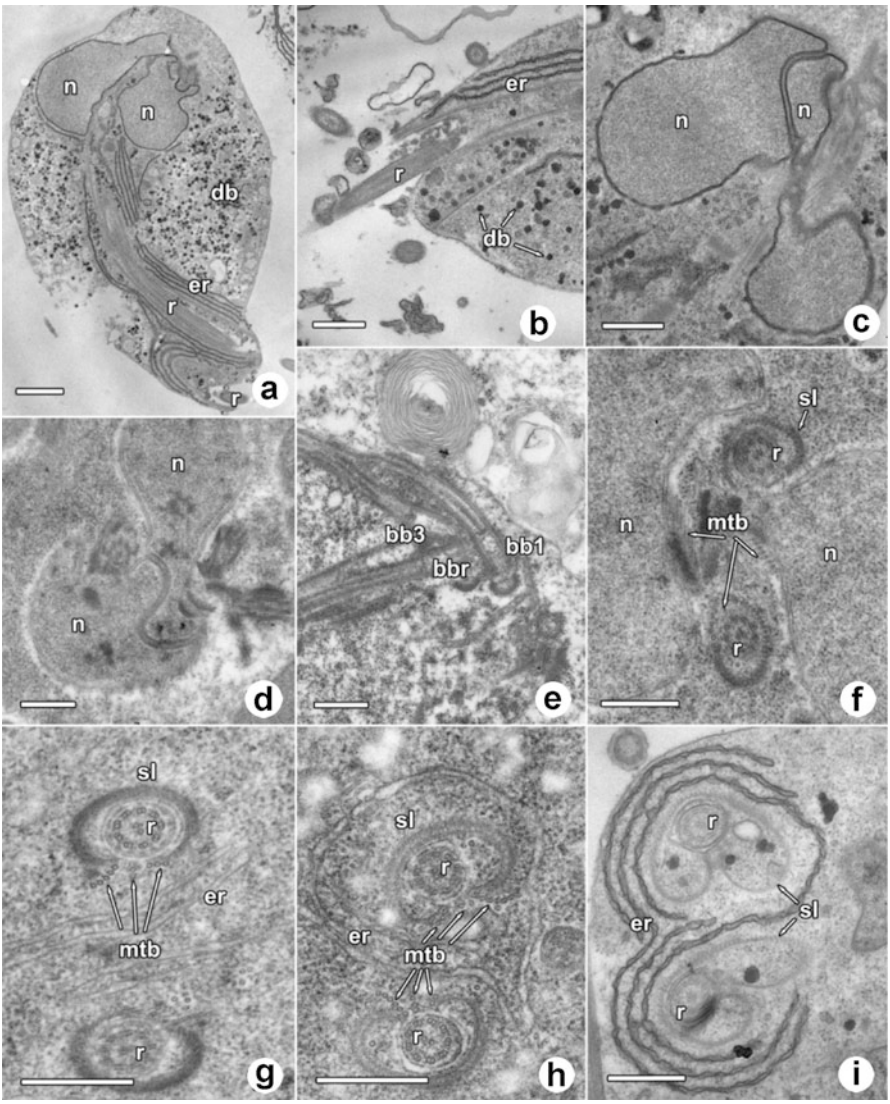

Fig. 2 Transmission electron micrographs of *Spironucleus salmonicida* isolated from a muscle abscess and the liver of farmed Arctic charr *Salvelinus alpinus* (L.). Scale bars = 500 nm except **a**; scale bar for ***a*** = 1 μm. (**a**) Longitudinal section showing two anterior nuclei (*n*) and two recurrent flagella (*r*). Note deep basal body pockets in the anterior end of one of the nuclei, the abundant endoplasmic reticulum (*er*) along the recurrent flagella, and the cytoplasm filled with dense bodies (*db*). (**b**) Longitudinal section of the posterior end showing an emerging recurrent flagellum (*r*). (**c, d**) Longitudinal and transverse sections of the nuclei showing intimate contact between the anterior ends of the nuclei. (**e**) Longitudinal section through one of the basal body complexes. The three visible basal bodies/flagella (bb1, bbr, bb3) are arranged in the same plane. (**f**) Transverse section through the posterior part of the nuclei showing that the recurrent flagella pass between the nuclei. Note the striated lamina (*sl*) and microtubular bands (mtb) along and between the recurrent flagella.

body shape but sucking disk less than half the cell length and an elongated claw-shaped median body that lies across the cell.

A subsequent work in defining species has concentrated on the large group of organisms found with the morphologic type described by Filice (Filice 1952) as *Giardia duodenalis* (Figs. 3, 4, and 5). Morphological differences observable at the EM level accompanied by DNA sequence data were used to describe separate species found in psittacine birds (*Giardia psittaci*) (Erlandsen and Bemrick 1987) and herons (*Giardia ardeae*) (Erlandsen et al. 1990) and voles (*Giardia microti*) (van Keulen et al. 1998). The group of organisms left within the *Giardia lamblia* group after separating out these species is found exclusively within mammals but is comprised of at least eight distinct genetic groups (assemblages or genotypes), each with a distinct molecular type and with at least some degree of host specificity (Caccio and Ryan 2008; Lasek-Nesselquist et al. 2010). It is likely that at least some of these genotypes will eventually be accepted as separate species, thus returning us partially to the original descriptions based on host of origin. In the current chapter, we have used the name *Giardia lamblia* to refer to all eight of these genotypes. Only genotypes A (Nash Groups 1 and 2; (Nash and Keister 1985; Nash et al. 1985) and B (Nash Group 3) are found in humans, and these two are so different that they have already been proposed as separate species (Adam et al. 2013; Nash and Keister 1985; Nash et al. 1985). The current phylogenetic grouping of *Giardia* species and *Giardia lamblia* genotypes argues against a strict application of host-parasite coevolution in that *Giardia muris* is actually far more distant from *Giardia lamblia* than the more recently identified bird species, *Giardia psittaci* and *Giardia ardeae* (Abe et al. 2012; van Keulen et al. 1993).

Giardia lamblia was not generally accepted as a human pathogen until the 1960s, when it was associated with a series of waterborne outbreaks of human diarrhea. Axenic cultivation of *Giardia lamblia* was first achieved in 1970 (Meyer 1970) and has been followed by axenization of several other diplomonad species (see below). The subsequent decades have seen an explosion of our knowledge of *Giardia*, culminating in the publication of the *Giardia* genome in 2007 (Morrison et al. 2007), which was done using the Genotype A isolate WB (Smith et al. 1982). Subsequently, the Genotype B isolate GS (Nash and Keister 1985; Nash et al. 1985) has been sequenced in a genome survey (Franzen et al. 2009) followed by a more complete genome sequence (Adam et al. 2013). Our knowledge of other genera is rudimentary in comparison, although recent genomic (Andersson et al. 2007; Roxstrom-Lindquist et al. 2010; Xu et al. 2014), biochemical (Lloyd and Williams 2014; Millet et al. 2011a), and structural (Millet et al. 2013) analyses of *Spironucleus* species have been published.

Fig. 2 (continued) (**g–i**) Transverse sections through the recurrent flagella at the anterior, middle, and posterior parts of the cell. Note the striated lamina surrounding the flagella and the three microtubular bands (*mtb*) accompanying each of the flagella. Abbreviations: *bb* basal body, *db* dark body, *er* endoplasmic reticulum, *mtb* microtubular band, *n* nucleus, *r* recurrent flagellum, *sl* striated lamina (From Jorgensen and Sterud 2006)

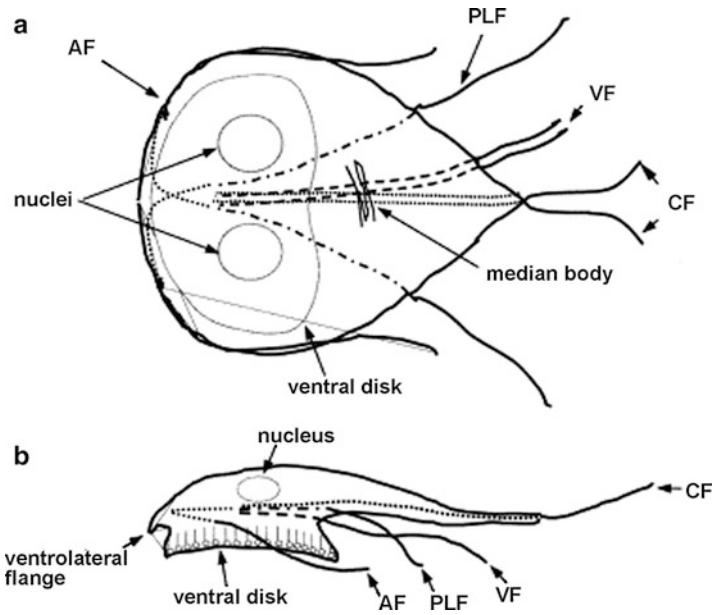

Fig. 3 Schematic representation of key structural features of a *Giardia lamblia* trophozoite. (**a**) The parasite is viewed dorsally, with lighter and dotted lines indicating internal structures. (**b**) The parasite is viewed laterally through the ventral groove. *AF* anterior flagella, *CF* caudal flagella, *PLF* posterior lateral flagella, and *VF* ventral flagella (From Elmendorf et al. 2003)

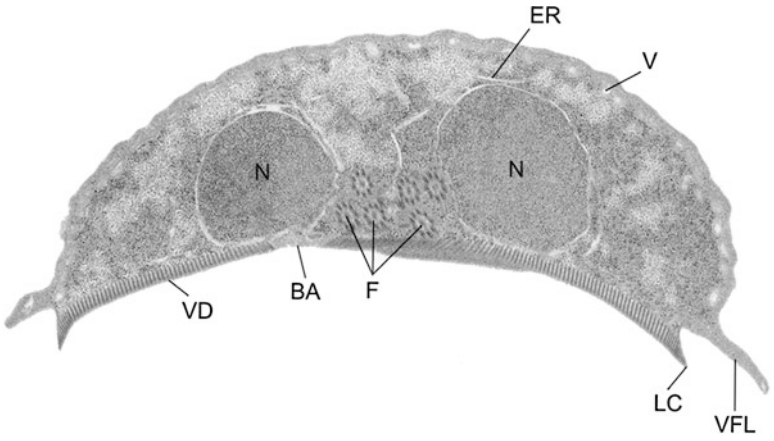

Fig. 4 Transmission EM of *Giardia lamblia* – A coronal view of a trophozoite demonstrates the nuclei (*N*), endoplasmic reticulum (*ER*), flagella (*F*), and vacuoles (*V*). The ventral disk (*VD*) attaches to an intestinal or glass surface. Components of the ventral disk include the bare area (*BA*), lateral crest (*LC*), and ventrolateral flange (*VLF*) (From Adam 2001)

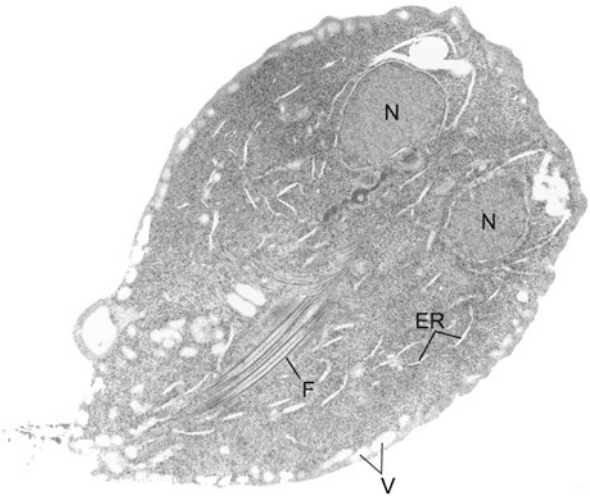

Fig. 5 Transmission EM of *Giardia lamblia* – A cross-sectional view of a trophozoite demonstrates the nuclei (*N*), flagella (*F*), vacuoles (*V*), and endoplasmic reticulum (*ER*) (From (Adam 2001)

Flagella and Cytoskeleton

The morphology of the flagella and cytoskeleton is the central defining feature of the diplomonads. There are typically three microtubular fibers that emerge from the basal bodies, although the extent of the fibers varies among genera. The supranuclear fiber arises from basal body 1 (= basal body 2 under terminology that is universal to other protists (Simpson 2003)) and passes in front of or over the nucleus. In *Trepomonas*, these fibers are well developed and curve to the rear to reinforce the anterior crests. In *Giardia*, the two supranuclear fibers fuse to form the striated disk that supports the ventral disk (see below). The infranuclear fiber originates at the recurrent flagellum (= flagellum 1 in universal numbering). In *Trepomonas*, *Hexamita*, and *Spironucleus*, the two infranuclear fibers cross over beneath the nuclei and appear to maintain them in their anterior position; these fibers taper at their distal end where they reinforce the cytostome on the opposite side. They are absent in *Giardia*, *Octomitus*, and *Brugerolleia* (Desser et al. 1993).

The third band of microtubules (direct fiber) also arises from the base of the recurrent flagellum but keeps to its own side of the cell body. This fiber borders the flagellar depression (*Enteromonas*) or the cytostome (*Trepomonas*; *Hexamita*, Fig. 1; *Spironucleus*; Fig. 2) or runs alongside the intracellular axoneme (*Octomitus*, *Giardia*). A lamina with periodic structure also arises from the base of the recurrent flagellum and supports the cytostome in the Hexamitinae; although it is present in *Octomitus*, it is lacking in *Giardia.*

Locomotion

Giardia species have four pairs of flagella, anterolateral (sometimes called anterior), posterolateral, caudal, and ventral, each emerging from a single basal body. Interestingly, the dyad symmetry of the flagella does not match the symmetry of the basal

bodies (Nohynkova et al. 2006). In *Giardia*, the trophozoites spend most of their life attached to intestinal villi, where the ventral flagella beat continually, leading, historically, to the hydrodynamic model of trophozoite attachment (see below). Conversely, the anterolateral (sometimes called anterior) flagella beat asynchronously only during swimming and appear to mediate forward motion (Holberton 1973). The caudal flagella may provide direction for the swimming trophozoite (Elmendorf et al. 2003).

The two powerful F1s are the locomotory flagella of *Trepomonas* and propel the organism rapidly through the water, while F2, F3, and FR lie in the oral groove (under universal terminology, these flagella are F2, F3, F4, and F1, respectively, i.e., the recurrent flagellum, FR becomes F1 (Simpson 2003). In the other common free-swimming diplomonads and in *Enteromonas*, F1, F2, and F3 participate in locomotion, but this is less rapid in the parasitic forms. The recurrent flagella of *Spironucleus* and *Octomitus* beat to assist locomotion, but those of *Hexamita* and *Giardia* usually trail passively.

Attachment to Host Surface

Giardia species replicate in the intestine of the host without tissue or cell invasion. Thus, a mechanism is required to allow the trophozoite to attach to the intestinal wall to avoid being swept downstream, while at the same time, facilitating nutrition acquisition. The attachment to the intestinal wall is facilitated by the ventral adhesive disk, which bites into the microvillar border of the host's epithelium. This disk is a unique attachment organelle supported by a complex cytoskeleton and delimited by a ridge, the lateral crest (Figs. 3, 4, and 5). The cytoskeleton is composed of a single layer of M1 microtubules wound in a clockwise spiral from the K1 basal bodies and initially connected to the disk's cytoplasmic membrane by short filamentous processes. From each microtubule, a "dorsal ribbon" extends into the cytoplasm. The ribbons have a periodicity of 12–15 nm and are each composed of two sheets of globular subunits separated by a fibrous inner core. The ribbons contain tubulin and beta-giardin (Brugerolle 1991), but alpha-giardins (annexins) and gamma-giardins are probably not part of the ventral disk (Elmendorf et al. 2003). The mechanism by which the ventral disk mediates attachment to the intestinal lumen is not yet certain. Lectin-mediated attachment has been proposed (Inge et al. 1988), but the presence of lectins on all surfaces of the cell and the ability of the cell to attach to inanimate surfaces such as glass argue that if lectins play a role, it is a secondary one. A hydrodynamic model in which the ventral flagella cause a fluid flow that generates negative pressure under the disk (Holberton 1974) does not easily explain the lack of correlation of flagellar beating with attachment and detachment (Elmendorf et al. 2003). At this time, the best hypothesis appears to be that attachment is mediated by contraction of the lateral crest and ventrolateral flange. The *Giardia* genome contains a single actin gene, but myosin and other classic microfilament-associated proteins are absent (Morrison et al. 2007).

Cytostome and Feeding

Endocytosis occurs through a specialized site or cytostome in the bacteriophagous genera. In *Enteromonas*, there is a shallow oral gutter beneath the recurrent flagellum; in *Trepomonas*, this groove or pocket is broad, and flagella F2, F3, and FR (= F3, F4, and F1) lie inside it, drawing bacteria into a cytostomal pit at the base of the flagella. In *Hexamita* and *Spironucleus*, the FR (= F1) on either side of the cell lies in an intracellular channel which opens at the posterior extremity of the body (Fig. 1); the two channel openings serve as cytostomes and bacteria pass along them to be engulfed near the basal body. Bidirectional cyclosis of food vacuoles has been demonstrated (Eyden and Vickerman 1975) in *Trepomonas agilis* and may occur in other phagotrophic forms. The pathways of cyclosis follow the direct fibers and infranuclear fibers (referred to as the M2 and M3 microtubular bands, respectively, by Eyden and Vickerman (1975)); in flattened specimens, vacuoles apart from these bands are stationary. A cytoproct is situated at the posterior extremity of the cell (Brugerolle 1975). In the nonphagocytic genera *Octomitus*, *Giardia*, and *Brugerolleia*, there is no special cytostome and pinocytosis can occur from the surface of the body. In these taxa, the FRs (F1s) pass to the posterior extremity as intracellular axonemes and not in cytostomal channels.

Contractile Vacuole

Present in free-living freshwater species only, this organelle differs from that of other protists in that systole takes place at a site distant from that of diastole. In *Trepomonas agilis*, small clear vacuoles carried in the cyclosis streams fuse to form a large contractile vacuole just behind the post-nuclear vortex of the two streams. The diastole of the stationary vacuole is followed by its transport along a median pathway to the posterior extremity, where systole occurs at the cytoproct site. Several contractile vacuoles may be observed at any one time in a given organism; systole occurs at 30–50 s intervals (Eyden and Vickerman 1975).

Nuclear Structure and Replication

Most of the advances of the last two decades have been through studies of *Giardia lamblia* because of its status as a human pathogen and the ability to culture the organism axenically. Therefore, the following will concentrate primarily on what is known about *Giardia*. Trophozoites have five chromosomes ranging in size from approximately 1–4 Mb, for a total haploid genome size of 12 Mb (Adam et al. 1988; Perry et al. 2011). Trophozoites are approximately tetraploid, as demonstrated by size variants of chromosome homologues (Adam 1992; Adam et al. 1988; Hou et al. 1995), by heterozygosity of repeat-containing alleles (Yang and Adam 1994, 1995; Yang et al. 1994), and by quantitative ascertainment of DNA content of individual organisms (Bernander et al. 2001). However, cytogenetic data suggest some degree of aneuploidy, with individual trophozoites containing from 19 to 21 chromosomes (expected number 20) (Tumova et al. 2007). DNA replication occurs relatively early, and trophozoites spend most of their time in the G2 phase of replication; these organisms actually contain eight copies of each chromosome (Bernander et al.

2001). Trophozoites replicate by binary fission with semi-open mitosis (Poxleitner et al. 2008) with the two nuclei replicating at approximately the same time (Wiesehahn et al. 1984), and both are transcriptionally active (Kabnick and Peattie 1990). Left/right nuclear asymmetry is maintained during trophozoite replication, such that each daughter trophozoite receives one right and one left nucleus; however, it is not as clear whether the nuclei switch from right to left at each division (Ghosh et al. 2001; Sagolla et al. 2006) or not (Yu et al. 2002), although more recent data suggests alternating sides (Poxleitner et al. 2008). When trophozoites are transfected with an episomal plasmid, the plasmid replicates in a single nucleus and is never found in both nuclei, indicating lack of transfer of DNA between nuclei during trophozoite replication (Poxleitner et al. 2008; Yu et al. 2002). The lack of genetic exchange makes it difficult to understand the extremely low degree of allelic sequence heterozygosity found in the genome isolate, WB (Morrison et al. 2007), since sequence difference should accumulate within and between nuclei; yet the degree of allelic heterozygosity for the WB isolate is less than 0.01%, while the allelic heterozygosity for the DH (Genotype A2) and GS (Genotype B) isolates is 0.037% and 0.425% (Adam et al. 2013). These levels of heterozygosity are all in the range expected for sexually reproducing organisms, but the reason for the greater than two-log difference in heterozygosity levels among genomes has not been studied. The suggestion of sexual reproduction has also supported by the finding that the genes known to be required for meiosis are present in *Giardia* (Ramesh et al. 2005). In addition, population genetic data in a region highly endemic for giardiasis near Lima, Peru, identified patterns that suggested recombination between isolates of a single genotype (Cooper et al. 2007, 2010). One study suggested recombination among *Giardia* isolates (Lasek-Nesselquist et al. 2009), but recombination among genotypes has not been supported by other studies (Cooper et al. 2010; Xu et al. 2012). These observations suggest the possibility of sexual reproduction within genotypes, but not between genotypes, and provide support for the designation of these genotypes as separate *Giardia* species. However, it remains possible that sexual reproduction in *Giardia* is parasexual rather than meiotic (Birky 2010), as has been shown for the yeast *Candida albicans* (Butler et al. 2009; Forche et al. 2008). All the findings to date could be explained by either meiotic or parasexual reproduction, so further studies will be required to distinguish which is occurring in *Giardia*.

Encystation and Excystation

Giardia cysts are oval-shaped and 5 by 7–10 μm in diameter. Light microscopic examination reveals two to four nuclei, depending on the maturity of the cyst, as well as flagellar axonemes and ventral disk segments. Electron microscopic examination of the axonemes reveals the 9 + 2 microtubule arrangement and that the periphery of the cyst has multiple vacuoles (Feely et al. 1990).The outer portion of the cyst wall is formed from four major proteins as well as galactosamine (Adam 2001).

Giardia cysts are relatively inert, with a metabolic rate about 10–20% that of trophozoites and are able to survive for up to a month outside the host in a cool moist environment. The survival in cool environments may explain the frequent

occurrence of waterborne transmission of *Giardia* in colder climates (some of the well-documented outbreaks have been from Norway, Russia, and Canada). The cyst survives its passage through the gastric acid of the host, and excystation into trophozoites may be induced by exposure to an acidic environment in vitro (Bingham and Meyer 1979). However, excystation may also be induced in a neutral pH environment (Feely et al. 1991). Encystation of some trophozoites occurs in the small intestine as a result of exposure to bile salts (Gillin 1987) or from cholesterol deprivation (Lujan et al. 1996). Although it is rather difficult, in vitro-derived cysts may be excysted to again form trophozoites (Schupp et al. 1988).

Each cyst is formed from a single trophozoite by mitotic division rather than by the fusion of two trophozoites (Carpenter et al. 2012). During encystation, nuclear anterior/posterior separation of the two nuclei is followed by nuclear replication. During the entire process, the dyad symmetry is maintained so that each daughter trophozoite receives a left and a right nucleus, rather than two right or two left nuclei (Carpenter et al. 2012). This observation rules out instant homogenization of the nuclear pairs. However, there is evidence that after nuclear migration, nuclear fusion may occur in which one progeny from each of the two nuclei fuses their membranes and exchanges genetic material (diplomixis) (Carpenter et al. 2012; Jirakova et al. 2012; Poxleitner et al. 2008). This nuclear fusion involves only two of the four nuclei but provides a mechanism by which the nuclear contents can be homogenized. Whether this exchange involves whole chromosomes and/or homologous recombination has not yet been determined (Carpenter et al. 2012). After the fusion, the nuclei separate, forming a cyst with four nuclei. Then during excystation, cytokinesis is completed followed by a round of nuclear replication and cytokinesis, resulting in four trophozoites (Bernander et al. 2001).

Cysts have been documented in a number of other diplomonad species, including *Trepomonas* (Brugerolle 1975) and many but not all *Spironucleus* species. Cysts have been identified in *S. meleagridis* (Wood and Smith 2005) and *S. muris* (Januschka et al. 1988). In addition, the survival of *S. vortens* in feces outside the host for 30 days suggests the possibility of a cyst form for this species (Williams et al. 2013). The *Giardia* and *Spironucleus* cysts are very similar at the light microscopy level, although the *Spironucleus* cysts are somewhat smaller. At the TEM level, *Giardia* cysts have parts of the ventral disk and median body, while *Spironucleus* cysts have a striated rootlet fiber, a flagellar sheath, and glycogen rosettes (Januschka et al. 1988). A genomic analysis of *S. salmonicida* has demonstrated orthologs of the genes known to be involved in the encystation process for *G. lamblia* (Xu et al. 2014). In addition, the *S. salmonicida* ortholog of *G. lamblia* cyst-wall protein-1 (CWP-1) gene encodes a protein that functions as a cyst wall protein in a *G. lamblia* encystation assay.

Biochemistry and Metabolism

As a group, the diplomonads are anaerobic organisms with moderate aerotolerance (Lloyd and Williams 2014). Those that have been specifically studied include *Spironucleus vortens* (Millet et al. 2011a, 2013) and *Hexamita inflata* (Biagini et al. 2003). *Giardia* trophozoites are facultatively anaerobic and, in vitro, are

grown in an oxygen-deprived environment (Adam 2001). Glucose is the major carbohydrate source of energy, which is generated by anaerobic glycolysis. *Giardia* has the nearly universal pyruvate kinase, but also has pyrophosphate-dependent pyruvate kinase, found in bacteria and other anaerobic protists (*Entamoeba* spp. and *Trichomonas* spp.), which can generate five rather than two ATPs from one molecule of pyruvate. In vitro (Hrdy et al. 1993) and in vivo (Feng et al. 2008) studies have suggested that this is the predominant pathway for energy synthesis from pyruvate.

Arginine metabolism is also a potentially important source of energy in *Giardia* (Edwards et al. 1992; Schofield et al. 1990) via an arginine dihydrolase pathway. Alanine and ethanol are the major end products of metabolism under anaerobic conditions, while acetate is the major product during aerobic conditions (Paget et al. 1990). More limited studies of energy metabolism have been done with the free-living organism *Hexamita inflata* (Biagini et al. 1998, 2003). These studies have demonstrated activity of the arginine dihydrolase pathway enzymes and have indicated that ethanol, alanine, acetate, and lactate are the predominant end products of energy metabolism, with a predominance of ethanol in a nutrient-rich medium with reduced oxygen tension (Biagini et al. 2003). It appears that arginine metabolism is favored under anaerobic conditions, while sugar fermentation (in this case, maltose) predominates under more aerobic conditions (Biagini et al. 1998). A genome survey of *Spironucleus salmonicida* identified the arginine dihydrolase pathway enzymes, but biochemical studies of *Spironucleus vortens* indicated the lack of the arginine dihydrolase pathway, at least during growth in rich medium. However, glutamate can act as a carbon source for *S. vortens* (Lloyd and Williams 2014). *Spironucleus vortens* also contrasts with *Giardia* in the predominant antioxidant system. While *Giardia* utilizes cysteine as the major thiol compound, glutathione is the predominant nonprotein thiol in *S. vortens* (Lloyd and Williams 2014).

As noted above, none of the diplomonads has typical eukaryotic mitochondria (Embley and Martin 2006). The canonical eukaryotic mitochondria are characterized by a separate genome, a double membrane, and the components of a citric acid cycle for generation of ATP. As early as 1973, hydrogenosomes had been reported from *Trichomonas vaginalis* (Lindmark and Muller 1973) and named because of the substantial production of hydrogen. Subsequent studies identified the hydrogenosome as an organelle with some but not all characteristics of a canonical eukaryotic mitochondrion. These organelles have double membranes and have proteins that are characteristic of mitochondria such as HSP60, mitochondrial HSP70, and HSP10 (Bui et al. 1996). Although ATP is produced, it uses pyruvate as the primary substrate and lacks a citric acid cycle, cytochromes, or DNA (Martincova et al. 2012; Shiflett and Johnson 2010). Thus, hydrogenosomes are one of several types of mitochondria-related organelles (MRO). Recent studies have demonstrated substantial levels of hydrogen generation in *Spironucleus* species, at a level comparable to that of *T. vaginalis*. Double-membrane organelles about 500 nm in size contain PFOR and FeFe-hydrogenase. These hydrogenosomes have been identified both in *S. vortens* (Millet et al. 2013) and in *S. salmonicida* (Jerlstrom-Hultqvist et al. 2013). Subsequent phylogenetic analysis has suggested that hydrogenosomes were

present in a common ancestor of the Fornicata (containing diplomonads) and parabasalids (e.g., *Trichomonas vaginalis*) (Jerlstrom-Hultqvist et al. 2013).

In 2003, a much more limited form of MRO, called the mitosome, was reported from *Giardia* (Tovar et al. 2003). The *Giardia* mitosome retains only the iron-sulfur cluster assembly function of canonical mitochondria and contains just 20 proteins, including nine that form the FeS cluster (Lloyd and Williams 2014). Unsurprisingly, it differs substantially from the *Spironucleus* MRO at a proteomic level (Martincova et al. 2015). These observations suggest that *Giardia* mitosomes represent an example of extreme reductive evolution.

Giardia has no carbohydrate, fatty acid, or nucleic acid synthesis and the only amino acid synthesis appears to be related to energy generation. Correspondingly, the genes for these processes are absent from the genome (Morrison et al. 2007). The growing information on genomics and biochemistry of the *Spironucleus* species will allow informative comparisons and contrasts with the synthetic processes of *Giardia* spp. (Lloyd and Williams 2014; Xu et al. 2014).

Protein Transport

The diplomonads lack a conventional Golgi apparatus that can be visualized using electron microscopy, so the mechanism of protein transport in these organisms is of interest. Despite the lack of conventional Golgi, *Giardia* trophozoites have perinuclear structures that co-localize the ER and Golgi functions (Lanfredi-Rangel et al. 2003). There are peripheral vacuoles that fulfill the functions of the endosomes and lysosomes (Lanfredi-Rangel et al. 1998; Rivero et al. 2012; Touz et al. 2012). Furthermore, relatively conventional Golgi complexes actually do appear during the process of encystation (Reiner et al. 1990) and are involved in protein sorting, which includes the sorting of cyst wall proteins into encystation-specific vesicles (ESV) followed by their transport to the cell membrane. In addition, protein sorting occurs in vegetative trophozoites and can be inhibited by brefeldin A, an inhibitor of Golgi function (Lujan et al. 1995). It is of note that Golgi-specific genes have been identified in *Giardia lamblia* as well as in *S. barkhanus* (Dacks et al. 2003).

VSP Genes and Antigenic Variation

Giardia lamblia trophozoites display on their surfaces a repertoire of variably expressed proteins, called variant-specific surface proteins (VSPs). The WB genome encodes a repertoire of approximately 270 genes encoding these VSPs (Adam et al. 2010), while the *vsp* gene repertoires of other *Giardia lamblia* genotypes range from about 120–500 (Adam et al. 2013). One *vsp* gene is expressed at a time and expression switches from one VSP to another by a mechanism that does not require DNA rearrangements or sequence changes (Yang and Adam 1994; Yang et al. 1994) and is epigenetic in nature (Kulakova et al. 2006). There is evidence for a role of microRNAs in the control of *vsp* gene expression (Li et al. 2012; Prucca et al. 2008). However, the expression of a *vsp* gene from only one of the four alleles, despite the identical sequence of all four alleles (Yang and Adam 1994), suggests additional mechanisms involved in the control of expression. In addition to the spontaneous switching seen in individual trophozoites, a whole population may switch during the

process of encystation and excystation (Svard et al. 1998). In humans and in animal models of infection, switching may correlate with the antibody response of the infected host, but in infected gerbils, switching does not continue to occur after the first week (Aggarwal and Nash 1988), while it does in immunodeficient mice (Gottstein and Nash 1991). Therefore, it is not yet clear whether antigenic variation is primarily or secondarily responsible for evasion of the host immune response. The role and function of the VSPs are also not known, but the high level of expression, the devotion of 3–6% of the genome to the VSP repertoire, and the maintenance of antigenic variation all suggest the great importance of the VSPs. The observation that different antigen types have different protease susceptibilities raises the possibility that these proteins are involved in helping the organisms adapt to different intestinal environments (Nash et al. 1991). The genome contains an additional 61 cysteine-rich protein genes (HCMP) (Davids et al. 2006) that are not related to the VSPs; their function is not yet known.

A genome survey of *Spironucleus salmonicida* also identified a large repertoire of cysteine-rich protein genes (Andersson et al. 2007). Further analysis of the *S. salmonicida* genome has demonstrated the presence of three groups of cysteine-rich protein genes (Xu et al. 2014). The first, encoding CRMP-1 (cysteine-rich membrane protein 1) is a family of 125 genes and has similarities with the *Giardia vsp* genes, notably including the frequent CXXC motif. Whether these proteins undergo antigenic variation has not yet been reported. The second, CRMP-2 is a family of 195 genes and has similarities with *Giardia* HCMP. There is a third group of 52 genes (CRP) with similarity to neither of the above.

Maintenance and Cultivation

Giardia lamblia cultures can be established by direct culture of organisms acquired from the intestine, or by excystation of fecally derived cysts, either directly or after passage through laboratory animals. Trophozoites were first cultured axenically in HSP1 medium (Meyer 1976), and are currently grown in modified TYI-S-33 (Keister 1983), in which the trophozoites have a generation time of about 8–12 h. Each 100 ml of modified TYI-S-33 medium contains 100 mg K_2HPO_4, 60 mg KH_2 PO_4, 2.0 g trypticase, 1.0 g yeast extract, 1.0 g glucose, 200 mg NaCl, 200 mg cysteine-HCl monohydrate, 20 mg ascorbic acid, 2.28 mg ferric ammonium citrate, and 50–100 mg dehydrated bovine bile with 10% bovine serum. The pH is adjusted to 7.0–7.2 and the medium is filter-sterilized. Trophozoites are grown in small sealed tubes at an incline at 37 °C and form confluent layers at the top of the tube, reaching densities of up to 10^6 per ml. Trophozoites can be cloned by limiting dilution in 96-well plates enclosed in sealed plastic with an anaerobic generator. *Giardia lamblia* is the only *Giardia* species that has been cultured axenically, and axenic culture has not been reported for the genotypes of *Giardia lamblia* that are not found in humans (genotypes C through H). *Giardia muris* has not been cultivated in vitro but can be maintained in the laboratory by serial passage through mice.

Hexamita inflata, a free-living organism, can be grown axenically at 25 °C in 2% trypticase, 0.1% yeast extract, 0.5% maltose, 1% L-cysteine, 10 mM K phosphate buffer, and 10% fetal calf serum at pH 7.2, supplemented with gentamicin 50 μg/ml to prevent bacterial growth (Biagini et al. 1997).

Trepomonas agilis (ATCC 50336) can be grown xenically in ATCC medium TYGM-9 (Cavalier-Smith and Chao 1996). For 1 l of medium, 2.8 g K_2HPO_4, 0.4 g KH_2PO_4, 2.0 g casein digest, 1.0 g yeast extract, 7.5 g NaCl, and 2.0 g gastric mucin are added to 970 ml distilled water and autoclaved. Heat-inactivated bovine serum (3%) and 0.5 ml filter-sterilized Tween 80 in absolute ethanol are added and the medium is added to 8 ml capped tubes. This is followed by the addition of 0.15–0.40 ml 5% rice starch solution which has been heated at 150 °C for 2 h and brought to pH 7.4 in phosphate-buffered saline. Some *Trepomonas* species (e.g., *Trepomonas steini*) have been grown in Sonneborn's *Paramecium* medium with cerophyll (ATCC #802) (Kolisko et al. 2008).

Spironucleus vortens trophozoites have been isolated from the small intestines of fish and grown in modified TYI-S-33 (Keister 1983; Millet et al. 2011b; Poynton et al. 1995). A more detailed evaluation of the in vitro growth requirements for *S. vortens* revealed an optimum pH of 6.5–7.5 and an optimum temperature of 28 °C (acceptable range 22–31 °C) (Sangmaneedet and Smith 2000). Parasites grew better without bile supplementation. *Spironucleus salmonicida* has been grown under similar conditions, although without as much detail in the determination of optimal growth requirements (Sterud 1998).

Species of *Enteromonas* can be cultivated with bacteria in the biphasic media formerly used for intestinal amoebae, e.g., HSre – a slope of heat-coagulated horse serum overlain by egg white diluted 1:10 with mammalian Ringer solution (Dobell and Laidlaw 1926); (Kolisko et al. 2008). Cultures initiated with fresh feces show abundant growth within 24 h at 37 °C, and for several days, cysts may be produced after 2–4 days. Subculture is difficult but once established with a suitable bacterial microbiota, strains can be maintained indefinitely. *Trimitus* spp. have also been grown xenically in the Dobell and Laidlaw medium (Kolisko et al. 2005).

Evolutionary History

The diplomonads lack some of the usual eukaryotic features, such as peroxisomes, as well as conventional Golgi apparatus and mitochondria. Therefore, they were proposed as members of the Archezoa (Cavalier-Smith 1983) which were thought to be the earliest branching members of the eukaryotic tree, predating the acquisition of these key eukaryotic features, especially mitochondria. This hypothesis was supported by early analyses of SSU rRNA sequence data from *Giardia lamblia* (Sogin et al. 1989). However, subsequent findings have identified remnants or reduced forms of mitochondria in diplomonads, and similar findings have been made for the other main groups proposed to be Archezoa.

The initial evidence against diplomonads being pre-mitochondriate organisms was the identification of the mitochondrial cpn60 gene in *Giardia lamblia* (Roger

et al. 1998) and *Spironucleus barkhanus* (Horner and Embley 2001). Subsequent studies identified mitosomes in *Giardia lamblia* trophozoites (Tovar et al. 2003) and hydrogenosomes in *Spironucleus vortens* (Millet et al. 2013) and *Spironucleus salmonicida* (Jerlstrom-Hultqvist et al. 2013) (see section on "Biochemistry and Metabolism"). Thus, these organisms are no longer considered to be pre-mitochondriate (Morrison et al. 2007); rather, they have reduced forms of these canonical eukaryotic organelles. Likewise, cell biology studies have identified the presence of other eukaryotic organelles, including Golgi in encysting *Giardia lamblia* trophozoites (Reiner et al. 1990) and peripheral vacuoles that fulfil the functions of endosomes and lysosomes (see "Protein Transport" section).

The presence of alternative genetic code in some but not all diplomonads is very useful in rooting the diplomonad tree. This altered codon usage has been documented in numerous Hexamitinae genera, but in none of the Giardiinae. Two of the three canonical termination codons (TAA and TAG) are used instead to encode glutamine in *Spironucleus* spp. and *Hexamita inflata* (Keeling and Doolittle 1996) as well as in *Enteromonas* and *Trimitus* species (Kolisko et al. 2008). Since the acquisition of an alternative genetic code is an exceedingly rare event, it is likely that this change occurred after the divergence of *Giardia* spp. but is ancestral to the divergence of these other genera.

One controversial question in the evolutionary history of Diplomonadida has been the relative placement of single vs. binucleate organisms. Some hypotheses have placed the enteromonads (*Enteromonas* and *Trimitus*), which have single nuclei, in one clade and the binucleate diplomonads in another (Levine et al. 1980). However, molecular phylogenetic analyses have indicated that the enteromonads do not form a single clade but that all of them fall inside Hexamitinae, where they are closely related to *Trepomonas* and *Hexamita*. This has led to the alternative proposal that reduction from two nuclei to one (or alternatively an increase from one to two) has occurred multiple times (Kolisko et al. 2005, 2008).

A second controversial question has been the relative placement of the free-living and parasitic organisms. The free-living diplomonads do not segregate neatly from the parasitic organisms in molecular phylogenies. Such analyses usually divide the Hexamitinae into three groups (Groups II to IV of the diplomonads; the Giardiinae form Group I) (Xu et al. 2016). *Spironucleus* species are found in Groups II, III, and IV, and all free-living species identified to date belong to Group IV. Because the complexity of feeding and metabolism is greater for the free-living organisms, the common assumption has been that the usual evolutionary direction is that parasitism arises from the free-living state, but if so, the phylogeny of diplomonads suggests a large number of independent adoptions of parasitism/commensalism. Genomic evidence suggests that the reverse may have occurred within the diplomonads, resulting in the transition of *Trepomonas* from a parasitic to a free-living state, in part by acquiring essential genes from bacteria by lateral transfer (Xu et al. 2016). On the other hand, other molecular and genomic comparisons have found substantial variability among the *Spironucleus* species (Andersson et al. 2007; Jorgensen and Sterud 2007; Roxstrom-Lindquist et al. 2010).

Phylogenetic and morphological assessments of organisms within the diplomonads, and comparisons with other protists, have contributed substantially to our understanding of adaptation to parasitism as well as free-living states, as well as how complex organelles can be reduced as organisms fill certain niches. It is likely that further structural and genomic approaches involving additional species of these organisms will lead to further improvement in our understanding of these phenomena.

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Retortamonadida (with Notes on *Carpediemonas*-Like Organisms and Caviomonadidae)

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Abstract

Retortamonadida (retortamonads) is a group of bacterivorous metamonads belonging to Fornicata, currently represented by the single family Retortamonadidae, with two genera, *Retortamonas* and *Chilomastix*, and about 60 species. They are adapted to low-oxygen environments and live predominantly as endocommensals in intestines of animal hosts, including humans. Two species were reported to be potential pathogens causing diarrhea in humans (*C. mesnili*) or unadapted avian hosts (*C. gallinarum*). One species (*C. cuspidata*) is free-living in hypoxic water sediments. Retortamonads are typical excavates with a single karyomastigont possessing four basal bodies and two or four flagella. One flagellum is recurrent, has two or three lateral vanes, and is associated with a ventral feeding groove. Double-membrane-bounded organelles without cristae, assumed to be mitochondrial derivatives, were found in *Chilomastix*. Retortamonads reproduce by binary division and produce a resistant cyst stage. The cysts of endobiotic species are discharged with feces and serve to spread the infection. Recent phylogenetic analysis and ultrastructural observations indicate that *Retortamonas* species from insects are close relatives of *Chilomastix*, while species from vertebrates appear to be relatives of Diplomonads and should be excluded from this genus. *Carpediemonas*-like

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organisms (CLOs) comprise a few species of small, free-living Fornicata. They are typical excavates and are biflagellated, though usually possess three or four basal bodies in the mastigont. CLOs form a paraphyletic grade in phylogenetic trees, having retortamonads, diplomonads, and Caviomonadidae (i.e., the rest of Fornicata) nested within them. Caviomonadidae is a group of morphologically reduced uniflagellates that were thought to belong to Diplomonadida until recently. Instead, they are closely related to the CLO genera *Hicanonectes* and *Aduncisulcus*. Caviomonadidae includes three endobiotic species and one undescribed free-living, marine isolate.

Keywords

Bacterivore • *Carpediemonas*-like organisms • Caviomonadidae • Excavata • Flagellate • Protozoa • Retortamonadida

Contents

Summary Classification

- **Fornicata**
  - ***Carpediemonas***
  - ***Ergobibamus***
  - ***Aduncisulcus***
  - ***Hicanonectes***

*Likely polyphyletic with its present species composition – see text.
**See "▶ Diplomonadida."

Introduction

General Characteristics

Retortamonads are small (5–20 μm) bacterivorous protists with two or four flagella. They possess a large ventral feeding groove elaborated into a conspicuous cytostome-cytopharynx complex supported by microtubules and other cytoskeletal components (Kulda and Nohýnková 1978). One of the flagella, equipped with vanes, is directed posteriorly and runs through or is confined to the cytostomal cavity. Motion of this flagellum generates the feeding current. Retortamonads lack classical mitochondria, peroxisome-like organelles, and a morphologically developed Golgi apparatus, but possess double-membrane-bounded organelles that resemble hydrogenosomes in their morphology (Hampl and Simpson 2008). The life cycle of retortamonads includes two stages: the motile trophozoite stage, which multiplies by longitudinal binary fission, and the resting cyst stage, which is protected by a thick cyst wall (Kulda and Nohýnková 1978). Because no biochemical or pertinent genomic data are available, the physiology and cell biology of retortamonads are largely unknown. No sexual processes have been observed. At present only one family, Retortamonadidae, and two genera, *Retortamonas* and *Chilomastix*, are recognized, with about 60 species described (Kulda and Nohýnková 1978). Molecular phylogenetic analyses show that Retortamonadida is not monophyletic with its current composition (Cepicka et al. 2008; Takishita et al. 2012).

Occurrence

Retortamonads typically exist as endocommensals in the digestive tracts of vertebrate and invertebrate animals; two species *Retortamonas intestinalis* and *Chilomastix mesnili* are found in the human large intestine (Kulda and Nohýnková 1978). *Chilomastix cuspidata* is a free-living species of this group that inhabits hypoxic water sediments (Bernard et al. 2000).

Literature

General information on retortamonads can be found in protozoology books such as Doflein and Reichenow (1952) and Levine (1973). The most complete data can be found in Grassé (1952) and Kulda and Nohýnková (1978). Brugerolle and Mignot (1990) and Brugerolle and Lee (2000) published comprehensive chapters on these organisms. Numerous earlier studies provide information on the morphology, taxonomy, and cell division of different retortamonad species based on light microscopy. Of these, publications by Bělař (1921), Boeck and Tanabe (1926), Bishop (1931, 1935), Wenrich (1932), Geinman (1935), Nie (1948, 1950), Kirby and Honigberg (1950), Moskowitz (1951), and McDowell (1953) deserve particular attention. The ultrastructure of *Retortamonas* and *Chilomastix* was examined by Brugerolle (1973, 1977) and Bernard et al. (1997). Molecular phylogenetic analyses based on sequences of the small subunit ribosomal RNA (SSU rRNA) gene were presented by Silberman et al. (2002) and Cepicka et al. (2008), and a multigene phylogenetic analysis was presented by Takishita et al. (2012).

History of Knowledge

Grassi (1879) described the first species of *Retortamonas* in the intestine of the mole cricket *Gryllotalpa.* The generic names *Embadomonas* and *Waskia* were used thereafter for several species described in insects and vertebrates (e.g., Brug 1922; Hegner and Schumaker 1928; see also Wenrich 1932). Later, the three names were treated as subjective synonyms, and only a single genus of biflagellate retortamonads, *Retortamonas*, was recognized (Wenrich 1932). Alexeieff established the quadriflagellate genus *Chilomastix* in 1912. Synonyms of *Chilomastix* include *Macrostoma*, *Fanapepea*, *Tetrachilomastix*, *Cyathomastix*, and *Difamus*. Both Alexeieff (1912) and Mackinnon (1915) observed the morphological similarity between *Retortamonas* and *Chilomastix*, but Alexeieff (1917) was the first to unite these genera into a single family, designated Embadomonadidae. Wenrich (1932) confirmed the relationship, restored the name *Retortamonas* for the genus possessing two flagella, and established the family Retortamonadidae. This classification, however, was not generally accepted, because it conflicted with the traditional grouping of flagellates in Protomonadida and Polymastigida, based on the number of flagella. Accordingly, *Retortamonas* (possessing two flagella) and *Chilomastix* (four flagella) were usually assigned to different orders of Mastigophora. Grassé (1952) dismissed this rigid concept and proposed a more natural grouping based on comparative morphology. He introduced several new orders of flagellates, including Retortamonadida, which comprised Wenrich's family and again brought together *Chilomastix* and *Retortamonas*. Subsequent electron microscopic observations (Brugerolle 1973, 1977) confirmed this relationship.

During the period 1926–1938, investigators published valuable observations on the morphology of members of the Retortamonadida based on light microscopy. The

introduction of the protargol staining method in the early1950s (see Nie 1950 for a workable protocol) allowed for a better resolution of karyomastigont structures, thus contributing to the definition of taxonomically sound morphological characters. The work of investigators from the Wenrich and Kirby schools during this period represents the best light microscopic morphology studies of the retortamonads and other intestinal flagellates. Electron microscopic studies by Brugerolle (1973, 1977, 1991) and Bernard et al. (1997) revealed the subcellular organization of both *Chilomastix* and *Retortamonas* and provided useful morphological data for phylogenetic considerations of retortamonads in the context of other "excavate taxa" (Simpson 2003). Early molecular phylogenetic analyses based on the SSU rRNA gene and involving several strains of *Retortamonas* from vertebrates (Silberman et al. 2002) showed a close relationship between retortamonads and diplomonads. When *Chilomastix* SSU rRNA gene sequences were included in analyses (Cepicka et al. 2008; Takishita et al. 2012), the monophyly of the Retortamonadida, though expected on the basis of ultrastructural observations (Brugerolle 1973, 1977), was not supported. As mentioned by Cepicka et al. (2008), these discrepancies may result from different taxon sampling, as all *Retortamonas* strains subjected to molecular phylogenetic analyses were isolated from vertebrates, whereas ultrastructural studies were performed on species from insects only. It is therefore likely that the genus *Retortamonas* is polyphyletic, which should lead to substantial taxonomic revision of the group in the future.

Practical Importance

Most members of the Retortamonadida are assumed to be harmless commensals; however, two species, *Chilomastix mesnili* in humans and *Chilomastix gallinarum* in poultry, have been reported as potential pathogens.

The medical importance of *Chilomastix mesnili* is marginal. The parasite is distributed worldwide and shows a low incidence in standard surveys (0.2–1%; e.g., Waikagul et al. 2002; Jacobsen et al. 2007; Nasiri et al. 2009). No marked differences in incidence have been observed in surveys performed in different geographic areas; however, an increased prevalence of 11–40% has been reported in specific groups of people suffering from diarrhea, including a closed community of institutionalized children (Červa and Větrovská 1958), military troops deployed on overseas missions (Oyofo et al. 1997), and selected groups of diarrheic patients (Felsenfeld and Young 1946). Although the association of *Chilomastix* infection with diarrhea does not necessarily reflect a causal relationship in all reported cases, the pathogenic potential of this species cannot be ruled out (Westphal 1939; for further information, see Kulda and Nohýnková (2006)). No specific treatment is recommended for asymptomatic infections; successful treatment of symptomatic patients by carbasone (Červa and Větrovská 1958) or metronidazole (Barnham 1977) has been reported. Because the infection is acquired by swallowing cysts released from the feces of an infected person, good sanitation and personal hygiene are the most effective preventive measures.

Chilomastix gallinarum, a common commensal in the ceca of chicken, turkeys, pheasants, and ducks, is potentially pathogenic to unadapted avian hosts, such as quail. Davis et al. (1964) described an outbreak of acute diarrhea and mortality in young pen-raised quail involving 35,000 birds. *Chilomastix*, which was abundant in the lower digestive tract of the affected birds, was identified as the etiologic agent, and its pathogenicity in quail was experimentally confirmed.

Habitat and Ecology

A common feature of the retortamonads is their adaptation to low-oxygen environments. Most retortamonad species live in the intestines of various animals; species of both *Retortamonas* and *Chilomastix* can be found in the human intestine. It seems that the typical association of retortamonads with particular hosts is commensalism (Kulda and Nohýnková 1978). Transmission among susceptible hosts occurs via ingestion of resistant cysts discharged into the environment from the feces of infected individuals. Most retortamonad species are most likely host specific, but a few of them may exhibit a wider host spectrum, including either related animals or members of a common biotope.

Species of the genus *Retortamonas* live in the intestines of both vertebrates and invertebrates. The typical habitat is the midgut or hindgut of insects, such as mole crickets, cockroaches, water-dwelling larvae of crane flies and beetles, and some termites (Grassé 1952; Brugerolle 2006). Their primary habitat in vertebrates is in the cecum and colon of mammals and the cloaca of amphibians and reptiles (Kulda and Nohýnková 1978). The ostrich *Struthio camelus* is the only avian host of *Retortamonas* reported thus far (Martínez-Díaz et al. 2001). *Retortamonas intestinalis* is an infrequent nonpathogenic inhabitant of human cecum, with low prevalence (up to 2%) even in populations with poor hygiene standards. Species of *Chilomastix* have been recorded in numerous vertebrates, including humans (Kulda and Nohýnková 1978), and in a few invertebrates (the horseleech *Haemopis sanguisuga* and some termites). The localization of these species in vertebrates is similar to that of *Retortamonas*. The free-living species C*hilomastix cuspidata* has been reported in hypoxic sediments of marine, brackish, and freshwaters in Australia, the United States, Northern Ireland, and Denmark (see Bernard et al. 1997 for pertinent references).

Recognition and Characterization

The Trophozoite

Light Microscopy

Trophozoites of both retortamonad genera are pyriform or carrot-shaped cells that are rounded anteriorly and tapered posteriorly, with a posterior spike in some species. In *Chilomastix*, the cell is ventrally flattened, dorsally convex, and

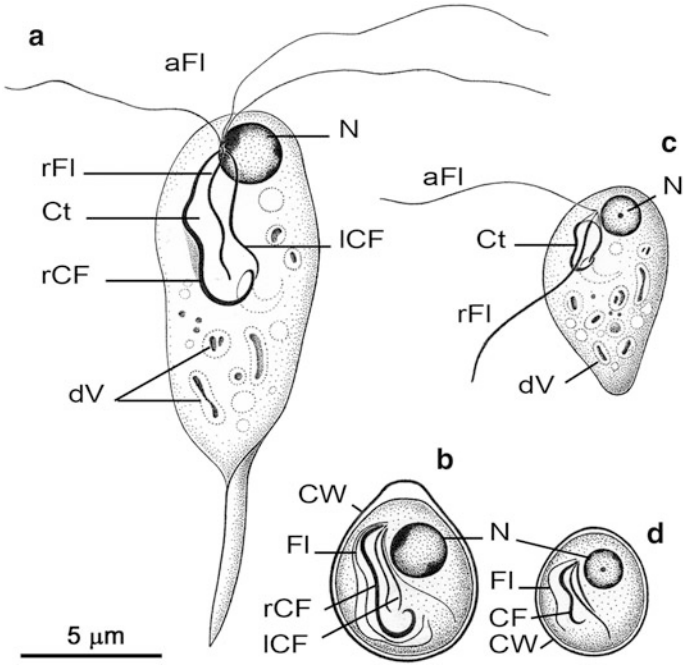

Fig. 1 Diagrammatic representation of *Chilomastix* and *Retortamonas* trophozoites and cysts, based on light microscopic observations. (**a**) *Chilomastix* trophozoite. Ventral view. The cell bears four flagella, three anterior (*aFl*), and one recurrent (*rFl*) that is located in the cytostomal pouch (*Ct*). There are two cytostomal fibers, the right (*rCF*) and the left one (*lCF*) outlining the margins of the cytostome on each side. The more prominent right cytostomal fiber is hooked posteriorly. All flagella and both fibers originate in the basal body complex situated close to the lower right margin of the nucleus (*N*). The cytoplasm contains numerous digestive vacuoles (*dV*) with ingested bacteria. (**b**) The pear-shaped *Chilomastix* cyst is enveloped by a thick cyst wall (*CW*) and contains a single nucleus (*N*), both cytostomal fibers (*RCF*, *LCF*), and internalized flagella (*Fl*). (**c**) *Retortamonas* trophozoite. The cell bears two flagella inserted near the nucleus (*N*). One is directed anteriorly (*aFl*); the other is recurrent (*rFl*), passing through the cytostomal pouch (*Ct*) and extending outside by its distal portion. Digestive vacuoles (*dV*) containing bacteria are present in the cytoplasm. (**d**) The cyst of *Retortamonas* showing a cyst wall (*CW*), nucleus (*N*), internalized flagella (*Fl*), and cytostomal fibers (*CF*)

sometimes twisted in its posterior portion. The most conspicuous feature is the cytostome, a large pouch-like oral aperture on the ventral side (Fig. 1a, c). The cytostomal cavity extends into a tubelike cytopharynx through which food particles enter the interior of the cell and are endocytosed. After protargol or hematoxylin staining, two fibers supporting the lips that surround the cytostome become evident. The right fiber is more prominent and longer than the left. The former is curved posteriorly and forms a hook along the cytopharynx.

The spherical nucleus is located near the anterior margin of the cell, dorsally, and to the left of the cytostome. The flagella and both cytostomal fibrils originate in a basal body complex above the arched anterior margin of the cytostome, close to the

nucleus. In both genera, one flagellum is directed backward into the cytostome, and the others emerge anteriorly as free flagella. There are three anterior flagella in *Chilomastix* (Fig. 1a), but only one in *Retortamonas* (Fig. 1c). The recurrent flagellum (also known as the cytostomal flagellum) is usually short and entirely located in the cytostomal cavity in C*hilomastix* (Fig. 1a), but the recurrent flagellum of *Retortamonas* is proportionately longer, extending through the cytostomal groove and emerging outside with a free terminal portion (Fig. 1c). The beating of anterior flagella moves the cell, and undulations of the recurrent flagellum propel food into the cytopharynx. Detailed descriptions of some species were published by Wenrich (1932) and Nie (1948).

Ultrastructure

Cytoskeleton

Although the two genera of retortamonads differ in the number of flagella, electron microscopy reveals that the kinetids are tetrakont in both, possessing four basal bodies (kinetosomes) arranged in two orthogonal pairs. Whereas all basal bodies in *Chilomastix* bear flagellar axonemes (Fig. 3c), one basal body in each pair is barren in *Retortamonas* (Figs. 2 and 5a). All flagella have the typical 9 + 2 organization of axonemal microtubules. The cytostomal (recurrent) flagellum is modified by two or three lateral vanes stiffened by paraxonemal lamellae (Figs. 2, 3a, e, and 5c, d). Electron microscopy provides unequivocal evidence that the recurrent/cytostomal flagellum of retortamonads does not form an undulating membrane attached to one of the cytostomal lips, thus correcting earlier interpretations based on light microscopy (Boeck and Tanabe 1926; Nie 1948).

The basal bodies also give rise to several different fibrillar roots, most of which integrate into the complex cytoskeleton of the cytostome. The most prominent is the microtubular root that descends from the basal body of the recurrent flagellum (root 2, according to the nomenclature recommended by Yubuki et al. 2013), a major support of the right cytostomal lip. In the kinetosomal area, this root originates as a hooked row of microtubules that is rolled into an incomplete cylinder on its left side (Fig. 2). The cavity of this "gutter" (Fig. 5b) opens posteriorly into the cytostomal groove (Fig. 5b, d). The curved band of the gutter microtubules separates from the main row to form the inner subroot (the hooked band), which extends into the cytopharynx as its microtubular support (Figs. 2 and 3b). The right portion of the root gradually expands into a flat ribbon through the addition of microtubules and serves as a major support of the right wall of the cytostomal groove (Figs. 2 and 3b). The second microtubular root originating at the recurrent basal body descends to the left wall of the groove, strengthening the left cytostomal lip (root 1, according to Yubuki et al. 2013; Figs. 2 and 5d).

Another prominent structure originating at the kinetosomes is the striated (paracrystalline) lamella (composite fiber), which is adjacent to the inner face of the microtubular root of the right cytostomal wall and extends posteriorly into the cytopharynx (Figs. 2, 3a, b, e, f, and 4). Both lips of the cytostome are connected at the top of the groove by an arched microfibrillar bundle, the

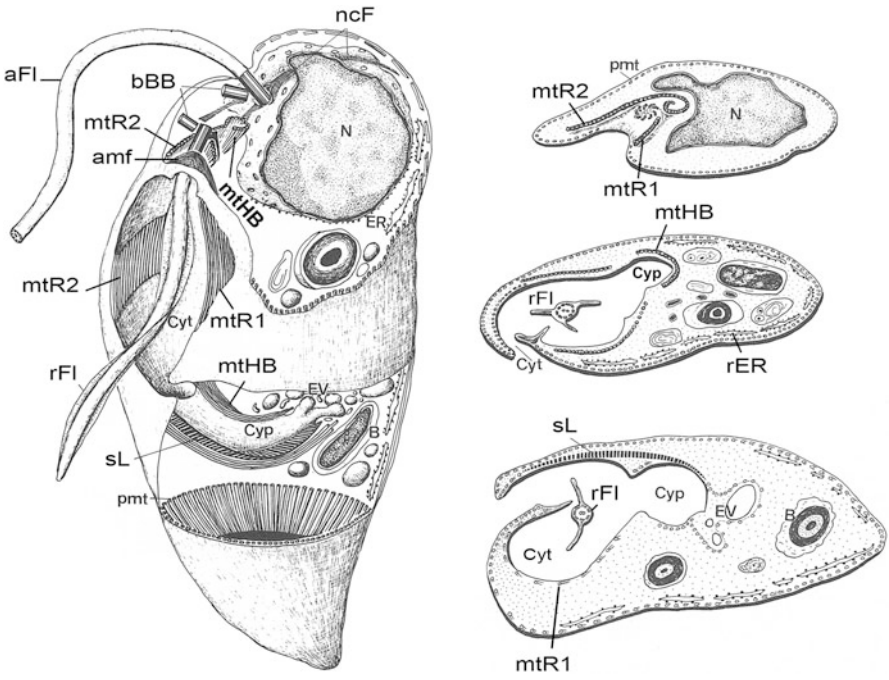

Fig. 2 Diagrammatic reconstruction of the *Retortamonas* trophozoite ultrastructure. The cell is surrounded by a corset of subpellicular microtubules (*pmt*). Four basal bodies, arranged in two pairs, are positioned near the nucleus (*N*). Only one basal body from each pair gives rise to a flagellum; the other two are barren (*bBB*). The free anterior flagellum (*aFl*) extends from the cell subapically. The recurrent flagellum (*rFl*) enters the cytostomal cavity (*Cyt*) and continues its course backward, extending out of the cytostome posteriorly. The left and right cytostomal lips are connected at the top by the arched bundle of microfilaments (*amf*) and maintained by microtubular roots (*mtR1*, *mtR2*). The root of the right cytostomal wall (*mtR2*) is reinforced by a striated lamina (*sL*) which extends along the cytopharyngeal region (*Cyp*). The hooked left side of the mtR2 root is rolled anteriorly into an incomplete cylinder and eventually separates as a hook band (*mtHB*) reinforcing the cytopharyngeal tube. The striated fiber "nuclear connector" (*ncF*) extends from kinetosomal complex to the top of the nucleus. Endocytotic vesicles (*EV*) are mainly formed at the end of the cytopharynx. The endoplasmic reticulum (*ER*) occurs along the subpellicular microtubules. Numerous endocytic vesicles (EV) and food vacuoles containing ingested bacteria (*B*) occur in the cytoplasm (From Brugerolle and Mignot 1990, slightly modified)

"arched fiber" (Figs. 2 and 3a). A fine striated fiber (the "nuclear connector") extends from the basal body complex to the proximal surface of the nucleus (Figs. 2 and 5a).

Both retortamonad genera possess a complete corset of interconnected "subpellicular" microtubules that maintain cellular rigidity (Figs. 2, 3d, and 5b, d). These microtubules originate in an electron-dense rim ("lapel") that extends dorsally around the site of flagellar emergence (Fig. 3c). Further information on cytoskeletal components of *Chilomastix* and *Retortamonas* (the latter from insect hosts only) are provided by Brugerolle (1973, 1977, 1991) and Bernard et al. (1997). Simpson

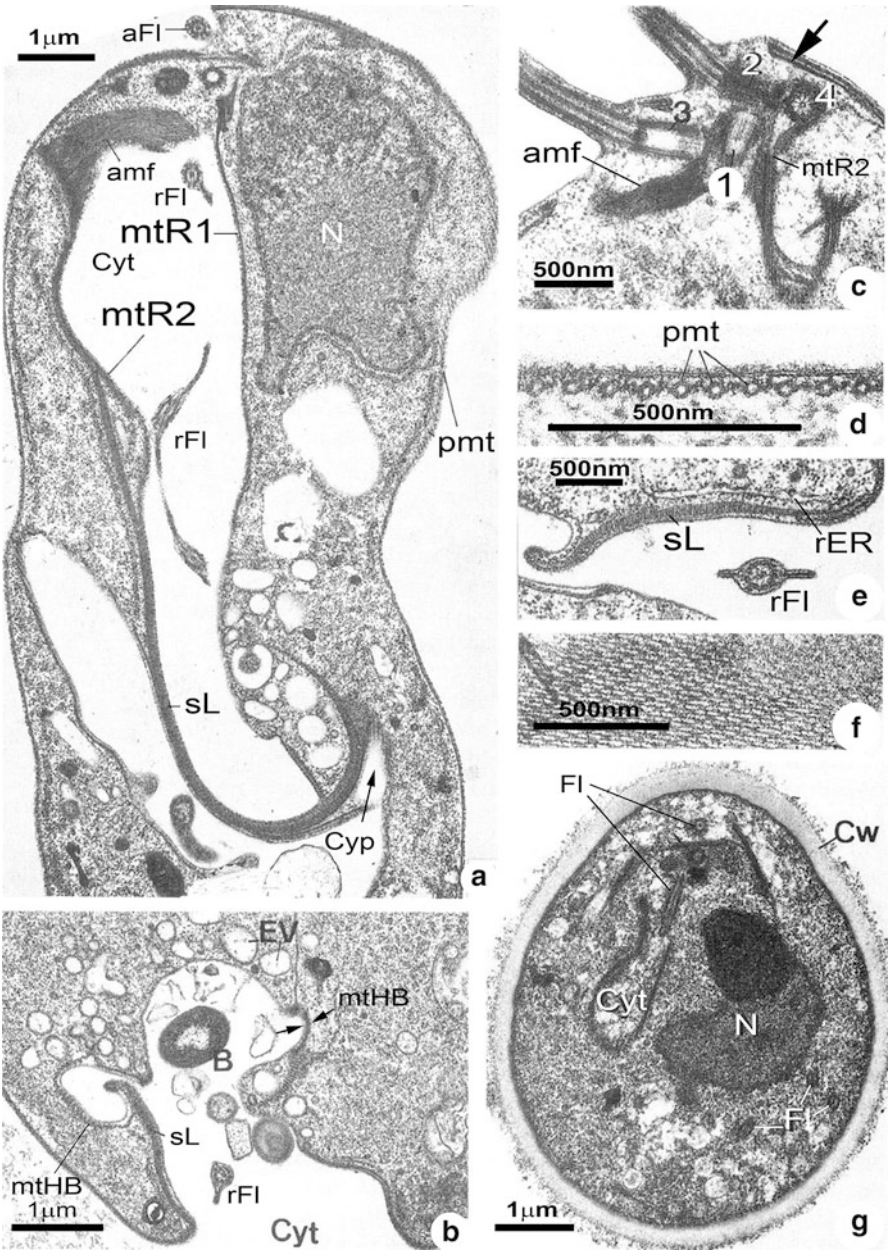

Fig. 3 The ultrastructure of trophozoites of *Chilomastix caulleryi* from a toad. (**a**) Longitudinal section through the cytostomal cavity (*Cyt*) showing the recurrent flagellum (*rFl*) with lateral vanes, the microtubular roots (*mtR1*, *mtR2*) bordering the left and right cytostomal lips, and an arched bundle of microfilaments (*amf*) connecting both lips at the top of the cytostome ("arched fiber"). The striated lamina (*sL*) adjacent to the mtR2 is curved posteriorly around the cytopharyngeal region (*Cyp*). One

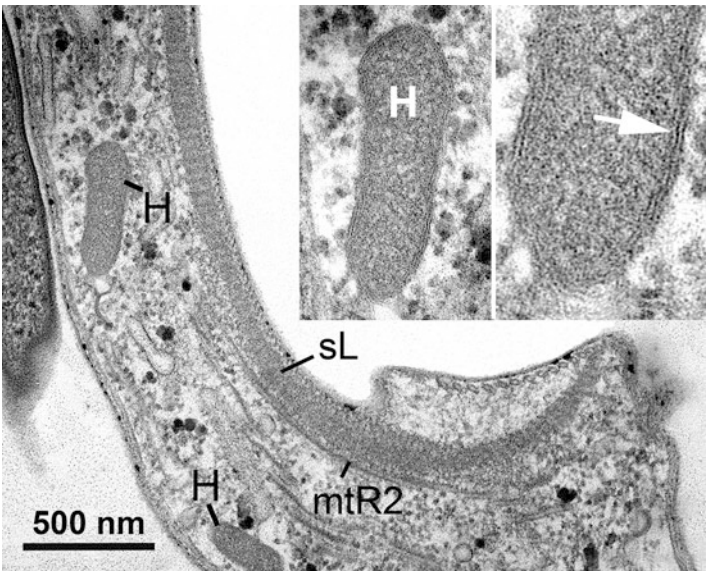

Fig. 4 Hydrogenosome-like organelles of *Chilomastix caulleryi*. Transmission electron micrograph of section through area close to the right cytostomal lip shows the elongate hydrogenosome-like organelles (*H*). Also shown is the striated cytostomal lamina (*sL*) adjacent to microtubules of the mtR2 root. As apparent at higher magnification (inset), the organelles are limited by two closely adjacent membranes (*arrow*) and lack cristae

(2003) published a proposal of universal terminology for flagellar roots and fibers in excavates, which was revised by Yubuki et al. (2013).

Weerakoon et al. (1999) examined the localization of centrin in *Chilomastix cuspidata* cells using conventional and confocal immunofluorescent microscopy.

Fig. 3 (continued) anterior flagellum (*aFl*), the subpellicular microtubules (*pmt*), and the nucleus (*N*) are also visible. (**b**) Transverse section through the cytopharyngeal region where the endocytosis occurs. Note the naked cytoplasmic membrane between the cytoskeletal structures of the cytopharynx, numerous pinocytic vesicles in the neighboring cytoplasm, and an engulfed bacterium (*B*) in the lumen of the cytopharynx. The derivatives of the hook band microtubules (*mtHB*) can be seen at both sides of the cytopharyngeal tube. Also shown is the striated lamina (*sL*) and a part of the cytostomal compartment (*Cyt*) with the recurrent flagellum (*rFl*). (**c**) The mastigont of *Chilomastix*, with two pairs of basal bodies and associated fibers. Basal bodies *2*, *3*, and *4* underlie the anterior flagella; the recurrent one (*1*) gives rise to recurrent/cytostomal flagellum. The arrow points to the lapel, the nucleating site of subpellicular microtubules. (**d**) Transverse section through the layer of subpellicular microtubules (*pmt*) interconnected by side arms. (**e**) Transverse section through the striated lamina (*sL*) of the right wall of the cytostomal pouch underlain with rough endoplasmic reticulum (*rER*) and through the recurrent flagellum (*rFl*) with two lateral vanes. (**f**) Tangential section of the striated lamina showing its paracrystalline structure. (**g**) *Chilomastix aulastomi* cyst from the horseleech, surrounded by a thick cyst wall (*Cw*) and containing the nucleus (*N*), internalized flagella (*Fl*), and cytostomal fibers (*Cyt*) (From Brugerolle and Mignot 1990)

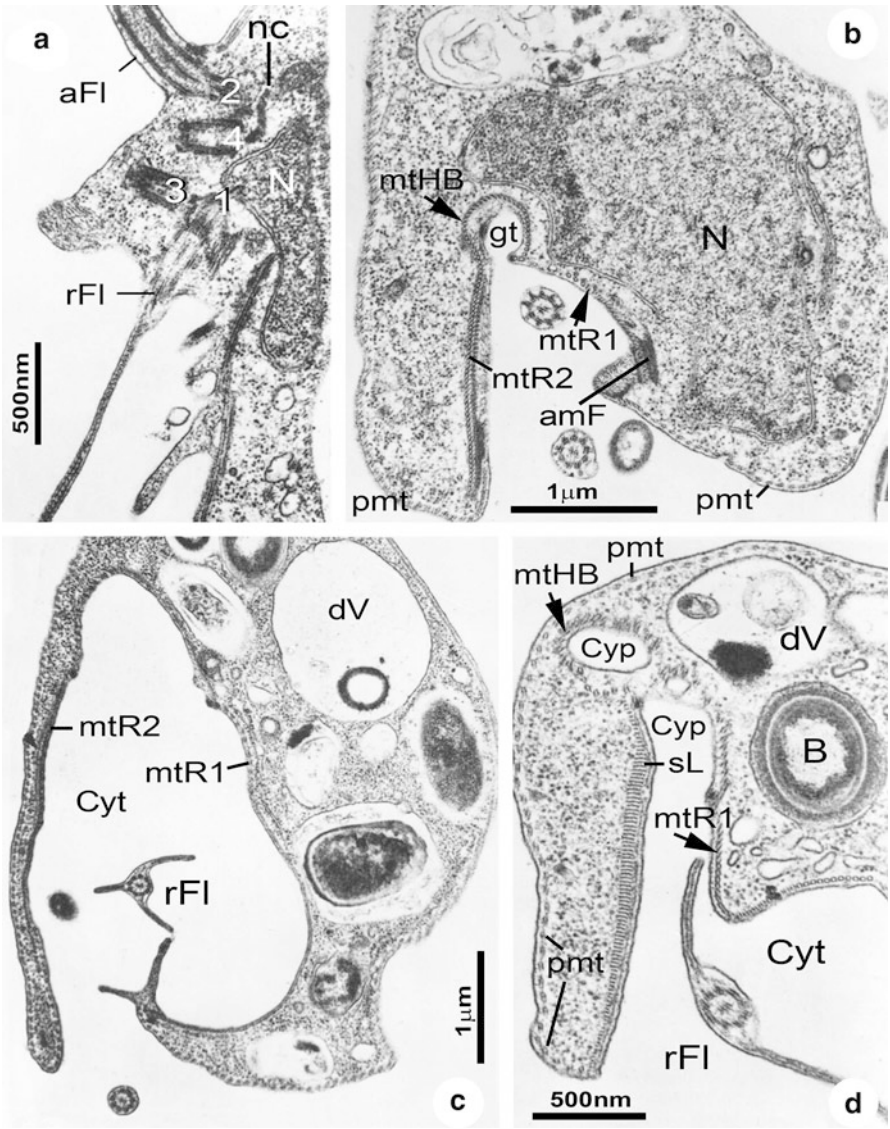

Fig. 5 The ultrastructure of *Retortamonas* from crane fly larvae. (**a**) Basal body arrangement in *Retortamonas*. The basal bodies, localized close to the nucleus (*N*), are grouped in two pairs: the anterior pair (*2*, *4*) and the posterior pair (*3*, *1*). Basal body 2 gives rise to the anterior flagellum (*aFl*); the recurrent flagellum (*rFl*) originates from basal body 1. The basal bodies 3 and 4 are barren. The origin of the nuclear connector fiber (*nc*) can be seen at the base of the basal body 2. (**b**) Transverse section through the upper part of the cytostomal pocket, showing the opening of the gutter (*gt*) into the cytostomal cavity to form the cytopharyngeal tube. The density attached distally to mtR1 microtubules is an anchoring point for microfilaments of the "arched fiber" (*amF*). The cell membrane of *Retortamonas* is supported by a corset of subpellicular microtubules (*pmt*). (**c**) Transverse section through the central part of the cytostomal pocket showing the recurrent

The authors found that in addition to its localization in the basal body area, the centrin signal colocalized with the tubulin signal along a microtubular root that passes through the cytostomal pouch. The major site of centrin association was most likely the hook band subroot of the cytostomal skeleton. Centrin is a calcium-binding protein that is a known component of the pericentriolar complex, but it is also involved in contractility and a variety of signal functions. Weerakoon et al. (1999) speculated that root-associated centrin in the *Chilomastix* cytostome might be involved in signaling related to the capture and endocytosis of food particles in the cytopharynx.

Nucleus and Cytoplasmic Organelles

The nucleus is always located in the most anterior part of the cell, close to the basal bodies (Figs. 1a, c, 2, and 3a). Its shape is almost spherical, with some depressions in the area facing the basal bodies and the intracytoplasmic portion of the recurrent flagellum axoneme. Condensed material visualized by transmission electron microscopy in the posterior part of the interphase nuclei of *Retortamonas* and *Chilomastix* (Brugerolle 1973, 1977) most likely represents the nucleolus and apparently corresponds to the membrane-associated chromatic plaques that give a negative result in Feulgen reactions, as described by Nie (1948).

The cytoplasm of the retortamonads contains numerous pinocytotic vesicles and large digestive vacuoles with ingested bacteria. Endocytosis occurs in the terminal parts of the cytopharynx, where the cytoskeletal armature is discontinuous. The rough endoplasmic reticulum is present, but it is usually not abundant. A single or double layer of reticulum cisternae is typically located at the cell periphery and around the nucleus (Figs. 2 and 3a, e). No Golgi apparatus is apparent (at least in the form of organized stacks of dictyosomes), and peroxisome organelles are absent. Brugerolle (1973) observed in *Chilomastix* a circular reticulum of smooth cisternae beneath the nucleus, but the function of this structure is unknown. Retortamonads do not possess typical mitochondria; however, double-membrane-bounded organelles without cristae that resemble hydrogenosomes have been demonstrated in *Chilomastix cuspidata* (Hampl and Simpson 2008) and other *Chilomastix* species (Fig. 4). Additional cytoplasmic components of retortamonads include free ribosomes and inclusions of multigranular glycogen.

Fig. 5 (continued) flagellum (*rFl*) with three vanes and the microtubular roots supporting the right (*mtR2*) and the left (*mtRl*) wall of the cytostome (*Cyt*). Digestive vacuoles (*dV*), some with ingested bacteria, are present in the cytoplasm. (**d**) Transverse section through the posterior part of the cytostome (*Cyt*) and the cytopharynx (*Cyp*) showing the recurrent flagellum (*rFl*) with two vanes sectioned, the microtubules of the left cytostomal wall (*mtR1*), the striated lamina (*sL*) which extends to the cytopharyngeal tube, and the cytopharyngeal derivatives of the hooked band microtubules (*mtHB*). A digestive vacuole (*dV*) and an endocytosed bacterium (*B*) are shown in the cytoplasm. Note the corset of subpellicular microtubules (*pmt*) (Pictures by courtesy of G. Brugerolle)

The Cyst Stage

The cyst is the resting stage that is resistant to unfavorable environments and serves to transmit infection. Retortamonad cysts are ovoid, pyriform, or lemon-shaped and exhibit a thick cell wall made of filamentous material (Figs. 1b, d and 3g). There is no operculum or pore in the cyst wall. The interior of the cyst contains basal bodies with internalized flagella, the retained cytoskeletal armature of the cytostome and cytopharynx, glycogen granules, and a single nucleus (Fig. 3 (12)). Brugerolle (1973) observed precystic stages of *Chilomastix* with flagella withdrawn into the cytoplasm, partly disorganized "subpellicular" microtubules, and numerous secretory vesicles trafficking cyst wall material to the cell surface. Retortamonads do not divide inside the cyst. A previous description of within-cyst division by Kofoid and Swezy (1920) has not been confirmed by subsequent investigators (Geiman 1935).

Reproduction

Trophozoites of retortamonads reproduce by binary fission, but the details of their mitosis and cell division are not well known. Somewhat conflicting observations by early authors and electron microscopic evidence (Brugerolle 1973, 1977) suggest that the process is similar to that observed in diplomonads (Brugerolle 1974). Mitosis proceeds inside the nucleus, which is enveloped by the persisting nuclear membrane, with the aid of intranuclear microtubules, but the mitotic spindle nucleates outside the nucleus as a "hemispindle" (i.e., the process is a type of semi-open mitosis). The two pairs of basal bodies (kinetosomes) separate during prophase and localize to the nuclear poles (Boeck and Tanabe 1926). The spindle microtubules then nucleate in the perikinetosomal area of the polar basal bodies (see Fig. 7 in Brugerolle 1973). The formation of a "paradesmose" (the extranuclear spindle characteristic of mitosis in Parabasalia), reported by some investigators (Bishop 1934, 1935; Geiman 1935), does not in fact occur in retortamonads.

The chromosomes condense in early prophase; six have been detected in *Chilomastix intestinalis* by the Feulgen reaction (Nie 1948). The cytostome of the parent cell disintegrates by the end of the prophase, and the de novo assembly of the daughter organelles is completed during telophase (Nie 1948). The daughter mastigonts are fully reorganized during the final phase of cytokinesis. The parent flagella are probably retained and redistributed to the daughter mastigonts (Bishop 1931, 1934). Further studies employing advanced methodology are necessary to gain better insight into mitosis and cell division in retortamonads.

Taxonomy

Grassé (1952) established the order Retortamonadida and placed it in the superorder Metamonadina, which at the time encompassed oxymonads, retortamonads, and several orders of parabasalids. Metamonads were later transferred to Archezoa (Cavalier-Smith 1983, 1998; see below). Alternatively, Simpson and Patterson (1999) proposed an "excavate hypothesis" grouping flagellates equipped with a

ventral groove associated with the recurrent flagellum and a characteristic cytoskeleton. After several revisions of the metamonad grouping, Cavalier-Smith (2003) returned to something similar to Grassé's concept, which he modified by including diplomonads and the free-living anaerobe *Carpediemonas*. Cavalier-Smith (2003) also abandoned Archezoa as a taxon and placed the Metamonada as a phylum of the Excavata infrakingdom. Meanwhile, Simpson (2003) proposed Fornicata as a taxon to include a phylogenetically coherent subset of metamonads, consisting of retortamonads, diplomonads, and *Carpediemonas* (and later including the subsequently characterized *Carpediemonas*-like organisms; see below and Kolisko et al. 2010). In the revised classification of eukaryotes proposed by the International Society of Protistologists (Adl et al. 2012), Retortamonadida, together with Diplomonadida and *Carpediemonas*-like organisms, are placed in the Fornicata Simpson 2003, a subgroup of Metamonada Cavalier-Smith 1987, as emended by Cavalier-Smith (2003), within the supergroup Excavata Cavalier-Smith 2002, as emended by Simpson (2003).

Order: Retortamonadida Grassé 1952

Retortamonadida are Fornicata with a single karyomastigont possessing four basal bodies and two or four flagella. One flagellum is recurrent, has two or three lateral vanes, and is associated with the ventral cytostomal pouch. In the phylogenetic tree of Fornicata (Fig. 6), Retortamonadida is split into two different clades, indicating that Retortamonadidae is currently polyphyletic (see section "Evolutionary History").

Family: Retortamonadidae, Wenrich 1932

Characteristics as for order Retortamonadida

Genus: *Retortamonas* Grassi 1879

Objective synonym: *Plagiomonas* Grassi 1881

Subjective synonyms: *Embadomonas* Mackinnon 1911; *Waskia* Wenyon and O'Connor 1917.

Members of the genus possess one anterior and one recurrent flagellum. The recurrent flagellum is directed into the cytostomal pocket, from which a free distal portion of the flagellum extends. Distinctive morphological characters among species include body shape and size, the size of the posterior spike, the relative length of the flagella, the length of the free portion of the cytostomal flagellum, and the size of the cytostome. All known species are intestinal endobionts of insects and vertebrates.

Type species:

Retortamonas gryllotalpae Grassi 1879 [Synonym: *Plagiomonas gryllotalpae* Grassi 1881], from the intestine of the mole cricket. The species was redescribed by Wenrich (1932).

More than 20 species of *Retortamonas* have been described. The retortamonads from crane fly larvae, *Retortamonas agilis* (Mackinnon 1911) and *Retortamonas*

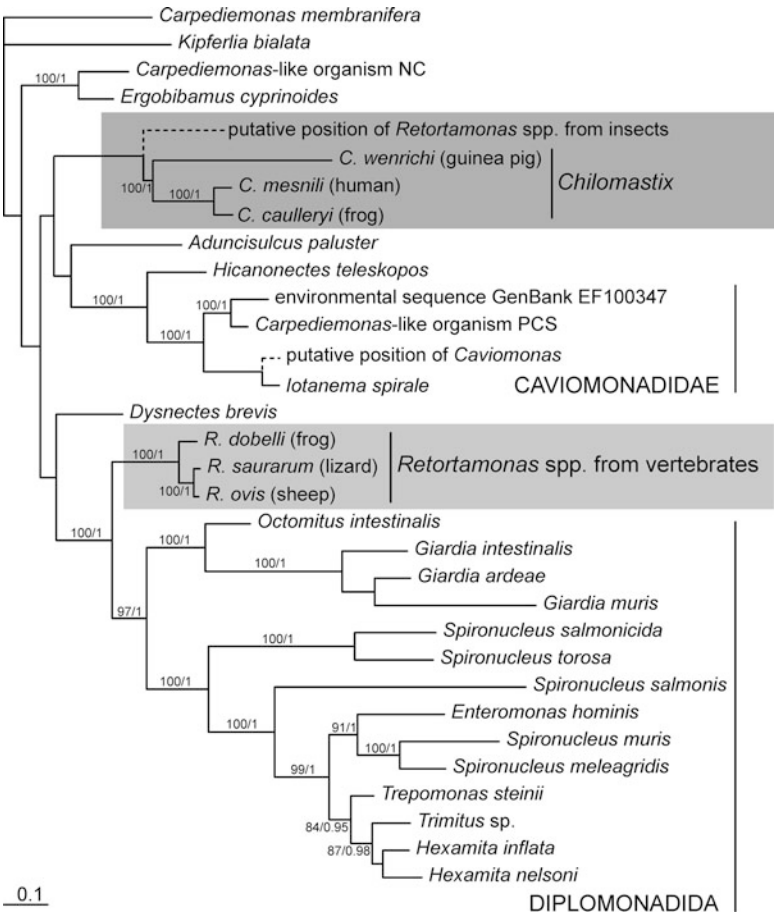

Fig. 6 Phylogenetic tree of Fornicata based on SSU rDNA sequences. The tree was constructed by the maximum likelihood method in RAxML (GTRGAMMAI model). The values at the branches represent statistical support in bootstrap values (RAxML)/posterior probability (MrBayes); support values below 50/0.95 are not shown. The putative position of *Retortamonas* from insects is shown in the tree

alexeieffi (Mackinnon 1912), were subjected to relatively detailed electron microscopic studies (Brugerolle 1977) and thus represent the model species of the genus, though Brugerolle (2006) also briefly described the ultrastructure of *R. hodotermitis*. Other *Retortamonas* species were found in larvae of beetles (*R. caudacus*, *R. phylophagae*), in roaches (*R. blattae*), termites (*R. termitis*, *R. hodotermitis*), water bugs (*R. belostomae*), amphibians (*R. dobelli*), reptiles (*R. boae*, *R. saurarum*, *R. testudae*), and various mammals including humans (e.g.,

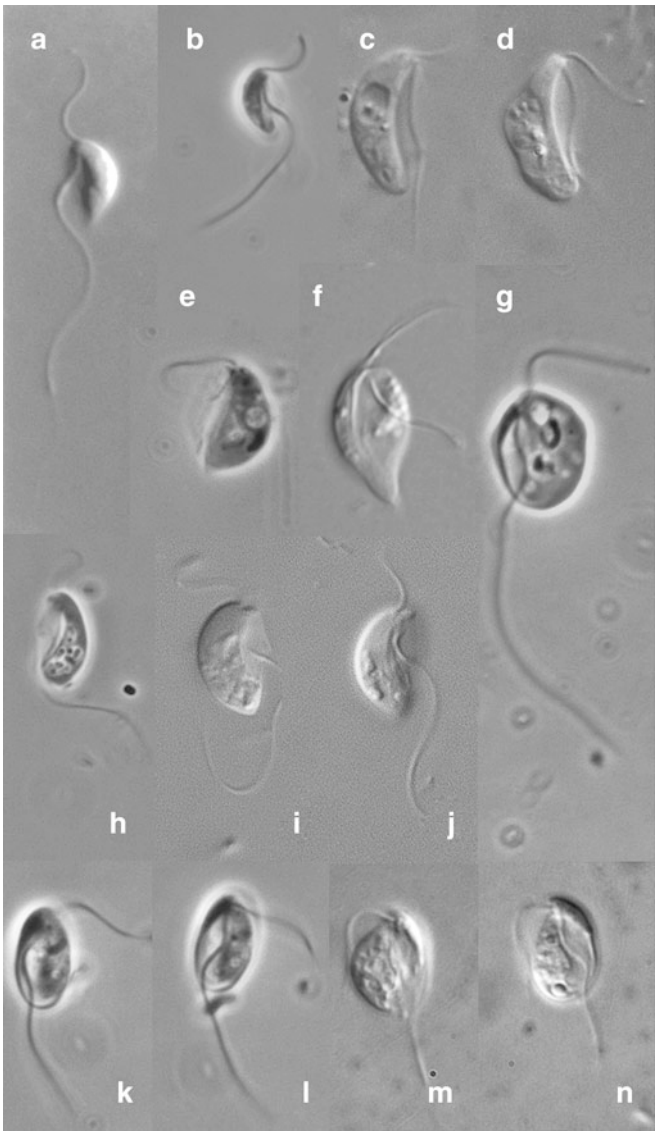

Fig. 7 Light microscopic photographs of free-living *Carpediemonas*-like organisms. (**a**), (**b**) *Carpediemonas membranifera.* (**c**), (**d**) *Kipferlia bialata*. (**e**) *Dysnectes brevis.* (**f**) *Dysnectes* sp. SIVOTA. (**g**) *Hicanonectes teleskopos*. (**h**) *Ergobibamus cyprinoides*. (**i**), (**j**) *Carpediemonas*-like organism NC. (**k**), (**l**) *Aduncisulcus* sp. PCE. (**m**), (**n**) *Aduncisulcus paluster*. Scale bar is 5 μm for all figures (From Kolisko et al. 2010, with permission of the Society for Applied Microbiology and Blackwell Publishing Ltd.)

R. intestinalis, *R. bradypi*, *R. cuniculi*, *R. mitrula*, *R. ovis*, *R. caviae*, *R. ruminantium*). The ostrich *Struthio camelus* is only avian host of retortamonas reported (MartinezDiaz et al. 2001). For an almost complete list of *Retortamonas* species, see Ansari (1955).

Genus *Chilomastix* Alexeieff 1912

Objective synonym: *Macrostoma* Alexeieff 1909

Subjective synonyms: *Fanapepea* Prowazek 1911; *Cyathomastix* Provazek and Werner 1915; *Difamus* Gäbel 1914; *Tetrachilomastix* da Fonseca 1915.

Members of this genus are endobiotic or free-living Retortamonadidae with three anterior and one recurrent flagellum. The recurrent flagellum is located in the cytostomal pocket and does not extend from it. Distinctive features among species include body size and shape; the relative length of the posterior spike; the relative length of the anterior flagella; the size, shape, and position of the cytostome; and, in some species, the supposed host specificity. Members of the genus are intestinal endobionts of many classes of vertebrates (including humans) and some invertebrates. One species is free-living.

Type species:

Chilomastix caulleryi (Alexeieff 1909) [*Macrostoma caulleryi* Alexeieff 1909]

This species is commonly found in the cloaca of amphibians. The original generic name *Macrostoma* was rejected because it is a junior homonym of *Macrostoma* Risso 1826.

Over 30 species of *Chilomastix* have been described. Light microscopic studies of general importance were published by Boeck and Tanabe (1926), Geiman (1935), Bishop (1935), Nie (1948, 1950), McDowell (1953), and Russel Gabel (1954). The ultrastructure of the genus was studied by Brugerolle (1973) and Bernard et al. (1997).

Examples of *Chilomastix* species:

Chilomastix mesnili (Wenyon 1910) [Synonyms: *Fanapepea intestinalis* Prowazek 1911, *Cyathomastix hominis* Prowazek and Werner 1914, *Difamus tunensis* Gäbell 1914, *Tetrachilomastix bengalensis* Chatterjee 1923] occurs in the cecum and colon of humans, monkeys, and pigs. The susceptibility of monkeys and pigs to human isolates was confirmed experimentally (Kessel 1924, 1928). The potential pathogenicity of this species in humans is discussed above.

Other *Chilomastix* species with mammalian hosts were described from horses, goats, cattle, hares, and rabbits. The major rodent species, with relatively wide host spectra, are *Chilomastix bettencourti* da Fonseca 1915 and *Chilomastix intestinalis* Kuczynski 1914. The former is commonly found in laboratory and wild rats, as well as mice, hamsters, voles, and gerbils. The latter occurs in guinea pigs, hamsters, and rabbits. Another frequent inhabitant of guinea pig cecum is *Chilomastix wenrichi* Nie 1948.

Chilomastix gallinarum Martin and Robertson 1911 is a common avian species present in the ceca of chicken, ducks, turkeys, pheasants, quails, and partridges. Boeck and Tanabe (1926) and McDowell (1953) published morphological studies of this species. Its pathogenic potential is discussed above. Three *Chilomastix* species were recorded in marine fishes (Lavier 1936), and *C. bursa* Moskowitz 1951 in the intestines of frogs.

The type species of the genus *C. caulleryi* (Alexeieff 1909) is found in the cloaca of anuran and urodelan amphibians. This large species can apparently infect the horseleech *Haemopis sanguisuga* in shared biotopes; however the autochthonous species of the horseleech, *C. aulastomi* Bělař 1921, is also identifiable by morphology.

The host list, with pertinent references and further information on *Chilomastix* species from vertebrates, is available from Kulda and Nohýnková (1978). Species living in invertebrates such as termites and sea urchins are listed by Grassé (1952).

The free-living species *Chilomastix cuspidata* (Larsen and Patterson 1990), originally assigned to the genus *Percolomonas*, is widely distributed in anoxic marine, brackish, and freshwater habitats (Bernard et al. 1997, 2000).

Maintenance and Cultivation

Various species of the Retortamonadida from vertebrates, including humans, can be isolated and maintained in xenic cultures (i.e., cultures containing a concomitant mixture of unidentified bacteria). None of the retortamonad isolates have been grown axenically, and no attempts to obtain monoxenic cultures (or any other type of gnotobiotic cultures) have been reported. Some isolates of *Retortamonas* and *Chilomastix* spontaneously form cysts in culture.

The crude biphasic media originally developed for the cultivation of intestinal amoebae, such as Locke-egg-serum medium (Boeck and Drbohlav 1925) or serum-Ringer-egg medium (Dobell and Laidlaw 1926), are still the best choices for the isolation of primary cultures of endobiotic species. Cultures established in biphasic media can usually be transferred and maintained in simple liquid media, such as Loeffler's serum-Ringer medium (Wenrich 1947) or TYSGM medium (Diamond 1982). The optimal pH for the cultivation of retortamonads is 7.2–7.4, and the recommended temperatures for mammalian and avian species are 35–37 °C and 38–40 °C, respectively. Under these conditions, cultures should be transferred at 2- to 3-day intervals. Isolates from amphibians, reptiles, and some invertebrates grow at room temperature (18–25 °C), and transfers at 1- or 2-week intervals are recommended.

The free-living species C*hilomastix cuspidata* can be maintained in sea-water-Cerophyl medium (ATCC Medium 1525, American Type Culture Collection, www.attc.org). The medium consists of a 1:1 mixture of filter-sterilized artificial

seawater and an autoclaved solution prepared by boiling rye grass Cerophyl or its equivalent.

In our experience, isolates of retortamonads can be cryopreserved in the presence of 5% dimethyl sulfoxide using a standard procedure and stored indefinitely in liquid nitrogen.

Reports on the cultivation of retortamonads from humans and animals were published by Hogue (1921), Boeck (1921), Boeck and Tanabe (1926), Collier and Boeck (1926), Bishop (1934), and Dobell (1935); brief procedures are also available in publications of Silberman et al. (2002), Cepicka et al. (2008), and Takishita et al. (2012).

Chilomastix cuspidata and two *Retortamonas* cultures can be obtained from the American Type Culture Collection; several isolates of *Retortamonas* and *Chilomastix* from mammals, amphibians, and reptiles are available in the culture collection of the Department of Parasitology, Charles University in Prague.

Evolutionary History

Retortamonads were once thought to belong to the "Archezoa," a group of putatively primitive eukaryotes that diverged before the acquisition of mitochondria by an ancestor of all living eukaryotes with this organelle (Cavalier-Smith 1983, 1987). Famously, the "archezoa hypothesis" has declined in popularity in the twenty-first century, as organelles interpreted as mitochondrial derivatives (e.g., hydrogenosomes and mitosomes) have been found in most putative archezoans (see Lindmark and Müller 1973; Simpson and Roger 2004; Tovar et al. 1999, 2003; Tachezy and Šmíd 2007). Double-membrane-bounded organelles resembling hydrogenosomes have been found also in *Chilomastix cuspidata* (Hampl and Simpson 2008) and some other *Chilomastix* species (Fig. 4). However, aside from gross morphology, no data are currently available on the biogenesis and function of these organelles in retortamonads.

The retortamonads are currently classified within the taxon Fornicata (Simpson 2003). According to phylogenetic analyses based on the SSU rRNA gene and a concatenate of several protein-coding genes (Takishita et al. 2012; Cepicka et al. 2008), the genera *Retortamonas* and *Chilomastix* form two unrelated clades. *Retortamonas*, represented by species from vertebrates, appears to be the closest relatives of diplomonads, while *Chilomastix* branched within the paraphyletic grade of *Carpediemonas*-like organisms. Cepicka et al. (2008) suggested that *Retortamonas* might be polyphyletic as well, because species from insects possess a complete microtubular corset, similar to that of *Chilomastix*, while the species from vertebrates seem to lack it (Cepicka et al. 2008). Accordingly, sequence data from *Retortamonas* species from insects, presented by Smejkalová et al. at the International Congress of Protistology in Vancouver, Canada (2013), indicate that insect retortamonads are closely related to *Chilomastix*, thus confirming the polyphyly of the genus *Retortamonas* (see also Fig. 6). These findings indicate that taxonomic revision of Retortamonadida can be expected soon.

Coda: *Carpediemonas*-Like Organisms and Caviomonadidae

Introduction

Carpediemonas-like organisms (CLOs) are a small assemblage of poorly known excavates. CLO cells are usually biflagellate, with one anterior and one recurrent flagellum, and possess a longitudinal ventral groove. All CLOs are anaerobic and almost all are free-living, occurring in marine hypoxic sediments. They seem to be relatively common in such environments and have been isolated worldwide. Various media for anaerobic free-living marine protists were shown to be suitable for free-living CLOs (see Kolisko et al. 2010). It was shown only recently that the endobiotic genus *Caviomonas*, which had been previously thought to be a highly reduced diplomonad, belongs to CLOs, being closely related to the endobiotic genus *Iotanema* and free-living, marine isolate PCS (Yubuki et al. 2016). Because caviomonads substantially differ from other CLOs by possessing a single flagellum and a highly reduced flagellar apparatus, they will mainly be treated in a separate section (see below). Together with diplomonads and retortamonads, the CLOs form the clade Fornicata. Recent molecular phylogenetic analyses showed that CLOs are paraphyletic.

The first known CLO was *Kipferlia bialata*, described by Ruinen (1938) as *Cryptobia bialata*. This species was, however, neglected for more than 50 years and was not recorded again before the end of the twentieth century (Fenchel et al. 1995). Two species of *Caviomonas* were described in 1950 and 1970, respectively (Nie 1950; Navarathnam 1970). *Carpediemonas membranifera*, the best known CLO, was described by Larsen and Patterson (1990) as *Percolomonas membranifera*, but was later transferred by Ekebom et al. (1996) to their newly created genus *Carpediemonas*. An ultrastructural study of *C. membranifera* by Simpson and Patterson (1999) was crucial for the formulation of the so-called excavate hypothesis. The morphological similarity of *Carpediemonas membranifera* and *Cryptobia bialata* was noted by Lee and Patterson (2000), who transferred the latter species to the genus *Carpediemonas*. The remaining species of free-living CLOs, *Dysnectes brevis*, *Hicanonectes teleskopos*, *Ergobibamus cyprinoides*, and *Aduncisulcus paluster*, were all described recently, as was the caviomonad *Iotanema spirale* (Yubuki et al. 2007, 2016; Park et al. 2009, 2010). At the same time, it was shown by SSU rRNA gene phylogenies that *Carpediemonas membranifera* and *C. bialata* are not closely to each other, and the genus *Kipferlia* was therefore created for *C. bialata* (Kolisko et al. 2010); the distant relationship between *Carpediemonas* and *Kipferlia* was corroborated by a multigene phylogenetic analysis of Fornicata (Takishita et al. 2012) and an ultrastructural study of *Kipferlia bialata* (Yubuki et al. 2013).

Light Microscopy

CLOs are small flagellates (5–18 μm) with two heterodynamic flagella and a conspicuous ventral groove (but see below for Caviomonadidae). The trophozoites are naked and possess a single, anteriorly located nucleus. The flagella are inserted slightly

subapically, on the ventral side of the cell, close to the nucleus. One flagellum (anterior flagellum, F2) is directed anteriorly and is approximately the same length as the cell. The other flagellum (recurrent, R, F1) is directed posteriorly, runs through the ventral groove, and continues behind the cell. This flagellum is usually substantially longer than the cell; in *Dysnectes*, however, the recurrent flagellum is shorter than the cell body. The recurrent flagellum beats in the ventral groove and creates a current that draws bacteria into the posterior part of the groove where they are ingested. The longitudinal groove occupies almost the whole ventral side of the cell.

Slow movement with little rotation is typical of *Carpediemonas* and *Ergobibamus*; occasional and very slow swimming is characteristic of *Dysnectes*, which usually lies on the substratum and rocks back and forth by moving the anterior flagellum. *Kipferlia* also swims slowly and often adheres to the substratum (Yubuki et al. 2013), whereas *Hicanonectes* rotates rapidly while moving (Park et al. 2009).

Flagellar Apparatus

In general, the organization of the flagellar apparatus of CLOs is similar to that of retortamonads (but see below for Caviomonadidae). Most CLOs possess two flagella (members of Caviomonadidae possess a single flagellum; see below), the recurrent flagellum (F1, R) and the anterior flagellum (F2) (Fig. 8a–e). Transmission electron microscopy shows that they differ in the number of basal bodies, however; in addition to the two basal bodies bearing flagellar axonemes (i.e., basal bodies 1 and 2), there are barren basal bodies: one in *Carpediemonas*, and two in *Kipferlia* (Fig. 8b), *Ergobibamus*, *Aduncisulcus*, and *Hicanonectes* (while the caviomonads *Caviomonas* and *Iotanema* have one and three barren basal bodies respectively; see below). Ultrastructurally, the basal bodies and the flagellar axonemes have a typical eukaryotic organization. As in retortamonads, the anterior flagellum of CLOs is smooth with no accessory structures, whereas the recurrent flagellum has one to three longitudinal vanes, each supported by a paraxonemal lamella with different ultrastructures and unknown biochemical composition (though these have been lost in Caviomonadidae). The ventral vane is always the broadest and is the only vane present in *Kipferlia* (Fig. 8c).

The two flagella-bearing basal bodies are arranged almost perpendicularly or at a slightly obtuse angle (Fig. 8a). The basal body of the anterior flagellum (basal body 2) is associated with the anterior root which consists of a single (*Kipferlia*, *Dysnectes*) (Yubuki et al. 2007, 2013) or several microtubules (e.g., two in *Carpediemonas*, five in *Aduncisulcus*, six in *Ergobibamus*, and nine in *Hicanonectes*; Simpson and Patterson 1999; Park et al. 2009, 2010; Yubuki et al. 2016). The anterior root (also known as root 3; see Yubuki et al. 2013) originates from the anterior side of basal body 2 and curves to run posteriorly down the left side of the cell (except in *Hicanonectes*, where the root travels anteriorly). In *Aduncisulcus*, *Carpediemonas*, *Ergobibamus*, and *Hicanonectes*, the anterior root is closely associated with microtubules of a dorsal fan, which supports the dorsal cell membrane, much like the subpellicular microtubules in *Retortamonas* and

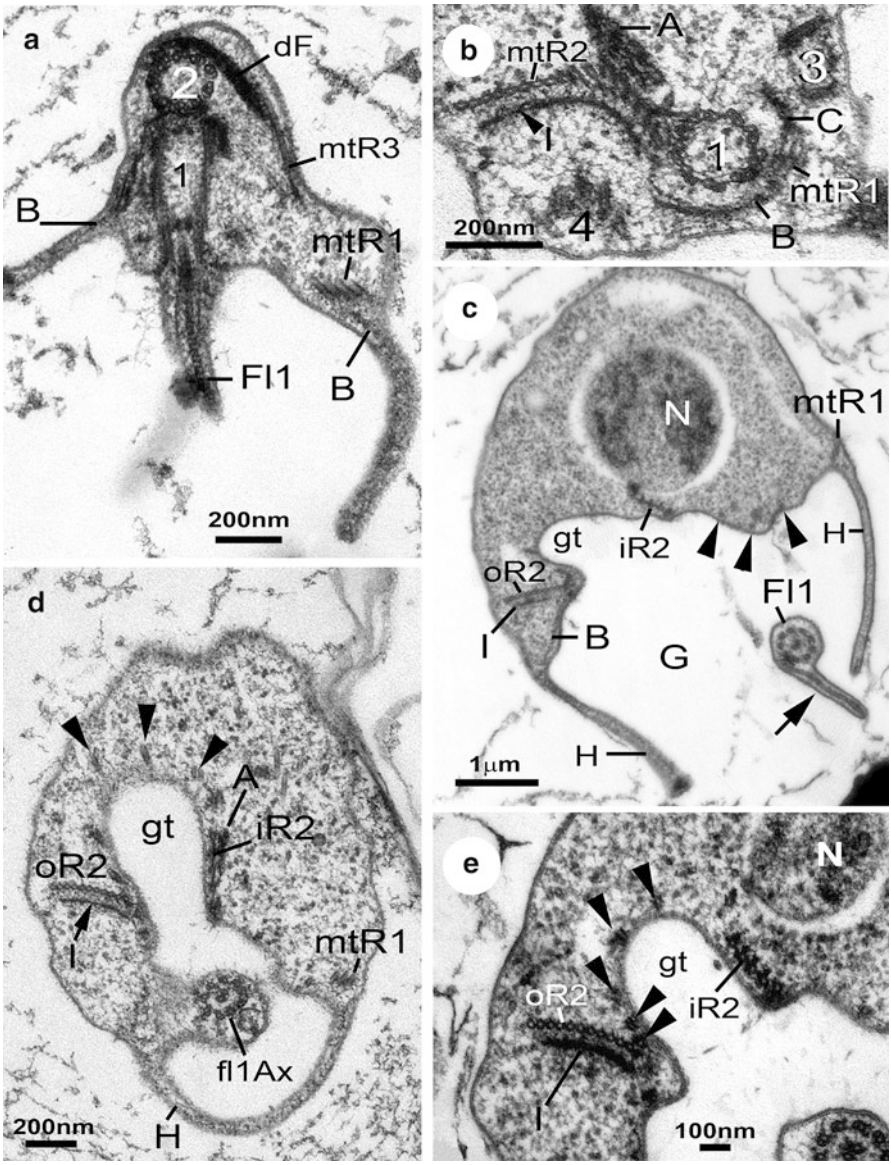

Fig. 8 The ultrastructure of *Kipferlia bialata*. (**a**) A longitudinal section through the anterior part of the cell showing perpendicularly arranged basal bodies of the recurrent flagellum (*1*) and anterior flagellum (*2*) and a dense fiber (*dF*) originating dorsally to basal body 2 and extending along the microtubular root 3 (*mtR3*). The recurrent flagellum (*Fl1*), microtubules of the left root (*mtR1*), and the B fiber can also be seen. (**b**) A transverse section through the anterior part of the cell showing the basal body of the recurrent flagellum (*1*), the barren basal bodies *3* and *4*, and associated structures of the mastigont including the left and the right microtubular roots (*mtR1* and *mtR2*), and the *A*, *B*, *C*, and I fibers. The I fiber (*arrowhead*) reinforces the ventral face of mtR2. The B fiber stretches from *mtR1* to *mtR2* (and the I fiber), around the ventral side of the recurrent basal body (*1*). (**c**) Transverse section

Chilomastix. The extent of the fan differs across CLO genera and is entirely absent in *Kipferlia* and *Dysnectes* (Yubuki et al. 2007, 2013).

The basal body of the recurrent flagellum (basal body 1 or R) is associated with cytoskeletal structures that support the ventral groove, namely, the left microtubular root (R1 or LR), the right microtubular root (R2 or RR), the singlet microtubular root, and several non-microtubular fibers (A, B, C, and I fibers) (Fig. 8b).

The right root originates from the right (or right dorsal) side of the basal body 1 as a single concavely curved row of 15–18 microtubules (Fig. 8b) that splits into two branches near the distal end of the basal body 1: a narrow inner branch (iR2 or IRR) and a more conspicuous outer branch (oR2 or ORR) (Fig. 8c–e). Both branches extend posteriorly. In most CLOs, the outer branch expands through additional microtubules and forms a ribbon supporting the right wall of the groove; the inner branch is associated with the floor of the groove. In *Kipferlia*, the only CLO with a deep gutter within the ventral groove, the outer and inner root branches support the right and left walls of the gutter, respectively (Fig. 8c–e).

The right root is accompanied by two fibers: the short A fiber (on the dorsal side) and the layered I fiber closely associated with its ventral side through fine fibrous connections between the fiber and the root microtubules (Fig. 8b). Posterior to the root splitting, the I fiber continues with the outer branch only and usually terminates halfway down the groove, whereas the root microtubules extend farther. *Kipferlia* and *Hicanonectes* cells have a prominent, posteriorly located cytopharynx, and this is supported by several microtubules ultimately derived from the right root.

The left root originates from the left side of basal body 1. It is formed from a single row of closely adjacent microtubules, which extend posteriorly to support the left margin of the ventral groove. The left root is associated with two fibers. The C fiber is found on the dorsal side of the left root (Fig. 8b). The fiber is short, terminating at the level of the groove opening, except in *Ergobibamus* in which the C fiber runs farther along the left root. The B fiber connects the left and right roots. The fiber traverses the ventral side of the basal body of the recurrent flagellum (Fig. 8b). The left region is always short and located ventral to the left root. The right region, beneath the cell membrane, descends the right side of the groove with the outer branch of the right root. In *Kipferlia*, the B fiber is the main support for the hood, a

Fig. 8 (continued) through the anterior part of the cell showing the ventral groove (*G*) bordered by the hood (*H*) and the gutter (*gt*) on the groove floor. The mtR1 root and individual microtubules (*arrowheads*) support the left wall of the groove. Note two microtubular bands (*oR2* and *iR2*) resulting from splitting of the right root. The I fiber reinforces the oR2 band. The recurrent flagellum with a prominent vane (*arrow*), the nucleus (*N*), and the B fiber supporting the hood are also visible. (**d**) Transverse section through the anterior part of the gutter and its associated structures: the microtubular bands oR2 and iR2, microtubules (*arrowheads*) beneath the gutter membrane, and the fibers I and A. The left root supports the left wall of the apical part of the ventral groove covered by the hood. The axoneme of the recurrent flagellum (*fl1Ax*) is visible shortly before the flagellum descends into the ventral groove. (**e**) A detailed view of the gutter region (more posteriorly than in *d*) to show organization of cytoskeleton that supports the wall of the gutter. *oR2* and *iR2*, five individual microtubules (*arrowheads*), and the I fiber are shown (Pictures courtesy of N. Yubuki)

membranous lip covering the anterior region of the cell above the anterior flagellum and extending down the left and right margins of the ventral groove (Fig. 8c; Yubuki et al. 2013).

Flagellar Transformation

During the cell division of *Kipferlia bialata*, the parental anterior flagellum (F2) becomes the recurrent flagellum (F1) in one daughter cell, while the parental recurrent flagellum (F1) remains recurrent in the other daughter cell. In both daughter cells, the anterior flagellum is formed de novo (Yubuki et al. 2013). Thus, the flagella in *K. bialata* are inherited in a semiconservative way, and the recurrent flagellum is older than the anterior flagellum. *Kipferlia* thus represents the first "typical excavate" in which flagellar transformation has been thoroughly documented (see Nohýnková et al. (2006) for flagellar transformation in the related, but atypical *Giardia intestinalis*).

Nucleus and Cytoplasmic Organelles

The nucleus of the CLOs is always located at the anterior part of the cell, in close association with the basal bodies. Its shape is mostly spherical except for the ovoid nucleus of *Ergobibamus*. The nucleus lacks a central nucleolus. Instead, electron-dense material forms a large mass located either subcentrally (*Carpediemonas*) or on one side of the nucleus (*Hicanonectes* and *Aduncisulcus*); alternatively this mass may form several small aggregates beneath the nuclear envelope, as in *Ergobibamus* (Simpson and Patterson 1999, Park et al. 2009, 2010).

Typical mitochondria are absent; however, double-membrane-bounded organelles that resemble hydrogenosomes are present in all CLO genera, at an abundance of several per cell. The limiting membranes of these organelles are very closely adjacent and do not form cristae. In most genera, these organelles are rounded and are 300–500 nm in diameter, though in *Carpediemonas*, they are elongated or dumbbell-shaped (Simpson and Patterson 1999; Park et al. 2009, 2010; Yubuki et al. 2007, 2013, 2016).

No discrete Golgi apparatus was observed in most CLOs. The exception is *Carpediemonas membranifera*, in which a single Golgi dictyosome with three to four cisternae is placed left of the ventral groove and ventral to the nucleus (Simpson and Patterson 1999).

Food vacuoles are usually located in the posterior two-thirds of the cell.

Ultrastructure of Cysts

The cyst stage of *Hicanonectes teleskopos* and *Iotanema spirale* (the latter a member of Caviomonadidae - see below) was observed by TEM (Park et al. 2009; Yubuki

et al. 2016). The rounded cyst of *Hicanonectes* is covered by a relatively thin, single-layered cyst wall, which is separated by empty space from the cytoplasm. The nucleus, hydrogenosome-like organelles, and internalized flagellar axonemes are located within the cytoplasm. Only a single cyst of *Iotanema spirale* was documented, and almost no details were observed (Yubuki et al. 2016).

Taxonomy and Evolutionary History

The CLOs are members of the Fornicata. To date, six species of free-living CLOs have been described (see below for Caviomonadidae), each currently belonging to a separate genus: *Aduncisulcus paluster*, *Carpediemonas membranifera*, *Dysnectes brevis*, *Ergobibamus cyprinoides*, *Hicanonectes teleskopos*, and *Kipferlia bialata* (Ekebom et al. 1996; Kolisko et al. 2010; Larsen and Patterson 1990; Park et al. 2009, 2010; Ruinen 1938; Yubuki et al. 2007, 2016). This assignment of known CLOs to described genera is now replacing a more informal division into clades labeled CL1-CL6 (Kolisko et al. 2010).

Cavalier-Smith recently (2013) divided CLOs into three families: Carpediemonadidae Cavalier-Smith 2003 (*Carpediemonas*, *Hicanonectes*, and *Ergobibamus*), Kipferliidae Cavalier-Smith 2013 (*Kipferlia*), and Dysnectidae Cavalier-Smith 2013 (*Dysnectes*). However, the family Carpediemonadidae sensu Cavalier-Smith 2013 is paraphyletic. *Aduncisulcus* has not been accommodated in any family (Yubuki et al. 2016).

Almost all CLOs were unknown before the end of the twentieth century, and consequently their phylogenetic affinities were barely considered. Simpson and Patterson (1999) recognized the so-called excavate taxa, to which they assigned *Carpediemonas membranifera* (among others), based on the presence of the ventral groove and the associated cytoskeleton. The specific relationship of CLOs, diplomonads, and retortamonads was revealed in 2002, when sequence data for *Carpediemonas membranifera* became available (Simpson et al. 2002); the three groups were subsequently united in the taxon Fornicata (Simpson 2003). It was later shown by analyses of the SSU rRNA gene that the CLOs are diverse and likely represent several distinct lineages (Kolisko et al. 2010; Park et al. 2009; Yubuki et al. 2007). Notably, *Dysnectes brevis* appeared to be the closest relative of the Diplomonadida + vertebrate *Retortamonas* clade (except for the report of Yubuki et al. 2007 in which this species branched more basally than *Carpediemonas membranifera*), but the SSU rRNA gene showed little power to resolve interrelationships within the Fornicata. A multigene analysis (Takishita et al. 2012) confirmed that the CLOs are paraphyletic, with diplomonads and retortamonads nested within them. Briefly, Fornicata split into four clades without resolved interrelationships: (1) *Carpediemonas*, (2) *Ergobibamus*, (3) *Hicanonectes* and *Aduncisulcus* (and Caviomonadidae, see below), and (4) *Chilomastix*, *Kipferlia*, *Dysnectes*, *Retortamonas*, and Diplomonadida. Within the latter *Dysnectes* was closely related to the *Retortamonas* + Diplomonadida lineage.

Recently it was established that the endobiotic genus *Caviomonas*, which had previously been considered an enteromonad diplomonad, actually descended independently from CLOs (see below).

Morphology, Ultrastructure, and Ecology of Caviomonadidae

Family Caviomonadidae, originally created by Cavalier-Smith (2013), currently consists of genera *Caviomonas* and *Iotanema* and an undescribed isolate PCS. Members of Caviomonadidae are small uniflagellate organisms with a single nucleus and no clear ventral groove (Fig. 9a–d). Preliminary electron microscopy of *Caviomonas* and *Iotanema* shows a simple flagellar apparatus, with two or four basal bodies, respectively (Brugerolle and Regnault 2001; Yubuki et al. 2016). One basal body (B2) bears the single flagellum, which was identified as F2 (i.e., homologous to the anterior flagellum of CLOs and other excavates). One (*Caviomonas*) or three (*Iotanema*) basal bodies are barren, including B1, which bears the recurrent flagellum F1 in other fornicates. The cytoskeleton associated with the basal bodies is simple and consists of a single microtubular fiber (called the nuclear fiber) and a dorsal fan of microtubules. The nuclear fiber is accompanied by a multilayered fiber and a connecting fiber (both are non-microtubular; the latter was observed only in *Iotanema*). It has been putatively homologized with root R1 of typical excavates, though more data are needed (see Yubuki et al. 2016). Mitochondria have not yet been detected in Caviomonadidae.

Genus *Caviomonas* comprises two endobiotic species, *C. mobilis* and *C. frugivori*, found in the large intestine of rodents (Nie 1950; Navarathnam 1970); they are considered harmless commensals. The only species of *Iotanema*, *I. spirale*, was obtained from feces of a gecko (Yubuki et al. 2016). By contrast, the organism "PCS" is free-living and was isolated from marine anoxic sediments

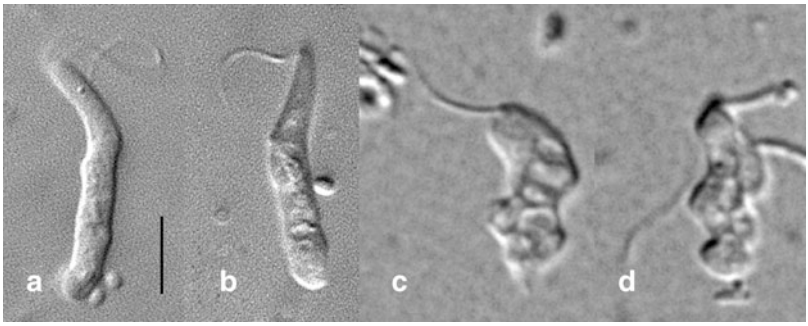

Fig. 9 Light microscopic photographs of Caviomonadidae. (**a**), (**b**) Free-living strain PCS. (**c**), (**d**) *Iotanema spirale*. Scale bar is 5 μm for all figures ((**a**), (**b**) from Kolisko et al. 2010, with permission of the Society for Applied Microbiology and Blackwell Publishing Ltd.; (**c**), (**d**) by courtesy of N. Yubuki)

(Kolisko et al. 2010). *Iotanema spirale* was successfully cultured in serum-Ringer-egg medium (Dobell and Laidlaw 1926); see Kolisko et al. (2010) for suitable culture medium for PCS.

Phylogenies of the SSU rRNA gene of *Iotanema* and PCS show a close relationship with the CLO *Hicanonectes* (Yubuki et al. 2016); sequence data from *Caviomonas* are still unavailable. It was concluded that Caviomonadidae are descended from CLOs, losing most of the typical excavate features and, in the case of *Caviomonas* (and almost certainly *Iotanema*), becoming endobionts. Therefore, they represent the third origin of the endobiotic lifestyle in Fornicata, independent of both retortamonads sensu stricto and the diplomonads + "vertebrate *Retortamonas*" lineage.

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Amoebozoan Lobose Amoebae (Tubulinea, Flabellinea, and Others)

35

O. Roger Anderson

Abstract

The amoebozoans included here are amoeboid protists that locomote by forward flowing of the internal cytoplasm and protrusion of peripheral, fingerlike or fan-shaped pseudopodia, excluding the myxomycetes and other slime molds, and Archamoebae, which lack classical mitochondria. These lobose Amoebozoa are an eclectic collection of amoeboid organisms. Some are naked without any surface covering, while other species may have a thin organic surface coat (glycocalyx) or delicate scales deposited on the outer cell membrane, with shapes that are species specific. Lobose testate amoebae are enclosed within an organic or mineralized shell (test) with an oral aperture where the tubular pseudopodia emerge. The lobose amoebozoans consume prey (e.g., bacteria, algae, smaller protists, yeast, etc.) by phagocytosis. They are widely distributed globally in aquatic and terrestrial environments. They become dormant cysts under unfavorable conditions, such as lack of adequate food or drying of the environment, but excyst and become active when environmental conditions improve (or form freeze-resistant, winter resting stages that are not encysted in some soil-dwelling amoebae in temperate regions). The amoebozoan lobose amoebae are significant members of aquatic and terrestrial microbial communities and serve as important linkages in food webs between microbes and higher organisms, such as invertebrates. Like other Amoebozoa, the lobose amoebae typically have tubular mitochondrial cristae, which partially distinguish them from the heterolobosean

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amoebae, with discoidal/flattened cristae. Molecular phylogenetic evidence indicates that Amoebozoans are monophyletic, with most, but not all, lobose amoebae falling into one of two subclades: Tubulinea (which includes the lobose testate amoebae or Arcellinida) and Discosea.

Keywords

Biogeography • Ecology • Evolution • Fine structure • Molecular phylogenetics • Naked amoebae • Protozoa • Taxonomy • Testate amoebae

Contents

Summary Classification

●**Amoebozoa**
●●**Tubulinea**
●●●**Euamoebida** (e.g., *Amoeba*, *Cashia*, *Hartmannella*, *Saccamoeba*)
●●●**Leptomyxida** (e.g., *Rhizamoeba*, *Flabellula*, *Leptomyxa*, *Paraflabellula*)
●●●**Arcellinida** (e.g., *Arcella*, *Difflugia*, *Cryptodifflugia*, *Nebela*)
●●**Discosea**
●●●**Flabellinia** (e.g., *Neoparamoeba*, *Paramoeba*, *Vannella*, *Vexillifera*)
●●●**Himatismenida** (e.g., *Cochliopodium*, *Ovalopodium*)
●●●**Stygamoebida** (e.g., *Stygamoeba*)
●●●**Longamoebia** (e.g., *Acanthamoeba*, *Sappinia*, *Stenamoeba*, *Thecamoeba*)
●●**Variosea**
●●●**Gracilipodida** (e.g., *Arachnula*, *Filamoeba*, *Flamella*)

[Note: Only taxa of Amoebozoa covered extensively in this chapter are listed here.]

Introduction

General Characteristics

The Amoebozoa broadly include amoeboid organisms, with or without an enclosing shell or test, that locomote largely by extension of pseudopodia and internal cytoplasmic streaming. Only the lobose amoebae with pseudopodia that are tubular and finger shaped (Figs. 1 and 2) or anteriorly broad and fan shaped, sometimes bearing extensions (e.g., subpseudopodia, Fig. 1c), are treated here. Other members of the Amoebozoa that are not (exclusively) lobose amoebae are treated in other chapters (see ▶ Archamoebae, ▶ Dictyostelia, ▶ Myxomycetes and ▶ Protosteloid Amoebae (Protosteliida, Protosporangiida, Cavosteliida, Schizoplasmodiida, Fractoviteliida, and Sporocarpic Members of Vannellida, Centramoebida, and Pellitida)). In some species of lobose amoebae, locomotion is by protoplasmic streaming of the cytoplasm within the body of the amoeba that continuously propels the amoeba forward; while in others the elongated pseudopodia attach to the substrate and provide traction, drawing the body of the amoeba forward.

The lobose amoeboid protists were, until recently, included in the taxon Rhizopoda, defined originally by Von Siebold (1845) and described in the twentieth century by Levine et al. (1980) as protozoa that locomote "by lobopodia, filopodia or by protoplasmic flow without production of pseudopodia." The assemblage included the naked lobose amoebae, shell-bearing testate amoebae, Heterolobosea (amoeboid organisms with flagellated life stages), Foraminifera (with branching and anastomosing granular rhizopodia), and other rhizopodal amoeboid organisms (e.g., Margulis and Schwartz 1988). Prior classification schemes were based substantially on the morphology of the pseudopodia, including the Levine et al. (1980) system. However, fine structural and molecular genetic evidence confirms that these characteristics are not indicative of natural groups and in some cases are clearly a result of convergent evolution, thus leading to a substantial revision of the taxonomy based on more conservative features. Modern research has considerably refined our knowledge of the natural affinities among amoeboid organisms, and newer classifications no longer recognize Rhizopoda as a higher-level taxonomic group. The lobose amoeboid protists are currently included in the Amoebozoa (e.g., Adl et al. 2005, 2012). Additional more detailed taxonomic treatments of some of the other pseudopod-bearing organisms, based on modern revisions, are presented in other chapters of this book.

In this chapter, much of the focus will be on naked amoebae, with some attention to the lobose testate amoebae and their relatives. The naked amoebae, previously categorized as "gymnamoebae," lack a substantial cell covering but may be enclosed by a thin or thickened organic surface coat (e.g., Page 1976, 1981, 1983, 1988), a variety of vertical, towerlike glycostyles (Page 1976, 1983), or in some cases mineral or organic scales adhering to a flexible organic matrix (Kudryavtsev 2006; Page 1983, 1988). The testate amoebae are enclosed by an aperture-bearing test or shell.

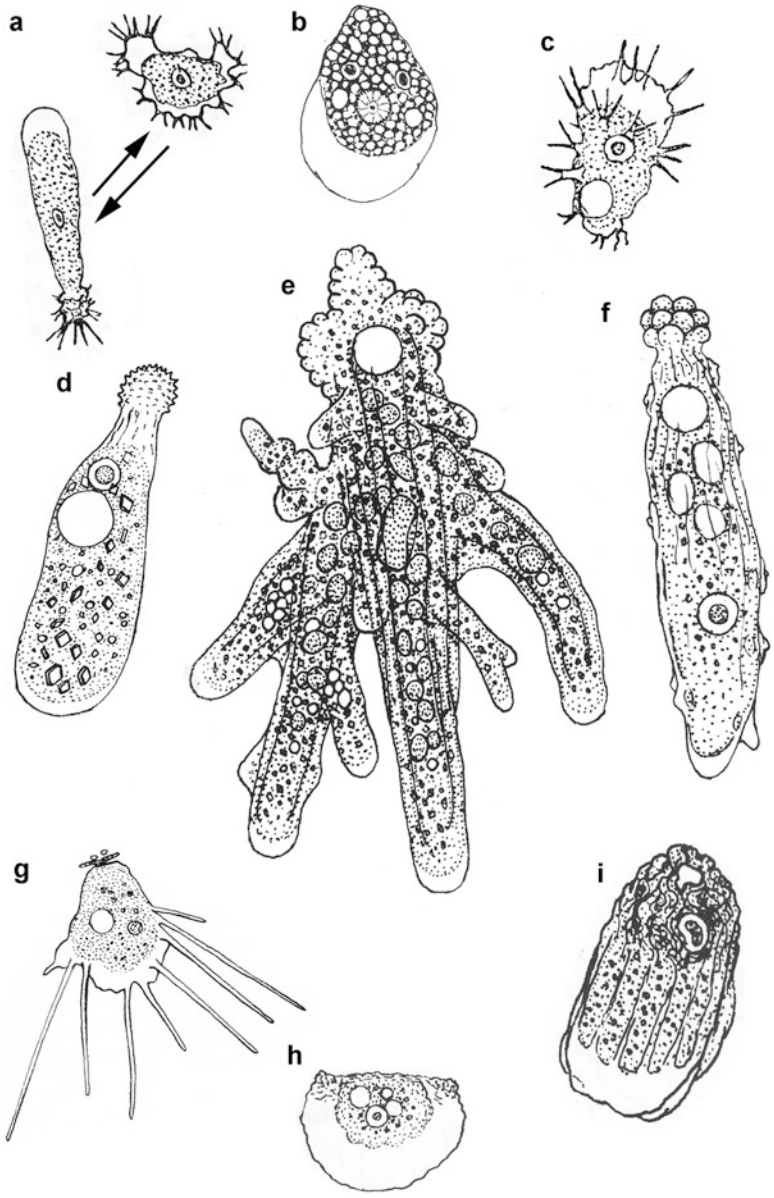

Fig. 1 Diagrammatic illustrations of some naked lobose amoebae. (**a**) *Rhizamoeba polyura*, showing an elongated motile stage and contracted stationary stage with fine lateral-radiating pseudopodia. (**b**) *Entamoeba histolytica* (actually a member of Archamoebae). (**c**) *Acanthamoeba castellanii*, bearing diagnostic bifurcated peripheral pseudopodia. (**d**) *Saccamoeba lucens*, a monopodial amoeba with prominent uroid. (**e**) *Amoeba proteus*, a polypoidal species with several lateral lobose pseudopodia. (**f**) *Mayorella limacis* in locomotion. (**g**) *Vexillifera lemani*, with characteristic of a triangular body and long tapered anterior pseudopodia. (**h**) *Vannella miroides*, a flattened

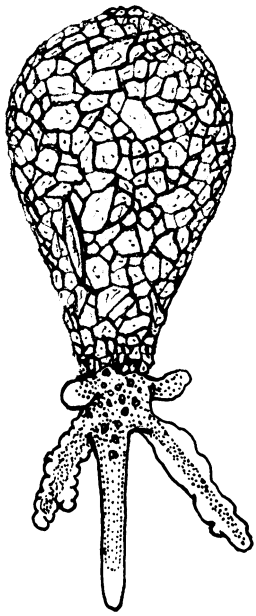

Fig. 2 Diagram of the lobose testate amoeba *Difflugia pyriformis*, with extended pseudopodia (Adapted from Bovee (1985a) with permission (International Society of Protistologists))

The morphology of the test is species specific and its composition is of diagnostic value. In some species it is composed of organic subunits cemented together. In others it is simply an enclosing leathery coat, or a more complex matrix with mineral components embedded within it (Bovee 1985a, b; Clarke 2003; Ogden and Hedley 1980). In some cases, the mineral components such as sand grains, diatom shell fragments, or other mineral particles are collected from the environment and attached to the organic matrix of the test. Typically, a single opening in the test (oral aperture) provides continuity between the internal cytoplasm and the protruding pseudopodia that extend into the surrounding environment (Fig. 2). There are two major groups: the lobose testate amoebae with lobopodia (e.g., Bovee 1985a; Smirnov 2008) and the filose testate amoebae with filopodia (e.g., Bovee 1985b). The latter are now included in the Cercozoa, within the subgroup Rhizaria, and are not considered here (see above).

Occurrence: Habitat, Distribution, and Abundance

Amoeboid protists are found in most habitats where other protists have been observed, including all major terrestrial habitats at low and high latitudes, freshwater

Fig. 1 (continued) fan-shaped amoeba. (**i**) *Thecamoeba sphaeronucleolus*, exhibiting characteristic of longitudinal surface ridges. Illustrations are not to scale; some are enlarged relative to others to display significant morphological features (Adapted from Bovee (1985a) with permission (International Society of Protistologists))

ponds and bogs, brackish marshes and estuaries, and open ocean at near surface or great depth. Typically, distinctions are made during research between soil-dwelling, freshwater, and marine species. This distinction may be more a matter of convenience, as a way to focus and delimit a research agenda, rather than being a necessary restriction of habitat diversity. In general, however, there is good evidence that many marine species are stenohaline and dwell only in marine environments. Estuaries are of particular interest because the periodic tidal fluxes create markedly varied salinity gradients, thus subjecting microbiota to strong selection pressures and leading to wide salinity tolerances. Some species collected from extreme environments, including extreme cold as in arctic and Antarctic locales, often are obligate cryophiles. They exhibit rapid evidence of distress and soon die when introduced to more moderate temperatures. Some amoeboid protists are extremophiles that are found in highly polluted environments with low pH and/or high levels of potentially toxic minerals or industrial waste products (e.g., Amaral Zettler et al. 2003). Their mechanisms of survival are of increasing interest as evidence of the remarkable adaptive capacity of some protists and perhaps as guides to the properties of lifeforms that may be found on other planets with more extreme environments ("exobiology").

Earlier research on amoeboid protists has provided substantial information on their habitats and distribution (e.g., Bovee 1979, 1985a; Kudo 1966; Leidy 1879; Loeblich and Tappan 1964; Page 1988). The distribution and adaptation of terrestrial naked and testate amoebae have been reviewed by Cowling (1994) and more recently for testate amoebae by Smith et al. (2008). Naked amoebae abundances, expressed as number per g soil dry weight, have been reported in the range of 10^5–2×10^6 g^{-1} for pine forest soil (Clarholm 1981), 10^2–5×10^3 g^{-1} in upland grassy plots in the USA (Anderson 2000), and with similar richness in grassland soils and the UK (Brown and Smirnov 2004). However, lower numbers (79–585 g^{-1}) were observed in sandy beach soil (Cowling 1994). Anderson (2009) reported data on the abundance of naked amoebae associated with major groups of plants, including moss (3.5×10^3–3.6×10^4 g^{-1}), ferns (2×10^3–4×10^6 g^{-1}), and seed plants (2×10^3–2×10^6 g^{-1}). He also reported similar data for testate amoebae, i.e., moss (3×10^2–6×10^3 g^{-1}), ferns (90–300 g^{-1}), and seed plants (10^4–4×10^5 g^{-1}). More recently, robust amoeba communities have been reported to be associated with terrestrial lichens (e.g., Anderson 2014), including other reports of the possible importance of testate amoebae (particularly the filose testate amoebae) in the silica biogeochemical cycle within lichen communities (Wilkinson et al. 2015). The distribution and abundance of terrestrial testate amoebae have been substantially investigated, in part because their tests persist for some time in the soil, especially in water-saturated sediments of peat bogs and marshes. Their diversity and abundance in soil strata provide evidence of their ecological and soil environmental histories (e.g., Smith and Coupe 2002). The abundance of testate amoebae of all kinds varies substantially within and across terrestrial sites but is generally in the range of 10^6–10^7 m^{-2} for

forest and sphagnum-rich soils (e.g., Cowling 1994; Foissner 1987; Lousier 1982; Miesterfeld 1977).

Recently, abundances of naked amoebae in freshwater and marine habitats have been more extensively recorded, e.g., Rogerson and Laybourn Parry (1992) reported an annual mean abundance in the Clyde Estuary (Scotland) of 8300 amoebae L^{-1}. Similarly, Anderson and Rogerson (1995) examined the annual abundances of naked amoebae in the Clyde, a more productive estuary, and the Hudson, a more turbid and less productive estuary. They found that maximum summer abundances in the Clyde were approx. 16,000 L^{-1}, while only 7000 L^{-1} were found in the Hudson. Moreover, increasing evidence indicates that naked amoebae in the Hudson may be major predators on bacteria, sometimes competing significantly with other bacteria-consuming protists in the food webs (e.g., Lesen et al. 2010). Naked amoebae can be particularly abundant in freshwater biofilms (Anderson 2013). In a freshwater pond in northern New York, biofilm amoeba densities ranged from 109 to 136 cm^{-2} biofilm surface area and 285 to 550 mg^{-1} biofilm dry weight. Sizes ranged from 13 to 200 μm. C-biomass ranged from 64 to 543 ng C cm^{-2} and 125 to 1700 μg C g^{-1} dry weight. Thirty morphospecies were identified, including very large amoebae in the range of 100–200 μm. Large amoebae (>50 μm) accounted for the largest proportion of the C-biomass.

With increasing interest in high-latitude biota, Mayes et al. (1998) examined naked amoeba abundances in the water column of two coastal sites off Eastern Antarctica. In general, numbers in the water column were highly variable (below detection to 2000 amoebae L^{-1}). There were no clear seasonal trends. Highest abundances, up to 2626 amoebae L^{-1}, were recorded at the ice-water interface. Abundance and diversity of amoebae in Alaskan tundra soils and their relationships to other terrestrial microbes in the carbon cycle and respiration of organic-rich, high-latitude soils have been reported by Anderson (e.g., Anderson 2012). Highly productive freshwater ponds support substantial numbers of naked amoebae, reaching densities close to 2000 mL^{-1} during the most productive periods in spring and early autumn when water temperatures are more favorable for growth (e.g., Anderson 1997). Organic-rich sediments are also highly favorable habitats for naked amoeba growth. The abundance and diversity have been reported in a variety of locales including brackish sediments of Niva Bay on the Baltic Sea (Smirnov 2002; Smirnov and Thar 2003, 2004) and calcareous sand sediments of coastal bays at Bermuda (Anderson 1998). Algal mats, fronds of thallose algae, and suspended floc are also favorable surfaces supporting diverse and/or abundant communities of naked amoebae (e.g., Armstrong et al. 2000; Rogerson 1991; Rogerson et al. 2003). The colony-forming cyanobacterium *Trichodesmium* supports a rich community of microbes, including naked amoebae (Anderson 1977), and as much as 50% of sampled colonies in the Sargasso Sea contained naked amoebae, among other eukaryotic microbes (Sheridan et al. 2002). Suspended floc in Antarctic lakes (e.g., Crooked Lake, Antarctica) may also support rich microbial communities that include attached amoebae and other eukaryotic microbes (Laybourn-Parry et al. 1992).

Practical Importance

Although most naked amoebae are free-living, some have also become pathogenic in humans and other primates. The amoeba *Entamoeba histolytica* (which is, phylogenetically speaking, a member of Archamoebae) invades the gut and causes amebic dysentery (a serious diarrhea), and in more pronounced morbid pathologies, it invades other organs and can be fatal. It is estimated to infect about 50 million people worldwide. More information on the biology of *Entamoeba* is included in the treatment of ▶ Archamoebae. *Balamuthia mandrillaris*, *Acanthamoeba* sp., and *Sappinia* sp. can invade the central nervous system, causing serious amoebic encephalitis, particularly in individuals with compromised immune systems (Visvesvara et al. 2007). *Acanthamoeba* also causes a local serious infection of the eye (*Acanthamoeba* keratitis; e.g., Auran et al. 1987).

Morphology and Taxonomy

Light Microscopic and Fine Structural Morphology

The classification of naked amoebae, based on light-microscopic morphology, relies largely on their shape, size, mode of locomotion, and rate of movement (e.g., Jepps 1956; Page 1976, 1983; Patterson et al. 2002; Rogerson and Patterson 2002; Sawyer 1980; Smirnov et al. 2011). One of the defining morphological features of the Amoebozoa is their shape and pattern of locomotion. Some amoebae that are fan-shaped with a broad anterior lobe often lack subpseudopodia extending from the anterior margin and move largely by differences in cytoplasmic pressure from posterior to anterior that propels the amoeba forward by constantly expanding the anterior margin and retracting the posterior portion. Likewise, anterior extension of the lobopodia occurs through forward flow of the cytoplasm that expands the tip of the pseudopodium in the direction of motion, as the main body of the amoeba is drawn forward. In some species, the subpseudopodia extending from the anterior surface can become quite long and tapered relative to the body of the amoeba (e.g., Fig. 1g). In addition to the overall shape of the amoeba and its pattern of locomotion, other defining features include the presence or absence of a uroid. A uroid is a posterior projection of the amoeba cell and may be rather rounded, sometimes smooth, or with fine posterior cytoplasmic extensions (Fig. 1a); it may exhibit small surface pustules (Fig. 1d) or be decorated with larger surface protrusions (Fig. 1f) which can become quite elaborate in certain species (Fig. 1e). In some species the uroids are adhesive, that is, they become momentarily attached to the substratum as the amoeba moves forward.

Identification of naked amoebae based on their morphology and mode of locomotion requires expert knowledge. In some cases, the subtle distinctions among some species make clear identification difficult. Moreover, broad and overlapping variations in morphology sometimes make discrimination difficult among closely related species, especially for differences in testate amoebae based on shell

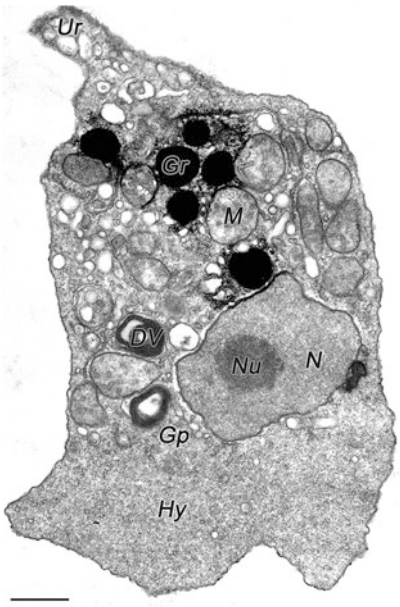

Fig. 3 Transmission electron microscopic image of a section through the nuclear region of a small naked amoeba showing the nucleus (*N*) with a denser nucleolus (*Nu*). The surrounding granular cytoplasm exhibits prominent vacuoles, including a digestive vacuole (*DV*) containing the non-digestible wall remains of a prey organism. Scale bar = 1 μm

morphology (Lahr and Lopes 2006). Consequently, in some cases, morphotypic categories are used, when appropriate for the research objectives. For example, Anderson and Rogerson (1995) used a typology with four types: Type 1, amoebae with lobose or filose protruding pseudopodia and/or locomotion by cytoplasmic streaming; Type 2, limax (worm-shaped) amoebae with steady, noneruptive locomotion; Type 3, limax amoebae with anterolateral bulging pseudopodia and eruptive locomotion; and Type 4, discoid or fan-shaped flattened amoebae. Additionally, identified genera and species were included as subcategories of each morphotype. Subsequently, a refined typology with 16 morphotypes based on more detailed features was published by Smirnov and Brown (2004).

Fine structure features shown by electron microscopy (e.g., Fig. 3) are typical of eukaryotic cells, including a prominent nucleus (N, Fig. 3) surrounded by a nuclear envelope, sometimes including a denser nucleolus (Nu, Fig. 3). The cytoplasm contains membrane-enclosed organelles, including mitochondria (M, Fig. 3), digestive vacuoles (DV, Fig. 3), and a variety of smaller vacuoles. Mitochondria in most species have branched tubular internal cristae, but some variations occur, including more flattened cristae; however, the mitochondria are not enclosed by rough endoplasmic reticulum as found in most Heterolobosea. In some cases food reserves are present as electron-dense granular deposits (Gr, Fig. 3). In species with a broad anterior region, the cytoplasm is largely composed of very fine contractile filaments and is designated as hyaloplasm (Hy, Fig. 3) compared to the more granular cytoplasm (Gp, Fig. 3) that contains most of the membrane-bounded organelles. The hyaloplasm is the region of the cell that is continuously expanding as the amoebae move forward. Amoebozoa dwelling in freshwater also have contractile

vacuoles that accumulate excess water from the cytoplasm and undergo rhythmic contractions to expel the water through a surface pore and into the surrounding environment.

Fine structure evidence has substantially improved our understanding of the cellular basis for making distinctions among taxa. For example, the amoeba's surface coat, if present, has been used to distinguish among genera. A full account is beyond the scope of this chapter, but some examples are given. The surface coat of *Mayorella* spp. is a multilayered organic lamina and differentiates them from the morphologically similar *Korotnevella* (syn.: *Dactylamoeba*) spp. that possesses organic oval to "boat-shaped" complex surface scales. *Vexillifera* has distinctive hexagonal peg-like surface glycostyles, while the members of the "vannellid group" bear either pentagonal towerlike glycostyles (*Vannella*) or less-prominent hexagonal prismatic projections (previously, *Platyamoeba*). Currently, however, molecular genetic evidence indicates that the fine structure of the scales is not a valid basis for distinguishing between species of *Vannella* and *Platyamoeba*. Therefore, it has been recommended that *Platyamoeba* species should be merged into the genus *Vannella* (Smirnov et al. 2007). In some cases, the surface coat is uniformly electron dense (e.g., *Thecamoeba* spp.) or thicker with chevron-like internal electron-dense structures (e.g., *Dermamoeba* spp.). The organization of the nucleus and the structure of mitochondrial cristae (tubular or flattened) are also important distinguishing characteristics (e.g., Page 1976, 1983, 1988). Internal inclusions such as crystals are also of significance, as are the arrangements of fibrillar substances and of microtubules.

The fine structure of the organic matrix and composition of surface components in the tests of testate amoebae have substantially enhanced our understanding of their morphology and systematics (e.g., Ogden and Hedley 1980), particularly in clarifying differences between surface components produced from within the cytoplasm (idiosomes) versus surface components gathered from the natural environment (xenosomes) (e.g., Anderson 1987, 1988a; Lahr and Lopes 2007; Miesterfeld 2002a, b).

Taxonomy

Modern taxonomy, based increasingly on fine structural and molecular phylogenetic evidence, is firmly rooted in the earlier systematics based largely on light microscopy. The literature base is substantial. Only some representative examples can be referenced here (e.g., Bovee 1985a, b; Cash et al. 1905/1909/1915; Chatton 1953; Deflandre 1953; De Saedeleer 1932; Page 1976, 1983; Penard 1902; Schaeffer 1926; Schaudinn 1899). Schaeffer's seminal publication (Schaeffer 1926) contains exquisite black ink-rendered illustrations produced by his own hand and, as he declared, with such attention to detail and lifelike features that they should look as though they could crawl off of the page. Our understanding of amoeboid protist systematics is still rapidly expanding, particularly with increasing insights from molecular genetics. Rogerson and Patterson (2002) identified 55 genera within 14 families in their

survey of naked amoebae (gymnamoebae). In the same publication, 71 genera of lobose testate amoebae were reported (Miesterfeld 2002a, b). In addition to those genera, further discoveries have been made including new naked amoebae: *Vermistella* (Moran et al. 2007) isolated from Antarctica, morphologically similar to *Stygamoeba*, but presently not grouping with it in molecular phylogenetic analyses; *Pellita*, an amoeba with an unusual thickened surface coat (Smirnov and Kudryavtsev 2005; Kudryavtsev et al. 2014); *Squamamoeba*, a small scale-bearing species (Kudryavtsev and Pawlowski 2013); *Cunea*, with two species of small triangular marine amoebae (Kudryavtsev and Pawlowski 2015); and new species of *Cochliopodium* (e.g., Tekle et al. 2013). A novel, filose pseudopod-bearing, multinucleated amoeba (*Telaepolella tubasferens*) assigned to the Gracilipodida (Amoebozoa), a taxonomic group that also includes the genus *Flamella* (see Adl et al. 2012), has been described by Lahr et al. (2012), and more recently its molecular phylogenetic position has been further clarified (Berney et al. 2015; Kudryavtsev et al. 2009).

A number of recent publications have addressed improved classification schemes using modern evidence (e.g., Adl et al. 2005, 2012; Lee et al. 2002; Smirnov et al. 2005, 2007, 2011). The classification of Adl et al. (2005), published by the International Society of Protistologists, is used here with modifications. However, for a more detailed updated hierarchical classification, especially for higher-level groups of amoebae, see Smirnov et al. (2011) and Adl et al. (2012). Within the following text, relevant categories included in the Adl et al. (2012) classification are also cited. Only the naked amoebae (without stages producing flagella or fruiting bodies) and the lobose testate amoebae are considered here. In this scheme, the lobose naked and testate amoebae are included in the supergroup Amoebozoa (and placed in turn in the higher-order group Amorphea by Adl et al. (2012)). However, further research is needed to validate the phylogenetic validity of the supergroups (e.g., Pawlowski 2009; Yoon et al. 2008).

Taxonomic Outline

Some examples of taxa included in major subcategories of Amoebozoa within the classification scheme listed prior to the introduction are briefly described, including illustrative genera.

Tubulinea. Amoebae with tubular or finger-shaped pseudopodia. The major morphological features and some illustrative genera are presented.

Euamoebida. Naked amoebae with subcylindrical pseudopodia in locomotion (or the entire cell is monopodial and subcylindrical); without alteration of the locomotive form to a flattened expanded and branched one; no adhesive uroid. *Amoeba*, *Cashia*, *Chaos*, *Deuteramoeba*, *Hartmannella*, *Hydramoeba*, *Saccamoeba*, and *Trichamoeba*

Leptomyxida. Naked, locomotive that forms a flattened expanded or reticulate one, becoming subcylindrically monopodial when in rapid movement or under

specific conditions; adhesive uroid; uninucleate, tending to have more, or always multinucleate in *Leptomyxa*. *Flabellula*, *Gephyramoeba*, *Leptomyxa*, *Paraflabellula*, and *Rhizamoeba*

Arcellinida. Testate amoebae with an organic or mineral extracellular test composed of either internally secreted components or mineral particles gathered from the natural environment and bounded together. Test with a single main opening. *Arcella*, *Cryptodifflugia*, *Difflugia*, *Nebela*, and *Pharynugula*

Disocosea. Flattened naked amoebae, never with tubular or subcylindrical pseudopodia and never altering the locomotive form, and cytoplasmic flow polyaxial (protruding outward around the periphery) or without a pronounced axis; subpseudopodia short or absent.

Flabellinia. Flattened, generally fan shaped, and discoid or irregularly triangular, never with pointed subpseudopodia; no centrosomes. *Korotnevella*, *Gocevia*, *Pellita*, *Trichosphaerium*, *Paramoeba*, *Vannella*, and *Vexillifera*

Himatismenida. Dorsal surface containing a rigid coat without a defined aperture, ventral surface naked. *Cochliopodium*

Stygamoebida. Flattened, elongate amoebae resembling slender toothpicks or splinters, temporarily with a forked or branched form; extended area of anterior hyaloplasm. *Stygamoeba*

Longamoebia. Flattened and elongated amoeba with pointed subpseudopodia and cytoplasmic centrosomes in one lineage. *Acanthamoeba*, *Balamuthia*, *Dermamoeba*, *Mayorella*, *Sappinia*, *Stenamoeba*, and *Thecamoeba*

Gracilipodida. Amoebae without cilium or centrosomes; flattened, fan shaped, or irregularly branched, with short conical subpseudopodia or fine hyaline, hair-like subpseudopodia; cysts with smooth single-layered enclosing wall. *Arachnula*, *Filamoeba*, *Flamella*

Comment

There are a number of taxa previously included in earlier published treatises on the naked and testate amoebae that are not accommodated in the current classification scheme, largely due to uncertainties about their molecular phylogenetic affinities, lack of clear evidence whether they produce stages with flagella or not, and other issues pertaining to the fine structural characteristics such as the presence or absence of identifiable mitochondria versus their possibly derived organelles such as hydrogenosomes (Yarlett and Hackstein 2005). Moreover, until recently the Amoebozoa have been relatively undersampled in molecular phylogenetic studies, and with increasing attention to their phylogeny, classification systems will undoubtedly undergo significant revisions to better accommodate the emerging evidence of their natural affinities. Hence, the classification scheme outlined here will undoubtedly be modified as additional evidence is available.

Life Histories and Ecology

Most lobose Amoebozoa are free-living amoebae inhabiting aquatic and terrestrial environments. Reproduction is by mitosis. Nuclear division (karyokinesis) precedes cytoplasmic division (cytokinesis). Sexual reproduction has not been documented in naked lobose amoebae but is reported in the testate amoebae. More recently, a form of parasexual activity (cell fusion followed by nuclear fusion and subsequent cell division without a meiosis stage) has been reported in *Cochliopodium* spp. (Tekle et al. 2014). In general, the organization of the nucleus and its transformation during mitosis can be a taxonomic diagnostic feature. Vesicular nuclei have a single central nucleolus (e.g., Fig. 3) or two or more portions (often, but not always, joined) in a parietal (lateral) position. The other principal type is the ovular or granular nucleus with many nucleoli, typically but not always in a parietal layer. Intermediate conditions exist including a moderate number of rather small nucleolar bodies. Mitotic patterns include open mitosis where the nuclear membrane disintegrates during metaphase, or closed mitosis where the nuclear membrane persists and may assist in the separation of the chromosomes during nuclear division.

Most of the amoeboid protists included here are exclusively heterotrophic, consuming bacteria, algae, or other small eukaryotes as prey. During ingestion, the prey is surrounded by the anterior pseudopodia and engulfed in intracytoplasmic digestive vacuoles (e.g., Fig. 3, DV). Some amoeba species contain intracellular algal symbionts (e.g., *Mayorella viridis*), but their role in host nutrition has not been established (Cann 1981). Bacterial endobionts are also present in some species, but their role also has not been described. However, an interesting example of co-adaptation has been reported in *Amoeba* by Jeon and Jeon (1976) that progressed from pathogenic bacterial infection (Jeon and Lorch 1967) to a mutually dependent status within several years, where the bacteria were required for the survival of the amoeba host. This relatively rapid evolution from a destructive to a mutually dependent relationship can be used as a model for the endosymbiotic origin of cellular organelles such as mitochondria (Margulis 1981).

Many terrestrial free-living Amoebozoan species are not obligate soil-dwelling biota and also are found in freshwater habitats. Some aquatic species are euryhaline, with a broad salinity tolerance. Others are strictly marine, or dwell in strong brackish water. Some species are cryophilic and grow only in cold temperatures, sometimes near the freezing point. Others require more moderate temperatures, and some thermophiles tolerate remarkably elevated temperatures, including those found in warm springs or shallow ponds subjected to elevated summer temperatures (e.g., Kyle and Noblet 1986, 1987). The capacity to form walled cysts, which resist desiccation, during unfavorable growth conditions (e.g., drought or insufficient food), especially for terrestrial and freshwater species, has enhanced the survival value of many amoeboid species and permitted widespread dispersal by wind or other transport mechanisms. Under favorable growth conditions, the encysted individuals excyst and emerge as actively feeding trophonts.

Earlier investigations on the habitats, feeding behavior, population growth dynamics, and life histories of pseudopodial-bearing protists (e.g., Bamforth 1985;

Bovee 1985a, b; Chatton 1953; Heal 1964; Sandon 1927) established a firm foundation for modern research on their life histories and ecology (e.g., Anderson 1988b; Fenchel 1985; Rodriguez-Zaragoza 1994; Smirnov 2008). Major advances have been made in our understanding of the significant ecological role of amoebae. Some recent representative studies on the life histories and ecology of amoeboid protists from aquatic and terrestrial environments are reviewed here within three broad ecological themes: (i) environmental variables, (ii) successions and seasonal abundances, and (iii) interactions with algae or plants, including biofilms.

Aquatic Ecology

Environmental variables. Temperature is a major variable determining the species composition and biogeographic distribution of Amoebozoa (e.g., Bonilla-Lemus et al. 2014). More generally, among other significant physicochemical variables, salinity is a major forcing function, segregating strictly freshwater amoebae (Page 1988) from marine species (Page 1983). In marine coastal marshes and estuaries, however, there are substantial populations of euryhaline amoebae (e.g., *Acanthamoeba Cochliopodium*, *Hartmannella*, *Mayorella*, *Vannella*, and *Vexillifera*) that have adapted to the diel cycles of tidal flushing where salinities may vary seasonally from 0 to 12 parts per thousand (e.g., Anderson and Rogerson 1995). The salinity tolerances of naked amoebae collected from widely different geographic sites, ranging from approximately 0 parts per thousand to 160 parts per thousand, were compared in laboratory experiments by Hauer and Rogerson (2005). Seven species were identified with remarkably wide tolerances in a range of 0 to 127 parts per thousand and six marine isolates that grew in the range of 2 to 127 parts per thousand. Further evidence of wide salinity adaptive tolerances of marine naked amoebae was reported by Cowie and Hannah (2006) who found substantial resilience to rapid salinity changes, including survival down to seven parts per thousand for the most resilient species.

Among other factors supporting naked amoeba population growth, the size, composition, and amount of suspended organic particles and floc in the water column are important variables. Naked amoebae must attach to surfaces while feeding on bacteria and other prey. Hence, suspended floc may be essential to support substantial planktonic populations of amoebae (e.g., Rogerson et al. 2003). Flocs may be "hot spots" for surface-dwelling eukaryotic microbes, especially amoebae (e.g., Anderson 2011), and represent significant centers for remineralization of nutrients through predation on bacteria (Arndt 1993; Zimmermann-Timm et al. 1998; Juhl and Anderson 2014).

Aquatic successions and seasonal abundance. The abundance of naked amoebae during seasonal successions is positively correlated with water temperature as exemplified by annual variation in abundances in some estuaries and ponds (Anderson and Rogerson 1995; Anderson 1997) with correlation values in the range of $r = 0.8$. Rivers and tidal estuaries offer unique environments to examine the effects of seasonal and tidal forcing functions on protists. Kiss et al. (2009) reported

maximum abundances of 3300 individuals L^{-1} in the Danube, particularly in April to July, with a secondary peak in October and November. Similar evidence of spring/summer and autumn blooms of naked amoebae was reported by Anderson and Rogerson (1995) for the Hudson Estuary and also in a shallow freshwater pond on the palisades above the Hudson Estuary (Anderson 1997, 2007). At maximum values, the amoeba carbon accounted for approximately 75% of the combined total carbon in the amoebae and ciliate fractions. Significant differences, however, may exist in the amoeba densities in sediments compared to the water column of some river systems, with amoebae dominating abundances in the sediment and ciliates in the water column (e.g., Gu et al. 1988). Weisse and Müller (1998), summarizing a 10-year analysis of seasonal standing stock of plankton in Lake Constance, reported that ciliates were found to be the single most important group, but naked amoebae were found in relatively high numbers and biomasses during phytoplankton peaks. A successional study of biofilms in a less-hospitable environment (the highly polluted Rio Tinto river, pH approx. 2) during 1 year by Aguilera et al. (2007) showed that amoebae and small flagellates were among the major eukaryotes after 1 month of biofilm development. Overall, the results suggest that some amoeboid eukaryotes are remarkably resilient, with potentials to adapt to highly mineral-polluted and low-pH environments.

Interactions within biofilms and with submerged phytobiota. Relatively little is known about the interactions of naked amoebae with prey bacteria in aquatic biofilms, but recent evidence suggests that naked amoebae may exert major top-down controls on biofilm bacteria (e.g., Anderson 2013; Zhang et al. 2014). Although ciliates are the most efficient predators in reducing bacterial biomass in the open water, amoebae can have a significant long-term negative effect on bacterial biomass both in the open-water phase and biofilms. Alga lamina and submerged stems and roots of plants, as well as floating colonies of algae (Anderson 1977), provide organically rich surfaces to support communities of naked amoebae, with surfaces of seaweeds supporting especially robust growth of potential bacterial prey (Armstrong et al. 2000). Additional studies of microbial populations on the surfaces of mangrove plant prop roots covered in epibiont film were reported by Maybruck and Rogerson (2004). No clearly discernible temporal pattern was detected throughout a 1-year sampling program, although naked amoebae were the second-most abundant group after flagellates. Some experimental trials comparing the growth of protozoa on tightly and loosely associated bacteria indicate that amoebae are more capable of removing tightly associated bacteria than are other micrograzers. Attached bacteria are likely to be significantly involved in the degradation of mangrove carbon; hence, predatory amoebae may serve an important ecological role in the film community.

Sediment and Soil Ecology

Environmental variables. The wide variation in the size and composition of organic and mineral particles in sediments and soils, as well as the intricate pore spaces,

produces a highly complex environment of microbial microniches, especially for amoeboid protists that typically attach to, or locomote upon, the elaborate surfaces of the solid substratum. Microniches have been characterized in sediments from Niva Bay (Baltic Sea) by Smirnov (2002) and subsequently extended to include oxygen analyses of the microenvironments within the microniches by Smirnov and Thar (2003). Naked amoebae were most abundant and diverse in the upper 1 cm of sediment. Their number and diversity decreased with increasing depth in the sediment. Species composition and abundance were highly heterogeneous, even at spatial scales of several centimeters, indicating the presence of microhabitats selectively occupied by particular suites of species. Amoebae were recovered from oxygenated upper layers as well as deeper anoxic layers. Some of the small sediment samples contained "hot spots" of amoebae biodiversity, with up to four species co-occurring in the same area. These may be loci of particularly favorable environmental growth conditions.

The distributions and biomass of amoebae and other protists in marine, brackish, and freshwater sediments were also examined by Lei et al. (2014) at 15 littoral stations across a relatively wide range of latitudes (arctic to European and North American sites). Amoeba abundance ranged from 0 to 937 cells mL^{-1} and biomass from 0 to 4.71 μg C mL^{-1}. Some of the highest naked amoebae densities were observed at marine tidal flats and contained only naked forms, whereas in a freshwater lake, only testate amoebae were observed. On an arctic ice floe, only naked amoeba forms were observed, and they contributed an average of more than 96% of the total protozoan abundance or biomass. At the other stations, both naked and testate amoebae were found.

Similar evidence of microhabitats (microbiocoenoses) in temperate forest soil has been reported by Anderson (2002). Samples of soil from four sites of varying soil porosity were analyzed in the laboratory, either unamended (NN) or amended (NE) with glucose solution to increase the organic content. Generally, the abundance of naked amoebae tended to increase with increasing soil particle size for both NN and NE treatments, possibly indicating that abundances increase with increasing porosity of the soil and the concurrent differences in physical and chemical properties that characterized the soil types. The NE cultures, moreover, showed consistently higher abundances and diversity of naked amoebae compared to the NN cultures. There also was evidence of growth "hot spots" where localized environmental conditions, such as sporadic nutrient loading or other favorable conditions, may have fostered proliferation of the amoebae.

Further evidence of the complexity of small-scale patchiness was obtained in a study carefully documenting the variation in numbers of amoeba morphotypes in small soil samples (Anderson 2003) to yield a mathematical model of biocomplexity using Euclidean spatial analysis. Three indices of amoeba abundance and distribution in the small volume samples were plotted as a three-dimensional graph: morphospecies richness (mean number of morphospecies counted in each small soil subsample), morphospecies diversity (number of morphospecies occurring in only one of the small soil subsamples but in no others), and morphospecies patchiness (the degree of aggregation or nonuniform distribution of morphospecies among

the soil subsamples). Soil samples were obtained from a freshwater bog, freshwater marsh, salt marsh, stream margin, and deciduous forest floor. Samples from the marsh rhizosphere were the most biocomplex, followed in decreasing order by the stream edge, salt marsh, and bog. Finally, forest soil samples and those from a nearby ravine were least complex.

In general, there is evidence that amoeba abundance decreases with depth in sediments (e.g., Decamp et al. 1999; Smirnov 2002), probably due to increasing anoxia and reducing conditions. However, there may be less stratification in soils, at least in the uppermost organically rich layers, especially if they are sufficiently hydrated (e.g., Bischoff 2002). Nonetheless, abundance usually declines within deeper layers of most stratified soils (Cowling 1994). Moisture content is seldom a major environmental variable in sediments, which are water-saturated in many cases. However, moisture is a major factor in determining naked amoeba abundance in terrestrial environments, as well as the proportion that is active versus encysted (e.g., Anderson 2000; Bischoff 2002).

Further evidence of the effects of soil water availability on terrestrial protists, including amoebae, was obtained by Geisen et al. (2014) using soil cores maintained under controlled environmental conditions in the laboratory. Total protist abundance differed eightfold between the two most extreme moisture treatments, and the higher number of total individuals was mainly attributed to an increased abundance of amoebae, which was 7.2-fold higher in the most moist treatment compared to the most dry. Some taxa reached highest numbers only in fully water-saturated soils and readily decreased when the habitable pore spaces became smaller, whereas other taxa were more resistant to decreasing water and only decreased at a later stage of water stress when the maximum size of water-filled pores (Pmax) was <60 μm. Overall, as the authors hypothesized, the largest protist species decreased with increasing soil dryness, but in particular nanoamoebae dominated in the dry soil, not flagellates as was initially predicted.

Successions and seasonal abundance. Abundances of terrestrial naked amoebae are typically lowest in winter and increase during early spring and summer, but precipitation and available moisture are much more significant factors than season (e.g., Anderson 2000; Bischoff 2002). Precipitation accounts for more variance in abundance than local organic content of the soil or its temperature at time of sampling, at least in a study of a temperate upland grassy site (Anderson 2000). Abundances of amoebae during mild winters with substantial precipitation may be comparable to those during warmer seasons of the year (Anderson 2000). Available water and water table depth in swamp and bogs may also be more important factors than is season in accounting for abundances of sphagnum-dwelling testate amoebae (Warner et al. 2007). Likewise, biological and microscale environmental factors may be important in explaining seasonal changes in testate amoebae, as documented above for soil-dwelling naked amoebae.

Testate amoebae serve an important role at the inception of succession on land. Wanner et al. (2008) examined the succession of testate amoebae in litter bags in four different soils that varied in nitrogen and phosphorous nutrients to document the early colonization (within less than 55 days) and establishment of testate amoebae

communities. Substrates at the nutrient-poor sites were colonized more rapidly than reference sites where colonization was later and in lower densities. Both small-sized (r-strategist) and larger (K-strategist) species occurred in remarkably high densities on all sites. During later stages of colonization, the influencing environmental factors became more complex, and the composition of the testate communities changed from variability to stability.

The ability of amoebae to encyst and excyst relatively rapidly has contributed to their survival capacity, especially in temperate terrestrial environments where soils are subjected to intervals of drying and protracted periods of freezing during winter. However, the dynamics of encystment and excystment have not been extensively investigated during the development of successions following recovery from winter conditions. Anderson (2010) obtained winter soil samples from an organically rich swamp site and a less-moist mineral soil beneath a stand of pine and observed the dynamics of excystment in laboratory microcosms during warming simulating spring temperatures. The proportion of active and encysted naked amoebae was documented for 10 days during the ensuing succession (Fig. 4). The pine stand sample (ambient 18% moisture and organic content 6%) overall had lower initial total densities of naked amoebae and proliferated to lower total levels after 10 days compared to the swamp sample (ambient 47% moisture and organic content 15%). However, the proportion of encysted to total amoebae was more informative. In the drier pine stand sample, the proportion of encysted relative to total amoebae increased markedly during the 10-day rising from an initial 60% to a final 100% (Fig. 4a), whereas, the proportion of encysted to total amoebae in the swamp sample,

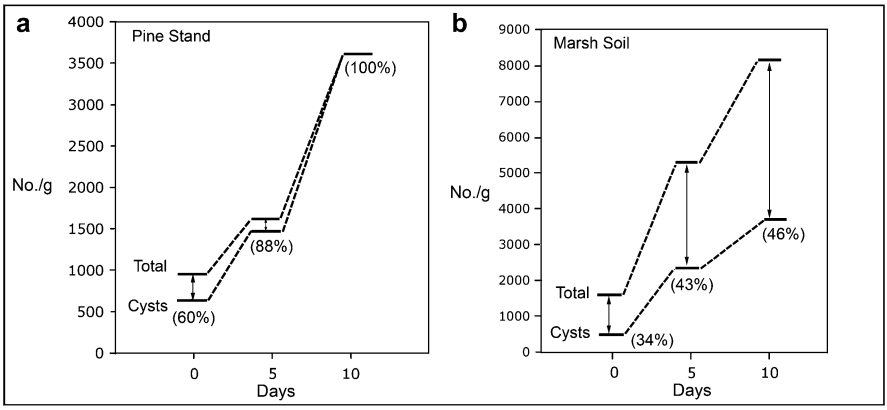

Fig. 4 Comparative plot of the densities of total amoebae (*upper graph*) and cysts (*lower graph*) including (percent encysted) at 0, 5, and 10 days in a laboratory microcosm study of a succession when winter soil was warmed to simulate spring temperatures. Plot of relative densities of total amoebae and encysted stages for a pine soil sample (**a**) and marsh soil sample (**b**) showing the gradual progression toward total encystment in the pine soil preparation and a more steady-state lesser ratio of encysted to total amoebae in the marsh soil preparation (Reproduced from Anderson (2010) with permission of the publisher (*Acta Protozoologica*))

though increasing moderately from 34% to 46% over 10 days, was much more stable (Fig. 4b). The increase between day 5 and day 10 for the swamp sample was 43–46%, which is probably near a constant carrying-state value.

Overall, in both samples there is clearly a dynamic relationship between active and encysted stages, with evidence of substantial interconversion of active and encysted stages during the succession. More recently Anderson (2016) showed that, in addition to encysted stages, amoebae in temperate soil environments are capable of forming freeze-resistant resting stages without forming cysts. These "resting cells" are able to rapidly resume active feeding and metabolism when the soil unfreezes, either intermittently during winter or with the onset of spring, thus providing a more rapid exploitation of the environment than can be achieved if the amoebae had encysted. However, soil moisture must be sufficient at the time of freezing to support active amoebae; otherwise, they will encyst rather than forming resting stages.

In general, the typical circular cycle of alternation between encysted and active stages portrayed in textbooks, and some scientific treatises, should be expanded to include recognition of the dynamic balance in the alternation of the two stages during early succession. Moreover, further refinement is needed to include winter freeze-resistant resting stages that may provide more rapid resumed growth compared to cysts, requiring a more prolonged time for excystment in response to favorable growth conditions.

Given an increasing interest in high-latitude environments and climate change, Anderson (2008) examined the seasonal abundance of naked and testate amoebae during a succession from spring (June) to summer (August) at a tundra site (Toolik Lake, Alaska). Naked amoebae abundance (number per g of soil dry weight) increased from 2×10^4 to 3×10^4, and testate amoebae abundance increased more markedly from 1000 to 6000 during the seasonal succession. Interestingly, in terms of carbon content, the testate amoebae accounted for a larger part of the biotic carbon fraction than naked amoebae. Testate amoebae comprise a significant part of the microbial communities in moss-rich, high-latitude environments.

With evidence of global warming, what is the likely effect on these significant microbial communities? Beyens et al. (2009) examined the potential effects of global warming on the structure of testate communities by experimentally simulating a heat wave in Greenland arctic soils. Although the experimental heating of the soil was sufficiently severe to induce significant leaf mortality in the aboveground vegetation, overall there was no detectable effect on testate amoebae abundance. However, transient shifts in species populations occurred in the heated plots during the acute exposure, followed by increases in species richness weeks after the experimental heat wave had ended. Lobose pseudopod-bearing testate amoebae were more resistant to the heating and its associated desiccation than filose amoebae.

Interactions with plants. In general, substantially more information has been gathered on the interaction of testate amoebae with a wide variety of plant types, especially mosses, largely because their tests are more easily preserved and counted in samples (e.g., Cowling 1994). Naked amoebae are known to be abundant in the rhizosphere (root zone) of plants (e.g., Clarholm 1981), and there is increasing

evidence that naked amoeba abundance is higher in the rhizosphere and soil beneath plants compared to surrounding bare soil in a variety of ecosystems, including agricultural soils (Cowling 1994; Zwart et al. 1994, p. 102), arid lands (Robinson et al. 2002), and deserts (Rodriguez-Zaragoza and Mayzlish 2005; Rodriguez-Zaragoza et al. 2005). There is some variation, depending on the precipitation patterns and time of year. Within the limitations of methodological error, the ratio of protozoan biomass in the rhizosphere to that in bulk soil is in the range of 4–6 (Zwart et al. 1994). This is attributed in part to the organic exudates released from plant roots and possibly also the higher moisture content of soil immediately surrounding the roots. The complex interactions of protists, including amoebae, with the plant rhizosphere have attracted considerable experimental research attention (e.g., Zwart et al. 1994) including a spatial analysis of the number of active and encysted amoebae in relation to the distance along the root axis (e.g., Coûteaux et al. 1988).

With evidence of increasing atmospheric carbon dioxide concentrations, there has been an interest in documenting how atmospheric CO_2 affects plants, and, in turn, what effects (if any) there may be on rhizosphere microbial communities. Anderson and Griffin (2001) grew wheat plants in containers in controlled climate chambers with ambient and elevated carbon dioxide concentrations. Plant dry biomass was higher in the elevated CO_2 treatment (4.4 g/plant) compared to the ambient treatment (2.8 g/plant). The rhizosphere mean abundance of flagellates, ciliates, and amoebae, expressed as number/g dry weight, was greater in the elevated CO_2 treatment compared to the ambient treatment, with an approximate twofold difference in amoeba abundances. Comparable results using pot-grown wheat plants were reported by Rønn et al. (2003), who found that soil from pots with plants grown in elevated CO_2 had higher abundances of protozoa (especially bacterivorous amoebae) but similar abundances of bacteria. The bacteria may have been under grazing pressure by the predators, thus controlling their numbers.

The interactions of the protozoan and bacterial communities with mycorrhizal fungi in the soil may be complex (Rønn et al. 2002). In the absence of fungi, protozoan abundance was enhanced under elevated CO_2 treatments, but when fungi were present, the abundance of protozoa was reduced, possibly by adverse competitive effects of the fungi on the growth of food bacteria. Similar results were found in the natural environment for soil microbiota in grasslands exposed to elevated CO_2 (Hungate et al. 2000). Although the biomass of active fungi and flagellates increased, there was no difference in the abundance of ciliates and naked amoebae between the ambient and elevated treatments. In sum, there appear to be at least short-term effects of elevated atmospheric CO_2 concentrations on plant growth and root proliferation, and consequently increased sources of organic nutrients to support microbial communities, including an increased abundance of amoebae. However, complex interactions in the microbial communities, especially with fungi, may moderate these effects. Moreover, in some cases the relative peak in protozoan abundance during the first several weeks in the elevated CO_2 treatment was not sustained for longer time intervals. The reasons are not clear, but changes in trophodynamics, including increased top-down predation on the protozoa, may account for their decline in abundance with time.

Increasing evidence that rhizosphere eukaryotic microbes enhance plant growth has led to some interesting experimental studies to better understand the synergistic interactions. Bonkowski et al. (2001) examined the effects of amoebae on growth of Norway spruce seedlings in experimental cultivation. Spruce seedlings, cultivated with or without an ectomycorrhizal fungus, were grown for 10 months in microcosm chambers with defaunated forest soil, either supplemented with naked amoebae or without amoebae. The presence of amoebae resulted in the development of a more complex root system by increasing root length (51%), length of fine roots (64%), and number of root tips (43%). The effects of the amoebae were more pronounced in the absence of mycorrhizae. The explanation for enhanced growth of plants in the presence of protozoa is not fully determined, although the most direct effect is likely the remineralization of nutrients by predation on bacteria and perhaps by activation of bacteria that break down complex molecules into smaller, more available sources of plant nutrition.

In a more novel perspective, Bonkowski and Brandt (2002) evaluated the hypothesis that rhizosphere protozoa enhance plant growth by a grazing-induced stimulation of plant growth-promoting rhizobacteria that release plant growth substances (phytohormones). They investigated changes in root morphology of watercress seedlings and effects on the composition of the rhizosphere bacterial community, by adding *Acanthamoeba* sp. to the experimental treatments. They found that the presence of *Acanthamoeba* sp. induced changes in root morphology of watercress seedlings as soon as the root protruded from the seed, i.e., it was greater and more branched. These changes resembled hormonal effects and were accompanied by an increase in the proportion of auxin-producing rhizosphere bacteria. Evidence showed that the auxin (indole-3-acetic acid, IAA) did not originate from amoebal metabolism but resulted from changes in the composition and activity of the prokaryotic microbial community. They proposed a new mechanism based on hormonal effects of protozoa on root growth: protozoa function as "bacteria-mediated mutualists" promoting plant growth by hormonal feedback mechanisms and, as previously proposed, also due to nutrient effects based on nutrient release from grazed bacterial biomass, i.e., the microbial loop. There are undoubtedly multiple synergistic effects in the plant-protozoan association, but the preponderance of evidence, both experimental and from field studies, indicates that there is a mutual enhancing effect through the association of these two very diverse biotas.

Maintenance and Cultivation

Detailed instructions for collecting and laboratory cultivation of amoebae have been published by Page (1988). Some general information is presented here. A good source for collecting amoebae is the organic debris and decaying plant material usually present in the sediment of shallow ponds. Collect some of the debris from the pond using any convenient container such as a small plastic pitcher with a handle, or a large cup. Gently suspend the debris in the water and pour small portions into shallow culture dishes (e.g., 9 cm plastic or Pyrex Petri dishes). Keep the dishes

covered to prevent excessive evaporation and possible increase in solute and nutrient concentrations. Maintain a temperature in the range of 25°C. Avoid direct sunlight to prevent overheating. After several days, when the preparation has become more stable, add a small segment of a heat-killed wheat seed or a small rice grain to serve as a source of nutrients for food bacteria for the amoebae (wheat seeds and contaminant-free rice grains are available from organic food stores or from biological supply houses). After about 1–2 weeks, withdraw some water from the bottom of each dish and examine it with a microscope. Phase-contrast microscopes are preferred for visualization of smaller flattened amoebae. A 40x objective is usually necessary. If you find that there are sufficient amoebae to be visualized within a few milliliters of water, you can transfer aliquots into new culture dishes. Try to include some of the organic debris when making the transfer so there will be an initial source of food for the amoebae, and add a freshly prepared portion of a wheat seed, or rice grain, to the new dish. Usually, within a week to 2 weeks, you should obtain a fairly robust culture that can be maintained by periodic transfer of aliquots to new culture dishes prepared as above. Sometimes, a better yield of amoeba growth is obtained if you use a cube of nutrient-enriched agar to promote bacterial growth, instead of, or in combination with, wheat seed. Prepare the agar as follows: fully dissolve 0.1 g of malt extract and 0.1 g of yeast extract in 1 L of water from the culture site, or a good grade of noncarbonated bottled springwater may also suffice. For convenience, take a 100 mL portion and add 1.5 g of non-nutrient agar. Gently heat until the agar becomes a sol (a microwave oven is often preferable to prevent overheating the agar). Care must be taken not to allow the agar to froth and boil over. The agar sol is poured into a clean or preferably sterile Petri dish to about ¼ depth and solidified. The Petri dish can be wrapped in plastic film and kept in the refrigerator until needed. Portions about 1 cm square are cut from the solidified malt/yeast agar preparation and added to your culture dishes as a source of nutrients for food bacteria. You may increase the concentration of the malt and yeast extract twofold if you want a slightly more robust source of nutrients.

An improved yield of amoeba growth may be obtained by using one of several mineral media (Page 1988) such as modified Neff's amoeba saline. Prepare each of the following stock solutions by dissolving in 100 mL of glass-distilled water.

NaCl	1.20 g
$MgSO_4 \cdot 7H_2O$	0.04 g
$CaCl_2 \cdot 2H_2O$	0.04 g
Na_2HPO_4	1.42 g
KH_2PO_4	1.36 g

Prepare the final dilution by adding 10 mL of each stock solution to enough glass-distilled water to make 1 L. The very slightly saline solution reduces osmotic stress for some amoebae, but the culture medium must be prepared exactly as prescribed. You may be able to obtain a high quality of bottled distilled water at a local pharmacy or food store, but caution must be exercised to ensure that the water is as pure as possible.

Living amoeba cultures are available from biological supply houses and some culture collections. If you prefer to pursue your own collections, a key to success is to try collecting from a variety of sites to ensure as much diversity as possible. Avoid contaminating the cultures with toxic substances introduced in unclean containers or from impure water used to make the culture media. Persistence often leads to success if a good natural source of sample material is located. If you choose to sample brackish or marine sites, prepare your culture media using water from the source, again trying to find samples from rich organic sediments or where you see organic floc or plant debris. In general, whatever your source for samples, do not put too much debris in your culture dishes when you transfer your suspension; otherwise, an overgrowth of bacteria may make the culture medium too acidic and/or too anoxic for good amoeba growth.

Evolutionary History: In Light of Molecular Phylogenetics

The naked amoebae, without a substantial enclosing test or shell, leave no trace in the microfossil record, and therefore their evolutionary history must be inferred from other evidence, including interpretations based on comparative morphology, fine structure, life histories (e.g., Schönborn 1989; Schuster 1979, 1990), and, more recently, the significant insights obtained from molecular phylogenetics (e.g., Minge et al. 2009; Tekle et al. 2008). Testate amoeba tests are well preserved in some environments and provide a fairly robust microfossil record (Deflandre 1953). Classical evidence (e.g., Bradley 1931; Loeblich and Tappan 1964) has also been augmented by modern molecular phylogenetic analyses. Some of the most ancient microfossil specimens are from the middle Eocene epoch and are similar to extant species, including approximately 24 species (e.g., Schuster 1990). Moreover, fossils resembling the tests of lobose testate amoebae have been reported from the 740 MYA Chuar formation (Porter and Knoll 2000; Porter et al. 2003).

Although the evolutionary roots of the naked amoebae remain obscure, there is emerging strong molecular genetic evidence that they arose from flagellated ancestors (Cavalier-Smith et al. 2014, 2015; Minge et al. 2009; Paps et al. 2013), as previously inferred in earlier treatises (e.g., Bovee and Jahn 1973; Schuster 1990). Whether the naked amoebae (Amoebozoa) are monophyletic or polyphyletic has been a topic of considerable debate (e.g., Bovee and Jahn 1973; Chatton 1953; Page 1976). Currently, there is increasing evidence that the Amoebozoa are monophyletic (e.g., Cavalier-Smith et al. 2015; Lahr et al. 2011; Tekle et al. 2008). The order of evolutionary emergence of the major Amoebozoan groups is not fully resolved, but Tekle et al. (2008) position the Tubulinea at a deeper level in the phylogenetic tree than Flabellinea, and more recently, Cavalier-Smith et al. (2015) place the Discosea near the base of the Amoebozoa.

The Arcellinida (lobose testate amoebae) are grouped within the Tubulinea, with fairly good evidence of monophyly based on ribosomal RNA analyses, but not on actin analyses. The tree of Tekle et al. (2008) places *Echinamoeba* in the Tubulinea,

basal to Leptomyxida, followed by Arcellinida, Hartmannellidae, Amoebidae, and Thecamoebidae. Acanthamoebidae is basal relative to Dactylopodida and Vannellidae. However, this is a rapidly developing field, and further refinements and adjustments are to be expected. Although our knowledge of phylogeny of testate amoebae is advancing, the origin of the test during evolution remains unclear.

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Protosteloid Amoebae (Protosteliida, Protosporangiida, Cavosteliida, Schizoplasmodiida, Fractoviteliida, and Sporocarpic Members of Vannellida, Centramoebida, and Pellitida)

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Frederick W. Spiegel, Lora L. Shadwick, George G. Ndiritu, Matthew W. Brown, Maria Aguilar, and John D. Shadwick

Abstract

Protosteloid amoebozoans, formerly called protostelids, are a non-monophyletic assemblage of Amoebozoa where, at one point in their development, an amoeba rounds up on the surface of a substrate and develops into a subaerial fruiting body, or sporocarp. The sporocarp consists of a noncellular, microscopic stalk bearing one to a few terminal spores. Amoeboid states of protosteloid amoebae vary considerably in morphology, and many species have life cycles that include both amoeboflagellates, a cell type that can reversibly transform from amoeba to flagellate, and obligate amoebae, a cell type that exists only as an amoeba. Protosteloid development was first recognized in *Protostelium mycophaga* and has been observed in perhaps 100 species, roughly 40 of which are formally named. Protosteloid amoebae are predators of decomposer bacteria and fungi in terrestrial ecosystems. They are global in distribution. Most are quite easy to isolate and to bring into culture, thus facilitating developmental studies. Sporocarpy, as a mode of development, is found in protosteloid amoebae and in myxogastrids; it is exclusive to Amoebozoa. This raises the question whether it is

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a homologous process or whether it has arisen independently multiple times within Amoebozoa. If the former proves to be the case, it would suggest that the last common ancestor of Amoebozoa must have had a sporocarp in its life cycle.

Keywords

Amoeba • Terrestrial environments • Fruiting • Sporocarp • Stalk development • Spore development • Amoeboflagellate

Contents

Taxonomic Summary

●**Amoebozoa**
●●**Variosea**
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●●●**Fractoviteliida** (*Soliformovum*)
●●●**Schizoplasmodiida** (*Schizoplasmodium*, *Nematostelium*, *Ceratiomyxella*)
●●●**Cavosteliida** (*Cavostelium*, *Schizoplasmodiopsis*, *Tychosporium*)
●●**Macromycetozoa**

●●●**Protosporangiida** (*Protosporangium*, *Clastostelium*, *Ceratiomyxa*)
●●●**Myxogastria** (*Echinostelium bisporum* only)
●●**Discosea**
●●●**Centramoebida** (*Acanthamoeba pyriformis*, *Luapeleamoeba*)*
●●●**Pellitida** (*Endostelium*)**
●●●**Vannellida** (*Protosteliopsis*)**

[Other protosteloid Amoebozoa (incertae sedis): *Microglomus*, *Echinosteliopsis*]

Note: Only groups with protosteloid members are listed; only protosteloid example genera are listed.

* – part of Longamoebia, as covered in ▶ Amoebozoan Lobose Amoebae (Tubulinea, Flabellinea, and Others)

** – part of Flabellinia, as covered in ▶ Amoebozoan Lobose Amoebae (Tubulinea, Flabellinea, and Others)

Introduction

General Characteristics

Protosteloid amoebozoans, formerly called protostelids (see Shadwick et al. 2009b), are a non-monophyletic assemblage of terrestrial protists found in both major subgroups of Amoebozoa, Conosa, and Lobosa (sensu Smirnov et al. 2011; Cavalier-Smith et al. 2015). They are considered together in this chapter because of their morphological and ecological similarity. All have an amoeboid trophic stage, or stages, that live primarily as predators of decomposer microorganisms in terrestrial habitats, and one of those amoeboid stages can develop into a stalked, spore-dispersal structure, or fruiting body, called a "sporocarp" in the terminology of Olive (1975). Sporocarps of protosteloid amoebae typically are morphologically similar, consisting of a microscopic stalk bearing a single spore or a few spores, while the amoebae are quite morphologically variable from taxon to taxon (Figs. 1, 2, 3, and 4). Sporocarpy, where a fruiting body develops from a single amoeboid cell, is a form of development that is only seen in Amoebozoa.

The taxon Eumycetozoa (sensu Olive 1975, Adl et al. 2012) was established to include the monophyletic and sporocarpic plasmodial slime molds, Myxogastria or myxomycetes (see ▶ Myxomycetes), the monophyletic and sorocarpic dictyostelid cellular slime molds, Dictyostelia (see ▶ Dictyostelia), and what Olive described as a paraphyletic assemblage of sporocarpic mycetozoans that he called Protostelia, or protostelids. The unifying characters of Eumycetozoa according to Olive were (1) amoeboid trophic cells with acutely pointed subpseudopodia, (2) mitochondria with tubular cristae (Olive 1975; Dykstra 1977), and (3) the ability to fruit by producing stalked, spore-bearing structures (though stalks are absent in some myxomycetes). Of the Eumycetozoa, sensu Olive, protostelids have the simplest fruiting bodies, or sporocarps (Olive 1975; Spiegel 1990; Spiegel et al. 2004; Shadwick et al. 2009). They develop from single amoebae or from nucleated fragments of

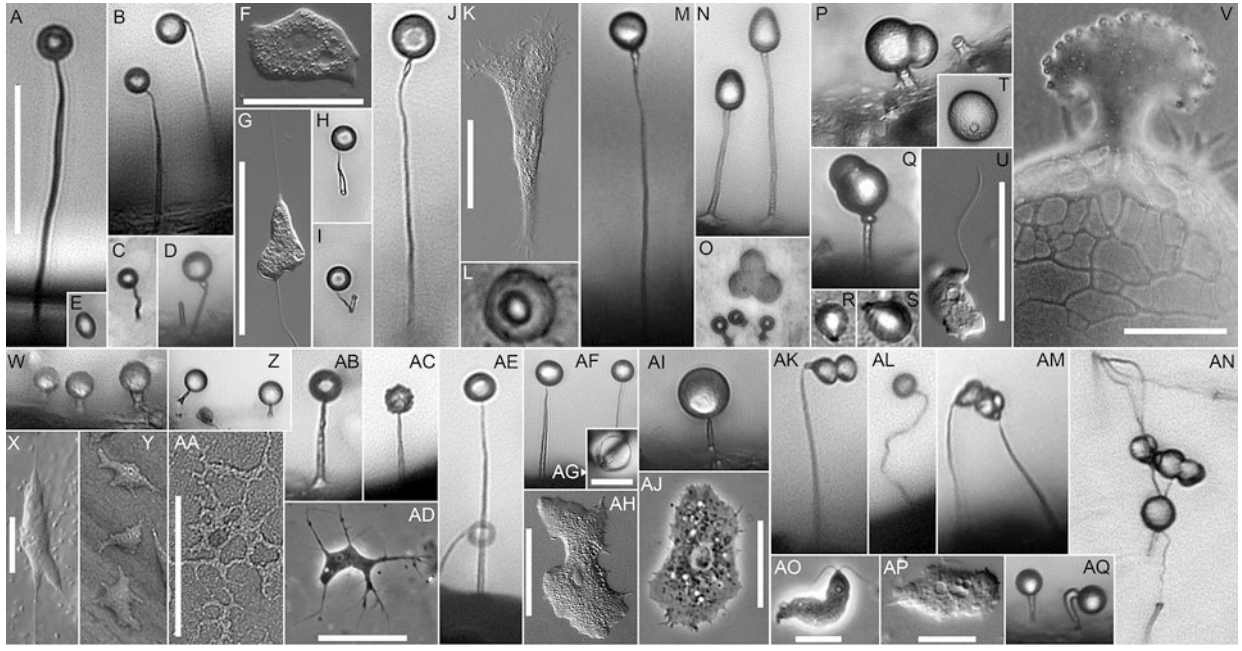

Fig. 1 All presently described species of protosteloid amoebae in Variosea (**a–aj**) and the simple protosporangiid Macromycetozoa (**ak–aq**). **a–g** Protosteliida. (**a)** Sporocarp of *Planoprotostelium aurantium*. (**b)** Sporocarps of *Protostelium mycophaga*. (**c)** Sporocarp of *P. nocturnum*. (**d)** Sporocarp of *P. okumukumu* with stalk base to its left. (**e)** Vertical view of early prespore cell of *P. nocturnum* showing morphology typical of *Protosteliida*. (**f)** Amoeba of *P. mycophaga* showing morphology typical of Protosteliida. (**g)** Biflagellated amoeboflagellate of *Pl. aurantium*. (**h–l)** Protosteloid Fractovitelliida. (**h**, **i)** Two views of sporocarps of *Soliformovum expulsum* showing bipartite stalk. (**j)** Sporocarp of *S. irregularis*. (**k)** Locomoting amoeba of *S. irregularis* showing morphology typical of genus. (**l)** Early prespore cell of *S. irregularis* showing morphology diagnostic of genus. (**m–v)** Schizoplasmodiida. (**m)** Sporocarp of *Ceratiomyxella tahitiensis*. Sporocarps of this species and *Nematostelium gracile* are indistinguishable from each other. (**n)** Sporocarps of *N. ovatum*. (**o)** Plasmodial obligate amoeba of *C. tahitiensis* cleaving into three prespore cells (*top*) and three rising sporogens (*below*). This mode of development is characteristic of all Schizoplasmodiida.

multinucleate amoebae and consist of a microscopic stalk that supports one or a few walled spores (Figs. 3 and 4). In the last edition of this Handbook, Spiegel (1990) suggested that most protostelids were members of a paraphyletic assemblage in a monophyletic Eumycetozoa but held out the possibility that some purported protostelids were not closely related to other fruiting amoebae at all. Initial molecular phylogenies, which included only single species of protostelids, gave some support to the taxon Eumycetozoa that contained protosteloid amoebae, myxomycetes, and dicytostelids (Spiegel et al. 1995a; Baldauf and Doolittle 1997; Baldauf et al. 2000). However, phylogenetic work with more species of purported protosteloid amoebae and a large set of other amoebozoans shows that organisms that had been called protostelids appear in several lineages of the taxon Amoebozoa (Shadwick et al. 2009b; Fiore-Donno et al. 2010; Lahr et al. 2011b; Kudryavtsev et al. 2014). For that reason it is now preferred to designate sporocarpic amoeboid organisms that have simple, microscopic, stalked sporocarps as "protosteloid amoebae" (Shadwick et al. 2009b). Regardless of their phylogenetic affinities, all protosteloid amoebae occur in

Fig. 1 (continued) When large plasmodia cleave, gregarious fruiting occurs. (**p**) Complete sporocarp of *Schizoplasmodium cavostelioides* with spore expulsion droplet on right side. Stalks from which spores have been expelled are visible to top right and lower left of intact sporocarp. All species of *Schizoplasmodium,* as used in this chapter, have the droplet-mediated forceful expulsion of spores. (**q**) Sporocarp of *S. seychellarum* with spore expulsion droplet on upper left of spore. (**r, s**) Expelled spore of *S. obovatum* on agar surface. Stalks are identical to those of *S. cavostelioides*. (**t**) Shed spore of *S. cavostelioides* is oriented such that the annular hilum associated with the spore/stalk articulation is visible. This hilum is characteristic of all schizoplasmodiids. Sporocarps of all schizoplasmodiids have highly deciduous spores such that they are often first recognized by shed spores such as these. (**u**) Amoeboflagellate of *C. tahitiensis.* (**v**) Plasmodial amoeba of *S. seychellarum*, as typical of all schizoplasmodiids. Upper flabellate portion is free of bacteria, and highly reticulate veins are immersed in colony of bacteria. This is the only trophic stage reported in *Schizoplasmodium* spp. and *Nematostelium* spp. as here defined. It represents the obligate amoeba stage in the life cycle of *C. tahitiensis.* (**w–aj**) Cavostellida. (**w**) Sporocarps of *Cavostelium apophysatum.* (**x**) Triflagellate amoeboflagellate of *C. apophysatum*. (**y**) Obligate amoebae of *C. apophysatum.* (**z**) Sporocarps of *Schizoplasmodiopsis pseudoendospora.* (**aa**) Highly reticulate plasmodial amoeba of *S. pseudoendospora.* This can be distinguished from schizoplasmodiid plasmodia because it is always reticulate and never flabellate. (**ab**) Sporocarp of *S. vulgare.* (**ac**) Sporocarp of *S. reticulata.* (**ad**) Amoeba of *S. vulgaris*, typical also of *S. reticulata*, showing highly branched pseudopodia that may form reticulations. Amoebae may have less extended pseudopodia under some conditions. (**ae**) Sporocarp of *Schizoplasmodiopsis micropunctata*. (**af**) Sporocarps of *Tychosporium acutostipes.* (**ag**) Spore of *T. acutostipes* showing craterlike hilum, typical also of *S. micropunctata*. (**ah**) Amoeba of *T. acutostipes*, also typical of *S. micropunctata.* (**ai**) Sporocarp of *Schizoplasmodiopsis amoeboidea*. (**aj**) Amoeba of *S.* amoeboidea. Note diffuse nucleolus. (**ak–aq**). Protosporangiida, Protosporangiidae. (**ak**) Two-spored sporocarp of *Protosporangium articulatum* with two spherical spores. (**al**) Two-spored sporocarp of *P. bisporum* with two hemispherical spores. (**am**) Four-spored sporocarp of *P. conicum*. (**an**) Four-spored sporocarps of *P. fragile.* (**ao**) Amoeboflagellate of *P. articulatum*, typical of morphology of all members of Protosporangiidae. This example has two apical kinetids with the long flagellum two of each one apparent. Supernumerary kinetids are common in this group. (**ap**) Obligate amoeba of *P. articulatum*, typical for the Protosporangiidae. (**aq**) Sporocarps of *Clastostelium recurvatum*

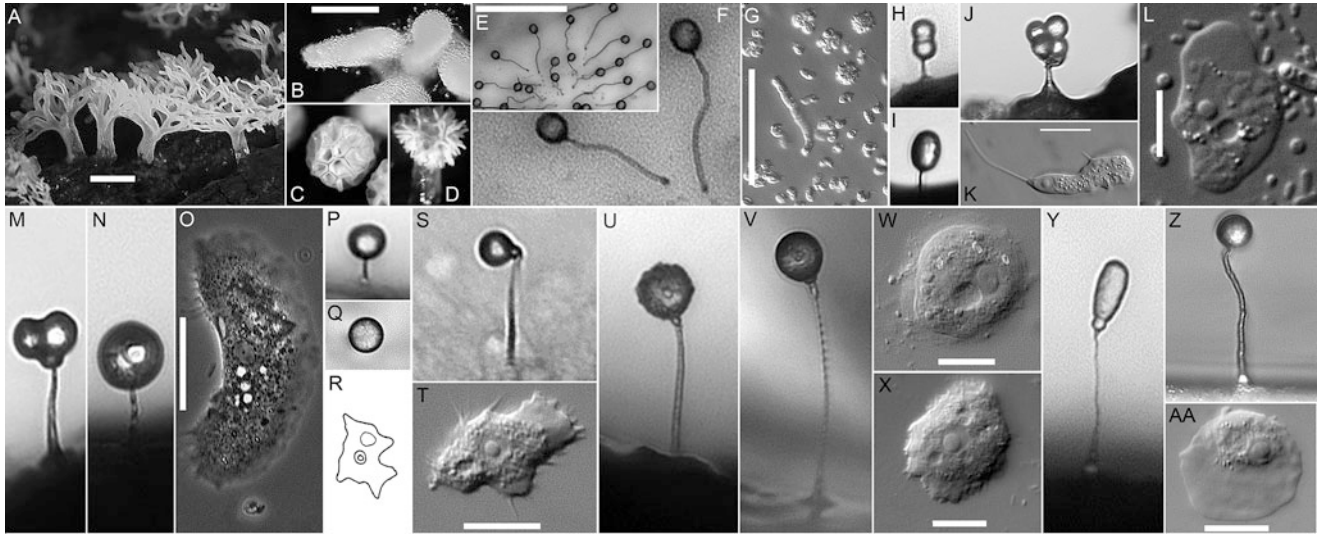

Fig. 2 All presently described species of *Ceratiomyxa*, Protosporangiida, Macromycetozoa (**a–g**), protosteloid and protosteloid-like Myxoagastria, Macromycetozoa (**h–l**), protosteloid Amoebozoa incertae sedis (**m–r**), and protosteloid Discosea (except *Luapeleamoeba hula*) (**s–aa**). (**a–g**) Protosporangiida, *Ceratiomyxa*. (**a**) Macrophotograph of fructification of *C. fruticulosa* showing multiple, arborescent slime columns. (**b**) Detail of slime columns of *C. fruticulosa* showing silhouettes of individual protosteloid sporocarps. (**c**) Fructification of *C. morchella*. (**d**) Fructification of *C. sphaerocephalum*. (**e**) Portion of simple fructification of *C. hemisphaerica* showing gregarious sporocarps on small, subtle slime mound. (**f**) Two individual sporocarps of *C. hemisphaerica*. (**g**) Low power overview of stages of spore germination and amoeboflagellate development in *C. fruticulosa*. In this developmental process, typical of the genus, the tetranucleate germling emerges from the spore as a vermiform cell, rounds up, undergoes a round of mitosis, and then forms eight lobes that separate as individual amoeboflagellates. (**h–l**) Protosteloid Myxogastria, *Echinostelium*. (**h**) Sporocarp of *E. bisporum* with the sheath (peridium) dehydrated showing two spores of sporangium in linear array. (**i**) Sporocarp of *E. bisporum* with hydrated sheath making it difficult to see the spores of the sporangium. (**j**) Sporocarp of the nonprotosteloid myxogastrid, *E. lunatum*, for comparison. (**k**) Flagellated amoeboflagellate of *E. bisporum*. (**l**) Amoeboflagellate of *E. bisporum* in the nonflagellate condition. (**m–r**) Incertae sedis protosteloid amoebozoans. (**m–o**) *Echinosteliopsis oligospora*. (**m**) Four-spored sporocarp with dehydrated sheath. (**n**) Sporocarp with hydrated sheath. (**o**) Amoeba. (**p–r**) *Microglomus paxillus*. (**p**) Sporocarp. Note faint vertical line delimiting spores in the sporangium.

similar habitats and fruit with sporocarps that are morphologically quite simple when compared with most myxogastrids or dictyostelids. However (see below), because being protosteloid is a morphological state and not a taxonomically significant designation, there are some myxogastrids that are also protosteloid, and they are thus included in this chapter (Fig. 2h, i, k, l).

Because species of protosteloid amoebae are difficult to identify with any certainty in the trophic, i.e., amoeboid, stage of the life cycle, the most effective way to find a protosteloid amoeba is to observe its sporocarps on natural substrates under a microscope (Spiegel et al. 2005). The sporocarp develops as follows: a trophic cell rounds up to become one or more prespore cells (Figs. 3d and 4m), rises at the tip of a delicate stalk during the culminating sporogen stage (Figs. 3e and 4n), and

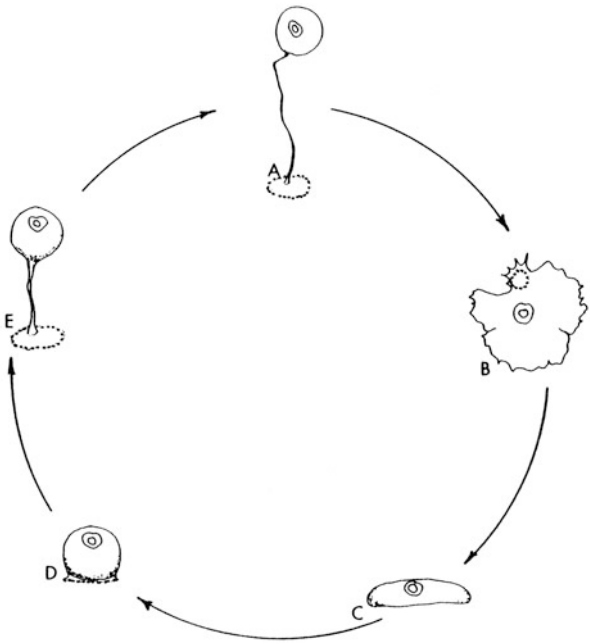

Fig. 3 Simple life cycle of *Protostelium mycophaga*: (**a**) sporocarp; (**b**) amoeba; (**c**) early prespore cell; (**d**) prespore cell just prior to formation of stalk; (**e**) culminating sporogen

Fig. 2 (continued) (**q**) Vertical view of sporangium showing faint outline of four spores. (**r**) Cartoon of amoeba from Spiegel (1990). (**s–aa**) Protosteloid Discosea. (**s, t**) Protosteloid Centramoebida I, *Acanthamoeba* (*Protostelium*) *pyriformis*. (**s**) Sporocarp with obpyriform spore. (**t**) Amoeba with acanthopodia. (**u–w**) Pellitida, protosteloid *Endostelium*. (**w**) Amoeba of *E. zonatum*. (**x, y**). Protosteloid Centramoebida II, *Luapeleamoeba* (*Protostelium*) *arachispora*. (**x**) Sporocarp. (**y**) Amoeba. Note that subpseudopodia are short and not acanthapodial. **z, aa** Protosteloid Vannellida, *Protosteliopsis fimicola*. (**z**) Sporocarp. (**aa**) Amoeba. Scale bars for Figs. 1 and 2. All sporocarps and prespore cells except Figs. 1o and 2e, 50 μm. Figs. 1f, k, o, u, ad, ah, aj and 2o, 25 μm. Fig. 1v, y (scale in Fig. 1aa), aa and 2e, 100 μm. Fig. 1X, 1ag, 1ao, 1ap, 2k, 2t, 2w, 2x, 2aa, 10 μm. Fig. 2a: 1 mm. Fig. 2b, c, d, 250 μm. Fig. 2g, l, 5 μm

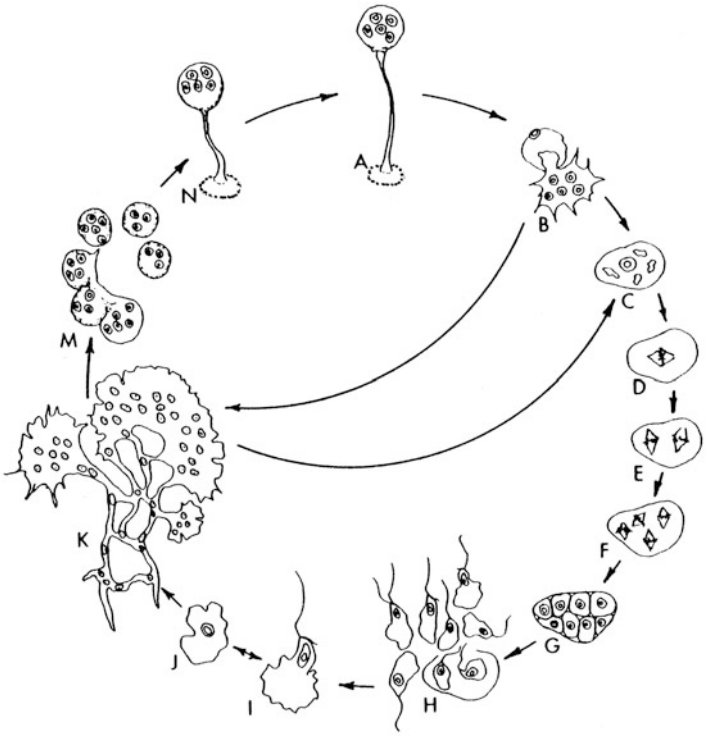

Fig. 4 Complex life cycle of *Ceratiomyxella tahitiensis*: (**a**) sporocarp; (**b**) germinating protoplast; (**c**) all but one of the nuclei degenerating in protoplast, or portion of plasmodium, which is converting into zoocyst; (**d–f**) three nuclear divisions in zoocyst; (**g–h**) eight or fewer flagellate cells cleaving and germinating from zoocyst; (**i–j**) amoeboflagellate stage; (**k**) plasmodium; (**m**) plasmodium cleaving into prespore cells; (**n**) rising sporogen. (**n, b**) the simple life cycle of *Nematostelium gracile* and all other schizoplasmodiids would jump from stage c to stage k with no development of an amoeboflagellate

develops a cell wall to form one or more spores at the apex of the mature stalk (Figs. 1, 2, 3a, and 4a). As stated above, sporocarps alternate in the life cycle with an amoeboid trophic stage, and the life cycle may be simple, with only one type of trophic cell (Fig. 3), or complex, with several types of trophic cells (Fig. 4). In complex life cycles, there is alternation between an amoeboflagellate (amoebomastigote) state and an "obligate amoeba" state (see Spiegel et al. 1995a), with the former associated with spore germination and the latter developing from the amoeboflagellate and subsequently producing the prespore cells. Such a life cycle is consistent with sexuality. Sex is confirmed (sensu, Lahr et al. 2011a; Spiegel 2011) in myxogastrids but has yet to be fully confirmed in other sporocarpic amoebozoans. However, synaptonemal complexes, structures associated with meiosis, have been reported in *Ceratiomyxa* (Furtado and Olive 1971) and *Protosporangium* (Bennett 1986a). Possible syngamy has been reported in *Ceratiomyxa* (Olive 1975; Spiegel 1981a). *Cavostelium* and *Ceratiomyxella* appear likely to be sexual, but direct

Table 1 Phylogenetic grouping of protosteloid Amoebozoa. Clades in *bold* according to the classification scheme of Smirnov et al. (2011). Other clades according to Adl et al. (2012)

Amoebozoa			
Conosa		**Lobosa**	
Variosea + Macromycetozoa	**Archamoebae**	**Tubulinea**	**Discosea**
Protosteliida* (V)	No known protosteloid members	No known protosteloid members	
Fractovitelida (V)			
Schizoplasmodiida* (V)			Centramoebida
Cavosteliida (V)			Pellitida
Protosporangiida* (M)			Vannellida
Myxogastria* (M)			
Conosa incertae sedis – *Microglomus*, *Echinosteliopsis*			

[a]Groups whose known members are all sporocarpic

evidence has yet to be found. In simple life cycles with a single type of amoeba, that amoeboid state may be derived from an amoeboflagellate or from an obligate amoeba (see Spiegel et al. 1995). Most protosteloid amoebae with simple life cycles appear to be asexual, but one taxon with a simple life cycle, *Microglomus*, has been reported to have synaptonemal complexes in its developing sporocarp, indicative of meiosis and a possible sexual life cycle (Olive et al. 1983).

As of this moment, there are 37 morphologically defined species of protosteloid amoebae that have been described formally. Of these 37, 33 are microscopic species (Figs. 1 and 2), i.e., their fruiting bodies can only be adequately resolved with the compound microscope (Spiegel et al. 2007). There are 19 genera, and protosteloid species are found among nine well-supported clades of Amoebozoa (Table 1). Five of these clades are exclusively sporocarpic, while the remaining four contain both sporocarpic and non-fruiting taxa. Two protosteloid species are incertae sedis. Revisionary work still underway suggests that several species may be complexes of cryptic species and that several genera are non-monophyletic as originally conceived. In addition, there are new protosteloid amoebae constantly being found that need formal descriptions. Therefore, the taxonomy of these organisms is still fluid, and it is likely that over 100 species will eventually be described.

Occurrence

Protosteloid amoebae occur as predators of bacteria and fungi on decaying plants in both terrestrial (see Spiegel et al. 2004) and freshwater (Lindley et al. 2007; Tesmer and Schnittler 2009) ecosystems, though they are only known to fruit subaerially, i.e., at the substrate/air interface. They have been found in all habitats where there are decaying plants, ranging from the Antarctic Peninsula to the subarctic (Fig. 5), and they appear to be most abundant in North Temperate to tropical latitudes. Microhabitats in which they occur include aerial, dead primary tissues of plants (i.e., dead pieces still attached to a standing plant), similar tissues in the ground litter, bark of

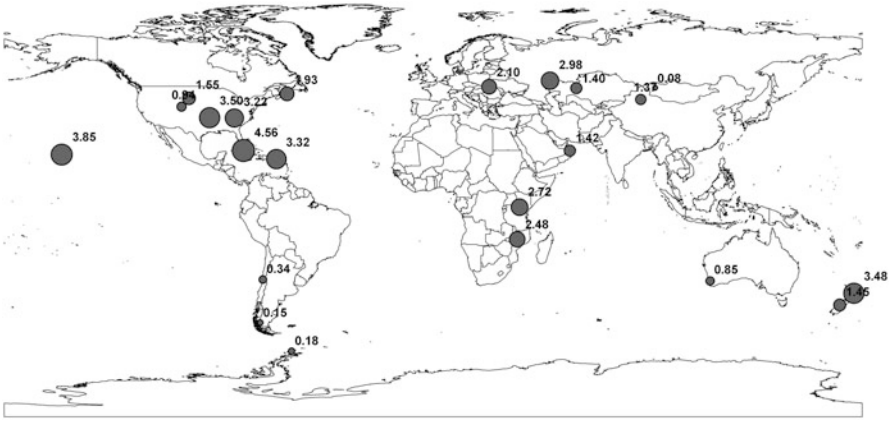

Fig. 5 Map of global collections showing mean number of species of protosteloid amoebae per collection. Though collection effort per country or state varied, there are enough collections associated with each to give at least a qualitative impression that protosteloid amoebae are ubiquitous and that they are more common in some parts of the world than in others

living trees, bark on decaying logs, decaying wood on both standing and fallen trees, herbivore dung, decaying plant parts submerged in freshwater, occasionally the surfaces of living leaves, and occasionally soil.

The most extensive collections of protosteloid amoebae are housed in the F.W. Spiegel lab at the University of Arkansas and the M.W. Brown lab at Mississippi State University, where both active and liquid nitrogen-frozen cultures are maintained. In addition, a number of species are available at the American Type Culture Collection's Eumycetozoan Special Collection, and others are available at the Culture Centre for Algae and Protozoa in the United Kingdom. There are plans to submit additional cultures to CCAP.

Literature

Most of the literature on the taxonomy and systematics of protosteloid amoebae is from the lab of the late L.S. Olive (see Olive 1967, 1970, 1975, 1982) and the lab of F.W. Spiegel (see Spiegel 1984, 1991; Spiegel et al. 1994, 1995a, b, 2004, 2006; Shadwick et al. 2009b, 2016; Adl et al. 2012; Schnittler et al. 2012; Tice et al. 2016). Prior to 1975 most work on the development and ultrastructure of protosteloid amoebae was done by Olive's group (see Olive 1975). After 1975, important publications on ultrastructure and development were carried out by M.J. Dykstra, K.D. Whitney, and members of the Spiegel lab, which are all cited in Spiegel et al. (1995a) and Shadwick et al. (2009b). Ecological research on protosteloid amoebae was pioneered by D.L. Moore, and this work is summarized in Spiegel et al. (2004). After 2004, most work on ecology has been done in association with the Spiegel lab and collaborators (Tesmer et al. 2005; Aguilar et al. 2007, 2011; Lindley et al. 2007;

Ndiritu et al. 2009; Shadwick et al. 2009a; Zahn et al. 2014). No work has been published on the physiology, biochemistry, or genetics of protosteloid amoebae. The best web resource for protosteloid amoebae is the *Eumycetozoan Project* (http://slimemold.uark.edu). It includes identification guides, guides to literature, culturing guides, and interactive maps.

History of Knowledge

The protosteloid amoebae were first recognized by L.S. Olive and C. Stoianovitch in 1959 when they discovered *Protostelium mycophaga*, along with the heterolobosean sorocarpic amoeba *Acrasis rosea* (Olive and Stoianovitch 1960). This was the beginning of a collaboration that lasted almost 25 years, during which the protosteloid amoebae were recognized to be morphologically diverse and ubiquitous members of the decomposer community. Most work on protosteloid amoebae that was not carried out by Olive and Stoianovitch has been done by his students, M.J. Dykstra, K.D. Whitney, W.E. Bennett, and F.W. Spiegel. Since the early 1980s, much work has involved Spiegel's students S.C. Gecks, L.D. Smith, R.N. Bortnick, D.L. Moore, J.D. Shadwick, L.L. Shadwick, G. Ndiritu, and M.W. Brown and collaborators, J. Feldman, D.E. Hemmes, S.L. Stephenson, J. Tesmer, M. Schnittler, C. Lado, and M. Aguilar. Recently, M.W. Brown has set up a lab at Mississippi State University that has become an important site of research on protosteloid amoebae, as well as Amoebozoa in general.

Practical Importance

To date, protosteloid amoebae have been treated as a somewhat esoteric group of relatively little importance because none are known to be pathogens of plants or animals. However, protosteloid amoebae are ideal organisms for studying cell motility and the evolution of cell motility systems (Spiegel, 1981b, 1982a, b; Spiegel et al. 1979, 1986). The actin-myosin-driven process of sporocarp culmination (Spiegel et al. 1979) is a useful model for contractile systems in non-muscle cells, and the variations in kinetid (i.e., flagellar apparatus) structure provide a model for relating rootlet structure to function (Spiegel 1981a, b, 1982a; Spiegel et al. 1986; Spiegel and Feldman 1989). Variations in nuclear division may be useful in understanding structure/function relationships in the mitotic spindle (Spiegel 1982a; Spiegel et al. 1986; Spiegel and Feldman 1986). They are also potentially useful for studying the evolution of amoeba morphology (Spiegel and Feldman 1985; Spiegel et al. 1995a). These organisms appear to make up a major portion of the amoeboid component of the decomposer community, where they feed upon bacteria and fungi (see Spiegel et al. 2004). It is quite possible that they may be involved in controlling populations of bacteria (Olive and Whitney 1982) and fungi (Feest 1987), some of which may be pathogenic. Certainly, much more work is needed to determine the practical ecological importance of protosteloid amoebae. The wide

distribution of amoebozoans with protosteloid fruiting is particularly interesting to understand. If protosteloid fruiting, a phenomenon found only in Amoebozoa, were to be demonstrated to be homologous, that would suggest that this developmental process was found in the last common ancestor of extant amoebozoans (Shadwick et al. 2009; Adl et al. 2012; Spiegel 2016).

Habitats and Ecology

A great deal has been learned about several aspects of the ecology of protosteloid amoebae since the last version of this Handbook (Spiegel 1990). There is still much to learn, however.

Protosteloid amoebae typically are found by bringing potential substrates into the laboratory, keeping them in moist chambers, and examining them under the microscope for the presence of sporocarps (Olive 1975; Spiegel et al. 2004, 2005, 2007). This technique is necessary because all species are microscopic, with the exception of the three larger species of *Ceratiomyxa*. However, Olive (1975) and Olive and Stoianovitch (1972) reported seeing blooms on rotting wood that microscopic examination showed to be large numbers of sporocarps of *Protosporangium* spp.

Substrates that have proven to yield protosteloid amoebae consistently include aerial portions of dead or dying plants that have begun to decay but have not fallen to the ground, decaying plants in the litter, herbivore dung, bark of living and dead trees, and rotting wood. In such substrates, there are large numbers of bacteria and fungi present to serve as a food source. The vast majority of described species of protosteloid amoebae have been found on dead plant parts made up of primary tissues, in both the aerial litter and ground litter microhabitats (see Spiegel et al. 2004). However, in some habitats the assemblages of species occurring in the two microhabitats may differ considerably (see Spiegel et al. 2004; Shadwick et al. 2009). Another source of protosteloid amoebae is the bark of living trees (Olive 1975; Best and Spiegel 1984; Spiegel et al. 2004). Pieces of bark from trees with coarse bark from North Temperate regions kept in a moist chamber for several days will almost always have one or more species present. However, smooth-barked trees, trees from the tropics, and trees from South Temperate regions appear to be much less likely to support protosteloid amoebae. Species of *Protosporangium* are most commonly found on bark. Unfortunately, bark-inhabiting protosteloid amoebae are very difficult to isolate and maintain in cultures; this is true even for species that are easy to isolate when they occur on other substrates (Olive 1975). Of the other substrates, one species, *Protosteliopsis fimicola* (which will shortly be reassigned to *Vannella*; see Shadwick et al. 2009b), is relatively common on herbivore dung. *Protosporangium fragile* and *Ceratiomyxa* spp. occur on rotting wood. In fact, the macroscopic *Ceratiomyxa fruticulosa*, which occurs on rotting wood, is probably the most commonly encountered slime mold (in the broad sense) on earth (see ▶ Myxomycetes). Protosteloid amoebae are rarely reported in humus and soil (Olive 1975; Feest 1987).

Protosteloid amoebae have been recorded from all major types of terrestrial biomes and from all continents (Fig. 5). Early collecting by Olive focused primarily on the Eastern United States and the tropical Pacific, with various additional collections in Australia, New Zealand, Southeastern Asia, Africa, and Western Europe. Other than mention of the detailed locations for nomenclatural types, most discussion of distribution of these early collections is very general. More detail is available in Olive's unpublished collecting notes at the Southern Collection of the Library at the University of North Carolina at Chapel Hill, though there are no truly quantitative data. The first quantitative data on protosteloid amoebae were presented by Best and Spiegel (1984) for temperate habitats in southwestern Ohio, followed by the work of Moore and several coworkers (see Spiegel et al. 2004) who studied the rates at which sterilized substrates were colonized by protosteloid amoebae when introduced to the environment. At present the Spiegel lab is in the process of a global biodiversity inventory of all purportedly eumycetozoan organisms, including all the protosteloid amoebae. Records of several thousand observations from around the world are available with interactive maps at the *Eumycetozoan Project* website (http://slimemold.uark.edu).

As large collection databases are being developed, it is becoming possible to discern some information about the biogeography of protosteloid amoebae in general (Fig. 5 and see Table 2 in Ndiritu et al. 2009):

1. In essentially all latitudes where dead, decaying vegetation occurs in terrestrial (and freshwater) habitats, at least some species of protosteloid amoebae can be found (Fig. 5).
2. Geographic isolation seems to be no barrier to their potential dispersal, since all described microscopic species have been found in high abundance in the Hawaiian Islands (Fig. 5), the most remote archipelago on earth. They have also been collected throughout Polynesia and Micronesia (Olive 1975) and in subantarctic islands (Spiegel and Stephenson 2000).
3. In general, species richness appears to be highest at tropical and temperate latitudes (Fig. 5), with lower species richness at high latitudes. However, species richness is lower than might be expected in southern South America. It is also low in parts of Central Asia.
4. Though species richness seems relatively uniform, the abundance of protostelids appears to be quite variable (Ndiritu et al. 2009). The average number of species per collection is highest in the moist tropics and forested, mesic habitats in North America, relatively high in western Eurasia, lower in drier to arid parts of the Northern Hemisphere, and lowest in southern South America. Until careful ecological analyses of these collections are completed, these patterns cannot be confidently explained.
5. Collections taken from around the world from above an altitude of 3000 m almost never yield protosteloid amoebae. There is presently no explanation for this phenomenon.

Table 2 Media useful for isolating and cultivating protostelids

Medium	Ingredients added to 1 L distilled water
wMY[a, b]	0.002 g malt extract, 0.002 g yeast extract, 0.75 g K_2HPO_4, 15–20 g Difco Bacto agar (Either the malt extract or yeast extract or both may be left out to little effect. Too much of either is more of a problem than too little.)
HI[b, c]	2.5 g hay infused in water, remove hay, adjust volume back to 1 L, add 15–20 g Difco Bacto agar
OB[b,c]	2.5 g white oak bark infused in water, treat as above
CM+	17.5 g Difco cornmeal agar, 2.0 g glucose, 2.25 g yeast extract
Liquid medium[d]	2.5 g peptone, 0.5 g yeast extract, 1.0 g glucose, 2.25 g KH_2PO_4, 0.5 g K_2HOP_4, 0.2 g $MgSO_4 \cdot H_2O$

[a]The standard medium in the Spiegel lab
[b]pH may be adjusted as desired, usually with lactic acid and NaOH
[c]Infusions may be made of other plants and types of bark
[d]The salts from this medium make a useful buffer

Olive (1975) stated that some species were abundant, others common, others occasional, and some rare. However, until the work of Best and Spiegel (1984), there were no quantitative data to support these conclusions. Even examination of Olive's unpublished collection records suggests that his group did not keep detailed information on the frequency with which species were observed; rather, they recorded only the occurrences that had particular interest to them. With the advent of the ecological studies of Moore (see Spiegel et al. 2004), a standard for describing species as abundant, common, occasional, or rare was established. An abundant species is one in which at least 10% of collections support the species, common >5–<10%, occasional >1–<5%, and rare <1%. From the set of 3535 collections used to generate Fig. 5, the two most abundant morphological species, globally speaking, are *Protostelium mycophaga* (39%) and *Schizoplasmodiopsis pseudoendospora* (34%). Other globally abundant species are *Schizoplasmodiopsis amoeboidea* (24%), *Soliformovum irregularis* (22%), *Nematostelium gracile* (including *Ceratiomyxella tahitiensis*) (22%), *Cavostelium apophysatum* (13%), *Schizoplasmodiopsis vulgare* (11%), *Luapeleamoeba* (as *Protostelium*) *arachisporum* (11%), and *Nematostelium ovatum* (10%). Globally, common species are *Protostelium nocturnum*, *Soliformovum expulsum*, *Echinosteliopsis oligospora*, *Schizoplasmodium cavostelioides*, *Tychosporium acutostipes*, *Endostelium zonatum*, *Acanthamoeba* (as *Protostelium*) *pyriformis*, and the myxogastroid *Echinostelium bisporum*. Because the vast majority of these collections consist of primary plant tissues from the aerial and litter microhabitats, bark specialists and dung specialists are underrepresented. Of the bark-preferring species, *Protosporangium articulatum* is the most common, while *Protosteliopsis fimicola* is the most common species found on herbivore dung.

However, although the ability of protosteloid amoebae to be dispersed to isolated archipelagoes is established, and although it is possible to make global generalizations about the abundance of particular species of protosteloid amoebae (Ndiritu et al. 2009), it is becoming clear that many species show biogeographic patterns. For

instance, even though *Protostelium mycophaga* (Figs. 1b and 3) is the most common species of protosteloid amoebae globally, it is not equally common everywhere. It occurs with high frequency in temperate North America and western Eurasia, Hawaii, East Africa, and New Zealand; however, it occurs at much lower frequency in southern South America, Western Australia, Oman, and Central Asia. Where it is uncommon, it is likely that there is no single explanation. For instance, in southern South America, all protosteloid amoebae are very uncommon, yet *P. mycophaga* is the most frequently found species, while in Oman and Central Asia, *P. mycophaga* is found much less frequently than other species. Another example of an abundant species with a distinct biogeographic pattern is *Nematostelium ovatum* (Fig. 1n). This species is most frequently found in Northern Hemisphere sites, especially in temperate forested areas and tropical sites. The only major habitat where it is not found abundantly is open grassland (see also Moore and Spiegel 2000c), though it is not uncommon in some deserts. However, with the exception of the North Island of New Zealand, *N. ovatum* is rare or absent from high southern latitudes, with the southern end of the North Island of New Zealand being the southernmost point where it is found. Our collecting efforts are reaching a level where it is possible to identify the biogeographic patterns of at least the abundant and common protosteloid amoebae even if we do not yet have any explanation for these patterns. Other information on other species is covered below in the section "Characterization and Recognition."

On a finer scale, Olive's work (see Olive 1975) suggested that various species were likely to be found in some microhabitats more than others. This was first borne out quantitatively in the work of Best and Spiegel (1984) which showed that some species were much more likely to occur on aerial dead plant parts than on bark, while others, such as species of *Protosporangium*, almost exclusively inhabit bark, as had been suggested by Olive's work. Moore (see Spiegel et al. 2004) was first to show that aerial and ground litter microhabitats from the same habitat could yield consistently different assemblages of protosteloid amoebae. As a rule, work over the last 20 years has shown general patterns for at least the common and abundant species that are covered below in section "Characterization and Recognition."

Spores of protosteloid amoebae are readily dispersed. Work by Moore and Spiegel (see Spiegel et al. 2004), which monitored colonization of sterilized wheat straws placed in the field, showed that dead, primary plant tissues could be colonized by protosteloid amoebae and their prey microorganisms in as little as 1 week (see Spiegel et al. 2004), and it has been noticed subsequently that some substrates can be colonized within 24 h (J. Shadwick, unpublished). Spores of protosteloid amoebae may be deciduous or nondeciduous. It is likely that the deciduous species are air-dispersed, at least in part. Tesmer et al. (2005) present evidence that strongly suggests that air dispersal is sufficient to account for the colonization of European beech leaves by *Protostelium mycophaga*. Many species of protosteloid amoebae produce forcibly ejected and dispersed spores, using a number of different mechanisms (see Spiegel et al. 1994, 2006). It is likely that these dispersal mechanisms serve to lift spores above the boundary layer of the substrate. In fact, all deciduous species with sporocarps under 40 μm tall appear to have forcible spore discharge.

Sporocarps of nondeciduous species, and perhaps spores and sporocarps of some deciduous species, may be dispersed by small arthropods and other invertebrates, by myxomycete plasmodia, or by water dispersal. All these mechanisms have been seen in culture plates and "primary isolation plates" (see section "Maintenance and Cultivation", below). Small invertebrates moving about on plates often become covered with spores and sporocarps and deposit them as they move about. During a bad mite infestation in working cultures that occurred in the Spiegel lab, the mites carried species from one plate to another. In one case, a millipede captured in the field and allowed to walk across an agar plate was found to be carrying *Schizoplasmodiopsis vulgare* (M.W. Brown, unpublished). Myxomycete-facilitated dispersal occurs when sporocarps of protosteloid amoebae float up upon the outer surface of plasmodia that migrate into them and then are carried some distance until they are deposited in the slime trail the plasmodium leaves behind (Spiegel, unpublished). Water dispersal is effected by sporocarps floating on their hydrophobic basal disks and being carried along by the flow (Spiegel, unpublished).

If one collects substrates throughout the year, it is possible to find species of protosteloid amoebae on them. However, if one introduces sterile substrates into the environment, then one finds that their dispersal is seasonal. Moore and Spiegel (see Spiegel et al. 2004) found that sterilized wheat straws were colonized about four times as frequently during the summer as the winter in Northwest Arkansas. This suggests that although protosteloid amoebae are present in winter, they are most likely to be in dormant states and that they are active and producing dispersal states, presumably fresh spores, only in warmer months.

Future work should include environmental sequencing because it may yield additional detail on the ecology of protostelids that cannot be discovered if they are not observed to be fruiting.

Characterization and Recognition

The *sine qua non* of protosteloid amoebae is the sporocarp, consisting of a delicate stalk that bears one to four spores or sometimes more (Figs. 1 and 2). These organisms are found by direct observation of their sporocarps that have developed on bits of natural substrate on primary isolation plates (see section "Maintenance and Cultivation"). Although sporocarps are superficially similar to the simple sporangia and conidiophores of some fungi, they may be easily distinguished because the fungal structures arise from hyphae and protosteloid sporocarps rest upon basal disks (Olive 1975; Spiegel et al. 2004, 2007). Their appearance is distinct enough that one can quickly learn to recognize protosteloid fruiting bodies on natural substrates. With a few weeks' practice, one can recognize almost all species of protosteloid amoebae by their sporocarps (Figs. 1 and 2; see also Spiegel et al. 2007). This makes it relatively easy to survey the biota of these organisms (see Spiegel et al. 2004; Tesmer et al. 2005; Aguilar et al. 2007).

However, though most species can be identified by fruiting body morphology, life histories must be taken into account both when confirming that an apparent

sporocarp is a protosteloid amoeba and when classifying species into higher groups (see Spiegel et al. 1995a).

For confirmation, especially with apparently undescribed species, spores must be germinated and/or an amoeba must be observed to fruit to confirm that what looks like a sporocarp is actually the fruiting stage of a protosteloid amoeba. Several microfungi produce sporulating structures similar to protosteloid sporocarps (see Olive 1975; Spiegel et al. 2007). Though culturing from spore to spore is ideal, these observations may still be made if culture proves difficult. Often, it is possible to identify the amoebae that produce sporocarps or to recognize the amoebae that germinate from spores on primary isolation plates. In other cases, spore germination can be observed on culture slides or culture plates, even if the whole life cycle cannot be completed in culture. In the latter case, great care must be taken to make frequent observations to be certain that any amoebae observed on a slide or plate actually germinated from the spores in question (Spiegel et al. 2005).

As our knowledge of the molecular signatures of protosteloid amoebae increases, and as our ability to acquire sequences from single spores improves, it will become possible to confirm that fruiting bodies belong to amoebozoans even if amoebae are never seen. For example, it was confirmed that *Endostelium amerosporum* is indeed congeneric with other species of culturable *Endostelium* spp. by acquiring sequence data from single spores (Kudryavtsev et al. 2014). Recent work in M.W. Brown's lab indicates that single-spore sequencing from primary isolation plates has great promise for expanding the ability to identify/confirm new species of protosteloid amoebae from sporocarp observations alone.

At present we accept 37 validly described species of protosteloid amoebae. Most are microscopic in all stages of the life cycle. Thirty-five of the described species fall into well-supported monophyletic groups of amoebozoans that have both morphological and molecular identities (Shadwick et al. 2009b; Lahr et al. 2011a; Adl et al. 2012), and two are considered Amoebozoa of the taxon Conosa, incertae sedis. These are listed below, and their places in the amoebozoan phylogeny-based classification are shown in Table 1:

1. Conosa/Variosea/Protosteliida (Fig. 1a–g) has four species, three in the genus *Protostelium* and one in the genus *Planoprotostelium* that are formally named, and three more that are being described (J. D. Shadwick et al., unpublished). All known species fruit. It corresponds to Group I of Spiegel (1990), minus *Acanthamoeba* (*Protostelium*) *pyriformis*.
2. Conosa/Variosea/Fractovitelliida (Fig. 1h–l) has two named protosteloid species in the genus *Soliformovum*, with one being described (J.D. Shadwick, unpublished). This group also includes the non-fruiting taxon *Grellamoeba* (not covered here). *Soliformovum* corresponds to Group III of Spiegel (1990).
3. Conosa/Variosea/Schizoplasmodiida (Fig. 1m–v) has six named protosteloid species in three genera, *Schizoplasmodium*, *Nematostelium*, and *Ceratiomyxella*. It corresponds to Group II of Spiegel (1990). All known members of the taxon fruit.

4. Conosa/Variosea/Cavosteliida (Fig. 1w–aj) has seven named species and at least two needing to be described. It presently consists of three genera, *Cavostelium*, *Schizoplasmodiopsis*, and *Tychosporium*. It corresponds to Group IV of Spiegel (1990), with the addition of *Tychosporium acutostipes*. All known species fruit.
5. Conosa/Macromycetozoa/Protosporangiida (Figs. 1ak–2g) has nine named species, all of which fruit. It is divided into two clades, Protosporangiidae (Fig. 1ak–aq), with five species, and *Ceratiomyxa*, with four (Fig. 2a–g). It corresponds to Group Va of Spiegel (1990). All known species fruit. The three macroscopic species of *Ceratiomyxa* were traditionally considered to be unusual members of the myxogastrids prior to the discovery of protosteloid amoebae and the careful observations of L.S. Olive (see Olive 1975; Olive and Stoianovitch 1979), and they are also discussed briefly in the chapter on ► Myxomycetes.
6. Conosa/Macromycetozoa/Myxogastria (Fig. 2h, i, k, l) has one protosteloid member, *Echinostelium bisporum*. The whole of the myxogastrids are covered in a separate chapter (► Myxomycetes). It corresponds to Group Vb of Spiegel (1990). Some preliminary evidence suggests that *Echinoseliopsis* (see 7 below) may also belong here.
7. Conosa (incertae sedis) includes two protosteloid species for which there are not yet adequate molecular data, *Echinosteliopsis oligospora* (Fig. 2m–o) and *Microglomus paxillus* (Fig. 2p–r). Ultrastructure (Lindley et al. 2006; Olive et al. 1983) does not suggest an obvious affinity for either species. The few preliminary molecular data for *E. oligospora* suggest it may belong in Myxogastria, and there are no molecular data for *M. paxillus.*
8. Lobosa/Discosea/Centramoebida (Fig. 2s, t, x, y), a taxon known mainly for its non-fruiting members, also contains the protosteloid species *Acanthamoeba* (*Protostelium*) *pyriformis* (Tice et al. 2016; Fig. 2s, t), *Luapeleamoeba hula* (illustrated in Shadwick et al. 2009b, 2016), and *Luapeleamoeba* (*Protostelium*) *arachispora* (Fig. 2x, y; Tice et al. 2016). The first was included in Group I of Spiegel (1990), and the last in Group VII, Eumycetozoa incertae sedis, of Spiegel (1990). *Acanthamoeba pyriformis* is the first confirmed sporocarpic species in this genus (Tice et al. 2016), and *Luapeleamoeba* is sister to the non-fruiting genus *Protacanthamoeba* (Shadwick et al. 2009b, 2016; Tice et al. 2016).
9. Lobosa/Discosea/Pellitida (Fig. 2u–w) has two protosteloid species in the genus *Endostelium*. One new member of this genus has not been observed to fruit (Kudryavtsev et al. 2014). *Endostelium* (*Protostelium*) *zonatum* was included in Group VII of Spiegel (1990). Pellitida also includes the non-fruiting amoebozoan genera *Pellita* and *Gocevia* (Kudryavtsev et al. 2014; Adl et al. 2012; see also Lahr et al. 2011a).
10. Lobosa/Discosea/Vannellida (Fig. 2z, aa). *Protosteliopsis fimicola* was shown to be a vannellid by Shadwick et al. (2009b). Spiegel (1990) included it in Group VI. It must still formally be transferred to *Vannella.* No other members of *Vannella* have ever been reported to fruit.

The described species are listed below and illustrated in Figs. 1 and 2 with kinetids for the appropriate groups diagrammed in Figs. 6 and 7. The groups to which they belong are based, in most cases, on the taxa listed in Adl et al. (2012). Earlier classifications are summarized in Shadwick et al. (2009b) and Spiegel (1990). Taxonomic revision is underway in several of the groups and will be published elsewhere; however, there will be some comments on the directions the revisions will be going. Those species that are in need of revision at the generic level have genus names in quotation marks. Sporocarps, except for the macroscopic *Ceratiomyxa* spp., are all illustrated at the same scale in Figs. 1 and 2. *Luapeleamoeba hula* is not illustrated here but is illustrated in Shadwick et al. (2009b, 2016), and Tice et al. (2016). It should be remembered that the overall proportions of sporocarps tend to remain the same but that size can vary within a species by at *least* twofold (and sometimes more). Amoeboid and prespore cells are shown for their salient characteristics, not for their details. "Amoeba" will be used for cells that never become flagellated, and "amoeboflagellate" (synonym "amoebomastigote") will be reserved for cells that can reversibly produce flagella (referred to as cilia in Spiegel 1990; Adl et al. 2012), consistent with Spiegel (1990).

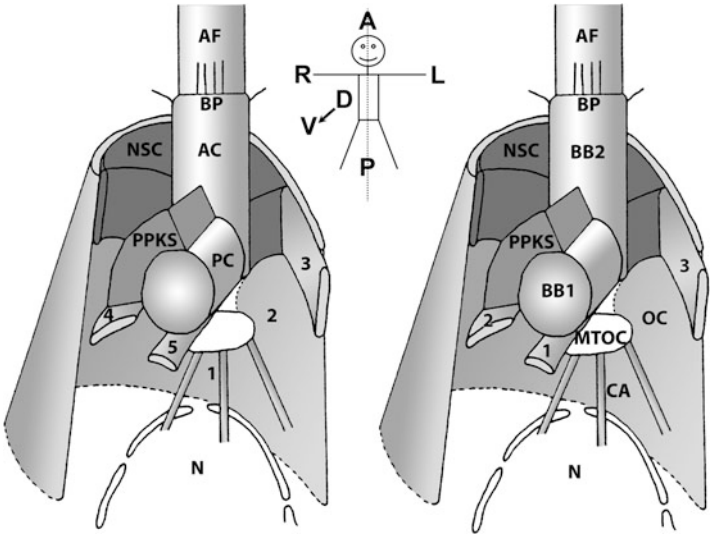

Fig. 6 Cartoon of ventral view of kinetid of *Echinostelium bisporum* (and all *Myxogastria*). comparing elements using the nomenclature of Spiegel (1990) (*left*) and the "universal" nomenclature for the eukaryote kinetid (e.g., Yabuki and Leander 2013; Heiss et al. 2013) (*right*). Abbreviations: *AC* anterior centriole, *BB2* basal body 2, *PC* posterior centriole, BB1 *basal body 1*, *BP* (*both*) basal plate, *N* (*both*) nucleus, *MTOC* (*both*) microtubule-organizing center, *PPKS* (*both*) posterior parakinetosomal structure, *NSC* (*both*) nonstriated connective; microtubular elements: 1 *(left)*, MTA-1 = CA, conical array (note this is attached by an unlabeled stalk to the proximal end of BB2); 2 (*left*), MTA-2 = OC (*right*), outer cone of microtubules (designated as F in Heiss et al. 2013); 3 (*left*), MTA-3 = 3 (*right*) microtubular root 3; 4 (*left*) MTA-4 = 2 (*right*), microtubular rootlet 2; 5 (*left*), MTA-5 = 1 (*right*), microtubular rootlet 1 (Redrawn from Spiegel (1990))

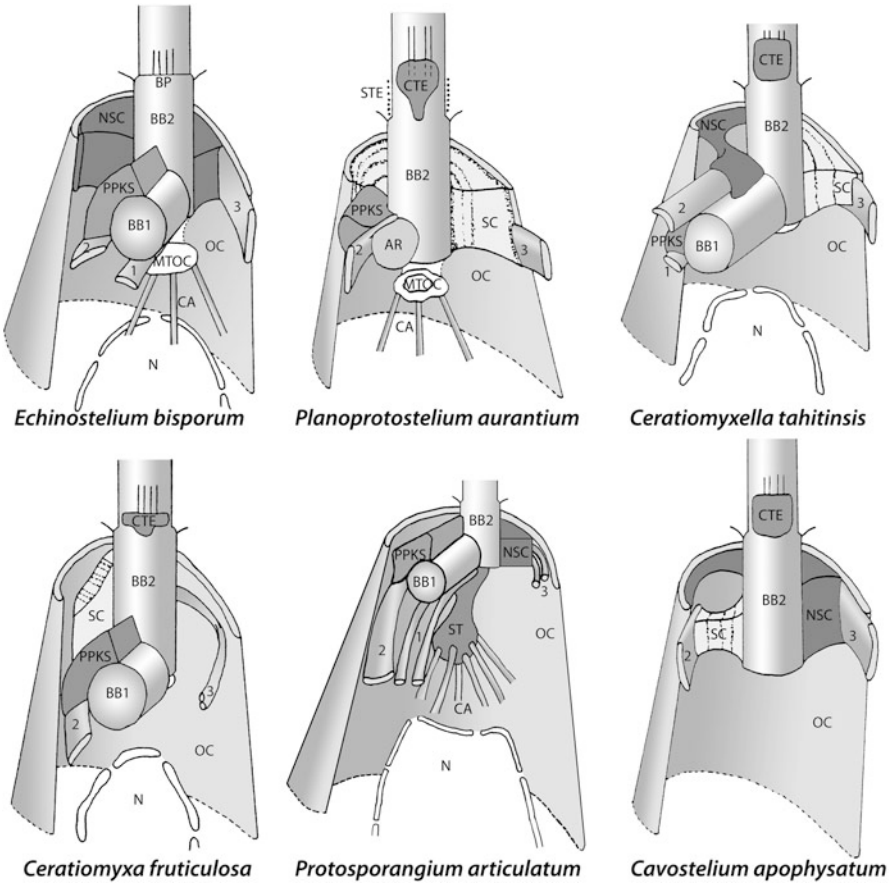

Fig. 7 Cartoons of ventral views of kinetids of all known amoeboflagellates of sporocarpic amoebozoans. Abbreviations: Same as Fig. 6 plus *STE* spiral transitional element, *CTE* cylindrical transitional element, *SC* striated connective, *ST*, stalk connecting proximal end of BB2 to CA. All redrawn from Spiegel (1990) except *P. articulatum* adapted from Spiegel et al. (1986)

Cyst stages have not been illustrated. References are provided for taxa that have been described since 1990. Readers are referred to the first edition of this Handbook for earlier references (Spiegel 1990). For more illustrations of sporocarps, see also Spiegel et al. (2007). Statements concerning the occurrence of these species are based on information from Spiegel et al. (2007) and Ndiritu et al. (2009).

Protosteliida

This clade contains the genus *Protostelium* s.s. and the monotypic *Planoprotostelium aurantium* (Spiegel et al. 1994, 2006) (Figs. 1a–g, 3, and 7). All species

have single spores; they may be deciduous or nondeciduous. The prespore cell of all species, when viewed from above, starts out lozenge-shaped before becoming circular in outline (Fig. 1c). *Protostelium okumukumu* (Spiegel et al. 2006) and *P. nocturnum* have forcibly discharged spores, the former through bursting of the swollen stalk apex (Spiegel et al. 2006) and the latter through the sudden disappearance of the entire stalk. *Protostelium mycophaga*, a probable species complex, is under revision. Most members of this nominal species have spores that fall passively from the stalk, though the degree of deciduousness can vary from strain to strain. "*Planoprotostelium aurantium*" belongs in this complex (see Shadwick et al. 2009) and typically has nondeciduous spores. All have uninucleate amoebae with lamellopodia that bear acutely pointed subpseudopodia (Figs. 1f and 3b) and contain orange-pigmented lipid droplets. The trophic cell of "*P.*" *aurantium* is an amoeboflagellate (Fig. 1g) that can reversibly transform from the amoeba typical of the group to a flagellate with one to several unikont kinetids, one of which is diagrammed in Fig. 7. "*Planoprotostelium aurantium*" nests within the *Protostelium* complex in molecular phylogenies and will have to be reassigned as a species of *Protostelium* (Shadwick et al. 2009). Trophic cells can either encyst as microcysts (not shown) or develop into ellipsoid prespore cells. On a global scale, *P. mycophaga* s.l. is the most abundant microscopic protosteloid amoeba; *P. nocturnum* is common to occasional, and the other species are rare. All tend to be found more often on aerial litter than any other microhabitat.

Fractovitelliida

One sporocarpic taxon, *Soliformovum*, and one non-fruiting taxon, *Grellamoeba* (Lahr et al. 2011a), make up this clade (Fig. 1h–l). *Soliformovum* was segregated from *Protostelium* by Spiegel et al. (1994) as had been suggested in Spiegel (1990). It has two described species, but at least two other rare, undescribed protosteloid amoebae probably belong to the genus based on prespore cell morphology. *Soliformovum irregularis* (Fig. 1m) has long straight, persistent stalks with a hastate tip and a single, spherical, deciduous spore, while *S. expulsum* (Fig. 1h, i) has a bipartite, reflexed stalk that bursts to forcibly discharge the single, spherical spore. The uninucleate amoebae of all species are broad and thin, and often flabellate, with lamellopodia bearing numerous short, acutely pointed subpseudopodia (Fig. 1k). The nucleus has a diffuse nucleolus consisting of several nucleolar bodies (Spiegel et al. 1994). An amoeba may either encyst (not shown) or develop into a rounded prespore cell that in its earliest stages has a raised refractile mound in the center (Fig. 1l). The "fried egg" appearance of the prespore cell is the basis for the genus name (Spiegel et al. 1994). *Soliformovum irregularis* is one of the most abundant species globally, preferring the aerial litter microhabitat over ground litter, while the common-to-occasional *S. expulsum*, though widespread, seems more likely to be found in the tropics on both aerial and ground litter.

Schizoplasmodiida

The schizoplasmodiids presently contain three described genera with a total of six species, all of which have an amoeba that is a large, multinucleate plasmodium that fragments into multinucleate prespore cells (Fig. 1o) (Figs. 1m–v, 4, and 7). The sporocarps of all species consist of a stalk that is topped with a cuplike swelling, or apophysis, on which sits a single spore with a ringlike hilum that fits like a socket over the apophysis (Fig. 1m, n, p–t). All species have deciduous spores. In *Schizoplasmodium* (Fig. 1p–t), the spores are forcibly discharged when a droplet is produced laterally and then bursts (Fig. 1p, q). *Schizoplasmodium cavostelioides* has a short stalk with a large, spherical spore, while *S. obovatum* has an obovate spore, and *S. seychellarum* has a moderately long stalk with a spherical spore. The other two genera, *Nematostelium* and *Ceratiomyxella*, have very long stalks (Fig. 1l, m). In *Schizoplasmodium* spp. and the two *Nematostelium* spp., the plasmodium (Fig. 1v) is the sole amoeboid state. In *Ceratiomyxella tahitiensis*, there is a complex, potentially sexual life cycle (Fig. 4) where amoeboflagellates (Figs. 1u and 4) can alternate with the plasmodium. The bikont flagellar apparatus of *C. tahitiensis* is diagrammed in Fig. 7. The sporocarps of *N. gracile* and *C. tahitiensis* are morphologically identical, so identification of these species from collections requires observation of spore germination. Once a more complete molecular phylogenetic study is available, a decision may be made to include all species in the genus *Schizoplasmodium*, the name that has nomenclatural priority. This is because of the homogeneity of the plasmodial obligate amoeboid stage and the very similar sporocarp topology. *Nematostelium gracile/C. tahitiensis* is abundant to common in tropical and midlatitudes and less common at higher latitudes. This complex is found on aerial and ground litter and on bark in the tropics, more commonly on the latter two microhabitats at mid-to-high latitudes. *Nematostelium ovatum* (see above) is abundant to common in the tropics, northern mid latitudes, and occasional in northern high latitudes. It is occasional in southern midlatitudes and absent in southern high latitudes. Its microhabitat preferences are similar to *N. gracile*. *Schizoplasmodium cavostelioides* is common to occasional in the tropics, abundant to occasional in midlatitudes, and rare at high latitudes. It is found most commonly on aerial litter. *Schizoplasmodium seychellarum* and *S. obovatum* are both very rare.

Cavosteliida

The cavosteliids are a morphologically diverse group, but the traits that appear to be universal among them are sporocarps with uninucleate, nondeciduous spores (Fig. 1w, z, ab, ac, ae–ag, ai); the spores bear some type of ornamentation that may or may not be visible with light microscopy (see Spiegel 1990) (Figs. 1w–ah and 7). All have amoebae that are flat and relatively transparent, and these amoebae often display long, thin subpseudopodia. *Cavostelium apophysatum* (Fig. 1w–y) has very short stalks with goblet-like apophyses and spherical spores with warty and micropunctate sculpturing that is easily visible with light microscopy (Fig. 1w).

It has a complex, potentially sexual life cycle where an amoeboflagellate, with one to several unikont kinetids (Figs. 1x and 7), alternates with a flat, transparent obligate amoeba with branched pseudopodia (Fig. 1y). *Schizoplasmodiopsis pseudoendospora* (Fig. 1z, aa) has short to very short delicate stalks with small, spherical spores (Fig. 1z). The spores are covered with minute spines that are seen clearly only with transmission electron microscopy (Dykstra 1978). The sporocarps often are gregarious when they have developed from a large plasmodium (Fig. 1aa). *Schizoplasmodiopsis vulgare* (Fig. 1ac, ae) and *S. reticulata* (Fig. 1ad) both have relatively long stalks when seen in primary isolation plates. The stalks are somewhat thick and coarse at the base and taper markedly to a relatively blunt apex where the stalk and spore are connected. Relative stalk length can be variable in *S. vulgare*, ranging from about two times the spore diameter to more than four times the spore diameter, especially in culture. The spores of *S. vulgare* and *S. reticulata* are both covered with a reticulum of raised ridges (Dykstra 1978; Spiegel and Feldman 1993). This is readily apparent in the light microscope with *S. reticulata*, even at low magnifications, but is only apparent under higher magnifications in *S. vulgare*. The three species, *S. pseudoendospora*, *S. vulgare*, and *S. reticulata*, make up the genus *Schizoplasmodiopsis* s.s. They all have amoebae that branch, which possess long, thin subpseudopodia that anastomose and become reticulate. These amoebae do not round up during mitosis, and they produce uninucleate prespore cells that are circular in outline. In *S. pseudoendospora* the uninucleate amoeba that germinates from a spore develops into a multinucleate, highly reticulate plasmodium (Fig. 1aa) that may exceed 1 cm in its longest dimension. In *S. vulgare* and *S reticulata*, the amoebae (Fig. 1ad) tend to stay uninucleate to plurinucleate, though large multinucleate masses that we interpret as culture artifacts are reported (Olive and Stoianovitch 1975). "*Schizoplasmodiopsis*" *micropunctata* (Fig. 1ae) and *Tychosporium acutostipes* Spiegel et al. (1995b) (Fig. 1a f, a g) are very similar, and we are currently investigating if they might be the same species (see also Spiegel et al. 1995a). Both have long, thin stalks that suddenly taper to a fine point and support turbinate-to-spherical spores that have a craterlike hilum at the point where they attach to the stalk (Fig. 1ag). Under oil immersion optics, the spores of both species can be seen to have numerous micropunctate markings. We usually assign a protosteloid organism to "*S.*" *micropunctata*, when its stalk length is more than five times its spore diameter and the thinning of the stalk apex is very pronounced, or to *T. acutostipes* when the stalk length is less than five times the spore diameter and the thinning of the stalk apex is more gradual. These two species typically have uninucleate amoebae with lamellopodia bearing short, narrow, acutely pointed subpseudopodia (Fig. 1ah) though some large, multinucleate forms may appear in cultures (Spiegel et al. 1995b). The prespore cells (not illustrated, but see Spiegel et al. 1995b) are similar in their development to those of Protosteliida. In fact, *Tychosporium* was originally hypothesized to be a member of the Protosteliida because of its prespore cells (Spiegel et al. 1995b); however, molecular systematics places it in the cavosteliids (Shadwick et al. 2009b). "*Schizoplasmodiopsis*" *amoeboidea* has a sporocarp (Fig. 1ai) that, at first, appears to be a larger version of *S. pseudoendospora*. Its spore has minute spines visible with TEM (Olive and

Whitney 1982), but its stalk is usually more tapered and has a more noticeably narrowed apex than *S. pseudoendospora* when viewed directly from the side. Its amoebae (Fig. 1ah) are broad and flat and may branch, but it does not have anastomosing subpseudopodia. In addition, the nucleus of the amoeba has a more diffuse-looking nucleolus than is seen in other members of this group, which have the typical, dense central nucleolus seen in most protosteloid amoebae. On a global scale, *S. pseudoendospora*, "*S.*" *amoeboidea*, *and S. vulgare* are among the most abundant species. Both *S. pseudoendospora* and *S. vulgare* are more likely to be found on ground litter, and *S. amoeboidea* is common on all both aerial and ground litter. Bark is also a common substrate for *S. pseudoendospora* and "*S.*" *amoeboidea.* Ground litter from cool, moist habitats is where *S. vulgare* is often the most commonly found species. *Cavostelium apophysatum* is often abundant in the tropics and common to occasional at mid-to-high latitudes. It occurs on aerial and ground litter and on bark. *Tychosporium acutostipes* morphotypes are common to occasional worldwide on aerial and ground litter. The other two species in the cavosteliids, *S. reticulata* and the "*S.*" *micropunctata* morphotype, are both rare.

Protosporangiida

The protosprorangiids fall into two groups, the Protosporangiidae that includes the genera *Protosporangium* and *Clastostelium* and its sister group, the genus *Ceratiomyxa* (see Spiegel 1991; Adl et al. 2012) (Figs. 1ak–2g and 7). Shadwick et al. (2009b) demonstrated the close relationships among the genera of Protosporangiidae, and subsequent unpublished analyses in our lab show its fully supported position as sister to *Ceratiomyxa.* All species have a complex, sexual or potentially sexual life cycle where the spore set contains more than one nucleus, usually two or four; the germling of the spore divides to produce additional amoeboflagellates (see Fig. 2g for the extreme example) which appear not to divide further. Microtubular rootlet 3 of the flagellar apparatus consists of only two microtubules (Fig. 7), and the amoeboflagellates are covered with a cell coat that consists of fine hairs that are branched at the apex. Also, when it has been observed, meiotic prophase is seen in the prespore cell and presumptive meiosis is completed during spore formation (Furtado and Olive 1971; Bennett 1986a). The genus *Protosporangium* consists of four species, *P. articulatum* (Fig. 1ak), *P. bisporum* (Fig. 1al), *P. conicum* (Fig. 1am), and *P. fragile* (Fig. 1an), which all have long, delicate stalks. The first two species most often have two spores, though *P. articulatum* may have four or even eight. The spores are nearly spherical and usually uninucleate in *P. articulatum*, and hemispherical and binucleate in *P. bisporum*. The stalk of *P. articulatum* typically has a distinct joint and bend about 1/2–2 spore diameters from its apex, while the stalk of *P. bisporum* is flexuous along its length. The latter two species typically have four uninucleate spores; *P. conicum* has three apical, spherical spores subtended by an obconic spore that articulates with the stalk, and *P. fragile* has a spore mass where the spores are compressed to each other to form quarter-spheres, or they may bulge out slightly

to give the spore mass a subtly lobed appearance. The stalk of *P. conicum* is fairly straight to moderately bent, and on the short end of length for the genus, while the stalk of *P. fragile* is long and bent at several articulations along its length, such that the stalk often looks collapsed. *Clastostelium recurvatum* (Fig. 1aq) has a bipartite stalk with a short conic base and an inflated, banana-shaped apical portion that supports two hemispherical to subspherical, uninucleate spores, usually in a side-by-side configuration. The fluid-filled apical portion of the stalk slowly begins to straighten and then suddenly bursts, propelling the spore mass away while leaving the base intact. The spores of both *Protosporangium* ssp. and *C. recurvatum* germinate as amoeboflagellates that divide shortly after germination to produce more amoeboflagellates (Fig. 1aq), which all have an essentially identical kinetid (Fig. 7). Then, with little or no division to produce further amoeboflagellates, they develop into obligate amoebae by a process that has not yet been observed (Fig. 1ar). The obligate amoebae are uni- to plurinucleate and have essentially identical morphology and microtubular cytoskeletons (see Spiegel 1991). The species of *Protosporangium* are most common on bark of living trees, though they may also be found on rotting wood. *Protosporangium articulatum* also can be found occasionally on dead primary plant tissues. *Clastostelium recurvatum* is most often found on aerial or ground litter. *Protosporangium articulatum* and *P. conicum* may be rare to common depending upon the habitat and are often among the few species found in arid habitats worldwide. The other two species of *Protosporangium* are rare, as is *C. recurvatum*. *Ceratiomyxa* spp. all produce fructifications where several to several thousands of individual sporocarps arise on a microscopic pad or on macroscopic columns of extracellular slime that is deposited by the plasmodium prior to its division into uninucleate prespore cells. A series of nuclear divisions, which is interpreted as meiosis, starts with prophase in the prespore cell and ends in the spore, with all nuclei surviving (Furtado and Olive 1971). The deciduous, tetranucleate spore germinates as a worm-shaped cell that eventually rounds up into a tetrad with one nucleus in each of its four lobes. Usually, each nucleus divides and the tetrad develops into an octet of eight lobes, and then each lobe differentiates into an amoeboflagellate (Fig. 2g). The amoeboflagellates never subsequently divide, though they have been observed to fuse, similarly to the plasmogamy seen between gametic amoeboflagellates of myxogastrids (Spiegel 1981b). Somehow, the amoeboflagellates or zygotes are involved in plasmodial development; however, this has never been observed. The few observations of plasmodia in the genus suggest that it is small (<1 mm) to extensive (>1 m), reticulate, and lacks regular shuttle streaming (Olive 1982; Olive and Stoianovitch 1979). Three species produce macroscopic fructifications: *C. fruticulosa* (Fig. 2a, b), *C. morchella* (Fig. 2c), and *C. sphaerosperma* (Fig. 2d). By contrast, *C. hemisphaerica* (Fig. 2e, f) has microscopic fructifications. Species are recognized on the basis of fructification morphology and spore shape. All species have single ellipsoidal spores except *C. sphaerosperma*, which has spherical spores. *Ceratiomyxa fruticulosa* has extensive, columnar to branched, white to brightly colored fructifications; *C. morchella* and *C. sphaerosperma* have white fructifications with colorless stipes and white, sporocarp-bearing heads, the former being pitted and the latter having radiating

columns; and *C. hemisphaerica* has small, clear, microscopic slime mounds. The macroscopic species of *Ceratiomyxa* fruit mainly on decaying woody substrates (including shells of tropical nuts in the case of *C. sphaerosperma* (A. Rollins, personal communication)), while *C. hemisphaerica* has also been found on aerial and ground litter. *Ceratiomyxa fruticulosa* may be the most commonly encountered macroscopic slime mold in moist to mesic forests worldwide. The other two macroscopic species are less common and mostly restricted to the tropics. *Ceratiomyxa hemisphaerica* is very rare and has been found exclusively in forests on various substrates.

Because the macroscopic species of *Ceratiomyxa* are easily seen and similar in size to myxogastrids, they were known well before it was recognized that there were other protosteloid amoebae. Therefore, until the 1970s (see Olive 1975), they were considered to be a morphologically unusual order-level taxon in the myxogastrids (Ceratiomyxales – see ▶ Myxomycetes). However, though their amoeboflagellates are superficially similar to myxogastrids, they differ considerably with respect to cell coats and kinetid structure (Figs. 6, and 7, see Spiegel 1991). Their plasmodia do not show the shuttle streaming typical of myxogastrid plasmodia (see Olive and Stoianovitch 1979). Their fructifications are macroscopic because of extracellular, secreted slime columns, not because of macroscopic spore-containing structures typical of the macroscopic myxogastrids. There is no equivalent to the slime columns among any other amoebozoans. They are unique to *Ceratiomyxa* and are one synapomorphy that distinguishes the genus from the rest of the protosporangiids. The individual sporocarps are protosteloid, and the spores are smooth-walled and not sculptured as in the myxogastrids, and meiosis appears to be completed before spore wall maturation (rather than after, as is the case in myxogastrids; see Olive 1975; Olive and Stoianovitch 1979; Spiegel 1991). Thus, the inclusion of the genus in the myxogastrids is no longer tenable with the discovery and understanding of the other protosteloid amoebozoans.

Myxogastria

The two-spored myxogastrid *Echinostelium bisporum* (Figs. 2h, i, k, l, 6, and 7) was originally described as a protosteloid amoeba and is most often found by workers looking for protosteloid amoebae. Because "protosteloid" refers to sporocarp appearance, rather than to a phylogenetic affinity, *E. bisporum* is treated here as a protosteloid amoeba (Figs. 2h–l, 6, and 7). Its sporocarp (Fig. 2h and see Spiegel and Feldman 1989) consists of a short, nearly solid stalk with a flared apophysis that articulates with a ring-shaped hilum on the bottom spore. There are matching hila on each of the spores at their point of articulation. The spores are covered with minute, hollow spines, and the slime sheath (= peridium) may become hydrated such that the spores appear to be suspended in a drop of liquid (Fig. 2i). The spores of *E. bisporum* germinate as amoeboflagellates (Fig. 2k, l) that are identical in general morphology and kinetid structure (Figs. 6 and 7) to other myxogastrids. Unlike all other myxogastrids, the only free-living amoeboid state is the amoeboflagellate (Fig. 2k, l);

the transition to anything similar to a plasmodial ultrastructure does not occur until well after fruiting is underway (Spiegel and Feldman 1989). Though never treated as a protosteloid amoeba, *E. lunatum* (Fig. 2j), with a sporangium of four to eight spores and a columella shaped like a half cup, may be encountered when collecting for protosteloid amoebae and be mistaken for a four- to eight-spored *E. bisporum.* Since *E. lunatum* has a complete myxogastrid life cycle that includes a plasmodium and a more complex sporocarp, we choose not to consider it to be protosteloid. *Echinostelium bisporum* is occasional to common in the tropics and midlatitudes and may be found on aerial or ground litter or occasionally on bark; *E. lunatum* is rare and almost always found on bark.

Conosa, Incertae Sedis

All of the above protosteloid organisms are sporocarpic amoebozoans that are members of molecularly and morphologically supported groups that are known best from their fruiting members (Fig. 2m–r). The following two taxa are accepted as amoebozoans, and probably conosans, because their sporocarps and amoebae show some similarity to the conosan protosteloid amoebae listed above. However, their morphology does not readily suggest any affinities to other amoebozoans.

Echinosteliopsis oligospora

This species has a sporocarp with a moderately long stalk and four (to eight) nearly spherical, smooth-walled spores that may not all be of equal size (Fig. 2m) (Fig. 2m–o). They are usually arranged such that one is attached to the stalk and the others form a group that sits around the basal spore's apex. As a result, only three of the four spores are usually visible in one view. The sheath is hygroscopic, such that the spores may appear suspended in a drop (Fig. 2n). The amoeba may have one to several nuclei. In overall appearance, the amoeba (Fig. 2o) is similar to the small protoplasmodia found in some myxomycetes; however, the nucleus is unique in that it has several discrete nucleoli (Lindley et al. 2006). It occurs most frequently on aerial or ground litter and is abundant to common in the tropics and common to occasional in midlatitudes and rare at high latitudes. There is no amoeboflagellate state. Phylogenies of elongation factor 1α and β-tubulin genes (L. Shadwick unpublished) and preliminary phylogenomic analyses performed in M.W. Brown's lab suggest that this taxon may be an unusual myxogastrid.

Microglomus paxillus

This small, short-stalked species has varieties that may be four-spored or two-spored (Fig. 2p, q). While it may look superficially like *S. pseudoendospora*, careful examination shows that it is sporangial and that the sporangium is often slightly

ellipsoid (Fig. 2p) (Figs. 2p–r). High magnifications reveal the demarcation between the tightly packed spores (Fig. 2q). The short stalk supports the spores within a wall (= peridium?) that has minute spines visible with TEM. Olive et al. (1983) interpreted the fruiting body to have a single two- to four-celled spore rather than multiple spores in a sporangium. Each sporocarp has nuclei in meiotic prophase during early development, and meiosis is thought to be completed during sporogenesis. This is the only protosteloid amoeba with a simple life cycle that shows evidence of sexuality. Germination results in the release of amoebae that eventually become uninucleate. The amoebae have conic pseudopodia that may have long acutely pointed subpseudopodia (Fig. 2r). No cultures of this species are currently available, and we have not been able to examine amoebae directly. This species is occasional to rare worldwide and is most commonly encountered on bark in arid regions but is also present on aerial and ground litter. It has recently been seen in primary isolation plates in M.W. Brown's lab, and there is hope to have some sequence data for it in the near future.

Protosteloid Amoebozoans from Taxa Known Primarily for Their Non-fruiting Members

The following protosteloid amoebae have been found to be members of groups of amoebozoans that are classically thought of as "naked" amoebae (Fig. 2s–aa). Two described species, *Acanthamoeba pyriformis* and "*Protosteliopsis*" *fimicola*, are placed in existing genera of "naked" amoebae, and the remaining species are placed in more inclusive taxa. All are found within the amoebozoan clade with the taxon name Discosea.

Centramoebida: *Acanthamoeba* (*Protostelium*) *pyriformis*

This species was tentatively treated as a member of the genus *Protostelium* by Spiegel (1990) (Fig. 2s, t). However, recent sequence data (Tice et al. 2016), as well as its morphology and ultrastructure (Bennett 1986b, Spiegel, unpublished), clearly demonstrate that it is a sporocarpic member of the genus *Acanthamoeba.* It has an obpyriform, uninucleate spore with a basal invagination that fits over the apex of the stalk (Fig. 2s). This allows the spore to flag (wave back and forth) easily in air currents. The apex of the stalk has a small, knob-like swelling, the apophysis, which is visible when the spore is shed. The stalk is relatively long and slightly tapered. During development, the apical portion of the forming stalk is enclosed in a sheathlike invagination of the rising sporogen. The amoeba stage is identical in appearance to non-fruiting species of *Acanthamoeba*, with respect to acanthopodia (Fig. 2t), cysts (Bennett 1986b), and the microtubule-organizing center (Bennett 1986b). Also, like some other species of *Acanthamoeba*, cells in late prophase to early anaphase display short, radiating pseudopodia (Spiegel, unpublished). Different strains that have been cultured have sporocarps that differ in size and cyst

morphology. This suggests that *A. pyriformis* may be a species complex. Until recently no viable cultures were available, but one isolate has recently been cultured and sequenced. Further success at isolation of other strains will make it possible to see if indeed there are other similar, cryptic species. This species is occasional worldwide and found about equally commonly on ground and aerial litter and sometimes on bark.

Centramoebida: *Luapeleamoeba*

Shadwick et al. (2009b) studied a protosteloid amoeba designated LHI05M5g-1 that has recently been described as a new species in a new genus, *Luapeleamoeba hula* (species not illustrated, see Shadwick et al. 2016; Tice et al. 2016) (Fig. 2x, y). Subsequent study (Tice et al. 2016) shows that *Protostelium arachisporum* should also be included in the same new genus, as *Luapeleamoeba arachispora*. It was something of a surprise that this taxon is not closely related to *Endostelium*, as hypothesized by Spiegel (1990). *Luapeleamoeba hula* has a sporocarp with a short stalk and obpyriform spore. The stalk apex has a knob-like apophysis that is inserted into an invagination at the base of the spore. It becomes visible when the deciduous spore is shed. While the spore is supported by the stalk, it constantly changes shape, suggesting that it either has a very thin, flexible spore wall or possibly no wall at all. Sporocarp development is similar to that seen in *Endostelium* spp. and *A. pyriformis*. As in these two taxa, the stalk develops inside an invagination of the rising sporogen (Shadwick et al. 2016). *Luapeleamoeba arachispora* is somewhat variable in many respects and probably will be segregated into several species. Nonetheless, it is fairly easy to recognize (Fig. 2y). Its stalk may range from less than the length of the spore to more than two times the spore's length. It always has a small, knob-like apophysis, and the base flares out noticeably just above the basal disk. The spore is usually elongate, often with one or more constrictions along its length such that it looks like a peanut, but it may be only slightly elongate and ellipsoid. The deciduous spores flag readily while attached to the stalk. We have noticed that spores can slowly change shape while attached to the stalk. This may be because the spore wall is very thin. The uninucleate amoeba of both species is relatively thick from top to bottom and round to elongate in outline (Fig. 2x). The pseudopodium is often broad and supports numerous short subpseudopodia which are blunt or pointed, with broad bases. *Luapeleamoeba arachispora* is abundant to common in the tropics and common at midlatitudes on both aerial and ground litter, while *L. hula* is rare.

Pellitida: *Endostelium*

Recent work by Kudryavtsev and others (Kudryavtsev et al. 2014) and Lahr et al. (2011a) has shown that *Endostelium* is closely related to the non-fruiting taxa *Gocevia* and *Pellita* (Fig. 2u–w). There are two fruiting species in the genus and one species that has not been reported to fruit. Both fruiting species, *E. amerosporum* (Fig. 2u) and

E. zonatum (Fig. 2n), have large, coarse sporocarps with large deciduous spores. The former has a smooth stalk with a knob-like apophysis, upon which sits a spherical or subspherical spore that is often covered with irregularities which appear to be bacteria adhering to its surface. The spores do not flag noticeably in air currents. *Endostelium zonatum* has a series of regularly spaced swellings along the length of the stalk, and the apex of the stalk is inserted into an invagination in an extension of the obpyriform-to-turbinate spore. The spore in this species flags readily in air currents. Both species have large, thick, amoebae that are round to slightly flabellate in outline, and they have a thick slime sheath. The pseudopodia are broad and have short, peg-like subpseudopodia extending forward and projecting downward from the ventral surface when the amoeba is locomoting (Kudryavtsev et al. 2014). The rising sporogens, analogous to *A. pyriformis* and *L. hula*, form an invagination in which the developing stalk is inserted. There appears to be a lot of variation in the morphology of different fruitings of *E. zonatum* with respect to overall size, spore shape, and the degree to which the regular swellings appear on the stalk. Sometimes, they even appear to have smooth stalks. With further work, it is likely that *E. zonatum* will eventually be split into several species. *Endostelium zonatum* is common in tropical and midlatitudes and equally common on both aerial and ground litter, and occasionally found on bark, while *E. amerosporum* is rare enough that no microhabitat preference has been determined.

Vannellida: "*Protosteliopsis*" *fimicola*

The long stalk of this species is relatively wide, somewhat contorted and appears waterlogged or gelatinous (Fig. 2z) (Fig. 2z, aa). Its base is noticeably broader than the rest of the shaft. It supports a single, nondeciduous, uninucleate spore (Fig. 2z). The amoeba may be elongate or flabellate with a broad, hyaline pseudopodium. Its amoeba (Fig. 2aa) is indistinguishable from other species of *Vannella*, and molecular data place it deep within the tree of *Vannella* (Shadwick et al. 2009b). It has yet to be formally renamed but will be reassigned soon. It is occasional worldwide and represents the protosteloid amoeba most likely to be found on herbivore dung, though it may also present in rich ground litter or even aerial litter.

Maintenance and Cultivation

Protosteloid amoebae are relatively easy to find on natural substrates (Spiegel et al. 2004, 2005, 2007). Substrates include pieces of dead plant parts from standing plants or litter, rotting wood, bark, herbivore dung, or humus/soil. Substrates are collected in the field and placed in paper bags, and location and ecological data are recorded. If the substrates are to be stored before plating, they must be air-dried. In this condition they can be stored for up to 18 months at room temperature. In the laboratory, the substrates are cut into small pieces and placed on plates of weakly nutrient agar such as wMY, HI, or OB (Table 2; this is the same as Table 3 in the first edition of this

Handbook, except that the recipe for wMY agar has been changed to **0.002 g malt extract** and **0.002 g yeast extract**. We have begun to leave malt extract and/or yeast extract out of the wMY medium with no ill effect. Our standard practice is to lay bits of substrate in eight radiating lines on wMY agar and then to wet them with sterile distilled water. We refer to these as "primary isolation plates." Other preparative methods include suspending substrates in water and then spreading them on an agar plate, a method that works with ground litter and soil, or placing substrates on moistened absorptive paper in a dish as a moist chamber.

After 2–3 days, microscopic examination of the sample with either a compound or dissecting microscope should reveal the first sporocarps of protosteloid amoebae. The first sporocarps will most likely be found directly on the dead plant material, but after several days, more should be seen on the surrounding agar. Primary isolation plates may be examined daily for 2–4 weeks because the species composition will change during this period. Species such as *Protostelium mycophaga*, *Soliformovum irregularis*, and *Cavostelium apophysatum* will appear within the first few days, while species of schizoplasmodiids may take well up to a week to appear. Macroscopic *Ceratiomyxa* spp. are best collected as showy white "fructifications" on rotting logs, as they rarely appear in primary isolation plates of substrate collections (Olive and Stoianovitch 1979).

In many cases it is necessary to culture a protosteloid amoeba that appears on a primary isolation plate. Most protosteloid amoebae are relatively easy to isolate and culture from nature (Spiegel et al. 2005). Exceptions, which will be discussed below, are species from bark and *Ceratiomyxa* spp.

Plates of weakly nutrient media should be spotted with several yeasts and bacteria to serve as possible food sources; then spores or other cells of the protosteloid amoeba to be isolated should be picked off the substrate plate with a fine, sterile metal or glass needle or an eyelash glued to a stick (W.E. Bennett, personal communication) and transferred to each spot of food organisms on the isolation plate. Single-spore isolation should be avoided until the culture is established. Spores may be picked off free of contaminants most easily; cysts and trophic cells will carry a number of other organisms. The use of a handheld needle or eyelash takes some practice, and, if available, a micromanipulator may be used instead (Olive 1975). An effort should be made to include one spot of bacteria and/or yeast from the primary isolation plate near the species being isolated, to serve as food on the initial culture plate. A researcher should try several types of tools and settle on the method that works best in his/her particular case.

Protosteloid amoebae should be allowed to grow for several days on the initial culture plate. They should then be transferred to culture plates of one of the agar media listed in Table 2, though we find that wMY works almost universally. The plate should be streaked with the food organism(s) on which the protosteloid amoeba has grown most vigorously (and formed sporocarps), and an inoculum of the species should be placed at one end of the streak. It is often difficult to get a protosteloid amoeba isolated cleanly on the first attempt; patience and several transfers are usually necessary. Species with ballistospores and deciduous spores may be cleaned up quite easily by inverting an agar block containing sporocarps on the lid of a

culture plate and letting the spores drop onto the agar below (Olive 1975). Some species grow very slowly when first isolated. These must be watched carefully and transferred quickly if contaminants appear on the plate. If one carefully observes the amoeboid cells that germinate from a spore and follows their development, a trustworthy culture may be established even if a strain will not fruit.

Bark-inhabiting protosteloid amoebae are usually very difficult to culture (Olive 1975). Much experimentation with agar hardness and pH and food organism combinations may be necessary to get a "barkie" to grow. Often, pieces of sterile bark must be scattered on the agar to get the protosteloid amoeba to form sporocarps, even if amoebae have grown (C. Stoianovitch, personal communication). No species of *Ceratiomyxa* has been grown in culture, but spores can be germinated by sprinkling them onto a plate of agar flooded with water (see Fig. 2g).

Once a protosteloid amoeba is in culture, it should be maintained with its food organism(s) on one of the weakly nutrient agars listed in Table 2; CM+ may be used for hardy species such as *Protostelium mycophaga* and *Planoprotostelium aurantium*. Liquid medium can be used to culture large numbers of trophic cells of some species (Spiegel 1982a). We have also found that any medium can be converted to a liquid medium by leaving out the agar. We find it easiest to keep cultures in plastic Petri dishes that allow microscopic inspection of the unopened culture at low power. Cultures are maintained at a room temperature below 25 °C (because some species are quite heat sensitive) and transferred once a month, although transfers may be made successfully from cultures that are over 6 months old. Most species can be frozen in liquid nitrogen and stored indefinitely.

Established cultures of most species are kept in the University of Arkansas and Mississippi State culture collections, and several species are available in the Eumycetozoan Special Collection at the American Type Culture Collection and the Culture Centre for Algae and Protozoa.

Evolutionary History

The fossil record for potential amoebozoans is best for arcellinids and appears to extend back at least 740 mya (Porter and Knoll 2000). Arcellinids are tubulinids, a highly derived group that contains no sporocarpic members. Their fossil record, therefore, serves only to place the last common ancestor of amoebozoans, as a whole, at some time earlier than about three quarters of a billion years ago. There are no known fossils that can be assigned to protosteloid amoebozoans. Therefore, their history, and that of amoebozoans in general, must be inferred from comparative morphology and comparative molecular studies.

Molecular work supports Amoebozoa as a major monophyletic lineage among eukaryotes (see Adl et al. 2012). This lineage contains several well-supported clades (see Adl et al. 2012; Lahr et al. 2011a; Shadwick et al. 2009b), but the deeper relationships among these clades have yet to be discovered. There are no data that support a monophyletic subclade that contains all, or even most, of the sporocarpic taxa to the exclusion of most non-fruiting amoebozoans, as espoused in the concept

of Eumycetozoa by Olive (1975, 1982) and by Spiegel (1990, 1991; Spiegel et al. 1995a). Rather, there are several independent, well-supported lineages that contain sporocarpic, protosteloid members (Table 1). One study has suggested, with weak support, uniting the protosporangiid taxon *Ceratiomyxa*, the myxogastrids, and the dictyostelids into the taxon Macromycetozoa (Fiore-Donno et al. 2010). This clade was not recovered in other studies (Lahr et al. 2011a); however, preliminary unpublished molecular work from the labs of M.W. Brown and F.W. Spiegel, based on a greater sample of amoebozoans, suggests more support for Macromycetozoa, and we have chosen to accept it in this chapter. Many protosteloid amoebae are not located in Macromycetozoa but instead in its apparent sister group, Variosea (Shadwick et al. 2009b; Fiore-Donno et al. 2010; Lahr et al. 2011a; Cavalier-Smith et al. 2015; Berney et al. 2015). Further, Berney et al. (2015) illustrated several new taxa of non-fruiting variosean amoebozoans with trophic cells that are very similar to schizoplasmodiids, fractoviteliids, and cavosteliids. There are also several discosean protosteloid amoebae.

Given that simple sporocarpy is the classical hallmark of protosteloid amoebozoans, the question to be asked is: How many times has sporocarpy arisen? Ultrastructure of mature and developing protosteloid sporocarps (see Spiegel 1990; Spiegel et al. 1995a; Spiegel, unpublished data) is consistent with the hypothesis that protosteliids, fractoviteliids, schizoplasmodiids, cavosteliids, protosporangiids, myxogastids, *Endostelium*, *A. pyriformis*, and perhaps the sorocarpic dictyostelids all had a common sporocarpic ancestor. However, not enough work has been done to rule out several origins of sporocarpy, with convergence upon a similar morphology. The lack of sporocarpy outside of Amoebozoa compared to the wide range of sorocarpy among eukaryotes (see Adl et al. 2012; Brown et al. 2011) tempts one to lean toward a single origin of sporocarpy.

We are at the beginning of a transcriptomic study of amoebozoans which may yield results that can help determine whether the underlying genetic basis for sporocarp development is the same, or different, across the wide phylogenetic range of protosteloid amoebozoans. Should the results indicate considerable homology for the fruiting process, this would lend support to a single origin of sporocarpy. Thus, it would suggest that sporocarpy was a trait of the last common ancestor of amoebozoans and that it has been independently lost several times in several lineages. A corollary of that would be the conclusion that all extant amoebozoans are the descendants of a terrestrial ancestor and that exclusively freshwater-aquatic and marine lineages of amoebozoans are derived.

Sporocarpy is not the only character protosteloid amoebae have in common; there is also the rest of the life cycle to consider. When one looks at life cycles with respect to amoebozoans as a whole, the history of the entire "supergroup" appears to reflect a loss of characters that were present in a morphologically and developmentally complex last common ancestor.

Because amoeboid cells of the different groups of protosteloid amoebae are so variable (see Spiegel 1990; Spiegel et al. 1995a), Spiegel was skeptical that sporocarpy had a common origin and was unwilling to accept it as a potentially apomorphic character of the protosteloid amoebae. However, many protosteloid

amoebae have a life cycle that includes an amoeboflagellate stage – there are flagellated members in the protosteliids, schizoplasmodiids, cavosteliids, protosporangiids, and myxogastrids. While the morphology and swimming behavior of amoeboflagellate cells is variable among these groups (Figs. 1g, u, x, ao and 2k; see also Spiegel 1991; Spiegel et al. 1995a); flagellar apparatus ultrastructure (Figs. 6 and 7; see Spiegel 1991; Spiegel et al. 1995a) is very consistent, suggesting a common ancestry for all these organisms. In addition, the morphology of amoeboflagellates when they are not flagellated (see Spiegel et al. 1995a) and the details of mitosis in amoeboflagellates (see Spiegel 1991; Spiegel et al. 1995a) all point to the conclusion that all of these organisms share a common ancestry. It was more the characters of amoeboflagellates than the characters of sporocarpy that led Spiegel (1990, 1991) and Spiegel et al. (1995a) to conclude that the protosteliids, schizoplasmodiids, cavosteliids, protosporangiids, and myxogastrids (at least; of the sporocarpic organisms) form a monophyletic group. Spiegel (1990, 1991) was one of the first protistologists to suggest that complex flagellar apparatuses within a purported clade were probably more primitive than simpler ones because the different groups with simple flagellar apparatuses lacked different elements that were present in groups with complex ones. Spiegel (1991) was also one of the first protistologists to suggest that the elements of the complex flagellar apparatus of one major lineage were homologous with topologically similar elements in another major lineage of eukaryotes, thus implying that the early ancestors shared by the major lineages had a complex flagellar apparatus. This is a view that is more widely held today (see Spiegel 2012, 2016; Yubuki and Leander 2013; Heiss et al. 2013). Other features of the amoeboflagellates of the sporocarpic amoebozoans, such as a ventral groove for the posterior flagellum, also appear to be widespread among eukaryotes (Spiegel 2012).

Sex is a symplesiomorphy of amoebozoans (Lahr et al. 2011b; Spiegel 2011) since it is a synapomorphy of extant eukaryotes. Thus, the last common ancestor of amoebozoans must have been sexual. Sex is present or suspected in the schizoplasmodiids, cavosteliids, protosporangiids, and myxogastids, all of which have complex life cycles with amoeboflagellates and obligate amoebae (Lahr et al. 2011b). *Microglomus* is the only suspected sexual sporocarpic amoeba that has a simple life cycle.

The presence of sporocarpy, the characters of amoeboflagellates, and similar potentially sexual life cycles suggested that organisms that share these characters should be closely related (see Spiegel et al. 1995a), but in-depth molecular phylogenetic studies clearly imply that there is no support for a single clade that includes all sporocarpic amoebozoans nor support for a single, exclusive subclade that includes all flagellated amoebozoans. Nonetheless, these characters strongly suggest that the organisms that express them are closely related. Therefore, the hypothesis must be considered that the last common ancestor of extant amoebozoans was sexual, had an amoeboflagellate stage with a ventral groove and a complex bikont kinetid, and was sporocarpic. Since sporocarps appear to be adaptations for dispersing propagules of terrestrial amoebozoans, it is likely that this ancestor was terrestrial. This hypothesis is offered so that it can be tested. More comparative light

microscopy and ultrastructural work combined with comparative genomics and comparative developmental genetics should provide the data to support or reject this hypothesis.

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Archamoebae

37

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Abstract

Members of the Archamoebae comprise free-living and endobiotic amoeboid flagellates, amoeboflagellates, and amoebae, with distinctive hyaline cytoplasm and bulging pseudopodia. They live in anoxic or microoxic habitats and are anaerobes, lacking typical mitochondria, as well as Golgi stacks, plastids, and normal peroxisomal microbodies. They have a distinctive flagellar apparatus present in all flagellated members of the group. Life cycles of individual species can include flagellates, amoebae of various sizes, and cysts. In recent years, the group has been divided into five separate families, Mastigamoebidae, Entamoebidae, Pelomyxidae, Tricholimacidae, and Rhizomastixidae, whose interrelationships have not been completely resolved. Here, we clarify the composition of these groups and the circumscription of genera in the Archamoebae.

Keywords

Archamoebae • Pelobiont • Amoebozoa • *Mastigamoeba*, *Pelomyxa*, *Entamoeba*

Contents

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Summary Classification

●**Archamoebae**
●●**Pelobiontida**
●●●**Mastigamoebina**
●●●●**Mastigamoebidae** (*Mastigamoeba*, *Iodamoeba*, *Endolimax*)
●●●●**Rhizomastixidae** (*Rhizomastix*)
●●●**Pelomyxina**
●●●●**Pelomyxidae** (*Pelomyxa*, *Mastigella*)
●●**Entamoebida**
●●●**Entamoebidae** (*Entamoeba*)

[Archamoebae incertae sedis: *Mastigina*, Tricholimacidae (*Tricholimax*), *Endamoeba*]

Introduction

General Characteristics

The Archamoebae is a group of amoebae, amoeboid flagellates, and amoeboflagellates (i.e., organisms with both flagellates and amoebae in their life cycle), with distinctive hyaline cytoplasm and bulging pseudopodia (Fig. 1). They comprise three

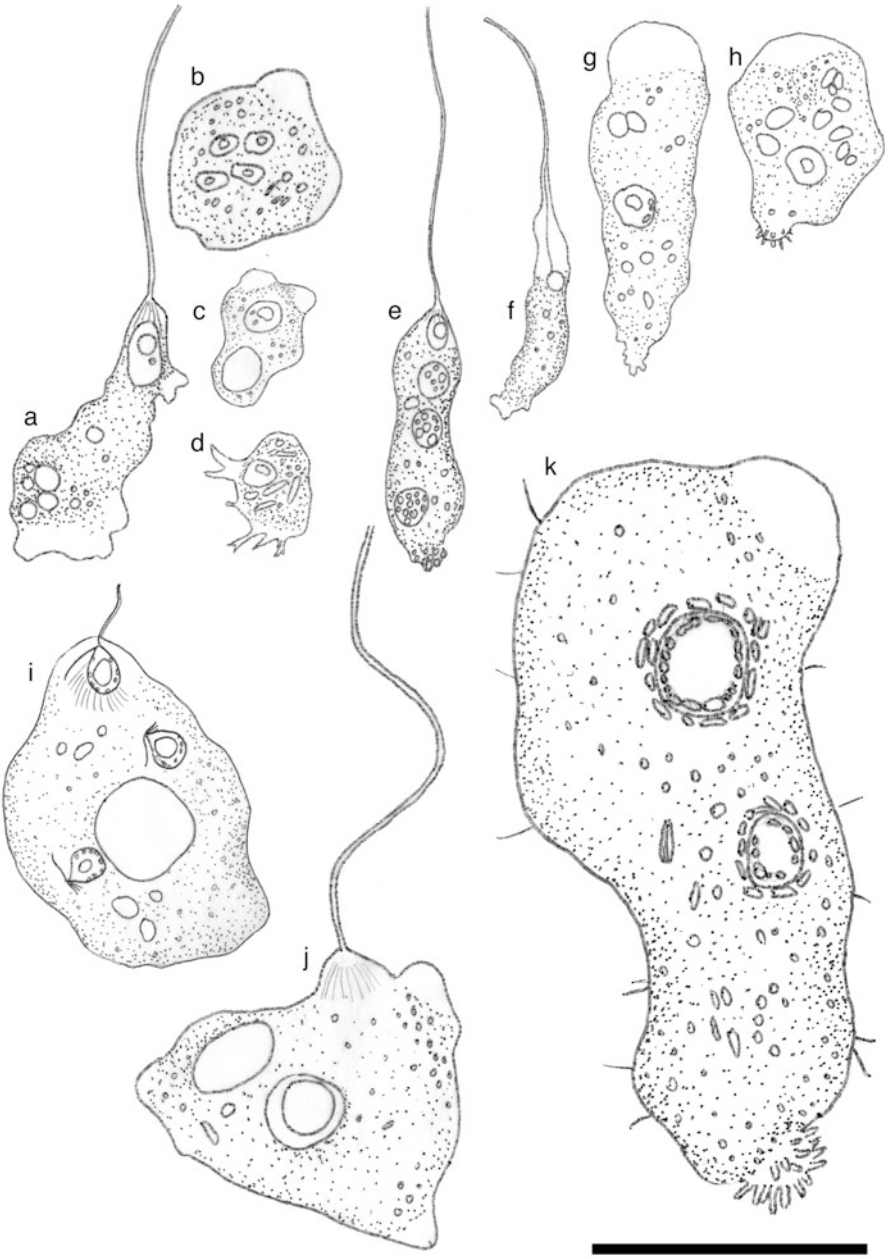

Fig. 1 General appearances of genera in the Archamoebae. (**a**) In the dominant flagellated trophic stage, ***Mastigamoeba*** has a microtubular cone that connects the flagellar base to the nucleus. (**b**) ***Mastigamoeba*** also typically has amoeboid stages, which may be multinucleate. (**c**) ***Iodamoeba*** contains small aflagellate amoebae with hyaline lobopodia. (**d**) ***Endolimax*** forms small aflagellate amoebae with variable pseudopodia similar to those in *Mastigamoeba*. (**e**) ***Mastigina*** contains

lineages that have collectively been referred to as pelobionts: mastigamoebids, pelomyxids, and *Tricholimax*, as well as *Rhizomastix* and entamoebids. All Archamoebae are anaerobic (some more aerotolerant than others) and lack normal mitochondria (which are reduced to remnants), Golgi stacks, plastids, and normal peroxisomal microbodies. Many contain endosymbiotic bacteria and archaea. Flagellated taxa have a distinctive cytoskeleton.

Mastigamoebids currently include *Mastigamoeba*, *Endolimax*, and *Iodamoeba*: these include mostly free-living amoeboflagellates as well as endobiotic amoebae; many have distinctively hyaline cytoplasm and move with pseudopodia when attached to a substrate. In flagellate mastigamoebids, the flagellum is functional and has a conventional 9 + 2 microtubular arrangement, but there is a distinctively "languid" or slow flagellar beat arising from the lack of outer dynein arms in the flagellar axoneme. The base of the flagellum gives rise to a cone of microtubules that connect to the nucleus. Molecular phylogenies divide mastigamoebids into two clades, "A," containing large species with a broad flagellar apparatus (e.g., *Mastigamoeba balamuthi*), and "B," containing small species with a narrow flagellar apparatus and trailing pseudopodia (e.g., *Mastigamoeba simplex*) as well as *Endolimax* and *Iodamoeba* (Ptáčková et al. 2013; Pánek et al. 2016). Members of *Endolimax* and *Iodamoeba* have entirely lost the flagellar apparatus and were historically classified within entamoebids. They have only recently been transferred to mastigamoebids on the basis of molecular-phylogenetic analyses (Cavalier-Smith et al. 2004; Ptáčková et al. 2013; Stensvold et al. 2012; Poulsen and Stensvold 2016). Historically, the flagellated *Mastigella* has usually been thought of as a mastigamoebid, but it is more closely affiliated with *Pelomyxa* (Zadrobílková et al. 2015).

Pelomyxids include members of *Mastigella* and *Pelomyxa*. *Mastigella* includes amoebae and flagellated amoebae where the base of the flagellum gives rise to a microtubular cone that is not connected to the nucleus. *Pelomyxa* includes large (up to several millimeters) amoebae, with nonfunctional, short flagella that have a disordered microtubular arrangement and a microtubular cone that is unconnected to the nucleus or nuclei. Recent phylogenetic analyses have shown that *Mastigella* is closely related to *Pelomyxa* (Zadrobílková et al. 2015).

Rhizomastix was recently placed in the Archamoebae (Čepička 2011; Ptáčková et al. 2013). It includes amoeboflagellates with a single anterior flagellum, which live

Fig. 1 (continued) amoeboid flagellates with a limax appearance and no lateral pseudopodia. (**f**) ***Rhizomastix*** contains small amoeboid flagellates that have a microtubular rhizostyle connecting the flagellar base to the nucleus. (**g**) ***Entamoeba*** contains amoebae with hyaline lobopodia and granular cytoplasm. (**h**) ***Endamoeba*** includes amoebae with a characteristic pattern of nuclear chromatin and no strong distinction between hyaline and granular areas of cytoplasm. (**i**) ***Tricholimax*** includes a single species of multinucleate amoeboid flagellate with a short, nonfunctional flagellum. (**j**) ***Mastigella*** contains amoeboid flagellates where the flagellar base is not connected to the nucleus by the cone of microtubules; the dominant trophic stage may be an aflagellate amoeba. (**k**) ***Pelomyxa*** contains large amoebae with multiple nuclei and very short, nonfunctional flagella. Scale bar = 20 μm

endobiotically in the intestines of insects and amphibians or are free-living in freshwater sediments. In *Rhizomastix* the cone of microtubules arising from the base of the flagellum has been modified into a tapering microtubular tube, the "rhizostyle."

Entamoebids are aflagellated, mostly endobiotic amoebae that were, until relatively recently, classified among other amoebae and taxonomically separated from the pelobionts. They currently formally include the genus *Entamoeba* (historically also *Endamoeba*, *Endolimax*, and *Iodamoeba*) and have in the past also included several genera of currently uncertain phylogenetic position, such as *Schizamoeba*, *Hydramoeba*, and *Malpighamoeba*. Their morphological similarities to pelobionts were recognized several decades ago (Cavalier-Smith 1983, 1987a, b) and since confirmed with molecular phylogenetics. Most entamoebids are probably harmless commensals of the digestive tract in invertebrates or vertebrates, including humans (e.g., *Entamoeba moshkovskii*; Heredia et al. 2012); a recent description reports a free-living or commensal marine species (Shiratori and Ishida 2016). The most important is the parasitic *Entamoeba histolytica*, which causes the dangerous amebic dysentery of humans.

Mastigina is a poorly known genus, with few sightings and no molecular data, currently classified as *incertae sedis* (Pánek et al. 2016) though likely to be a member of the mastigamoebids. It has many similarities to *Mastigamoeba* but has a limax body shape where pseudopodia emerge only at the anterior or posterior ends. Its identity has historically been confused with that of *Tricholimax* (see section "Systematics and Taxonomy", below).

Tricholimax hylae, a large multinucleate amoeba with a short, nonfunctional flagellum, is endobiotic in the hindgut of frog tadpoles. The phylogenetic placement of *Tricholimax* is unknown, in the absence of molecular data, so it is classified as Archamoebae incertae sedis (Pánek et al. 2016); nonetheless, it shows considerable similarity to *Mastigella* and *Pelomyxa*. Its identity has historically been confused with that of *Mastigina*.

Endamoeba is a poorly known genus; it includes aflagellate Archamoebae found in insects. Its morphological similarity to *Entamoeba* suggests it is likely to be a member of Entamoebidae, but it is currently classified as Archamoebae incertae sedis in the absence of molecular data (Pánek et al. 2016).

History of Knowledge and Literature

Taxonomic History of the Archamoebae as a Group

The name Archamoebae was introduced and used by Cavalier-Smith (1983, 1987a, b) and Cavalier-Smith et al. (2004) to group the pelomyxids, entamoebae, and mastigamoebids. The grouping of entamoebae, pelomyxids, and mastigamoebids was later supported by molecular phylogenetic analyses with complex evolutionary models (Cavalier-Smith et al. 2004; Edgcomb et al. 2002; Kudryavtsev et al. 2005; Milyutina et al. 2001; Nikolaev et al. 2006; Pánek et al. 2016; Ptáčková et al. 2013; Stensvold et al. 2012). The taxonomic concept of Archamoebae has been used at

ranks of infraphylum and class (Cavalier-Smith 1998, 2013; Cavalier-Smith et al. 2004) and has been compositionally unstable (Cavalier-Smith 1991, 1997; Cavalier-Smith and Chao 1995). It is currently ranked as a class, Archamoebae Cavalier-Smith, 1983 (Pánek et al. 2016).

In its recent incarnations (e.g., Cavalier-Smith 2013; Ptáčková et al. 2013; Zadrobílková et al. 2015, 2016), Archamoebae has included four main clades, the entamoebae, pelomyxids, mastigamoebids, and *Rhizomastix*, with *Tricholimax* sometimes treated as a fifth clade (Cavalier-Smith 2013) or regarded as incertae sedis but probably part of the pelomyxids, as discussed above (Frolov 2011; Pánek et al. 2016; Ptáčková et al. 2013; Zadrobílková et al. 2016). Relationships between clades have recently been resolved using multigene phylogenetics, dividing the group clearly into entamoebids (*Entamoeba*) and pelobionts (pelomyxids; mastigamoebids and *Rhizomastix*), and the high-level taxonomy has been updated to reflect this, as presented below here (Pánek et al. 2016).

Flagellated mastigamoebids and pelomyxids have historically been considered together as "pelobionts." The Order Pelobiontida was originally introduced to include only the genus *Pelomyxa* (Page 1976, 1987) and has occasionally been used at other ranks, e.g., Class Pelobionta (Krylov et al. 1980). Its trophic form being a large amoeba, *Pelomyxa* was until recently more usually classified with lobose amoebae (e.g., Bovee 1972; Bütschli 1880; Chatton 1925, 1953; Page 1976; Reichenow 1952; Siemensma 1987) but with increasing recognition that it was distinct from other large amoebae (Whatley and Chapman-Andresen 1990). Griffin (1988) revised the Order Pelobiontida to include *Mastigamoeba*, *Mastigella*, *Mastigina*, and *Dinamoeba*, on the basis of the ultrastructural evidence for flagella in *Pelomyxa* (Griffin 1979, 1988). Cavalier-Smith (1987a, b) created a different concept of pelobionts consisting of pelomyxids and entamoebids. However, this composition of the pelobionts has only rarely been used (e.g., Cavalier-Smith et al. 2004). The term "pelobiont" has since been used to encompass mastigamoebids and pelomyxids to the exclusion of entamoebae (Pánek et al. 2016).

The name Entamoebae was created to group aflagellate Archamoebae (*Entamoeba*, *Endamoeba*, *Endolimax*, and *Iodamoeba*). However, *Endolimax* and *Iodamoeba* have been removed from entamoebids, *Endamoeba* is currently regarded as Archamoebae incertae sedis, and the loss of the flagellum has occurred at least twice independently in Archamoebae. Thus, the name Entamoebae is confusing and should ideally only be used in the future with specific clarification as to its composition, noting that this is different from that of recent years.

History of Genera in the Archamoebae

Mastigamoeba was the first genus created to house species with a flagellum and an amoeboid body, with a hyaline cytoplasm unlike that of other superficially similar taxa such as the cercomonads (Kent 1880; Klebs 1892; Schulze 1875b; Stokes 1886, 1888, 1890). Frenzel (1897) then created *Mastigella* as a vehicle for species with similar characteristics but no (direct) connection between the nucleus and the

flagellum and *Mastigina* for flagellated species that had a limax shape and few lateral pseudopodia. These three genera have historically been described collectively as "mastigamoebids" (i.e., hyaline amoeboid flagellates), though the composition of this informal group has recently changed (see "General Characteristics", above). Subsequent revision of *Mastigina* from Goldschmidt (1907a) added an apical, spherical nucleus as a distinguishing criterion, as well as fountain-flow cytoplasm, though this is not present in all described species of *Mastigina* (Frenzel 1897) and is present in species of other genera that had already been described at that time, e.g., *Pelomyxa palustris*, *Mastigamoeba aspera*, and *Tricholimax hylae* (Frenzel 1897; Greeff 1874; Leidy 1879; Schulze 1875a, b). This had the effect of narrowing the circumscription of *Mastigamoeba* to include only hyaline amoeboid flagellates with a connection between the flagellum and the nucleus, with lateral pseudopodia, and with elongated nuclei. *Tricholimax hylae* (the sole species of *Tricholimax*) was then also treated as belonging to *Mastigina* (Brugerolle 1982; Brugerolle and Patterson 2000; Collin 1913; Goldschmidt 1907a; Wickerham and Page 1970), leading to some confusion about the distinguishing criteria of *Mastigina* (Frolov 2011).

Through the twentieth century, over 200 nominal species of *Mastigamoeba* and *Mastigella* were created on the basis of shape and size, pseudopodial form, and contractile vacuole number and location (e.g., Goldschmidt 1907a, Lemmermann 1914; see list of names in Ptáčková et al. 2013). Early studies of life cycles (Goldschmidt 1907a, b) and more recent culture-based studies (Bernard et al. 2000; Chystjakova et al. 2012; Ptáčková et al. 2013; Simpson et al. 1997; Walker et al. 2001; Zadrobílková et al. 2015) have shown that a single individual over time can express a very wide range of size, mode of amoeboid movement, gliding, swimming, and/or pseudopodial form, meaning that many previously described species are difficult to recognize with any confidence. However, recent molecular phylogenetic work has demonstrated that the ephemeral characteristics previously used to distinguish species can be used successfully as taxonomic characters, given a sufficiently detailed description (Ptáčková et al. 2013; Zadrobílková et al. 2015, 2016). Molecular phylogenetics has also shown that there are two main clades within *Mastigamoeba*, which have different ultrastructural characteristics (Pánek et al. 2016; see below).

What are now the type species of *Endolimax* and *Iodamoeba* were described in 1917 and 1912, respectively, as two *Entamoeba* species from humans (originally *Entamoeba nana* and *Entamoeba buetschlii*, respectively). *Endolimax* was created in 1917 (Kuenen and Swellengrebel 1917), and *Iodamoeba* in 1919 (Dobell 1919). Species have subsequently been assigned to each genus mostly based on morphology, with some debate about the degree of host specificity leading to fluctuating numbers of nominal species. Recent molecular phylogenetic studies suggest that the diversity encompassed by these descriptions is highly inconsistent from one nominal species to the next. Both genera are in need of revision (Stensvold et al. 2012; Poulsen and Stensvold 2016).

Species description in *Pelomyxa* has followed a similar pattern to that of *Mastigamoeba*. The amoeba now known as *Pelomyxa palustris* was first found by Greeff in 1870 and was named *Pelobius* (a name already occupied by an insect); it

was subsequently redescribed in more detail under its present name, *Pelomyxa palustris* (Greeff 1874). Greeff recognized that this "new, large freshwater rhizopod" was distinctively different from members of the genus *Amoeba* and emphasized the great morphological variability of the species. Leidy (1879) was also aware of this variability and suggested that the different forms of *Pelomyxa* might all be shown later to be different stages in the life cycle of the same species. During the twentieth century, various authors assigned numerous species to *Pelomyxa* (e.g., Penard 1902), but the prevailing view by the end of the twentieth century was that there was one or a few highly polymorphic species (Whatley and Chapman-Andresen 1990). Subsequently, as studies have employed light and electron microscopy and molecular phylogenetics, the number of species has begun to increase again (Berdieva et al. 2015; Chystjakova and Frolov 2011; Chystjakova et al. 2014; Frolov et al. 2004, 2005a, b, 2006, 2011; Ptáčková et al. 2013). The concept of *Pelomyxa* as a flagellated amoeba is relatively recent (Griffin 1979, 1988), but its similarities to mastigamoebids have long been noted (Bütschli 1880; Goldschmidt 1907a; Kudo 1939; Page 1970; Penard 1936; Schulze 1875b).

The genus *Endamoeba* was created in 1879 by Leidy for his newly described species, *Endamoeba blattae*, from cockroaches. Without noticing this, Casagrandi and Barbagallo (1895) created the genus *Entamoeba* and transferred into it the human parasitic species known previously as *Amoeba coli*. Since the names *Entamoeba* and *Endamoeba* are very similar, they were often confused, leading to their homonymization and the formal suppression of *Entamoeba* in 1928. The human pathogen *Entamoeba histolytica* can be, therefore, found under name *Endamoeba histolytica* in the older literature. This concept was, however, challenged by many authors (e.g., Kirby 1945) who argued that *E. blattae* and *E. coli* were not congeneric. The *Entamoeba*/*Endamoeba* problem was settled in 1954 when the International Commission on Zoological Nomenclature refuted the homonymization. Since that time, *Entamoeba* and *Endamoeba* have universally been considered distinct genera. The phylogenetic placement of *Endamoeba* remains unknown: although it is assumed to be related to *Entamoeba*, the cases of *Endolimax* and *Iodamoeba* now being placed in Mastigamoebidae suggest that *Endamoeba*'s position should be regarded as incertae sedis for now.

Since *Entamoeba histolytica* is an important human parasite, it is by far the best-known member of the Archamoebae. It was first reported by Lösch in 1875 and has since been intensively studied (see Martínez-Palomo (1993) and Wenyon (1926) for pertinent citations). The research into cell biology and biochemistry of *Entamoeba* was greatly facilitated when methods of its axenic culture became available (Diamond 1961). *Entamoeba histolytica* was one of the first putatively amitochondriate eukaryotes whose mitochondrial derivative (mitosome in this case) was discovered (Tovar et al. 1999). The genome sequence of *E. histolytica* was published more than 10 years ago (Loftus et al. 2005). Genome sequences of several other *Entamoeba* species are currently available (http://amoebadb.org/amoeba/). Several hundred papers on *E. histolytica* have been published annually during the last few years.

The genus *Rhizomastix* was described in 1911 by Alexeieff for his species *Rhizomastix gracilis* from the intestines of an axolotl. *Rhizomastix gracilis* was soon found also in larvae of crane flies (Mackinnon 1913), and its morphology was studied in detail by Ludwig (1946). Although a few additional *Rhizomastix* species have been described from insects and amphibians (see Čepička (2011) and Zadrobílková et al. (2016) for pertinent references), the phylogenetic position of this genus remained unclear until the recent molecular phylogenetic study of Ptáčková et al. (2013), which showed that *Rhizomastix* belongs to Archamoebae. The diversity of the genus has recently been studied using cultures and molecular phylogenetics (Zadrobílková et al. 2016; Pánek et al. 2016).

Practical Importance

Endobiotic Taxa Most described endobiotic Archamoebae are poorly known, and their relationships with their hosts have not been determined. Nevertheless, most species are probably harmless commensals.

The situation is much clearer in case of the few human symbionts. Several species have been reported from the intestine of humans (*Entamoeba histolytica*, *E. coli*, *E. dispar*, *E. polecki*, *E. hartmanni*, *E. moshkovskii*, *Endolimax nana*, and *Iodamoeba buetschlii*) and oral cavity (*Entamoeba gingivalis*). Although most of them are considered non-pathogenic, *Entamoeba histolytica* is an important human pathogen, which is responsible for 500 million new cases and 100,000 deaths annually. The disease caused by *E. histolytica* is called amebiasis or amebic dysentery and is distributed worldwide, though most cases are reported in developing countries. Amebiasis is also commonly diagnosed in travelers returning from tropical countries. The infection by *E. histolytica* usually occurs by ingestion of cysts with food or water. The primary site of infection is the large intestine where the amoebae may invade the mucosa and cause colitis that itself may be lethal. Occasionally, the amoebae spread via blood to various internal organs (usually the liver), and extra-intestinal amebiasis (with significant mortality) develops. For more information on amebiasis, see Martínez-Palomo (1993) and Haque et al. (2003).

Entamoeba dispar is morphologically indistinguishable from *E. histolytica* and was historically considered its non-pathogenic variant. It has been, however, shown that the two organisms represent separate species (see Diamond and Clark 1993). The pathogenity of *E. moshkovskii* is currently under debate (Heredia et al. 2012). *Entamoeba invadens* is known to cause severe reptilian amebiasis (see Reavill and Schmidt 2010).

Endolimax has been considered to be pathogenic by some authors, possibly causing diarrhea, intestinal inflammation, polyarthritis, or urticaria, but evidence for this is inconclusive (Poulsem and Stensvold 2016).

Free-Living Taxa Pelobionts and rhizomastixids are of unknown practical importance. While they have a worldwide distribution, they are not known to contribute to

any specific agricultural or pathogenic processes. They have historically been regarded as extremely "primitive" or deep-branching eukaryotes but are no longer regarded as such (see "Evolutionary History", below).

Habitats and Ecology

Occurrence/Distribution in Nature

Free-living pelobionts and rhizomastixids are found in the upper layers of mud or sand, mostly in freshwater rivers, lakes, bogs, or pits, and usually in stagnant or near-stagnant water, which creates a low-oxygen environment. The ideal place to find them is among undisturbed algal growth at the water-sediment interface, in shallow low-oxygen ponds (about 10–20% atmospheric oxygen) with relatively low pH (Bernard et al. 2000; Chystjakova et al. 2012; Frolov 2011; Ptáčková et al. 2013; Whatley and Chapman-Andresen 1990). Several papers have described them from sewage treatment works (e.g., Lackey 1923, 1932). Some taxa have been also described from low-oxygen intertidal marine sediments (Bernard et al. 2000; Bovee and Sawyer 1979; Larsen and Patterson 1990; Lee and Patterson 2000; Page 1983; Simpson et al. 1997; Shiratori and Ishida 2016; Zadrobílková et al. 2015).

Pelobionts have been described from all over the world (e.g., Larsen and Patterson 1990; Lee and Patterson 2000). The majority of reports in the literature are from temperate Europe and North America, due to the intensity of study in these locations in the last 200 years.

Endobiotic Archamoebae are widely distributed among various vertebrates and invertebrates. However, host specificity of particular species is only poorly understood and has been partially elucidated only in human parasites. For example, humans seem to be the primary host of *Entamoeba histolytica* though it has also been isolated from nonhuman primates and dogs. Similarly, *Entamoeba coli*, another species from the intestines of humans, can infect also nonhuman primates, dogs, and marsupials (see Thompson and Smith 2011). *Entamoeba moshkovskii* is most probably both endobiotic and free-living (Heredia et al. 2012; our observations).

Characterization and Recognition: Light-Microscopical Features

Recognizing Archamoebae

Most of the nominal species among Archamoebae have been described from a few cells seen on a single occasion (e.g., Calaway and Lackey 1962; Lackey 1923; Larsen and Patterson 1990; Penard 1902, 1909; Stokes 1886, 1888, 1890; Skvortzkov and Noda 1976). Many species can exist as flagellates, uninucleate amoebae, multinucleate amoebae, and cysts, a situation referred to as polymorphism.

Also, individual amoebae and flagellates change their appearance and are therefore pleomorphic. As most well-studied pelobionts are both polymorphic and pleomorphic, it would be tempting to assume that only static taxonomic characters that transcend any ephemeral changes can be used to distinguish species, such as the spines on the outside of *Mastigina setosa* (Goldschmidt 1907a), the extranucleolar "dot" in *Mastigamoeba punctachora* (Walker et al. 2001), or the doubled nucleus in *Mastigamoeba schizophrenia* (Simpson et al. 1997). But recent molecular work combined with observations of cultures (Ptáčková et al. 2013; Zadrobílková et al. 2015, 2016) confirms that – while transcendent characters make distinctions easier – ephemeral characters such as pseudopodial shape do correlate with molecular phylogenetic differences. Under these circumstances, it is important to not only observe variation in size and shape, flagellar length, speed of swimming, cytoplasmic movement, or separation into inner and outer layers but also to observe what percentage of the time characters are expressed.

General Appearance of the Cell

Archamoebae are chiefly recognized by being obviously amoeboid or amoeboid flagellates but are differentiable from other, similar taxa (e.g., myxomycete or protostelid swarmers, breviates, cercozoans, lobose amoebae) by having hyaline (clear) cytoplasm, which gives rise to "eruptive" or "bulging" hemispherical pseudopodia, usually at the anterior end of a moving cell (Fig. 1). The range of pseudopodial shapes reported in the literature also includes other morphologies – lateral or trailing pseudopodia may be rarely fine and filose or, more often, tapering finger shaped, broadly conical, or broadly lobate (e.g., Chystjakova et al. 2012; Frolov 2011; Frolov et al. 2004, 2006; Ptáčková et al. 2013; Zadrobílková et al. 2015, 2016). A uroid is frequently present at the posterior end of the cell; though in some taxa, particularly *Mastigamoeba simplex* and other members of the "Mastigamoebidae B" clade, and some *Mastigina* species, trailing pseudopodia are present instead (e.g., Goldschmidt 1907a; Pánek et al. 2016; Walker et al. 2001). *Rhizomastix* displays various pseudopodial shapes (Čepička 2011; Ptáčková et al. 2013; Zadrobílková et al. 2016). *Pelomyxa* has larger cells, and short flagella, but has many of the amoeboid characteristics seen in *Mastigamoeba*, as do members of *Mastigella* (Zadrobílková et al. 2015). Cells of *Entamoeba* spp. move by eruptive, clear lobopodia, though trailing filaments may be rarely formed. Although *Endolimax* is superficially similar to *Entamoeba*, its cells often form short tapering pseudopodia, similar to those seen in some species of *Mastigamoeba* (see Figs. 1, 3, and 4).

In some archamoebae, the cytoplasm is arranged in a distinct separation between "endoplasm" (interior of the cell body, where organelles are located) and the "ectoplasm" – a clear peripheral layer of cytoplasm, directly underneath the plasma membrane, which remains distinct from the agglomeration of nuclei, vacuoles, and endomembrane system in the center of the cell. This is the layer from which eruptive

pseudopodia form, into which the central cell contents then roll. The differentiation of ectoplasm has been described in mastigamoebids, including *Mastigamoeba scholaia* (Klug 1936), *Mastigella nitens* (Penard 1909), and *Mastigina setosa* (Goldschmidt 1907a; Skibbe and Zölffel 1991).

The ectoplasm may also be involved in "fountain flow" cytoplasmic streaming, as the outer part of the cytoplasm that runs backward, relative to the forward motion of the middle of the cell. Cytoplasmic streaming has mostly been described in *Pelomyxa* (Blochmann 1894; Hollande 1945; Rhumbler 1905; inter alia) but has also been reported in *Mastigamoeba aspera* (Chystjakova et al. 2012; Schulze 1875b), *Tricholimax hylae* (Becker 1928; Brugerolle 1982; Collin 1913; Frenzel 1897), and *Mastigina setosa* (Goldschmidt 1907a; Skibbe and Zölffel 1991).

Polymorphism and Life Cycle

Polymorphism is well-documented and a defining feature in pelobiont Archamoebae, as well as being necessary for completion of the life cycle in parasitic entamoebae. Life cycles have also been studied in several species. However, the actual cytological processes accompanying changes from one form to another remain mostly unknown, except in *Entamoeba* (Frolov 2011).

Recent descriptions of members of *Mastigamoeba*, *Rhizomastix*, and *Mastigella* have usually employed laboratory culture conditions that do not permit a study of the life cycle under normal environmental conditions. The general picture is that mastigamoebid Archamoebae can produce some or all of the following stages: amoeboid flagellates, aflagellate amoebae, large multinucleate amoebae, and cysts. All of these forms have been documented in detailed studies of *Mastigamoeba balamuthi* (Chavez et al. 1986; Pánek et al. 2016) and *M. schizophrenia* (Simpson et al. 1997), while flagellates and amoebae have been documented in *M. aspera* (Chystjakova et al. 2012), *M. simplex* (Bernard et al. 2000), *M. punctachora* (Bernard et al. 2000), *M. lenta*, *M. abducta*, *M. guttula*, *M. errans*, and *Rhizomastix libera* (Ptáčková et al. 2013); *Mastigella erinacea* and *Mastigella rubiformis* (Zadrobílková et al. 2015); flagellates and cysts in *Mastigella nitens* (Frolov 2011), *Rhizomastix bicoronata*, *R. tipulae*, and *R. elongata* (Zadrobílková et al. 2016); and flagellates, amoebae, and cysts in *R. vacuolata* (Zadrobílková et al. 2016).

Earlier studies of members of *Mastigamoeba* and *Mastigella* (e.g., Frenzel 1897; Goldschmidt 1907a; Schulze 1875b) employed microcosms rather than typical modern laboratory culture conditions, mimicking normal environmental conditions and allowing some observations of succession of polymorphism, from which life cycle details could be inferred. Flagellates reproducing by mitosis, aflagellate amoebae, and large, multinucleate division stages giving rise to uninucleate cells were documented in *Mastigella vitrea* and *Mastigina setosa* (Goldschmidt 1907a, b).

A series of studies of *Pelomyxa* species has also employed microcosms as a culture method, permitting extensive observation of the life cycle of natural populations (Whatley and Chapman-Andresen 1990; Frolov 2011). *Pelomyxa palustris* progresses in spring from cysts containing four nuclei, to small binucleate amoebae (100 μm long), then to larger multinucleate amoebae with or without endosymbionts, and then in summer to large elongate multinucleate amoebae (up to 5 mm long) with 30–60 nuclei, endosymbionts, flagella, and a posterior uroid. In autumn, they progress to large, spherical amoeboid cells with bacteria around the thousands of nuclei; these giant cells then divide by plasmotomy to form cysts or small amoebae over winter (Frolov 2011; Frolov et al. 2007; Schirch 1914; Whatley and Chapman-Andresen 1990). Other life-cycle descriptions exist for *P. corona* (Frolov et al. 2004), *P. gruberi* (Frolov et al. 2006), *P. binucleata* (Frolov et al. 2005a; Frolov 2011), *P. flava* (Frolov et al. 2011), and *P. paradoxa* (Chystjakova et al. 2014).

Cysts

The structure of the cyst has been best characterized in *Entamoeba invadens* (see Samuelson and Robbins 2011). The cyst wall is composed mainly from chitin and various glycoproteins. Cysts of particular *Entamoeba* species are rounded and differ in the number of nuclei present, e.g., the uninucleate *E. polecki*; *E. histolytica*, *E. hartmanni*, and *E. marina* with four nuclei; and *E. coli* and *E. muris* with up to eight nuclei (recently summarized in Shiratori and Ishida 2016). During the encystation of *Entamoeba* trophozoites, so-called chromatoid bodies are formed in the cytoplasm. These are elongated bars with rounded ends and are formed by ribosomes arranged in helices. The chromatoid bodies disappear from the older cysts (Rosenbaum and Wittner 1970; Schuster 1990).

Apart from *Entamoeba* spp., cysts with various numbers of nuclei have been documented in some other members of Archamoebae: uninucleate cysts in *Mastigamoeba balamuthi* (Chavez et al. 1986), *M. punctachora* (Bernard et al. 2000), *M. schizophrenia* (Simpson et al. 1997), and *Iodamoeba buetschlii*, binucleate cysts in *Rhizomastix* spp. (Čepička 2011; Zadrobílková et al. 2016), and four-nucleated cysts in *Endolimax* spp. and *Pelomyxa palustris* (Frolov 2011; Frolov et al. 2007; Griffin 1988; Ptáčková et al. 2013; Whatley and Chapman-Andresen 1990). The cysts of *Endamoeba* are multinucleate. *Tricholimax* varies between two and four nuclei according to the age of the cyst (Collin 1913). The composition of the cyst wall of non-*Entamoeba* Archamoebae is still unknown. In *Pelomyxa*, the cyst has a central vacuole containing endosymbionts, and the cyst wall has three layers. The laminated structure of cyst walls would be better revealed by cryofixation techniques, which have not yet been employed, than by chemical fixation, which has been used hitherto except on cysts of *Rhizomastix* (Zadrobílková et al. 2016). The cyst of *Iodamoeba buetschlii* contains a characteristic glycogen-containing inclusion in the cytoplasm (see Fig. 5).

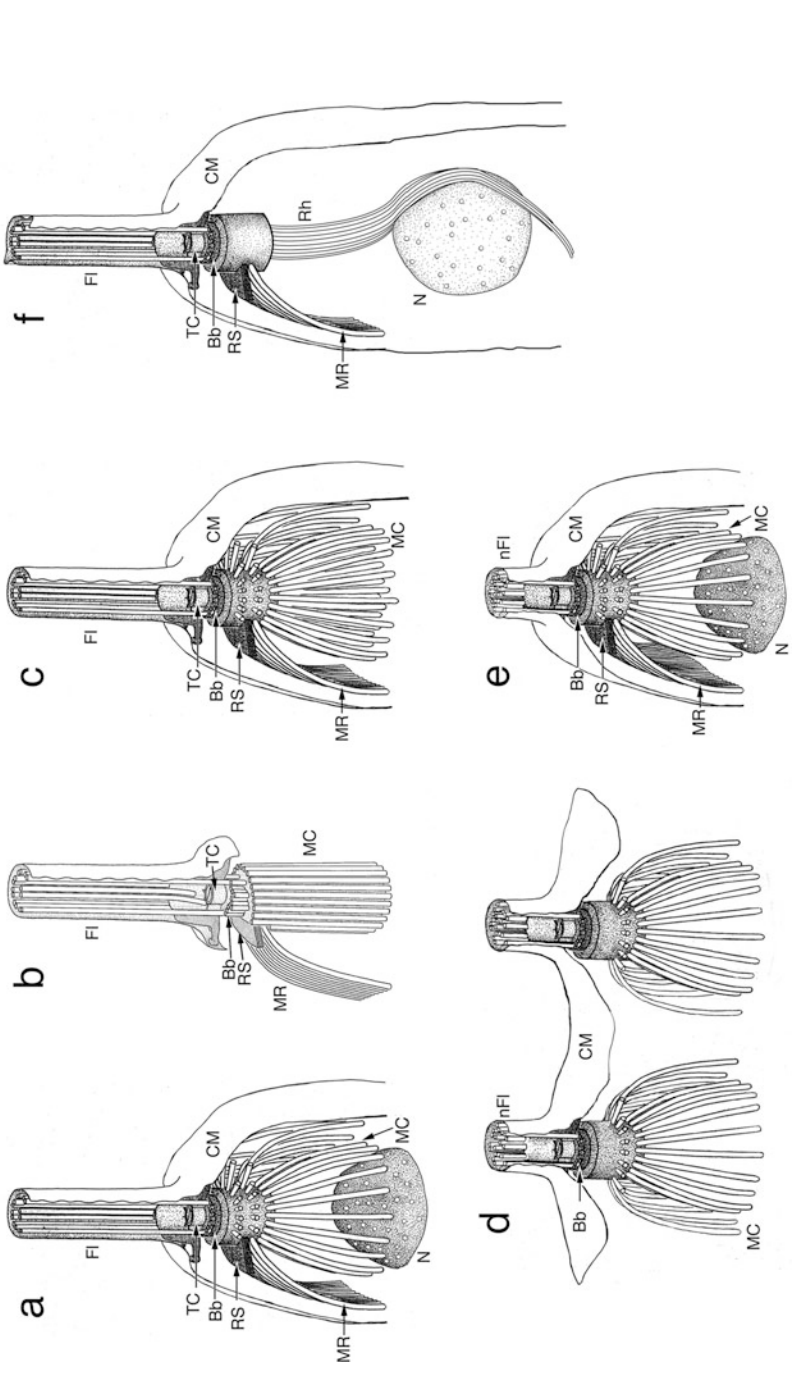

Fig. 2 Flagellar apparatus ultrastructure defines the identities of genera of flagellated Archamoebae. The features common to all flagellated Archamoebae include a single basal body (*Bb*), from which arises a flagellum (*Fl*) and a cone (*MC*) or rhizostyle (*Rh*) of microtubules, and (in most taxa) a

Ultrastructure

Flagellar Cytoskeleton

Flagella in Archamoebae appear to have degenerated in several groups independently, either to structures with dysfunctional axonemes or to the complete absence of a flagellar apparatus (discussed in Zadrobílková et al. 2015). Members of *Entamoeba*, *Endamoeba*, *Endolimax*, and *Iodamoeba* have no flagellar apparatus (El-Hashimi and Pitman 1970; Morris 1936; Rosenbaum and Wittner 1970; Zaman et al. 1998, 2000) and are not referred to further in the sections below.

Flagellated Archamoebae have a relatively simple microtubular cytoskeleton (see Fig. 2). Among the flagellated taxa that have been studied by TEM (see Table 1), the flagellar apparatus consists of one or more "monokinetids" – single, flagellated basal bodies – giving rise proximally to a cone or cylinder of microtubules, as well as (in most taxa) a lateral root of microtubules with a bilaminar "root sheet" at the origin of the root on its distal face. Members of *Mastigamoeba*, *Mastigella*, *Mastigina*, *Tricholimax*, and *Rhizomastix* typically have only one flagellum per cell, though the type species of *Mastigella*, *M. polymastix*, has up to four flagella per cell (Frenzel 1897) and *Tricholimax* has multiple nuclei, each with its own attached (internal) kinetid (Becker 1925; Brugerolle 1982, 1991; Collin 1913). The flagellar apparatus in *Mastigamoeba* varies between a wide distribution of microtubules, seen in Mastigamoebidae "A" (as defined in Ptáčková et al. 2013, Pánek et al. 2016; see also Table 1 and Figs. 2a and 3), and a very narrow single layer of microtubules, seen in members of Mastigamoebidae "B" (as defined in Ptáčková et al. 2013; Pánek et al. 2016; see also Table 1 and Figs. 2b and 3).

Fig. 2 (continued) transitional cylinder (*TC*). In most cells, the flagellar apparatus is usually directed perpendicularly to the cell membrane (*CM*), but the cone of microtubules can sometimes run parallel to it. (**a**) ***Mastigamoeba*** has a microtubular cone (*MC*) that connects to the nucleus; a microtubular root (*MR*) also arises from the basal body, immediately proximal to a bilaminate root sheet (*RS*); typically there is only one monokinetid flagellum per cell. This shows a representative flagellar apparatus from the group "Mastigamoebidae A" where the cone of microtubules (*MC*) arises laterally from both the sides and the base of the basal body; the flagellar transition zone is long. *Fl* flagellar axoneme, *TC* transition zone cylinder, *Bb* basal body. *RS* bilaminar root sheet, *MR* microtubular root. (**b**) ***Mastigamoeba*** showing a representative of group "B" where the cone of microtubules (*MC*) arises longitudinally from near the base of the basal body, and the flagellar transition zone (*TZ*) is short and contains no extra elements. (**c**) ***Mastigella*** has a microtubular cone that does not connect to the nucleus. There may be up to four long flagella per cell in some species. (**d**) ***Pelomyxa*** has numerous nonfunctional, short, monokinetid flagella (*nFl*) with "n + n" arrangement of microtubules. There is no obvious microtubular root or root sheet. (**e**) ***Tricholimax*** has a flagellar apparatus similar to that of *Mastigamoeba*, but it has nonfunctional "n + n" flagella (*nFl*), and it frequently has several nonflagellated monokinetids connected to nuclei in the cytoplasm. (**f**) ***Rhizomastix*** has a rhizostyle (*Rh*) – a tapering bundle of microtubules that extends from the base of the basal body, to wrap around the nucleus. Its flagellum has two vanes, which may contribute to its fast, jerky swimming movement. Scale bar = 250 nm (Figures (**a**) and (**c**) redrawn with modifications from Walker et al. 2001, and Figure (**b**) reproduced from Walker et al. 2001 with permission from Elsevier)

Table 1 Flagellar apparatus details for taxa described by TEM. Further details are provided in the taxonomic section. Where a value is given as "?," there is no data for that feature in a published account. Where a value is given as "(?)," the interpretation of data here differs from that given in the published account

Larger group/ species	Flagellum	9 + 2 axoneme	Dynein arms missing	Transition zone length	Transitional dense column or spiral	Transition zone cylinder	Cartwheel in base of Bb	Bb length	Lateral root of MT	Bilaminar root sheet	MT of cone emerging from sides of Bb	MT of cone emerging from Bb base	Electron-dense material around Bb	Electron-dense MTOC below Bb	References
Rhizomastixidae															
Rhizomastix elongata	Long (1 × cell length)	?	?	"Short"	Spiral	Yes	?	250 nm	Yes	No	No	Rhizostyle	No	Dense material at base	Zadrobílková et al. (2015)
Rhizomastix libera	Long (1 × cell), with vanes	9 + 2	?	"Short"	?	Yes (?)	?	250 nm	Yes	No	No	Rhizostyle	No	Dense material at base	Ptáčková et al. (2013)
Mastigamoebidae "A"															
Mastigamoeba aspera	Long (>2 × cell)	9 + 2	?	"Short"	?	?	?	250 nm	Yes	?	Many of cone MT at sides	Most of cone	No	Triangle	Chystjakova et al. (2012)
Mastigamoeba balamuthi	Long (>2 × cell)	9 + 2	Outer ones	700 nm	DC	Yes	?	250 nm	Yes	Yes	Most of cone arises at sides	Few	No	No	Chavez et al. (1986), Brugerolle (1991), Pánek et al. (2016)
Mastigamoeba punctachora	Long (>2 × cell)	9 + 2	Outer ones	1000 nm	DC	Yes	No	250 nm	Yes	Yes	Most of cone arises at sides	Few	No	Possibly?	Bernard et al. (2000), Walker et al. (2001)
Mastigamoeba schizophrenia	Long (>2 × cell)	9 + 2	Outer ones	700 nm	DC	Yes	Yes	250 nm	Yes	Yes	Single layer arises at base, lateral	No	No	No	Simpson et al. (1997)
Mastigamoeba sp.	Long (>2 × cell)	9 + 2	?	700 nm	Spiral + central filament	Yes	?	250 nm	Yes	Yes	Single layer arises at base, lateral	No	No	Ring	Brugerolle (1991)
Mastigamoebidae "B"															
Mastigamoeba simplex	Long (>2 × cell)	9 + 2	Outer ones	200 nm	No	Yes	Yes	250 nm	Yes	Yes	Single longitudinal-axis layer	No	No	No	Walker et al. (2001)
Mastigamoeba guttula	Long (>2 × cell)	9 + 2	?	200 nm	No	Yes	?	250 nm	Yes	Yes	Single longitudinal-axis layer arises at base	No	No	No	Ptáčková et al. (2013), Pánek et al. (2016)

Pelomyxidae															
Mastigella commutans	Long (>2 × cell)	9 + 2	Outer ones	200 nm	No	Yes	No	250 nm	Yes	Yes	Yes	Yes	No	No	Walker et al. (2001)
Mastigella rubiformis	Long (>1 × cell)	9 + 2	Outer ones	200 nm	?	?	?	250 nm	Yes	Yes	Yes	Yes	No	No	Zadrobílková et al. (2015)
Pelomyxa palustris (short group[a])	Short (ca. 10 μm)	n + n	All arms	200 nm	No (?)	Yes	?	250 nm	No (?)	?	Most of cone arises at sides of BB	Few	No	Ring	Seravin and Goodkov (1987a, b, d), Griffin (1988), Goodkov and Seravin (1991), Frolov (2011), Ptáčková et al. (2013)
Pelomyxa belevskii (short group)	Short (ca. 10 μm)	?	All arms	?	?	?	?	500 nm	?	?	Many of MT arise at sides	Many	No	Probably	Frolov (2011), Ptáčková et al. (2013)
Pelomyxa binucleata (short group)	Short (ca. 10 μm)	n + n	All arms	200 nm	No	Yes	No	200 nm	?	?	Most of cone arises at sides	Few	No	Ring	Frolov et al. (2005a), Frolov (2011)
Pelomyxa corona (? group)	?	?	All arms	?	?	?	?	?	?	?	?	Probable	?	?	Frolov et al. (2004)
Pelomyxa flava (long group)	Short (ca. 10 μm)	n + n	All arms	250 nm (?)	Spiral + central filament	No (?)	Yes (?)	700 nm	Yes	Yes	Yes	Yes	No	No	Frolov et al. (2011)
Pelomyxa gruberi (long group)	Short (ca. 10 μm)	2n + n	All arms	400 nm	DC	Yes	?	600 nm (?)	Yes (?)	Yes (?)	2/3 of cone arises at sides	Yes, 1/3	Yes	No	Frolov et al. (2006), Frolov (2011)
Pelomyxa stagnalis (short group)	?	n + n	All arms	200 nm	No	No (?)	?	150 nm	?	?	Layer of MT arises laterally at base	Yes (?)	No	?	Chystjakova and Frolov (2011); Ptáčková et al. (2013)
Pelomyxa paradoxa (intermediate form)	Medium (20–50 μm)	?	?	?	?	?	?	200 nm	Yes	Yes (?)	Layer of MT arises laterally at base	Yes	Yes	No	Chystjakova et al. (2014)
Pelomyxa prima (long group)	Short (ca. 10 μm)	?	All arms	?	?	?	?	700 nm	?	?	Many MT arise from sides	Many	Yes	Triangle	Frolov et al. (2005b), Frolov (2011)

(*continued*)

Table 1 (continued)

Larger group/ species	Flagellum	9 + 2 axoneme	Dynein arms missing	Transition zone length	Transitional dense column or spiral	Transition zone cylinder	Cartwheel in base of Bb	Bb length	Lateral root of MT	Bilaminar root sheet	MT of cone emerging from sides of Bb	MT of cone emerging from Bb base	Electron-dense material around Bb	Electron-dense MTOC below Bb	References
Pelomyxa secunda (? group)	None seen	?	?	?	?	?	?	?	?	?	?	?	?	?	Berdieva et al. (2015)
Pelomyxa schiedti (probable short group)	Short (ca. 5 μm)	9 + 1	All arms	?	?	?	?	200 nm	Yes	?	?	?	No?	?	Zadrobílková et al. (2015)
Pelomyxa tertia (short group)	Short (ca. 10 μm)	?	All arms	?	?	?	?	600 nm	Yes (?)	?	Yes (?)	Yes (?)	?	?	Frolov (2011)
Incertae sedis															
Tricholimax hylae	Short (ca. 10 μm)	2n + n	All arms	<400 nm	No	Yes (?)	?	500 nm	Yes	Yes	Few MT arise from sides, most from base	Many	No	Yes	Brugerolle (1982, 1991) (as *Mastigina hylae*)

Bb single basal body, *MT* microtubules, *MTOC* microtubular organizing center

[a]"Short group," "intermediate form," and "long group" refers to the categorization by basal body length and microtubular cone type, in Chystjakova et al. (2014)

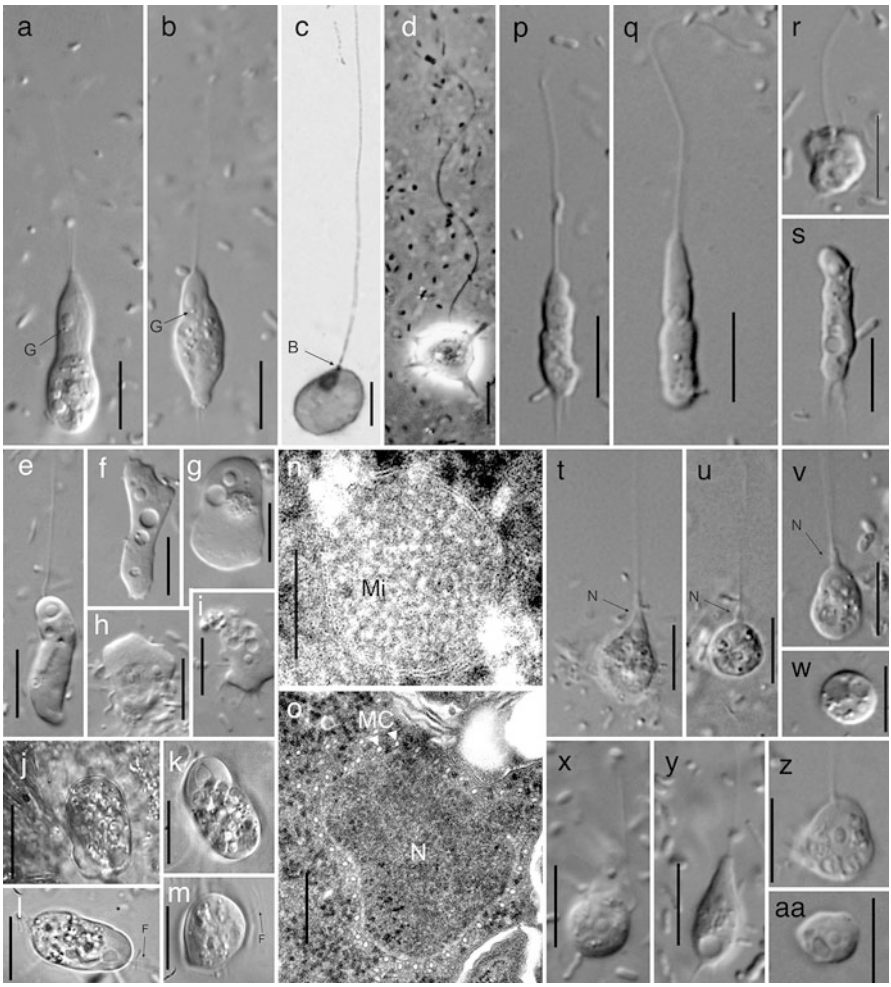

Fig. 3 ***Mastigamoeba***. All images are differential interference contrast light microscopy (*DIC*), unless otherwise specified. (**a**–**o**)**: Representatives of Mastigamoebidae A.** (**a**–**c**) *Mastigamoeba punctachora*, G = extranucleolar granule. (**c**) Bright field microscopy with protargol staining; B = basal body, immediately adjacent to the stained nucleus. (**d**) *Mastigamoeba balamuthi* swimming cell, phase contrast LM. (**e**–**i**) *Mastigamoeba errans*. In e, gliding flagellate cell; f–i, aflagellate cells. (**j**–**m**) *Mastigamoeba* sp. *ex Pelomyxa belevskii*. F = flagellum. In j, bright field light microscopy. (**n**) *M. punctachora* transmission electron microscopy. Double-membraned, mitochondrion-related organelle (*Mi*). (**o**) *M. punctachora* TEM. Nucleus (*N*) surrounded by microtubules of the cone (*MC*) cut in transverse section. (**p**–**aa**)**: Representatives of Mastigamoebidae B**. (**p**–**s**) *Mastigamoeba simplex*. In p–r, gliding flagellates; in s, aflagellate cell. (**t**–**w**) *Mastigamoeba scholaia*. In t, u, gliding cells; in v, gliding aflagellate cell; in w, aflagellate cell. N = neck. (**x**–**aa**) *Mastigamoeba guttula*. In x–z, flagellates; in aa, aflagellate cell. Scale bars in **a–c**, **e–i**, **p–aa** =10 μm; in d = 20 μm, in j–m = 50 μm, in n, o = 200 nm (Figures a–c, e–m, and p–aa all reproduced from Ptáčková et al. 2013 with permission from Elsevier)

Flagella are usually positioned at the anterior end of the cell and used in movement, though particularly amoeboid cells in *Mastigella* may move predominantly by pseudopodia, with the poorly functional flagellum pointing in a different direction (Frenzel 1897; Frolov 2011; Goldschmidt 1907a; Walker et al. 2001, Zadrobílková et al. 2015). The flagellum of *Mastigella* is generally slower beating and less motile than that seen in *Mastigamoeba* and may be an intermediate stage that gave rise to the extreme flagellar reduction seen in *Pelomyxa* (discussed in Zadrobílková et al. 2015).

Giant amoeboid cells of *Pelomyxa* have numerous separate monokinetids, bearing (usually) short, nonfunctional flagella with a disorganized axoneme. Typically, a microtubular cone is formed, but this varies between a dense cone and a single layer of microtubules, with or without a lateral root (Fig. 2d; see Table 1 for further details). The number of monokinetids varies between ca. 50 and ca. 700 in *P. palustris* (Griffin 1988). Likewise, their density varies more than tenfold (Griffin 1988), and their abundance and distribution over the cell's sides or uroid also varies (e.g., compare *P. flava*, *P. paradoxa*, and *P. stagnalis*; Chystjakova and Frolov 2011; Chystjakova et al. 2014; Frolov et al. 2011). Whether basal bodies form flagella or merely "buds" can vary widely through the life cycle (e.g., *P. paradoxa* vs. *P. gruberi*; Chystjakova et al. 2014; Frolov et al. 2006). Flagella are typically not found on anterior pseudopodia in *Pelomyxa* (Frolov 2011) and have not been seen at all in *P. secunda* (Berdieva et al. 2015).

For further details of the flagellar cytoskeleton, see Fig. 2 and Table 1.

Nuclei and Cell Division

Numbers of nuclei are one of the main features that distinguish "pelomyxid" Archamoebae from "mastigamoebid" or "entamoebid" Archamoebae. In many pelomyxids, the usual state is cells with tens or hundreds of nuclei, whereas in other Archamoebae, the usual state is one or few nuclei, though stages with tens of nuclei may exist.

A uninucleate flagellate is the main trophic form in *Mastigamoeba*, some members of *Mastigella*, *Mastigina*, and *Rhizomastix*. Reproduction of uninucleate flagellates by mitosis has been documented only in *Mastigella* and *Mastigina* (Goldschmidt 1907a, b). The nuclei of *Mastigamoeba schizophrenia* appear in "pairs" of two adpressed nuclei, but the extent of differentiation within these pairs is unknown (Simpson et al. 1997). Some "flagellate" taxa may typically have more than one nucleus, such as *Tricholimax hylae*, which has 1–6 (Becker 1925; Brugerolle 1982, 1991; Collin 1913; Frenzel 1897), and members of *Mastigella* (Zadrobílková et al. 2015).

As discussed above, a few "flagellate" species also have distinct, large amoeba stages with more than one nucleus: *Mastigamoeba schizophrenia* has 2–10 (i.e., 1–5 pairs; Simpson et al. 1997); *M. punctachora* has 1–8 (Bernard et al. 2000); and *M. aspera* has two or more nuclei in its amoeba form, which would previously have

been described as a different species, *Dinamoeba mirabilis* (see Chystjakova et al. 2012). In at least one such species, *Mastigamoeba balamuthi*, the multinucleate amoebae (with on average 15 nuclei but up to 46), are the dominant trophic stage, reproducing while multinucleate by mitosis and subsequent plasmotomy (i.e., cytokinesis not preceded by mitosis), which results in large and asymmetric numbers of nuclei in daughter cells.

"Budding" plasmotomy, from multinucleate amoebae to uninucleate flagellates, only happens rarely and only under specific trophic conditions (Chavez et al. 1986). Production of small uninucleate flagellates, from a large, rounded amoeboid cell that is filled with smaller cells, has also been described from *Mastigina setosa* and *Mastigella vitrea* (Goldschmidt 1907a, b); whether this is a form of plasmotomy or more akin to sporogony is unclear. It resembles the processes described in *Pelomyxa palustris* (Frolov 2011; Whatley and Chapman-Andresen 1990).

In *Pelomyxa*, the main described trophic "adult" form is a large, multinucleate amoeba, which also has flagella (see above). This "flagellated" trophic form, typically a cylindrical amoeba, may in some cases later transform into an immotile, rounded amoeboid form with hundreds of nuclei (e.g., *P. corona*, *P. gruberi*; Frolov 2011; Frolov et al. 2004, 2006). It is unclear whether this rounded form is homologous to the large multinucleate amoebae seen in *Mastigamoeba schizophrenia* (Simpson et al. 1997) and *M. balamuthi* (Pánek et al. 2016) or to the large rounded forms that divide by plasmotomy in *Mastigella vitrea* and *Mastigina setosa* (Goldschmidt 1907a), as they are only rarely seen and have not been studied by electron microscopy. Species of *Pelomyxa* can be divided into taxa that have hundreds of nuclei in the adult motile form (e.g., *P. palustris*, *P. corona*, *P. prima*: Frolov 2011; Whatley and Chapman-Andresen 1990) and taxa with tens (e.g., *P. belevskii*, *P. stagnalis*; Frolov 2011; Ptáčková et al. 2013) or one or two nuclei in mature cells (e.g., *P. binucleata*, *P. flava*, *P. paradoxa*, *P. schiedti*; Chystjakova et al. 2014; Frolov 2011; Frolov et al. 2005a; Zadrobílková et al. 2015). Division from multinucleate adult cells to smaller juvenile cells is typically by unequal plasmotomy (Frolov 2011; Ptáčková et al. 2013; Schulze 1875a; Whatley and Chapman-Andresen 1990).

The trophozoites of *Entamoeba* spp. are uninucleate, and no multinucleate trophic forms are usually created. Mitosis of *Entamoeba histolytica* was studied, for example, by Solis and Barrios (1991) and by Chávez-Munguía et al. (2006).

Nuclear Chromatin

Patterns of nuclear chromatin appear to distinguish different species in Archamoebae, though there is not a clear taxonomic pattern above species level. Chromatin patterns can be a peripheral arrangement of small clumps of chromatin around the edge of the nucleus or a single large nucleolus or a few chromatin clumps joined together in the center of the nucleus (described further in Frolov 2011). Peripheral arrangements are common in *Entamoeba*, pelomyxids, and *Rhizomastix* (Čepička 2011; Chystjakova et al. 2014; Frolov 2011; Frolov et al. 2004, 2005a, b,

2006; Ptáčková et al. 2013; Zadrobílková et al. 2015, 2016) and are also present in "*Mastigina trichophora*," *Mastigella eilhardi*, and *Mastigella nitens* (Frolov 2011; Penard 1909; Zadrobílková et al. 2015).

Nuclear chromatin is used as a diagnostic feature in *Entamoeba* species, with *E. histolytica*, *E. dispar*, and *E. marina* showing a fine peripheral ring with a small central dot, *E. hartmanni* showing a large central nucleolus and thick peripheral clumps of chromatin, and *E. coli* showing peripheral clumps only (Shiratori and Ishida 2016). The majority of members of *Mastigamoeba* have large nucleoli, as do *Tricholimax hylae*, *Pelomyxa palustris*, *P. gruberi*, *P. stagnalis*, and *Rhizomastix* species. In both *Iodamoeba* and *Endamoeba*, the nucleolus is large and central, and there is no peripheral ring of chromatin (see section "Systematics and Taxonomy", below).

There are cajal-body-like stacked inclusions in the central nucleolus in *Pelomyxa stagnalis* (Chystjakova and Frolov 2011; Ptáčková et al. 2013), and *P. belevskii* has a loose stacked arrangement of "vermiform bodies" of chromatin around the edge of the nucleus, similar in size to the inclusion in *P. stagnalis* (Ptáčková et al. 2013).

Cell Coverings

As reviewed in Frolov (2011), most pelobionts are covered in a thin layer of amorphous or filamentous organic material. The extracellular layer may be irregularly, thinly distributed over the cell surface (e.g., Brugerolle 1982; Chavez et al. 1986; Simpson et al. 1997; Walker et al. 2001), often being thickest at the posterior end of the cell, particularly in pelomyxids (Frolov 2011). Finely filamentous cell coverings may be present, with filaments apparently perpendicular to the cell surface (e.g., *Pelomyxa palustris*, *P. gruberi*, *Mastigella nitens*: Frolov 2011) or parallel to the surface in the case of very thick coverings, which can reach up to 1 μm thickness (e.g., *P. flava*, *Mastigamoeba aspera*; Chystjakova et al. 2012; Frolov 2011; Frolov et al. 2011). These fine extracellular layers are rarely preserved well with chemical fixation for electron microscopy; cryofixation would be appropriate for further study.

Numerous species in pelobionts have been described with spines on the outside of the cell, with varying degrees of packing, from the dense, highly refringent layer of very tightly packed spines in *Mastigina chlamys* (Frenzel 1897; Seckt 1922), through regularly arranged but obviously discrete spines in *Mastigina setosa* (Goldschmidt 1907a; Skibbe and Zölffel 1991), to irregular and loosely packed spicules in *Mastigamoeba trichophora* (Lauterborn 1901), *Mastigamoeba pilosa* (Schoudeten 1907), and *Mastigina spicata* (Penard 1909). Frolov (2011) shows electron micrographs of "*Mastigina trichophora"* where hollow spicules are each placed in "boat-shaped" scales, which are packed in a tight layer over the extracellular glycocalyx. It is unclear whether these spines are made only of organic material (Frolov 2011) or are mineralized as well, as seen in the euglenid *Trachelomonas* (Conforti et al. 1994; Preisig 1994).

Mitochondrial Remnants

Like other anaerobic groups in the eukaryotes, Archamoebae are "secondarily amitochondriate." In almost all cases, such anaerobic eukaryotes retain reduced mitochondrion-related organelles (MROs) which have usually lost DNA, genome maintenance proteins, and a typical ATP-producing electron transport chain. They often still produce ATP by partial anaerobic oxidation of pyruvate (hydrogenosomes), but some have lost energy metabolic function entirely (mitosomes), preserving only a few other functions (such as aspects of mitochondrial type iron-sulfur cluster assembly machinery) and acquiring the ability to import ATP (Chan et al. 2005; Maguire and Richards 2014).

Both kinds of double-membrane-bound MROs have been described from Archamoebae (Barberà et al. 2007; Hampl and Simpson 2008). Mitochondrial cpn60 function has been demonstrated in *Entamoeba histolytica*, and localized, to the mitosomes in *E. histolytica* (Chan et al. 2005; Clark and Roger 1995; Leon-Avila and Tovar 2004; Tovar et al. 1999), and to hydrogenosome-like organelles in *Mastigamoeba balamuthi* (Gill et al. 2007; Hampl and Simpson 2008). Iron-sulfur cluster assembly proteins are closely related to those of epsilon proteobacteria both in *Mastigamoeba balamuthi* (Gill et al. 2007) and *Entamoeba histolytica* (Maralikova et al. 2010; van der Giezen et al. 2005), implying a single lateral transfer event where mitochondrial Fe:S assembly appears to have been lost entirely and replaced with an Fe:S assembly system that is not homologous to the standard mitochondrial system and is now cytosolic (Nývltová et al. 2013). The acquisition of alternatives to normal mitochondrial pyruvate dehydrogenase may have happened more than once, with *M balamuthi* and *E. histolytica* each possessing copies of pyruvate ferredoxin/oxidoreductase (Embley and Martin 2006; Gill et al. 2007); but *M. balamuthi* also possesses a pyruvate formate lyase that was laterally transferred from a firmicute (Stairs et al. 2011). Mitosomes are extremely abundant in *E. histolytica* implying that some functions are yet to be uncovered in detail (Aguilera et al. 2008) – an example being the synthesis of sulfur compounds required for encystation (Mi-ichi et al. 2015). The MROs of Archamoebae are discussed in the context of other MROs by Maguire and Richards (2014).

Ultrastructural reports also exist for double-membrane-bound organelles in *Mastigamoeba simplex*, *Mastigamoeba punctachora*, and *Mastigella commutans* (Walker et al. 2001), *Mastigella rubiformis*, *M. ineffigiata* (Zadrobílková et al. 2015), *Endolimax piscium* (Constenla et al. 2013), *Rhizomastix libera* (Ptáčková et al. 2013), *R. elongata* (Zadrobílková et al. 2016), and *Pelomyxa palustris* (Seravin and Goodkov 1987c). As previously discussed (Zadrobílková et al. 2015), there is a notable lack of reports of MROs in EM studies of *Pelomyxa*. This may be because of the difficulty of serially sectioning a large amoeba, a misidentification of MROs as symbionts, or, alternatively, the real absence of MROs in all examined species of *Pelomyxa* except *P. palustris*.

Golgi

Canonical Golgi dictyosomes have not been reported in the Archamoebae so far, though related elements of the endomembrane system have been shown to be functionally present in *Entamoeba histolytica* (Bredeston et al. 2005) and *Mastigamoeba balamuthi* (Dacks et al. 2004), with all "core" functions being present (Mowbrey and Dacks 2009). However, methods for detecting endomembrane system function and Golgi dictyosomes are diverse, and many eukaryotes do not possess canonical Golgi stacks that are easy to recognize by transmission electron microscopy. Tantalizing reports exist suggesting that *Entamoeba* may possess Golgi stacks that are only visible with the application of cryofixation techniques (Chavez-Munguia et al. 2000; Ghosh et al. 2000). A similar situation may hold in pelobionts: a few reports exist of Golgi-like stacks of membranes, but in each case, these may be poorly fixed endoplasmic reticulum, and further ultrastructural work, using different techniques, is required. The multi-membrane organelle seen in *Rhizomastix libera* (Ptáčková et al. 2013) is positioned close to the flagellar base, as per the Golgi dictyosomes of other superficially similar amoeboid taxa with flagella (e.g., Heiss et al. 2013; Walker et al. 2003), but the published preservation of *Rhizomastix* is not sufficiently good for any clear identification of the organelle, and it is not seen in *R. elongata* under better fixation conditions (Zadrobílková et al. 2016). Similar, though bigger and more organized stacked structures have been reported from *Pelomyxa palustris* (Seravin and Goodkov 1987b) and *P. corona* (Frolov et al. 2004) as well as reticulate multimembranous organelles from *P. flava* (Frolov et al. 2011). These have subsequently been interpreted as endoplasmic reticulum (Frolov 2011), as have reports of stacked structures in *Mastigamoeba schizophrenia* (Simpson et al. 1997), *M. punctachora* (Walker et al. 2001), and *Rhizomastix elongata* (Zadrobílková et al. 2016).

Peroxisomes

While Archamoebae have traditionally been considered to lack peroxisomes (Žárský and Tachezy 2015), an unpublished thesis reports peroxisomal proteins in both *Mastigamoeba balamuthi* and *Entamoeba histolytica* (Žárský 2012), which would be consistent with secondary loss of peroxisomal function in Archamoebae.

Symbionts

The best-known symbionts of Archamoebae are the three morphologies of prokaryotes that surround the nuclei of *Pelomyxa* species (Berdieva et al. 2015; Chystjakova et al. 2014; Frolov 2011; Frolov et al. 2006; Whatley 1976; Whatley and Chapman-Andresen 1990). There is one large bacterium with a distinctive axial cleft and up to two smaller methanogenic archaea, one of which is known as *Methanobacterium formicicum* DSM3637 (Frolov et al. 2004, 2005a, b, 2006, 2011; Griffin 1988; van Bruggen et al. 1988; Whatley 1976). *Methanobacterium*

formicicum DSM3637 was isolated from *P. palustris* (van Bruggen et al. 1983, 1988), and a draft of its genome has been published (Gutierrez 2012). Endobiotic prokaryotes also exist in *Mastigella* species (Frolov 2011; van Bruggen et al. 1985; Walker et al. 2001; Zadrobílková et al. 2015) and *Rhizomastix libera* (Ptáčková et al. 2013). The organic layer covering *Mastigamoeba aspera* also contains numerous small, rod-shaped prokaryotic ectobionts of uncertain identity (Chystjakova et al. 2012; Goldschmidt 1907a; Kudo 1950; Lemmermann 1914; Page 1970; Penard 1902, 1909, 1936; Schulze 1875b; Siemensma 1987).

The physiological role played by these endo- and ecto-symbionts has not been clearly demonstrated, but numerous cases exist of symbiosis between methanogenic archaea (which presumably derive hydrogen from within their hosts) and anaerobic eukaryotes with mitochondria that have been reduced to hydrogenosomes (Embley et al. 1995; Fenchel and Finlay 1995; Martin and Müller 1998; van Bruggen et al. 1983; van Hoek et al. 2000).

Endobiotic mastigamoebids have also been described as emerging from *Pelomyxa* (Greeff 1874; Hollande 1945; Whatley and Chapman-Andresen 1990); recent observations including sequence data confirm that this is not a life-cycle stage of *Pelomyxa* (Ptáčková et al. 2013).

Systematics and Taxonomy

Class Archamoebae Cavalier-Smith, 1983
Anaerobic/microaerophilic Amoebozoa with reduced mitochondria. May exist as amoebae, amoeboflagellates, or cysts. Ancestrally with a single apical flagellum, arising from a single basal body that gives rise proximally to a microtubular cone and laterally to a microtubular root arising immediately proximal to a double-layered "root sheet" sitting perpendicular to the axis of the basal body. Secondarily aflagellate or multiflagellate, with disordered flagellar axoneme, or with no flagellar apparatus. Amoeboid movement with eruptive lobopodia. Free-living or endobiotic.

Order Pelobiontida Page, 1976
For the current composition, see Pánek et al. (2016). The clade including mastigamoebids, pelomyxids, and rhizomastixids; using the branch-based definition introduced and discussed by Pánek et al. (2016), the clade consisting of *Mastigella eilhardii* Bürger 1905 and all organisms that share a more recent common ancestor with *M. eilhardii* than with *Entamoeba histolytica* Schaudinn 1903.

Suborder Mastigamoebina Frenzel, 1897
Rank changed from order to suborder by Pánek et al. (2016). The clade including Mastigamoebidae and Rhizomastixidae; using the node-based definition introduced and discussed by Pánek et al. (2016), the least-inclusive clade consisting of *Mastigamoeba balamuthi* (Chávez et al. 1986) Simpson et al. 1997, *Mastigamoeba abducta* Ptáčková et al. 2013, and *Rhizomastix libera* Ptáčková et al. 2013.

Family Mastigamoebidae Goldschmidt, 1907
Archamoebae with trophozoites which are uninucleate to multinucleate, with single motile anterior flagellum associated with microtubular cone, or aflagellate. Amoebae flattened, amoeboid movement slow, typically with multiple pseudopodia. Free-living or endobiotic. Current composition as per Ptáčková et al. (2013).

Mastigamoeba Schulze, 1875 (Fig. 3)
Mastigamoeba contains amoeboid cells where the flagellated basal body and the anterior nucleus are immediately adjacent to each other during movement. The basal body and nucleus are joined by a cone of microtubules.

Circumscription Archamoebae with a uniflagellated trophic stage, in which the nucleus and flagellum are connected by a cone of microtubules that arises from the base and sides of the single (flagellated) basal body; a cylinder is present in the transition zone of the flagellum. A single root of microtubules arises from the side of the basal body, and the root has a bilaminar "root sheet" on its anterior edge (Fig. 2a, b). Basal bodies usually have nine triplets of microtubules, but *M. schizophrenia* has nine doublets (Simpson et al. 1997). The flagellum has a conventional eukaryotic "9 + 2" arrangement of microtubules but lacks outer dynein arms, giving rise to a distinctively languid flagellar beat. The flagellates may, at least in some species, transform to amoebae with one, few, or many nuclei. Both flagellate and amoeboid forms may transform into cysts. Nuclei are usually single, but are paired in *M. schizophrenia*, and have vesicular nucleoli. In *M. punctachora*, the nucleus contains a small extranucleolar "dot" of chromatin (Bernard et al. 2000). The outside of the cell is usually naked, but in *M. aspera*, there are ectobiotic bacteria (Chystjakova et al. 2012) and spined species have been described. Cells have been found in soils and freshwater and marine habitats. Phylogenetic analyses divide this genus into two separate groups (as defined in Ptáčková et al. 2013; Pánek et al. 2016): "A" with a broad multilayered microtubular cone radiating laterally from the sides of the basal body, a long flagellar transition zone, and, in some taxa, a transitional column or spiral, microtubules arising from the base of the basal body or an MTOC immediately proximal to the basal body (Fig. 2a; Table 1) and "B" with a single-layered microtubular cone extending posteriorly from sides the basal body, and a short transition zone, and none of the extra features seen in some members of "A (Fig. 2b; Table 1)." There is little morphological variation at the light microscopical level in "B" (Fig. 3).

Type Species *Mastigamoeba aspera* Schulze, 1875
(See Chystjakova et al. 2012 for a recent description and discussion of synonymy with *Dinamoeba*; discussed further in Ptáčková et al. 2013)

Remarks Based on recent phylogenetic analyses (Stensvold et al. 2012; Ptáčková et al. 2013), the flagellum-lacking entamoebid genera *Endolimax* and *Iodamoeba* form an internal branch of *Mastigamoeba*, making the latter genus paraphyletic. Recent analyses also divide *Mastigamoeba* into "A" and "B" clades, as discussed

above. The full scope and character of *Mastigamoeba* is uncertain, as recent phylogenetic analyses do not include most of the previously described taxa in *Mastigamoeba*, and we lack electron microscopical data for many taxa, as well as molecular data on the phylogenetic position of *M. aspera*, the type species. The degree of overlap between *Mastigamoeba*, *Mastigina*, and *Tricholimax* has long been unclear, leading to confused and inconsistent taxonomy of some species (Frenzel 1897; Frolov 2011; Goldschmidt 1907a; Lemmermann 1914). Other genera that fall within the circumscription of *Mastigamoeba* include *Dinamoeba* Leidy, 1874 (*D. mirabilis* has long been regarded as a synonym of *M. aspera* – as most recently discussed in Chystjakova et al. 2012; Ptáčková et al. 2013) and *Phreatamoeba* Chávez et al. 1986 (transferred to *Mastigamoeba* by Simpson et al. 1997).

Endolimax Kuenen and Swellengrebel, 1917 (Fig. 4)
Endolimax contains aflagellate uninucleate amoebae reminiscent of *Entamoeba*. All are intestinal symbionts of various insects and vertebrates including humans.

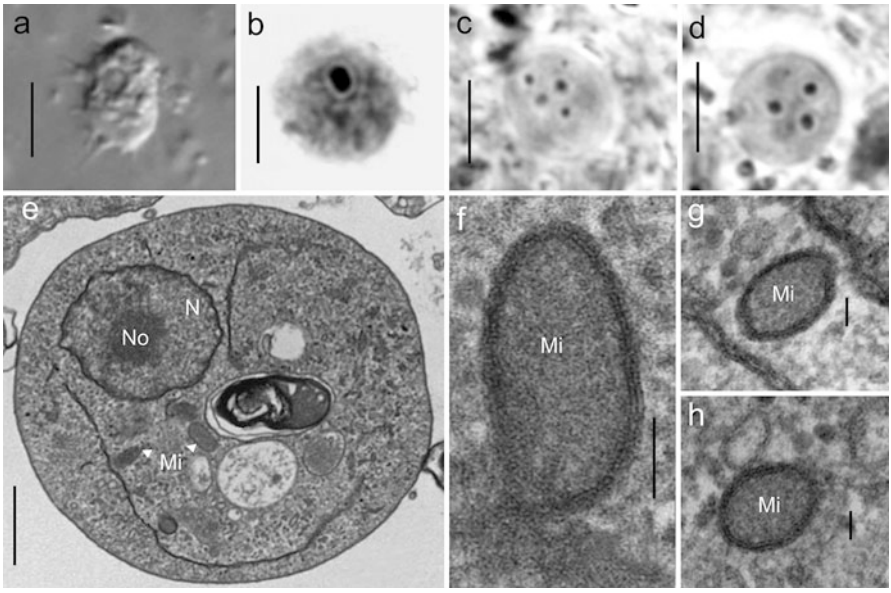

Fig. 4 ***Endolimax.*** (**a**) *Endolimax nana* live trophozoite (amoeba) showing characteristic finger-shaped pseudopodia and a single nucleus with a vesicular nucleolus. Differential interference contrast light microscopy. (**b**–**d**) *E. nana* prepared slides, stained with iron hematoxylin, bright field light microscopy: (**b**) trophozoite, showing a large spherical nucleolus in the nucleus. (**c**, **d**) Cysts with four nuclei. (**e**–**h**) Transmission electron micrographs of *Endolimax piscium* from Constenla et al. 2013: (**e**) Whole cell, showing nucleus (*N*) with central nucleolus (*No*), and mitochondrion-related organelles (*Mi*, *arrowheads*). (**f**–**h**) Mitochondrion-related organelles (*Mi*), with double membranes. Scale bars in a, c, d = 5 μm; in b = 10 μm, in e = 500 nm, in f, g, h = 50 nm (Micrographs in **e, f, g, h** reprinted from *Journal of Fish Diseases* 37, Constenla et al., *Endolimax piscium sp. nov. (Amoebozoa), causative agent of systemic granulomatous disease of cultured sole, Solea senegalensis Kaup,* pp. 229–240, Copyright (2013), with permission from John Wiley & Sons)

Circumscription Trophozoites are aflagellate amoebae measuring up to 20 μm. Actively moving cells form a single broad, hyaline, and eruptive lobopodium. Morphologically, *Endolimax* closely resembles *Entamoeba*, except that the nucleus of *Endolimax* does not contain peripheral heterochromatin (Figs. 4 and 9). There is a single large central nucleolus in the nucleus. Nucleoli in nuclei of cysts are often elongate. Cysts are rounded or elongate, with one to eight nuclei (usually four; rarely many). Approximately 20 *Endolimax* species have been described, all being commensals of the lower intestine of various animals (e.g., *E. nana* from humans, *E. caviae* from rodents, *E. gregariniformis* from birds, *E. clevelandi* from reptiles, *E. ranarum* from amphibians, *E. leptocoridis*, and *E. termitis* from insects). *Endolimax piscium* was reported from lesion in the muscle tissue of a fish (Costenla et al. 2013).

Type Species *Endolimax nana* (Wenyon and O'Connor 1917) Kuenen and Swellengrebel, 1917 (basionym *Entamoeba nana* Wenyon and O'Connor 1917).

Remarks Because it is aflagellate, *Endolimax* was traditionally considered a member of Entamoebidae. Based on SSU rRNA phylogeny, Cavalier-Smith et al. (2004) removed *Endolimax* from Entamoebidae and created the family Endolimacidae for it. Since *Endolimax* forms an internal branch of *Mastigamoeba*, Ptáčková et al. (2013) transferred it to Mastigamoebidae. The genus as a whole is relatively understudied (Poulsen and Stensvold 2016).

Iodamoeba Dobell, 1919 (Fig. 5)
Iodamoeba contains aflagellate uninucleate amoebae reminiscent of *Entamoeba* and *Endolimax* that are intestinal commensals of vertebrates.

Circumscription Trophozoites are aflagellate amoebae measuring up to 20 μm. The cells move slowly by hyaline lobopodia. The nucleus contains a single large nucleolus surrounded by globules, though these do not lie directly beneath the nuclear membrane as in *Entamoeba*. Cysts are often irregularly shaped and possess a single nucleus with an eccentric nucleolus. Typically, a large glycogen granule is present in the mature cyst. Four *Iodamoeba* species have been described from lower intestines of mammals (e.g., *I. buetschlii* from humans) and reptiles (*I. testudinis*).

Type Species *Iodamoeba buetschlii* (Prowazek 1912) Dobell, 1919 (basionym *Entamoeba buetschlii* Prowazek 1912).

Remarks Because it is aflagellate, *Iodamoeba* was traditionally considered a member of Entamoebidae. On the basis of recent molecular phylogenetic work (Stensvold et al. 2012; Ptáčková et al. 2013), it was recently removed from Entamoebidae and transferred to Mastigamoebidae together with *Endolimax* (Ptáčková et al. 2013).

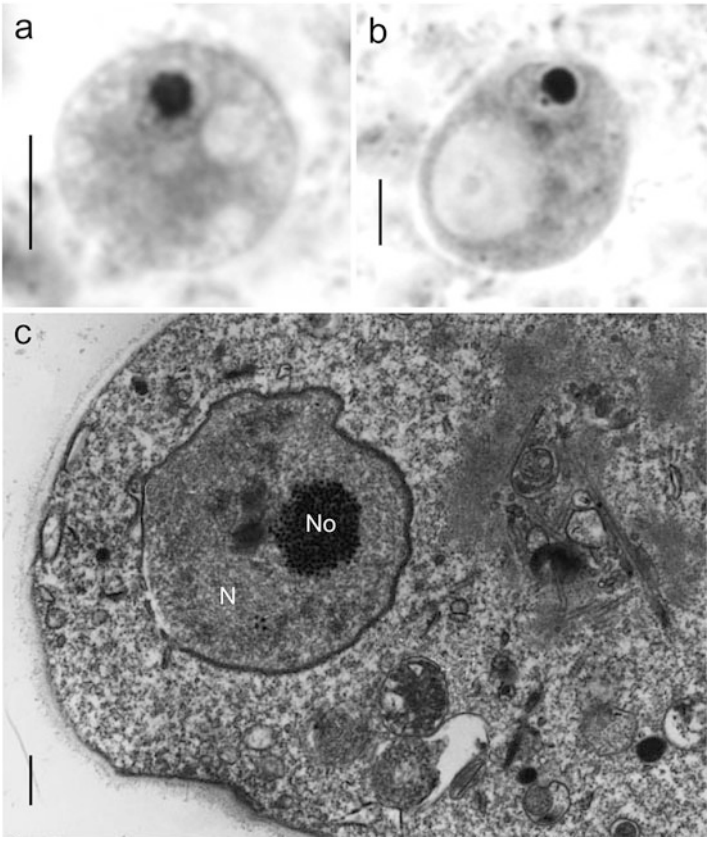

Fig. 5 ***Iodamoeba*** (**a**) Trophozoite of *Iodamoeba buetschlii* showing nucleus with central nucleolus and granules of chromatin; prepared slide, stained with iron-hematoxylin. (**b**) Cyst of *I. buetschlii* with a single vacuole; prepared slide, stained with iron-hematoxylin. (**c**) Transmission electron micrograph of cyst, showing the structure of nucleolar material (*No*) and chromatin in the nucleus (*N*). Scale bar in a = 5 μm, in b = 10 μm, in c = 1 μm (Micrograph in **c** reprinted from *Parasitology Research* 84, Zaman et al., *Ultrastructure of the Iodamoeba bütschlii cyst,* pp. 421–422, Copyright (1998), with permission from Springer)

Family Rhizomastixidae Ptáčková et al. 2013
Amoeboflagellate Archamoebae. Trophozoites with single anterior flagellum. Microtubular cone modified into the "rhizostyle." Amoeboid movement slow. See below and Ptáčková et al. (2013), for discussion of the *nomen nudum* Rhizomastigidae.

Rhizomastix Alexeieff, 1911 (Fig. 6)
Rhizomastix contains amoeboid uniflagellate or biflagellate species whose microtubular cone has been modified into a tube that extends through the cell. Most species are endobiotic, but two (and possibly a third) free-living species have been described as well.

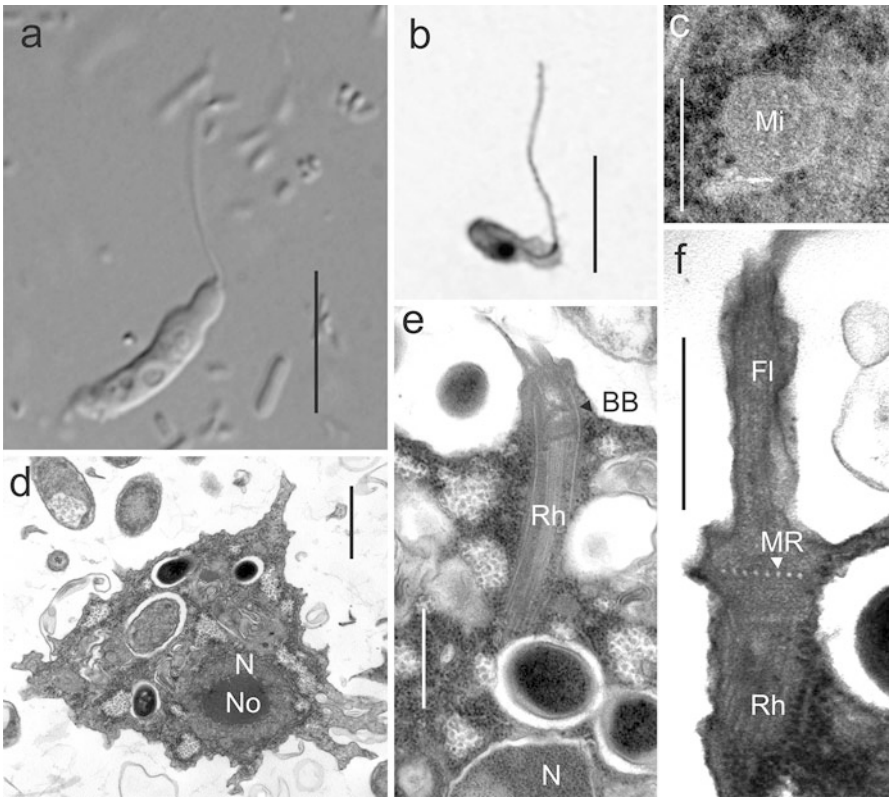

Fig. 6 ***Rhizomastix***. (**a**–**f**) *Rhizomastix libera*. (**a**) Light micrograph of flagellate showing central, rounded nucleus and nucleolus and posterior food vacuoles (DIC). (**b**) Protargol-stained cell showing the rhizostyle connecting the anterior flagellum to the central nucleus. (**c**–**f**) Transmission electron microscopy. (**c**) Mitochondrion-related organelle (*Mi*) with double membrane. (**d**) Whole cell, showing large nucleus (*N*) and nucleolus (*No*) and food vacuoles. (**e**, **f**) Details of the flagellar apparatus showing the flagellum (*Fl*), basal body (BB) with proximally extending microtubular rhizostyle (*Rh*), and laterally extending microtubular root (*MR*). *N* nucleus. Scale bar in a, b = 10 μm, in c, f = 200 nm, in d = 1 μm, and in e = 500 nm (Figures **c**, **d**, **e**, and **f** reprinted from *Protist* 164, E. Ptáčková et al., *Evolution of Archamoebae: Morphological and Molecular Evidence for Pelobionts Including Rhizomastix*, *Entamoeba*, *Iodamoeba*, *and Endolimax*, Pp. 380–410, Copyright (2013), with permission from Elsevier)

Circumscription Archamoebae with a uniflagellate (biflagellate in *R. biflagellata*) trophic stage. A single-layered cylindrical bundle of microtubules, the "rhizostyle," extends from the bottom edge of the basal body through the cell. A second microtubular element, arising from the base of the basal body and running parallel to the rhizostyle, may also be present. A single-layered root of microtubules arises from the side of the basal body (Fig. 2f). Fine structure of the basal body is unknown; a cylinder is present in the transition zone of the flagellum. The flagellum has a conventional eukaryotic "9 + 2" arrangement of microtubules, but it is unclear

whether it lacks outer dynein arms. Flagellar movement is relatively fast in comparison with *Mastigamoeba*. The nucleus is similar to that of *Entamoeba*, having a single central (large) nucleolus and peripheral heterochromatin granules at least in some species. The flagellates may transform to uninucleate amoebae. Binucleate cysts have been reported in some species. Sixteen *Rhizomastix* species have been described. Most of them are intestinal commensals of vertebrates and insects (e.g., *R. gracilis*, *R. biflagellata*, and *R. bicoronata*), three are described as free-living (*R. libera*, *R. borealis*, *R. varia*); and some are of uncertain status (*R. elongata*) (Ptáčková et al. 2013; Zadrobílková et al. 2016).

Type Species *Rhizomastix gracilis* Alexeieff, 1911.

Remarks The genus *Rhizomastix* was recently transferred into the Archamoebae and a new family Rhizomastixidae established to accommodate it (Ptáčková et al. 2013). *Rhizomastix* was classified with *Mastigamoeba* and *Mastigella* by Kudo (1939, 1977); Čepička (2011) suggested it might be related to pelobionts; its position was clarified on the basis of molecular and ultrastructural data (Ptáčková et al. 2013; Zadrobílková et al. 2016). The name Rhizomastigidae has historically been used for today's Mastigamoebidae (e.g., Bütschli 1880, 1884; Calkins 1901; Lepşi 1965; Reichenow 1952). The name was created by Bütschli (1884) as Rhizomastigina and later standardized to Rhizomastigidae by Calkins (1901); however, as it was not based on and often did not include *Rhizomastix*, Rhizomastigidae is regarded by some as a *nomen nudum* (Loeblich and Tappan 1961). The composition of Rhizomastigidae has typically been very confused (e.g., Cavalier-Smith and Scoble 2013), leading Ptáčková et al. (2013) to create Rhizomastixidae as the family containing *Rhizomastix*.

Suborder Pelomyxina Starobogatov, 1980
See Pánek et al. (2016) for current composition. The clade containing *Pelomyxa* and *Mastigella*; using the branch-based definition introduced and discussed in Pánek et al. (2016), the clade containing *Pelomyxa palustris* Greeff, 1874, and all organisms sharing a more recent common ancestor with *P. palustris* than with *Mastigamoeba balamuthi* (Chávez et al. 1986) Simpson et al. 1997.

Family Pelomyxidae Schulze, 1877
Anaerobic or microaerophilic flagellated amoebae with slow-beating monokinetid or immobile polykinetids. See Zadrobílková et al. 2015 for current composition.

Pelomyxa Greeff, 1874 (Fig. 7)
Pelomyxa was originally described as a large multinucleate amoeba, with a division of the cytoplasm into an inner layer containing organelles displaying fountain-flow movement and a clear hyaline outer layer from which pseudopodia can "roll" out and with a posterior uroid attaching the amoeba to the substrate. Later reports extended the description to refer to prokaryotes that coexist endosymbiotically in the cell (van Bruggen et al. 1988) and to non-motile flagella (Frolov 2011; Griffin 1979, 1988; Seravin and Goodkov 1987a).

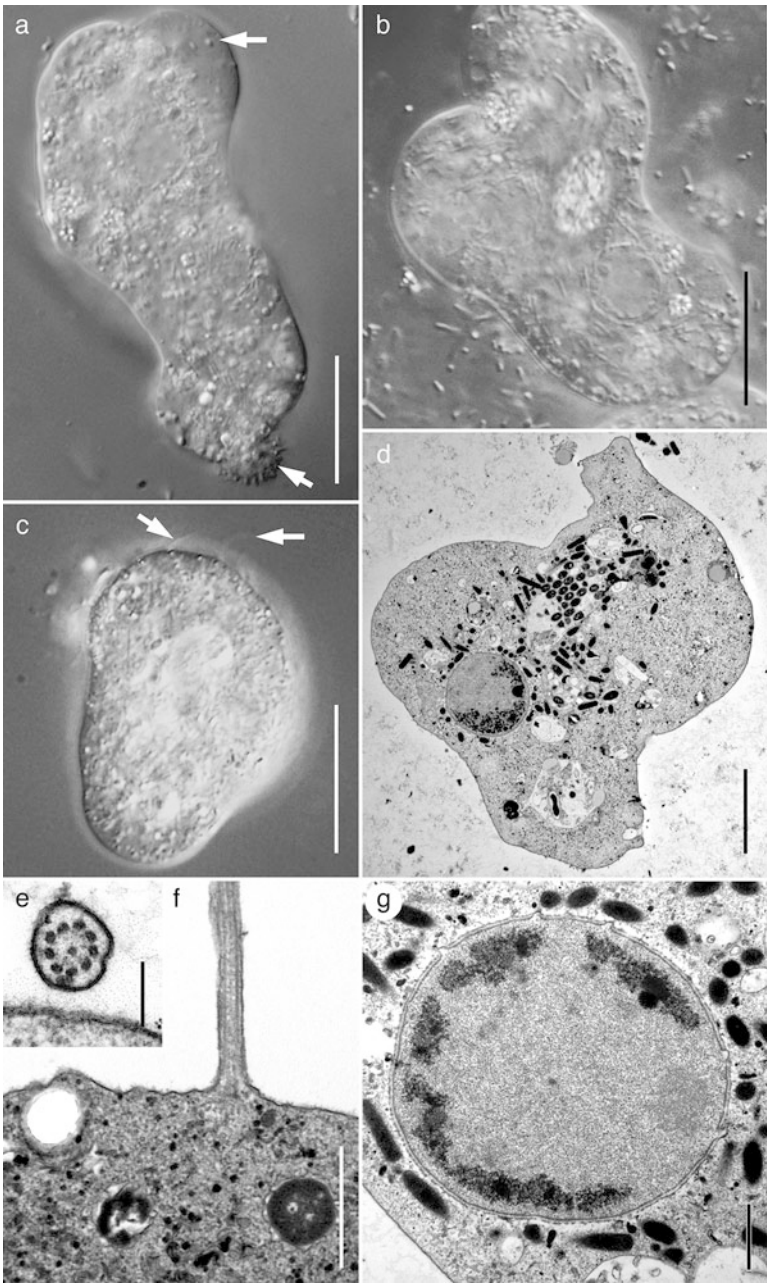

Fig. 7 ***Pelomyxa***. (**a**–**g**) *Pelomyxa schiedti*, strain SKADARSKE in (**a**, **b**), strain TIWI in (**c**), strain WACT07 in (**d**–**g**). (**a**–**c**) Differential interference contrast showing cells filled with granules and endosymbionts, distinctive nuclear structure (in **b**), leading pseudopodium (in **a**, *arrow upper right*), a bulbous, villous uroid-like area (in **a**, *arrow lower right*), and multiple immobile poorly

Circumscription *Pelomyxa* is distinguished by the form most usually encountered: a large multinucleate amoeba, with many inactive flagella. It includes polymorphic species, with a large multinucleate amoeboid feeding form up to 5000 μm long, with poorly motile or non-motile flagella that insert into a clear "hyaline" outer cytoplasmic layer, which encloses an inner layer filled with organelles, with endosymbiotic bacteria, and sometimes with refringent cytoplasmic granules (sand); movement is directed by fountain-flow cytoplasm and an anterior monopodium. The posterior uroid is active in food uptake. Cysts about 100 μm in diameter are known from at least one species. *Pelomyxa* species can be divided into two groups on the basis of the organization of the flagellar apparatus: the first group is characterized by a long basal body and very numerous radiating microtubules, while the second is characterized by a short basal body associated with a very reduced number of radiating microtubules. There is at least one intermediate state between these groups (Chystjakova et al. 2014). Cells have many flagella, each arising from a single basal body; each basal body gives rise proximally to a cone of microtubules, which has not been shown to extend to the nucleus (though several species do have microtubules surrounding the nucleus). The transition zone of each flagellum contains a transitional column. Flagella are with or (in most cases) without a 9 + 2 arrangement of microtubules in flagellar axoneme; the pattern of microtubules may be unstable in individuals in some species, particularly in the central pair or group of axonemal microtubules. The flagella insert into the hyaline outer cytoplasmic layer, which is devoid of organelles (ER, nuclei, vacuoles). Nuclei show distinctive, diagnostic patterns of chromatin condensation and may be surrounded by endosymbiotic bacteria. Morphological and molecular data suggest that *Pelomyxa* may have evolved from *Mastigella* by nuclear and flagellar multiplication (Zadrobílková et al. 2015).

Type Species *Pelomyxa palustris* Greeff, 1874.

Remarks *Pelomyxa* has been widely reported (e.g., Greeff 1874; Griffin 1988; Grüber 1885; Schirch 1914; Schulze 1875a; Whatley and Chapman-Andresen 1990; Frolov 2011). *Pelomyxa palustris* is currently relatively poorly known, and descriptions of it (e.g., Griffin 1988; Whatley and Chapman-Andresen 1990) probably encompass the variation of multiple species (Frolov et al. 2004; Goodkov et al. 2004). On the other hand, very few of the light microscopy-based species

Fig. 7 (continued) visible flagella (in **c**). (**d**–**g**) Transmission electron microscopy; (**d**) section through the cell showing the amoeboid body, single nucleus, and endosymbionts. (**e**) Transverse section of the flagellum with aberrant arrangement of microtubules. (**f**) Longitudinal section of the flagellar apparatus. (**g**) Detail of the nucleus, showing peripheral chromatin and small nucleolus. Scale bars in a–c = 20 μm; d = 5 μm; e = 200 nm; f and g = 1 μm (Figures **a**–**g** reprinted from *Protist* 164, E. Ptáčková et al., *Evolution of Archamoebae: Morphological and Molecular Evidence for Pelobionts Including Rhizomastix*, *Entamoeba*, *Iodamoeba*, *and Endolimax*, pp. 380–410, Copyright (2013), with permission from Elsevier)

descriptions currently available can be differentiated from the life cycle described by Whatley and Chapman-Andresen (1990). All the previous accounts of species probably describe real variation; however it is not currently possible to assign most of this variation to different species in any consistent or clear way, on the basis of the published descriptions. Species are currently distinguished mainly on characteristic patterns of nuclear chromatin, the thickness of the outer cytoplasmic layer, and the flagellar apparatus (Berdieva et al. 2015; Chystjakova and Frolov 2011; Chystjakova et al. 2014; Frolov 2011; Frolov et al. 2005a, b, 2006; Griffin 1979, 1988; Seravin and Goodkov 1987a; Ptáčková et al. 2013; Zadrobílková et al. 2015).

Mastigella Frenzel, 1897 (Fig. 8)
Mastigella contains amoeboid cells with a flagellated basal body with a cone of microtubules, but there is no connection between the cone and the nucleus. Endosymbiotic prokaryotes are present in several species.

Circumscription Archamoebae with a uniflagellated trophic stage, in which the nucleus is not connected to a cone of microtubules that arises from the base and sides of the single (flagellated) basal body; a cylinder is present in the transition zone of the flagellum. A single root of microtubules arises from the side of the basal body, and the root has a bilaminar sheet on its anterior edge. Basal bodies have nine triplets of microtubules. The flagellum has a conventional eukaryotic "9 + 2" arrangement of microtubules but lacks outer dynein arms. The flagellum may contribute to cell movement, but its beating is typically slower and less effective than that seen in *Mastigamoeba*. The flagellates may, at least in some species, transform to amoebae with one, few, or many nuclei. The nuclei may contain a distinctive distribution of chromatin; endosymbiotic bacteria may be present; the outside of the cell may be covered with irregular spines. Both flagellate and amoeboid forms may transform into cysts. Cells are from 3 to 150 μm long with one to four flagella up to 150 μm long. Reported from anoxic or low-oxygen freshwater or marine sediments (Walker et al. 2001; Zadrobílková et al. 2015).

Type Species *Mastigella polymastix* Frenzel, 1897.

Remarks *Mastigella* was introduced to describe an amoeboid flagellate with multiple long flagella extended from the cell body on small "necks" that wandered over the cell body and were not attached to the nucleus (Frenzel 1897). Subsequently, the concept of the genus changed to one of amoeboid flagellates without a connection between the flagellum and nucleus (Goldschmidt 1907a, b; Lemmermann 1914). Following from Goldschmidt's (1907a, b) informal group "Mastigamöben," *Mastigamoeba* and *Mastigella* were grouped as the Mastigamoebidae by Chatton (Chatton 1925; Kudo 1939, 1977) and have traditionally been thought of as sister taxa. This was initially supported by phylogenies that included the GenBank sequence AF421219: it had been ascribed to *Mastigella commutans* but belongs to *Mastigamoeba punctachora* (Ptáčková et al. 2013), meaning that these phylogenies

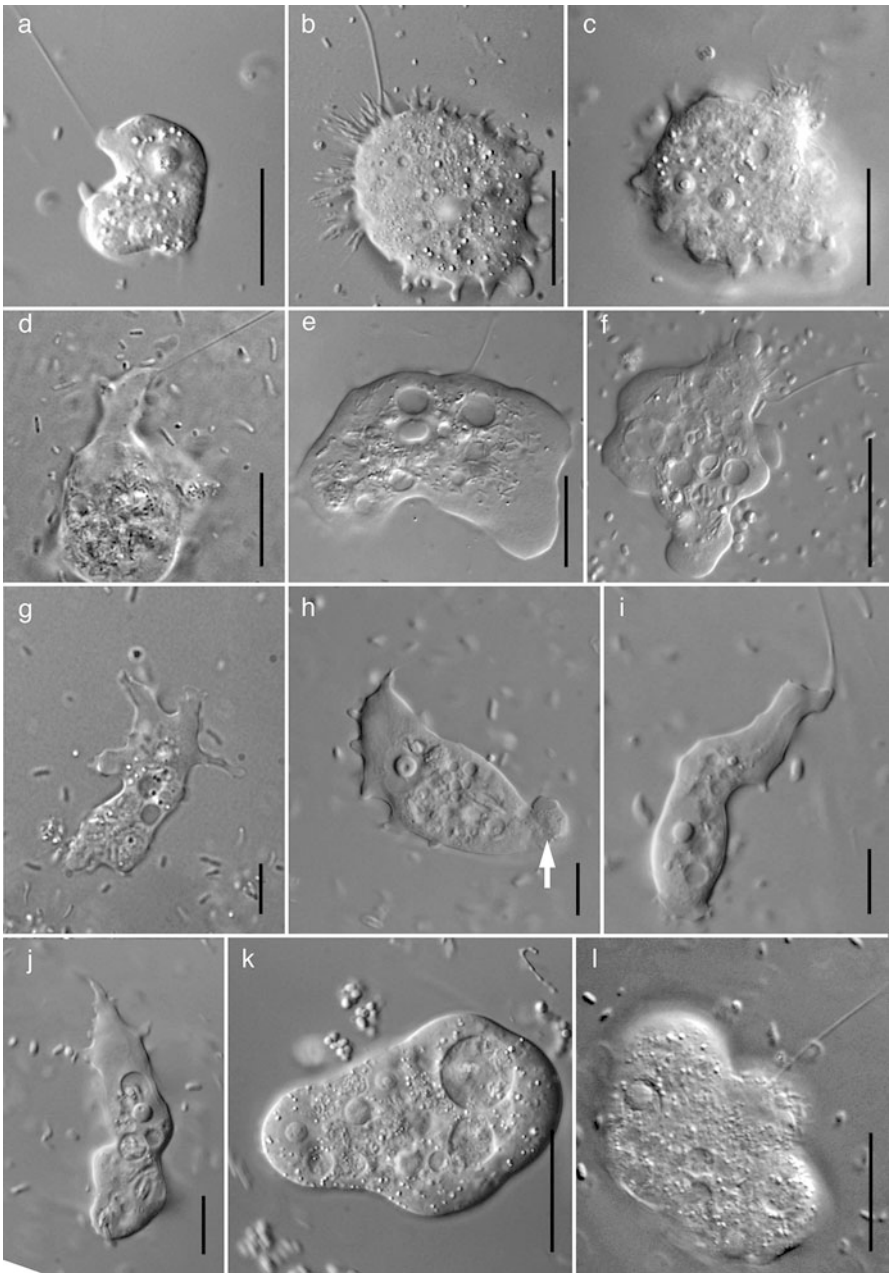

Fig. 8 ***Mastigella***. (**a**–**l**) Differential interference contrast. (**a**–**c**) *Mastigella erinacea* strain TOLEDO, showing binucleate cells with distinctive "fried-egg" nucleus and granular nucleolus, endosymbiotic bacteria, and highly variable villous, lobate or finger-shaped pseudopodia. (**d**, **e**) *Mastigella ineffigiata* strain OLB6AN, showing "shapeless" morphology and conspicuous

are silent on the placement of *Mastigella*. However, cell form and movement, nuclear structure, and the presence of endosymbiotic prokaryotes resembling methanogenic archaea (Frolov 2011; van Bruggen et al. 1985; Walker et al. 2001; Zadrobílková et al. 2015) all suggest a relationship of *Mastigella* with *Pelomyxa*. This hypothesis was supported by recent phylogenetic analyses where *Pelomyxa* forms an internal branch of *Mastigella*, while *Mastigamoeba* is phylogenetically more distant (Zadrobílková et al. 2015). Other nominal genera that fall within the circumscription of *Mastigella* include *Limulina* Frenzel, 1897; *Micromastix Frenzel*, 1897 (Frenzel 1897) and *Mastigamoebula Fantham*, 1919 (Fantham 1919).

Order Entamoebida Cavalier-Smith, 1993
Using the branch-based definition introduced by and discussed in Pánek et al. (2016), the clade consisting of *Entamoeba histolytica* Schaudinn, 1903 and all organisms that share a more recent common ancestor with *E. histolytica* than with *Mastigella eilhardi* Bürger, 1905.

Family Entamoebidae Chatton, 1925
Aflagellate Archamoebae. Flagellar apparatus completely reduced. Amoeboid movement typically monopodial and relatively fast. See Cavalier-Smith 1993 for current composition.

Entamoeba Casagrandi and Barbagallo, 1895 (Fig. 9)
Entamoeba contains most of the species of aflagellate Archamoebae. They are usually intestinal commensals of various animals, though at least three are likely to be free-living. A few species, including *E. histolytica* from humans, are pathogenic for their hosts.

Circumscription Trophozoites are aflagellate, uninucleate amoebae measuring up to 60 μm. The flagellar apparatus of *Entamoeba* is completely absent, and no cytoplasmic microtubules can be found in nondividing cells. Actively moving cells usually crawl using a single or several eruptive lobopodia; the hyaline lobopodia are clearly distinguishable from the granuloplasm. Uroidal filopodia have been rarely observed (Martínez-Palomo 1993). Nuclear structure is distinctive,

Fig. 8 (continued) endosymbionts. (**f**) *Mastigella rubiformis* strain HRAAN, showing cells with hyaline area, distinctive "*Pelomyxa*-like" nucleus, and prominent endosymbiotic bacteria. (**g**, **h**, **j**) *Mastigella eilhardi* strain ATCC 50342, showing pseudopodial variation; nucleus with "hollow," "donut-shaped" nucleolus; and endosymbiotic bacteria, with a posterior uroid (arrow). (**i**) *M. eilhardi* strain GO7 showing its characteristic swanlike long "neck" and posterior villous pseudopodia. (**k**) *Mastigella erinacea* strain KORISSION, showing binucleate or quadrinucleate cells with distinctive "fried-egg" nucleus with a granular nucleolus and villous pseudopodia from which the flagellum originates in some cells. Scale bars in a–f, i, k, l = 20 μm; g, h, j = 10 μm (Figures **a**–**l** reprinted from *Protist* 164, E. Ptáčková et al., *Evolution of Archamoebae: Morphological and Molecular Evidence for Pelobionts Including Rhizomastix*, *Entamoeba*, *Iodamoeba*, *and Endolimax*, pp. 380–410, Copyright (2013), with permission from Elsevier)

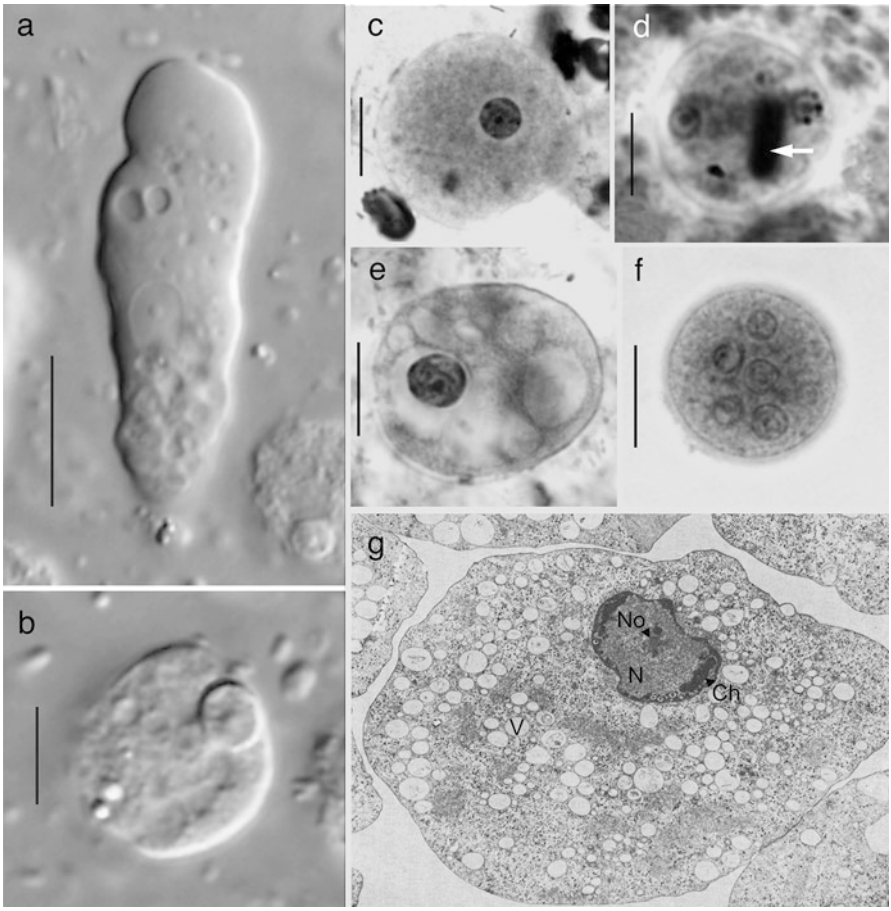

Fig. 9 ***Entamoeba***. (**a**) *Entamoeba moshkovskii* free-living trophozoite amoeba showing an anterior hyaline pseudopodium and central nucleus with the characteristic "dot" nucleolus (DIC). (**b**) *E. moshkovskii* resting cell, showing the nucleus with its characteristic "dot" nucleolus and peripheral chromatin (DIC). (**c**, **d**) Parasitic *Entamoeba histolytica* trophozoite (**c**) and immature cyst (**d**) with two visible nuclei and chromatoid body (*arrow*); prepared slide material, stained with iron-hematoxylin to show nuclei with a thick ring of peripheral chromatin and a small nucleolus. (**e**, **f**) Parasitic *Entamoeba coli* trophozoite (**e**) and cyst (**f**) with eight nuclei (five visible in the focal plane of the photograph); prepared slide material, stained with iron-hematoxylin to show nuclei with large chromatin granules. (**g**) Transmission electron micrograph of *E. histolytica*, showing numerous vacuoles (*V*) and the characteristic arrangement of nuclear chromatin (*Ch*) and nucleolus (*No*) in the nucleus (*N*). Scale bar in a, c, d, e, f = 10 μm; in b = 5 μm (Figure in **g** reprinted from *Parasitic Protozoa* Volume 3, 2nd Edition, J.P. Kreier & J.R. Baker, *Chapter 3: Parasitic amebas of the intestinal tract*., pp. 65–141, Copyright (1993), with permission from Elsevier)

with a single small, central nucleolus and conspicuous peripheral granules of heterochromatin (Fig. 9). The number and size of the granules differ between particular *Entamoeba* species. A cyst stage is documented for many species (see

above). The number of nuclei in the cyst is an evolutionarily important feature (Silberman et al. 1999). Cysts have been lost in *E. gingivalis*, which is found in the oral cavity of humans and transferred between hosts via saliva.

Many *Entamoeba* species have been described. The vast majority live as intestinal endobionts of various animals (e.g., *E. histolytica*, *E. dispar*, and *E. coli* from humans, *E. muris* and *E. cobayae* from rodents, *E. gallinarum* from birds, *E. invadens* from reptiles, *E. ranarum* from amphibians, *E. gadi* from fish, *E. aulastomi* from leeches, and *E. minchini* from insects). Some species are pathogenic, the best known being *E. histolytica*. Further information on endobiotic and parasitic species is given in the "Practical Importance" section.

Three *Entamoeba* species may be facultatively free-living. *Entamoeba moshkovskii* is relatively well known and has been repeatedly isolated from wastewaters, anoxic sediments, as well as from human stool (see Heredia et al. 2012; our observations). The second species, *E. ecuadoriensis*, has been isolated only once from sewage water (Clark and Diamond 1997). The third, *E. marina*, was isolated from marine tidal flats (Shiratori and Ishida 2016).

Type Species *Entamoeba coli* Grassi, 1879, Casagrandi and Barbagallo 1895 (basionym *Amoeba coli* Grassi 1879).

Remarks There was some uncertainty regarding the names of genera *Entamoeba* and *Endamoeba* during the first half of the twentieth century (see section "History of Genera in the Archamoebae"). *Entamoeba histolytica* can be, therefore, found under the name *Endamoeba histolytica* in the older literature. Since the 1950s, the concept of *Entamoeba* is stable, and *Endamoeba* is now considered a separate genus (e.g., Patterson et al. 2000). *Entamoeba* constitutes the family Entamoebidae. Historically, the other aflagellate Archamoebae, *Endolimax* and *Iodamoeba*, were classified within Entamoebidae as well but were recently removed from it (Cavalier-Smith et al. 2004; Ptáčková et al. 2013). *Endamoeba* is considered here as Archamoebae *incertae sedis*.

Archamoebae *Incertae Sedis*

Mastigina Frenzel, 1897 (Fig. 10)
Mastigina contains limax-shape amoeboid cells with a flagellum and no lateral pseudopodia and with a connection between the base of the flagellum and the rounded nucleus. Its microtubular ultrastructure has not been studied by electron microscopy.

Circumscription Archamoebae with a uniflagellated trophic stage, with limax amoeboid shape and no lateral pseudopodia, and in which the round nucleus is connected to the base of the flagellum, which has the distinctively languid flagellar beat typical of other pelobionts. One species, *Mastigina setosa*, has fountain-flow cytoplasmic movement (Goldschmidt 1907a). The outside of the cell is covered with

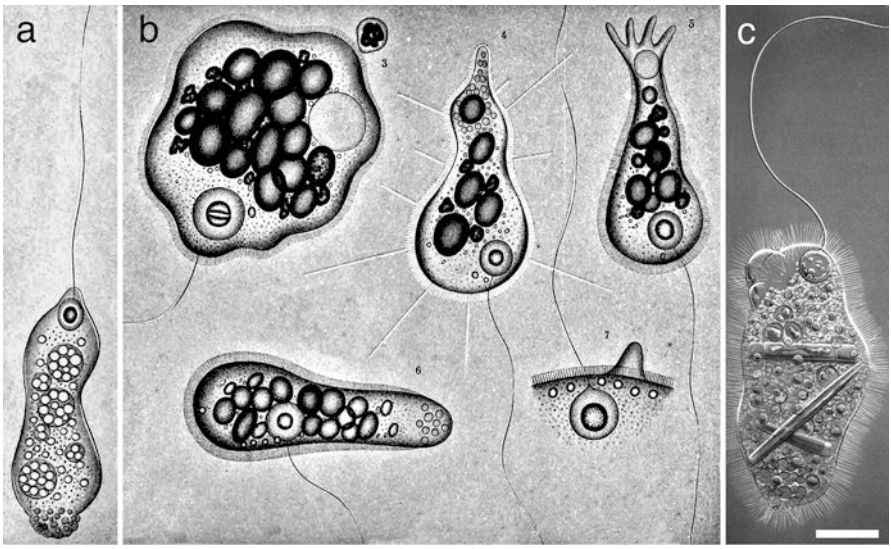

Fig. 10 ***Mastigina***. (**a**) Drawing of one of the two original species of *Mastigina*, *M. paramylon*, showing numerous starch granules inside the cell, connection between the long flagellum and the nucleus (with central vesicular nucleolus), and the lack of any lateral pseudopodia. (**b**) The type species of *Mastigina*, *M. chlamys*, showing a dense layer of short, refringent spines and in one case longer spines interspersed with the short ones, a connection between the long flagellum and the nucleus (with central nucleolus), and pseudopodia arising only at the anterior and posterior of the cell, not laterally. (**c**) *Mastigina setosa*, showing similar characteristics to *M. chlamys* but longer, less dense spines. Scale bar in a = 5 μm; in b, c = 20 μm (Drawings in **a** and **b** reprinted from *Untersuchungen über die mikroskopische Fauna Argentiniens*. *Erster Teil: Die Protozoen*. *I und II*, *Abteilung: die Rhizopoden und Helioamoeben*, J. Frenzel, (1897), Erwin Nägele, Stuttgart; with no known copyright restrictions. Drawing in **c** reprinted from *Archiv für Protistenkunde* Supplement 1, R. Goldschmidt, *Lebensgeschichte der Mastigamöben Mastigella vitrea* n.sp. *u*. *Mastigina setosa* n.sp., pp. 83–165, Copyright (1907), with permission from Elsevier)

closely packed spines in two species, *Mastigina chlamys* and *M. setosa* (Frenzel 1897; Goldschmidt 1907a; Skibbe and Zölffel 1991). Reported from anoxic or low-oxygen freshwater sediments.

Type Species *Mastigina chlamys* Frenzel, 1897.

Remarks *Mastigina* was not clearly distinguished from other pelobiont genera when it was introduced, in the original descriptions of *M. chlamys* and *M. paramylon* (Frenzel 1897). Goldschmidt (1907a) defined the genus on the basis of limax body shape with no lateral pseudopodia, round apical nucleus connected closely to the base of the flagellum (as opposed to elongated or drop-shaped, sometimes appearing removed from the base of the flagellum, in *Mastigamoeba*), characters that are adopted here. Goldschmidt (1907a) also regarded *Tricholimax hylae* as belonging to *Mastigina*, leading to later confusion about the characters

displayed by *Mastigina* species: he used fountain-flow cytoplasmic movement as a defining feature of *Mastigina*, despite it being present only in *M. setosa* and *T. hylae* and despite the two species in the original description of *Mastigina* not displaying it. Ultrastructural studies of *Tricholimax hylae* have been carried out under the name *Mastigina hylae* (Brugerolle 1982, 1991), and summaries of the genus have subsequently relied largely on descriptions of *T. hylae* (Griffin 1988; Brugerolle and Patterson 2000).

Perhaps on the basis that fountain-flow cytoplasm is also present in *Mastigamoeba aspera* (Chystjakova et al. 2012; Schulze 1875b), Lemmermann (1914) in turn regarded *Mastigina* as a junior synonym of *Mastigamoeba*, leading to further confusion about the distribution of fountain-flow cytoplasm and spines in pelobionts. Frolov (2011) redefined *Mastigina* as containing only taxa with spines on the surface, disregarding the criteria used by Frenzel (1897) and Goldschmidt (1907a). Although 50% of the species currently assigned to *Mastigina* do have spines, 50% do not, and there are also members of *Mastigamoeba* and *Mastigella* with spines.

In the absence of clear ultrastructural and phylogenetic data on any of these species, we see no reason to reject Goldschmidt's circumscription of the genus.

Family Tricholimacidae Cavalier-Smith, 2013

Monotypic family with the characteristics of the sole species. Using the diagnosis of Cavalier-Smith (2013), uniciliate (i.e., uniflagellate) endosymbiotic anaerobes with inactive cilium (i.e., flagellum) with numerous irregularly arranged doublets; no obvious transition zone cylinder; dense microtubule nucleating center attached to the proximal side of the single centriole (with doublets not triplets) nucleates (a) multilayered cone of microtubules that surround nucleus and (b) lateral rhizostyle, a broad band of microtubules with numerous underlying singlet and bundled microtubules.

Tricholimax Frenzel, 1897 (Fig. 11)

This monotypic genus has been described from the guts of amphibians. Cells have a limax shape with fountain-flow cytoplasmic movement and without lateral pseudopodia, and the anterior nucleus is connected to the short non-motile flagellum, which lacks normal 9 + 2 organization of the microtubular axoneme.

Circumscription The one species, *Tricholimax hylae*, exists mostly as flagellates measuring 50–135 μm. Flagellated amoeboid and cyst stages (up to 30 μm in diameter with up to four nuclei) may form. Flagellated cells have one to six nuclei, one of which is connected to the emergent flagellum. In the flagellar apparatus, there is a cone of microtubules arising from base and sides of the single basal body; these enclose the nucleus and connect to the nuclear envelope by microfibrils. A single root of 32 microtubules arises from the side of basal body, and there is a bilaminar sheet associated with the anterior edge. There is an "n + n" arrangement of microtubules in the flagellar axoneme, though no dynein arms are visible. There may be

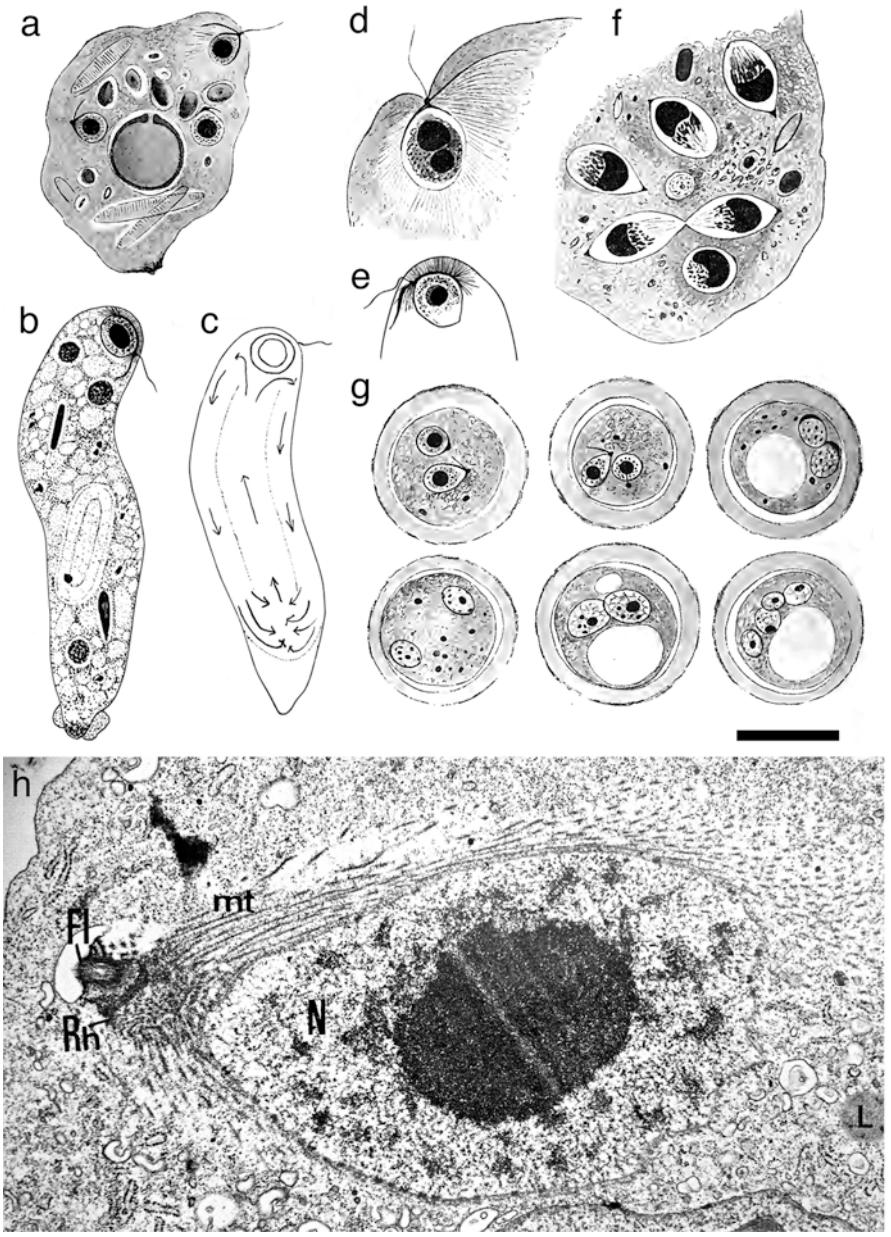

Fig. 11 ***Tricholimax hylae***. (**a**–**b**) Drawings of whole cells showing the microtubular connection between the short, nonfunctional flagellum and the large apical nucleus with its central nucleolus. Accessory nuclei, with attached kinetids, are visible in the cytoplasm in **a**, along with numerous food vacuoles. (**c**) Diagram showing the direction of fountain-flow cytoplasmic streaming, where the posterior uroid is not included. (**d**, **e**) The microtubular cone and lateral root (which are clearly visible under the light microscope, even without DIC optics). There is some variation in the

reduced internal basal bodies with cones of microtubules attached to extra internal nuclei (Brugerolle 1982, 1991).

Type Species *Tricholimax hylae* Frenzel, 1897.

Remarks This genus can be distinguished from *Mastigina* because of the atypical organization of the single non-motile flagellum. It has been widely described (Frenzel 1897; Goldschmidt 1907b; Collin 1913; Lemmermann 1914; Becker 1928; Sassuchin 1928; Ivanic 1936; Chatton 1953; Brugerolle 1982, 1991; Griffin 1988). The occurrence of the "n + n" flagellar organization and fountain-flow cytoplasm both here and in *Pelomyxa* has resulted in the placement of both genera in the Pelomyxidae, though this circumscription is now widened to also include *Mastigella*. No molecular phylogenetic information exists for *Tricholimax*, so its formal placement in the Pelomyxidae remains premature.

Endamoeba Leidy, 1879 (Fig. 12)
Poorly known genus containing aflagellate Archamoebae found in insects.

Circumscription Trophozoites measure up to 120 μm. They are aflagellate and move slowly by one or several lobopodia. Unlike in *Entamoeba*, there is no marked distinction between hyaloplasm and granuloplasm (Wenyon 1926). The nucleus of *Endamoeba* has a typical structure dissimilar from that of *Entamoeba* and other aflagellate Archamoebae (Wenyon 1926; compare Fig. 12 with Figs. 4, 5, and 9). Several species have been described from the hindgut of insects, such as *Endamoeba blattae* from cockroaches and *E. disparata* from termites.

Type Species *Endamoeba blattae* Bütschli, 1878 Leidy 1879 (basionym *Amoeba blattae* Bütschli, 1878).

Remarks Probable member of Entamoebidae. This classification is, however, tentative, since no sequence data from *Endamoeba* are currently available.

Fig. 11 (continued) presence of a single or a double nucleolus. (**f**) Nuclei dividing, showing a connection between the posterior poles of the nuclei, with anterior kinetids present. (**g**) Six cyst stages, showing variation in the number of nuclei from two to four, and gradual development of a central vacuole. (**h**) Transmission electron micrograph showing the flagellum (*Fl*), single basal body with microtubular root (*Rh*), and dense cone of microtubules (*mt*) connecting to the nucleus (*N*). Scale bar: in a, b, c = 30 μm, in d, e, f = 10 μm, in g = 20 μm, in h = 3 μm (Drawings in **a**, **d**, **f**, and **g** reprinted from *Archives de Zoologie expérimentale et générale* 51, B. Collin, Sur un ensemble de protistes parasites des batraciens (Note préliminaire). Pp. 59–76 (1913) with no known copyright restrictions. Drawings in **b**, **c**, **e** reprinted from *Journal of Parasitology* 11, E. R. Becker, The morphology of *Mastigina hylae* (Frenzel) from the intestine of the tadpole. Pp. 213–216, Copyright (1925) with permission from Allen Press. Micrograph in **h** reprinted, from *Protistologica* 18, G. Brugerolle, *Caractères ultrastructuraux d'une mastigamibe: Mastigina hylae (Frenzel)*, pp. 227–235, Copyright (1982), with permission from Elsevier)

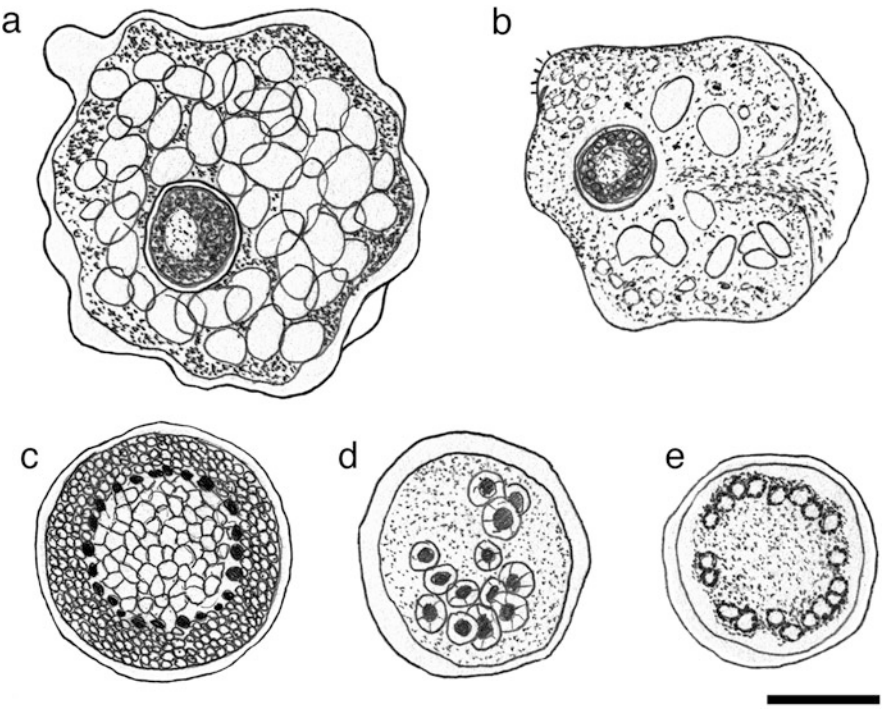

Fig. 12 ***Endamoeba*** **(a–e):** *Endamoeba blattae*, redrawn from Kudo (1960). (**a**, **b**) Trophozoites, showing the distinctive arrangement of chromatin in the nucleus and eruptive pseudopodia. Drawn from live material. (**c**) Nucleus, showing the distinctive ring of chromatin. (**d**) Multinucleate cyst, as seen when stained with iron-hematoxylin, showing changed nuclear structure, relative to (**e**) Cyst, showing distinctive ring of chromatin in the nuclei. Scale bar in a, b = 10 μm, in c = 4 μm, in d, e = 5 μm. Pictures redrawn from Kudo (1960)

Maintenance and Cultivation

Various free-living Archamoebae from the genera *Mastigamoeba*, *Mastigella*, and *Rhizomastix* can be isolated and maintained in xenic cultures with bacteria. Sonneborn's *Paramecium* medium (cereal grass infusion medium, ATCC medium 802; see www.atcc.org) is suitable for freshwater strains (Ptáčková et al. 2013). Seawater 802 medium (ATCC medium 1525; see www.atcc.org) can be used for the cultivation of marine pelobionts (Zadrobílková et al. 2015). The strains grow well at room temperature in sealed 15 ml tubes with 10 ml of medium and transfers every 1–3 weeks or in sealed tissue flasks filled with medium and transfers every 1–2 months. Although the Archamoebae are anaerobic and die when exposed to oxygen for a longer period, no special precautions are necessary, since bacteria present in the culture quickly consume the oxygen. Most strains grow well also under anoxic conditions.

Members of the genera *Entamoeba* (including the free-living *Entamoeba moshkovskii*) and *Endolimax* can be isolated and maintained in xenic cultures as well. Various media developed for cultivation of intestinal flagellates and amoebae, both biphasic (e.g., LE medium, Robinson's biphasic medium) and monophasic (TYSGM-9 medium, Robinson's monophasic medium) can be used (see Clark and Diamond 2002). Culture conditions depend on the origin of the organisms – strains from invertebrates and poikilotherm vertebrates are cultivated at room temperature with transfers once a week to once a month; strains from homoiotherms should be cultivated at 37 °C with transfers approximately twice a week.

Only a few species of *Entamoeba* and *Mastigamoeba balamuthi* have been successfully axenized. The process of isolation and axenization of *Entamoeba histolytica* is described in Clark and Diamond (2002). For maintenance, several complex media, such as TYI-S-33, YI-S, and LYI-S-2 (ATCC medium 2154), can be used (for preparation, see Clark and Diamond 2002 and www.atcc.org). Apart from *Entamoeba* spp., only a single strain of *Mastigamoeba balamuthi* has been axenized (Chávez et al. 1986). Chávez et al. (1986) described the process of the axenization in a detail. *Mastigamoeba balamuthi* can be maintained in PYGC (Chávez et al. 1986) or LYI-S-2 (see above) media.

So far, only one successful long-term cultivation attempt of *Pelomyxa* spp. has been published (Zadrobílková et al. 2015). Cells of *Pelomyxa* spp. can be maintained alive for several months in hermetically closed flasks filled with fresh water and sediments, or with Lozina-Lozinskii medium supplemented with boiled detritus (see Chystjakova and Frolov 2011; Frolov et al. 2011).

Cultures of Archamoebae can be cryopreserved in the presence of 5% dimethyl sulfoxide using a standard procedure and stored in liquid nitrogen. Detailed protocols for cryopreservation of *Entamoeba* cultures are described in Diamond (1995) and Samarawickrema et al. (2001).

Evolutionary History

Phylogeny and Evolution

Separately and together, members of the Archamoebae have been considered to be some of the most deep-branching eukaryotes, on the basis of lacking mitochondria and Golgi dictyosomes, and having a "simple" or nonexistent flagellar apparatus. Using the argument that some or all of these traits are primitive, it was suggested that the pelobionts represent a very early stage in the evolution of eukaryotes (Brugerolle 1993; Cavalier-Smith 1983; Griffin 1979, 1988; Margulis 1970; Patterson 1994; Patterson and Sogin 1992; Whatley 1976; Whatley and Chapman-Andresen 1990). This evolutionary significance was in the context of an evolutionary paradigm known as the Archezoa hypothesis (Cavalier-Smith 1983), where "amitochondriate" protists represented basal eukaryotic lineages that had diverged before the acquisition of the mitochondrial endosymbiont and other key eukaryotic innovations (e.g., introns, Golgi dictyosomes, peroxisomes).

The Archezoa hypothesis was supported by molecular phylogenetic trees of eukaryotes consisting of a "crown" (animals, plants, fungi, and many algae) and a ladderlike sequential divergence of amitochondriate parasites from the base (Sogin 1991; Sogin and Silberman 1998).

In the late 1990s, developments in phylogenetic methods and the identification of mitochondriate remnant organelles led to the rejection of the Archezoa hypothesis and the crown/base view of eukaryotic relationships (Roger 1999). The development of evolutionary models incorporating heterogeneity among taxa (of evolutionary rate or of base composition) led to the realization that systematic phylogenetic error, such as "long branch attraction," had had a major effect on the positioning of "archezoan" organisms in the tree (Dacks et al. 2002; Holder and Lewis 2003; Philippe and Germot 2000). The finding of gene sequences of

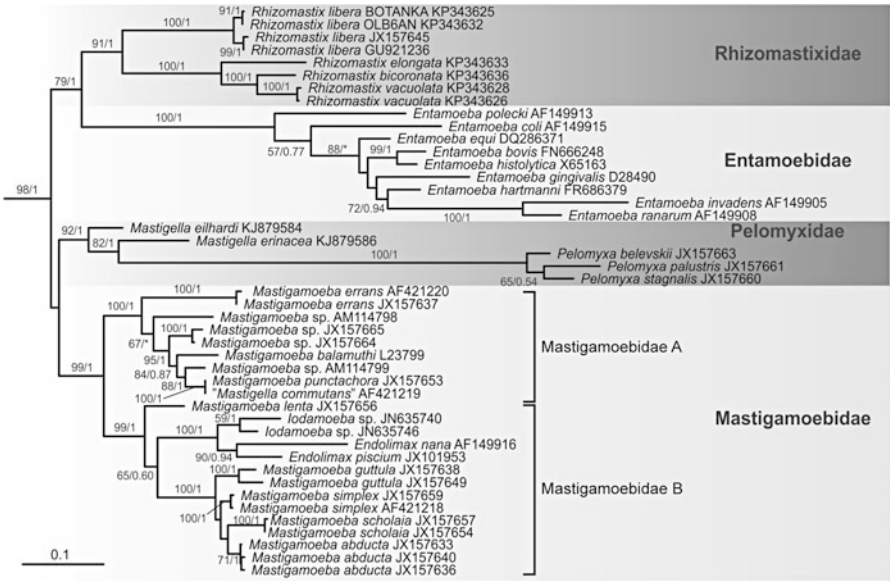

Fig. 13 Phylogenetic tree of Archamoebae based on SSU rRNA genes, showing generic relationships within families. *Mastigamoeba* is divided into two main clades, corresponding to "Mastigamoebidae A," with 100% ML bootstrap support and Bayesian posterior probability of 1, and "Mastigamoebidae B," with 99% ML bootstrap support and Bayesian posterior probability of 1. Note that the sequence labelled "*Mastigella commutans*" is thought to be from *Mastigamoeba punctachora* rather than *M. commutans* (see Zadrobílková et al. 2015). Support for relationships between families is not recovered from SSU rRNA data alone, requiring a multigene approach (Pánek et al. 2016). Sequences were aligned using the G-INS-I algorithm with default settings, on the MAFFT7 server (http://mafft.cbrc.jp/alignment/server/). The resulting alignment was manually edited to remove ambiguously aligned sites; the final dataset contained 1263 nucleotide positions. A maximum-likelihood phylogenetic tree was constructed in RAxML 7.2.3 (Stamatakis 2006) using the GTRGAMMAI model; the values at the branches represent statistical support in maximum likelihood (RAxML, 1000 pseudoreplicates)/Bayesian posterior probability (MrBayes, GTR + Γ + I + covarion model). Support values below 50/0.90 are not shown or are represented by an asterisk (*)

mitochondrial origin (*cpn60*, *HSP70*, *IscU*) and the localization of these gene products (by immuno-microscopy) to double-membrane-bounded organelles (Bui et al. 1996; Clark and Roger 1995; Roger et al. 1996, 1998; Tovar et al. 2003) have led to the identification of mitochondrion-derived organelles (i.e., hydrogenosomes and mitosomes) in almost all of the proposed "amitochondriate" organisms (van der Giezen 2009).

The identification of mitochondrial-remnant organelles (discussed above) and comparisons of ribosomal DNA sequences (Cavalier-Smith 1997; Hinkle et al. 1994; Milyutina et al. 2001; Morin and Mignot 1995; Silberman et al. 1999; Stiller and Hall 1999) and other genes (Arisue et al. 2002; Bapteste et al. 2002; Hannaert et al. 2000) now suggest that the Archamoebae are derived within the mitochondriate taxon Amoebozoa and are not deep-branching eukaryotes (Cavalier-Smith 1997, 1998; Edgcomb et al. 2002; Simpson and Roger 2004).

As discussed above, systematics within the Archamoebae is currently undergoing considerable flux, while many gene and protein sequences are obtained for many more representatives of the group. So far this has clarified that endobiotic taxa are distributed among the free-living taxa, showing that parasitism has clearly evolved multiple times in the Archamoebae (Pánek et al. 2016; Ptáčková et al. 2013; Stensvold et al. 2012; Zadrobílková et al. 2015, 2016). Obtaining multiple sequences from a single taxon can improve the robustness of placement of particular taxa, both at species level and at higher levels – for example the placement of *Pelomyxa* has long been unstable because the original sequence obtained was highly divergent (Milyutina et al. 2001; Ptáčková et al. 2013). Further sequences from *Mastigella*, *Tricholimax*, and *Mastigina* will be key to resolving relationships among the genera of the Archamoebae; and sequences from *Mastigamoeba aspera* would permit certainty in the proposed split of *Mastigamoeba* discussed above (Pánek et al. 2016) (Fig. 13).

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Myxomycetes

38

Steven L. Stephenson and Martin Schnittler

Abstract

The myxomycetes (class Myxogastria), also commonly known as plasmodial slime molds or acellular slime molds, are the most species-rich group within the Amoebozoa, with approximately 1,000 morphologically recognizable species having been described. These organisms are free-living predators of bacteria and other eukaryotic protists. Myxomycetes have been recorded from every terrestrial habitat investigated to date. The two trophic stages (amoeboflagellates and plasmodia) in the life cycle are usually cryptic, but the fruiting bodies are often large enough to be observed directly in nature. Fruiting bodies release airborne spores that are dispersed by air or, more rarely, animal vectors. Myxomycetes are associated with a wide variety of different microhabitats, the most important of which are coarse woody debris, ground litter, aerial litter, and the bark surface of living trees. Specimens can be obtained as fruiting bodies that have developed in the field under natural conditions or cultured in the laboratory. A substantial body of data on the worldwide biodiversity and distribution of myxomycetes has been assembled over the past 200 years, but there is a relative lack of molecular data, since myxomycetes are neither pathogenic nor of

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economic importance. However, recent studies have produced the first, albeit still incomplete, molecular phylogenies of the group. Moreover, there appears to be a much higher level of diversity on the molecular level than reflected in the number of morphospecies, with the latter often consisting of reproductively isolated populations which can be considered as biospecies.

Keywords

Amoebozoa • Biodiversity • Biospecies • Ecology • Introns • Plasmodial slime molds • Soil microbiology • Molecular phylogeny

Contents

Summary Classification

●**Myxogastria**
●●**Collumellidia**
●●●**Echinosteliales** (e.g., *Echinostelium*)
●●●**Physarales** (e.g., *Badhamia*, *Didymium*, and *Physarum*)
●●●**Stemonitales*** (e.g., *Meriderma* and *Stemonitis*)
●●**Lucisporidia**
●●●**Liceales*** (e.g., *Licea* and *Lycogala*)
●●●**Trichiales** (e.g., *Trichia* and *Hemitrichia*)

* Paraphyletic, based on molecular phylogenetic evidence; see Table 1 for comparison of traditional classification and groupings based on molecular phylogenetics.

Introduction

General Characteristics

One of the major branches of the eukaryotic tree of life consists of an assemblage of amoeboid protists referred to as the supergroup Amoebozoa, which are close relatives to the Opisthokonta (Holozoa and Holomycota) as indicated by Baldauf (2008) and Bapteste et al. (2002). Dictyostelid (cellular) and true (acellular) slime molds are part of the Amoebozoa (Pawlowski and Burki 2009) the myxomycetes (class Myxogastria) are one of the most diverse groups in the Amoebozoa. Myxomycetes (also known as plasmodial slime molds or myxogastrids) are a group of free-living terrestrial heterotrophs with complex life cycles. The unicellular forms are amoebae and flagellates (collectively, the "amoeboflagellate" stage). These develop, usually via sexual fusion, into a multinucleate "plasmodium" stage, which is also trophic. The plasmodium stage can produce fruiting bodies, which release airborne spores that are dispersed by air or, more rarely, animal vectors. The amoeboflagellates and plasmodia are usually cryptic, but the fruiting bodies are often large enough to be observed directly in nature. Myxomycetes have been recorded from every terrestrial habitat investigated to date. They are associated with a wide variety of different microhabitats, the most important of which are coarse woody debris, ground litter, aerial litter, and the bark surface of living trees. Specimens can be obtained as fruiting bodies that have developed in the field under natural conditions or cultured in the laboratory. A substantial body of data on the worldwide biodiversity and distribution of myxomycetes has been assembled over the past 200 years, but there is a relative lack of molecular data, since myxomycetes are neither pathogenic nor of economic importance.

Since their discovery, the myxomycetes have been variously classified as plants, animals, or fungi. Because they produce aerial spore-bearing structures that resemble those of certain fungi and typically occur in some of the same ecological situations as fungi, myxomycetes have traditionally been studied by mycologists (Martin and Alexopoulos 1969). Indeed, the name most closely associated with the group, first used by Link (1833) more than 175 years ago, is derived from the Greek words *myxa* (which means slime) and *mycetes* (referring to fungi). However, abundant molecular evidence now confirms that they are amoebozoans and not fungi (Yoon et al. 2008). Interestingly, the fact that myxomycetes are protists was first pointed out by de Bary (1864) more than a century and a half ago, and he proposed the name Mycetozoa (literally meaning "fungus animal") for the group. However, myxomycetes continued to be considered as fungi by most mycologists until the latter half of the twentieth century and are still governed by the Botanical Code of Nomenclature.

Other Similar Microorganisms

The myxomycetes are the most prominent representatives of a guild of sometimes unrelated nonpathogenic microorganisms that share a number of ecological features

(Schnittler et al. 2006). For this reason, some of these non-related forms can be confused with myxomycetes (see below). All of these organisms have a free-living, predatory lifestyle and a life cycle that begins with solitary amoeboid cells. The latter increase their biomass by aggregation of cells or by undergoing nuclear divisions without cell division (e.g., the plasmodia of myxomycetes) and convert this biomass into typically stalked fruiting bodies that can develop within hours or days. These fruiting bodies are produced not as a true growth process but by rearrangement of the available biomass, ultimately to release propagules for (potentially, at least) long-distance dispersal. The production of airborne propagules is the key innovation that enables these microorganisms to colonize terrestrial habitat islands with a locally higher density of microbes serving as prey (Schnittler and Tesmer 2008).

Myxomycetes are neither pathogenic nor of economic importance. Only a few model species, especially *Physarum polycephalum* and *Didymium iridis*, have been used to investigate cell division and developmental biology in myxomycetes (Hüttermann 1973) or the importance of mating type genes (Collins 1979) and the distribution of group I introns in these organisms (Wikmark et al. 2007; Feng and Schnittler 2015).

Other non-related members of this guild include the prokaryotic myxobacteria (a group consisting of perhaps 40–60 species), which produce fruiting bodies that in some species can reach a height of as much as 1 mm (Reichenbach 1993). Their spores are distinctly smaller than the smallest myxomycete spores, which usually fall within the range of (4–)7–12(–22) μm. Eukaryotic microorganisms with a similar lifestyle are the sorocarpic amoebae formerly known as the acrasid cellular slime molds or Acrasea (Olive 1975; Stephenson 2014). This is a group of approximately 20 species now known to be polyphyletic, containing aggregating, fruiting body-forming amoebae of different supergroups, with most not belonging to the Amoebozoa (Brown et al. 2009, 2010, 2012). Examples include the genera *Acrasis*, *Copromyxa*, *Guttulinopsis*, and *Fonticula* (Dykstra and Keller 2000; Brown et al. 2012). All these genera form fructifications by the aggregation of amoebae; *Acrasis* possesses a cellular stalk, whereas the others form sessile fruiting bodies. The ciliate genus *Sorogena* (Colpodea) produces stalked fruiting bodies strikingly similar to those found in myxomycetes, but the spores contain both a micro- and a macro-nucleolus (Bardele et al. 1991; Sugimoto and Endoh 2008).

Other Eumycetozoan Slime Molds

The eumycetozoans as defined by Olive (1975) include the Myxogastria (true or acellular slime molds, myxomycetes), the paraphyletic protosteloid amoebae (protostelids; see ▶ Protosteloid Amoebae (Protosteliida, Protosporangiida, Cavosteliida, Schizoplasmodiida, Fractoviteliida, and Sporocarpic Members of Vannellida, Centramoebida, and Pellitida)), and the Dictyostelia (dictyostelid cellular slime molds or dictyostelids; see ▶ Dictyostelia). There are approximately 160 species known for the Dictyostelia (Romeralo et al. 2011) and about 35–40 species for the protosteloid amoebae (Spiegel et al. 2004), whereas at least 1,000 morphologically recognizable species of myxomycetes have been described (Lado 2005–2016).

Fig. 1 Fruiting bodies of *Ceratiomyxa fruticulosa*, the most commonly encountered species *Ceratiomyxa*. What is recognized as *C. fruticulosa* is most likely a species complex, with one of the morphotypes producing exclusively cylindrical fruiting bodies as it can be observed in this image. These consist, in contrast to all other myxomycetes, of a slimy matrix and solitary spores which develop on tiny stalks, giving the surface of the fruiting body a fur-like appearance (Photograph by M. Schnittler)

As recognized by Olive (1975), both the Myxogastria and protosteloid amoebae are sporocarpic, with fruiting bodies ultimately derived from a single amoeboid cell. In contrast, the fruiting bodies in the Dictyostelia are derived from an aggregation of amoebae. Both the Myxogastria and Dictyostelia appear to represent monophyletic groups (Fiore-Donno et al. 2010a; Schaap et al. 2006), whereas the protosteloid amoebae are found in several lineages throughout the Amoebozoa, although apparently restricted to the Conosa (Shadwick et al. 2009; Adl et al. 2012).

In all but the most recent treatments of the myxomycetes, the four described species of the genus *Ceratiomyxa* were considered as part of the Myxogastria as the sole members of its own order, Ceratiomyxales (Fig. 1). However, these organisms differ by exogenous spore development (solitary spores are formed individually on stalks emerging from a joint matrix) from all other myxomycetes (in which spores develop inside a fruiting body surrounded, at least in the early stages, by a peridium). It has been suggested that they should be regarded as a sister group to the Myxogastria (Fiore-Donno et al. 2008, 2010a), and there are as well treatments which consider them with the protostelids (Olive 1970, 1975; Olive and Stoianovitch 1979; Adl et al. 2012), a group better referred to as the protosteloid amoebae (Shadwick et al. 2009). Chapter 36, ▶ Protosteloid Amoebae (Protosteliida, Protosporangiida, Cavosteliida, Schizoplasmodiida, Fractoviteliida, and Sporocarpic Members of Vannellida, Centramoebida, and Pellitida) assigns *Ceratiomyxa* to the taxon Protosporangiida (and does not employ the taxon Ceratiomyxales). Nevertheless, they are mentioned in this chapter because of their long history of study as myxomycetes. Other than *Ceratiomyxa*, all of the organisms assigned to the myxomycetes constitute a well-defined monophyletic group traditionally placed into five different taxonomic orders (Echinosteliales, Liceales, Trichiales, Stemonitales, and Physarales; Martin and Alexopoulos 1969).

Occurrence and Distribution

Myxomycetes can be detected directly in the field by fruiting bodies (about 60% of all known species). Over the past 200 years, a substantial body of data on their worldwide diversity and distribution has been assembled (Stephenson et al. 2008). More recent studies have incorporated the use of moist chamber cultures (Stephenson and Stempen 1994), and about 40% of all species of myxomycetes are known primarily or even exclusively from specimens appearing in moist chamber (or sometimes, agar) cultures (Schnittler et al. 2015). Checklists are available for a number of regions of the world, such as arctic and boreal zones (Stephenson et al. 2000), Africa (Ndiritu et al. 2009), and the Neotropics (Lado and Wrigley de Basanta 2008). This is quite unlike the situation that exists for most other protists, for which distributional data are often very limited. Based on recordable occurrence of fruiting bodies, methods of community ecology can be applied to study these organisms (e.g., Stephenson 1988; Stephenson et al. 1993; Schnittler 2001b; Rojas and Stephenson 2011) and have shown surprisingly narrow ecological niches for some species.

It seems certain that the trophic stages of myxomycetes, especially the amoebae, have a much wider distribution in nature than reflected by the occurrence of fruiting bodies. In fact, some species may have lost the ability to fruit altogether. For example, molecular phylogeny shows the free-living amoebae formerly treated as *Hyperamoeba* are instead several different lineages of myxomycetes (Fiore-Donno et al. 2010b). These have been recovered from artificial as well as natural aquatic environments, including the coelomic cavity of sea urchins (Karpov and Mylnikov 1997; Zaman et al. 1999). An RNA-based study (Urich et al. 2008) identified the amoebae of eumycetozoans as a key group of soil microbes. Studies that have used environmental PCR to investigate the presence of myxomycetes in alpine soils (Kamono et al. 2012; Clissmann et al. 2015; Fiore-Donno et al. 2016) recovered numerous sequences hitherto not known from fruiting bodies.

Due to their dormant stages (spores can survive for decades, microcysts and sclerotia for months to years), myxomycetes are capable of surviving under rather severe environmental conditions, even the extremely xeric conditions found in the Atacama Desert (Lado et al. 2007; Wrigley de Basanta et al. 2012), parts of the Arabian Peninsula (Schnittler et al. 2015), and Mongolia (Novozhilov and Schnittler 2008). In theory, long-distance dispersal by means of spores (Kamono et al. 2009) would seem to provide myxomycetes with the potential to occur anywhere on the earth, but the actual distribution of most species is usually determined by the availability of suitable microhabitats for their establishment, growth, and development (Schnittler et al. 2000). However, global patterns of distribution do appear to exist as well, since some species are predominantly subtropical to tropical, whereas others are restricted to temperate regions of the world (Stephenson et al. 2008). Temperature certainly limits the formation of fruiting bodies in tropical species, which sometimes appear in Europe in greenhouses. However, habitat preferences are currently known only from fruiting bodies. Future studies that make use of environmental PCR (as noted above) may provide a very different picture of myxomycete distribution.

History of Knowledge

Since Linnaeus provided the first descriptions of a few organisms now known to be myxomycetes (e.g., *Lycoperdon epidendrum*, the original name for the common species *Lycogala epidendrum*), the nomenclatural starting point for the taxonomy of the group is the publication of *Species Plantarum* in 1753. The first noteworthy taxonomic treatment of the myxomycetes was published by de Bary (1859), who was the first to conclude that these organisms are protists and not fungi. Rostafinski, a student of de Bary, is credited with producing the first relatively comprehensive monograph (Rostafinski 1873, 1874–1876), albeit in Polish. However, much of the information in the monograph was made available in English publications by Cooke (1877) and Massee (1892).

The single most significant pre-twentieth century publication on the myxomycetes was the first edition of Arthur Lister's *A Monograph of the Mycetozoa* (Lister 1894). This monograph, revised and expanded versions were published by his daughter Gulielma Lister (1911, 1925), became the standard reference to the myxomycetes during the early part of the twentieth century. Thomas Macbride published the first edition of his book *The North American Slime-Moulds* in 1899 and followed this with a greatly expanded second edition in 1922. These two works (Macbride 1899, 1922) are of particular importance because they were the basis of yet another work, *The Myxomycetes*, which Macbride coauthored with George Martin (Macbride and Martin 1934). Several decades later, Martin collaborated with Constantine Alexopoulos to produce their comprehensive world monograph, *The Myxomycetes* (1969). The Martin and Alexopoulos monograph, published by the University of Iowa Press, still remains the single most definitive treatment for the myxomycetes.

Until recently, identification of myxomycetes was based almost exclusively upon morphological characters of the fruiting body (Martin and Alexopoulos 1969), and keys and descriptions to the various morphospecies have been provided in a number of monographs over a period of almost a century and a half (e.g., Rostafinsky 1874–1876; Lister 1894, 1911, 1925; Martin and Alexopoulos 1969; Nannenga-Bremekamp 1991; Neubert et al. 1993, 1995, 2000; Ing 1999; Stephenson 2003; Poulain et al. 2011). However, recent molecular phylogenies (Fiore-Donno et al. 2012, 2013) show that the classical system of classification used for myxomycetes is in need of revision (see Table 1).

Practical Importance

Myxomycetes are neither pathogenic nor of economic importance. Only a few model species, especially *Physarum polycephalum* and *Didymium iridis*, have been used to investigate cell division and developmental biology in myxomycetes (Hüttermann 1973) or the importance of mating type genes (Collins 1979) and the distribution of group I introns in these organisms (Wikmark et al. 2007; Feng and Schnittler 2015).

Table 1 Comparison between the traditional classification of myxomycetes followed in most monographs and groupings emerging from molecular phylogenetics. Only important genera (e.g., isolated position or species-rich) are listed

Groups supported by molecular phylogenies	Traditional classification
Myxogastria[a]	Class Myxogastria (myxomycetes)
Dark-spored basal clade/Collumellidia[b]	
Echinosteliid superclade (*Echinostelium*)	Order Echinosteliales
Fuscisporoid superclade	
Meridermid clade (*Meriderma*)	Order Stemonitales *pro parte (p.p.)*[d]
Stemonitid clade (*Stemonitis*, *Comatricha*)	Order Stemonitales *p.p.*[e]
Lamprodermid clade (*Badhamia*, *Physarum*, *Didymium*, *Lamproderma*)	Orders Physarales, Stemonitales *p.p.*[f]
Bright-spored basal clade/Lucisporidia[c]	
Cribrarioid superclade (*Cribraria*)	Order Liceales *p.p.*[g]
Trichioid superclade	
Reticularioid clade (*Lycogala*, *Reticularia*, *Tubifera*)	Order Liceales *p.p.*[h]
Liceoid clade (*Licea*)	Order Liceales *p.p.*[i]
Trichoid clade (*Arcyria*, *Hemitrichia*, *Trichia*)	Order Trichiales[j]

[a]The genus *Ceratiomyxa*, highly distinct from all other members of the group, is probably best excluded from the Myxogastria, which is supported by current molecular investigations (Kretzschmar et al. 2016). This would make endogenic spore formation a uniting character for all Myxogastria. *Ceratiomyxa* shows affinities to some of the protostelids, which are not a monophyletic group (Shadwick et al. 2009, ▶ Protosteloid Amoebae (Protosteliida, Protosporangiida, Cavosteliida, Schizoplasmodiida, Fractoviteliida, and Sporocarpic Members of Vannellida, Centramoebida, and Pellitida))

[b]Dark-spored myxomycetes sensu Cavalier-Smith (2013): spores with melanin (except for *Echinostelium*), therefore usually violaceous brown in color

[c]Bright-spored myxomycetes sensu Cavalier-Smith (2013): spores with various other pigments (yellowish or reddish colors)

[d]*Meriderma* was split off from *Lamproderma* and forms a distinct clade within the dark-spored myxomycetes; the peridium, which fragments into tiny pieces, distinguishes the genus from *Lamproderma*

[e]The classical Stemonitales include all dark-spored myxomycetes with non-calcareous fruiting bodies. However, molecular phylogenies (Fiore-Donno et al. 2012) show the classical Physarales nested within the Stemonitales

[f]*Lamproderma* shows closer affinities to the classical Physarales, defined by calcareous fruiting bodies, even if calcareous structures are absent or reduced to little splinters on the peridium

[g]The order Liceales, with only the absence of a capillitium as the unifying character, were long thought not to be monophyletic (Eliasson 1977, 2015), but *Cribraria* forms a highly distinct clade in molecular phylogenies (Fiore-Donno et al. 2013)

[h]The genera *Lycogala*, *Reticularia*, and *Tubifera* form a monophyletic clade, but the latter does not include *Dictydiaethalium*, which shows a closer relationship to the traditional Trichiales (Leontyev et al. 2014)

[i]*Licea*, as the largest genus of the traditional Liceales, is not monophyletic, since at least some species show closer affinities to the traditional Trichiales

[j]This order, defined by free elaters as capillitial structures, is best maintained in the light of molecular investigations, although the traditional boundaries between genera do not seem to reflect natural relationships

Habitats and Ecology

Myxomycetes have been recorded from every major type of terrestrial ecosystem examined to date (Stephenson et al. 2008), and at least a few species have been recovered from aquatic habitats (Lindley et al. 2007). Temperature and moisture are thought to be the main factors limiting the occurrence of myxomycetes in nature (Alexopoulos 1963), and species richness tends to increase with increasing diversity and biomass of the associated vegetation giving rise to the plant detritus that supports the bacteria and other microorganisms upon which both trophic stages feed (Madelin 1984; Stephenson 1989). Some species of myxomycetes (e.g., *Badhamia utricularis* and *Fuligo septica*) are known to excrete exoenzymes, thus enabling them to literally consume the fruiting bodies of fungi. The pH of the substrates potentially available to myxomycetes in a particular habitat also represents an important factor influencing their distribution (Harkönen 1977; Stephenson 1989; Wrigley de Basanta 2000; Mosquera et al. 2000; Rojas et al. 2010). Although many myxomycetes appear to have a relatively wide pH tolerance, this is not the case for all species. For example, some species of *Paradiacheopsis* are found almost exclusively on bark that is quite acidic (Schnittler et al. 2016), whereas numerous species in the Physarales are restricted largely to substrates with a pH >5.0 (Schnittler and Stephenson 2002).

Microhabitats

Virtually all knowledge we have about myxomycete ecology and distribution is based only upon the occurrence of fruiting bodies. A few studies employing environmental PCR to detect myxomycete sequences in various types of substrates (Clissmann et al. 2015: bright-spored myxomycetes in wood; Fiore-Donno et al. 2016: dark-spored myxomycetes in soil) indicated that amoebal populations seem to be more widely distributed than data on fruiting body occurrence would suggest. In temperate regions of the world, where the fruiting bodies of myxomycetes appear to be most abundant, these organisms are associated with a number of different microhabitats. These include coarse woody debris, the bark surface of living trees, ground litter, and aerial portions of dead but still standing herbaceous plants. Each of these microhabitats tends to be characterized by a distinct assemblage of species (Stephenson 1988, 1989; Stephenson and Stempen 1994). The myxomycetes associated with coarse woody debris are the best known, since the lignicolous (wood-inhabiting) species typically occurring in this microhabitat tend to be among those characteristically producing fruiting bodies of sufficient size to be detected with the naked eye in the field (Martin and Alexopoulos 1969). Many of the more common and widely known myxomycete taxa, including various species of *Arcyria*, *Lycogala*, *Stemonitis*, and *Trichia*, are predominantly lignicolous. The assemblage of myxomycetes present on coarse woody debris changes with the stage of decomposition (Takahashi and Hada 2009). For example, some taxa (e.g., *Badhamia*) are restricted largely to the early stages when bark is still present. Several hundred species of myxomycetes are predominantly or completely lignicolous,

including most of the species with large compound fruiting bodies. As such, it is one of the most diverse microhabitats for myxomycetes.

Moist Chamber Cultures

The myxomycetes associated with the bark surface of living trees and with ground litter tend to be much less conspicuous and more sporadic in their occurrence and are thus difficult to detect in the field. However, the moist chamber culture technique as it applies to myxomycetes (Gilbert and Martin 1933) provides a convenient method of supplementing field collections (see, e.g., Novozhilov et al. 2017) when studying such microhabitats as bark and litter. It essentially involves blind collection of substrates with populations of amoebae, microcysts, and/or spores present and incubating these with at first abundant and then decreasing moisture conditions. The technique has been used with considerable success by many researchers (e.g., Keller and Brooks 1976; Blackwell and Gilbertson 1980; Harkönen 1981; Stephenson 1989) and works best in arid habitats (Schnittler et al. 2015). More than 200 species of "corticolous" (bark-associated) myxomycetes have been reported from bark in the field and/or in moist chamber culture (Mitchell 1980; Snell and Keller 2003). Many of these species are also known to occur in other microhabitats, but at least some species appear to be restricted to the bark of living trees. Prominent examples include various species of *Echinostelium*, *Licea*, and *Macbrideola* (Alexopoulos 1964; Mitchell 1980) with small fruiting bodies.

Ground litter supports an exceedingly diverse assemblage of myxomycetes, with approximately 400 species having been reported from this microhabitat, including many members of the Physarales that can be cultured. It seems likely that many myxomycetes fruiting on the upper litter layers actually inhabit the soil-litter interface as amoebae (Stephenson et al. 2011). A number of special microhabitats support rare assemblages of myxomycetes with seemingly specialized species present. In tropical regions, myxomycetes have been reported from epiphyllous liverworts growing on living leaves (Schnittler 2001a) and on decaying portions of the inflorescences of large tropical herbaceous plants, especially members of the order Zingiberales, which provide a highly basic pH (Schnittler and Stephenson 2002). An additional microhabitat in temperate regions supports about two dozen species of bryophilous (bryophyte-inhabiting) myxomycetes, which are found associated with mosses covering the surface of rocks, usually sandstone, in moist cool gorges (Schnittler et al. 2010). Likewise, about 25 species, some with specially adapted thick-walled spores, are known from dung (coprophilous myxomycetes, Eliasson and Keller 1999). In deserts, decaying portions of succulent plants represent another special microhabitat, from which about 50 species of "succulenticulous" myxomycetes have been reported (Lado et al. 1999). The amoebae of these myxomycetes probably prey on yeasts, and their spores are likely to be dispersed by fruit flies (*Drosophila* spp., Stephenson 2010).

The amoebae of myxomycetes are exceedingly abundant in most arable soils (Madelin 1984). Environmental PCR approaches that target the 18S rRNA (gene)

are problematic because so-called universal primers are poorly suited to detecting myxomycetes (Stephenson et al. 2011; Schnittler et al. 2017). However, in a large molecular data set for the soil microbial community obtained using a metatranscriptomic approach, Urich et al. (2008) found that myxomycetes indeed represent a major component of total protozoan soil biodiversity. The occurrence of myxomycetes in soil was discussed in detail by Stephenson et al. (2011) and Stephenson and Feest (2012).

Characterization and Recognition

General Life Cycle

The myxomycete life cycle (Fig. 2) includes two very different trophic stages, one consisting of uninucleate haploid amoebae, with or without flagella (the term "amoeboflagellate" encompasses both types of cells), and a distinctive multinucleate

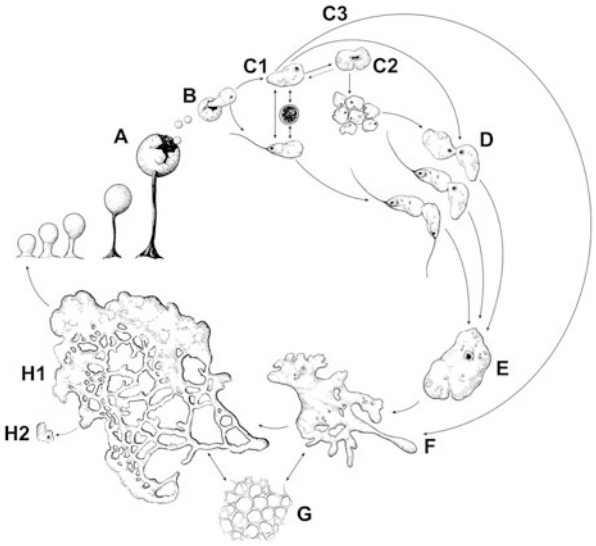

Fig. 2 Life cycle of a myxomycete. A fruiting body (*A*) releases spores (*B*) that germinate to produce uninucleate amoebae (*C1*), which can convert into resistant microcysts (middle structure) or flagellated forms (lower structure). The uninucleate cells divide (*C2*) to build up often large populations. The sexual cycle involves syngamy of two compatible uninucleate cells (*D*) to produce a zygote (*E*). [An additional hypothetical life cycle involves a uninucleate cell developing directly into a plasmodium.] The zygote gives rise to a plasmodium (*F*). The latter increases in size by phagocytosis and subsequent nuclear divisions to develop into a larger structure (*H1*). It has been reported that small portions of the plasmodia can separate as amoebae (*H2*). Under adverse conditions a plasmodium can transform into a resistant sclerotium (*G*). The segregation of a plasmodium into fruiting bodies (left side of the figure) completes the life cycle (Drawing by A. Mele)

Fig. 3 Phaneroplasmodium of a myxomycete. This is one of three different types of plasmodia produced by these organisms. The phaneroplasmodia of some species of myxomycetes can reach more than a meter in total extent (Photograph by R. Darrah)

Fig. 4 Group of solitary fruiting bodies of *Didymium bahiense* var. *microsporum* (Physarales). Such fruiting bodies usually develop by segregation of a larger plasmodium into smaller portions (Photograph by M. Poulain)

structure, the plasmodium (Martin et al. 1983). Plasmodia (Fig. 3) are motile and in some species can reach a size of more than a meter across. Large plasmodia contain many thousands of synchronously dividing diploid nuclei. Under suitable conditions, the plasmodium gives rise to one or (in most species) many fruiting bodies (also referred to as sporocarps for the Myxogastria or sporophores in *Ceratiomyxa*) containing haploid spores (Figs. 4 and 5). The spores represent the most durable of the three dormant stages in the life cycle, with the others being microcysts (derived from amoebae) and sclerotia (derived from plasmodia).

The fruiting bodies produced by myxomycetes are somewhat suggestive of those produced by certain dicaryan fungi (Eumycota), but they are considerably smaller (usually no more than 1–3 mm tall) and totally different in structure, since all visible components, except for the spores, are composed of extracellular material and thus do not show a cellular structure. Presumably, the spores are wind dispersed and

Fig. 5 Fruiting bodies of *Leocarpus fragilis* (Physarales). This is one of the most distinctive of all myxomycetes (Photograph by M. Schnittler)

complete their life cycle by germinating to produce uninucleate amoebae or flagellate cells (both forms are convertible; Stephenson et al. 2008). These feed and divide by binary fission to build up large clonal populations in the various microhabitats in which these organisms occur. Ultimately, this stage in the life cycle gives rise to the plasmodium, usually following gametic fusion between mating-type compatible amoeboid cells. Presumed apomictic strains occur in culture (Collins 1980, 1981; Clark and Haskins 2013); to what extent these occur in nature is unknown (Feng et al. 2016). Bacteria apparently represent the main food resource for both trophic stages, but plasmodia are also known to feed upon yeasts, eukaryotic microalgae, and fungal spores and hyphae (Stephenson and Stempen 1994; Smith and Stephenson 2007).

Plasmodium

Plasmodia are characterized by often colorful pigments (including white, yellow, or orange to red tints), but possess only a few characters useful in distinguishing among species of myxomycetes. It is possible to recognize three fundamentally different types (Alexopoulos 1960). These are protoplasmodia, aphaneroplasmodia, and phaneroplasmodia. Protoplasmodia are microscopic structures with only a few nuclei present, whereas aphaneroplasmodia and phaneroplasmodia are larger, multinucleate structures that are essentially giant cells. Aphanoplasmodia, characteristic of those myxomycetes assigned to the Stemonitales, are thin, transparent, and difficult to observe in nature; they generally become evident only when emerging from a particular substrate (e.g., a decaying log) just prior to the formation of fruiting bodies. Phaneroplasmodia are more robust and often highly pigmented and represent the type of plasmodium usually observed in nature. Plasmodia are extremely flexible structures and are capable of penetrating even very solid wood, most likely through the pits present in the dead cells making up the wood (Feest et al. 2015). Both aphanoplasmodia and phaneroplasmodia go through a stage that resembles a

protoplasmodium in the earliest stages of development. As a result of active cytoplasmic streaming, portions of a plasmodium are able to reach relatively distant food sources (Nakagaki et al. 2007).

Fruiting Body

Myxomycete fruiting bodies are morphologically very diverse (see Stephenson and Stempen 1994 or Schnittler et al. 2012 for a summary of morphological terms and characters; Neubert et al. 1993–2000, Poulain et al. 2011, www.slimemold.uark.edu for images showing their diversity). In *Ceratiomyxa*, fruiting bodies produce external spores on separate stalks, which is one of the characters that distinguishes the four members of this genus from all of the "true" myxomycetes. In spite of the fact that "slime mold" is the most widely used common name applied to the myxomycetes, *Ceratiomyxa* is the only genus in which the fruiting body actually has a slimy appearance at maturity. All of the true myxomycetes possess stalked or sessile fruiting bodies with internally formed spores (Fig. 6). Large aphaneroplasmodia and phaneroplasmodia primarily segregate into subportions by plasmotomy, with each subportion developing into a fruiting body (usually referred to as a sporocarp), often with a hypothallus at the base. Although possession of a stalk seems to be an

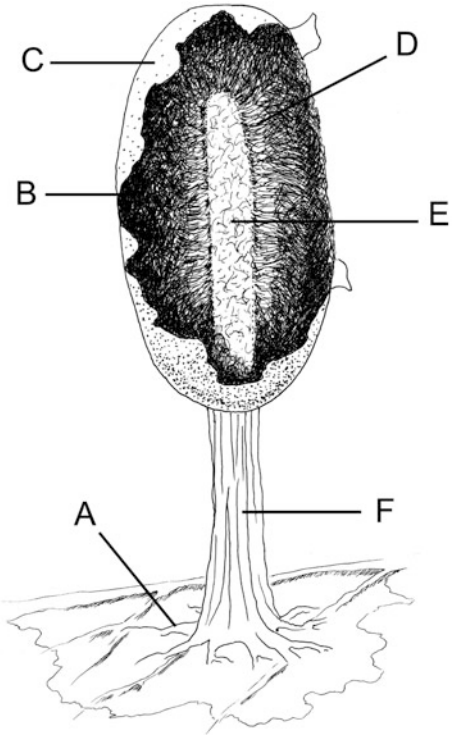

Fig. 6 Morphological features of the fruiting body of a myxomycete. (**a**) Hypothallus, (**b**) spores, (**c**) peridium, (**d**) capillitium, (**e**) columella, and (**f**) stalk (Adapted from Stephenson (2003))

Fig. 7 Compound fruiting body of *Tubifera montana* (Liceales), with evidence of the individual fruiting bodies still apparent. This type of compound fruiting body evolved, most likely independently, in several different groups of myxomycetes (Photograph by M. Schnittler)

ancient character (Fiore-Donno et al. 2012), in the majority of genera, sessile species exist beside stalked ones. In some species several fruiting bodies may share a common stalk, which seems to be the first step in the evolution of compound fruiting bodies. Large compound fruiting bodies, which are most often sessile, have evolved independently within several different lineages (Fig. 7). In some of these, single fruiting bodies are still recognizable (pseudoaethalia), but in other instances (aethalia) they are not.

The stalk, if present, is always acellular (although it can be filled with spore-like cells in some members of the Trichiales) and is secreted externally (Spiegel and Feldman 1989). In the dark-spored orders Echinosteliales and Stemonitales, the stalk forms as an invagination into the developing fruiting body, and the fruiting body rises upwards on it. Stalks formed in such a fashion usually extend into the spore mass as a central continuation, called a columella, which often diverges into many fine branches. In the other myxomycetes, the visible stalk emerges by constriction of the external surface of the plasmodial mass from which the fruiting body is derived. All structures holding the spore mass and allowing it to dry out slowly are referred to as a capillitium (Figs. 8 and 9). In the case of internal stalks, these are the branches of the columella, which is connected with the peridium in some taxa (Echinosteliales, genus *Meriderma*) but is not in others (most other Stemonitales). Capillitial structures are thus either extensions of the stalk (Echinosteliales and Stemonitales), tubular threads that are often stuffed with lime (Physarales), or free, threadlike structures called "elaters" that are often ornamented with spiral bands (Trichiales). In compound fruiting bodies, peridial remnants from the individual fruiting bodies may form a pseudocapillitium (found in some members of the Liceales).

Fruiting bodies are usually surrounded by an extracellular layer (peridium), although it may often be evanescent. In the latter situation, the peridium is simple and membranous, but it can as well be multilayered and covered with organic material or lime which shows different degrees of crystallization (Physarales). Spores are usually dispersed by air in nearly all species with solitary, stalked sporocarps, but dispersal may also occur by means of insects, especially in taxa

Fig. 8 Capillitial structures in *Lamproderma echinosporum* (Stemonitales), showing the stalk extending into the spore mass as a columella, where the capillitium branches off. Scale bar = 100 µm (Photograph by Y. K. Novozhilov)

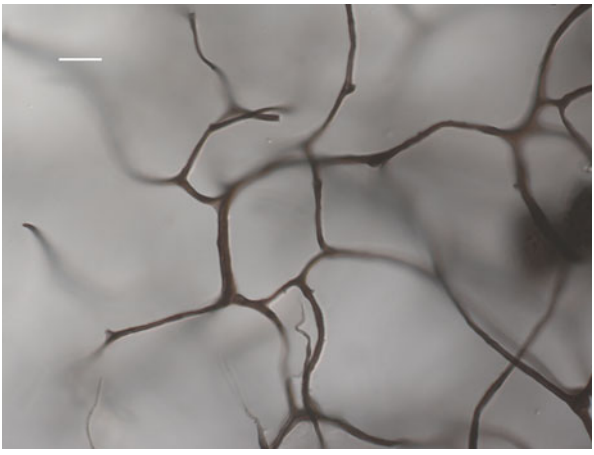

Fig. 9 Expanded view of the outer capillitial structures in *Lamproderma echinosporum*. Scale bar = 10 µm (Photograph by Y. K Novozhilov)

with compound fructifications (e.g., *Fuligo*, *Tubifera*, or *Reticularia*), or from the impact of falling raindrops (*Lycogala* or *Reticularia*). The latter are an example of convergent evolution with some of the gasteromycetes (e.g., puffballs) in Basidiomycetes (Estrada-Torres et al. 2005). Similar to many gasteromycetes, these

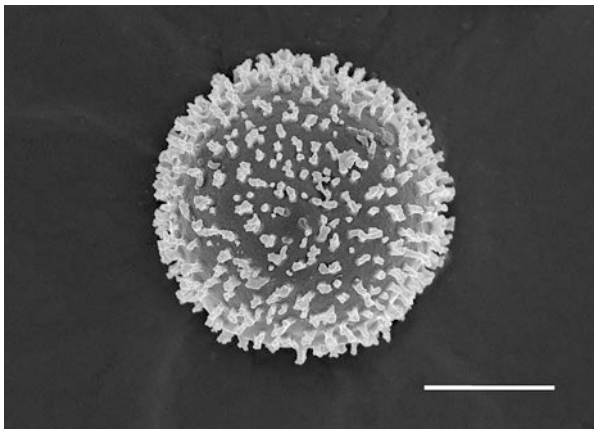

Fig. 10 A single spore of *Meriderma spinulisporum* (Stemonitales) as observed by scanning electron microscopy. Typically, myxomycete spores are nearly completely spherical, lack a hilum, and are ornamented with warts, spines, or ridges which sometimes form a more or less complete reticulum. Scale bar = 5 μm (SEM micrograph by A. Ronikier)

myxomycete species possess spores with an extremely hydrophobic ornamentation composed of a reticulum of ridges (Hoppe and Schwippert 2014).

Spore number per fruiting body ranges from just two in *Echinostelium bisporum* to 10^4–10^6 (but up to 10^{11}) in large compound fruiting bodies (Schnittler and Tesmer 2008). The spores of the vast majority of myxomycetes are spherical and range from 4 to 22 μm in diameter, with most species producing spores 10 ± 2 μm in diameter. Except for *Ceratiomyxa*, these spores lack a microscopic indentation (hilum) due to their internal development and are rarely smooth but more often ornamented with hydrophobic warts, spines, or elevated ridges (Fig. 10). Spores with yellow, reddish, or brown pigments (Trichiales: naphthoquinones; Iwata et al. 2003) occur in the bright-spored myxomycetes (Blackwell and Busard 1978; Rebhahn et al. 1999), whereas the dark-spored Stemonitales and Physarales have more uniform brown to nearly black spores pigmented by melanin (Loganathan et al. 1989; Dembitsky et al. 2005). Except for peridia with thick outer layers of organic material or lime, spore color determines the color of the fruiting body as a whole. In addition, false silvery to blue colors may also occur, as is the case for *Diachea leucopodia* or many species of *Lamproderma*. These false colors derive from interference of light reflected on the outer and inner surface of extremely thin peridia.

Sexual and Asexual Reproduction

Myxomycetes should be expected to be primarily sexual (Lahr et al. 2011; Spiegel 2011), as sex is a general attribute of eukaryotic life (Speijer et al. 2015). However, experiments on monosporic cultures suggest that they include a mixture of heterothallic (sexual) strains, where fusion of amoebae leads to the formation of a diploid plasmodium, and non-heterothallic presumably asexual strains, where single amoeboflagellates can mature into haploid plasmodia (Clark and Haskins 2010). Heterothallic isolates reproduce sexually, and fusion of compatible amoebae is controlled by mating type genes. As such, monosporic cultures, grown from a single

spore, usually do not form plasmodia. In contrast, non-heterothallic isolates can form plasmodia in monosporic cultures; most likely the life cycle can be completed by means of automixis (a degenerated meiosis or coalescence of meiotic products leading to diploid spores). In this case, the life cycle should be completed in the diploid stage (Clark and Haskins 2013). In addition, in the model organism *Physarum polycephalum*, the (temperature-dependent) diploidization of a haploid plasmodium has been identified as a possible initial event (Schaap et al. 2016). Conversion from heterothallic (sexual) to non-heterothallic (presumably automictic) forms within a species was reported by Collins (1980). Figure 1 in Feng et al. (2016) presents and discusses possible reproductive options.

Apart from cultivated stains, which are mostly limited to members of the Physarales, our knowledge about the occurrence of these reproductive modes in natural populations is very limited. A molecular investigation of bryophilous (bryophyte-associated) species of *Lamproderma* did not exclude the possibility of asexual reproduction (Fiore-Donno et al. 2011). Feng and Schnittler (2015) found that the distribution of introns in the 18S rRNA gene of the morphospecies *Trichia varia* was consistent with the existence of three sexual but reproductively isolated cryptic species. A third case study in *Meriderma* spp. (Feng et al. 2016) suggested predominant sexual reproduction. As such, we must assume that natural populations of myxomycetes consist mostly of clonal strains of amoebae, but the development of fruiting bodies is predominantly coupled with a sexual event.

The chromosomes of myxomycetes are small and difficult to count (Hoppe and Kutschera 2013). Ribosomal RNA genes that are most important for barcoding in this group of organisms are located in a few to several hundred copies on extrachromosomal plasmids (Torres-Machorro et al. 2010) and do not show Mendelian inheritance (Ferris et al. 1983). The only relatively complete myxogastrid genome sequence to date is that of an axenic culture of *Physarum polycephalum*, which shows extremely long stretches of single-sequence repeats together with large homopolymeric tracts, hampering assembly (Schaap et al. 2016).

Systematics

Recent molecular phylogenies have found a monophyletic clade (referred to as the "macromycetozoa"; Fiore-Donno et al. (2010a)) composed of the Dictyostelia, Myxogastria, and *Ceratiomyxa* (Pawlowski and Burki 2009). The Myxogastria is monophyletic but deeply divided into two groups (Fiore-Donno et al. 2010b), the bright-spored myxomycetes and the dark-spored myxomycetes; this division corresponds largely to the occurrence of melanin in spore walls. Cavalier-Smith (2013) recently proposed the formal names Lucidisporidia and Columellidia, respectively. Detailed phylogenetic relationships within the two groups have yet to be resolved; therefore, current knowledge does not allow the arrangement of all myxomycete genera into a natural system. Most of the traditional orders seem not to be

monophyletic, as shown by the contrasting traditional and informal classifications provided in Table 1.

Particularly problematic is the circumscription and sometimes the systematic position of a number of genera in several of the orders (Erastova et al. 2013). This suggests that morphological characters that are easy to observe tend to be overweighed (Schnittler and Mitchell 2000). These include traits like spore arrangement (single versus clustered). There are several rare cluster-spored species which essentially differ only in this character from more common single-spored species. The same is true for solitary versus compound fruiting bodies and the presence or absence of fruiting bodies with stalks (i.e., stalked versus sessile). In contrast, molecular data suggest that characters such as the structure of the peridium and the type of connection it has with the capillitium are evolutionarily conservative and appear to be seriously underweighted.

Maintenance and Cultivation

Only a small percentage (about 70 species, Clark and Haskins 2010, 2011) of the approximately 1,000 morphologically described species of myxomycetes can currently be induced to complete their life cycle in cultures with an appropriate bacterium present as a food source. Even fewer have been cultured under axenic conditions. The vast majority of these are litter-inhabiting members of the order Physarales. Media typically used to culture myxomycetes include weak nutrient agar to which various substrate decoctions have been added (Haskins and Wrigley de Basanta 2008). Fruiting can often be induced by adding sterile oatmeal flakes to a particular culture. Groups with specialized growth requirements, such as the nivicolous myxomycetes, are often difficult or impossible to culture (Shchepin et al. 2014). From these experiments, an independent biological species concept was developed (Clark 2000), which is not necessarily consistent with the prevailing morphological species concept (see discussion in Feng et al. 2016; Walker and Stephenson 2016).

For diversity studies, the moist chamber culture technique (Stephenson and Stempen 1994) is often used. For this simple technique, which is very convenient as well for demonstrations and school experiments (Keller and Braun 1999), samples of various types of dead plant material are placed on filter or toilet paper in sterile Petri dishes and allowed to soak with water. During the slow desiccation of the cultures, myxomycetes (particularly corticolous species) are regularly induced to fruit.

Spiegel et al. (2004) provided a synopsis of the eumycetozoans, with special regard to the methods used for carrying out inventories, various culturing techniques, and the preservation of specimens. A relatively nontechnical description of all of the techniques involved in collecting and studying myxomycetes is given in Stephenson and Stempen (1994).

Evolutionary History

A complete molecular phylogeny of the myxomycetes is gradually being developed (Fiore-Donno et al. 2008, 2010a, b, 2012, 2013). Many genes (Schaap et al. 2016) and especially rRNA sequences are rich in introns and extremely divergent, which makes it difficult if not impossible to develop universal primers. As is the case for other groups of protists (Adl et al. 2014), the most promising sequence for barcoding seems to be the first part of the 18S rRNA gene (SSU, Feng and Schnittler 2017; Schnittler et al. 2017). In contrast to the fungi, the ITS region is extremely variable even among closely related species of myxomycetes. The 18S region contains several insertion sites for group I introns (ten are currently known), which makes the myxomycetes an interesting model system for studying these structures (Johansen et al. 1993, 1997; Haugen et al. 2003). Introns may be independently acquired even within closely related biospecies (Feng and Schnittler 2015) and can contain homing endonuclease genes, seemingly following the Goddard-Burt cycle of intron acquisition and loss (Goddard and Burt 1999).

Due to the fragile nature of the fruiting body, fossil records of myxomycetes are exceedingly rare. Domke (1952) described a species of *Stemonitis* and Dörfelt et al. (2003) a species of *Arcyria* from Baltic amber dating from the Eocene. The maximum age that could be assigned to either of these fossils is about 50 million years, which is older than that of the few records of fossil spores that appear to be those of myxomycetes, which date only from the Oligocene and Pleistocene (Graham 1971). Molecular dating analyses that have considered eumycetozoans seem to indicate that the sorocarpic ancestors of myxomycetes may have existed even before the colonization of land by plants (Fiz-Palacios et al. 2013), but the highly divergent 18S rRNA gene sequences point as well to recent speciation events (Aguilar et al. 2013; Feng and Schnittler 2017).

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Dictyostelia

39

Sandra L. Baldauf and Joan E. Strassmann

Abstract

Dictyostelia are common amoebae, mostly known from forest soil and litter. They respond individually to adverse conditions by forming simple amoeboid cysts (microcysts) or en masse by aggregation. Aggregates can include >100,000 amoebae and culminate in either thick-walled highly resilient macrocysts (zygocysts), the sexual cycle of Dictyostelia, or multicellular fruiting bodies (sorocarps). In contrast to Myxomycetes, Dictyostelia form pseudoplasmodia, and sorocarp formation in most species includes cellular differentiation and cell death (~20% of the aggregate). Traditional classification recognized three genera based on sorocarp morphology. However, these morphologies are now known to have evolved multiple times and thus correspond to morphotypes rather than phylogenetic taxa. Acytostelid morphotypes (traditional *Acytostelium* spp.) have tiny delicate sorocarps with acellular stalks (no cell death). Polysphondylid morphotypes (traditional *Polysphondylium* spp.) have cellular stalks bearing regularly spaced whorls of side branches. Dictyostelid morphotypes (traditional *Dictyostelium* spp.) also have cellular stalks but with diverse morphologies ranging from solitary sorocarps with a single sporehead to sorocarps with multiple sporeheads on irregularly spaced side branches and/or various arrangements of clustered sorocarps. There are ~150 described species, which molecular

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phylogeny places into eight distinct divisions, and the taxonomy of group is now being formally revised accordingly. The best-known species is *Dictyostelium discoideum*, an important model organism widely used to study, e.g., cell signaling, cellular differentiation, and social behavior. Other taxa are also under development as models, including full genome data from all major divisions. Given its age (~600 myr), molecular depth, and small number of described species, it is expected that substantial dictyostelid diversity remains to be discovered.

Keywords

Amoeba • Soil • Aggregation • Development • Sorocarp • Acrasin • cAMP • Microcyst • Macrocyst • Social behavior

Contents

Summary Classification

●**Dictyostelia**
●●**Acytosteliales** (= "Group 1 + 2")
●●●**Cavenderiaceae** (= "Group 1"; *Cavenderia*)
●●●**Acytosteliaceae** (= "Group 2"; *Acytostelium*, *Heterostelium*, *Rostrostelium*)
●●**Dictyosteliales** (= "Group 3 + 4 + violaceum complex + polycephalum complex")
●●●**Raperosteliaceae** (= "Group 3"; *Hagiwaraea*, *Raperostelium*, *Speleostelium*, *Tieghemostelium*)
●●●**Dictyosteliaeae** (= "Group 4 + violaceum complex"); *Dictyostelium*, *Polysphondylium*)
●●●***Coremiostelium*** (= "polycephalum complex")
●●***Synstelium*** (= "polycarpum complex")

Introduction

General Characteristics

Dictyostelia is a monophyletic group of sorocarpic terrestrial amoebae also known informally as cellular slime molds or social amoebae. Their closest well-known relatives are Myxomycetes (Fiore-Donno et al. 2010; ▶ Myxomycetes), from which they most notably differ in forming pseudoplasmodia (instead of true plasmodia) and fruiting bodies (sorocarps) with differentiated cell types. Dictyostelid amoebae feed primarily on bacteria (Potts 1902; Cavender 1973; Vadell 1993) and are distributed worldwide (Raper 1984; Cavender 1990), although they have been isolated primarily from forest soils and soil litter. Amoebae probably exhibit some preference in their bacterial prey (Singh 1947b; Depraitere and Darmon 1978; Nasser et al. 2013), including carrying them along with spores to new locations ("farming"; Brock et al. 2011). Thus, dictyostelids are important components of soil communities, potentially affecting the size and composition of the soil microbiota (Stout 1973; Landolt et al. 1992).

Dictyostelids were the first discovered (and are still the best-studied) organisms with aggregative multicellularity, whereby a single-celled trophic stage alternates with a multicellular (developmental) dispersal stage (Fig. 1). This is especially well studied in the model organism *Dictyostelium discoideum*, and much of what is known is based on studies of this species. The trophic stage (vegetative cycle; Fig. 1) is strictly unicellular, consisting of independent amoebae (often referred to as myxamoebae) feeding on bacteria (Raper and Smith 1939; Depraitere and Darmon 1978; Vadell 2000; Kessin 2001). Amoebae multiply by binary fission, which occurs roughly every 8–10 h under optimal growth conditions in the lab (Escalante and Vicente 2000). The population grows until food becomes scarce, at which point cells can switch to one of the three alternative pathways resulting in the formation of microcysts, macrocysts, or fruiting bodies (sorocarps; Fig. 1). Sorocarps consist of a stalk, which may be cellular or acellular, supporting a bolus of spores (sorus). However, not all responses are found in all species – for example, microcysts are unknown in *D. discoideum* and its immediate relatives (the redefined genus *Dictyostelium*; see below). Of the three responses, sorocarp formation is the best studied because of its multicellular nature and central importance for species identification.

The long-standing traditional taxonomy of Dictyostelia recognized three genera, which correspond to three general sorocarp morphologies (Fig. 2) – dictyostelid (traditional *Dictyostelium* spp.), polysphondylid (traditional *Polysphondylium* spp.), and acytostelid (traditional *Acytostelium* spp.) (Olive 1975; Raper 1984; Hagiwara 1989). More recently, morphological (Swanson et al. 2002) and molecular analyses (Schaap et al. 2006) reject this traditional classification, and molecular phylogeny in particular identifies instead up to eight major divisions, none of which correspond to the three genera (Fig. 3). Thus, the traditional genus-level designations are now understood to refer to morphotypes rather than phylogenetic taxa. The taxonomy of

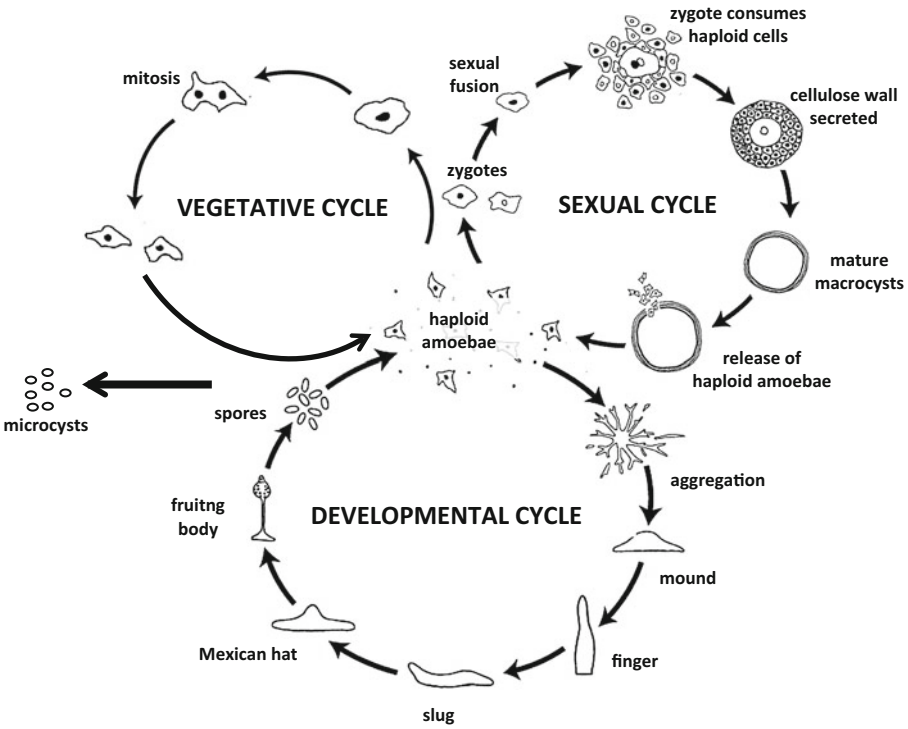


Fig. 1 Life cycle of Dictyostelia, based on *Dictyostelium discoideum*. The trophic stage (vegetative cycle) consists of haploid amoebae, which actively pursue their bacterial prey and which reproduce asexually. Various stimuli, particularly scarcity of food, lead to either the sexual or the social (developmental) cycle. In the developmental cycle, amoebae aggregate by the 10s–100s of thousands and then cooperate to form a multicellular fruiting body (sorocarp). The fruiting body consists of a ball of live spores supported by an inert stalk, which in all but acytostelids is cellular, consisting of the dead remains of ~20% of the aggregate cells. In the sexual cycle, amoebae aggregate, two cells of opposite mating type fuse, and the resulting zygote consumes the remaining aggregate. The result is a giant, hardy macrocyst with a thick multilayered cell wall. The macrocyst eventually undergoes recombination and meiosis and then hatches hundreds of recombinant, haploid progeny (Courtesy of S. Sheikh, D. Brown, and J. Strassmann)

the group is being formally revised, and the new proposed taxonomy is shown in Fig. 3. The new taxonomy is used here but is relatively easily mapped back to the traditional taxonomy by noting that nearly all new genera correspond to traditional *Dictyostelium* spp. with the exception of most *Heterostelium* spp. and *Rostrostelium ellipticum* (Fig. 3, Table 1)

Occurrence

The first dictyostelid to be formally described was *Dictyostelium mucoroides* (Fig. 4a), reported in 1869 by the German mycologist Oscar Brefeld, who was

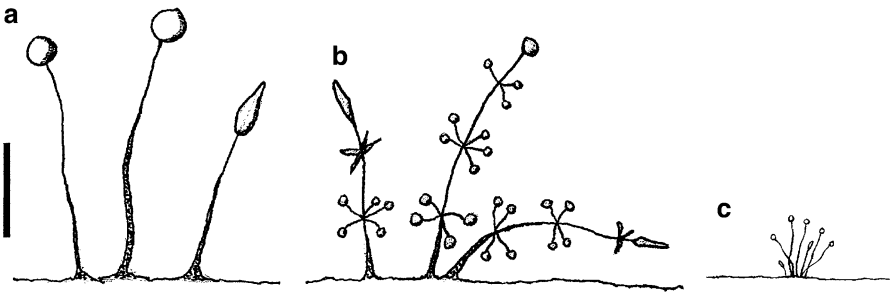


Fig. 2 Three Dictyostelia morphotypes. Representative schematics are shown for the three general sorocarp morphologies (morphotypes) occurring in Dictyostelia: (**a**) dictyostelid, (**b**) polysphondylid, and (**c**) acytostelid. Scale bar = 1.0 mm (Reproduced with permission from Swanson et al. (2002))

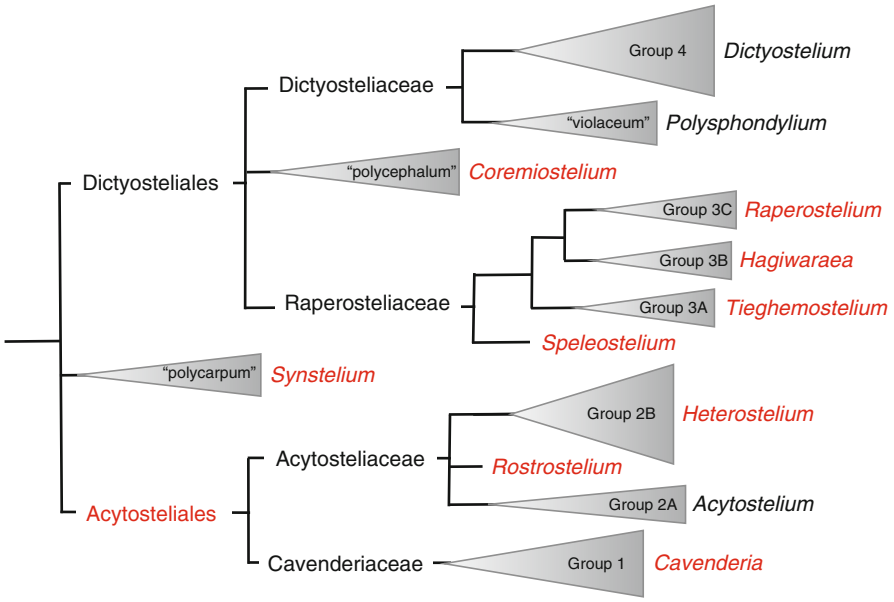


Fig. 3 Schematic phylogeny of Dictyostelia showing the major molecular groups and proposed new taxonomy. Informal names based on the molecular phylogeny are shown enclosed in shaded triangles (Schaap et al. 2006; Romeralo et al. 2011; Singh et al. 2016). Recently proposed names are shown in *red* (Sheikh et al. in press)

studying substrates such as animal dung and decomposing plant material looking for new microorganisms. Brefeld originally found *D. mucoroides* sorocarps on horse dung, leading to the idea that dictyostelids are coprophilous. Although later studies showed that their primary habitat is forest soils (Cavender and Raper 1965a; Raper 1984; Hagiwara 1989), they have also been found in agricultural soils (Agnihothrudu 1956), prairies (Smith and Keeling 1968; Rollins et al. 2010) and

Table 1 Species referred to in the text and figures, including new and former names

Genus	Species	Former name[a]	Genus	Species	Former name
Acytostelium	*A. anastomosans*		*Hagiwaraea*	*Ha. coeruleostipes*	*D. coeruleo-stipes*
	A. irregulosporum			*Ha. vinaceofuscum*	*D. vinaceo-fuscum*
	A. leptosomum				
	A. longisorophorum		*Heterostelium*	*He. arachnoides*	*P. arachnoides*
	A. magnuphorum			*He. boreale*	*D. boreale*
	A. serpentarium			*He. colligatum*	*P. colligatum*
	A. subglobosum			*He. filamentosum*	*P. filamentosum*
				He. flexuosum	*P. flexuosum*
Cavenderia	Ca. *antarctica*	*D. antarcticum*		*He. oculare*	*D. oculare*
	Ca. *aureostipes*	*D. aureo-stipes*		*He. pallidum*	*P. pallidum*
	Ca. *bifurcatum*	*D. bifurcatum*		*He. pseudocolligatum*	*P. pseudocolligarum*
	Ca. *boomeransporum*	*D. boomeransporum*			
	Ca. *deminutivum*	*D. deminutivum*		*He. stolonicodeum*	*P. stolonicodeum*
	Ca. *fasciculatum*	*D. fasciculatum*			
	Ca. *mexicanum*	*D. mexicanum*	*Polysphondylium*	*P. violaceum*	
	Ca. *multistipes*	*D. multi-stipes*		*P. laterosorum*	*D. laterorosum*
	Ca. *myxobasis*	*D. myxobasis*			
	Ca. *stellatum*	*D. stellatum*	*Raperostelium*	*Ra. australe*	*D. australe*
				Ra. lacteum	*D. lacteum*

Coremiostelium	*Co. polycephalum*	*D. polycephalum*		*Ra. minutum*	*D. minutum*
				Ra. tenue	*D. tenue*
Dictyostelium	*D. ammophilum*				
	D. aureum		*Rostrostelium*	*Ro. ellipticum*	*A. ellipticum*
	D. austroandinum				
	D. discoideum		*Speleostelium*	*Sp. caveatum*	*D. caveatum*
	D. gargantuum				
	D. giganteum				
	D. implicatum		*Synstelium*	*Sy. polycarpum*	*D. polycarpum*
	D. mucoroides				
	D. leptosomopsis		*Tieghemostelium*	*T. lacteum*	*D. lacteum*
	D. purpureum			*T. menorah*	*D. menorah*
	D. rosarium				
	D. septentrionalis		*Incertae sedis*	*D. roseum*	
	D. sphaerocephalum				
	D. valdivianum				

[a]For all former names, *A. Acytostelium*, *D. Dictyostelium*, *P. Polysphondylium*

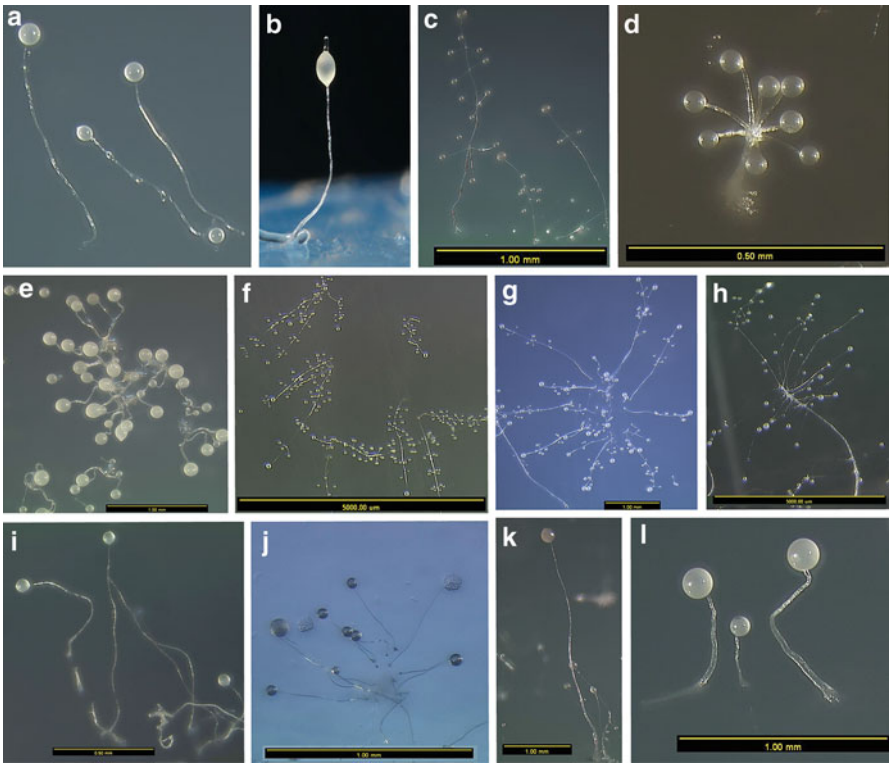


Fig. 4 Examples of sorocarp morphologies: (**a**) *D. mucoroides*, (**b**) *D. discoideum*, (**c**) *P. violaceum*, (**d**) *Co. polycephalum*, (**e**) Ca. *bifurcatum*, (**f**) *He. colligatum*, (**g**) *He. pallidum*, (**h**) *He. arachnoides*, (**i**) *A. magnuphorum*, (**j**) *A. subglobosum*, (**k**) *D. purpureum*, and (**l**) *D. sphaerocephalum* (Photographs courtesy of Ushman Bashir (b), James Cavender (f, h), and Andrew Swanson and Frederick Speigel (a, c–e, g, i–l))

deserts (Benson and Mahoney 1977). Further species have been found on dung (Raper 1984) as well as under-decomposed grasses, on mushrooms and wood (Hagiwara 1992), and on epiphytes in tropical forests (Stephenson and Landolt 1998). Recent surveys extending to high latitudes and altitudes, poorer soils, and even forests bordering on subarctic sand dunes (Romeralo et al. 2010) have resulted in the isolation of many new species, indicating that the habitat tolerance of dictyostelids is wider than previously suspected.

Lyophilized collections of type cultures are available at the American Type Culture Collection (ATCC – Rockville, Maryland, USA). The Dicty Stock Center (dictybase.org/StockCenter/StockCenter.html) also maintains frozen spores of almost all natural isolates. All species isolates from the first molecular survey of Dictyostelia (Schaap et al. 2006) are also available from the University of Dundee (p.schaap@dundee.ac.uk), while most of the species isolated from 2006 to 2016 are

available from Uppsala University (sandra.baldauf@ebc.uu.se). Kenneth Raper's original collection of dictyostelids is maintained at the University of Wisconsin (Madison) by M. Filutowicz (msfiluto@wisc.edu).

Literature

E. W. Olive (1902): "*Monograph of the Acrasieae*" is the first work that includes the dictyostelids as a group.

Bonner (1967): "*The Cellular Slime Molds*" includes an introduction to the group and detailed treatments of some of the best-known species. The book also includes information on other groups thought at the time to possibly be related to dictyostelids, such as labyrinthulids, plasmodiophorids, and acrasids (now placed within Stramenopila, Rhizaria, and Heterolobosea, respectively).

L. S. Olive (1975): "*The Mycetozoans*" is an excellent condensed introduction to mycetozoan morphology, ecology, and life history, including detailed descriptions of dictyostelids as well as myxogastrids and some protostelids (see ▶ Protosteloid Amoebae (Protosteliida, Protosporangiida, Cavosteliida, Schizoplasmodiida, Fractoviteliida, and Sporocarpic Members of Vannellida, Centramoebida, and Pellitida)). In addition to labyrinthulids and plasmodiophorids, the book also includes descriptions of acrasids (▶ Heterolobosea) and other sorocarpic taxa now recognized as non-amoebozoan such as *Fonticula alba* and *Guttulinopsis vulgaris* (Brown et al. 2012).

K. B. Raper (1984): "*The Dictyostelids*" is an in-depth overview and currently the most comprehensive single resource available on the group. It deals almost exclusively with the dictyostelids, including detailed descriptions of 50 species. There is also information on dictyostelid ecology, distribution and culture conditions, as well as dichotomous keys for the three "traditional genera." It also includes some discussion of the acrasid cellular slime molds, sensu L. S. Olive (1975).

H. Hagiwara (1989): "*The Taxonomic Study of Japanese Dictyostelid Cellular Slime Molds*" covers all Japanese species known at the time, many of which are missing from Raper's book. New morphological characters for identification are included, such as base and tip morphology, aggregation shape, and growth pattern.

R. H. Kessin (2001): "*Dictyostelium*: *Evolution*, *Cell Biology*, *and the Development of Multicellularity*" is a comprehensive book with a focus on cell and molecular biology of *D. discoideum*.

W. F. Loomis (2012): "*Dictyostelium*: *a developmental system*" is a collection of chapters by different authorities on the different development stages and their evolution, plus molecular techniques and genomics.

M. Romeralo et al. (2015):"*The Dictyostelids*" is the most recent collection of essays by leaders in the field.

Websites:	http://www.discoverlife.org/mp/20q?guide=Dictyostelids
	http://cosmos.bot.kyoto-u.ac.jp/index.html
	http://dictybase.org/

History of Knowledge

The first persons to describe dictyostelids were a mycologist (Brefeld 1869) and a botanist (van Tieghem 1880). Although the unusual dictyostelid life cycle soon captured the interest of developmental biologists, it was not until the isolation of axenic strains of *D. discoideum* (Fig. 4b) that their potential as an experimental model began to be realized (Sussman and Sussman 1967). Brefeld described the first dictyostelid (*D. mucoroides*, Fig. 4a; Brefeld 1869) as well as the first polysphondylid (*Polysphondylium violaceum*, Fig. 4c; Brefeld 1884). It was also Brefeld who suggested the generic name *Dictyostelium*, which combines *Dictio-* (from gr. δίκτυον, net), used in botany to refer to something forming netlike structures, and *stelium* (tower), referring to the presence of a stalk formed by a network of cells. Brefeld thought that the aggregated amoebae fused to form a true plasmodium; however van Tieghem soon realized that the amoebae remain independent throughout the life cycle and denoted the aggregate as a pseudoplasmodium (van Tieghem 1880). His published accounts of acrasids and dictyostelids, which together he called Acrasiées, provided the criteria for their eventual taxonomic removal from the Myxomycetes because of the lack of a true plasmodium, although the acrasids are now removed from Amoebozoa altogether and placed in Heterolobosea (see below; Adl et al. 2012). van Tieghem's experiments with dictyostelids also led him to anticipate the role they would play in the field of developmental biology.

The phylogenetic position of Dictyostelia has been controversial for most of its scientific history, due to various similarities with fungi, plants, acrasids, and even animals (e.g., Loomis and Smith 1990). Traditionally, the group Dictyostelia was most often placed with fungi based on superficial similarities between their sorocarps and fungal fruiting bodies (e.g., Cappuccinelli and Ashworth 1977). However, E.W. Olive noted as early as 1902 that dictyostelids lack hyphae, and he placed them instead with acrasids (family Acrasidae; Olive 1902; ▶ Heterolobosea) using a name first proposed by van Tieghem (1880). L. S. Olive (1975) and Raper (1984) later suggested that acrasids were probably not closely related to Dictyostelia based on morphological and behavioral differences between their amoebae. Dictyostelids also differ from acrasids in their developmental cycle, including aligned streaming of amoebae and highly developed sorocarps with cellulosic stalks. Page and Blanton (1985) were the first to suggest splitting *Acrasis* and also *Pocheinia* from Dictyostelia and placing them instead in Heterolobosea. This was eventually confirmed by molecular phylogeny (Roger et al. 1996; Baldauf et al. 2000), and *Acrasis* (+ *Pocheinia*) and Dictyostelia are now recognized to be extremely distantly related, being members of Discoba and Amoebozoa, respectively (Adl et al. 2012).

Modern experimental study of Dictyostelia arguably began with the discovery of *D. discoideum* strain NC4 in forest soils of North Carolina (Raper 1935). It was Raper's subsequent slug-grafting experiments with this strain which demonstrated that the stalk and spores develop from the front and rear of the migrating

pseudoplasmodium, respectively, in this and related species, experiments that are considered classics in the field (Raper 1940). In another classic series of experiments, Bonner demonstrated the existence of a chemotactic agent responsible for cell aggregation, which he termed an "acrasin" (Bonner 1947). Subsequent identification of the *D. discoideum* acrasin as cyclic AMP (cAMP), also in Bonner's lab (Konijn et al. 1967), was the first demonstration of extracellular signaling by cAMP, an important intracellular signaling molecule across eukaryotes (e.g., Ravnskjaer et al. 2016). The role of cAMP as an acrasin was later found to be restricted to *D. discoideum* and its parent taxon (the redefined *Dictyostelium s.s.*; Fig. 3), while a variety of small molecules serve as acrasins in other Dictyostelia (e.g., glorin, folate, and pterin). Subsequent work showed that extracellular cAMP signaling also plays a central role in post-aggregative developmental signaling, a role that is probably universal among and ancestrally present in Dictyostelia (reviewed in Schaap 2016).

The sexual stage of *D. discoideum* was discovered in the early 1970s (Fig. 5; Clark et al. 1973; Erdos et al. 1973), before which it was thought that Dictyostelia were exclusively asexual and haploid. Although the existence of the macrocyst had been known for some time, its relationship to sexuality was not firmly established until later (Clark et al. 1973; Erdos et al. 1973). This began with the discovery that macrocyst formation in certain species depends upon mixing amoebae of opposite mating type. This lead eventually to the discovery that the macrocyst is a diploid resting phase (Fig. 5). However, work with macrocysts was still hampered by problems with germination and recovery of the zygote (Katz 1978; Newell

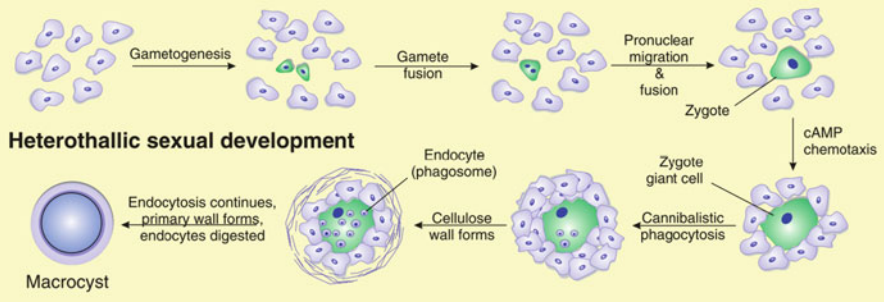


Fig. 5 Sexual development of heterothallic *D. discoideum* in mixed mating type (type I/type II) cultures. Small, motile gametes fuse to produce a binucleate cell that increases in size to become a binucleate giant cell. As growth continues, the pronuclei swell, migrate together, and fuse producing a true zygote giant cell (ZGC). Amoebae are chemically attracted to the ZGC, which begins to ingest and convert them to endocytes. Meanwhile, aggregate cells begin to build a multilayered, loose precyst sheath. Sexual phagocytosis continues until all of the amoebae are ingested as endocytes. The endocytes are gradually digested by the zygote as the macrocyst matures and the macrocyst wall is formed (O'Day and Keszei 2012) (Reproduced with permission of authors and journal)

1978b), until the development of temperature-sensitive mutants and the application of parasexual genetics (Kessin et al. 1974). Macrocysts occur throughout Dictyostelia (Fig. 3), indicating that a sexual phase formed by aggregating cells also evolved very early (Schaap et al. 2006; O'Day and Keszei 2012; Du et al. 2015).

Genetic studies in *D. discoideum* began with the selection of morphogenetic mutants by Sussman (1956). This research then accelerated with the creation by mutagenesis of the first axenically growing strain, AX2, making it possible to grow *D. discoideum* in the lab on simple defined growth media (Sussman and Sussman 1967). This facilitated the isolation of additional mutants to be used as genetic markers (Franke and Kessin 1977). *Dictyostelium discoideum* can now be transformed with foreign DNA (Escalante and Vicente 2000) and manipulated with a suite of sophisticated molecular tools and techniques (reviewed in Schaap 2011). Full genome sequences are also now available from representatives of all major phylogenetic groups (Fig. 3; Glöckner et al. 2016; Singh et al. 2016) as well as 20 separate clones of *D. discoideum* (Ostrowski et al. 2015) facilitating both macro- and microevolutionary studies.

Practical Importance

Dictyostelium discoideum is an important, widely used, and well-developed experimental model for the study of processes at the cellular level such as cell motility, chemotaxis, cytokinesis, and phagocytosis (Schaap 2011; Romeralo et al. 2013). At the multicellular level, it is also used to study, e.g., cell-cell communication (Newell 1978a), cellular differentiation, development, and competition (Maeda et al. 1997; Kessin 2001; Escalante and Vicente 2000; Strassmann et al. 2000; Loomis 2014; Romeralo et al. 2015). Dictyostelids have a number of important features that have contributed to their popularity for experimental study – they are easy to isolate and grow, are nonpathogenic, have a small genome, and readily complete their life cycle under laboratory conditions. This has been augmented by the development of molecular tools such as whole genome sequences, transcriptome profiles, genetic transformation, and targeted mutagenesis (Loomis 2013; Faix et al. 2013). With the development of a comprehensive phylogeny of Dictyostelia (Schaap et al. 2006; Romeralo et al. 2011; Sheikh et al. 2015), it is now also possible to study these phenomena within a robust evolutionary framework (Fig. 3)

Dictyostelium discoideum has a long history of use in medical research (reviewed in Romeralo et al. 2012). Comparative genomics of *Dictyostelium* and pathogenic Amoebozoa such as certain species of *Entamoeba* and *Acanthamoeba* allow the identification of amoebozoan-specific genes with potential applications in controlling amoebic diseases (Du et al. 2015). *Dictyostelium* is also used as a model for studying aspects of the mammalian immune response, as it displays basic similarities in traits such as lymphocyte motility and macrophage phagocytosis (Jin et al. 2009; Tatischeff 2013; Cosson and Lima 2014). It has also been used to study other disease processes, and *D. discoideum* is used as a model for a number of human disease-related proteins.

Habitats and Ecology

The primary habitat of dictyostelids appears to be the surface layers of forest soils and the soil litter. These substrates have yielded most of the ~150 known species, including such diverse types as *Coremiostelium polycephalum* (Fig. 4d), *Cavenderia bifurcatum* (Fig. 4e), *Heterostelium colligatum* (Fig. 4f), *Heterostelium arachnoides* (Fig. 4h), *Acytostelium magnuphorum* (Fig. 4i), *Acytostelium subglobosum* (Fig. 4j), and *Dictyostelium sphaerocephalum* (Fig. 4l). Although the common species *D. mucoroides* (Fig. 4a), *P. violaceum* (Fig. 4c), *Heterostelium pallidum* (Fig. 4g), and *Dictyostelium purpureum* (Fig. 4k) were first isolated from animal dung, these are now known to be widespread members of the soil microbial community (Romeralo et al. 2013). Other species isolated from dung include the much more rare *Dictyostelium aureum* (Olive 1902) as well as "*Dictyostelium roseum*" (van Tieghem 1880), one of a number of species that have only been isolated once and are only known by their description (Raper 1984). Otherwise, no dictyostelids are known to be strictly coprophilic. Perhaps the closest to this is *Speleostelium caveatum* (Raper 1984), which lives in the dung of cave-dwelling bats and preys on other dictyostelid amoebae (Waddell and Vogel 1985). This fascinating and highly molecularly distinct species (Schaap et al. 2006; Romeralo et al. 2011), which can disrupt the developmental cycle of other dictyostelids (Waddell and Vogel 1985; Nizak et al. 2007), has also never been re-isolated. Some dictyostelids have also been isolated from decaying plants and fungi, such as *Tieghemostelium lacteum*, first isolated from a decaying mushroom (van Tieghem 1880), and L. S. Olive frequently isolated species from rotting wood (Olive 1975). Recent explorations of new habitats have also yielded new species such as *Dictyostelium ammophilum* isolated near the Great Kobuk Sand Dunes at the Kobuk Valley National Park, Alaska, USA (Romeralo et al. 2010).

Geographically, dictyostelids are distributed worldwide, from Alaska (Romeralo et al. 2010) and northern Sweden (Perrigo et al. 2013) to the tropics (Cavender 1973; Vadell 1993; Cavender et al. 2016). In general, diversity appears to increase toward the equator and lower altitudes (Cavender 1973; Hagiwara 1984; Swanson et al. 1999) and, as in many groups of plants and animals, appears to be highest in the tropics (Cavender 1978; Kawabe 1980). However, there are species that appear to be endemic to temperate (Cavender 1978; Hagiwara 1982) as well as subalpine zones (Traub et al. 1981). The neotropical region in particular seems to be a center of diversification (Vadell and Cavender 2007). The most widely encountered species are *D. sphaerocephalum* (Fig. 4l) and *D. mucoroides* (Cavender 1983) (Fig. 4a) followed by *P. violaceum* (Fig. 4c) and *He. pallidum* (Fig. 4g), although the latter is now known to be a species complex (Fig. 9; Romeralo et al. 2011). Species such as *D. discoideum*, *D. purpureum*, and *Raperostelium minutum* are more restricted in distribution, while the crampon or basally digitate species (*Hagiwaraea* spp.) are some of the most restricted.

The first studies on the ecology of dictyostelids were conducted by Raper (1939), who grew *D. discoideum* with different bacteria as food. Later, Singh (1947a, b) studied the influence of humidity on dictyostelid growth. Dictyostelids inhabit soils

over a wide spectrum of moisture regimes, although amoebal density is greatest at intermediate moisture levels (Cavender and Raper 1965b) and decreases with decreasing moisture (Rollins et al. 2010). Overall the abundance of dictyostelid amoebae can be quite high, ranging from hundreds to thousands per gram of soil (Cavender and Raper 1965b).

Forest soils, preferably slightly acidic ones, are the best habitat for dictyostelids, in terms of numbers of amoebae recovered and species diversity (Cavender and Raper 1965b; Landolt et al. 2006). However, some species are also tolerant of alkaline or neutral conditions, and a few, such as *D. mucoroides* (Fig. 4a), are tolerant of a wide range of pH. The numbers of dictyostelid spores and amoebae also decrease progressively with soil depth. Early studies indicated optimal growth temperatures of 20–25 °C, with considerable overlap among species (Raper 1984). However, species isolated from higher latitudes prefer lower temperatures (Romeralo et al. 2010). In temperate zones, there are also seasonal fluctuations with spring and fall peaks in spore and amoebal numbers (Cavender and Raper 1965b; Frischnecht-Tobler et al. 1979). A relationship has been demonstrated between vascular plants and dictyostelid species, such that different dictyostelids seem to preferably associate with certain plant species (Cavender and Raper 1965b, 1968; Cavender and Kawabe 1989). Most dictyostelids also appear to be phototactic during the slug and rising sorogen stage of the life cycle (Fig. 1; Raper 1984; Bonner 2006), possibly to aid the aggregate in finding an exposed microsite to erect the sorocarp and thus increase its dispersal opportunities.

Escherichia coli and *Klebsiella pneumoniae* (or *K. aerogenes*) are the preferred laboratory food sources for all examined species. Experiments conducted in the field (Kuserk 1980) suggest that food plays a prominent role in shaping the local diversity of dictyostelids. There is also some evidence of bacterial prey preference in the wild (Singh 1947a; Depraitere and Darmon 1978; Nasser et al. 2013), and it has been shown recently that *D. discoideum* spores can cotransport selected bacterial prey (Brock et al. 2011). Response of the larger species to light and differential temperatures (Raper 1940; Bonner et al. 1950; Kessin 2001), humidity (Bonner and Shaw 1957), gases (Bonner and Dodd 1962; Bonner and Lamont 2005), and solutes (Slifkin and Bonner 1952) is quite dramatic during migration and development, indicating great environmental sensitivity. Smaller species may be even more sensitive to some of these factors since they are more difficult to culture, for example, *Rostrostelium ellipticum*, *Tieghemostelium menorah*, *Cavenderia stellatum* (Fig. 10c), and *Heterostelium oculare* (Fig. 10g). The use of these organisms as monitors of the soil environment (e.g., Vadell 2004) could potentially rival their popularity as tools for developmental biology.

To summarize, species diversity and composition change with forest type (Cavender and Raper 1965c), soil moisture gradient (Sutherland and Raper 1978), vegetational diversity (Hagiwara 1976), altitude (Hagiwara 1976; Cavender 1983; Romeralo and Lado 2006), and latitude (Cavender 1973). Optimal conditions for dictyostelid development are moderate temperature, high soil oxygen, medium humidity, and sufficient bacteria (Cavender and Raper 1968; Raper 1984). However, there are reasons to suspect that the diversity of Dictyostelia is still largely unknown.

Sampling efforts have mostly used a narrow set of culture conditions that tend to favor larger, faster-growing species. Meanwhile more recent studies that focused on tiny and/or slower-growing species have identified many new ones (Fig. 10; Cavender and Vadell 2000; Cavender et al. 2013). Dictyostelia is also ancient (>600 mya; Fiz-Palacios et al. 2013) and of extreme molecular depth (Schaap et al. 2006) despite the small number of described species. Although dictyostelids are largely absent from culture-independent (metagenetic) surveys, this is probably due to the strong AT bias and generally divergent nature of their rRNAs (Romeralo et al. 2011). In fact, preliminary metagenetic sampling with Dictyostelia-specific rRNA primers suggests hidden diversity at all taxon levels (SLB, ms in prep).

Kin Recognition and Cheating

How Dictyostelia interact with each other and other organisms in the soil is only beginning to be understood, mostly based on studies in *D. discoideum*. Dictyostelids recognize kin using two membrane proteins with highly polymorphic extracellular domains, TgrB1 and TgrC1 (see below), which also help induce competence for post-aggregative cell differentiation (Hirose et al. 2011). The fact that dictyostelid spores are dispersed as a unit should increase the likelihood that nearby amoebae will be clonal and thus produce clonal fruiting bodies. However, genetically distinct clones of *D. discoideum* have been shown to co-occur in nature (Fortunato et al. 2003a) and to give rise to a small proportion of mixed-clonal fruiting bodies (Gilbert et al. 2007). Genetically distinct clones can also form fruiting bodies together in the lab. Moreover, in some of these cases, certain clones may be overrepresented in spore compared to stalk, a phenomenon known as cheating (Strassmann et al. 2000). Cheating appears to be due to a combination of preset clonal characters (fixed cheating) and inter-clonal interactions (facultative cheating) (Buttery et al. 2009), the contributions of which vary along a linear gradient (Fortunato et al. 2003b).

Cheating appears to have costs and benefits. Chimeric slugs move less distance toward light than clonal slugs (Foster et al. 2002). However larger slugs also move farther than smaller ones, and joining with others may sometimes be the only way to become large (Foster et al. 2002). This advantage of forming a larger slug may extend to joining with amoebae from a different species entirely, e.g., between the distantly related *D. purpureum* and *D. discoideum* (Fig. 9). Interestingly, in the latter case, the resulting fruiting bodies take on the form of one or the other species rather than being intermediate (Jack et al. 2008). Although each clone contributes some cells to each type, there are predictors as to which clone is likely to prevail in contributing more to spore than stalk. In general, stronger amoebae are more likely to become spores, whether they are stronger because they did not divide recently or because they were fed on a high-sugar diet (Castillo et al. 2011; Gomer and Firtel 1987; Thompson and Kay 2000). The cells that initiate the aggregation are also more likely to end up as spores (Huang et al. 1997; Kuzdzal-Fick et al. 2010). It also appears that not all cells participate in aggregation. These "loner" cells may represent

a form of "bet-hedging," able to take advantage of an improved environment in situ (Dubravcic et al. 2014; Tarnita et al. 2015; Wolf et al. 2015).

Some evidence that cheating, and protection from it, are important aspects of dictyostelid evolution comes from experimental evolution. A single clone passaged through 30 generations in the lab resulted in many lines of cheaters, some of which could not even form fruiting bodies on their own (obligate social parasites; Kuzdzal-Fick et al. 2011). Meanwhile, non-obligate social parasites from the same evolved population were less vulnerable to these cheaters than were naïve clones, indicating that they were evolving resistance to cheating (Levin et al. 2015). Mutation accumulation experiments, where each new generation is initiated with a randomly selected clone, show reduced cheating. This suggests that there is selective pressure to maintain competitive behavior (Hall et al. 2013).

Dictyostelids distinguish kin from non-kin using the tiger genes, *tgrB1* and *tgrC1* (Benabentos et al. 2009; Hirose et al. 2011; Ho et al. 2013). Clones with different *tgrB1*and/or *tgrC1* alleles show clear signs of sorting early in the developmental cycle (Ostrowski et al. 2015). However, sorting is not complete, possibly because mixed tiger genes are a useful marker of chimerism and promote competition within the slug. Kin selection appears to be stronger in *D. purpureum* than in *D. discoideum*, showing that it varies among species (Mehdiabadi et al. 2009; Mehdiabadi et al. 2006). Environmental structure and local growth patterns can also cause much sorting even before the recognition genes would come into play (Buttery et al. 2012; Gilbert et al. 2012; Smith et al. 2014). *Dictyostelium discoideum* also shows molecular evolutionary signatures of social selection. Single gene knockout experiments identify over a hundred genes that cause cheating when knocked out (Santorelli et al. 2008). Social genes also show molecular signatures of frequency-dependent selection, suggesting that rare types are prevailing over more common ones (Ostrowski et al. 2015).

Farming may also influence interactions among clones in Dictyostelia. Some *D. discoideum* clones have enduring symbioses with *Burkholderia* bacteria (Fig. 6), and dictyostelid spores may carry food bacteria, which they release after spore dispersal to generate new food populations (Brock et al. 2011; DiSalvo et al. 2015). Moreover, this appears to be a complex interaction apparently driven by a third non-prey bacterial partner, which is also a *Burkholderia* sp. (Fig. 6; DiSalvo et al. 2015). Thus, the non-prey carried bacteria may perform a protective function, being harmful to other dictyostelid clones without harming their host (Brock et al. 2013; Stallforth et al. 2013). There is also evidence of coevolution between the symbiont bacteria and *D. discoideum* (Brock et al. 2015).

Characterization and Recognition

Taxonomy

Dictyostelid amoebae are essentially indistinguishable among species, and therefore dictyostelid taxonomy is based on aggregation patterns and sorocarp morphology, now augmented with molecular phylogeny. One of the main distinguishing traits is

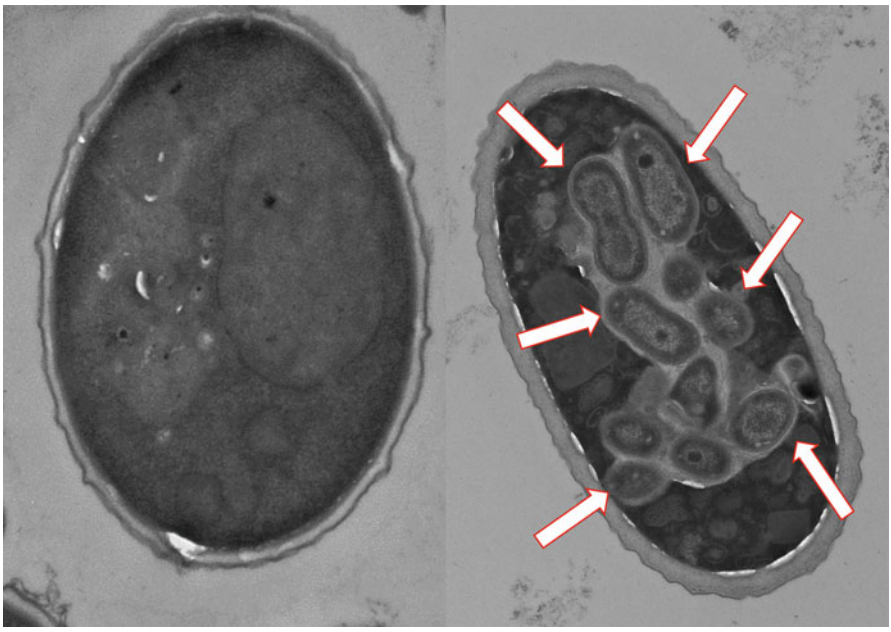


Fig. 6 Micrographs of spores from nonfarmer (*left*) and farmer (*right*) strains of *D. discoideum*. The farmer strain carries bacteria belonging to *Burkholderia* clade 2 (DiSalvo et al. 2015). *Arrows* indicate individual bacterial cells (Micrographs courtesy of Longfei Shu)

the presence (dictyostelids and polysphondylids; Fig. 7b, c) or absence (acytostelids; Fig. 7d) of a cellular stalk. Species with larger sorocarps may also have additional support structures such as a basal disc (e.g., *D. discoideum*; *Cavenderia mexicanum*; *Dictyostelium gargantuum*; Fig. 7e) or a crampon or holdfast base (e.g., *Hagiwaraea* spp.; Fig. 7f). Aggregation characters include acrasin identity, aggregation pattern (presence/absence/degree of streaming; Fig. 7g), the presence/absence of a migration stage (sorogen; Fig. 7h, i), the presence/absence of a stalk during migration (Fig. 7h, i), and patterns of early development (Fig.7j–l). Sorocarps vary in their general habits, such as the presence/absence/degree of clustering (Fig. 8a–d), and in specific sorocarp morphology, such as the presence/absence/degree and pattern of branching (Fig. 8e–i). At least eight acrasins have been identified so far (see above for examples), and acrasin identity remains unknown for most species (Bonner 1983; Schaap et al. 2006).

Spore characters used for identification include size, shape, and the presence/absence/organization of starch granules. Most species have elliptical spores (Fig. 8j, k), but all acytostelids except *Ro. ellipticum* have spherical spores (Fig. 8l, m), as do some dictyostelids (e.g., *Dictyostelium rosarium* and *T. lacteum*). Spore sizes can be as small as 1.5–2.0 × 3.5–5.0 μm but are most commonly in the range of 2.5–3.5 × 6.5–8.0 μm. However, spore size can change with cell ploidy – e.g. – *D. discoideum* spores are in the common size range when haploid but 3.0–4.0 × 10–13 μm

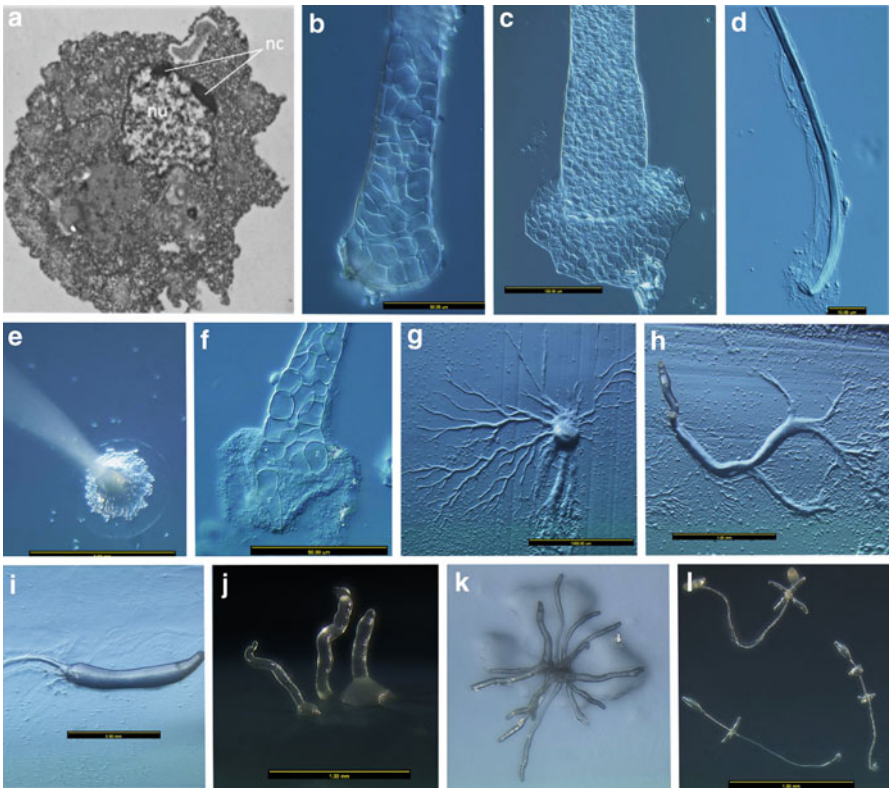


Fig. 7 Transmission electron microscopy (**a**) and light micrographic (**b**–**l**) images of (**a**) dictyostelid amoeba with prominent nucleus (nu) and lobed nucleolus (nc); sorocarp stalk types, (**b**–**c**) cellular (*D. purpureum*, *D. discoideum*), (**d**) acellular (*A. irregulosporum*); sorocarp support structures, (**e**) basal disc (*D. discoideum*), (**f**) crampon base (*Hagiwaraea vinaceofuscum*); aggregation patterns, (**g**) streaming (*D. discoideum*); cell migration behavior, (**h**) stalked (*Dictyostelium implicatum*), (**i**) stalkless (*D. discoideum*); and early sorocarp development, (**j**) Ca. *aureostipes*, (**k**) *Cavenderia multistipes*, (**l**) *He. pallidum* (Photographs courtesy of Longfei Shu (a) and Andrew Swanson and Frederick Speigel (b–l))

when diploid (Sussman and Sussman 1962). Spores can contain starch granules that may be concentrated in their polar regions. Polar spore granules can be either loosely distributed (unconsolidated; Fig. 8j) or compact (consolidated; Fig. 8k) (Traub and Hohl 1976; Hagiwara 1989).

Traditional classification of Dictyostelia recognized three morphologies, previously given the rank of genera (Fig. 2), although these are now recognized as non-monophyletic morphotypes (Fig. 3). Acytostelid types (*Acytostelium* spp. and *Ro. ellipticum*; Fig. 3, Fig. 4i, j, Table 1) produce an acellular stalk tube (Fig. 7d), and therefore all cells in the aggregate survive to form spores. In contrast, dictyostelid and polysphondylid morphotypes have differentiated stalk and spore cells, so that a substantial number of cells in the initial aggregate (~20% in *D. discoideum*; Raper

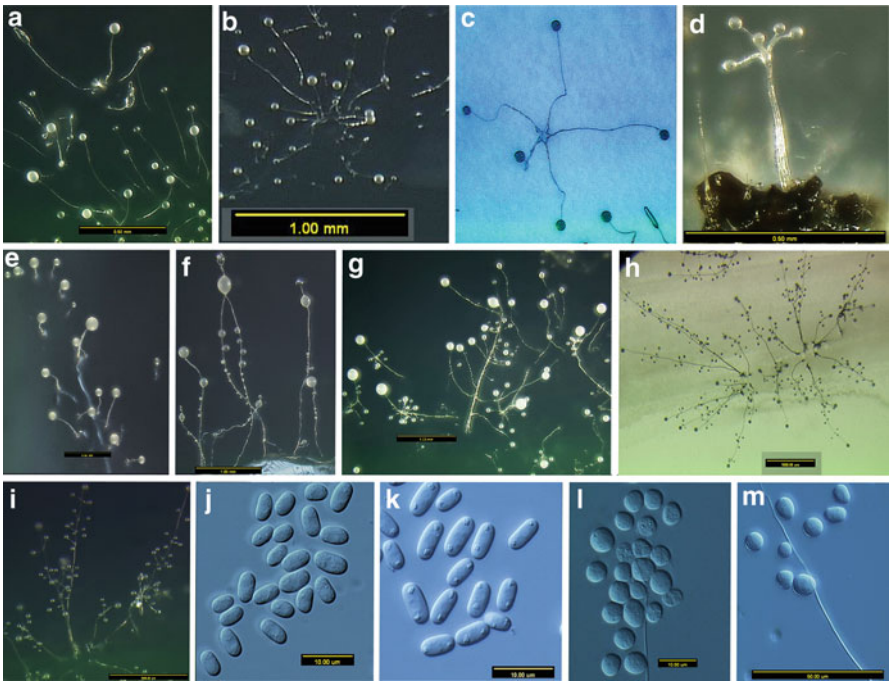


Fig. 8 Light microscopic images of dictyostelid sorocarp characters: habit, (**a**, **b**) gregarious (*A. magnuphorum*, *Ra. minutum*), (**c**) loosely clustered (*Ca. multistipes*), (**d**), coremiform (*Co. polycephalum*); branching patterns, (**e**) unbranched (*Ca. mexicanum*), (**f**) stalkless side branches (*D. rosarium*), (**g**) irregularly spaced side branches (*Ha. vinaceofuscum*), (**h**–**i**) regularly spaced whorled side branches (*He. colligatum*); spores, (**j**) oval without polar granules (*D. mucoroides*, K) oval with consolidated polar granules (*Ca. aureostipes*), (**l**) regular globular (*A. subglobosum*), (**m**) irregular globular (*A. irregulosporum*) (Photographs courtesy of Andrew Swanson and Frederick Speigel)

1984) are sacrificed to build the cellular stalk (Fig. 7b, c). Polysphondylid sorocarps (*Polysphondylium* spp. and many *Heterostelium* spp.; Fig. 3, Table 1.) are characterized by whorls of regularly spaced side branches (Figs. 4c, f–h, 7l, and 8h, i), while all other species with cellular stalks are considered to have dictyostelid-type morphologies (some or all members of all genera except *Acytostelium*; Table 1). Dictyostelid-type sorocarps may be unbranched (e.g., Figs. 4a, b, l, and 8e), irregularly branched (e.g., Fig. 8f, g), and/or consist of clusters of sori that may be gregarious (Fig. 8a, b), loosely clustered (Fig. 8c), or tightly clustered (coremiform; Figs. 4d and 8d).

Molecular phylogenetic analyses based on 18S rRNA and α-tubulin (Schaap et al. 2006) and 18S + ITS rRNA (Romeralo et al. 2011) were the first data to thoroughly reject the traditional classification system. These data divide Dictyostelia into eight distinct divisions, none of which correspond to the three traditional genera, and the taxonomy of the group is currently being revised to accommodate the new

phylogeny (Fig. 3, Table 1). Recent molecular analyses utilizing partial (Romeralo et al. 2013; Singh et al. 2016) and whole (Sheikh et al. 2015) genome data further divide Dictyostelia into two higher-order taxa. The new classification system elevates the two major divisions to the level of order, with the names Acytosteliales and Dictyosteliales, which correspond to, respectively, the molecular groups 1 + 2 and 3 + 4, the latter including also the violaceum and polycephalum complexes (Fig. 3). The major molecular groups are outlined below using the new taxonomy.

Cavenderiaceae (molecular Group 1) consists of a diverse set of dictyostelid morphotypes that are currently assigned to a single genus, *Cavenderia* (Figs. 9 and 10a–c). These species produce sorocarps that vary considerably in size and morphology, ranging from solitary to clustered, with or without side branches. However, the majority of Cavenderiaceae tend to have small sorocarps with irregularly spaced side branches (Romeralo et al. 2011). Although there are still a relatively small number of described species, they are widely distributed including species isolated from Antarctica, Mexico, and Thailand. These species were originally noted as having smaller spores than the other major taxa (Schaap et al. 2006), but two recent isolates, *Cavenderia boomeransporum* (Fig. 10a) and *Cavenderia myxobasis*, have some of the largest spores yet seen in Dictyostelia (Romeralo et al. 2011).

Acytosteliaceae (molecular Group 2) is the most morphologically diverse, including examples of all three morphotypes (Schaap et al. 2006). The family currently consists of three genera, *Acytostelium*, *Heterostelium*, and *Rostrostelium*. *Acytostelium* (molecular Group 2A; Figs. 9 and 10d–f) includes nearly all known acytostelid morphotypes. Thus, *Acytostelium* is the most homogeneous division of Dictyostelia, consisting entirely of small delicate species that do not differentiate stalk cells. In contrast, *Heterostelium* is extremely diverse and includes a mixture of dictyostelid and polysphondylid morphotypes (Figs. 9 and 10g–j). The deepest branches of the group are two molecularly very distinct dictyostelids, *He. oculare* (Fig. 10g) and *Heterostelium boreale*. These are then sister lineages to a dense cluster including all small, unpigmented polysphondylids (Figs. 4f–h, 8h, l, and 10h, i) and a further cluster of small, pale dictyostelids (Fig. 10j). The third genus of Acytosteliaceae is *Rostrostelium*, which consists of a single species, *Ro. ellipticum* (formerly *Acytostelium ellipticum*). Molecular sequences from this isolate place it either as the sister group to *Heterostelium* (Schaap et al. 2006) or the sister group to *Heterostelium* + *Acytostelium* (Singh et al. 2016), but never with the other acytostelid types (*Acytostelium* spp.). Thus, sorocarp morphology is especially plastic in Acytosteliaceae, including gain and loss of regular branching and probably also loss and gain of cellular differentiation.

Raperosteliaceae (molecular Group 3; Fig. 9) includes *Raperostelium*, *Hagiwaraea*, *Speleostelium*, and *Tieghemostelium*. This is morphologically a relatively conservative collection of species, as all assigned taxa have dictyostelid-type morphology. That is, all Raperosteliaceae have sorocarps with differentiated cell types and side branches that are either irregularly arranged or absent (Fig. 10k–m). Nonetheless, taxa vary greatly in size, ranging from species with very small sorocarps, such as *Ra. minutum*, to a distinct cluster of species with relatively

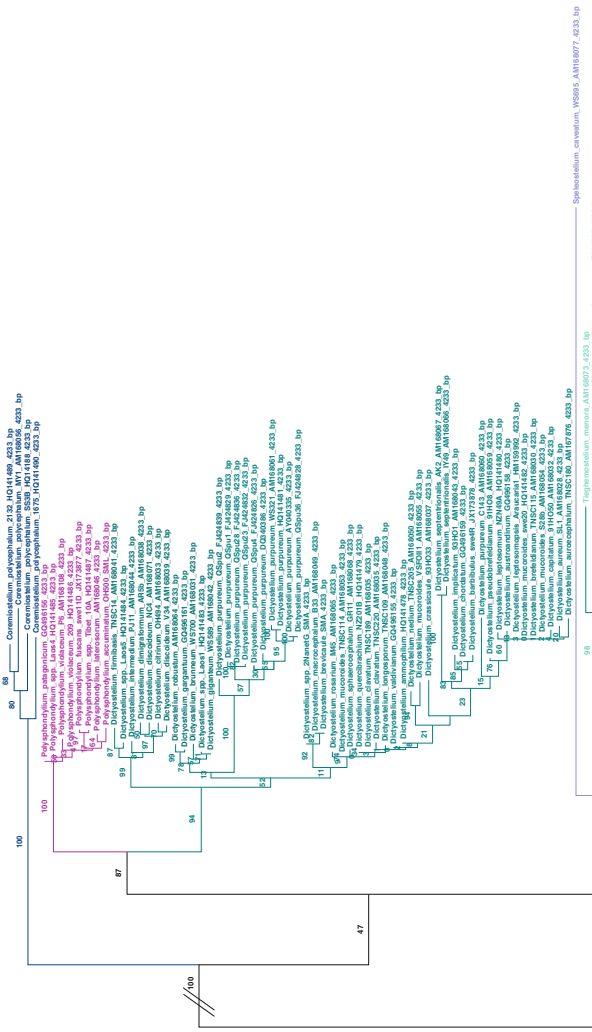
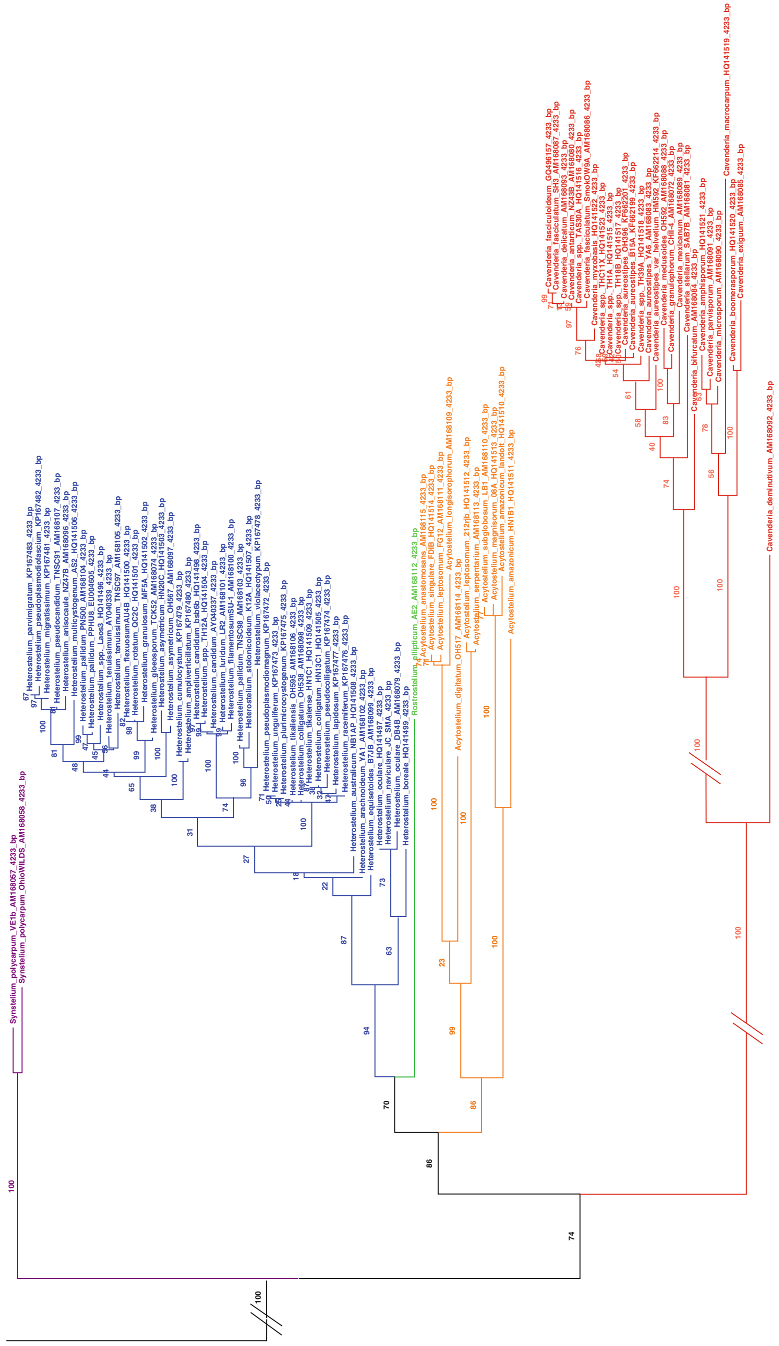


Fig. 9 (continued)



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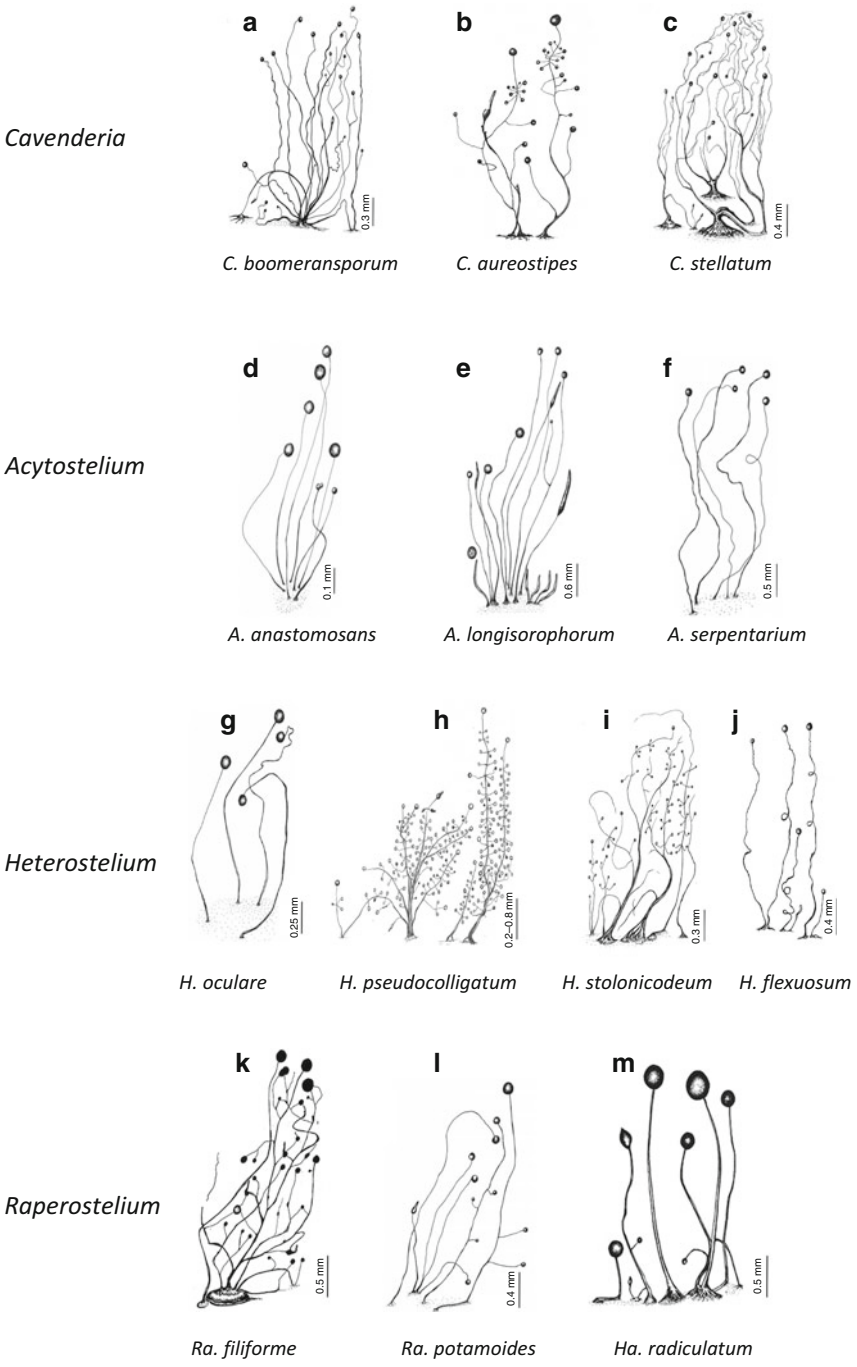
large sorocarps, the *Hagiwaraea* (molecular Group 3B). The latter are especially distinctive in having crampon-like bases to their sorocarps, which likely help to support these relatively large structures (Figs. 7f and 10m). One of the most intriguing members of Raperosteliaceae is *Sp. caveatum* (formerly *Dictyostelium caveatum*; Fig. 9). This is the only dictyostelid known to prey on the amoebae of other species (see above).

Dictyosteliaceae (*Dictyostelium*, *Polysphondylium*, and *Synstelium*) is again morphologically diverse, particularly the redefined *Polysphondylium* (Fig. 3), which includes both dictyostelid and polysphondylid morphotypes. However, unlike the polysphondylid types in *Heterostelium* (Acytosteliaceae; see above), which tend to be delicate and unpigmented, species in *Polysphondylium* tend to have large robust sorocarps with lavender-violet pigmented sori (Figs. 4c and 10q). In fact, the one described dictyostelid morphotype in this group (now, *Polysphondylium laterosorum*) also has lavender-pigmented sori. Meanwhile, *Dictyostelium* (molecular Group 4, Fig. 9) consists purely of dictyostelid morphotypes, including the type species of Dictyostelia, *D. mucoroides* (Fig. 4a), and the model organism *D. discoideum* (Fig. 4b). This is the most speciose genus of Dictyostelia and includes many of the more frequently encountered species (Fig. 10n–p). Isolates of *Dictyostelium* tend to have large aggregates, stalkless migration, and robust sorocarps, often with a single large sorus. However, more recent isolates include species with clustered or coremiform sorocarps, e.g., *Dictyostelium austroandinum* (Fig. 10n) and *Dictyostelium valdivianum* (Fig. 10p). A number of these new species also have polar granules in their spores, a feature previously thought to be universally absent from the group (Schaap et al. 2006). *Dictyostelium s.s.* also appear to be the only Dictyostelia that use cAMP as both a developmental signaling molecular and an acrasin.

Dictyostelia also includes two molecularly very distinct dictyostelid morphotypes whose phylogenetic affinities have been difficult to resolve. *Coremiostelium* (formerly the polycephalum complex; Fig. 3, Table 1) consists of four isolates that are morphologically almost indistinguishable but show as much molecular distance among them as almost any two species in the whole of *Dictyostelium* (Romeralo et al. 2011). Similarly, *Synstelium* (formerly the polycarpum complex; Fig. 3, Table 1) consists of two morphologically similar isolates, with a large molecular distance between them (Schaap et al. 2006; Romeralo et al. 2011). Recently multigene phylogenies place *Coremiostelium* as the first major branch of Dictyosteliales and *Synstelium* as sister to *Heterostelium* + *Rostrostelium* (Singh et al. 2016), although these results still rely on a small number of sequences and conflict with alpha-tubulin and 18S rRNA phylogeny (Schaap et al. 2006).

Fig. 9 A comprehensive phylogeny of Dictyostelia based on 18S rRNA. The tree shown was derived by maximum likelihood analysis of 4233 universally aligned 18S rRNA sequence positions using RAxML (version 7.2.8, GTRGAMMA substitution model) and 1000 bootstrap replicates. Only bootstrap values above 50% are shown. Taxon names are followed by their 18S rRNA GenBank accession number. Colours are used to indicate the different genera (Sheikh et al. in press)



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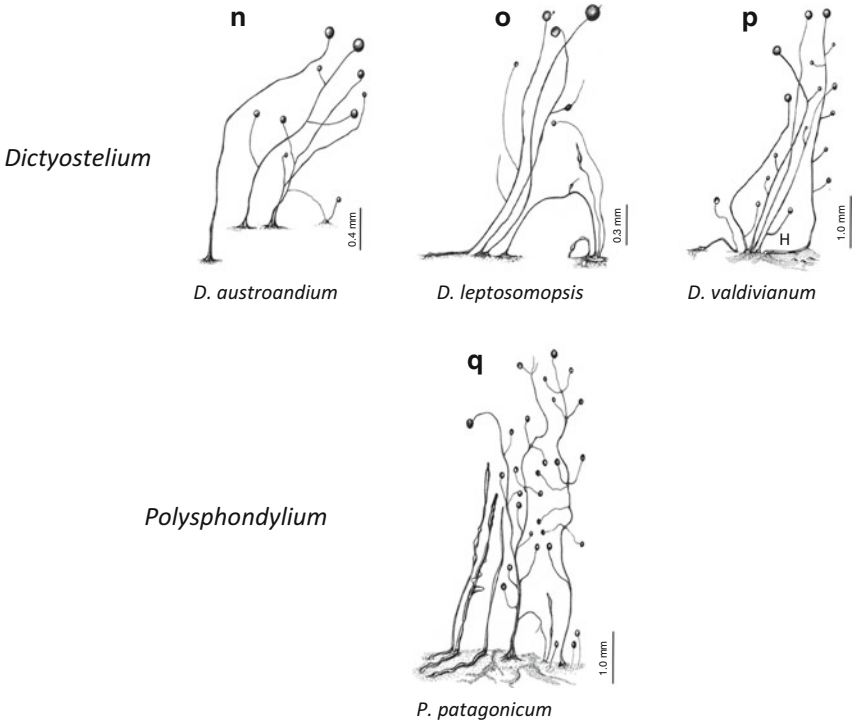

Fig. 10 Examples of recently described species from all major groups within Dictyostelia. Illustrations are reproduced with permission from Cavender et al. (2005) (**c**–**g**, **l**), Vadell et al. (2011) (**n**–**q**), Cavender et al. (2013) (**k**), Landolt et al. (2008) (**a**, **i**–**j**, **m**), Cavender et al. (2016) (**h**), and courtesy of E. Vadell (**b**) (Figure courtesy of S. Sheikh and D. Baldauf)

A reexamination of morphological data in light of molecular phylogeny shows that most of the characters that form the basis of traditional dictyostelid classification are phylogenetically unreliable (Schaap et al. 2006; Romeralo et al. 2011). Among the more broadly consistent characters are spore traits. *Cavenderia* and *Heterostelium* species tend to have oblong spores with tightly grouped (consolidated) polar granules (Fig. 8k), while the spore granules of Raperosteliaceae are loosely grouped (unconsolidated), and polar granules appear to be mostly absent in Dictyosteliaceae (Fig. 8j; Schaap 2007; but see Romeralo et al. 2011). Species in Acytosteliales and Raperosteliaceae often display a clustered or gregarious sorocarp habit, a character that is found dispersed throughout these groups. Meanwhile *Dictyostelium* species mainly form large solitary fruiting bodies, and branched forms are rare. However, sorocarp morphology also appears to be quite plastic, as the incidence of cryptic species seems to be very high (Mehdiabadi et al. 2009; Romeralo et al. 2011).

Recent attempts to isolate new species suggest that there are many Dictyostelia remaining to be discovered using standard isolation techniques alone. In fact, in the

last 10 years, the number of described species has almost doubled, all initially based on morphological characterization and confirmed by molecular phylogeny (Fig. 10). For example, 15 new species were recently isolated from samples collected at one of the most widely studied locations, the Smoky Mountains National Park in North Carolina, USA (Cavender et al. 2005). These include five new acytostelids (e.g., Fig. 10d–f) and ten new dictyostelids (e.g., Fig. 10c, 10g, 10l). Numerous species have been isolated from richly sampled locations such as Tikal National Park in Guatemala (35 new species; Vadell 1993). More recent expeditions to new locales have yielded many new species, such as the Iguazu region of Argentina (11 new dictyostelids, e.g., Fig. 10o, 10p; 3 new polysphondylids, e.g., Fig. 10q; Vadell and Cavender 2007; Vadell et al. 2011), the seasonal rain forests of Central America (ten new dictyostelids, e.g., Fig. 10k; Cavender et al. 2013), and three scattered locations in Australia (three new polysphondylids, six new dictyostelids, e.g., Fig. 10a, 10m; Romeralo et al. 2011). Much of this increased sampling has been supported by the Planetary Biodiversity Inventory (PBI) of Mycetozoa (National Science Foundation, USA; slimemold.uark.edu).

Life Cycle, Ultrastructure, and Genome

Life Cycle Detailed descriptions of the life cycle, development, and ultrastructure of *D. discoideum* are given in Olive (1975), Raper (1984), Kessin (2001), Schaap (2011), and Loomis (2012). Therefore, only minimal descriptions are given here (Fig. 1). The amoebae of *D. discoideum* and most other species emerge upon germination of the spores through a longitudinal split in the spore wall. The amoebae actively pursue their bacteria prey using short filose-like pseudopodia. Upon food depletion or under various other unfavorable environment conditions, amoebae respond by forming microcysts, macrocysts, or fruiting bodies (sorocarps; Fig. 1). Microcysts are a highly resilient resting stage, consisting of a dormant amoeba surrounded by a double-layered cell wall (Khan 2006). These structures differ considerably from dictyostelid spores, which have a three-layered cell wall and more condensed cytoplasm (Hohl et al. 1970; Kawabe et al. 2009), and macrocysts, which are highly resistant with a five-layered cell wall (Fig. 5; O'Day and Keszei 2012). Macrocyst formation represents the sexual cycle in Dictyostelia and leads to the formation of a diploid zygote that attracts and preys upon other haploid cells (O'Day and Keszei 2012). Sorocarp formation transforms free-living amoebae into what, in all but acytostelids, is arguably a true multicellular structure with distinct tissues, differentiated cell types, and a kind of programmed cell death (not homologous to apoptosis).

It is not fully understood how amoebae decide whether to form microcysts, macrocysts, or sorocarps. However, quorum sensing, the ability to detect the density and ratio of surrounding amoebae and prey (bacterial) cells, appears to be important (Du et al. 2015). Essentially, under conditions of low prey density, amoebae will aggregate if they sense the presence of sufficient numbers of fellow amoebae; otherwise they will encyst (Du et al. 2015). Factors affecting macrocyst formation

are more elusive (Raper 1984), and many species cannot be induced to form macrocysts in the lab. However, many species are also only available as a single isolate and may thus be clonal and lack complementary mating types (Schaap et al. 2006). However, in general, there seems to be a requirement for cool, moist conditions, and probably also food shortage and high amoebal density (O'Day and Keszei 2012).

Sorocarp Formation The sorocarpic cell cycle of *D. discoideum* in the lab takes about 72 h from spore to spore at an optimum growth temperature of 22–25 °C. Since there is no food ingestion once sorocarp development begins, the spore and stalk walls and slime sheath are composed largely of cellulose, thought to be derived by amino acid conversion (Freeze and Loomis 1977). However, EM data suggest that cells are full of glycogen early in fruiting, so the direct source of glucose for cellulose formation may in fact be glycogen (F Spiegel, pers. comm.). Lack of food induces amoebae to begin preparation for aggregation by synthesizing acrasins and the receptor proteins that enable them to respond to acrasin and other external chemical factors. The amoebae also synthesize species-specific cell adhesion proteins, particularly TgrB1 and TgrC1, whose polymorphic extracellular domains aid the amoebae in identifying close relatives (Strassmann and Queller 2011; Du et al. 2015). At the same time, ammonia and certain other environmental factors can halt or even reverse development and induce amoebae of some species to form microcysts instead (Lonski 1976). For this reason, some dictyostelids can be encouraged to fruit in the lab by placing small amounts of activated charcoal in the culture dish.

Aggregation is initiated in *D. discoideum* by a sharp increase in cyclic AMP (cAMP) production, called an acrasin pulse. The pulse, which is repetitive (every 6 min in laboratory conditions), produces directional pseudopodial responses in starving, receptive cells, whose receptor sites are concentrated at one end (polarised) (Swanson and Taylor 1982). Subsequent cell movement occurs along the acrasin gradient. Responding cells degrade the incoming acrasin and then release an acrasin pulse of their own. Thus, pulses occur periodically (Durston 1974), each followed by a refractory period during which background cAMP is cleared by phosphodiesterase (Gerisch and Hess 1974). This gives rise to waves of signal propagating outward from the aggregation center. As cells begin to contact each other, they adhere and form streams, creating aggregation patterns that vary among species. As the cells gather into a mound, the tip of the mound takes on the role of an organizing center, continuing to secrete pulses of acrasin and also cAMP (or additional cAMP if this is also the acrasin). This causes the cells within the mound to begin differentiating into prespore and prestalk cells (Schaap 2011).

As amoebae stream into the mound, the tip is forced upward until the whole pseudoplasmodial mass falls over and becomes a slug. The slug is a polarized multicellular unit with a specialized ("head") region capable of detecting signal and directing migration of the slug toward differences in temperature, relative humidity, solute concentration, and light. The slug moves as a unit by means of a coordinated helical motion of the individual cells inside a slime sheath (Clark and Steck 1979). It is organized from the tip (Rubin and Robertson 1975), which

continues to emit pulses of cAMP. Waves of cell contraction and elongation appear to proceed from tip to rear at regular intervals. The cells of the *D. discoideum* slug are partially differentiated (Bonner 1952), with the anterior approximately 20% (Bonner and Slifkin 1949) destined to become stalk cells and the remaining posterior cells to become spores, except for the rearguard cells, which form the disc (Raper 1940). It should be noted that many species lack a migratory phase, in which case the sorocarp arises directly from the mounded aggregate.

In some migrating species, the sorocarp stalk is secreted continuously during migration, while in others, such as *D. discoideum*, stalk formation only begins after migration stops. In the latter case, the prestalk cells begin by secreting a stalk tube that is brought to the agar surface by flattening of the slug ("Mexican hat" stage, Fig. 1; Raper and Fennell 1952). As the stalk tube forms, prestalk cells migrate down into the tube in an inverted fountain movement. Once inside the tube, these cells vacuolate, construct cellulose walls, and die. The rearguard cells of the slug, which form the basal disc, also vacuolate and die. The prespore cells then move up the growing stalk where they eventually differentiate into spores encompassed by slime (Fig. 1). Construction of the *D. discoideum* sorocarp takes about 8 h in the lab.

Dictyostelid sorocarps do not dry out and release individual spores for wind dispersal, as in ▶ Myxomycetes but rather stay together as a unit so that the spores are dispersed en masse. Thus, dictyostelid spores are probably not transported long distances by wind. Instead, sorocarps tend to be transported by water or animals such as insects, rodents, amphibians, bats, birds (Suthers 1985; Stephenson and Landolt 1992), and even large mammals (Perrigo et al. 2012). This is presumably aided by having small erect fruiting bodies (Loomis 2012; Huss 1989), and there may be a fitness trade-off between having large numbers of spores that can be distributed together (single large sporehead or "sorus") versus smaller numbers of spores potentially distributed to multiple locations (accessory sori, branched, or grouped sorocarps). It has also been postulated that clustered fruiting bodies may lessen the potential for desiccation in drier habitats (Romeralo et al. 2013).

The Macrocyst Formation of a zygotic cyst or "macrocyst" marks the sexual cycle in Dictyostelia (Fig. 5). Its function was not discovered until the 1970s (Erdos et al. 1973), based on ultrastructure studies showing the presence of a synaptonemal complex (Erdos et al. 1972). This was confirmed by genetic studies indicating the production of recombinant progeny (Erdos et al. 1975; Okada et al. 1986; Francis 1988) and further studies detailed in O'Day and Keszei (2012). Macrocyst formation requires fusion competence (Blaskovics and Raper 1957). Other important factors that have been identified include a combination of environmental factors, particularly darkness (Hirschy and Raper 1964), excess water (Weinkauff and Filosa 1965), ethylene (Amagai 1984), low phosphate, and the presence of calcium ions (O'Day and Keszei 2012). However, these undoubtedly vary between species so the list is far from complete, nor is it exclusive.

Macrocyst formation begins with the formation of fusion-competent cells (Blaskovics and Raper 1957; O'Day et al. 1987). In *D. discoideum*, a tripartite mating locus gives rise to three mating types, any one of which can mate with any

other (Bloomfield et al. 2010) resulting in heterothallic, homothallic, or bisexual fusions (Fig. 5; O'Day and Keszei 2012). However, *D. mucoroides*, *Ra. minutum*, and *P. violaceum* appear to be homothallic (O'Day and Keszei 2012), although the possibility of apomixis cannot be ruled out. Competent cells aggregate together, until two compatible cells meet, attach, and fuse to form a binucleate zygote. The two nuclei eventually fuse, producing a giant cell or zygote (McConachie and O'Day 1987). The zygote then attracts other cells to the aggregate by release of cAMP and additional chemical signals (pheromones) (O'Day 1979; Saga and Yanagisawa 1983; O'Day et al. 1987; Amagai 1984). This aggregation process shares similarities to pre-sorocarpic aggregation in *D. discoideum*, including cAMP pulses and cell streaming, but on a much smaller scale, as only ~200 cells contribute to the sexual aggregate (O'Day 1979).

Eventually, the collective becomes surrounded by a sheath, either secreted by the zygote or by the collective as a whole. This may serve to protect the developing aggregate but also effectively prevents the non-zygotic amoebae from escaping. The zygote gradually devours these captive cells, using the ingested material to grow increasingly larger as well as gradually building up a five-layered cell wall. The process, which can take several weeks, results in a dormant highly resilient macrocyst that can be difficult to germinate in the lab. Before germination occurs, the giant cell undergoes meiosis, from which only a single meiotic product survives. This is followed by multiple rounds of cell division so that upon germination, the macrocyst releases a mass of haploid trophic amoebae (Fig. 5; Okada et al. 1986).

Ultrastructure Solitary feeding amoebae have relatively broad hyaloplastic pseudopodia with acutely pointed subpseudopodia, sometimes referred to as filose (Fig. 7a; Olive 1975). These amoebae move in a slow, smooth manner, unlike the eruptive manner of acrasid amoebae (see ▶ Heterolobosea). Cells are somewhat rounded while feeding, with a size of about 10–15 μm in diameter, but they assume a distinctive elongated form during aggregation. The nucleus is characterized by a single peripheral nucleolus, which is digitate so that it appears as two to five dense masses of RNA devoid of dense chromatin and lying in tight contact with the nuclear membrane (Fig. 7a; Benichou et al. 1983).

Fructifications (sorocarps) may be clustered or branched or regularly whorled (Figs. 4, 8a–i, and 10). The aggregates of *Dictyostelium* (molecular Group 4) species usually remain together as a unit and give rise to a solitary fruiting body that is only rarely branched and, even then, mostly only sparely (Figs. 4a, b, and 8e). In contrast, other species throughout the tree tend to split up their aggregates into multiple sorogens, which may then subdivide even further to yield clustered fruiting bodies (Fig. 4e–i). The generally larger structures of *Dictyostelium* species are typically supported at their base by a basal disc or triangular supporter (Fig. 7c, e), which are derived from a third cell type, the anterior-like cells. In at least one *Dictyostelium* species, *D. discoideum*, this cell type diverges even further to produce two more structures, the upper and lower cup that serve to support the relatively large spore head. Thus *D. discoideum* and probably other *Dictyostelium s.s.* species differentiate five distinct cell types. The sorocarps of the crampon-based species of *Hagiwaraea*

also tend to be relatively large (Fig. 7f), suggesting a general correlation in Dictyostelia between sorocarp size and cell type diversity.

Genome The first dictyostelid genome, that of *D. discoideum*, was published in 2005 (Eichinger et al. 2005). It is 34 megabases (Mb) in size with six chromosomes encoding an estimated 12,500 proteins. Although most genes contain introns, these are small in size (150 base pairs on average), similar to many other microbial eukaryotes. The genome is extremely AT rich (70–80%). Many protein-coding genes carry long tracts of triplet repeats, and these are translated into repetitive amino acid tracts that are retained in the mature proteins (Eichinger et al. 2005). Such repeats are thought to contribute to evolutionary plasticity (e.g., Radó-Trilla et al. 2015), and while they are common in eukaryotes, including humans, the extent in *D. discoideum* is so far unrivaled (Scala et al. 2012). These sequences are generally poorly conserved, and there is little similarity in their size or location between *D. discoideum* and *D. purpureum* (Sucgang et al. 2011), which are both species of *Dictyostelium s.s.*, albeit distantly related (Fig. 9).

In addition to *D. discoideum*, full or advanced draft genome sequences are available for *D. purpureum* (Sucgang et al. 2011), *Ra. lacteum* (Du et al. 2015), *Cavenderia fasciculatum* (Heidel et al. 2011), *He. pallidum* (Heidel et al. 2011), and *A. subglobosum* (Urushihara et al. 2015). Thus, there are now genome data for at least one representative of each of the five largest molecular groups (Fig. 3). The genomes range in size from 31 to 34 Mb, except for *Raperostelium lacteum* which appears to be ~22 Mb (Du et al. 2015). The genomes seem to encode similar numbers of genes, and the differences in genome size are mostly due to varying levels of noncoding DNA such as intergenic spacers and introns (Du et al. 2015). It is interesting to note that these genomes are all considerably smaller than that of the solitary amoeba *Acanthamoeba castellanii*, which has a 45 Mb genome with 15,455 predicted genes (Clarke et al. 2013). Genome data have also recently been reported for representatives of the remaining major divisions of Dictyostelia – *Co. polycephalum*, *P. violaceum*, *Ro. ellipticum*, and *Synstelium polycarpum* (Singh et al. 2016).

Maintenance and Cultivation

Dictyostelids and their fruiting bodies are too small and sparse to be easily observed in nature. However, many are relatively easy to isolate and grow in the lab (reviewed in Douglas et al. 2013). The superficial layers (0–3 cm depth) of almost any forest soil will yield four to eight species that can be identified on isolation plates, although species have been found as deep as 20–30 cm (JES unpublished). Isolation involves placing diluted soil samples on nutrient-poor agar (e.g., hay infusion agar; Table 2), followed by incubation at room temperature for several days until aggregates and/or fruiting bodies begin to appear (slower-growing species, such as acytostelids, may require up to 6 days before aggregates appear).

Cavender (1990) lists five important factors for isolating dictyostelids from soil:

Table 2 Media used in culturing dictyostelids (Raper 1951)

Hay infusion (HI)		NTGY		Standard LP (0.1%)		MY	
Component	[g/li]	Component	[g/li]	Component	[g/li]	Component	[g/li]
Specific							
Weathered mature grass (*Poa* spp.) (filtered)	8.0	Tryptone	5.0	Peptone	1.0	Malt extract	0.002
$Na_2HPO_4 \cdot 12H_2O$	0.96	Glucose	1.0	Lactose	1.0		
General							
KH_2PO_4	1.5		1.0		–	(Optional)	0.75
Yeast extract	–		5.0		–		0.002
Agar (Difco)	15.0		15–20		15.0		15.0

1. A low nutrient medium lacking inhibitors
2. Some buffering capacity
3. A low aqueous soil dilution (< 1:50)
4. Provision of a pregrown bacterial food source
5. The use of fresh, undried, unfrozen surface soil and leaf mold
6. A few charcoal grains added to lids of inverted plates (optional)

Freezing kills trophic cells (Cotter and Raper 1968). However, this also provides a means of measuring percentages of active versus resting propagules. Dictyostelid species differ greatly in their abundance in soil and their sensitivity to culture media used for their isolation. According to Cavender (1990), dictyostelids fall into three general types in terms of ease and conditions for isolation.

Type A. Larger, More Vigorously Growing, and Easily Isolated Species These consume heavier growths of bacteria and tolerate higher concentrations of phosphate buffers and soluble nutrients. Examples are *D. discoideum*, *D. purpureum*, *D. sphaerocephalum*, *Dictyostelium giganteum*, *D. gargantuum*, and *P. violaceum*. It should be noted that it is possible to isolate some of these robust species of Dictyosteliaceae without added bacteria, as they may often carry their own (i.e., farming species; see above).

Type B. Much Smaller and More Delicate Species These consume only light growths of bacteria and are inhibited by nutrient and phosphate buffer concentrations suitable for type A species. Examples are *He. oculare*, *Co. polycephalum*, *Sy. polycarpum*, *Ca. stellatum*, *Ra. lacteum*, *Cavenderia deminutivum*, *Ra. minutum*, *Acytostelium leptosomum*, *Ro. ellipticum*, and *Acytostelium irregulosporum*.

Type C. Species of Intermediate Size and Sensitivity Examples include *D. rosarium*, *Cavenderia aureostipes*, *D. aureum*, Ca. *fasciculatum*, and *He. pallidum*. These grow best on conditions intermediate between types A and B.

A very weak hay infusion medium is recommended to isolate all three groups together (Table 2; Cavender and Raper 1965a; Douglas et al. 2013). It is preferable to use fresh agar plates (~24 h), although older plates (several weeks) have been used routinely for some *Dictyostelium* species (Fortunato et al. 2003a). Bacteria, typically *E. coli* (B/r, 281 K.B.R., or other common laboratory strain) or *K. aerogenes* (900 K. B.R.), are pregrown for 24 h at 30° on NTGY (*E. coli*) or SM medium (*K. aerogenes*). A 1:50 soil/water dilution is made using two dilutions (1:10, 1:25) to allow coarse particles to settle. Dilutions are gently shaken to avoid damage to trophic cells (Kuserk et al. 1977). An 0.5 ml aliquot of the 1:50 dilution is added to each hay infusion plate along with 0.4 ml of a heavy bacterial suspension (white in appearance). These are mixed over the surface by tilting, and the lid of the petri dish is set ajar until the excess water evaporates. If the agar surface is level, the suspension will remain uniformly spread.

Some species such as *A. leptosomum* and *Raperostelium tenue* need charcoal for optimal fructifications (Cavender 1990). Since charcoal is never detrimental to cultures of Dictyostelia, we recommend its routine use (Douglas et al. 2013). This seems to be related to the capacity of charcoal to absorb some gas, probably NH_3, which inhibits the formation of fruiting bodies (Bonner et al. 1986). Clones of cellular slime molds begin to develop in the bacterial lawn after 3–6 days at 22–25 °C. Overhead illumination at this time improves the development of dictyostelid clones, which are most distinct when other soil amoebae are limited in number (e.g., in acid forest humus). Media used to culture dictyostelids are shown in Table 2 (Raper 1951). Two-member culture methods have been reviewed by Raper (1984). A standard medium containing non-nutrient agar (NNA) is preferred to be able to compare morphological descriptions of all species.

Individual species are isolated by replating. To do this, the sorus of the desired isolate is touched with a glass needle and transferred to bacterial streaks made on a fresh NNA plate using a suspension of the host bacterium. Growth types A, B, and C all grow on NNA, although type B and C species generally respond favorably to charcoal crumbs placed in the lid of an inverted culture dish (Raper 1984). Culture plates are incubated in darkness or diffuse light at 20–25 °C. However, some species require different temperatures, such as *Dictyostelium septentrionalis* and *Raperostelium australe* (15–20 °C), *Co. polycephalum* (25–30 °C), and *Cavenderia antarctica* (15–18 °C), reflecting their environments of origin (Bonner and Lamont 2005). Submerged culture methods, first developed by Gerisch (1959), are routinely used for experimental studies. Axenic culture on defined media is possible for *He. pallidum* (Goldstone et al. 1966) and *D. discoideum* (Franke and Kessin 1977), allowing for isolation of auxotrophic mutants for genetic analyses. For suspension of pregrown amoebae, Bonner's salt solution (1947) is used.

Long-term preservation of spores is best achieved by freezing spores with glycerol buffer or with the medium HL5 (Franke and Kessin 1977). Spores can also be stored by lyophilization, dessicated in silica gel (Raper 1984), or frozen in glycerol. For further details on isolation and cultivation of dictyostelids, see Douglas et al. 2013.

Evolutionary History

The phylogenetic position of Dictyostelia has been controversial for much of its scientific history. Much of the early study of Dictyostelia was conducted by mycologists, who placed it within kingdom Mycetae (Fungi) (DeBary 1857). As a result, current dictyostelid nomenclature is based on the International Code of Nomenclature for Algae, Fungi, and Plants, and their systematics traditionally follows botanical rules. Early molecular studies were also problematic. The earliest 18S rRNA trees to include both *D. discoideum* and a myxogastrid, *Physarum polycephalum*, placed them as separate relatively early diverging branches of eukaryotes (Sogin et al. 1986). This was eventually recognized as an artifact due to a combination of

inadequate taxon sampling, uneven evolutionary rates, and the nucleotide compositional bias of *D. discoideum* (A+T rich) versus *Ph. polycephalum* (G+C rich) rRNAs (Spiegel et al. 1995; Baldauf and Doolittle 1997; Bapteste et al. 2002; Fiore-Donno et al. 2005). Taxon-rich multigene trees now unambiguously place Dictyostelia and Myxomycetes/Myxogastria together, in some cases with certain protostelid sister taxa (Baldauf and Doolittle 1997; Shadwick et al. 2009; Cavalier-Smith et al. 2016). The latter grouping was first designated as Eumycetozoa (Olive 1975), but this is now recognized as invalid because protostelids are not monophyletic see ▶ Protosteloid Amoebae). Dictyostelia is now confidently placed with Myxomycetes in Macromycetozoa (Fiore-Donno et al. 2010). This is a division of the Conosa branch of Amoebozoa, the bulk of whose diversity consists of solitary amoebae (Pawlowski and Burki 2009).

Within Dictyostelia, the acetyostelid morphotypes were long thought to be the earliest branch, diverging before the evolution of cellular differentiation. Meanwhile polysphondylids were thought to be very derived, because of the apparent complexity of their highly ordered sorocarps. However, molecular phylogeny places acytostelids embedded within a morphologically complex Acytosteliaceae, along with dictyostelid and polysphondylid morphotypes (Fig. 3). Thus, the simple morphologies of acytostelids appear to have evolved by loss of ancestral complexity. Meanwhile the polysphondylid morphology has evolved at least twice independently (within *Heterostelium* and Dictyosteliaceae; Fig. 3). Arguably the most "complex" dictyostelids are found in *Dictyostelium*; e.g., *D. discoideum* sorocarps contain at least five differentiated cell types (Schaap 2007).

The first rRNA phylogeny of Dictyostelia suggested molecular Group 1 (now, Cavenderiaceae) as the first major branch, albeit without significant statistical support (Schaap et al. 2006). This suggested a possible trend toward larger size and complexity in Dictyosteliaceae (Alvarez-Curto et al. 2005; Schaap 2007). However, due to the highly divergent nature of mycetozoan rRNAs, the distance between the ingroup and outgroup is immense. More recent attempts to root the tree with multigene data confidently place the root between Dictyosteliales and Acytosteliales (Romeralo et al. 2013; Sheikh et al. 2015; Singh et al. 2016). Although both families are morphologically diverse, Acytosteliales contains representatives of all three morphotypes including all acytostelids and most of the polysphondylids, while Dictyosteliales consists largely of dictyostelid morphotypes (Sheikh et al. 2015).

The fundamental split of Dictyostelia into Dictyosteliales and Acytosteliales indicates that the last common ancestor of Dictyostelia already possessed most of the notable traits of the group. These include streaming aggregation, acrasin signaling, cellular differentiation, and developmental regulation by extracellular cAMP signaling, microcysts, macrocysts, and a sexual cycle involving aggregation and cannibalism. Nonetheless, a few scenarios can be postulated with reasonable confidence. Microcysts were undoubtedly inherited from their amoebozoan ancestors, as this is a common survival trait among solitary amoebae (Du et al. 2015). Extracellular cAMP-controlled development probably evolved from intracellular cAMP signaling, which is used to regulate encystation in the single-celled amoebozoan

Acanthamoeba (Kawabe et al. 2015). Acrasin signaling may have evolved from prey detection pathways; e.g., the acrasin folate is also a common by-product of bacterial metabolism and promotes chemotaxis and phagocytosis in various Dictyostelia (Pan et al. 1972, 2016). The use of cAMP as an acrasin, however, appears to have evolved only in *Dictyostelium*, probably early in the evolution of the genus. This involved duplication of an ancestral cAMP cell surface receptor that is still used throughout Dictyostelia for developmental signaling (Alvarez-Curto et al. 2005).

Nonetheless, size has probably been an important force in dictyostelid fruiting body evolution. The most consistently large sorocarps are found among species of *Dictyostelium s.s.* These have the largest sori and the thickest and longest stalks. This larger size was probably facilitated by coevolution of additional cell types to help these larger sorocraps remain erect (Schaap 2007). The largest sorocarps in Raperosteliaceae, those of *Hagiwaraea*, have also evolved an additional support structure in the form of a digitate crampon-like base, which undoubtedly helps stabilize these structures. Increased branching or the presence of whorls may also help stabilize some larger sorocarps (Romeralo et al. 2013).

Among the major groups, *Dictyostelium* is of particular interest as it includes the model species, *D. discoideum.* The genus is molecularly shallow but species rich, and it is dominated by relatively robust species, many of which are abundant in a wide variety of habitats. The latter may be due to their generally robust sorocarps, which may better survive long dispersal times and/or avoid decomposition in humus soil (Schaap 2007). However, the frequent recovery of these species could also reflect, at least in part, their ease of cultivation in the lab. Another factor that may aid the development of larger sorocarps in *Dictyostelium* is stalkless migration, which in *D. discoideum* allows the slug to move a considerable distance away from its aggregation site without shedding cells along the way (Bonner 2006).

The evolution of the polyspondylid morphotype is intriguing, as this striking morphology has evolved at least twice independently (Schaap et al. 2006). The majority of polysphondylids, particularly the numerous species with small unpigmented sorocarps (e.g., *He. pallidum*), are now assigned to *Heterostelium*, where they are embedded within a scattering of small unpigmented dictyostelid morphotypes and possibly also acytostelids (Fig. 9). Meanwhile, the robust violet-colored polysphondylids, most notably the type species *P. violaceum*, are found clustered with a robust violet-colored dictyostelid (*P. laterosorum*) forming the genus *Polysphondylium* (Fig. 3; Romeralo et al. 2011). This suggests that pigmentation and sorocarp size are more evolutionarily conservative than branching pattern. The abundance of cryptic species throughout Dictyostelia (Romeralo et al. 2011) further suggests that few genes may be involved in specifying sorocarp branching patterns (Schaap et al. 2006).

The possibility that acytostelid simplicity is derived was first suggested by Bonner (1982). However, this scenario is further complicated by the fact that acytostelid morphotypes are not monophyletic, with *Ro. ellipticum* as the sister group to either *Heterostelium* (Fig. 9; Schaap et al. 2006; Romeralo et al. 2011) or to all other Acytosteliaceae (*Heterostelium* + *Acytostelium*; Singh et al. 2016). Either scenario requires that either acytostelid-type morphological reduction occurred twice

independently, once in an ancestor of *Acytostelium* and once on the lineage leading to *Ro. ellipticum*, or that multicellularity was re-invented early in the evolution of *Heterostelium*. Additional isolates and, if possible, closer relatives of *Ro. ellipticum* need to be examined to better understand what might have occurred and how.

Dictyostelia is an ancient and molecularly deep taxon. Understanding of the molecular, behavioral, and developmental biology of *D. discoideum* is very sophisticated, and this knowledge is now being extended across the diversity of the group. However, given the depth and antiquity of the taxon, there are still relatively few species known. This and the facts that the deepest branches tend to be occupied by small delicate elusive species and most localities across the world have been only sparsely sampled, if at all, suggest that much of the diversity of the group remains to be discovered. It will be especially useful to find taxa that break up some of the longer branches as well as relatives of the enigmatic *Co. polycephalum*, *Sy. polycarpum*, *Sp. caveatum*, and *Ro. ellipticum*, all of which occupy interesting junctures in the tree. New genomic data from these lineages (e.g., Singh et al. 2016) and the continued isolation of new species from the wild (e.g., Cavender et al. 2016) should help resolve their phylogeny and further understand evolutionary, ecological, and behavioral patterns in Dictyostelia.

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Choanoflagellatea

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Abstract

Choanoflagellates are a group of unicellular and colonial heterotrophic flagellates within the Opisthokonta. The characteristic choanoflagellate cell morphology, present in all species, is an ovoid to round protoplast with one apical flagellum surrounded by a collar of microvilli. Phylogenetic studies indicate that choanoflagellates are the closest known living relatives of animals, which has led to a focus on this group to reconstruct the history of animal evolution. Choanoflagellates display a worldwide distribution from the Arctic to Antarctica, in fresh, marine, and brackish water, and they have also been detected in soil ecosystems. In aquatic habitats they play an essential role in the microbial food web as highly efficient filter feeders. Historically, choanoflagellate taxonomy has been based on morphological features, but recent molecular sequence data have refined the taxonomy and revealed several prominent discrepancies. All choanoflagellates belong to one of two sister groups: the Acanthoecida, which construct a siliceous extracellular structure known as a lorica, and the Craspedida, which lack a lorica but may possess an organic extracellular structure called a theca. The loricate Acanthoecida are comprised of two well-described subgroups, distinguished by characteristic differences in how their loricae are constructed: the Acanthoecidae, or nudiforms, and the Stephanoecidae, or tectiforms. To date, two choanoflagellate genomes have been sequenced, both craspedids: *Monosiga brevicollis* and *Salpingoeca rosetta*. The life cycle of *S. rosetta* has been most thoroughly characterized, with sexual and

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asexual phases, sessile thecate single cells, slow and fast free-swimming cells with distinct morphologies, and both chain and rosette colony types.

Keywords
Choanoflagellate • Flagellate • Heterotroph • Bacterivore • Metazoa • Holozoa • Opisthokonta • Multicellularity • Lorica • Theca

Contents

Summary Classification

- **Choanoflagellatea**
- **Craspedida**
- **Salpingoecidae** (e.g., *Codosiga*, *Monosiga*, *Salpingoeca*)
- **Acanthoecida**
- **Acanthoecidae** (*Acanthoeca*, *Helgoeca*, *Polyoeca*, *Savillea*)
- **Stephanoecidae** (e.g., *Bicosta*, *Diaphanoeca*, *Pleurasiga*, *Stephanoeca*)

Introduction

General Characteristics

Choanoflagellates are unicellular and colonial microbial eukaryotes found ubiquitously in marine and freshwater environments, where they are prevalent in both pelagic and benthic communities. They are heterotrophic phagotrophs whose distinctive cell morphology is defined by a collar of microvilli surrounding a single apical flagellum. The undulation of the flagellum creates water currents that transport suspended food particles, primarily bacteria, to the collar, which is thought to serve as a filter (Lapage 1925; Pettitt et al. 2002).

There are two recognized groups within the Choanoflagellatea: the Craspedida (referred to as craspedids; roughly 210 described species) and the Acanthoecida (referred to as acanthoecids or loricates; roughly 150 described species). Craspedid cells either lack a firm extracellular investment, or they may be surrounded by a thin fibrillar coat called a glycocalyx, or they may possess an organic investment called a theca (Figs. 1a and 2). The theca is an organic structure, although its precise composition has yet to be characterized (Norris 1965; Leadbeater 1980; Parke and Leadbeater 1977). In contrast, all known acanthoecids produce a silica-based extracellular structure called a lorica, which is constructed from costal strips joined to form costae (Thomsen 1973; Leadbeater and Manton 1974) (Figs. 1b and 3). The acanthoecids are composed of two subgroups: the Stephanoecidae, or tectiforms, and the Acanthoecidae, or nudiforms. These two subgroups are distinguished on the basis of their morphology during cell division and on the point in the cell cycle when costal strips are accumulated and subsequently assembled into a lorica (Leadbeater 2008; see the section on "Life Cycle" below).

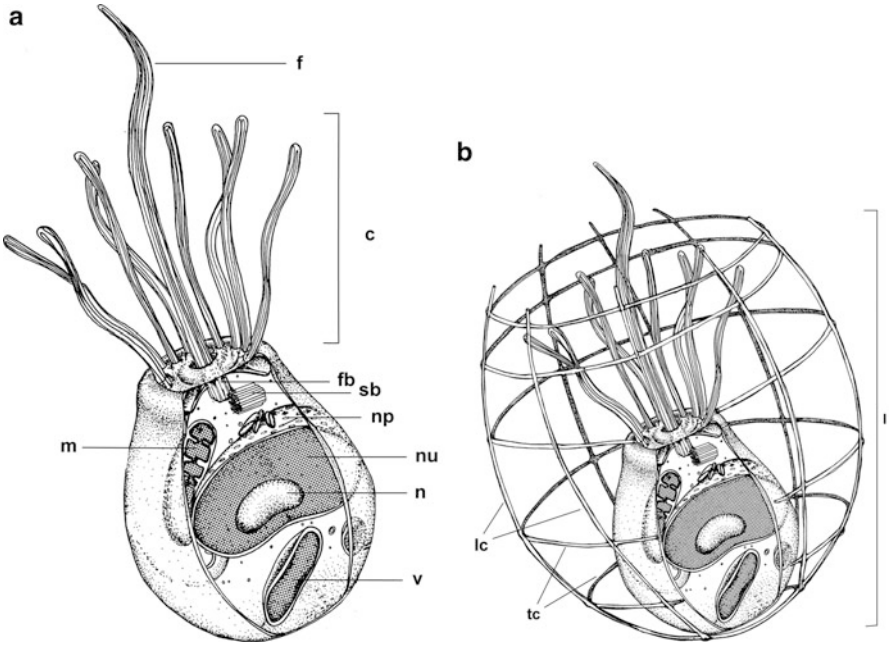

Fig. 1 Drawings of typical choanoflagellate cells. (**a**) Cell depicted with no extracellular structures. The basic features of the choanoflagellate cell are found in both craspedids and acanthoecids (loricates). *c* collar of microvilli, *f* flagellum, *fb* flagellar basal body, *m* mitochondrion, *n* nucleolus, *nu* nucleus, *np* nuclear pores, *sb* second basal body, *v* food vacuole. The Golgi apparatus, which in many species is found adjacent to the nonflagellar second basal body, is not depicted in this drawing. (**b**) An acanthoecid (loricate) cell. *l* lorica, *lc* longitudinal costae, *tc* transverse costae. Drawings adapted from the first edition of this book (Buck 1990), originally by Steven Alexander

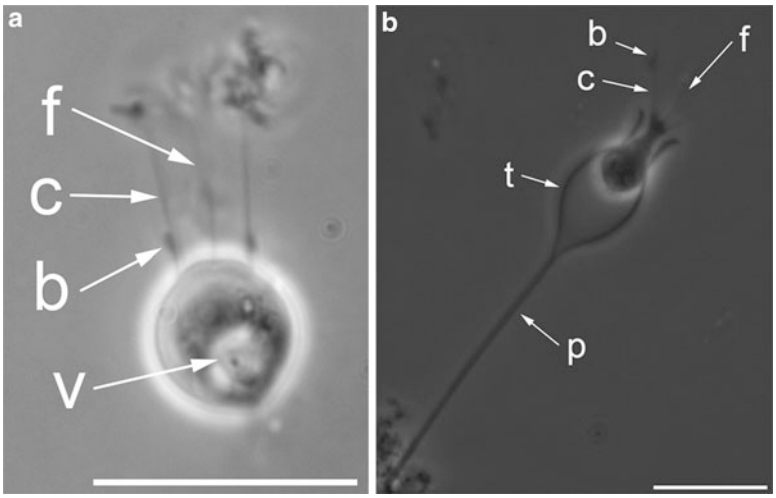

Fig. 2 Phase contrast light microscopy images of craspedid choanoflagellates. (**a**) *Codosiga hollandica*, a freshwater stalked species without a theca. (**b**) An undescribed freshwater flask-shaped thecate species from the River Rhine, Germany. *b* bacterium, *c* collar, *f* flagellum, *p* pedicel, *t* theca, *v* food vacuole. Scale = 10 μm

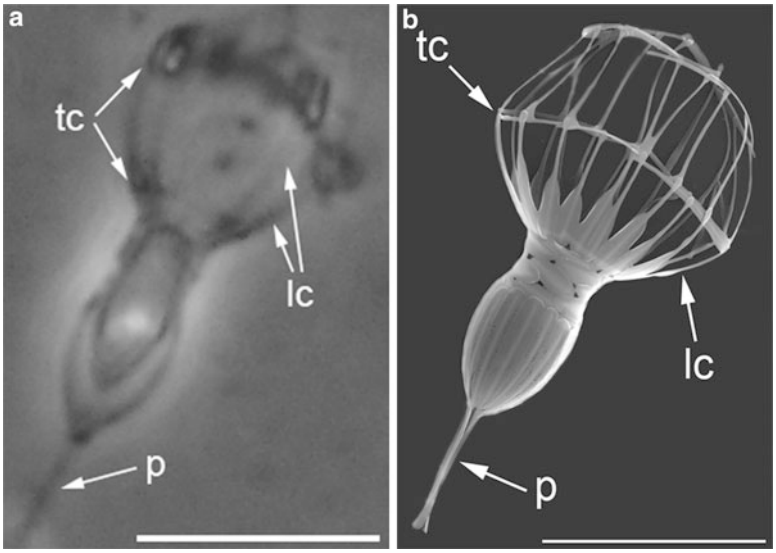

Fig. 3 Images of *Didymoeca elongata*, a marine tectiform loricate. (**a**) Phase contrast light microscopy of a living cell. (**b**) Scanning electron microscopy (SEM) of an empty lorica. *p* pedicel, *lc* longitudinal costa of the lorica, *tc* transverse costae of the lorica. Scale = 10 μm

Occurrence

Choanoflagellates are globally distributed. Some environments in which choanoflagellates have been detected by microscopy are: the North Atlantic (Ellis 1929; Leadbeater 1972a, b, 1980, 1983; Leadbeater et al. 2008; Thomsen 1973, 1976, 1982; Throndsen 1970, 1974; Manton et al. 1975, 1976; Tikhonenkov et al. 2006), Thailand (Thomsen and Boonruang 1983; Thomsen and Moestrup 1983), New Zealand and Australia (Al-Qassab et al. 2002; Moestrup 1979), Taiwan and Japan (Hara and Takahashi 1987; Hara et al. 1996, 1997; Nitsche and Arndt 2008), the Galapagos Islands (Manton et al. 1980), the Mediterranean Sea (Leadbeater 1973, 1974), the Red Sea (Thomsen 1978), the North Pacific (Booth et al. 1982; Booth 1990), the South Atlantic (Bergesch et al. 2008), and the Antarctic (Buck 1981; Buck and Garrison 1988; Chen 1994; Hara and Tanoue 1985; Takahashi 1981b; Marchant and Perrin 1990; Leakey et al. 2002; Nitsche et al. 2007).

Literature

Early literature on choanoflagellates was published largely in book format (e.g., Stein's *Der Organismus der Infusionsthiere* and Kent's *Manual of the Infusoria* (Stein 1878; Kent 1880-1882)) or in the *Quarterly Journal of Microscopical Science* (now known as the *Journal of Cell Science*). Subsequent characterizations of choanoflagellate ecology and new species descriptions have been published in journals focusing on marine science or on the biology of microbial eukaryotes, principally *Journal of the Marine Biological Association of the United Kingdom, Archiv für Protistenkunde* (now known as *Protist*), *European Journal of Protistology*, and *Journal of Eukaryotic Microbiology*. Within the past 15 years, interest in choanoflagellates as the sister group to animals has engendered a number of publications in molecular biology journals such as *Molecular Biology and Evolution*, *BMC Evolutionary Biology*, and *Developmental Biology* and in general interest science journals including *PLoS ONE*, *Current Biology*, *Science*, *Proceedings of the National Academy of Sciences of the USA*, and *Nature*. Several review articles, guides, and book chapters have been written on choanoflagellates, including those by Zhukov and Karpov (1985), Leadbeater and Thomsen (2000), and the previous edition of this book (Buck 1990).

A comprehensive book covering all aspects of choanoflagellate biology, ecology, and evolution has recently been published (Leadbeater 2015).

History of Knowledge

The first description of a choanoflagellate, *Codosiga botrytis* (originally named *Epistylis botrytis*), was made by Ehrenberg (Ehrenberg 1831, 1838). Further light microscopy studies from the late nineteenth and early twentieth centuries increased

the catalog of morphologically described choanoflagellate species (Kent 1880-1882; Ellis 1929). Norris (1965), drawing on evidence from electron microscopy, established the basis of the current systematic hierarchy within the group. Studies on mitochondrial morphology (Leadbeater 1974) and kinetid structures (Hibberd 1975; Karpov 1982) provided initial information on the phylogenetic relationship of choanoflagellates to other eukaryotes, placing them within the now-retired protist phylum Zoomastigina and not in the groups Craspedophyceae or Chrysophyceae. The advent of molecular methods conclusively established choanoflagellates as the sister group to animals (Wainright et al. 1993; Snell et al. 2001; Lang et al. 2002; Shalchian-Tabrizi et al. 2008; Philippe et al. 2005; Steenkamp et al. 2006; Ruiz-Trillo et al. 2008; Carr et al. 2008). Molecular phylogenetic studies of diverse choanoflagellates have mainly been based on ribosomal small subunit RNA gene sequences but have also extended to a small number of protein coding genes (Snell et al. 2001; Carr et al. 2008; Nitsche et al. 2011; del Campo and Massana 2011). Interest in metazoan evolution has led to increased molecular characterization of choanoflagellates, a significant component of which has been analysis of genome and transcriptome data sets, and comparison to animals (King and Carroll 2001; Fairclough et al. 2013; King et al. 2003; 2008; Richter and King 2013).

Practical Importance

Choanoflagellates, part of the size/nutritional class of heterotrophic nanoflagellates (2–20 μm), are components of an essential link within aquatic food webs, consuming bacteria and hence propagating resources to higher trophic levels (Arndt et al. 2002). To date, no parasitic or endosymbiotic species have been reported nor has any form of toxicity or pathogenicity been described within the choanoflagellates.

Habitats and Ecology

The habitats in which choanoflagellates have been found vary broadly, consistent with the hypothesis that they are ubiquitously distributed in the environment. For example, marine choanoflagellates have been reported as members of the aquatic surface microlayer (neustonic) community (Norris 1965), within sea ice (Buck 1981; Takahashi 1981b; 1981a; Thomsen et al. 1997) and as plankton of coastal waters (del Campo et al. 2015) and oceanic regimes in the deep sea (Nitsche et al. 2007). Although most reports on planktonic habitats have been from coastal areas, several open ocean transect studies have documented choanoflagellates at all sampled stations and depths (Booth et al. 1982; Leakey et al. 2002; de Vargas et al. 2015). Choanoflagellates have also been detected in soil (Ekelund and Patterson 1997; Ekelund et al. 2001; Geisen et al. 2015).

Craspedid choanoflagellates are present in both freshwater and marine environments. Loricate (acanthoecid) choanoflagellates were considered to be strictly

marine until recent reports describing two new species in freshwater lakes: one in Mongolia (Paul 2012) and the other in Samoa (Nitsche 2014). Marine choanoflagellates have been found in habitats where the potential for salinity variation is great, such as tide pools, sea ice, hypersaline lakes (van den Hoff and Franzmann 1986; Couradeau et al. 2011), and coastal regions (see above). Other environmental tolerances may differ among choanoflagellate species: *Parvicorbicula socialis* has been found at 25 °C in the Mediterranean and in both polar regions at temperatures as low as −1.8 °C (Buck 1980; Manton et al. 1975), while *Bicosta antennigera* has not been recorded at temperatures exceeding 10 °C (Manton et al. 1980). Choanoflagellates have been cultured from hypoxic water masses (Wylezich et al. 2012). A study of the craspedid morphospecies *Codosiga botrytis* showed that a single morphotype may contain different genotypes which reflect adaptations to different habitats like estuaries, rivers, or soil (Stoupin et al. 2012).

Although choanoflagellates are generally found at low abundance in most aquatic ecosystems (e.g., Buck et al. 1996), they can be a major component of plankton in some specific environments (for example, exceeding 10^6 cells per liter the ice edge region of the Antarctic Weddell Sea (Buck and Garrison 1983)).

The mode of nutrition of choanoflagellates is phagotrophy by suspension feeding that is generally interpreted as a form of filter feeding (Lapage 1925; Pettitt et al. 2002). Choanoflagellates create water currents via an undulating movement of their single apical flagellum, which transports particles to the outside of their collar. These particles consist mostly of bacteria, picoplanktonic algae, and debris. These particles are transported along the microvilli of the collar near to the protoplast, whereupon cytoplasmic pseudopodia extend to engulf the food, which is subsequently digested within a food vacuole (Fenchel 1982; Pettitt et al. 2002; Leadbeater and Manton 1974). Choanoflagellates can handle many food particles simultaneously (Boenigk and Arndt 2000), and multiple different modes of feeding have been observed in craspedids (Zhukov and Karpov 1985).

In the craspedid *Salpingoeca rosetta*, a sulfonolipid constitutively produced by a coisolated prey bacterium is capable of inducing colony formation (Alegado et al. 2012). While the ecological significance of this interaction is currently unknown, *S. rosetta* may interpret the bacterial signal as an indication of prey presence (Alegado et al. 2012; Alegado and King 2014) by forming colonies around which the rate of fluid flow, and therefore the rate of contact with potential prey, is higher than that of single cells (Orme et al. 2001; Roper et al. 2013).

Characterization and Recognition

General Appearance and Ultrastructure

Choanoflagellates are identified by the presence of a single apical flagellum, whose length may vary between species and within species based on life history stage (Leadbeater 2015), surrounded by a collar of 20–50 actin-filled microvilli. In the craspedid *Monosiga brevicollis*, these microvilli are about 0.1 μm wide, 0.1 μm apart

at their bases and 0.6 μm apart at their tips (Mah et al. 2014). No organisms or cell types possessing a single flagellum surrounded by a collar of microvilli have been reported outside of the choanoflagellates, with the exception of the choanocyte cell type found in animals (reviewed in Alegado and King 2014). The cell body is in a size range of 1.2–10 μm in length and 2–10 μm in width (Mah et al. 2014; Leadbeater 2015). A symmetric wing-like vane spanning the proximal two thirds of the flagellum has been described in some choanoflagellate species (Fjerdingstad 1961; Hibberd 1975; Karpov and Leadbeater 1998; Karpov 2000; Mah et al. 2014). In the craspedid *Codosiga botrytis*, the vane was observed to be approximately 2 μm wide on either side of the flagellum, and to contain two sets of overlapping fine fibrils (Hibberd 1975). In addition to a basal body directly associated with the structure of the flagellum, all choanoflagellates also feature a second nonflagellar basal body whose length varies by species; to date, only *C. botrytis* has been observed to contain more than two basal bodies (Hibberd 1975; Karpov and Leadbeater 1998; Leadbeater 2015). A stacked microtubular structure consisting of two or more layers (depending on species) extends from one third to one half the length of the cell and supports the bases of the collar microvilli (Hibberd 1975; Zhukov and Karpov 1985; Karpov and Leadbeater 1998). Choanoflagellate mitochondrial cristae are generally flattened in shape, as in other opisthokonts (although tubular cristae have recently been observed (Wylezich et al. 2012)), but mitochondrial location, size, and shape varies among species (Leadbeater 2015). Typically, the Golgi apparatus, whose size and structure differs among species, is located adjacent to the second, nonflagellar basal body (Leadbeater 2015). The nucleus is generally located below the Golgi apparatus, and nuclear division has been examined in detail in both craspedid and acanthoecid species (Karpov and Mylnikov 1993; Leadbeater 1994b; Karpov and Leadbeater 1997).

Many craspedid choanoflagellate species possess an extracellular organic structure known as a theca. Theca shape is currently considered to be diagnostic for species identification. Furthermore, thecae from different species have been divided into classes by shape: cup shapes such as *Salpingoeca rosetta* (Dayel et al. 2011), flask shapes such as *Salpingoeca urceolata* (Kent 1880-1882), and tube shapes such as *Salpingoeca gracilis* (James-Clark 1868), with other classes of shapes remaining to be discovered. In contrast to the Craspedida, the Acanthoecida possess a distinct morphological characteristic, the siliceous lorica, which has received considerable taxonomic attention during the past five decades due to the relative ease of preparation of the lorica for electron microscopy and the species-specific conservation of the critical details of lorica morphology (reviewed extensively by Leadbeater 2015). The electron microscopy study of Norris (1965) was the first to show that the costae that make up the lorica of the Acanthoecida were themselves constructed of component costal strips. These costae are formed intracellularly within silica deposition vesicles from incorporated environmental amorphous silicon dioxide and are subsequently exocytosed during lorica formation; the highly choreographed steps of lorica construction are subject to a universal set of rules that are implemented in a species-specific manner (Leadbeater and Davies 1984; Leadbeater 1985, 1989; Marron et al. 2013; Leadbeater 2015; Leadbeater et al. 2009).

Life Cycle

Several cell types/life history stages have been described within choanoflagellates, including: (i) cells attached to a substrate, either directly, by a long stalk or pedicel, or within a theca or lorica; (ii) cells passively suspended in the water column, either naked or within a lorica; (iii) "normal" swimming cells that closely resemble attached cells; (iv) fast-swimming cells with shorter collars, often differently shaped from slower swimming cells and sometimes referred to as swarmers; and (v) colonies, either free-swimming or attached to a substrate, in which cells may be connected by fine intercellular bridges (Hibberd 1975; Karpov and Coupe 1998; Dayel et al. 2011; Fjerdingstad 1961). As with the theca, the presence or absence of cell types or life history stages within a culture may be condition-dependent, and only in a few cases are these conditions beginning to be understood (e.g., Alegado et al. 2012). Whether or not morphologically similar cell types are homologous among choanoflagellate species is currently unknown.

Asexual reproduction in choanoflagellates is by means of longitudinal fission in most species (Karpov and Mylnikov 1993; Karpov and Leadbeater 1997). The two primary subgroups of acanthoecids differ in the mode of lorica formation associated with asexual reproduction (Leadbeater 2008). In the Stephanoecidae, the daughter cell is supplied with a whole set of costal strips from the mother cell when it departs the parent lorica (tectiform replication, e.g., Leadbeater 1994a). In contrast, in the Acanthoecidae, the daughter cell leaves the mother cell naked and subsequently produces the costal strips by itself (nudiform replication).

Sexual reproduction and evidence for genetic recombination have been observed in the craspedid thecate choanoflagellate *Salpingoeca rosetta* (Levin and King 2013). In *S. rosetta*, sexual transitions from a haploid to a diploid state occur in nutrient-limiting conditions, and the converse transition from diploidy to haploidy, presumed to occur via meiosis, takes place in nutrient-rich conditions. Fusion of anisogamous haploid gametes occurs via a smaller, rounded cell (the "male" gamete) fusing and then entering a larger, ovoid cell (the "female" gamete). Sex in *S. rosetta* can occur within clonal cultures and between genetically differentiated cultures derived from the same initial isolate. In addition, choanoflagellates possess conserved eukaryotic genes involved in meiosis (Carr et al. 2010).

Choanoflagellates are capable of forming cysts, although the environmental triggers and molecular pathways for cyst formation remain uncharacterized (Zhukov and Karpov 1985; Leadbeater and Karpov 2000; Stoupin et al. 2012).

Nuclear and Mitochondrial Genomes

Two craspedid choanoflagellate nuclear genomes have been sequenced: *Monosiga brevicollis* (King et al. 2008) and *Salpingoeca rosetta* (Fairclough et al. 2013). *Monosiga brevicollis* has an estimated genome size of 41.6 Mb, with a GC content of 55%, 9,171 predicted protein-coding genes, and 6.6 introns per gene of average length 174 bp. The genome of *S. rosetta* is larger, with an estimated size of 55.4 Mb,

a GC content of 56%, and 11,629 predicted protein-coding genes featuring 7.5 introns per gene with a mean length of 255 bp.

The circular mitochondrial genome of *M. brevicollis* is 75,568 bp, with a very low GC content of 14%, and encodes 55 predicted genes which comprise 47% of the genome (Burger et al. 2003).

Choanoflagellate genomes may be heavily influenced by horizontal gene transfer from their prey; approximately 4.4% of the genes in the *M. brevicollis* nuclear genome have been estimated to result from lateral transfers from bacterial or algal donors (Yue et al. 2013).

Systematics and Nomenclature

The formal taxon name for choanoflagellates is Choanoflagellatea Cavalier-Smith 1998, emended by Nitsche et al. 2011. All described species fall into two groups: Craspedida and Acanthoecida, both proposed by Cavalier-Smith (1997) and emended by Nitsche et al. (2011). The group Craspedida contains at present only one subtaxon, the Salpingoecidae Kent 1880-1882 emended sensu Nitsche et al. 2011, whose type genus is *Salpingoeca* James-Clark 1867. The group Acanthoecida consists of two subtaxa, the Stephanoecidae Leadbeater 2011 and the Acanthoecidae Norris 1965, emended sensu Nitsche et al. 2011. *Stephanoeca* Ellis 1929 is the type genus of Stephanoecidae, and *Acanthoeca* Ellis 1929 is the type genus of Acanthoecidae.

Several choanoflagellate genus and species names are known to require revision (Nitsche et al. 2011). Early catalogs of choanoflagellate species were based on light microscopy (Kent 1880-1882; Ellis 1929) and were later supplemented with electron microscopy data. However, both molecular phylogenetic evidence and detailed observations of clonal cultures have demonstrated that characteristics of protoplast (cell) morphology and life history are not reliable indicators of species identity (Carr et al. 2008; Dayel et al. 2011; Nitsche et al. 2011). For example, *Choanoeca perplexa* and *Proterospongia choanojuncta*, which were originally described as two distinct species, have since been recognized as unicellular and colonial life history stages of the same species (Leadbeater 1983; Carr et al. 2008). Therefore, instead of relying on protoplast morphology or a single life history stage as a means of species recognition, choanoflagellates are currently identified using a combination of three separate methods: (1) the morphology of their extracellular structures such as the theca or the lorica (if either is present); (2) characterization of multiple life history stages in clonal culture; and (3) molecular sequence data, where the highest sampling depth exists for small and large subunit ribosomal RNA gene sequences. In cases where the first two methods have produced ambiguous results, molecular data are used as the arbiter for final identification.

The relationship among species with similarly shaped thecae is less well understood. Species belonging to the same class of theca shape are likely to be more closely related to one another than to species in a different class, but the exact nature of the relationship among shape classes awaits clarification, due to the fact that only

a handful of species within each shape class have been studied in detail, and because a choanoflagellate species capable of producing a theca may not do so under certain natural or laboratory conditions (Dayel et al. 2011).

The introduction of molecular methods of phylogenetic analysis demonstrated that the two groups of loricate choanoflagellates (Stephanoecidae and Acanthoecidae, which are distinguished by their mode of replication, described below under "Life Cycle") are distinct (Carr et al. 2008; Nitsche et al. 2011). However, molecular data also indicated that a taxonomy based solely on lorica morphology is not sufficient. For example, although the morphology of the loricae of *Acanthocorbis unguiculata* and *Helgoeca* (formerly *Acanthocorbis*) *nana* are strikingly similar, phylogenetic analysis showed that they belong to different groups (Leadbeater et al. 2008). Therefore, although the lorica is a suitable characteristic to determine identity at the species level, higher taxonomic levels cannot necessarily be determined by lorica structure alone (Nitsche et al. 2011).

Maintenance and Cultivation

Choanoflagellate cultures can be isolated from the environment either using a micropipette or by dilution (King et al. 2009). Choanoflagellates cannot currently be cultured in the absence of bacteria, which serve as their food source (a 1970 report [Gold et al. 1970] of choanoflagellates grown without bacteria has not been replicated). The process of isolation generally produces a culture with a single clonal choanoflagellate and a mixed culture of dozens or more species of bacteria. A combination of antibiotic treatment and selective dilution techniques can be used to produce a "monoxenic" culture grown with one species of bacterium (King et al. 2008, 2009; Dayel et al. 2011).

Choanoflagellates are cultured in organically enriched medium of the appropriate salinity. Media are frequently enriched with infusions of cereal grass or by directly adding individual autoclaved pieces of rice or other grains (King et al. 2009). Frequency of passaging cultures into new growth flasks depends on the growth rate of the cells within the culture, but is generally conducted between once per day and once per several weeks. Choanoflagellates growing attached to culture flasks may be detached using a plastic cell scraper. The rate and method of passaging can also be used to influence the proportion of different cell types within the culture (Dayel et al. 2011). Choanoflagellate cultures can be stored long-term as frozen stocks in liquid nitrogen (King et al. 2009), and over 20 species are available from the American Type Culture Collection (ATCC).

Evolutionary History

There is currently no fossil record of choanoflagellates. The most likely candidates for preservation would be those with a siliceous lorica, but the combination of their microscopic size and the lack of knowledge about how they might be preserved, if at

all, have prevented their detection to date. Living cells of *Codosiga botrytis* have been retrieved from permafrost cores estimated to be between 28,000 and 32,000 years old (Stoupin et al. 2012).

Choanoflagellates have been demonstrated to be the sister group to animals (Snell et al. 2001; Lang et al. 2002; Shalchian-Tabrizi et al. 2008; Philippe et al. 2005; Steenkamp et al. 2006; Ruiz-Trillo et al. 2008; Carr et al. 2008), and molecular dating methods have estimated the divergence of animals and choanoflagellates to have occurred somewhere from 600 million to over one billion years ago (Peterson et al. 2004; Douzery et al. 2004; Hedges et al. 2004; Parfrey et al. 2011). Substantial interest has focused on studying the choanoflagellates as a means to reconstruct the evolutionary history of animals (reviewed in Alegado and King 2014; Richter and King 2013).

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Blastocladiomycota

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Martha J. Powell

Abstract

The Blastocladiomycota are posteriorly uniflagellated zoosporic fungi found as saprotrophs and parasites primarily in freshwater and soil. Once considered Chytridiomycota, phylogenetically they are a monophyletic group divergent from other zoosporic fungi, clustering among the nonzoosporic fungi. Their thalli range from monocentric, polycentric, tubular, to hyphal and are unusual among fungi in exhibiting alternation of a haploid gametophytic generation with a diploid sporophytic generation. Thick-walled resistant sporangia are the sites of meiosis and aid in the survival of the organism when environmental conditions become adverse. The hallmark of the group is the ultrastructural architecture of their zoospores, which includes a single nucleus proximal to the kinetosome, an aggregated cluster of ribosomes capping the nucleus anteriorly, and a lateral microbody-lipid globule complex (MLC). In addition to being the center for utilization of stored energy, the MLC has been implicated in rhodopsin-based photoreception and signal transduction in response to blue-green light. Invertebrates, plants, algae, oomycetes, and other blastoclads serve as hosts of parasitic members. For example, *Paraphysoderma* is a highly destructive pathogen of algae grown in mass cultures for biofuels and pharmaceuticals. As a pathogen of mosquitoes, *Coelomomyces* has been explored as a biocontrol agent, but its life cycle requirement for alternation of hosts makes this a difficult system to maintain. The saprotrophs *Allomyces* and *Blastocladiella* are emerging as model organisms in developmental biology, genetics, physiology, and genomics.

Keywords

Freshwater • Parasites • Phylogenetics • Saprotrophs • Soil • Ultrastructure • Zoosporic fungi

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●Blastocladiomycota
●●Physodermataceae
●●Paraphysodermataceae
●●Coelomomycetaceae
●●Catenariaceae
●●Blastocladiaceae
●●Sorochytriaceae

Introduction

General Characteristics

The Blastocladiomycota is a monophyletic phylum of zoosporic fungi (Hibbett et al. 2007; James et al. 2006a, b, 2014) commonly found as saprotrophs and parasites of vascular plants, algae, invertebrate animals, oomycetes, and other blastoclads in soil and freshwater (Sparrow 1960). Unwalled zoospores and zygotes function in dispersal and establishment of new thalli, typically surrounded by a chitin-containing cell wall. Thallus morphology ranges from a monocentric thallus composed of a single sporangium and attached rhizoids (Fig. 2c) to more extensive rhizomycelia (Fig. 3f) and hyphae (Fig. 2b) bearing multiple sporangia (Fig. 3a). Thalli are multinucleate (Fig. 2e) and aseptate, except for pseudosepta (Fig. 2a, e) that arise irregularly or septa that delimit sporangia and gametangia (Figs. 2c and 3a, c, d). The life cycle may include alternation between haploid gametothallus and diploid sporothallus generations, and in *Coelomomyces,* alternation of hosts as well. In sexual reproduction, pairs of uniflagellated gametes fuse, resulting in biflagellated

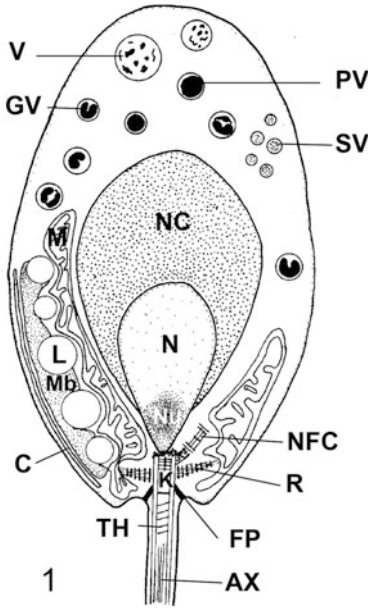

Fig. 1 Summary diagram of a longitudinal section of a blastoclad zoospore as visible with a transmission electron microscope. Characteristic structures include: the nuclear cap [(*NC*), the ribosomal aggregation surrounded by a cisterna continuous with the outer membrane of the nuclear envelope]; the cone-shaped nucleus (*N*) pointed toward the kinetosome (*K*); nucleolus (*Nu*); the nonflagellated centriole (*NFC*); a multilayered, striated rhizoplast (*R*); flagellar props (*FP*), spiral concentric fiber/transitional helix (*TH*); gamma-particle vesicles (*GV*); phosphate-containing vesicles (*PV*); secretory/adhesion vesicles (*SV*); vacuole (*V*); side body complex also known as the microbody-lipid globule complex [consisting of a backing membrane cisterna (*C*), microbody (*Mb*), lipid globules (*L*), and mitochondrion (*M*)]; flagellar axoneme (*Ax*). The anteriorly projecting cage of microtubules extending from the apical end of the kinetosome is not illustrated in the diagram

zygotes. In asexual reproduction, posteriorly uniflagellated zoospores (Fig. 1) are formed and released from thin-walled sporangia. One blastoclad is unusual in the production of amoeboid aplanospores rather than only flagellated zoospores (James et al. 2011; Letcher et al. 2016; Strittmatter et al. 2016). Resistant sporangia (=resting sporangia) usually germinate by an irregular cracking of the outer layer of their thick wall (Fig. 4a), releasing meiospores that give rise to gametothalli. Resistant sporangia are darkly pigmented with ornately sculptured (Figs. 2c and 3b), multilayered cell walls (Fig. 4a) and are highly characteristic of blastoclads.

The real hallmark of the Blastocladiomycota is its characteristic zoospore with distinguishing features visible with light microscopy and even more striking with electron microscopy (Fuller 1977). A prominent nuclear cap crowning a conspicuous cone-shaped nucleus pointed toward the posteriorly directed flagellum (Fig. 1) clearly distinguishes the elongate blastoclad zoospore from motile cells of other organisms. As a zoosporic group in the basal radiation of fungi from a protoctistian

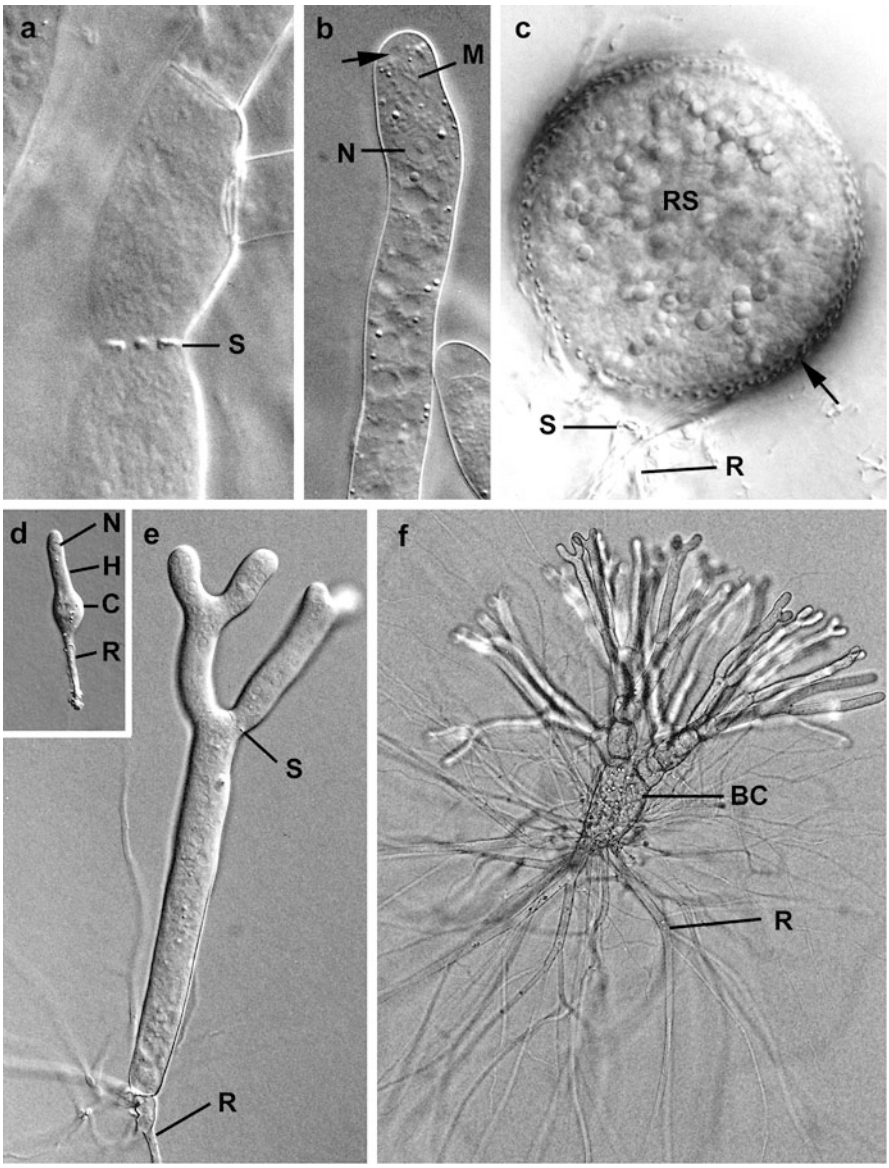

Fig. 2 (**a**) *Allomyces macrogynus* sporothallus. Hypha with pseudoseptum (*S*). The septum joins the outer wall like spokes of a wheel. In this surface view, consequently, the septum is incomplete. × 1,100. (**b**) *Allomyces macrogynus* sporothallus. Growing hypha with region of the Spitzenkörper (*arrow*) visible as an area devoid of organelles. Elongate mitochondria (*M*) cluster near the hyphal apex and a nucleus (*N*) is more distal. × 700. (**c**) *Blastocladiella britannica* monocentric, eucarpic thallus consisting of a resistant sporangium (*RS*) and basal rhizoids (*R*) separated from sporangium by a septum (*S*). Notice the characteristic loose enclosure of the resistant sporangium within the thallus wall (notice space between resistant sporangium and septum). The pitted surface of the

ancestor (James et al. 2006b; Medina et al. 2003; Steenkamp et al. 2006), the blastoclads share certain features with chytrids, different from higher fungi, but also share characteristics with higher fungi distinct from chytrids (see section on "Evolutionary History"). Reflecting an ancient shared ancestry with chytrid fungi, yet also an evolutionary divergence from this group (James et al. 2006a, b), the zoospores of blastoclads may abruptly change directions as they swim, as do chytrid zoospores; however, they typically move in a gliding pattern rather than the hopping motion typical of a chytrid zoospore.

Occurrence

With a global distribution, blastoclads are known from freshwater and terrestrial habitats, but none have been cultured directly from marine habitats (Powell 1993; Sparrow 1960; James et al. 2014). One genus, *Blastocladia*, is composed entirely of obligate fermenters that thrive in stagnant water (Emerson and Natvig 1981). In still water, they are frequently found on submerged, partially decorticated, water-logged twigs or decaying fleshy fruit, especially of the rose family (Whisler 1987). Some members, such as *Blastocladiella*, are most frequently collected in soils from southern latitudes (Sparrow 1960), and *Allomyces* is commonly collected from slowly air-dried soil (Willoughby 1984). Saprotrophs in soil and water are collected on a variety of substrates including seeds, pollen, cellulose, feathers, hair, and chitin (e.g., Czeczuga et al. 2004; Whisler 1987). Others occur as parasites on microinvertebrates in aquatic habitats (e.g., *Catenaria, Coelomomyces, Polycaryum*) or terrestrial habitats (*Catenaria, Sorochytrium*). Still others parasitize terrestrial and aquatic vascular plants (*Physoderma*), algae (*Paraphysoderma*), and other blastoclads (*Catenaria*).

Literature

The most comprehensive analysis of the molecular phylogeny of the Blastocladiomycota revealed that the current classification is in need of revision (James et al. 2014; Porter et al. 2011). Although most monographic works on the

Fig. 2 (continued) resistant sporangium (*RS*) is visible at the edge of the thallus (*arrow*). × 800. (**d**) *Allomyces macrogynus* sporothallus. Bipolar germination of a zoospore. The encysted zoospore is a rounded cell (*C*) from which a rhizoid (*R*) and hypha (*H*) emerge in opposite directions. A nucleus (*N*) is located at the growing tip of the hypha at this stage. × 500. (**e**) *Allomyces macrogynus* sporothallus. Thallus development with hyphal tip branching dichotomously as the tips take on a tuning-fork arrangement. A pseudoseptum (*S*) has formed, and the rhizoidal system (*R*) that anchors the basal cell becomes extensive. The hypha is coenocytic, and scattered nuclei with prominent nucleoli have a "fried egg" appearance. × 500. (**f**) *Allomyces macrogynus* sporothallus. Developing thallus with extensive rhizoids (*R*), a trunk-like basal cell (*BC*) from which sprouts numerous bushy hyphae with characteristic dichotomously branched tips. × 250

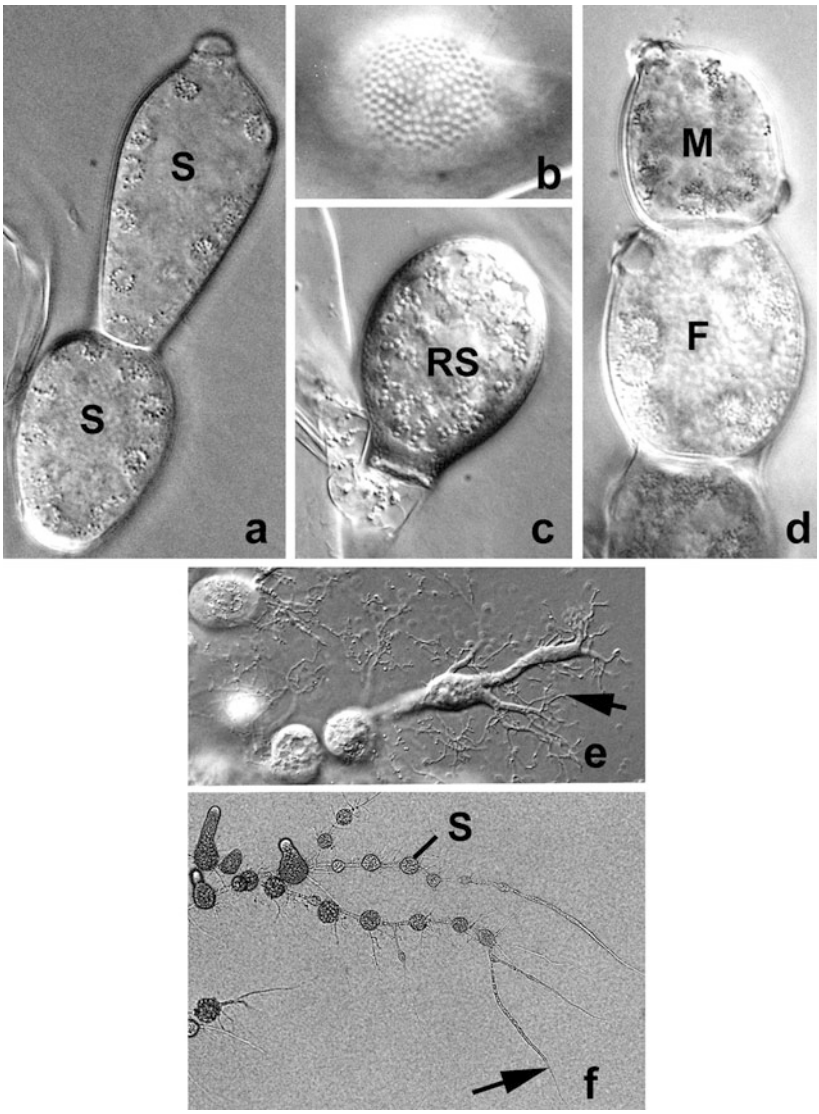

Fig. 3 (**a**) *Allomyces macrogynus* sporothallus. Two terminal sporangia (*S*) with "ring-like" arrangement of tiny lipid globules. A discharge papilla is visible at the apex of the terminal sporangium. × 500. (**b**) *Allomyces macrogynus* sporothallus. Surface view of resistant sporangium wall showing the regular arranged pores in the wall. × 500. (**c**) *Allomyces macrogynus* sporothallus. Terminal resistant sporangium (*RS*) with thickened wall. × 500. (**d**) *Allomyces macrogynus* gametothallus. Terminal male gametangium (*M*) is pigmented and smaller than the hyaline female gametangium (*F*) on which it rests. × 500. (**e**) Early development of rhizomycelium of *Catenaria anguillulae* demonstrating dendritic branching with many finely divided rhizoids (*arrow*) emerging from the main axis. × 300. (**f**) Rhizomycelium of *Catenaria anguillulae.* Notice the catenulate organization of sporangia (*S*) separated by isthmus cells. The growing tips of the rhizomycelium are finely tapered and bifurcated. × 100

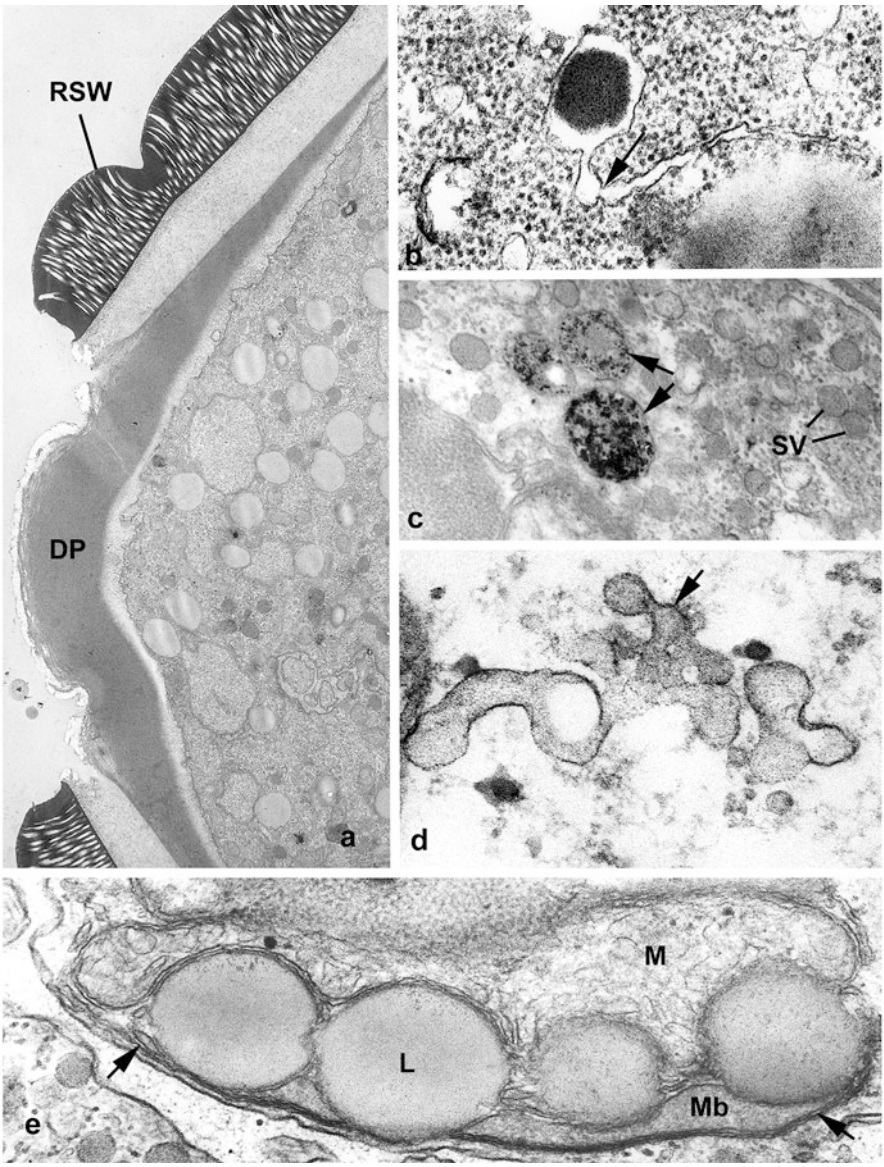

Fig. 4 (**a**) Electron micrograph of thin section through resistant sporangium of *Coelomomyces punctatus* illustrating the thick ornamented cell wall (*RSW*), inner layer of material, and lens-shaped discharge plug (*DP*) that has formed in the opened discharge crack in the wall. × 12,500. (**b**) Formation of a gamma-particle vesicle from a segment of rough endoplasmic reticulum (*arrow*) during zoosporogenesis in *Catenaria allomycis*. Smaller particles have coalesced into a spherical electron-opaque inclusion. × 75,000. (**c**) Cytochemical precipitation of free phosphate with 0.02 M lead nitrate, pH 5.2, into lead phosphate, a heavy metal that is detectable with electron microscopy. The scattered electron-opaque precipitates thus localize the presence of phosphorous in vesicles (*arrows*) in meiospores of *Coelomomyces punctatus*. Smaller secretory vesicles (*SV*) with a finely

Blastocladiomycota are dated, they still serve as invaluable reservoirs of knowledge. Sparrow's (1960) *Aquatic Phycomycetes* includes the most complete monograph of this phylum. In *Chytridiomycetarum Iconographia,* Karling (1977) described life histories, morphology, development, and characteristics of blastoclad species and presented a copious collection of drawings, helping to make this group tractable. Karling (1950) monographed the genus *Physoderma*, placing *Urophlyctis* into synonymy with *Physoderma*; but Sparrow (1962) later argued the morphological distinctions between these two genera. Emerson and Robertson (1974) provided keys to the five genera in the Blastocladiaceae and discussed issues with generic distinctions in this family. Couch and Bland's (1985) monograph of *Coelomomyces* is richly illustrated with light and scanning electron micrographs and is detailed in its review of knowledge of development of this obligate parasite of dipterans and copepods. The genus *Allomyces* has been extensively studied in developmental investigations and much of this work is summarized in Olson's (1984) "*Allomyces*-a different fungus." Longcore's "Chytrid Taxonomy since 1960" is a compilation of new taxa, combinations, and synonymies published since Sparrow's (1960) monograph and available at http://umaine.edu/chytrids/Chytrid-Bibliography/.

History of Knowledge

The recent recognition of the Blastocladiomycota as a Phylum distinct from the Chytridiomycota is based on molecular phylogenetic analyses and supporting biochemical and ultrastructural data (James et al. 2006b). As a relatively small group, it is not surprising that resurgent interest in biodiversity, fueled by molecular phylogenetics, is revealing new taxa and new taxonomic affiliations (Hoffman et al. 2008; James et al. 2014; Johnson et al. 2006a; Porter et al. 2011). The uniqueness of this group was first recognized when Petersen (1909) established the order Blastocladiales, based on a single genus, *Blastocladia* (Reinsch 1878), transferring it out of the Saprolegniales (oomycetes). The generic name is descriptive of the thallus and was derived from combining the Greek *blastos* (=germ or sprout) with *clados* (=branch). New genera were either added or described over the next 100 years until now there are 16 recognized genera (Table 1). Actually, the oldest recognized zoosporic fungus is *Physoderma* (Wallroth 1833), but its blastoclad affinity was not officially recognized until 1980 when, based on zoospore

Fig. 4 (continued) granular matrix are thought to function in cyst coat formation and adhesion during encystment. × 55,000. (**d**) A Golgi equivalent in *Allomyces javanicus* consisting of a fenestrated cisterna (*arrow*) from which vesicles appear to be forming. × 80,000. (**e**) Side-body complex (microbody-lipid globule complex = *MLC*) in meiospore of *Coelomomyces punctatus*. Four spherical lipid globules (*L*) are sandwiched between an elongate mitochondrion (*M*) and sheet-like microbody (*Mb*). A single cisterna (*arrows*; backing membrane or simple cisterna) extends over the microbody and lies just within the plasma membrane. × 65,000

Table 1 Zoospore ultrastructural studies in the Blastocladiomycota

Families	Genera	References on motile spore ultrastructure
Blastocladiaceae	*Allomyces* *Blastocladia* *Blastocladiella* *Blastocladiopsis*[a] *Microallornyces*	Aliaga and Pommerville 1990; Fuller and Olson 1971; Olson 1973, 1984; Robertson 1972 Lingle and Barstow 1983 Reichle and Fuller 1967
Physodermataceae	*Physoderma* *Urophlyais*	Lange and Olson 1979, 1980a, b; Olson and Lange 1978
Paraphysodermataceae	*Paraphysoderma*	Hoffman et al. 2008; Strittmatter et al. 2016 – (light microscopy only); Letcher et al. 2016 – (transmission electron microscopy)
Catenariaceae	*Catenaria* *Catenophlyctis*	Chong and Barr 1974; Lange and Olson 1979; Olson and Reichle 1978a; Olson et al. 1978
Coelomomycetaceae	*Coelomomyces* *Coelomycidium* *Callimastix*[a]	Federici and Lucarotti 1986; Lucarotti and Federici 1984; Martin 1971 Travland 1979; Whisler et al. 1972 Loubès and Manier 1974; Weiser and Zizka 1975 Manier and Loubès 1978
Sorochytriaceae	*Sorochytrium*[a]	Dewel and Dewel 1990
Incertae sedis	*Polycaryum*[b] *Myiophagus*[a,c]	Johnson et al. 2006a
Former blastoclad now chytrid	[*Catenomyces persicinus*[d]]	

[a]Indicates no molecular sequences available for this taxon
[b]Sister to clade containing *Catenophlyctis variabilis* and *Catenaria anguillulae* – Johnson et al. 2006a
[c]Structure of zoospore and resting spore suggests placement in Blastocladiomycota – Humber 2012; Karling 1948; Sparrow 1939
[d]In clade with Chytridiomycota in molecular analyses (previously classified in the Catenariaceae) – James et al. 2006b; Porter et al. 2011

ultrastructure, Lange and Olson (1980b) transferred it to the Blastocladiales in a new family. Similarly, *Catenaria anguillulae* was classified in the Chytridiales until Couch (1945) transferred it to the Blastocladiales and established a new family.

Practical Importance

Because of the range of substrates used and adaptations for growth in soils and water, blastoclads are important in the biodegradation of organic matter and recycling of nutrients. In addition, the use of parasitic blastoclads to control invertebrates, including nematodes and insects, is a fertile area of investigation. Many years ago, Couch (1945) proposed *Catenaria anguillulae* as a biocontrol agent against root knot nematode of tomatoes, and a recent study has actually demonstrated the efficacy of *Catenaria anguillulae* in controlling the destructive impact of root knot nematode on rice (Singh et al. 2007). Tribe (1977) similarly highlighted the potential of C.

auxiliaris to control sugar beet cyst-nematode, and Castillo and Lawrence (2011) discovered *C. auxiliaris* attacking reniform nematodes on cotton. Under moist environmental conditions, *Myiophagus* is a possible control of scale insects (Karling 1948). Use of *Coelomomyces* as a bioinsecticide against mosquitoes and other dipterans is complex because of its obligatory requirement for an intermediate microcrustacean host and the difficulties in mass producing inoculum. With increased insect resistance to conventional chemical control, attempts to develop *Coelomomyces* for biological control continue (Lucarotti and Shoulkamy 2000; Scholte et al. 2004).

Parasitic blastoclads can also negatively impact agricultural production. *Physoderma* is a parasite of several vascular plants; *P. maydis* causes brown spot and has a significant impact on the commercial production of corn (Karling 1950; Lange and Olson 1980a). *Paraphysoderma sedebokerense* (James et al. 2011) is a recently discovered algal parasite, sister to *Physoderma* and capable of devastating microalgae mass-cultured in large outdoor ponds for commercial production (Hoffman et al. 2008). *Allomyces* and *Blastocladiella* have been reported growing on fish (Chukanhom and Kishio 2004; Czeczuga and Muszynska 1999), but their role as a primary infective agent of vertebrates has not been verified. Perhaps more important in fish production, *Polycaryum laeve* infection of *Daphnia* can adversely influence the access of fish to *Daphnia* as a food source, the long-term availability of *Daphnia* (Johnson et al. 2006b, 2009), and the nutrient content available to other animals that prey on *Daphnia* (Forshay et al. 2008).

One of the most important uses of blastoclads is as popular experimental organisms to study gene regulation during development (e.g., Ojha and Barja 2002; Silva et al. 1987) because of the ease of growing *Allomyces* and *Blastocladiella* in synchronous cultures and their clearly defined developmental stages (germination; vegetative growth; sporulation; motile spore stages).

Assemblages of sequenced genomes of *Allomyces macrogynus, Blastocladiella emersonii,* and *Catenaria anguillulae* are providing a framework for comparative exploration of gene functions (Avelar et al. 2014; Idnurm et al. 2010; Krishnan et al. 2012), adding to insights gained earlier with sequencing and annotation of mitochondrial genomes from *Allomyces macrogynus* (Paquin and Lang 1996) and *Blastocladiella emersonii* (Tambor et al. 2008).

Habitats and Ecology

Blastoclads are important in aquatic and terrestrial ecosystems as decomposers of organic materials and also as parasites (Powell 1993). In having to compete with other soil fungi and bacteria, terrestrial saprotrophic blastoclads could be expected to produce antimicrobial compounds. Interestingly, a preliminary study demonstrated that several fast-growing isolates of *Allomyces* could inhibit the growth of *Streptococcus* and *Staphylococcus* in culture (Lorelli and Held 1983), a result that invites additional exploration. Blastoclads are themselves hosts to very few other organisms, but may be subject to RNA-virus infections (Khandjian et al. 1977) or attack

by other zoosporic fungi such as *Olpidium* and *Rozella* as well as the blastoclad *Catenaria* (Karling 1977). Whether blastoclads are able to mount effective defenses against invaders or whether the evolutionary radiation of parasitic groups has not exploited these organisms as hosts are fascinating questions.

Parasitic blastoclads may impact or control populations in terrestrial and aquatic habitats (Gleason et al. 2010). For example, *Catenaria* species parasitize nematodes, rotifers, and midges (Sparrow 1960), while *Sorochytrium milnesiophthora* infects tardigrades inhabiting clumps of moss (Dewel and Dewel 1990). In mesocosm experiments, Johnson et al. (2006b) discovered that *Polycaryum laeve* increased the visibility of *Daphnia* and as a consequence increased their susceptibility to predation by fish in waters low in dissolved organic carbon, effectively reducing population levels of *Daphnia*. Additionally, *Coelomomyces'* attacks on mosquito larvae (including the vector for malaria) impact adult mosquito populations. Typically mosquito larvae infected with *Coelomomyces* die before they pupate and morph into adults. But lightly infected larvae may mature into adult mosquitoes, in which case, in females the fungus invades interstitial spaces of ovary tissue, preventing egg formation. The female's blood meal and resulting hormonal changes trigger the transformation of *Coelomomyces* thalli into resistant sporangia the size of normal mosquito eggs. Thus, when the female mosquito tries to oviposit eggs, fungal-resistant sporangia are released instead, further dispersing this pathogen (reviewed in Lucarotti and Shoulkamy 2000; Powell 1993; Scholte et al. 2004).

Many blastoclads are adapted to their environment because of responses to chemical and environmental signals. For example, it is advantageous to individual blastoclads to colonize new substrates when original substrates become crowded. Obviously, individual blastoclad sporangia cannot move, but their zoospores can. During zoosporogenesis, and only during this stage in *Blastocladiella emersonii*, sporangia release a soluble compound that maintains zoospore motility and blocks the activity of agents which induce encystment. In this manner, zoospores are most likely to swim into new areas and colonize new substrates devoid of other blastoclads (Gottschalk and Sonneborn 1985). As another example, Deacon and Saxena (1997) have shown that zoospores of *Catenaria anguillulae* exhibit chemotaxis to exudates from excretory pores and the anus of their nematode hosts, sites where they can readily infect the host. The attraction is so great that numerous zoospores congregate and encyst in these areas, and the collective onslaught aids the blastoclad in overcoming host defense mechanisms (Jansson and Thiman 1992). Finally the movement of zoospores toward light may increase their access to plant organic matter. Zoospores of several species of *Allomyces* exhibit phototaxis to light (Olson 1984; Robertson 1972). Physiological (Saranek and Foster 1997), and comparative genomic studies (Avelar et al. 2014; Idnurm et al. 2010; Krishnan et al. 2012) reveal that animal-like type II rhodopsins, distinct from opsin pigment genes found in other fungal lineages, may be involved in light sensing responses in blastoclads. Recently protein localization at the surface of the microbody-lipid globule complex (MLC) has implicated the blastoclad "side-body complex" in rhodopsin-based photoreception and signal transduction in response to blue-green light (Avelar et al. 2014).

Characterization and Recognition

Morphology and Life Cycles

Within the Blastocladiomycota, there is a great range in thallus morphologies and complexities of life cycles. Thallus types may be monocentric (Fig. 2c), polycentric-rhizomycelial (Fig. 3e and f), or hyphal (Fig. 2a and b). Life cycles vary in complexity, even within the same genus, but all life histories are variations on a general pattern (reviewed in James et al. 2014). Unusual among fungi, blastoclads exhibit alternation of generations with two forms of thalli, and in *Coelomomyces*, alternation of hosts as well. Emerson (1941) first elucidated the differences in complexity of life cycles and divided *Allomyces* into three subgenera (an approach Karling [1973] later followed in *Blastocladiella)*: (1) Subgenus *Euallomyces* includes all species of *Allomyces* with a long-life cycle consisting of isomorphic gametothallus and sporothallus. (2) In subgenus *Cystogenes*, the gametothallus is reduced to a holocarpic cyst (= the encysted meiospore released from the resistant sporangium on the sporothallus), which is totally consumed when it discharges four gametes. (3) *Brachyallomyces* remains for any species of *Allomyces* for which gamete fusion has not been found.

In the *Euallomyces* long cycle, the diploid sporothallus (Fig. 2f) bears thin-walled multinucleate zoosporangia (Fig. 3a) and thick-walled pigmented resistant sporangia (Fig. 3b and c). The zoosporangium produces diploid zoospores (mitospores) which regenerate the sporothallus. Meiosis occurs in resistant sporangia (Lange and Olson 1980a; Olson 1984; Olson and Reichle 1978a, b), which subsequently produce haploid zoospores (= meiospores). After a period of motility, meiospores encyst and germinate into haploid gametothalli.

In *Allomyces*, male and female gametangia are formed on the same gametothallus (Fig. 3d), but in *Coelomomyces* distinct male and female gametothalli are formed (Whisler et al. 1975). Male and female gametes may differ in size as in *Allomyces* or be the same size as in *Catenaria* and *Coelomomyces*. Unlike their colorless female counterparts, male gametangia and male gametes are orange because they contain carotene. Pommerville (1977, 1978) elucidated the role of chemical attractants in gamete recognition in *Allomyces*. Female gametes produce a sesquiterpene-type pheromone, sirenin (Pommerville 1977) that specifically attracts the male gametes; and male gametes similarly produce a compound, parisin (Pommerville and Olson 1987), that specifically attracts female gametes. In the presence of the hormones, gametes' swimming patterns are altered into ever narrowing arc paths until contact is made between male and female gametes (Pommerville 1978). Gametes fuse in pairs and swim as a biflagellated zygote, eventually withdrawing their flagella into the spore body, encysting, and germinating into a sporothallus.

Zoospore Ultrastructure

All flagellated spores of blastoclads (mitospores, meiospores, gametes, or zygotes) have similar and characteristic architectures with compactly organized organelles

(Fig. 1). Spores are nonassimilative and depend solely on endogenous reserves for energy, with little or no protein synthesis. The molecular machinery needed for zoospore motility and for zoospore germination, including undulopodia withdrawal, cyst-wall formation, and germ-tube initiation, is already packaged within the spore (Silva et al. 1987). The flagellar apparatus is in the posterior end of the zoospore and typically consists of the kinetosome, a nonflagellated centriole (=secondary centriole) lying at an angle and anterior to the kinetosome, a multilayered striated rhizoplast (root) positioned perpendicular to the plane of flagellar beat and connected to the basal mitochondrion (Aliaga and Pommerville 1990), and microtubules typically arranged in nine groups of threes extending symmetrically from dense material around the kinetosome as a cage around the nucleus and nuclear cap. All ribosomes are aggregated into a nuclear cap and surrounded by a cisterna that is continuous with the outer membrane of the nuclear envelope (Fig. 1). Messenger RNAs, arrested at the elongation stage, are sequestered among the ribosomes of the nuclear cap and are not translated until the ribosomes in the nuclear cap disperse during zoospore germination (Jaworski 1987; Jaworski and Stumhofer 1981). Minor variations on this basic design are found, such as the absence/reduction of a prominent rhizoplast in zoospores of *Physoderma, Sorochytrium,* and *Coelomomyces,* the lack of a nonflagellated centriole in *Coelomomyces*, and microtubules evenly spaced in *Sorochytrium* (Table 1).

Most blastoclad zoospores store a large amount of lipids that is metabolized with the coordination between microbodies and mitochondria (Powell 1978a). First called the side-body complex because of its conspicuous location in zoospores (reviewed in Fuller 1977; Fuller and Olson 1971), electron microscopy determined that this structure was an assemblage of microbodies, lipid globules, mitochondria, and membrane cisternae (the microbody-lipid globule complexes = MLC), an energy generation unit (Powell 1978a). In the MLC, lipid globules are sandwiched between microbodies and mitochondria (Fig. 4e), which extend toward and branch around the kinetosome. Typically there is also a single membrane cisterna (the backing membrane) associated with the MLC. Examination of zoospores of a range of species reveals that the basic organization of the MLC may vary in different genera (Dewel and Dewel 1990), but the association between these organelles is a consistent feature. However, in zoospores of the anaerobic genus *Blastocladia*, mitochondria and lipid globules are sparse, and microbodies and the backing membrane are absent (Lingle and Barstow 1983). There is evidence of stored glycogen in blastoclad zoospores, appearing to be most abundant in anaerobic members (Lingle and Barstow 1983).

Zoospores contain several classes of vesicles. One type is the gamma-particle vesicle enclosing a large electron-dense cup-shaped or globular particle that is synthesized during zoosporogenesis in the rough endoplasmic reticulum (Fig. 4b) as small particles which later coalesce (Barstow 1979). Biochemical analysis of the gamma particle shows that it is composed of two major proteins with high basic amino acid content (Hohn et al. 1984). The compact structure of the gamma particle breaks down during germination of the zoospore, suggesting the use of stored proteins during this process. A second type of vesicle contains an electron-dense

globule and is sometimes confused with the gamma-particle vesicle. However, energy dispersive X-ray analysis (Aldrich et al. 1984) and cytochemistry (Fig. 4c) demonstrated that this inclusion contained phosphorus and calcium and may be polyphosphate granules. Another group of vesicles, secretory vesicles, has a finely granular and homogenous matrix (Fig. 4c) and is implicated in zoospore adhesion or coat formation during encystment (Dewel and Dewel 1990; Martin 1971).

Systematics and Representative Genera

Thallus forms, host, and nutrition are used as primary characters for delimitation of the six families and sixteen genera of the Blastocladiomycota (Table 1). Formal assignments of *Polycaryum* and *Myiophagus* to families have not been made and await more extensive molecular characterization. These two genera are therefore currently considered *incertae sedis* within the Blastocladiomycota (Table 1).

1. Physodermataceae includes obligate parasites of plants, often in aquatic or moist environments, and forms a clade sister to other members of Blastocladiomycota in molecularly based phylogenies (James et al. 2006b, 2014; Porter et al. 2011). Unlike other members of the Blastocladiomycota, Golgi cisternae are stacked into dictyosomes (Fig. 27 in Lange and Olson 1980a). Karling (1950) placed *Urophlyctis* into synonym with *Physoderma*. Molecular phylogenetic analyses from herbarium specimens of *Physoderma* and *Urophlyctis*, however, place representatives of each genus as sisters in monophyletic clades (James et al. 2014; Porter et al. 2011), supporting the distinctiveness and monophyly of each genus and Sparrow's (1962) questioning of the validity of the earlier synonymy of *Urophlyctis* with *Physoderma* (Karling 1950). *Physoderma* produces two types of thalli on hosts, an epibiotic-monocentric thallus and an endobiotic-rhizomycelial thallus. The rhizomycelium of *Physoderma* generates a variety of intercalary enlargements, and resistant sporangia are formed from spindle-shaped or turbinate swellings (Karling 1950; Sparrow 1962). Resistant sporangia are characteristically large with sculptured walls, and at germination a large operculum is pushed open or a crack forms as the inner sporangial wall layer protrudes through the opening (Lange and Olson 1980a). As Sparrow (1962) discusses, *Urophlyctis* unlike *Physoderma* stimulates gall formation in hosts and can possibly be distinguished based on subtle morphological differences.
2. Paraphysodermataceae. *Paraphysoderma sedebokerense,* an algal parasite that produces amoeboid aplanospores within a monocentric, eucarpic thallus, has recently been described (James et al. 2011), and the new taxon is phylogenetically sister to the *Physoderma/Urophlyctis* clade (Hoffman et al. 2008; James et al. 2014; Porter et al. 2011). Rapidly swimming uniflagellate zoospores have also been observed (Strittmatter et al. 2016), but their role in the life history of this organism is not yet resolved. *Paraphysoderma sedebokerense* is highly destructive on *Haematococcus pluvialis* when the green algal host is grown in mass culture for the commercial production of astaxanthin, the red ketocarotenoid used

as a pigment and antioxidant in food, cosmetic, and pharmaceutical industries (Guerin et al. 2003; Strittmatter et al. 2016). The same species infects the green alga *Scenedesmus dimorphus* grown in mass culture for biofuel production (Letcher et al. 2016). On its host, *Paraphysoderma* produces an epibiotic thin-walled sporangium with a single rhizoid axis, and under certain conditions, it also produces resting sporangia with thick walls (Letcher et al. 2016). Typical of resting sporangial germination in the Blastocladiomycota, the outer wall cracks open during germination and discharge of spores. A recent ultrastructural study demonstrated the presence of synaptonemal complexes in thick-walled resting sporangia, indicating the site of meiosis (Letcher et al. 2016). In an ultrastructural study, both nonflagellated and flagellated spores were observed in resting sporangia (Letcher et al. 2016); thus, details of the life history of this organism remain to be elucidated.

3. Members of the Coelomomycetaceae are obligate parasites of mosquitoes, flies, ostracods, and copepods. *Coelomycidium* and *Coelomomyces* in the Coelomomycetaceae diverge from the Physodermataceae in molecular analyses and are sister to a clade containing Catenariaceae and Blastocladiaceae (James et al. 2006b; Porter et al. 2011). The life cycle of *Coelomomyces* requires an alternation between a dipteran primary host housing the sporothallus and a copepod or ostracod secondary host housing the gametothallus. The resistant sporangium is the only stage with a thick cell wall (Fig. 4a) and able to survive adverse environmental conditions.

   Zygotes typically infect mosquito larvae during the first or second instar stages (Travland 1979). The thallus begins as an unwalled protoplast discharged from the casing of an encysted zygote and gains entry into the host through the cuticle and epidermis. The thallus eventually moves into the coelomic cavity where it produces unwalled but carbohydrate-coated hypogens and branching filaments (Couch and Bland 1985; Powell 1976, 1994). The fungus acquires nutrients from the haemocoel and adjoining fat bodies, but can also invade other host tissues, eventually filling the larva with thick-walled resistant sporangia and killing the host. Resistant sporangia crack open (Fig. 4a) and release meiospores that infect a secondary copepod or ostracod host (Federici and Lucarotti 1986). Within the secondary host, distinctive colorless female gametothalli and orange-male gametothalli grow (Whisler et al. 1975). At maturity, the gametothalli cleave into gametes (Lucarotti and Federici 1984). Following death of the host, gametes escape and fuse in pairs forming biflagellated zygotes (Travland 1979). Zygotes then infect mosquito larvae and reestablish the sporothallus stage. Lightly infected larvae may pupate and morph into adults, in which case the female mosquito distributes resistant sporangia of the parasite rather than her own eggs (reviewed in Lucarotti and Shoulkamy 2000; Powell 1993; Scholte et al. 2004).
4. The two genera classified in the Catenariaceae (Table 1) are nutritionally diverse and include parasites of insects (especially midge egg masses), rotifers, nematodes, copepods, sheep liver flukes, and the blastoclad *Allomyces,* as well as saprotrophic members utilizing keratin and plant substrates (Couch 1945; Karling

1965; Martin 1991; Tribe 1977). *Catenophlyctis* (Karling 1965) is a commonly found soil keratinophilic blastoclad that produces both monocentric and polycentric thalli. Its resistant sporangia are amber to dark brown, but unlike those of *Catenaria,* do not lie free within the thallus wall. *Catenaria* produces a dendritic rhizomycelial sporothallus (Figs. 3e), eventually consisting of tubular, sterile isthmuses adjoining spherical zoosporangia (Fig. 3f) and resistant sporangia held loosely within the thallus. Rhizoids emanate from the surface of either the sporangium or the isthmuses, and the pointed tips of the rhizomycelium branch dichotomously (Fig. 3e and f). Early development of *C. allomycis* and *C. anguillulae* differs. *Catenaria allomycis* is an obligate parasite of *Allomyces*. It releases an unwalled spherical protoplast directly into the host cytoplasm (Powell 1978b). The round-cell stage produces a granular cell wall and then begins to elongate into a walled rhizomycelium. In response to the infection, the host proliferates an endoplasmic reticulum investment around the developing sporothallus of the parasite (Powell 1978b). The life cycle of *C. allomycis* is of the *Cystogenes* type, with a reduced gametothallus represented by a single cyst which releases four isogametes (Couch 1945). *Catenaria anguillulae* is a facultative parasite of nematodes, sheep liver flukes, and copepods. In *C. anguillulae*, the encysted spore germinates in a monopolar manner and produces a walled rhizomycelium directly within the host cell. No fusion of gametes (*Brachyallomyces* type) is known for this species, but detection of synaptonemal complexes in resistant sporangia of *C. anguillulae* suggests that meiosis occurs prior to resistant sporangium germination and release of meiospores (Olson and Reichle 1978a). The site of diploidization is not resolved.

Phylogenetically *Catenophlyctis variabilis* clusters with isolates identified as parasites of nematodes, *Catenaria anguillulae. Catenaria spinosa* and *C. uncinata*, parasites of midge eggs, however cluster in another clade. These results suggest that the genus *Catenaria* as currently circumscribed is not monophyletic (James et al. 2014; Porter et al. 2011).

5. The Blastocladiaceae is the only family composed entirely of saprotrophs. Thalli of the five genera in this family (Table 1) range from eucarpic, monocentric forms (Fig. 2c) to mycelial forms with indeterminate growth (Fig. 2b and e). Germination of the encysted zoospore or zygote is bipolar (Fig. 2d), and studies have demonstrated antigenic differences between the rhizoidal and hyphal surfaces of *Allomyces macrogynus* (Fultz and Sussman 1966). *Allomyces* is mycelial and grows as a saprotroph on decaying plant and animal matter. It has a characteristic basal cell, anchored with rhizoids, that is most obvious early in development (Fig. 2e). The apex branches in a tuning-fork manner (sympodially or dichotomously) (Fig. 2e and f). Sporangia (Fig. 3a), resistant sporangia (Fig. 3b and c), or gametangia (Fig. 3d) are typically formed at the hyphal apex. Pseudosepta are obvious along hyphae (Fig. 2a and e) as is also an apical structure that corresponds to the location of the Spitzenkörper (Fig. 2b) (defined below). Species of *Blastocladiella* (Couch and Whiffen 1942) bear a single zoosporangium, resistant sporangium (Fig. 2c), or gametangium at the apex of an unbranched basal cell and can appear simply as a monocentric thallus (Fig. 2c). There may be some space

between the resistant sporangium and the thallus wall (Fig. 2c). *Blastocladia* has a similar thallus with a trunk-like basal cell attached to the substrate with rhizoids, but the apex of the basal cell is lobed or branched. On natural substrates, *Blastocladia* grows in crisp white tufts or pustules. Significantly this genus is an obligate fermenter and functions as a facultative anaerobe. It requires a carbohydrate source, such as found in fleshy fruits or twigs, for fermentation. However, unlike obligatory anaerobic rumen fungi, *Blastocladia* cells contain mitochondria and not hydrogenosomes (Gleason et al. 2002; Lingle and Barstow 1983). The Blastocladiaceae is in need of revision because it is polyphyletic in molecular phylogenetic analyses with representatives of *Blastocladiella* failing to place in the clade with *Allomyces, Blastocladia,* and *Microallomyces* (James et al. 2014; Porter et al. 2011).

6. The Sorochytriaceae contains a single species, *Sorochytrium milnesiophthora*, a parasite of the moss-inhabiting tardigrade, *Milnesium tardigradum* (Dewel et al. 1985). This blastoclad produces two thallus forms. In the living host, it forms the parasitic endobiotic colonial phase consisting of a sorus with numerous sporangia that scatter within the host body cavity. When the host dies, *Sorochytrium* grows externally as a saprotrophic rhizomycelium. Eventually, the whole body of the tardigrade becomes filled with orange thick-walled resistant sporangia.

Maintenance and Cultivation

Whisler (1987) gave an insightful summary of techniques widely used to collect and isolate blastoclads, building on more classical narratives (Sparrow 1960). Approaches for isolation of zoosporic fungi from soil and water can also be found in more general but still detailed descriptions (Bills et al. 2004; Fuller and Jaworksi 1987; Shearer et al. 2004). Because many blastoclads produce thick-walled resistant sporangia that withstand desiccation, use of slowly air-dried soils is an effective approach to select for genera such as *Allomyces, Blastocladiella*, and *Catenaria* (Willoughby 1984). It is sometimes necessary to store a dried sporothallus for a period of time and rehydrate it to induce germination of resistant sporangia. Many blastoclads (*Coelomomyces, Catenaria allomycis, Physoderma*) are obligate parasites and have only been grown in two-membered or mixed cultures. Barr and Babcock (1994) developed a method for cryopreservation and long-term storage of cultures that has proved in practice successful with recovery of cultures frozen 20 years previously.

Flooding about a teaspoon of soil with sterile pond water or distilled water and baiting with a variety of substrates (keratin, chitin, cellulose, pollen, oil-rich seeds) can result in growth of saprotrophic blastoclads on these substrates. This "enrichment" technique allows the proliferation of organisms which can then be brought into pure culture by streaking thalli or zoospores on nutrient media containing antibiotics (250 mg/L penicillin and streptomycin). Dilute nutrient media at 20–50% strength are commonly used for initial isolation media. With repeated transfers, isolates become adapted to richer media on which they survive for longer

times. Two common media (Whisler 1987) used for maintenance of blastoclads are YpSs (20.0 g soluble starch, 1.0 g yeast extract, 1.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 1 L distilled water, 20 g agar) and PYG (1.25 g peptone, 1.25 g yeast extract, 3.0 g glucose, 1 L distilled water, 20 g agar).

Because it is an obligate fermenter and facultative anaerobe, the genus *Blastocladia* is often collected on submerged twigs and fleshy fruits in small bodies of stagnant waters with reduced concentrations of dissolved oxygen (Gleason et al. 2002; Whisler 1987). A common method for collecting is to take a firm apple, prick its surface repeatedly with a needle, and place the apple in a wire cage. The cage is left submerged in a pond for several weeks to several months. The apple is then brought into the lab and inspected for the crisp white pustules of blastoclads. If pustules are present, the surface is washed vigorously to remove biofilms, and thalli are transferred with a fine needle to nutrient media containing antibiotics. To maintain *Blastocladia* in broth culture, it is necessary to neutralize the lactic acid it produces with a base. It is grown in broth in an Erlenmeyer flask with a side arm containing 0.5 M NaOH, added as needed to maintain the pH in the 6.7–7.0 range. Broth used contains 3.0 g glucose or glycerol, 1.25 g yeast extract, 1.25 g peptone, 1.36 g KH_2PO_4, 0.71 g Na_2HPO_4, 0.12 g $MgSO_4 \cdot 7H_2O$, 1.0 mg bromocresol purple, a color indicator of pH, 1 L distilled water (Gleason et al. 2002).

Evolutionary History

Early Devonian Rhynie Cherts in Aberdeenshire, Scotland, amassed some of the best preserved fossil remains of zoosporic fungi (Remy et al. 1994). Microscopic preparations of these materials have revealed the presence of thalli that resemble present-day blastoclads. For example, *Paleoblastocladia milleri* produced two types of dichotomously branching thalli bearing gametangia and sporangia in terminal chains. Thus, fully 400 million years ago blastoclads were present on earth and were in the process of their evolutionary radiation (Remy et al. 1994). Another approach to building the evolutionary history of fungi, which generally do not have as abundant fossil record as groups such as higher plants and animals, is the calibration technique (Berbee and Taylor 2007). In this method, groups of organisms with rich fossil records and with different times of first occurrence on earth are used to calibrate the evolutionary rates of base changes in genes through geological time. Estimates based on these models suggest that fungal and metazoan lineages diverged from a shared protoctistan ancestor over a billion years ago and that the basal zoosporic fungi radiated 800 million years ago (Berbee and Taylor 2007; Steenkamp et al. 2006). Thus, zoosporic fungi as members of the supertaxon Opisthokonta are more recent than oomycetes and other flagellated heterotrophs. The timing of the radiation of blastoclads suggests that the advent of plants and animals on land and in wetlands may have provided new hosts and sources of organic substrates for blastoclads, driving their evolution. Consistent with this view, in molecular-based phylogenies of blastoclads, members that are algal and plant parasites are basal (James et al. 2006b; Porter et al. 2011). Later divergences led to blastoclad

saprotrophs and blastoclad parasites of invertebrate animals (James et al. 2006b; Porter et al. 2011).

Numerous molecular phylogenetic studies have suggested that the Blastocladiomycota represent an early radiation of fungi from a protoctistian ancestor, diverging from other groups of zoosporic fungi including chytrids (James et al. 2006a, b; Steenkamp et al. 2006). Phylogenetic analyses of gene sequences place the filose-pseudopodial amoebae group, the nucleariids, as ancestors to the fungi and also diverging from a flagellated protoctistian ancestor (Medina et al. 2003; Steenkamp et al. 2006). Supporting this hypothesis, blastoclad zoospores are capable of amoeboid motion, even when they are undulopidated (Deacon and Saxena 1997). The recent description of a blastoclads capable of producing amoeboid spores (Hoffman et al. 2008; James et al. 2011) further supports the sister relationship of nucleariids to fungi.

As might be expected when techniques and approaches to analyses are still in refinement, alternate phylogenetic hypotheses exist, but consistently the blastoclads are resolved at the basal radiation of the fungi (Aleshin et al. 2007; Liu et al. 2006). Accordingly, in blastoclads and other fungi, cell walls contain chitin as a structural polysaccharide, the essential amino acid lysine is synthesized in the alpha-aminoadipic-acid pathway with distinct enzymes (Nishida and Nishiyama 2000; Vogel 1964), glycogen serves as the major storage carbohydrate, and mitochondria contain lamellate cristae. Consistent with their evolutionary radiation in a lineage leading to higher fungi and separate from chytrids, blastoclads have features found in higher fungi and not in chytrids. Similar to many higher fungi, the poles of the nuclear envelope remain closed during mitosis (Olson 1984) in contrast to open poles found in chytrids. For blastoclads with hyphal growth, the Spitzenkörper (Fig. 2b) is the organizational region for tip elongation (Vargas et al. 1993), but thus far a typical Spitzenkörper has not been identified in chytrids. It should be noted, however, that the Spitzenkörper of *Allomyces* consists of a granular/fibrillar matrix that excludes ribosomes and other organelles, whereas in regularly septate fungi, the Spitzenkörper contains an aggregation of apical and microvesicles (Vargas et al. 1993). Also similar to most fungi and unlike chytrids, most blastoclads (*Physoderma* and *Paraphysoderma* are exceptions) have been reported to produce Golgi equivalents (Fig. 4d; Bracker 1967) in their endomembrane system (Feeney and Triemer 1979; Sewall et al. 1989) rather than the stacked cisternae in dictyosomes characteristic of chytrids and most other eukaryotic organisms.

Blastoclads differ from chytrids in additionally significant ways. Meiosis in blastoclads is typically sporic (Lange and Olson 1980a; Olson 1984; Olson and Reichle 1978a, b), whereas in chytrids it is zygotic. Cleavage of blastoclad zoospores begins with the growth of the flagella into a flagellar vesicle followed by cytoplasmic cleavage (Renaud and Swift 1964), while in chytrids the zoospore body and flagella are cleaved at the same time. On the other hand, indicative of an ancient shared flagellar ancestor, both blastoclads and chytrids have cholesterol rather than ergosterol as their major sterol (Weete et al. 1989), nine flagellar props connect the zoospore kinetosome to the plasma membrane (Barr 1981; Barr and Hadland-Hartmann 1978), and the transition zone between the kinetosome and flagellum contains a spiral

concentric fiber sometimes called a transitional helix (Barr 1981, 1992) (Fig. 1). Morphological and developmental characteristics in Blastocladiomycota, thus, support the reliability of molecular-based phylogenetic hypotheses and define blastoclads as a zoosporic fungal group distantly related to, but distinctive from, chytrids.

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Chytridiomycota

42

Martha J. Powell

Abstract

The Chytridiomycota is a group of fungi characterized by the production of zoospores with a single posteriorly directed flagellum. The thalli of these fungi are typically microscopic and varied. The advent of molecular phylogenetics combined with analyses of zoospore ultrastructural characters has transformed our understanding of the relationship of these fungi and has revealed greater genetic diversity than expected. What was once the single order Chytridiales is now ten monophyletic orders. *Rozella*, *Olpidium*, and the Blastocladiomycota, organisms once thought to be within the Chytridiomycota, diverge in separate lineages and are no longer included in the Chytridiomycota. The Neocallimastigomycota are sister to Chytridiomycota, but they are distinct in their zoospore ultrastructure, behavior of nuclear envelope during mitosis, and specific adaptation as anaerobes to digestive systems of herbivores. Molecular characterization of environmental samples demonstrates that zoosporic fungi are widespread and can survive in a range of habitats, from oceans to freshwater and even harsh environments including under artic snow or in exposed soils in alpine regions. The importance of these organisms as parasites, saprotrophs of refractory materials, and components in food webs is discussed. Ways to collect, culture, and characterize these organisms are highlighted. Current classification based on molecular and ultrastructural analyses is described.

Keywords

Biodegradation • Food webs • Fungi • Molecular phylogenetics • Parasitism • Systematics • Zoospore ultrastructure

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Summary Classification

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●●●**Rhizophlyctidales** (*Arizonaphlyctis, Borealophlyctis, Rhizophlyctis, Sonoraphlyctis*)
●●●**Spizellomycetales** (e.g., *Geranomyces, Kochiomyces, Powellomyces, Spizellomyces, Triparticalcar*)
●●●**Chytridiales** (e.g., *Chytridium, Chytriomyces, Pseudorhizidium, Rhizoclosmatium, Irineochytrium*)
●●●**Cladochytriales** (e.g., *Catenochytridium, Cylindrochytridium, Endochytrium, Nephrochytrium, Nowakowskiella*)
●●●**Polychytriales** (e.g., *Arkaya, Karlingiomyces, Lacustromyces, Neokarlingia, Polychytrium*)
●●●**Lobulomycetales** (e.g., *Alogomyces, Clydaea, Cyclopsomyces, Lobulomyces, Maunachytrium*)
●●●**Mesochytriales** (e.g., *Mesochytrium*)
●●●**Gromochytriales** (e.g., *Gromochytrium*)
●●●**Synchytriales** (e.g., *Synchytrium, Micromyces, Endodesmidium*)

- ••**Monoblepharidomycetes** (e.g., *Gonapodya, Harpochytrium, Hyaloraphidium, Monoblepharella, Monoblepharis*)
- •**Neocallimastigomycota** (e.g., *Anaeromyces, Caecomyces, Neocallimastix, Orpinomyces, Piromyces*)

Introduction

General Characteristics

In the previous edition of *Handbook of Protoctista*, the Phylum Chytridiomycota *sensu lato* included all posteriorly uniflagellated zoosporic osmotrophs (Barr 1990). Multigene-based phylogenies, however, revealed unexpected genetic diversity among these organisms, as well as evidence that the Chytridiomycota was paraphyletic (James et al. 2006a, b). As a consequence, the Phylum Chytridiomycota was retained *pro parte* and redescribed, circumscribing a monophyletic group (Hibbett et al. 2007), and two new Phyla were established for excluded taxa. (1) The Phylum Neocallimastigomycota (neocallimastigos), a clade sister to the Chytridiomycota, was erected (Hibbett et al. 2007) for specialized obligate anaerobes that reproduce with zoospores bearing single to multiple posterior undulipodia and that inhabit the digestive systems of ruminate and other herbivores (Ho and Barr 1995; Mountfort and Orpin 1994; Trinci et al. 1994). (2) The order Blastocladiales was elevated to the Phylum Blastocladiomycota (James et al. 2006b) as a monophyletic lineage diverging among filamentous, non-zoosporic higher fungi, rather than with the Chytridiomycota. Morphological, developmental, and ultrastructural characters support the molecular distinctions of the three phyla of zoosporic osmotrophic opisthokonts, Chytridiomycota, Neocallimastigomycota, and Blastocladiomycota (Hibbett et al. 2007; James et al. 2006a, b; Powell and Letcher 2012, 2014a, b).

This chapter emphasizes the Phylum Chytridiomycota as currently described, including its two classes Chytridiomycetes (= chytrids) and Monoblepharidomycetes (= monoblephs) (Hibbett et al. 2007). Although Doweld (2001) raised monoblephs to a phylum, the group typically places sister to Chytridiomycetes in molecular phylogenetic analyses (Dee et al. 2015). Zoospore ultrastructure and the structure of the mitotic apparatus, with opening of the nuclear envelope only at the spindle poles (Dolan and Fuller 1985; McNitt 1973; Powell 1975, 1980; Roychoudhury and Powell 1991), further substantiate the relationship of these two classes. Thus, in this chapter we discuss monoblephs as a class in Chytridiomycota, following the precedent in Hibbett et al. (2007) and Dee et al. (2015).

This chapter also addresses general characteristics of the smaller but important sister group, the Phylum Neocallimastigomycota (= neocallimastigos) once classified in the Chytridiomycota. Zoospore structure of neocallimastigos is distinct (Gold et al. 1988; Heath et al. 1983; Li et al. 1991, 1993) from that of Chytridiomycota, and

in contrast to Chytridiomycota, the nuclear envelope remains intact during mitosis (Li et al. 1993).

Members of the Chytridiomycota characteristically produce thalli with coenocytic sporangia which discharge zoospores bearing single posteriorly directed flagella. Although basal to the higher fungi, their major sterol is cholesterol rather than ergosterol (Weete et al. 1989). Like fungi, Chytridiomycota gain nutrition as osmotrophs, store carbohydrates as glycogen, synthesize lysine by the α aminoadepic pathway (Vogel 1964), have mitochondria with flattened cristae (Fig. 1b), and construct cell walls containing the structural polysaccharide chitin. In the tree of life, Chytridiomycota are among the earliest radiation of fungi (Steenkamp et al. 2006). Over 1,000 species of Chytridiomycota have been described based on classical taxonomy and thallus structure, but recent molecular and ultrastructural studies indicate that the actual species richness of this group is largely untapped (Letcher et al. 2005, 2008a, b, c, 2012b). Adapting chytrids for survival in diverse habitats and substrates, a variety of thallus forms range from a holocarpic sporangium to eucarpic hyphae bearing numerous sporangia (see Characterization and Recognition). The most distinguishing trait of chytrids is their posteriorly, whiplash uniflagellated zoospore, deriving energy from stored lipid (Powell 1976b, 1978) and glycogen and swimming with a characteristic abruptly darting and hopping pattern, sometimes becoming amoeboid in its motion (Sparrow 1960). After a period of motility, zoospores retract their flagella, produce a wall (Fig. 1a), and develop into thalli that will eventually generate sporangia. For some orders of chytrids, such as the Spizellomycetales, sexual reproduction has never been reported; in others, such as the Chytridiales (Fig. 2c), events of sexual reproduction are well documented. Oogamous sexual reproduction characterizes the monoblephs; but some genera, such as *Harpochytrium,* appear to reproduce only asexually (Powell and Letcher 2012).

Occurrence

Karling's (1977) atlas of Chytridiomycota displays their astonishing shapes, forms, and seemingly unlimited modes of survival in and on living and decaying organisms. Monoblephs (Fig. 2h–j) are typically found in tropical and temperate regions, most commonly in permanent still bodies of freshwater with low silt. They occur primarily as saprotrophs on submerged, water-logged twigs; insect cadavers; and rosaceous fruits (Emerson and Natvig 1981; Emerson and Whisler 1968; Sparrow 1960; Whisler 1987).

Chytrids are essentially universal in aquatic and terrestrial habitats as saprotrophs, biotrophs, and necrotrophs (Fig. 2f) (Powell 1993), including living in higher plants and animals. They thrive in the temperate zone, especially in lakes, ponds, bogs, and acidic forest soils (Bills et al. 2004; Fuller and Jaworski 1987; Shearer et al. 2004; Sparrow 1960) and can readily be isolated from plankton tows, submerged muds, and floating plant and animal debris that accumulate along the shore line of lakes and ponds (Barr 1990, 2001; Sparrow 1960). Although less common than in freshwater,

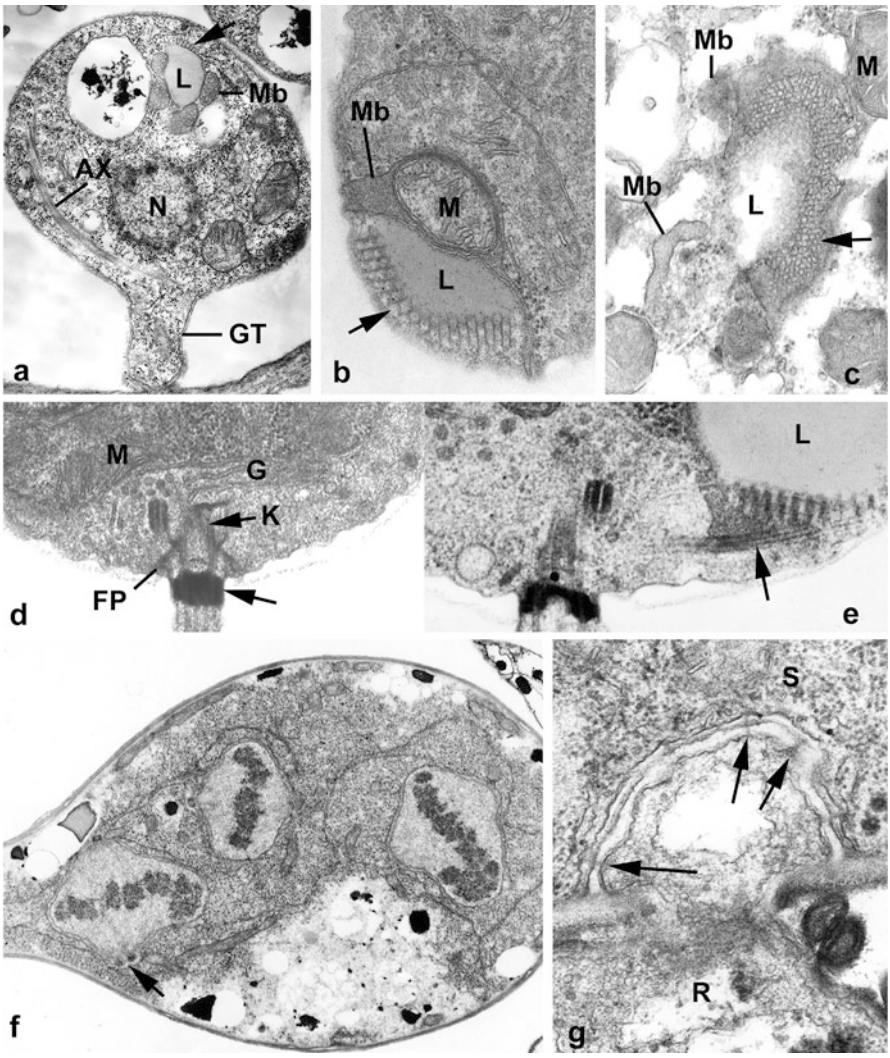

Fig. 1 (**a**) Electron microscopy of germination of a recently encysted zoospore that has generated a germ tube (GT). The nucleus (N) remains in the zoospore cyst. Remnants of the retracted axoneme (AX) microtubules are visible. Organelles in the microbody lipid globule complex have still not dispersed. Microbodies (Mb) are adjacent to the lipid globule (L). On the surface of the lipid the fenestrated cisterna (*arrow*) is still visible. ×13,000. (**b**) Microbody lipid globule complex (MLC) in zoospore of the chytrid, *Chytriomyces hyalinus*. The fenestrated cisterna (*arrow*) underlies the plasma membrane and is adjacent to the lipid globule (which is in contrast to the organization of the MLC of monoblephs where the fenestrated cisterna is adjacent to the microbody). A microbody (Mb) is appressed to the lipid globule (L) and is closely associated with the mitochondrion (M). Notice that mitochondria (M) have flattened cristae. The MLC is involved in energy production for zoospores. ×35,000. (**c**) Microbody lipid globule complex (MLC) in zoospore of the monobleph, *Monoblepharella*. Notice that the MLC organellar organization in the monobleph differs from that of the chytrid in (**b**). The fenestrated cisterna (= rumposome) (*arrow*) contacts the microbody

chytrids are also found in marine habitats, most frequently as parasites of algae (Johnson 1966; Lepelletier et al. 2014; Muehlstein et al. 1988; Müller et al. 1999; Nyvall et al. 1999). Reproduction with zoospores mandates that most chytrids depend upon water for dispersal. However, they are also adundant in dry soils, including halomorphic soils, tree–top canopy soils, grass land soils, and disturbed soil, (Booth 1971; Letcher et al. 2004a, 2014a; Longcore 2005; Lozupone and Klein 2002; Willoughby 1998). They are known to survive in harsh environments (Gleason 1976; Gleason et al. 2004, 2005; Letcher et al. 2004a, 2008b) and have been detected at the earth's Polan regions and high alpine mountains, even under blankets of snow or within sea ice (Freeman et al. 2009; Hassett and Gradinger 2016; Powell 1993; Schmidt et al. 2012; Simmons et al. 2009). With expanded exploration for life in extreme environments (Nagahama et al. 2011), it is expected that more chytrids, as ancient organisms, will be detected in these environments. Consistent with this view, chytrids are now known to inhabit herbivore dung (Davis et al. 2016b; Simmons et al. 2012; Wakefield et al. 2010).

Chytrids are microscopic and seldom observed directly from an environmental sample, other than one containing algae parasitized by chytrids. Thus, their occurrence is typically detected by incubating a sample in the laboratory with the addition of enrichment "baits" for a few days (Bills et al. 2004; Fuller and Jaworski 1987; Shearer et al. 2004). Intensive baiting of soil and aquatic samples has revealed an astounding amount of diversity of chytrids, even from a localized area (Davis et al. 2013). The phylogenetic tree generated from analysis of sequences from chytrids cultured in the Davis et al. (2013) survey had a tree topology corresponding with that from analyses of chytrids collected globally (North American, Latin American, Australia). Thus, these results demonstrate how widespread chytrids are, as well as the value and practicality of intensive sampling from a localized region (Davis et al. 2013).

Culture-independent molecular analyses of environmental samples have also revealed that chytrids are more abundant and more diverse than previously understood (Freeman et al. 2009; Lefèvre et al. 2007, 2008, 2012). PCR methodologies have unexpectedly recovered chytrid ribosomal genes (phylotypes) from novel sites, such as the intestinal system of mice (Scupham et al. 2006). Development of

Fig. 1 (continued) (Mb) instead of the lipid globule (L), but the microbody is still proximal to the lipid globule. ×35,000. (**d**) Transmission electron micrograph showing characteristics of systematic importance: mitochondrion (M) associated with kinetosome (K); plug in transitional region of flagellum (*arrow*), Golgi apparatus in posterior end of zoospore (G); presence of flagellar props (FP). ×60,000. (**e**) Transmission electron micrograph showing features of the zoospore of systematic importance. A microtubular root (*arrow*) extends from the side of the kinetosome, over the microbody which is adjacent to the lipid globule (L), and contacts fenestrated cisterna. ×78,000. (**f**) Typical synchronous mitotic division in multinucleate chytrid sporangium. Notice that at metaphase there is perinuclear ER. The nuclear envelope remains intact and opens only at the pole. Paired centrioles are at each end of the spindle poles (*arrow*). ×4,000. (**g**) Dome-shaped septum between sporangium (S) and rhizoid (R). Several plasmodesmata (*arrows*) traverse the septum. ×40,000

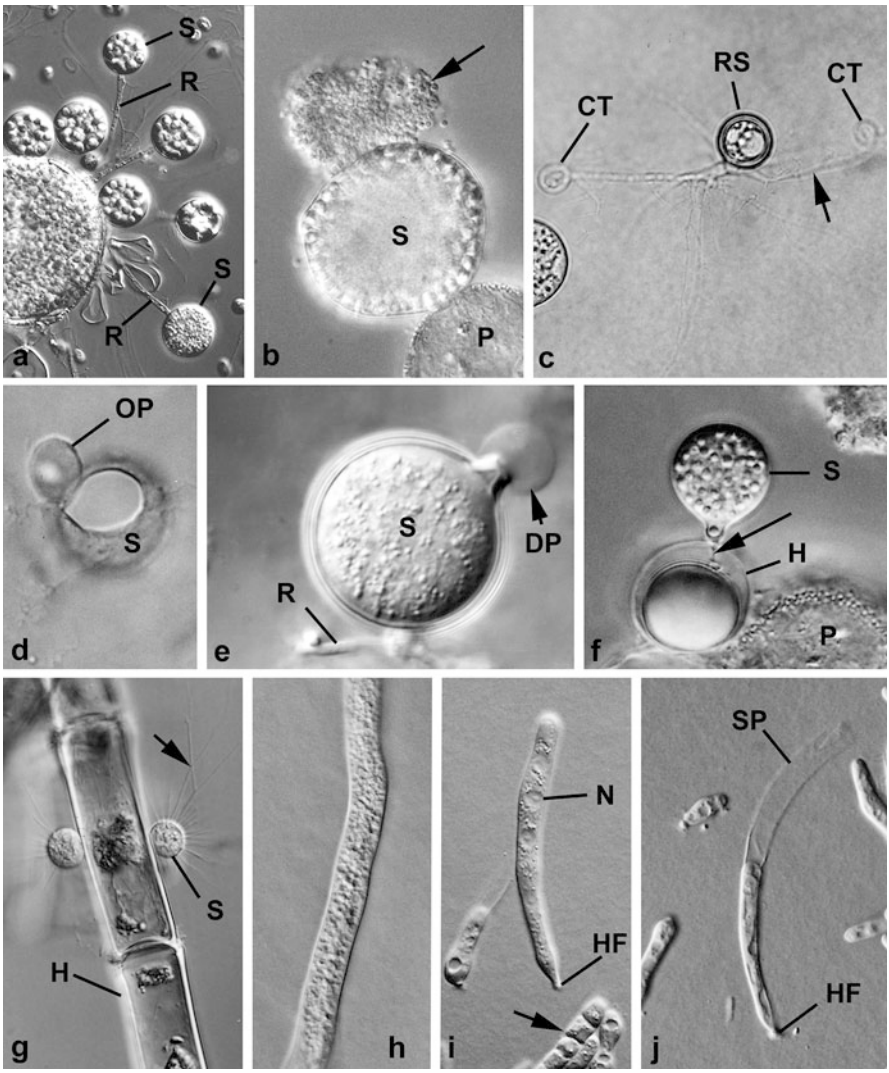

Fig. 2 (**a**) Epibiotic thalli on pollen. The sporangium (S) is on a long, pedicel-like rhizoid (R). ×600. (**b**) Monocentric thallus with a sporangium (S) grows on a pollen grain (P) and is releasing zoospores (*arrow*) in a cluster. The zoospores swarm in the vesicle, become active in swimming, and then they will swim away from the mass. ×800. (**c**) Sexual reproduction in *Chytriomyces hyalinus*. Rhizoids of two contributing thalli (CT) fuse and the contents of both pass through the rhizoids (*arrow*) and fuse. At the point of fertilization, a zygote forms and develops into a thick-walled resting spore (RS). ×700. (**d**) An operculum (OP) is folded back and opens the discharge pore in the sporangium (S). ×500. (**e**) A gelatinous discharge plug (DP) protrudes from an inoperculate discharge tube prior to zoospore discharge from a sporangium (S). Rhizoids (R) are at the base of the sporangium. ×1.000. (**f**) Chytrids may grow on other chytrids. The resting spore (H) of one chytrid is growing on a pollen grain (P), and another chytrid (S) grows on the resting spore, penetrating it with a delicate unbranched haustorium (*arrow*) ×1000. (**g**) Two thalli of a

molecular methods, such as colorimetric hybridization assays using species-specific peptide nucleic acid probes linked to dyes, enable rapid detection of individual chytrid species (Duy et al. 2015).

Literature

The most extensive monographic treatments of chytrids and monoblephs are Sparrow's (1960) *Aquatic Phycomycetes*, Karling's (1964) *Synchytrium*, and Karling's (1977) *Chytridiomycetarum Iconographia*. More recent approaches have focused on taxonomic revisions, as with *Diplochytridium* (Blackwell et al. 2002), or monographic synthesis of single genera, as with *Chytriomyces* (Letcher and Powell 2002a), *Karlingiomyces* (Blackwell et al. 2004), *Obelidium* (Blackwell et al. 2012), *Rhizophydium* (Letcher and Powell 2012), *Septosperma* (Blackwell and Powell 1991), and *Solutoparies* (Blackwell and Powell 1998). A summary of growing knowledge of chytrids based on molecular phylogenetics and comparative zoospore ultrastructure is *Chytrid Fungi on Line* http://nsfpeet.as.ua.edu/. Longcore (1996) maintains a compilation of taxonomic changes among Chytridiomycota since Sparrow's (1960) monograph at http://umaine.edu/chytrids/chytrid-bibliography/.

History of Knowledge

Sparrow (1960) provided a comprehensive historical account of the early systematics of chytrids and monoblephs. The history of chytrids began in 1851 with Braun's description of *Chytridium olla*, a monocentric, epibiotic biotroph of oogonia of *Oedogonium* (Sparrow 1960, 1973). The history of monoblephs began in 1871 when Cornu described *Monoblepharis*, a unique aquatic fungus with sexual reproduction involving nonmotile eggs in oogonia and motile sperms in antheridia. Because of its filamentous thallus, Cornu (1871) considered the new genus a member of the Oomycetes. For most of their history, Chytridiomycota were regarded as Phycomycetes (Fitzpatrick 1930), a collection of heterotrophic fungal and fungal-like organisms with coenocytic thalli that produced spores within sporangia. A major

Fig. 2 (continued) chytrid growing on a filamentous green alga. The chytrids have destroyed the host (H) cytoplasm and chloroplasts are clumped. The sporangium (S) of this chytrid is ornamented, with fine filaments (*arrow*). ×500. (**h**) Highly vacuolated hypha of *Monoblepharis*, illustrating the characteristic foamy appearance of hyphae of monoblephs. ×500. (**i**) Simple, unbranched filamentous thallus of the monobleph *Harpochytrium*. A holdfast (HF) attaches the thallus to a substrate, and nuclei (N) are arranged linearly. At the bottom of the picture a portion of a sporangium with cleaved zoospores is visible (*arrow*). ×900. (**j**) Thallus of *Harpochytrium* which has discharged zoospores, leaving the walls of the empty sporangium (SP) and the basal cell which retains its cytoplasm. Notice the hold fast (HF) of the thallus. ×1,000

advancement was made in understanding the relationships of zoosporic heterotrophs when Scherffel (1925) popularized the concept that the structure of the motile cell was of primary significance as a taxonomic character. Sparrow later adopted a classification scheme that grouped together organisms with posteriorly uniflagellate zoospores (Sparrow 1943), including chytrids and monoblephs as distinct orders (Sparrow 1960). Consistent with this earlier view of relationships, recent molecular and ultrastructural phylogenetic analyses support the related nature of chytrids and monoblephs as sister clades (Dee et al. 2015; James et al. 2006a, b); however, considering the striking differences in pattern of sexual reproduction, they are now classified as classes (Chytridiomycetes and Monoblepharidomycetes) in the Chytridiomycota (Hibbett et al. 2007). Doweld (2001) raised monoblephs to a phylum and created additional lower ranks (reviewed in Powell and Letcher 2012); but consistent with the most recent molecular phylogenetic understanding of monoblephs, we herein consider them as a class (Dee et al. 2015).

Practical Importance

Monoblephs are only known as saprotrophs, and none are recognized to form mutualistic or parasitic relationships. Some monoblephs, however, may be found growing as epiphytes on other organisms, such as snail shells, seeds, and algae (Emerson and Whisler 1968), but no other commensal relationships have been identified.

Chytrids are common in soil and aquatic habitats rich in organic substrates or suitable hosts where they play a vital role in nutrient recycling and control of populations of other organisms (Kagami et al. 2007, 2011, 2012; Powell 1993). The concept of the role of chytrids in a "mycoloop," where zoospores released from sporangia parasitizing inedible phytoplankton facilitate energy transfer as zooplankton feed on energy rich chytrid zoospores, highlights the importance of chytrids in aquatic food webs (Kagami et al. 2014). Chytrids can breakdown substrates that are resistant to decay by most other organisms, including cellulose, keratin, chitin, spores of mosses and ferns, and pollen. They are especially common as necrotrophs and biotrophs of cyanobacteria and algae (freshwater, terrestrial, and marine), oomycetes, protozoa, and microinvertebrates. The large genus *Synchytrium* contains obligate biotrophs of plants and algae (Karling 1964). *Synchytrium endobioticum*, the etiological agent of black wart on potatoes, is an especially troubling pathogen because its resting spores may remain viable for at least 30 years, rendering contaminated soils unsuitable for cropping with potatoes (reviewed in Powell 1993; Smith et al. 2014). This species also has the distinction of appearing on the United States Department of Agriculture Animal and Plant Health Inspection Service list as a "Select Agent" (Smith et al. 2014).

The most notorious chytrid is *Batrachochytrium dendrobatidis* (Bd), the first chytrid known to exploit vertebrate animals (Longcore et al. 1999, 2007). Attacking

frogs, toads, salamanders, newts, and caecilians, *B. dendrobatidis* causes chytridiomycosis and is associated with the alarming decline, extirpation, and extinctions of frogs (Daszak et al. 2004). Retallick et al. (2006) demonstrated that the same strain of Bd could infect both frogs and salamanders. The disease mechanism of Bd is not totally understood, but zoospores produce thalli within the keratinized epidermal cells of frog skins and tadpole mouth parts. Tadpoles do not appear to be adversely affected by the chytrid, but the skin in infected areas of adults becomes thickened (hyperkeratosis). Since amphibians depend in part on their skin for gas exchange and osmotic homeostasis, it is thought that Bd may produce toxic compounds or essentially suffocate the frog. To explore the molecular basis of infection, Rosenblum et al. (2008) compared stage-specific gene expression in sporangia versus zoospores using whole genome arrays. Significantly, they found that genes in a gene family associated with dermatophytous fungi, fungalysin metallopeptidase, was also expanded and differentially expressed in sporangia and zoospores.

Although awareness of *B. dendrobatidis* started in the 1990s, retrospective studies of museum preserved frog specimens indicate Bd has been active since 1932 in Africa where it coexisted with its host (Weldon et al. 2004). How genetically similar strains emerged at the same time all around the world is not known (Morehouse et al. 2003), but one theory is that Bd was carried to new sites with the increased spread of exotic species through the global pet trade (Daszak et al. 2004; Fisher and Garner 2007) and the worldwide distribution of *Xenopus laevis,* first for human pregnancy tests in the mid-1930s and later as a model research organism (Weldon et al. 2004). Infected frogs escaping captivity or interacting with other exotic frog species may have released the pathogenic chytrid onto highly vulnerable non-native hosts with no resistance to Bd. Since the pathogen seems to be most destructive in pristine areas, humans may also spread Bd from their own activities in studying these sites (Weldon et al. 2004). The frog chytrid does not survive high temperatures for long periods of time, growing between 4 °C and 25 °C, and temperature may restrict its spread in some areas (Piotrowski et al. 2004); although, it nonetheless continues to successfully spread on a global scale.

Comparative genomics of Bd strains from geographically diverse regions (Farrer et al. 2011) have revealed more genetic diversity than earlier detected (Morehouse et al. 2003) as well as ranges of pathogenicity, including a hypervirulent global panzootic lineage. Curiously, different strains contain different levels of heterozygosity, suggesting potential for recombination. Neither sexual reproduction nor resistant sporangia (which may be formed either asexually or sexually) has been discovered for this organism, although a case has been made that they do exist (Morgan et al. 2007). On the other hand, a polyploidy event of the total genome could have resulted in "hybrid vigor," which could contribute to its level of lethality.

Recently a second lethal species of *Batrachochytrium*, *B. salamandrivorans*, was discovered in the Netherlands infecting and causing mass die-offs of salamanders (Martel et al. 2014). This species poses a serious threat to the world's salamander and newt populations due to its virulence, host range, and the prevalence of international pet trade of salamanders (Martel et al. 2014).

Habitats and Ecology

Assessments of the importance of chytrids in the environment are increasing, including their roles in trophic transformations as a food source for microinvertebrates (Kagami et al. 2007, 2011, 2014), in the decline of zooplanktonic populations, in control of phytoplanktonic blooms (Beakes et al. 1988, 1993; Canter and Jaworski 1981; Doggett and Porter 1996; Ibelings et al. 2004), and in biodegradation of refractory materials and nutrient recycling (Midgley et al. 2006; Powell 1993).

Chytrid ecology is emerging as a new focus in studies of food webs and energy loops (Gleason et al. 2008; Kagami et al. 2007, 2014), especially in aquatic systems and with the use of molecular detection of chytrids (Lefèvre et al. 2007, 2008, 2012). Ecological studies suggest that chytrids can serve as a food source for microinvertebrates. In an investigation of *Daphnia*, one of the keystone crustacean species in aquatic habitats, researchers found that this filter-feeder readily consumed cholesterol-rich zoospores of chytrids which grew on diatoms. Thus, although the large, silicon-covered diatoms were not palatable to *Daphnia*, chytrids transformed the biomass of these primary producers into chytrid biomass, which *Daphnia* could readily utilize (Kagami et al. 2007). This is only one of multiple potential scenarios where chytrids may have a role in facilitating trophic energy transfer (Gleason et al. 2008).

Canter and colleagues (Beakes et al. 1988, 1993; Canter and Jaworski 1981) conducted foundational work and established the role of chytrids in the regulation of algal and other phytoplankton blooms (reviewed in Ibelings et al. 2004; Kagami et al. 2007; Park et al. 2004; Powell 1993). Light and nutrient levels directly impact the severity of infection, and under some circumstances chytrids can decimate algal populations (Ibelings et al. 2004). The complexity of these interactions (Kagami et al. 2007) is exemplified in Canter and Jaworksi's study (1981) on the requirement of light for zoospores of *Rhizophydium planktonicum* to adhere to their desmid algal host.

As saprotrophs, chytrids play a vital role in decay of refractory materials from plant and animal organic debris and in the recycling of nutrients. Molecular detection of chytrids, coupled with culture studies, will facilitate future studies of the distribution and abundance of chytrids in soil and aquatic habitats (Lefèvre et al. 2007, 2008, 2012). Most of our understanding of abundance and distribution of saprotrophic chytrids has come from baiting and culture studies from environmental samples. The most significant factors that influence chytrid distribution in soil are temperature, moisture, presence of essential ions, availability of substrate or host, pH, and salinity (Booth 1971; Gleason et al. 2004, 2005; reviewed in Letcher and Powell 2001). Certain chytrid species are cosmopolitan and grow within a broad continuum of environmental conditions (Letcher and Powell 2001, 2002b; Letcher et al. 2004a). Other species are more fastidious in their growth requirements, tolerating a narrower range and set of environmental conditions. For example, *Lacustromyces hiemalis* is isolated from ponds and lakes on submerged chitin bait most frequently when the temperature is below 11 °C, and the chytrid can tolerate temperatures only up to 23 °C (Longcore 1993).

Several survey studies have focused on the structure and dynamics of chytrid communities in terrestrial habitats. In a study of chytrid distribution in four forest communities with similar characteristics, Letcher and Powell (2001, 2002b) found that eight common species characterized the community structure. For each site, however, a number of other chytrid species were present but were scarce to rare. Although these less abundant species contributed to the species diversity of a community, they most likely occupied highly specific ecological niches and were more limited in nutrients that they could use. Supporting the concept that resource availability helps shape the pattern of chytrid distribution, Midgley et al. (2006) demonstrated that in soils containing limited amounts of orthophosphate chytrids differed in their abilities to use different forms of phosphorous. Whereas all chytrids studied grew on orthophosphate as the sole phosphate source, species exhibited varying capabilities to use other sources of phosphorous, such as phytic acid and DNA. Thus, limited sources of orthophosphate could affect the distribution of chytrid species in soils.

Other studies showed that distribution of chytrids in the soil is influenced by microhabitat and micro-landscape factors, rather than larger scale influences (Letcher and Powell 2002b). Chytrids are commonly found in soil under clumps of mosses, most likely due to the retention of moisture and accumulation of organic material. In a study of the distribution of chytrids under two species of mosses, significant differences in the diversity and abundances of chytrids were found (Letcher and Powell 2002b). Interestingly, chytrid diversity in soil proximal to the moss reflected the composition of chytrids under the mosses. Thus, chytrids under mosses may provide a point source of inoculum for soils adjacent to mosses, spreading when capillary water is present in the soil.

Potential mutualistic interactions of chytrids with other organisms are underexplored. What appears to be a cross-feeding interaction between a chytrid and alga results in improvement of growth for both organisms and suggests additional dynamics for chytrids in the environment (Picard et al. 2009, 2013). Differences in distribution of chytrids may also be related to the mechanism by which chytrid zoospores detect potential substrates or hosts. In aquatic systems, some, but not all, chytrid zoospores exhibit taxis to light and to specific food sources (Kazama 1972; Moss et al. 2008; Muehlstein et al. 1988). A positive chemotactic and phototactic response offers the advantage of bringing zoospores closer to potential usable plant and animal substrates. Whether or not zoospores of different chytrid species respond to different environmental clues, keeping them out of competition with other chytrid species, has not been explored.

Chacterization and Recognition

As Chytridiomycota, chytrids and monoblephs share numerous characteristics, including the basic architecture of their zoospores (Fig. 1b versus Fig. 1c) and the structure of their mitotic apparatus, which opens only at the spindle poles (Fig. 1f) and discards the mid-zone at telophase (Dolan and Fuller 1985; McNitt 1973; Powell

1975, 1980; Roychoudhury and Powell 1991). The oogamous mode of sexual reproduction among the monoblephs is a clear distinction from the variety of methods for genetic recombination found among the chytrids (Sparrow 1960).

Thallus Forms

Chytrids exhibit a range of microscopic thallus forms which adapt them for life as necrotrophs or biotrophs and as saprotrophs of organic materials. The simplest thallus form is the holocarphic thallus consisting solely of the sporangium (without rhizoids), which occurs within a host or substrate (Fig. 3e). The eucarpic thallus consists of both a sporangium and root-like structures called rhizoids. If the thallus has only a single sporangium, it is considered monocentric (Figs. 2a, b, e and 3c), but polycentric if there are several sporangia (Fig. 3h). The size of sporangia can range in diameter from less than 10 μm (Longcore et al. 2016) to over 100 μm (Sparrow 1960). The eucarpic thallus can be found internal (Fig. 3d) or external to its food source (Fig. 2b); and when epibiotic, the rhizoids anchor the thallus to its substrate (Fig. 2a). Rhizoids, thought to be involved in uptake of nutrients, can be minute and delicate (Fig. 3c) or extensive and coarse (Fig. 3f), increasing the capacity for food absorption. Rhizoids are rich in mitochondria, endoplasmic reticulum, vacuoles, and vesicles but they lack nuclei. When the thallus is developing, there is an unobstructed opening between the rhizoids and sporangium; but as the sporangium matures and comes closer to zoospore formation, a septum containing plasmodesmata (Fig. 1g) forms (Powell 1974; Powell and Gillette 1987). Thus, a route for nutrient uptake remains, but a portal for organellar movement is blocked at this point. The extent of the rhizoids for the eucarpic-monocentric thallus determines how expansive the thallus is (Fig. 3c versus f); consequently, the monocentric thallus has determinate growth and tends to be localized. Its ability to compete with filamentous forms in colonizing a substrate depends on numbers of thalli produced.

Some chytrids form a more complex thallus with multiple sporangia (polycentric) joined by mycelial/rhizoidal segments, the rhizomycelium (Fig. 3h). In contrast to the monocentric thallus, polycentric rhizomycelial growth is indeterminate where growth can be extensively branched and effective in radiating out into the substrate (Fig. 3g). Chytrids with rhizomycelia are common on cellulosic substrates but can be found on chitin and keratin as well (Longcore 1993; Longcore and Simmons 2012; Mozley-Standridge et al. 2009).

Most monoblephs produce a filamentous hyphal-like structure (Fig. 2h), often with a basal holdfast (Fig. 2i, j). The cytoplasm is highly vacuolated (Fig. 2h), giving the hypha a distinctive foamy appearance. Sporangia appear at the apex of these filaments. The thallus is greatly reduced in *Harpochytrium* (Fig. 2i, j) and *Oedogoniomyces*, consisting of a holdfast cell and sporangium (Fig. 2j). *Hyaloraphidium curvatum,* a recently recognized monobleph, earlier thought to be a colorless relative of algae (Forget et al. 2002; Ustinova et al. 2000), has a similarly simple thallus, but its sporangia produce non-motile spores (autospores).

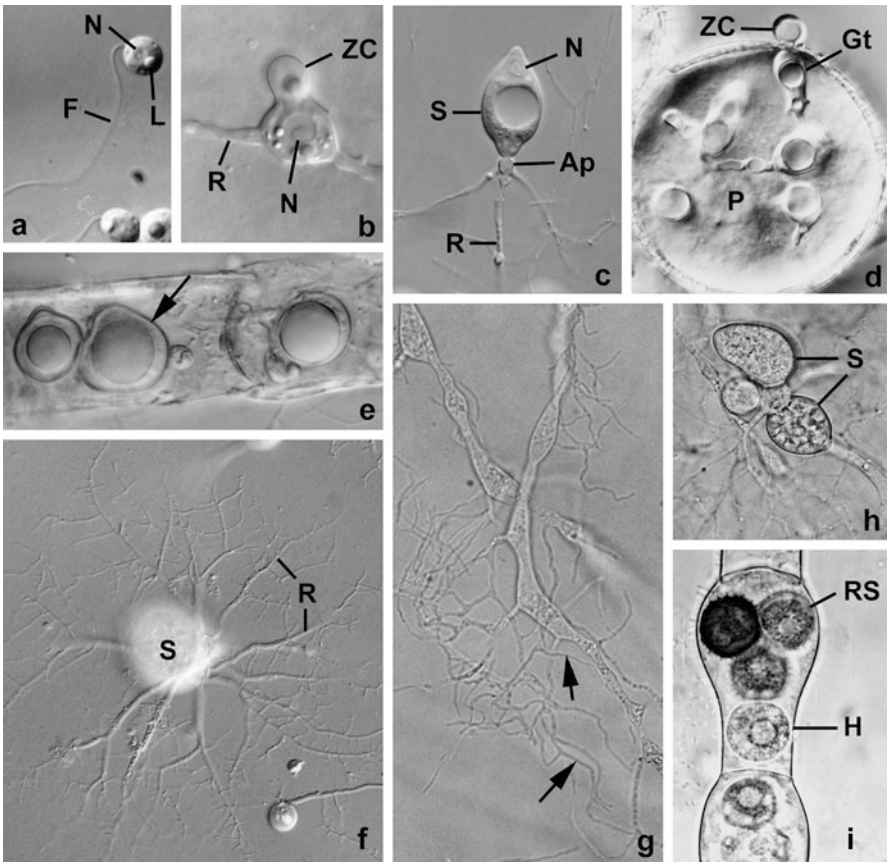

Fig. 3 (**a**) Chytrid zoospore with single nucleus (N), prominent single lipid globule (L), and single, posteriorly directed flagellum (F). Notice the symmetry of the flagellar beat is as a sine wave. ×1,200. (**b**) Thallus development in which the nucleus (N) has migrated from the zoospore cyst (ZC) into the germ tube. The germ tube is enlarging into a sporangium from which rhizoids (R) emanate. ×1,200. (**c**) Thallus development in which the nucleus (N) remains in the zoospore cyst which enlarges into the sporangium (S). The germ tube develops into the apophysis (Ap) and delicate rhizoids (R). ×800. (**d**) Formation of eucarpic, monocentric endobiotic thalli in a pollen grain (P). The zoospore cyst (ZC) is at the surface of a spherical pollen grain (P) and has germinated with a germ tube (Gt), which has penetrated the pollen grain and has just started enlarging into a sporangium as rhizoids branch at the end of the germ tube. The zoospore cyst does not enlarge. ×1,100. (**e**) Endobiotic, holocarpic thalli (*arrow*) within the filament of a green alga. The thallus consists only of the sporangium and no rhizoids are produced. ×750. (**f**) Eucarpic thallus with coarse and extensively branched rhizoids (R) extending from the spherical sporangium (S). ×500. (**g**) Rhizomycelium of *Polychytrium* sp. The area of absorption is enlarged with the fine bifurcations (*arrows*) radiating from the broadly tubular portion of the thallus. ×900. (**h**) Polycentric rhizomycelium of *Polychytrium* sp. with sporangia (S) developing. ×500. (**i**) Resting spores (RS) of *Rozella allomycis* have spiny walls and serve as the only walled stage in the life history of this organism. Notice the hypertrophy of the host (*Allomyces* sp.) hypha (H). ×500

Asexual Reproduction

Zoospores (Figs. 1b–e and 3a) discharged from sporangia (Fig. 2b) are the primary means of dispersal for Chytridiomycota. Zoospores contain a single nucleus (Fig. 3a) and are unwalled, but sometimes covered with a carbohydrate coat (Dorward and Powell 1983; Powell 1994). Zoospores are not an assimilative phase, but rather, they use their endogenous reserves stored as glycogen and prominent or smaller, scattered lipid globules for energy (Powell 1976b, 1978) (Figs. 1b, c and 3a). Powell (1976b) demonstrated that an assemblage of organelles, including microbodies, lipid globules, mitochondria, and a membrane cisterna (the microbody-lipid globule complex – MLC), had the capacity to transform the energy stored in lipid globules. Cytochemical studies showed that MLC microbodies in both monobleph and chytrid zoospores contained catalase (Dorward and Powell 1980; Powell 1976b, 1978), and biochemical analysis revealed that isolated microbodies contained marker enzymes for glyoxysomes (Powell 1976b). The close spatial association of organelles in the MLC and the proximity of the MLC to the flagellum suggested that the MLC served as a "battery" for zoospores, with glyoxysomes and mitochondria working in consort to transform the energy stored in lipid globules into ATP for zoospore motility. Additionally, cytochemical localization of calcium in the MLC cisterna and the proximity of the cisterna to the plasma membrane and flagellum suggested a role in signal transduction and the regulation of zoospore motility (Dorward and Powell 1982; Powell 1983, 1994).

Chytrid zoospores range in shape from spherical to oval and are characteristically small, in the 3–5 μm diameter range. However, some chytrids with oval zoospores, such as *Polyphagus* (Powell 1981a), and most monoblephs may be up to 13 μm in length. The swimming pattern of the zoospore is distinct and can be distinguished from flagellated cells of other organisms. Zoospores swim in a darting motion, abruptly changing directions in what can appear to be a hopping pattern. The single flagellum beats in a single plane with a sine-curve wave, and the zoospore body may spin on its axis while swimming or glide in a single plane.

After a period of swimming for what may be a few minutes to over 24 h, depending upon species, the zoospore contacts a suitable substrate or host, withdraws its flagellum by one of several mechanisms (Koch 1968), and secretes a wall around itself (Fig. 1a). Much of the differences in complexity of thalli depends on the fate of the nucleus after the zoospore encysts (Blackwell et al. 2006; Powell and Koch 1977a, b). The encysted zoospore develops into the thallus (Fig. 3b–d), and coenocytic sporangia produce numerous zoospores which are discharged from operculate (Fig. 2d) or inoperculate (Fig. 2e) discharge openings (Powell 1976a; Taylor and Fuller 1981).

Sexual Reproduction

Well-authenticated studies of sexual reproduction among Chytridiomycota are limited. Sparrow (1960, pp. 69–85) reviewed classical reports of sexual reproduction,

most based on cytological studies of nuclear behavior. Sexual reproduction among the monoblephs is oogamous. Fusion occurs between a nonmotile egg and a motile sperm in three of six genera of monoblephs. Sperms with single flagella are produced in antheridia, and oospheres are produced in oogonia. Sperms fertilize oospheres, and the zygote produces a thick wall and becomes an oospore, which functions in perennation. In *Monoblepharis*, the zygote is motile after fertilization, propelled by the sperm's flagellum; but zygotes of *Monoblepharella* and *Gonapodya* immediately produce thick walls and develop into oospores after fertilization. Favored temperature and light regimes regulate the reproductive cycle, typically with asexual reproduction in light and at lower temperatures (8–15 °C) and sexual in the dark at higher temperatures (Marek 1984; Sparrow 1943, 1960).

The best documented ultrastructural studies of chytrid sexual reproduction are of *Chytriomyces* and *Zygorhizidium*. The presence of synaptonemal complexes in meiotic nuclei at the time of zygote germination in *Zygorhizidium planktonicum* is evidence that the life cycle of chytrids involves zygotic meiosis (Doggett and Porter 1996). The demonstration of nuclear migration and fusion in a zygote leading to a resting spore in *Chytriomyces hyalinus* suggests that genetic recombination occurs (Miller and Dylewski 1981). Light microscopic studies of *Siphonaria variabilis* (Karling 1945) and *Polyphagus euglenae* (Wager 1913) give additional evidence that nuclear fusion precedes resting spore formation in chytrids. A range of strategies for genetic recommendation has been reported (Sparrow 1960) and include:

1. Fusion between motile gametes, as in plant pathogenic species of *Synchytrium*.
2. Gametangial copulation where one gametangium transfers its protoplasm to another gametangium, as described in *Sporophlyctis rostrata*.
3. Gametangial contact where the contents from one gametangium migrates through a conjugation tube into the other gametangium and the zygotic resting spore forms, as in *Zygorhizidium*.
4. Somatogamy with fusion between rhizoidal filaments, as in *Chytriomyces hyalinus* (Fig. 2c) and *Siphonaria variabilis*.

Systematics

Early classification of chytrids was controversial because researchers were inconsistent in assignment of priority to characters. Sparrow (1960) regarded the manner by which sporangia opened prior to the release of zoospores, either operculate or inoperculate, as most important and used this feature for two series of chytrid. On the other hand, Whiffen (1944) and Karling (1977) regarded mode of sporangial discharge secondary to the pattern of thallus development and complexity. Additionally, features of the rhizoids such as presence/absence of subsporangial swelling (Fig. 3c) (= apophysis; Sparrow 1960; Karling 1977), the extent of the rhizoid system, or tapered versus rounded tip morphology have been used in systematics (Barr 1980).

The physical appearance of the mature thallus is clearly a convergent feature. Chytrids with different patterns of development can produce thalli at maturity that look the same (Blackwell et al. 2006). Because of the limited reliability of thallus appearance and the great range of morphological plasticity in characters, such as the presence or absence of an apophysis, comparative zoospore ultrastructure has guided chytrid systematics since the 1970s (summarized in Barr 1978, 1990, 2001; Powell and Letcher 2014a). Molecular phylogenetic analyses substantiate that zoospore ultrastructure can be used reliably to place taxa into orders (James et al. 2006b; Letcher and Powell 2014; Letcher et al. 2006, 2008a, c). The chytrid zoospore is an intricately designed cell with remarkable diverse architectures. Koch (1961) was the first to clearly describe unique subcellular organization in chytrid zoospores and suggest zoospore characteristics as a more reliable source of phylogenetic valid characters. Electron microscopic studies gave the first fine view of the remarkable diversity in zoospore organellar architecture (Barr 1978, 1981a; Lange and Olson 1979; Powell 1978). Structure of organelles, microbody-lipid globule complex (MLC) (Fig. 1b, c) (Powell 1976b, 1978; Powell and Roychoudhury 1992), and ciliary apparatus (Fig. 1d, e) (Barr 1978, 1980, 1981a; Barr and Désaulniers 1988; Barr and Hadland-Hartmann 1978a; Powell and Letcher 2012; Roychoudhury and Powell 1992) were used to define zoospore types and to characterize orders. Barr acted on the view that a zoospore "type" could characterize an order and removed from the Chytridiales a new order, Spizellomycetales, for chytrids in which ribosomes were dispersed and the nucleus was spatially associated with the kinetosome (Barr 1980, 1984b). The advent of molecular phylogenetic analyses has provided a clearer picture of deeper branch relationships, and tree topologies validate the reliability of chytrid zoospore ultrastructure in systematics (Letcher and Powell 2014; James et al. 2006b). Results of molecular analyses have stimulated even greater scrutiny of ultrastructural details, resulting in new views of taxonomically important structural characters, such as kinetosome associated structures (KAS) and kinetosome to nonflagellated centriole bridges (Letcher and Powell 2014; Letcher et al. 2006, 2008a, c; Powell and Letcher 2014a).

Molecular phylogenetic analyses have also revealed that many of the morphological characters classically used as primary taxonomic characters actually arose numerous times in different chytrid lineages. Thus, both operculate and inoperculate members radiate among the Chytridiales (Letcher et al. 2005), Rhizophydiales (Letcher et al. 2006; Powell et al. 2011), Cladochytriales (Mozley-Standridge et al. 2009), and Lobulomycetales (Seto and Degawa 2015; Simmons et al. 2009). Monocentric and polycentric thalli may be found within the same lineage, such as the polycentric genus *Physocladia* and the monocentric genus *Chytriomyces* in the Chytridiales lineage (Letcher et al. 2005).

A significant outcome of molecular-based phylogenetic studies was the realization that Chytridiomycota was not monophyletic and represented a tremendous amount of untapped biodiversity. This realization stimulated increased investigations of chytrid diversity. Chytridales has been redefined (Hibbett et al. 2007; Letcher and Powell 2014; Letcher et al. 2005; Vélez et al. 2011), and new monophyletic orders have been established for most recognized clades (James et al. 2006b) in the

Chytridiomycetes (Fig. 4), each correlated with a distinct type of zoospore and a constellation of distinguishing characteristics (reviewed in Letcher and Powell 2014; Powell and Letcher 2012, 2014a, b).

The class Chytridiomycetes currently includes ten orders (Fig. 4), delineated based on molecular phylogenetics and comparative character analyses of zoospore structure:

1. Chytridiales (Barr and Hadland-Hartmann 1978a; Barr and Hartmann 1976; Davis et al. 2015; Dorward and Powell 1982, 1983; Leshem et al. 2016; Letcher and Powell 2005b, 2014; Letcher et al. 2005, 2012a, 2014a, b; Longcore 1992b, 1995; Picard et al. 2009; Powell 1983; Powell et al. 2013; Vélez et al 2011, 2013)
2. Cladochytriales (Barr 1986; Barr and Désaulniers 1986, 1987; Barr et al. 1987; Lucarotti 1981; Mozley-Standridge et al. 2009; Steiger et al. 2011)
3. Rhizophydiales (Barr and Hadland-Hartmann 1978b; Lepelletier et al. 2014; Letcher and Powell 2005a; Letcher et al. 2004b, 2006, 2008b, c, 2012b, 2015a; Longcore 2004; Longcore et al. 2011; McNitt 1974; Powell et al. 2011, 2015)
4. Lobulomycetales (Longcore 1992a; Seto and Degawa 2015; Simmons et al. 2009, 2012)
5. Rhizophlyctidales (Barr and Désaulniers 1986; Barr and Hartmann 1977; Davis et al. 2016a; Letcher et al. 2008a)
6. Spizellomycetales (Barr 1980, 1981b, 1984a, b; Barr and Allan 1981; Davis et al. 2016b; Longcore et al. 1995; Powell 1976b, 1978; Simmons 2011; Simmons and Longcore 2012; Wakefield et al. 2010)

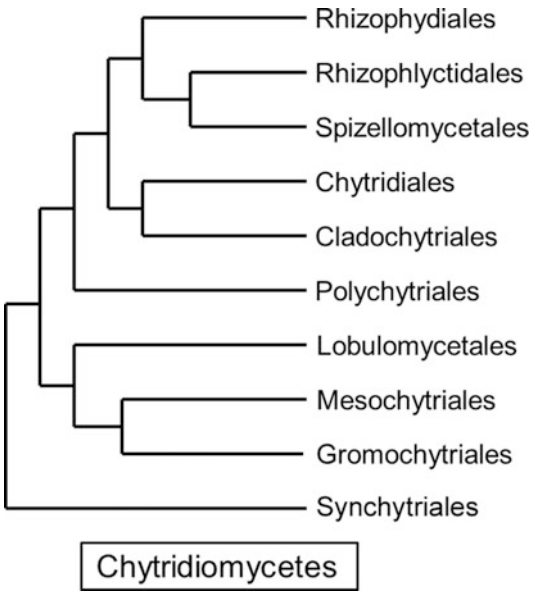

Fig. 4 Based on molecular and ultrastructural analyses, new orders have been described for monophyletic lineages within the Chytridiomycetes. The Rhizophlyctidales has been separated from the Spizellomycetales. The Rhizophydiales, Lobulomycetales, Cladochytriales, Polychytriales, and Synchytriales have been separated from the Chytridiales. The Mesochytriales and Gromochytriales are newly discovered sister lineages which include no previously described chytrid species

7. Polychytriales (Longcore 1993; Longcore and Simmons 2012)
8. Gromochytriales (Karpov et al. 2014a)
9. Mesochytriales (Karpov et al. 2010, 2014a)
10. Synchytriales (Lange and Olson 1978b; Longcore et al. 2016; Montecillo et al. 1980; Smith et al. 2014)

Several species of chytrids have been examined ultrastructurally, but their molecular phylogenetic placement is uncertain, and these remain as *incertae sedis*: *Caulochytrium* (Powell 1981b); *Entophlyctis apiculate* (Shin et al. 2001); *Polyphagus euglenae* (Powell 1981a); *Rhizophydium planktonicum* (Beakes et al. 1993); and *Zygorhizidium* (Beakes et al. 1988).

Because *Rozella allomycis* (Fig. 3i), *Olpidium brassicae,* and *O. bornovanus* (Barr and Hadland-Hartmann 1977; Barr and Hartmann 1977; Lange and Olson 1978a) place outside of the Chytridiomycota in molecular phylogenetic analyses (James et al. 2006b; Sekimoto et al. 2011), they are excluded from the phylum Chytridiomycota. The endophyte of oomycetes and chytrids, *Rozella* has recently been classified in Cryptomycota, a new phylum based primarily on phylotypes from environmental samples (Jones et al. 2011). As a clade sister to Fungi, the Superphylum Opisthosporidia (Karpov et al. 2014b) now includes Cryptomycota along with endoparasites of algal cells (Aphelida: Karpov et al. 2013; Letcher et al. 2013, 2015b) and animal cells (Microsporidia).

Recent molecular phylogenetic analyses (Dee et al. 2015) support three monophyletic orders in the class Monoblepharidiomycetes. Interestingly, the three hyphal monobleph genera (*Monoblepharis, Monoblepharella, Gonapodya*) place in a clade sister to nonhyphal members, supporting origin of hyphae in the monobleph lineage independent from other fungi. Electron microscopy of spores has also been studied in monobleph genera.

1. Harpochytriales (*Harpochytrium, Oedogoniomyces*) (Gauriloff et al. 1980a, b; Reichle 1972; Travland and Whisler 1971)
2. Monoblepharidales (*Monoblepharis, Monoblepharella, Gonapodya*) (Dorward and Powell 1980; Fuller and Reichle 1968; Gauriloff et al. 1980a; Mollicone and Longcore 1994, 1999; Reichle 1972)
3. Hyaloraphidiales (*Hyaloraphidium*) (Ustinova et al. 2000)

Maintenance and Culture

Fuller and Jaworski (1987) edited an excellent compendium of methods for manipulating development of a variety of chytrid taxa in culture. Techniques for isolating and culturing chytrids have been summarized for both aquatic and terrestrial chytrids (Barr 1983, 1987; Bills et al. 2004; Lange and Olson 1983; Shearer et al. 2004). Some chytrids, such as most species of *Synchytrium,* are obligate biotrophs and have not been grown outside their hosts (Barr 1983; Lange and Olson 1983).

The most common approach for bringing chytrids into pure culture is to add natural substrates (cellulose, chitin, keratin, pollen, heat-killed algae) directly to water collected containing organic material or to soil flooded with water. Unlike hyphal fungi, most chytrids do not exhibit filamentous growth and cannot grow appreciably away from their substrate and other contaminating organisms. The easiest method to obtain chytrids in pure culture is to observe chytrids on their bait through a microscope, and when zoospores are released, pipette up a 100 µl suspension and spread it on nutrient media containing antibiotics (0.5 g/L penicillin G and 0.5 g/L streptomycin sulfate). Large thalli and polycentric thalli can be dissected from the substrate on which they are growing and transferred to antibiotic-containing media. It is critical that incubation plates are observed closely for the first 48 h so that growing chytrids can be transferred onto clean nutrient plates, away from contaminating organisms.

Typically media low in nutrients is used for initial isolations, and with time, chytrids tend to grow better on richer media. Fuller and Jawaroski (1987) list numerous formulations for growth of chytrids and monoblephs, but a range of media may be needed to bring a chytrid into culture because some have unusual nutritional requirements (Gleason 1976) and media with other carbon, nitrogen, and ions are needed. There are a couple of multipurpose media on which most chytrids grow; these have the advantage of relative transparency, allowing clear observation of chytrids directly in culture: PmTG (1.0 g peptonized milk, 1.0 g tryptone, 5.0 g glucose, 10 g agar, 1 L water) or mPmTG (0.4 g peptonized milk, 0.4 g tryptone, 2.0 g glucose, 10 g agar, 1 L water). Other commonly used media include YpSs (15.0 g soluble starch, 4.0 g yeast extract, 1.0 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 20 g agar, 1 L distilled water) and ARCH (2.0 g peptone, 3.0 g malt extract, 5.0 g glucose, 8.0 g agar, 1 L water).

One of the challenges in culturing chytrids is long-term maintenance of cultures. Because chytrids do not preserve well with freeze-dry methods, cryomethods have proved the most reliable (Boyle et al. 2003). The Barr and Babcock (1994) Q-tip technique is the most time-tested cryomethod for chytrids, but other more recently developed protocols using other cryoprotectants and controlled freezing step-downs show promise of success (Boyle et al. 2003). In the Q-tip method (Barr and Babcock 1994), 2 cm lengths are cut from the tips and sterilized. Several severed Q-tip heads are then added to nutrient broth in an Erlenmeyer flask. Cotton fibers of the Q-tips increase the surface area on which chytrids grow and facilitate transfer of thalli into cryovials. Cultures are grown on rotary shakers after inoculation for 6–10 days until vigorous growth is achieved. One Q-tip segment is then sterilely transferred to each 2 ml cryotube containing 10% glycerol as a cryoprotectant and stored at −80 °C. Some cultures have been successfully stored for over 10 years. To recover cultures, cryopreserved cultures are warmed to 35 °C quickly (30–60 s), and the thawed Q-tip transferred to broth culture. Soil chytrids appear to recover from freezing at a greater rate than aquatic chytrids.

Evolution and History

Molecular calibration of age correlated with fossil records suggests that the lineage leading to the Chytridiomycota diverged from another opisthokont lineage leading to metazoans over 1 billion years ago (Berbee and Taylor 2007). The best preserved fossils of chytrids come from the 400 million year old Devonian Rhynie Chert (Taylor et al. 1992). Chytrids resembling extant chytrids were found in thin sections on a *Nitella*-like organism. Although both holocarpic and eucarpic forms were found, no polycentric forms were detected. Well-preserved fossil chytrids have also been identified from Pennsylvainian age coal balls and included epibiotic and endobiotic forms (Millay and Taylor 1978). Thus, it is clear that the capacity of chytrids to invade living host and refractory organic materials was already developed in the Paleozoic, suggesting the evolution of wide-ranging enzymatic capabilities.

Chytridiomycota are sister to the radiation leading to higher fungi (James et al. 2006a, b). Molecular phylogenetic analyses (James et al. 2006a, b) reveal that the lineage leading to the neocallimastigos, monoblephs, and chytrids forms a monophyletic group, which diverges from the clade containing blastoclads, zygos, *Olpidium*, and higher fungi. *Rozella* (Fig. 3i), once considered a member of the order Spizellomycetales (because of the connection or proximity of the zoospore nucleus to the kinetosome), is basal to this divergence and appears to radiate with Microsporidia and Aphelidea (Karpov et al. 2013, 2014b; Letcher et al. 2013, 2015b). Like Microsporidia and Aphelidea, *Rozella* infects its host as an unwalled protoplast (Held 1981). Interestingly, *Rozella* appears to phagocytize host protoplasm (Powell 1984) as reported among Aphelidea. Distinct from Microsporidia, *Rozella* produces zoospores with single flagella (Held 1975), contains mitochondria (rather than mitosomes), and does not infect animal cells (Held 1981).

During the evolutionary radiation of chytrids, spore motility has been lost repeated times along multiple lineages of chytrids and monoblephs. For example in the chytrid radiation, nonmotile spores or autospores are found in chytrids such as *Sporophlyctis rostrata* (Sparrow 1960) and in monoblephs such as *Hyaloraphidium curvatum* (Ustinova et al. 2000). *Septosperma* exhibits a reduction in dependency on zoospores as the sole means of dispersal. Commonly found in forest soils that experience periodic inundation with water from floods or rains, *Septosperma's* rocket-shaped resting spores disarticulate from their basal cells, which facilitate their passive transport as water percolates through soil (Powell and Blackwell 1991). Fossil evidence suggests that chytrids may have undergone evolutionary radiations at the same time as land plant and animal radiations (Taylor et al. 1992). The availability of new niches and motile hosts, as well as adaptation to drier terrestrial environments, may have been factors driving evolutionary loss of spore motility. Discovery and recognition of new organisms at the base of the fungal evolutionary radiation will help disentangle the roles of symbiosis and environment in driving adaptation and divergence of Chytridiomycota (James et al. 2006a).

Characteristics of the Neocallimastigomycota

The Neocallimastigomycota are obligate anaerobic symbionts living in the rumen and other portions of the gastrointestinal tract of herbivorous mammals and reptiles (Gruninger et al. 2014; Ho and Barr 1995; Mountfort and Orpin 1994; Orpin and Letcher 1979; Trinci et al. 1994). They produce monocentric (one sporangium) and polycentric (many sporangia) thalli typically with extensive rhizoids penetrating and dissolving refractory, cellulose-containing fibrous plant materials (Ho and Barr 1995). They release posteriorly, single to multiple flagellated, unwalled zoospores from sporangia. These zoospores vary in size, even in the same isolate, with single flagellated spores typically being smaller. Sexual reproduction with fusion of gametes has never been reported among these organisms. However, Wubah et al. (1991) found evidence of aero-tolerant, resistant structures in *Neocallimastix* sp. and potential diploidization during their formation.

Occurrence

These organisms are adapted for growth in the rumen and digestive tracts of animals including, sheep, goats, cows, horses, deer, elephants, camels, and buffalo. They are not restricted to growth in ruminant animals and have been found in non-ruminant herbivores including reptiles (Liggenstoffer et al. 2010). They are not typically aero-tolerant but can survive outside of their hosts in feces (Wubah et al. 1991) and have been detected in anoxic landfills rich in cellulosic materials (Lockhart et al. 2006). Molecular analyses of environmental samples reveal that the diversity of this group is much greater than currently characterized and includes undescribed novel lineages (Kittelmann et al. 2012; Liggenstoffer et al. 2010).

Literature

Ho and Barr (1995) monographed this group, providing a key to species of five genera. Light micrographs of thallus morphology "permit the functional identification of genera and species" (Ho and Barr 1995). Since this monograph, three new genera been described (Callaghan et al. 2015; Dagar et al. 2015; Ozkose et al. 2001); consequently, there are now a total of eight genera and just over 20 species. Chen et al. (2007) produced a maximum parsimony analysis of the group based on ITS1 gene sequences and provided a key to six genera in the group and to the two species of *Caecomyces*. Knowledge of this group is relatively recent, but because of their importance in feed-utilization in herbivores, their physiology and growth have been widely studied (reviewed in Mountfort and Orpin 1994; Tachezy 2008; Trinci et al. 1994).

History of Knowledge

Although known since the early 1900s, Orpin (1975) first recognized the systematic affinity of *Neocallimastix* to fungi because of the detection of chitin in their cell walls. Heath et al. (1983) formally classified them in the order Spizellomycetales among Chytridiomycetes based on their production of thalli consisting of sporangia and rhizoids and discharge of posteriorly flagellated zoospores from sporangia. Comparative ultrastructural and molecular analyses, however, demonstrated their striking distinctions from chytrids, and Li et al. (1993) raised this group to an order, Neocallimastigales. Later, multigene phylogenetic analyses revealed that they were sister to the Chytridiomycota (James et al. 2006a, b); and with the recent formal establishment of the now recognized phylum Chytridiomycota, they were elevated to a phylum, Neocallimastigomycota (Hibbett et al. 2007).

Practical Importance

Neocallimastigos are significant in feed efficiency of plant materials by herbivores. They interact with other rumen microbes in the breakdown of fibrous material that would otherwise be indigestible to the host. They are early colonizers, and their extensive rhizoidal system penetrates plant fibers helping to physically breakdown cellulose and other compounds in plant walls. They secrete a wide range of degradative enzymes, including esterases, endo- and exo- glucanases, hemicellulases, mannanases, proteases, and xylanases (Orpin and Letcher 1979). Although it has not been shown that they actually break down lignin, they can solubilize lignin-containing fibers, and their zoospores exhibit chemoattraction to phenolic compounds and to sugars (Orpin and Bountiff 1978). There is current focus on these organisms because of the diversity of polysaccharide-degrading enzymes they produce which are potentially useful in biofuel production as well as food and textile industries (Gruninger et al. 2014).

Characterization and Recognition

This is a relatively small group with eight genera (*Anaeromyces, Buwchfawromyces, Caecomyces, Cyllamyces, Neocallimastix, Oontomyces, Orpinomyces, Piromyces*) and just over 20 species (Callaghan et al. 2015; Chen et al. 2007; Dagar et al. 2015; Ho and Barr 1995; Ozkose et al. 2001). They are distinguished primarily based on thallus complexity (monocentric or polycentric), degree of rhizoidal development, and number of undulipodia on zoospores. The presence of a transitional helix (= concentric fiber, Heath et al. 1983; Li et al. 1991) is a symplesiomorphic character shared with chytrids and blastoclads. Although sister to the Chytridiomycota in most molecular phylogenetic analyses (James et al. 2006a, b), the Neocallimastigomycota

are distinctive in numerous features. For example, instead of mitochondria, they contain hydrogenosomes that produce ATP by substrate-level phosphorylation (van der Giezen et al. 2003). Significantly, zoospore flagella lack props and a secondary centriole (= nonfunctional centriole or nonflagellated centriole) is absent (Heath et al. 1983; Li et al. 1991). Unique kinetosome-associated structures are complex in organization and include circumciliary rings, a spur, struts, and scoop (Gold et al. 1988; Heath et al. 1983; Li et al. 1991). Microtubules radiate from the kinetosome in a fan-shaped array and underlie the plasma membrane. Rather than retracting their flagella when zoospores encyst as in chytrids, they shed them and discard their kinetosomes, carrying forward no organized centrioles into their vegetative cells. During development of the coenocytic sporangium, the nuclear envelope remains totally intact at metaphase (reviewed in Li et al. 1993). In addition, no plasmodesmata (Powell 1974) have been found in septa that delimit their rhizoids and sporangia (Heath et al. 1983). A final difference is that as the sporangium undergoes zoosporogenesis, flagella elongate into vesicles prior to cytoplasmic cleavage (Heath et al. 1983), rather than forming simultaneously with cytoplasmic cleavage as in chytrids (1974).

Cultivation

Neocallimastigos must be cultivated under anaerobic conditions. They are isolated from herbivore feces or from filtered rumen fluids collected through a fistula and cannula into the rumen. Rezaeian et al. (2004) have summarized in detail methods for culturing and maintaining these anaerobes.

Evolutionary History

The neocallimastigos are zoosporic opisthokonts and are evolutionarily a highly derived, secondarily amitochondrial group. The presence of molecular import mechanisms in their hydrogenosomes, similar to those of mitochondria, and reports of two surrounding membranes support the concept that their hydrogenosomes were derived from mitochondria. These organelles, however, possibly have not retained any of the mitochondrial genome (van der Giezen et al. 2003). Evidence also suggests that neocallimastigos share a common uniflagellated aerobic ancestor with chytrids but diverged from chytrids in a lineage that adapted them to an anaerobic habitat. In this divergence, some of their enzymes may have been acquired by horizontal gene transfer from rumen bacteria. For example, glycosyl hydrolases are important in the ability to degrade fibrous cellulosic plant material. Similarities between glycosyl hydrolases in neocallimastigos and those found in rumen bacteria suggest that they were gained by horizontal gene transfer (Garcia-Vallvé et al. 2000). The neocallimastigos are an important group for additional investigations to understand the basal radiation of zoosporic osmotrophic opisthokonts and to exploit their economic potentials.

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Microsporidia

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Abstract

Microsporidia are unicellular, obligate intracellular, spore-forming eukaryotes classified among the protists. As parasites, they have been reported from every major group of animals from other protists to mammals and man. They are economically and medically important and can be found environmentally in terrestrial, marine, and freshwater ecosystems. This phylum consists of over 200 genera and approximately 1,300 species producing benign to lethal infections. While they are extremely diverse, they all share the diagnostic and unique resistant spore. It contains a polar filament complex which begins the life cycle by extruding this filament injecting the spore contents, the sporoplasm, into a host cell. As intracellular parasites, they are dependent upon their host for access to nutritional products and have evolved several ways to obtain the required metabolites which in turn have reduced their need to produce many of the biochemicals necessary for their development. As a result of this reduced need to produce their own metabolites, there has been a reduction in their physiological machinery, as well as formation of unique organelles and biochemical pathways. Gene sequencing data has indicated diversity in genome size that ranges from 50+Mbp to the smallest eukaryotic genome reported to date (2.3 Mbp).

Keywords

Cryptomycota • Microsporidia • Mitosomes • Polar filament • Protista • Protozoa • Spore • Sporoplasm

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Contents

Summary Classification

The Microsporidia have undergone several changes in their taxonomic status. However, this group is now accepted as the Phylum Microsporidia Weiser 1977 (formerly known as Microspora and Microsporida) (Sprague and Becnel 1998). As a phylum of eukaryotic microorganisms, it has been considered a "sister" to the fungi (Weiss and Becnel 2014; Weiss 2005; James et al. 2006) but not in any fungal group. Microsporidiologists place them in Protista. Meiosis was first reported in 1976 (Loubes et al. 1976), and has subsequently been demonstrated several times, and has been reviewed in Vavra and Sprague (1976) and Weiss and Becnel (2014).

Introduction

The Phylum Microsporidia consists entirely of eukaryotic unicellular, obligate intracellular, spore-forming parasites. Their spores range in size from 1 to 20 μm but most are about 1–5 μm. Their hosts include all major animal phyla, even some protists, such as the phyla Ciliophora, Myxozoa, and Apicomplexa (in gregarines). Arthropods, then vertebrates, are their most common hosts. While they are known from all five classes of vertebrates, they are primarily reported in fish and mammals. Since the 1980s, they have been identified as significant opportunistic parasites of humans (Cali and Owen 1988; Didier and Weiss 2008; Weber et al. 1994; Weiss and Becnel 2014) with only a few reports prior to that time (Strano et al. 1976).

In general, the life cycle consists of three phases (Fig. 1): proliferative, sporogonic, and environmental. The proliferative phase is primarily responsible for the increase in numbers of organisms within each host cell; proliferative cells, sometimes referred to as meronts, divide repeatedly by binary or multiple fission. The sporogonic phase is composed of a division sequence called sporogony (three completely different sequences in the polymorphic genera). Meiosis, when it occurs, is initiated in sporont cells, prior to spore formation. Spores fill

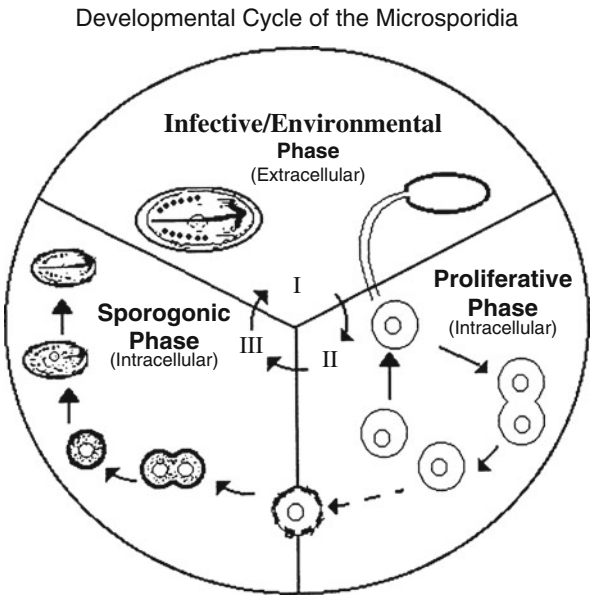

Fig. 1 A typical microsporidial developmental cycle can be divided into three Phases. Phase **I**, the infective/environmental phase, is the only extracellular part of the cycle. It is represented by mature spores shed into the environment from previously infected hosts. Under appropriate conditions, the spores germinate (e.g., if the spores are ingested by an appropriate host, they are activated by the digestive tract environment), this results in the explosive expulsion of the polar filament (which everts becoming a hollow tube). If the polar tube pierces a host cell, the spore contents, the sporoplasm, is injected into it and phase II begins. Phase II is the proliferative phase, the first phase of intracellular development. During this part of the microsporidian life cycle, organisms are usually in direct contact with the host cell cytoplasm or in a parasitophorous vacuole as they increase in number. The transition to Phase III, the sporogonic phase, represents the organisms' commitment to spore formation. In many life cycles this is morphologically indicated by parasite secretions through the plasmalemma producing a "thickened" membrane (many also form a surrounding sporophorous vesicle, SPOV). The number of cell divisions that follow varies, depending on the genus in question, and results in sporoblast cells that develop into spores (Reprinted from Cali and Takvorian 1999)

host cells (Fig. 2) and may either autoinfect after immediate germination within the infected host cell or they may require environmental exposure (environmental phase). These spores pass out into the environment during the life of the infected host, in its waste products or at its death, and are dispersed as a source of infections in new hosts.

Sporont cells are usually distinguished from proliferative stages (meronts) by the presence of an electron-dense surface coat secreted onto the plasma membrane during transition from proliferative cells to sporonts. In some species, an additional layer also develops around the sporonts. This layer forms the sporophorous vesicle (SPOV) enclosing the plasmodium which divides, forming groups of organisms that develop into mature spores. The SPOV and (usually) the presence of the thickened

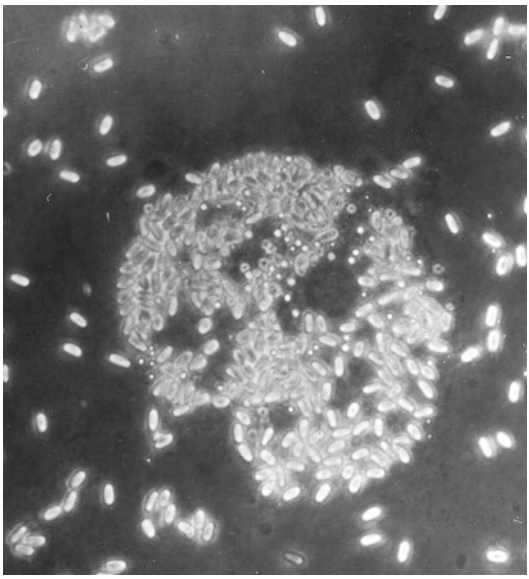

Fig. 2 *Nosema apis* spores in an intestinal epithelial cell of the honey bee. The spores appear highly refractile when observed in a fresh squash preparation by phase contrast microscopy. Spores are 4 × 2 μm (Reprinted from Cali and Owen 1988)

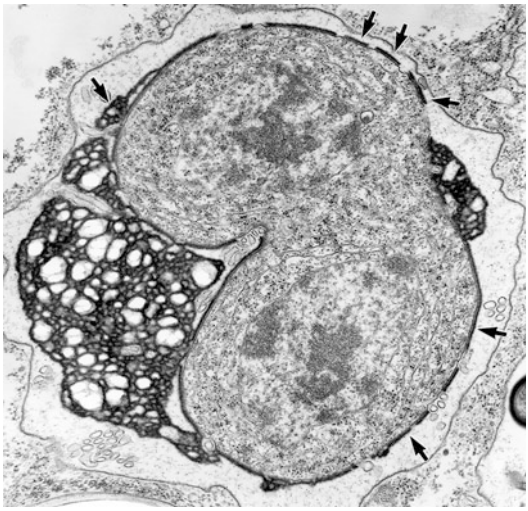

Fig. 3 *Vairimorpha necatrix.* Electron micrograph of a developmental cell in sporogony inside a SPOV. The SPOV contains a multinucleate sporogonial plasmodium surrounded by tubules. An electron dense coat is forming on its cell surface (*arrows*) (Reprinted from Mitchell and Cali 1993)

surface coat (Fig. 3) indicate that the cells are irreversibly committed to spore production and are thus sporonts. The products of sporont division are sporoblasts, which are cells that undergo morphogenesis, resulting in the formation of spores. The microsporidial spore, containing the unique polar filament complex (Fig. 4), is the diagnostic stage for the identification of organisms in this phylum. A glossary of

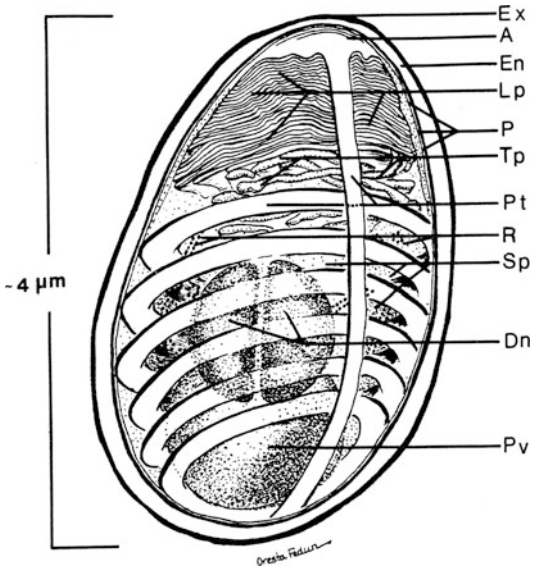

Fig. 4 Diagram of internal structure of a microsporidial spore. The spore coat has an outer electron dense region called the exospore (Ex) and an inner thicker electron lucent region, endospore (En). A unit membrane (P) separates the spore coat from the spore contents. The extrusion apparatus: Anchoring disc (A), polar tubule (Pt), lamellar polaroplast (Lp), and tubular polaroplast (Tp) dominate the spore contents and are diagnostic for microsporidial identification. The presence of a posterior vacuole (Pv) is variable. The spore cytoplasm is dense and contains ribosomes (R) in tightly coiled helical arrays. The nucleation may consist of a single nucleus or a pair of abutted nuclei, diplokaryon (D). The spore size depends on the species and varies from less than a micrometer long to over ten. The number of polar tube coils is also variable from a few to 30 or more (Reprinted from Cali and Owen 1988)

terms employed in describing microsporidial biology has been compiled by Sprague and Becnel (1999).

The mechanism, by which Microsporidia actively infect host cells is unique. It involves penetrating the plasma membrane without its destruction or the formation of a phagosome. A spore organelle, the polar filament (called polar tube after germination), is only 0.1 µm in external diameter and often exceeding 100 µm in length, within the intact spore it is coiled and anchored by the anterior attachment complex (Fig. 4). When the spore germinates, the activated polar structure is everted through the spore wall at this attachment. The sporoplasm (the infective agent) consisting of nucleus and cytoplasm bounded by a membrane passes through the tube and is inoculated into the host cell cytoplasm if the polar tube pierces a cell (Figs. 5 and 6). This process ensures that the parasite initially lies directly within the host cell cytoplasm, not in a phagosome vacuole derived from host plasma membrane, as is generally the case with parasites internalized by phagocytic processes. This provides protection against the lytic action of cells, but, even in cases where a vacuolar membrane is later formed around the dividing parasites, fusion of

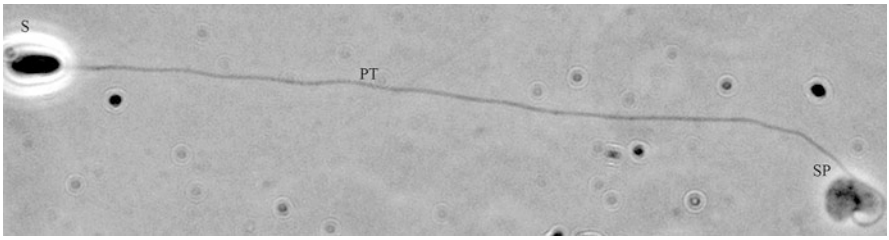

Fig. 5 *Anncaliia algerae.* Germinated spore (S) with the extruded polar tube (PT) and the discharged sporoplasm (SP) still attached (Reprinted from Cali and Takvorian 2001)

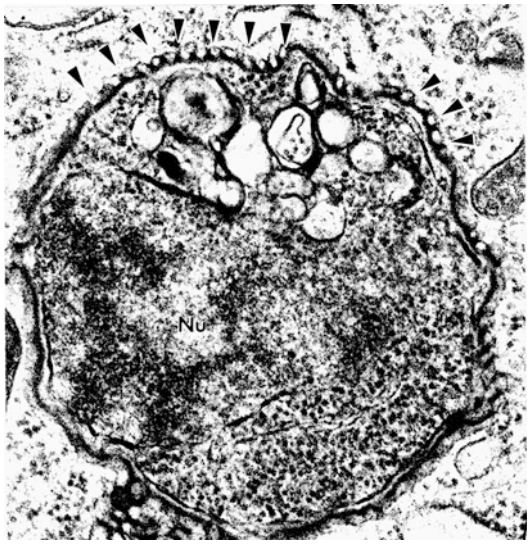

Fig. 6 Electron micrograph of an *Anncaliia algerae* sporoplasm in the cytoplasm of a host cell. The sporoplasm nucleus (Nu), some endoplasmic reticulum, numerous whorled vesicles, and a well-defined limiting membrane are present. The surface of the sporoplasm is adorned with fibrous protrusions and assorted vesicles (*arrowheads*) (Reprinted from Takvorian et al. 2005)

lysosomes does not occur. Additionally, a more traditional means of entry, via phagocytosis, has been reported. The entire spore may be engulfed by the host cell with subsequent spore activation and germination resulting in the polar tube extrusion and inoculation of another host cell (Cali and Takvorian 1999; Franzen 2004; Takvorian et al. 2005).

As obligate intracellular parasites, the microsporidia were considered primitive but are now accepted as evolved and well-adapted specialized organisms, with a lifestyle incorporating several unique features. A Golgi is present that while functionally similar (histochemically) to the classic organelle has evolved a very specialized function in the formation of the polar filament (Takvorian and Cali 1994, 1996) and a morphology resembling a vesicular mass (Beznoussenko et al. 2007). The microsporidia were considered amitochondriate until the early 2000s when it was discovered that more than a dozen genes encoding mitochondrion-derived proteins have been identified and localized to a body now identified as a mitosome (Vavra 2005; Vivares et al. 2002; Williams et al. 2002, 2008a) not resembling a

typical mitochondrion but suggesting a mitochondrial ancestry. The microsporidia are eukaryotic but have prokaryotic-sized ribosomes (being of the 70S sedimentation type with subunits of 23S and 16S). They also lack a 5.8S ribosome subunit, but its sequences are found at the beginning of the 23S subunit a feature found in bacteria, not eukaryotes (Weiss and Vossbrinck 1999). Centrioles, a usual feature of eukaryotes, are lacking in the microsporidia. During karyokinesis, the nuclear envelope remains intact and an intranuclear spindle forms, emanating from spindle plaques on the nuclear envelope. Phylogenetic analysis of their β-tubulin genes suggests a relationship between the microsporidial tubulin and the fungi (Akiyoshi et al. 2007; Edlind et al. 1994, 1996; Lee et al. 2008). However, their lineage remains challenging because their molecular sequences are so divergent and they have lost so many genes as well as the cellular and metabolic features that go with them, that they cannot be simply placed with them (Keeling and Fast 2002; Keeling et al. 2014). At 2.3 Mbp, the smallest eukaryotic genome known is that of the microsporidium, *Encephalitozoon intestinalis* (Peyretaillade et al. 1998). The microsporidial genome varies among their genera, from 2.3Mb to 50+Mbp (Keeling et al. 2014).

Occurrence

Microsporidia are commonly occurring parasites. There are approximately 1,200 known species distributed among 209 genera infecting virtually all major animal phyla. They exhibit varying degrees of host specificity from those infecting a narrow host range to some which can infect both invertebrate and vertebrate hosts. Appearance of infection can vary from no external signs of infection to macroscopic cysts causing deformities (Fig. 7a, b).

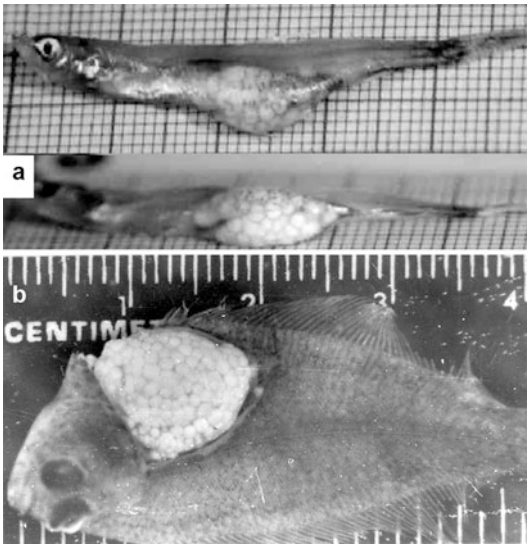

Fig. 7 The location and appearance of *Glugea* infections in fish vary depending upon parasite species and host, but all induce the formation of Xenomas (cyst-like structures). In smelt, they often cause deformities that can be seen externally, while in flounder, the Xenomas are not visible externally. (**a**) Young-of-the-year smelt infected with the microsporidium, *Glugea hertwigi* (Reprinted from Pekan-Hekim et al., 2005). (**b**) Young-of-the-year winter flounder infected with *Glugea stephani* (skin cut away)

Microsporidial percent occurrence within a given species can vary dramatically. The microsporidium, *Glugea hertwigi*, has been reported with prevalence that range from almost 100% in Baltic smelts (Weissenberg 1913) to 1–59% in northern Russian lakes (Anenkova-Khlopina 1920) in North America, smelt infection ranged from 4.6% to 100% and affected young-of-the-year as well as adults (Canning and Lom 1986). Experimental infection of young-of-the-year Winter flounder demonstrated 63% mortality from a single exposure to *Glugea stephani* (Cali et al. 1986) and 88% from two exposures (unpublished personal observations).

The Microsporidia are important parasites in many commercial economic and medical host groups. Although, the vast majority is known from insects and fish, currently 17 species have been identified in humans (Fayer and Santin-Duran 2014). The occurrence of human infecting microsporidia has resulted in studies generating a broader understanding of the geographic, zoonotic, epidemiologic, and environmental distribution of the Microsporidia and their interrelationships (Fig. 8) (Cali and Takvorian 2004). The spore stage is variable in its resistance and may survive years in the environment. Spores kept in the laboratory, in a water suspension or dry, may survive months or years, and other species may be stored in liquid nitrogen or lyophilized (Maddox and Solter 1996). Some Microsporidia are maintained in cell

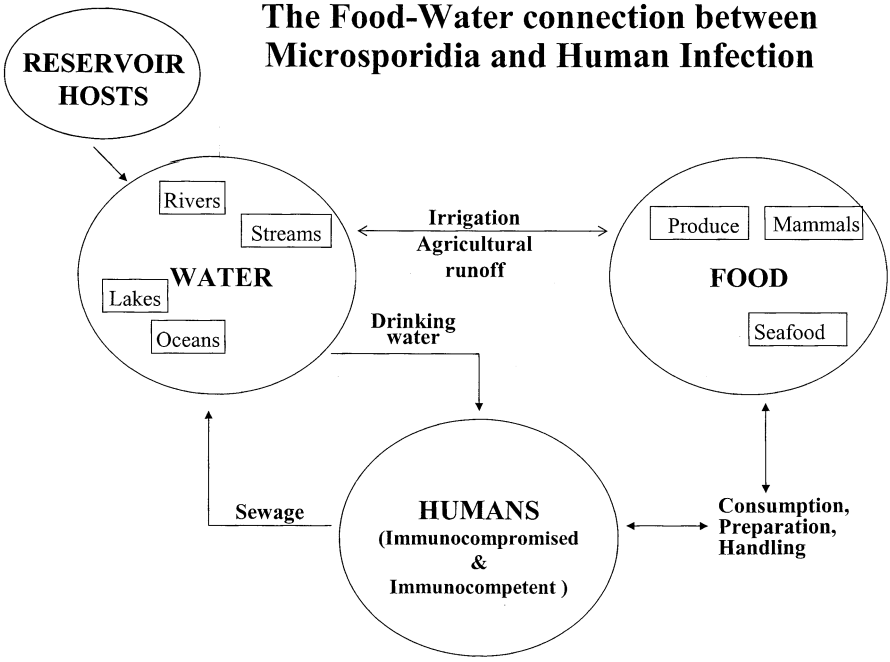

Fig. 8 The food-water connection between Microsporidia and human infection is important in helping to understand the occurrence of human-infecting Microsporidia. Epidemiological studies have provided a broader understanding of geographic, zoonotic, and environmental distribution of the Microsporidia and their interrelationships (Modified from Cali and Takvorian 2004)

cultures and are available from American Type Culture Collection (ATCC). Other species can be maintained in the laboratory in their natural or other related hosts.

Literature

In 1884, Balbiani published a book (*Lecons sur les Sporozoaires*) devoting a chapter to Microsporidies (pp. 150–168). Subsequently, in 1909, Stempell established Order Microsporidia with three families in a major review of the known microsporidial biology of that time (Stempell 1909). The monograph, by Kudo (1924), was the first volume devoted to general microsporidian biology and taxonomy. It was followed by four edited publications: one by Bulla and Cheng (1976, 1977 – two volumes); one edited by Beyer and Issi (1986); one edited by Wittner and Weiss (1999); and the most recent one edited by Weiss and Becnel (2014). These volumes contain articles by many authorities in the field.

There are numerous reviews and/or chapters on specific groups or aspects of the Microsporidia, including a treatise on the Microsporidia of insects by Weiser (1961 and updated in 1977), on infections in vertebrates by Canning and Lom (1986), in fish by Lom and Nilsen (2003), in mosquitoes by Andreadis (2007), and in insects by Solter et al. (2012). A few reviews/chapters on genera identification across the phylum include one by Larsson (1986) who reviewed the ultrastructure and adopted a cladistic approach to an analysis of the relationships of genera, by Sprague et al. (1992), by Larsson (1999), by Voronin (1999 in Russian), by Canning and Vavra (2000). Human infection reviews include Strano et al. (1976), Cali and Owen (1988), Bryan et al. (1991), Webber et al. (Weber et al. 1994), and Didier and Weiss (2008).

Journals carrying the bulk of original papers are *Journal of Invertebrate Pathology*, *Journal of Eukaryotic Microbiology (formerly J. Protozoology)*, *Protistologica*, *Věstník Československé Společnosti Zoologické*, *Acta Protozoologica*, *Folia Parasitologica* (Praha), and *Parazitologyia* (St. Petersburg). Increasing numbers of papers on fish Microsporidia are found in the *Journal of Fish Diseases*, and smaller numbers on general topics are found in *Zeitschrift für Parasitenkunde*, *Protist (formerly Archiv für Protistenkunde)*, *Parasitology*, and *Journal of Parasitology*. Japanese and Chinese sericultural and fisheries journals also carry some papers. Human infecting microsporidial reports are scattered through the medical as well as some of the abovementioned journals; however, the current computer search engines provide access to all current literature.

History of Knowledge

The first microsporidian to be named was *Nosema bombycis* Nägeli, 1857. He considered it to be a fungus but never classified it beyond the genus. It was the etiological agent of "pebrine" or silkworm disease, an epidemic of which occurred in Europe in the mid-nineteenth century, commanding the attention of many eminent

scientists, most notably Pasteur (1870). In spite of preventive measures, the disease played an important role in the decline of the silk industry in Europe.

The Microsporidia, as a distinct group for these organisms, was established in 1882 by Balbiani. They were grouped with the Myxosporea as Microspora in the Cnidosporidia, which at that time belonged in the class Sporozoa (Doflein 1901). A number of classification schemes were proposed for the Microsporidia; all were within the framework of the phylum Protozoa. Honigberg et al. (1964), in a major revision of the Protozoa, elevated them from an order to a class within the subphylum Cnidospora. In the1980 revision of the Protozoa (Levine et al. 1980), they concurred with the elevation of various groups (including the microsporidia) to the level of phylum.

Notable early workers include Thélohan, Gurley, Léger, Stempell, Hesse, Pérez, Fantham and Porter, Debaisieux, Duboscq, and Paillot. They concentrated on host/parasite relationships and on elucidating life cycles, but, because of the diminutive size of the organisms, many conflicting results were reported. This period of activity culminated in the monograph of Kudo (1924), summarizing the biology and taxonomy of the group as it was then known. Kudo listed 14 genera and about 150 species. In an annotated list compiled by Sprague (1977b) he recognized 44 genera, and he established the use of a collective group, *Microsporidium*, for ambiguous or uncertain forms. In the 1999 microsporidia volume edited by Wittner and Weiss, 143 genera were recognized, and in 2014 microsporidia volume edited by Weiss and Becnel, 200 genera were reported and currently (this chapter) 209 genera have been reported.

One of the most significant observations made in the early years was that the polar filament serves as a tube for the passage of the sporoplasm to the exterior of the spore (Oshima 1937). Oshima's observation was confirmed years later in stained smears by Kramer (1960) and in electron micrographs, e.g., Huger (1960), Kudo and Daniels (1963). The electron microscope has been instrumental in resolving early questions of parasite structure and host/parasite interface.

Another significant observation has been the demonstration of synaptonemal complexes in several microsporidial genera indicating that meiosis occurs (Loubes 1979). It has been demonstrated at both the light and electron microscopic level, in both single nucleated and diplokaryotic species. Some genera that utilize meiosis include *Amblyospora*, *Gurleya*, *Duboscqia*, *Parathelohania*, and *Vairimorpha*; however, it is not a general feature of the Microsporidia (Loubes et al. 1976; Maddox et al. 1981; Hazard and Brookbank 1984; Solter and Maddox 1998; Andreadis 2007). This subject has been reviewed in Vavra and Larsson (1999). Meiosis has been well documented and illustrated in the *Amblyospora connecticus* life cycle (Becnel and Andreadis 1999), and more recently (Fig. 9) in other *Amblyospora* spp. (Andreadis 2007).

A significant landmark in microsporidial biology is the genome sequencing of the microsporidium, *Encephalitozoon cuniculi* (Katinka et al. 2001). This has been followed by several other microsporidial genome sequences reviewed by Keeling et al. (2014). This has provided information for the application of genomic and proteomic molecular tools contributing to a better understanding of these organisms

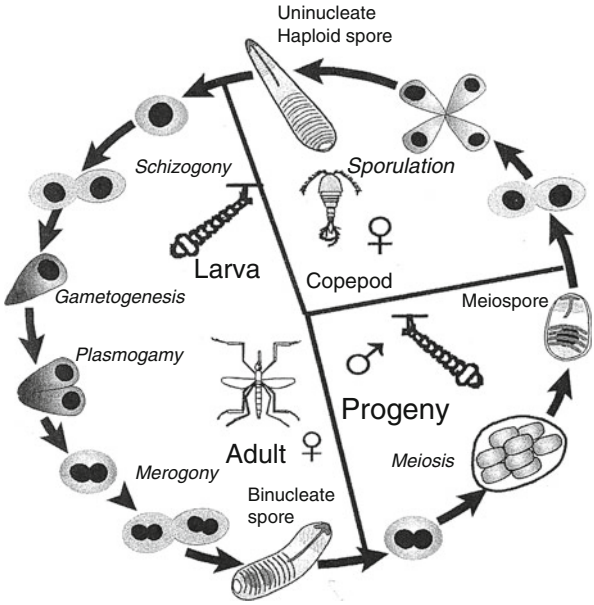

Fig. 9 Meiosis in Microsporidia: In *Amblyospora* spp., spores are produced in adult female mosquitoes and are passed by transovarial transmission to the next generation. The haploid uninucleate spores that develop in sporophorous vesicles produced in mosquitoe larvae are not infective to adults. These uninucleate spores are infective to copepods in which another cycle of development takes place, producing a third type of spore (diploid) infective to mosquitoes (Reprinted from Andreadis 2007)

(Williams et al. 2002, 2008a, b). Both morphology and molecular biology are currently in use for taxonomic purposes to define both genera and families. Consequently, depending on ones' approach, conflicting taxonomic schemes have been generated: "Molecular versus morphological approach to microsporidian classification" (Larsson 2005) and "Molecular phylogeny of the microsporidia: ecological, ultrastructural and taxonomic considerations" (Vossbrinck and Debrunner-Vossbrinck 2005). The time is approaching when a unified and integrated taxonomic system can be developed.

Practical Importance

The threat presented by microsporidia to hosts of economic importance, like silkworms, has already been mentioned. The same threat applies to bees infected with *Nosema apis* or *N. ceranae* and to fish, which are hosts to many species (Shaw and Kent 1999). Fish farming has increased the risks of epizootics. Alternatively, Microsporidia may play a beneficial role in nature: There is little doubt that some of the more virulent microsporidian pathogens in insects play a part in the natural control of host populations. There is encouraging evidence that some species can be exploited in biological control of pests, probably in conjunction with low levels of chemical insecticides: examples are *Nosema locustae* against grasshoppers (Henry 1971; Henry et al. 1973) now known as *Paranosema* (Sokolova et al. 2003) and/or *Antonospora* (Slamovits et al. 2004) and *Vairimorpha necatrix* against lepidopteran larvae (Maddox et al. 1981).

Identification of mammalian microsporidial infection is more recent: *Encephalitozoon cuniculi* Levaditi et al. 1923, was not accepted as a microsporidium until the 1960s and then it was placed in the genus *Nosema* (Weiser 1964). In 1970, its differences from the genus *Nosema* were demonstrated ultrastructurally and *Encephalitozoon* was made a microsporidial genus (Cali 1970). It has subsequently been recognized that it is quite widespread in mammals. *E. cuniculi* has been reported from over 30 different mammalian hosts including rodents, rabbits, carnivores, and nonhuman and human primates and is probably the most studied microsporidium (Cali and Owen 1988; Wilson 1979).

In humans, 17 species of microsporidia, causing a variety of pathologies and fatalities, have been identified (Cali and Owen 1988; Cali et al. 2011; Didier and Weiss 2008; Fayer and Santin-Duran 2014; Sobottka et al. 2012; Weber et al. 1994). They are opportunistic in that they are not self-limited in immunocompromised individuals. Worldwide prevalence rates have ranged from 0% to 50% depending on a variety of factors (Didier et al. 2004). While several organisms have been recognized, the majority of human infections are caused by four species of microsporidia in the two genera, *Enterocytozoon* and *Encephalitozoon.* The discovery of these parasites in AIDS patients has led to their identification in other immune deficiency situations such as organ transplants, rheumatoid arthritis, and cancer treatment. Since microsporidia are ubiquitous in nature and have a variable host range, reservoir hosts play an important role. Many animal hosts have been recognized for the genus *Encephalitozoon*, and more recently animal hosts have been documented for *Enterocytozoon* and many of the other human-infecting microsporidia (Bryan and Schwartz 1999; Cali et al. 2005; Coyle et al. 2004; Matos et al. 2004).

Habitats and Ecology

As obligate intracellular parasites, the microsporidial spores, the environmentally transmittable stage is the only extracellular stage in their life cycle, and they are very resistant in the external environment. Their survival outside the host varies according to species. In general, ultraviolet radiation and high temperatures are detrimental and quickly kill spores. Drought is also detrimental, more so for species parasitizing aquatic hosts than those in terrestrial hosts. Spores of the latter can often survive for weeks or months if protected by the dried-out cadavers or feces of their hosts. Spores in water can often survive for years. When spores enter a host and are stimulated to germinate they must inoculate a host cell with their sporoplasm in order to begin development. Their intracellular development is extremely variable and dependent upon the microsporidial genus.

The immediate environment for all microsporidial development is within the host cell. Initially the sporoplasm lies directly within the host cell cytoplasm, with the exception of a few fish and/or crustacean infecting genera: *Nucleospora*, *Enterospora*, and *Desmozoon* which develop within the host cell nucleus (Docker et al. 1997; Freeman and Sommerville 2009; Hedrick et al. 1991; Stentiford and

Bateman 2007; Stentiford et al. 2007). The others remain in the cytoplasm throughout their development, and they avoid intracellular lysis as normally occurs when lysosomes fuse with phagocytic vacuoles containing foreign organisms. Various methods are apparent by which Microsporidia avoid the lethal responses of their hosts.

The following section includes many variations of the intracellular environment (Table 1).

Table 1 Interfacial relationships of the microsporidia

Type I. Direct contact
The parasite plasmalemma is in direct contact with the host cell cytoplasm, e.g., *Nosema* and *Enterocytozoon*, or in the host cell nucleoplasm, e.g., *Nucleospora* and *Enterospora*
Type II. Indirect contact by parasite-produced isolation
The parasite secreted surface material present throughout parasite development, e.g., *Anncaliia*, *Brachiola*, and *Tubulinosema*
The parasite secreted elaborate envelope that surrounds parasite cells throughout development. It becomes an SPOV in sporogony when the parasite plasmalemma pulls away from the secreted envelope and then the plasmalemma thickens, e.g., *Pleistophora*
The parasite develops in direct contact with the host cell cytoplasm during early development, but then a parasite-formed membrane (SPOV) isolates the sporogonic stages from host cytoplasmic contact, e.g., *Vairimorpha*
The parasite appears in direct contact with the host cell cytoplasm during early development; however, the parasite produces an outer layer, somewhat like a glycocalyx, that provides for a zone of separation between the parasite plasmalemma and the host cell cytoplasm. This material "blisters" off the plasma membrane and pulls away, forming an SPOV in sporogony, e.g., *Pseudoloma*
Type III. Indirect contact by host-produced isolation
Host ER double membrane surrounds parasite cells throughout development. In the proliferative phase, the host ER double membranes follow the plasmalemma of the dividing cells so that no obvious vacuole is formed. In sporogony, the host ER does not divide with the sporonts and instead forms a double-membraned parasitophorous vacuole surrounding the cluster of organisms formed in sporogony, e.g., *Endoreticulatus*
Type IV. Indirect contact by host- and parasite-produced isolation
The host and parasite contribute to the formation of a thick interfacial envelope that surrounds all stages of parasite cells, e.g., *Trachipleistophora*
A host-formed parasite modified single membrane surrounding the developing parasite cell cluster, the parasitophorous vacuole. This is present during both the proliferative phase and the sporogonic phase; however, the parasite relationship to it changes, e.g., *Encephalitozoon cuniculi*
Host-formed parasitophorous vacuole surrounds the developing parasite cluster, and parasite-secreted material surrounds each parasite cell inside the parasitophorous vacuole, e.g., *Encephalitozoon intestinalis*
Host ER closely abuts the parasite plasmalemma in the proliferative phase (merogony). In sporogony, the parasite produces an SPOV. It may also contain tubules, e.g., *Loma* and *Glugea*
Parasite-induced infected host cell growth and hypertrophy, with parasite and host organelle proliferation combined with isolation by extracellular means, xenoma formation, e.g., *Glugea*, *Loma*, *Ichthyosporidium*, and *Microsporidium cotti* (In these genera a host response to the massively hypertrophying infected host cell includes host isolation of this cell by collagenous or other deposits around the cell. This complex is called a xenoma, ranging in size from microscopic to >5 mm in diameter.)

Some Microsporidia develop with their plasmalemma in direct contact with the host cell cytoplasm, e.g., *Nosema* and *Enterocytozoon* (Table 1 type I). They appear to interact with host cell organelles (Dionisio 2012). A more common relationship is parasite produced isolation, which is quite variable from a thickened plasmalemma to a secreted envelope (Table 1 Type II). The secretion from the parasite plasmalemma maintained on the surface of the parasite cells without separation occurs in some genera such as *Anncaliia*, *Brachiola*, and *Tubulinosema*. However, a phenomenon associated with many microsporidial genera is the separation of a secretion envelope of electron dense material external to the parasite's plasma membrane at the onset of sporogony. This produces a sporophorous vesicle, SPOV (Table 1 Type II), within which the plasmalemmal surface of the sporonts thicken and with various cell division cycles (characteristic of the genera) spores are produced, e.g., *Vavraia*, *Pleistophora*, *Trachipleistophora*, and *Vairimorpha*. The mechanism by which a second membrane can separate at the surface of an organism is obscure. Whatever its nature, after its separation it appears to function as a barrier to exchange between parasite and host cell. Frequently the cavity becomes filled with, tubules, fibers, and/or "metabolic products" of the parasite in the form of granules. The structure was known as the pansporoblast, but Canning and Hazard (1982) advocated the use of the term sporophorous vesicle (SPOV) for this microsporidial structure to make clear the distinction between it and the pansporoblast of the Myxozoa, which has a quite different origin and is composed of living cells.

An alternative is when the parasite cells may become secondarily invested by host membrane systems, such as the double membrane system of the host ER surrounding the developing parasitic organisms as they multiply, e.g., *Endoreticulatus* (Table 1 Type III).

In some microsporidia both the host and parasite contribute to the interface. A single membrane (possibly of both host and parasite origin) forms a parasitophorous vacuole, within which the parasites multiply and produce spores, e.g., *Encephalitozoon* (Table 1 Type IV). Although lysosomes are sometimes observed at the edge of the parasitophorous vacuole, lysosomal fusion is not triggered and the parasites are unaffected. *Encephalitozoon cuniculi*, a parasite of epithelial cells, endothelial cells, macrophages, and certain types of cells in the brain and kidney, provides the best example of intravacuolar development.

In genera such as *Glugea* and *Loma*, the host ER closely abuts the parasite plasmalemma in merogony but then the parasite starts production of "blisters" on its surface. An envelope forms, becoming the SPOV in sporogony (Table 1 Type IV). Inevitably, nutrients are provided for growth and multiplication of the parasites at the expense of the host cell cytoplasm. These different interfacial relationships demonstrate the diversity found among the many genera of the microsporidia (Fig. 10). Ultimately, there may remain little more of the host cell than its membrane around a large aggregate of spores (Fig. 2). Breakdown of heavily parasitized cells is common. If the cells are gut epithelial, excretory tubule or bladder cells, the spores can be discharged into the external environment. If there is no direct exit, spores released

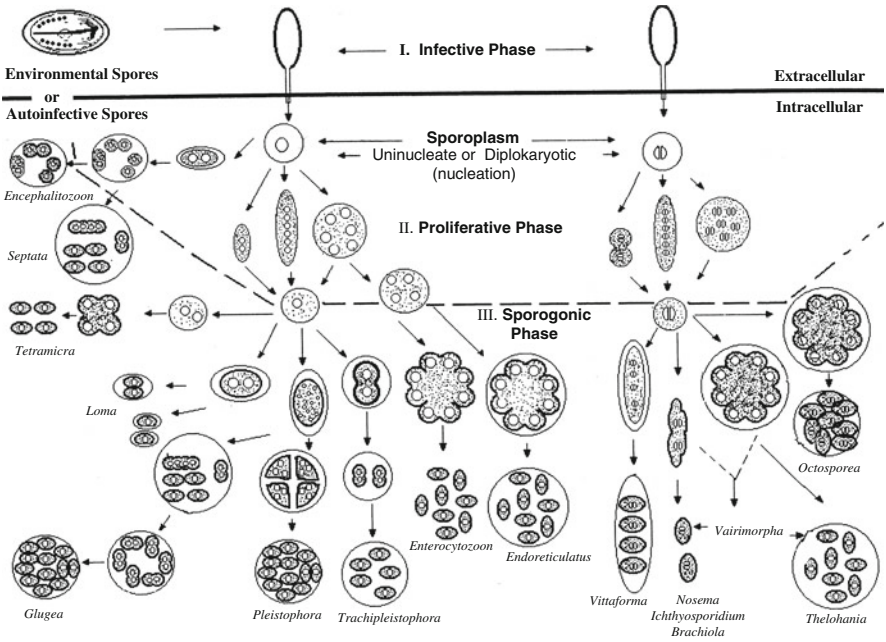

Fig. 10 Diagrammatic representation of some horizontally transmitted microsporidian life cycles, illustrating developmental diversity. In the **infective phase**, the proper environmental conditions are required to activate mature spores, resulting in polar tube extrusion. The polar tube of each spore is illustrated as piercing the host cell plasmalemma, represented by the solid black line. Below the line, is the intracellular cytoplasmic area. The sporoplasm travels through the everted polar tubule and is deposited inside the host cell. This begins the **proliferative phase** of development. The sporoplasm on the left is uninucleate and the cells that are produced from it represent the developmental patterns of several microsporidia with isolated nuclei. The sporoplasm on the right is diplokaryotic and it similarly produces the various diplokaryotic developmental patterns. Cells containing either type of nucleation will produce one of three basic developmental forms. Some cycles have cells that divide immediately after karyokinesis by binary fission (e.g., *Anncaliia* formerly *Brachiola*). A second type forms elongated moniliform multinucleate cells that divide by multiple fission (e.g., some *Nosema* species). The third type forms rounded plasmodial multinucleate cells that divide by plasmotomy (e.g., *Endoreticulatus*). Cells may repeat their division cycles one to several times in the proliferative phase. The intracellular stages in this phase are in direct contact with the host cell cytoplasm or closely abutted to the host ER. There are two types of exceptions. a) The proliferative cells of *Encephalitozoon* and *Septata* are surrounded by a host formed parasitophorous vacuole throughout their development (possibly *Tetramicra*). b) The proliferative plasmodium of the genus *Pleistophora* is surrounded by a thick layer of parasite secretions in the proliferative phase that separates and becomes the sporophorous vesicle in the sporogonic phase. Below the dashed line are the stages of the **sporogonic phase**. A few cycles maintain direct contact with the host cell cytoplasm in the sporogonic phase, *Nosema*, *Ichthyosporidium*, *Anncallia*, *Tetramicra*, and *Enterocytozoon*. The remaining genera form a sporophorous vesicle as illustrated by the circles around developing sporogonial stages. It should be noted that in the *Thelohania* cycle and the *Thelohania*-like part of the *Vairimorpha* cycle, the diplokarya separate and continue their development as cells with isolated nuclei (Reprinted from Cali and Takvorian 1999)

from cells are now vulnerable to the phagocytic processes of the host. This may provide the vehicle for transport to other parts of the host body as in disseminating Microsporidia such as the *Encephalitozoon* species in which the phagocytic cell does not kill the parasite; instead it multiplies in it (Orenstein et al. 1992). In insects, spores liberated into the hemocoele may be encapsulated by hemocytes and by subsequent deposition of melanin. In vertebrates, the aggregates of free spores are broken up by infiltrating cells and removed by phagocytes.

The relationship between parasite and host cell is not always a simple one of gain and loss, but a mechanism appears to operate by which the cell is imbued to a greater or lesser extent with the ability to compensate for the utilization of its cytoplasm by the parasites (Desjardins et al. 2015). There are indications that the parasites can, in some respects, control the metabolism of the cell, e.g., by the close relationship between the parasites and host mitochondria, endoplasmic reticulum, Golgi, and microtubules. In *Encephalitozoon*, host mitochondria accumulate close to the surfaces of parasites and lie at the edge of parasitophorous vacuoles, while in *Enterocytozoon* the host mitochondria appear to abut the parasite plasmalemma (Scanlon et al. 2004). Perhaps the most remarkable is the association between host endoplasmic reticulum and the merogonic stages in genera such as *Endoreticulatus* (Cali and Garhy 1991) and *Glugea* in which each meront is completely encased in a cisterna of endoplasmic reticulum and when meront division takes place, the endoplasmic reticulum follows the constrictions, which separate the offspring cells, and divides with them. This invariable association with host endoplasmic reticulum suggests that the host's proteins are being utilized by the parasite, but the manner in which they cross the membrane barriers is obscure. It has recently been demonstrated that in *Anncaliia* infected HeLa cells, microtubule organizing centers become disrupted resulting in fragmented Golgi associated with the developing Microsporidia (Santiana et al. 2015).

In genera such as *Glugea* and *Endoreticulatus* the host ER closely parallels the parasite plasmalemma. In general, the Microsporidia rely on the host organelles for nutrition. The host cell nucleus is rarely invaded; it is frequently hypertrophic and clearly survives long enough to exert control over synthesis of new proteins, which enables the host cell to enlarge and accommodate the growing parasites. Often the host cell survives, expands to accommodate the parasites, and, to all intents and purposes, itself becomes parasitic on the surrounding host tissues (Diamant et al. 2014). The term xenoma is used for this host/parasite cell complex. The xenomas induced by the *Glugea* species in fish show this interaction between host cell and parasite to a high degree. *Glugea* xenomas each represent a single greatly hypertrophic host cell (Takvorian and Cali 1981), reaching several millimeters in diameter (Fig. 7). Typically, the host reacts by producing a collagenous multilayered envelop, to isolate the xenoma. The plasma membrane of the *Glugea* xenoma displays pinocytotic activity in accord with its function to absorb nutrients from the surrounding tissue for its growth and that of the parasites within. The host nucleus becomes highly branched, ramifying through the peripheral layers of the cytoplasm, and numerous nucleoli, seen in every branch of the nucleus, are consistent with increased synthetic activity.

Different types of single-cell tumors are induced by other genera parasitizing fish. In *Ichthyosporidium giganteum* infections of *Leiostomum xanthurus*, the host cell is devoid of strengthening layers but is provided with extensive ramifications into surrounding host tissue (Sprague 1966). In *Loma morhua* parasitizing Atlantic cod, *Gadus morhua*, the host cell is enclosed by the thick basement membrane of the pillar system in the gills, and the plasma membrane interdigitates with it (Morrison and Sprague 1981). In *Spraguea lophii* infections of angler fish, *Lophius budegassa*, the infected ganglion cells are hypertrophic and surrounded in the ganglion by flattened cells. The colonies of parasites are strictly localized in the distal region of the ganglion cell body, close to the point of exit of the axon.

Characterization and Recognition

Phylum Microsporidia Balbiani 1882, *stat. nov.*, Weiser 1977.

Definition: Microsporidia are obligate intracellular parasites with spores of unicellular origin, containing a single uninuclear or binuclear sporoplasm, surrounded by a polar filament which becomes tubular upon discharge (eversion). As the spore germinates, the sporoplasm is transferred from the spore, via the polar tube, into a prospective host cell.

Microsporidia were once thought to be very primitive, subsequent research has demonstrated that, as parasites, they have degenerated/evolved, resulting in the loss or modification of some eukaryotic organelles, (see general characteristics for details). Additionally, their nucleation is represented by isolated (unattached) or abutted paired (attached) nuclei, called a diplokarya. During karyokinesis, their nuclear envelope remains intact. Typical centrioles, composed of microtubules are absent but centriolar plaques, consisting of electron-dense material deposited on the nuclear envelope, at the site of spindle attachment are present (Fig. 11). The plaques appear as 1–5 stacked bar–like structures that lie outside the nuclear envelope, with polar vesicles (mitosomes). These structures lie outside the nuclear envelope, but often in a depression of it. In genera containing meiosis, it occurs early in sporogony. Microsporidial spores are of single cell origin and are highly characteristic (diagnostic), containing a sporoplasm surrounded by a coiled single polar filament which is everted in the germination process, thus becoming tubular, allowing the passage of the sporoplasm through it, thus host cells may be infected by "inoculation" (Fig. 12). Due to their small size, electron microscopy is often used for confirmation of their microsporidial nature. The intracellular development consists of proliferative (merogony) and sporulation phases. In some genera, three different sporogonic sequences lead to a marked spore polymorphism.

Life Cycle

Microsporidial spores may survive in the environment for indeterminate periods of time and will not become activated until they encounter the proper stimuli necessary

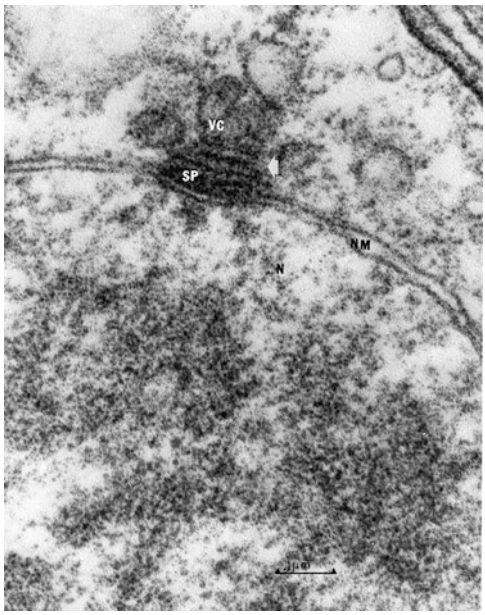

Fig. 11 Mitosome and spindle-plaque of *Glugea stephani.* The spindle-plaque (SP) the site of microtubule production and spindle attachment consists of stacked electron-dense bar-like structures on the cytoplasmic side of the nuclear membrane (NM). Membranous vesicles (VC) are the mitosome or relic mitochondria. Both of these structures are on the outside (cytoplasmic side) of the nucleus (N)

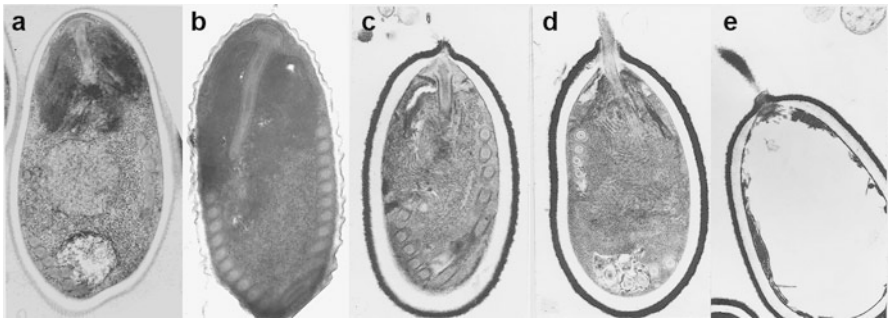

Fig. 12 Electron micrographs of mature spores comparing the morphological organization of inactive spores with those in the process of germination. (**a**) An inactive spore containing a very prominent anchoring disc followed by extensive lamellar polaroplast membranes and tubules in its anterior end. Below the polaroplast is the sporoplasm containing a well-defined nucleus in its cytoplasm. The posterior vacuole, not always present, is visible in this section. (**b**) An inactive spore illustrating the anterior straight portion of the polar filament as well as the cross-sectional views of the coiled portion in the medial to posterior part of the spore. (**c–e**) Changes associated with activation, polar filament eversion, forming the everted polar tube, and sporoplasm discharge. (**c**) Activated spore just starting to evert its polar filament through the rupturing apical portion of the spore. The polar filament coils are still present but are starting to reposition. (**d**) Activated spore with a large portion of its polar tube extruded and the remainder passing through the apical portion of the spore coat. The remaining part of the polar tube is repositioned. (**e**) Empty spore shell with the remnant of the polar tube still attached, the sporoplasm and associated structures have been transported out of the spore

for their particular germination (Fig. 12). This is most often in the digestive tract of a proper host. After activation of the spore, the germination process results in the transfer of its contents, the sporoplasm, into a host cell. If successful, the life cycle begins.

The Sporoplasm Sporoplasms (Fig. 6) have been observed in studies of emergence from spores (Fig. 5) in tissue culture (Cali et al. 2002; Takvorian et al. 2005; Weidner 1972; Weidner and Trager 1973). They have also been seen after spore germination in the natural host (Avery and Anthony 1983; Scarborough-Bull and Weidner 1985). Detailed studies of *Anncaliia (Brachiola) algerae* reveal the presence of a unique "network" MIN (multilayered interconnecting network) surrounding the cytoplasm and immediately inside the plasmalemma. It is connected to the polar tubule and disappears within minutes after extrusion, with only whorled vesicles remaining (Cali et al. 2002; Takvorian et al. 2005). The MIN has been identified as Golgi (Takvorian et al. 2013). These cells contain nuclei, little cytoplasmic differentiation, and whorled vesicles (Fig. 6). The surface membrane has been variously reported as simple, adorned with fibrous protrusions, or double, with the outer layer continuous with the polar tube sheath.

The proliferative phase (sometimes referred to as merogony) contains cells generally having a simple ultrastructural organization containing one to several single or abutted nuclei (diplokarya). In many Microsporidia, karyokinesis is not immediately followed by cytokinesis, resulting in multinucleate cells (Fig. 13).

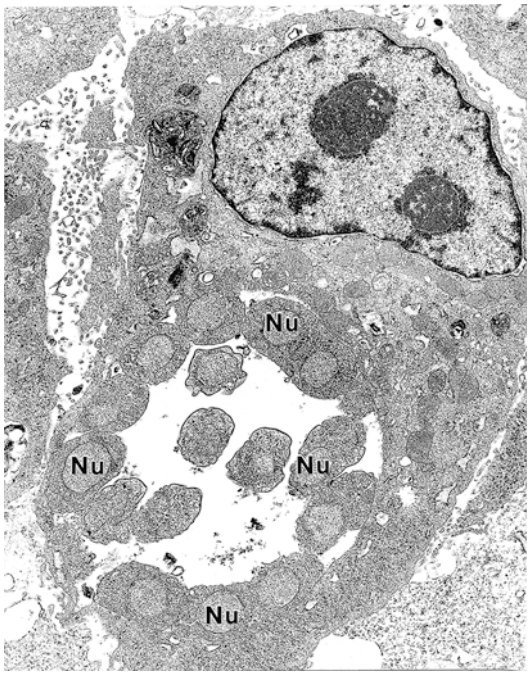

Fig. 13 *Encephalitozoon cunuculi.* Parasitophorous vacuole containing proliferative cells with large round isolated nuclei (Nu). Note that karyokinesis has not been immediately followed by cytokinesis, resulting in multinucleate cells tightly abutted to the periphery of the vacuole (Reprinted from Cali and Takvorian 1999)

These cells have a relatively simple cytoplasm containing some ER, ribosomes, and a medium dense appearance when viewed by electron microscopy. The plasma membrane is usually an unadorned unit membrane. In some microsporidia, the surface of the proliferative cells is covered with an electron-dense surface coat which is variously adorned with close-packed tubules, ridges, and/or vesicles (Cali et al. 1998), e.g., *Anncaliia* (*Nosema*, *Brachiola)* species (Fig. 14). An electron-dense surface coat is also present throughout the development of *Orthosomella*. In *Pleistophora* (*P. typicalis and P. ronneafiei*), there is a very thick amorphous coat, which divides with the proliferative cells but separates from the plasma membrane and becomes the sporophorous vesicle wall in sporogony (Fig. 15) (Cali and Takvorian 2003; Canning and Hazard 1982; Canning and Nicholas 1980).

Division of proliferative cells may be by binary fission of stages, which contain isolated nuclei (*Encephalitozoon*, *Unikaryon*) or diplokarya (*Nosema*, *Anncaliia*), and which elongate before constriction. Often they are multinucleate cylindrical (*Glugea*) or rounded plasmodia (*Alfvenia*, *Pleistophora*), also with diplokarya or isolated nuclei. The multinucleate stages may divide by simultaneous fission into unikaryotic or diplokaryotic products, or into smaller multinucleate segments by plasmotomy. In some taxa more than one morphological type of proliferation has been described. The heavy infections typical of most microsporidia demonstrate the abundance of some kind of multiplication process in proliferative development. The proliferative stages may not be morphologically distinguishable from sporonts; the proliferative phase may be short and linked to a cycle that includes autoinfective spores, extruding their polar tubes immediately after formation in an infected host

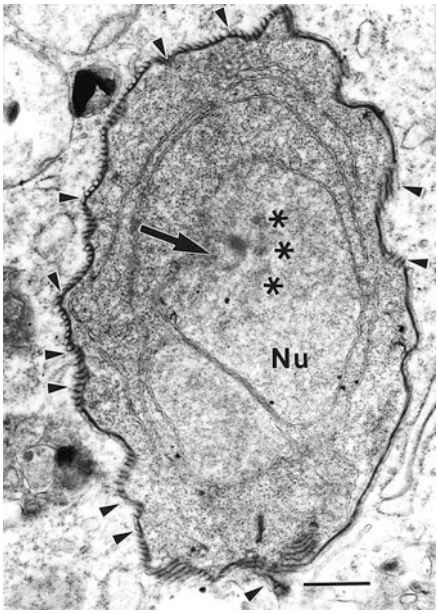

Fig. 14 *Anncaliia* (*Brachiola) algerae.* Proliferative cell in direct contact with the host cell cytoplasm. Parasite contains diplokaryotic nuclei (Nu) undergoing karyokinesis. Persistent nuclear membrane invaginates and contains a spindle plaque on the envelope (*arrow*) and chromosomes (*) within the nucleoplasm are present. Note the presence of vesiculotubular material (*arrow heads*) on the thickened plasmalemma (Reprinted from Takvorian et al. 2005)

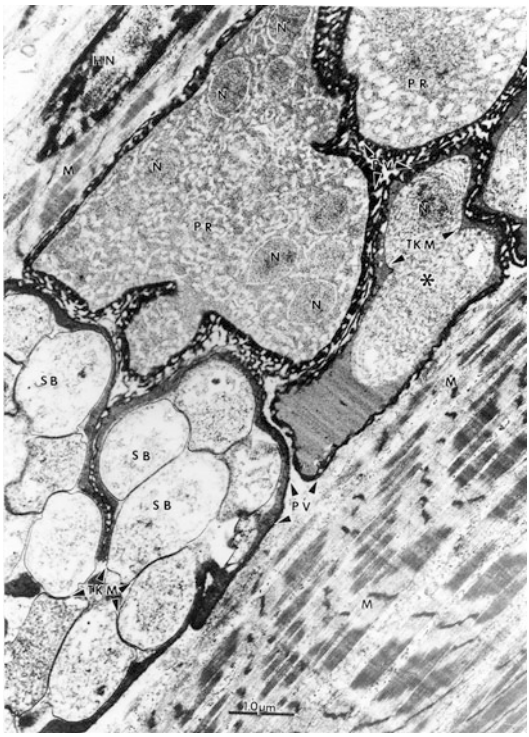

Fig. 15 Electron micrograph of *Pleistophora ronnefiei* developing in the skeletal muscle of a patient with AIDS. The proliferative stages (PR) and sporoblasts (SB) are enclosed in thick walled sporophorous vacuoles (PV). The proliferative forms are multinucleated, with many isolated nuclei (N). Early sporont has plasmalemma pulling away from the sporophorous vacuole (PV) and the plasmalemma has started to thicken (TKM) (Reprinted from Cali and Takvorian, 2003)

cell, and resulting in the infection of many more cells within the same host. Immediate germination of first-generation spores has been observed in several Microsporidia (Iwano and Ishihara 1989).

The Sporonts and Sporogony In those microsporidia that have a simple plasma membrane during their proliferative development, certain changes are observed which are associated with the entry into the phase of sporogony. These changes are morphologically characterized by secretions through the plasmalemma, giving it the appearance of an electron dense material that may first appear as "localized thickenings" like a scalloped surface (Fig. 16) and culminating in the formation of a "thickened membrane" (Fig. 17). Many genera produce appendages during this development or earlier. They vary in size and shape and are usually associated with the formation of the "thickened membrane" (Cali et al. 1998; Takvorian and Cali 1983). Uniquely, protoplasmic extensions have been demonstrated on *Brachiola vesicularum* (Cali et al. 1998) (Fig. 18). It is thought that these structures function in providing for nutritional needs after plasmalemmal thickening. Sporogony culminates in the production of spores, which are either (a) packaged in varying numbers within sporophorous vesicles or (b) are dispersed freely in the host cell cytoplasm.

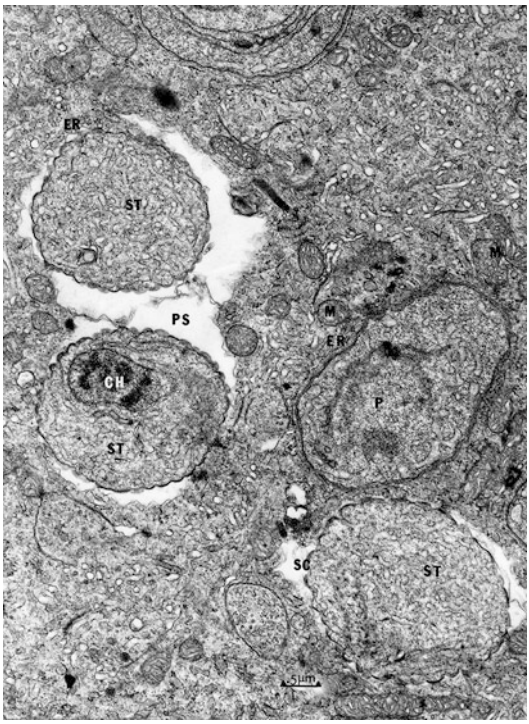

Fig. 16 *Glugea stephani* cells undergoing transition from proliferative (P) to sporogonic (ST) development. The most obvious morphological changes associated with the transition are a change from their association with the host cell cytoplasm and a concurrent change of the parasite cell surface. The proliferative plasmalemmal membrane is tightly embedded in the host cytoplasm, and the sporont plasmalemma is characterized by secretions of an electron dense material that appears as "localized thickenings" forming a scalloped surface (SC). As scalloping progresses, the plasmalemma becomes uniformly "thick" and the parasite cell is isolated from direct contact with the host cytoplasm by vacuole formation

(a) Development in Sporophorous Vesicles (formerly pansporoblasts)

In many of the genera, the parasite contracts within an envelope laid down de novo external to its plasma membrane (Fig. 19). Usually this is a fine, membrane-like structure as is present in *Vairimorpha* (Fig. 3) (Mitchell and Cali 1993); however, in *Pleistophora* and *Vavraia*, it is a thick layer already present during the proliferative phase (Canning and Hazard 1982), which acquires electron-dense additions, and then as the plasma membrane pulls away, it becomes a persistent sporophorous vesicle (Fig. 15).

The body within the sporophorous vesicle is the sporont which, when multinucleate, is referred to as the sporogonial plasmodium. The sporont acquires an electron-dense surface coat which later becomes the exospore layer of the spore wall (Fig. 15). This surface coat is thus an indicator of the commitment to sporogony. The sporont divides into sporoblasts. The division may be direct by binary fission

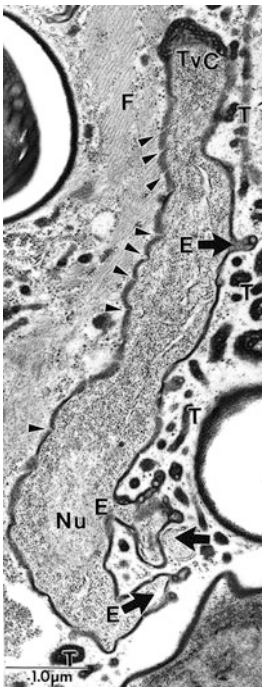

Fig. 17 *Brachiola vesicularum* produces protoplasmic extensions, unique to this parasite. They branch and terminate in extensive vesiculotubular structures, believed to function in providing for the parasite's nutritional needs (Reprinted from Cali et al. 1998)

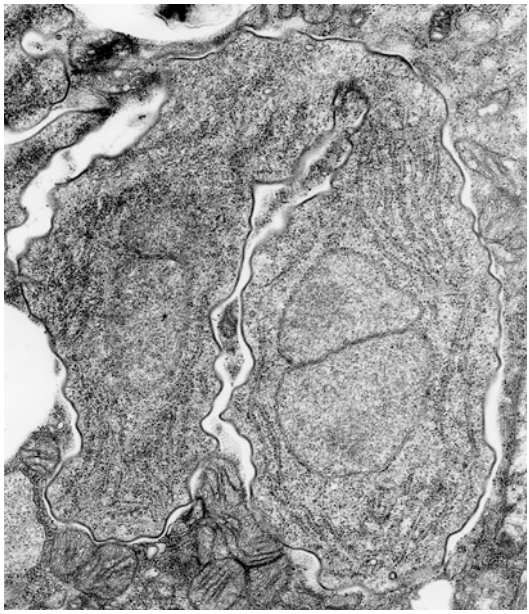

Fig. 18. *Nosema bombycis* sporont cell possessing a thickened membrane. The diplokaryon has undergone karyokinesis, and cytokinesis has commenced, but a connection between the two diplokaryotic parts of the cell is still present (Reprinted from Cali 1970)

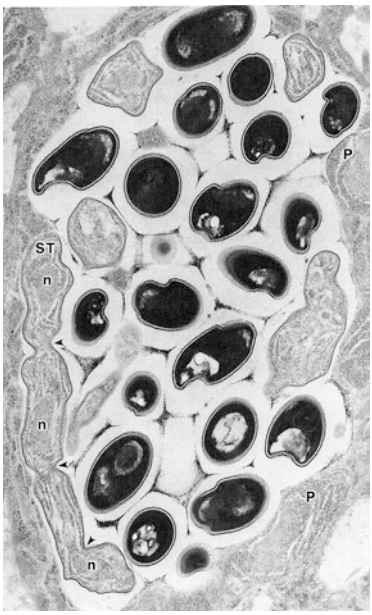

Fig. 19 *Encephalitozoon* (*Septata*) *intestinalis* in a parasitophorous vacuole with a fibrillar lamina separating the individual parasite cells. An elongated multinucleate (n) sporont (ST) cell is in the process of cytokinesis (*arrow head*). This cluster of parasite cells also contains many mature electron-dense spores as well as proliferative cells (P) (Reprinted from Cali et al. 1993)

(*Telomyxa*, *Berwaldia*) or multiple fission (*Gurleya*, *Amblyospora*, *Toxoglugea*). It may be a two-phase division whereby a multiple fission gives rise to uninucleate products (sporoblast mother cells), which undergo binary fission into sporoblasts (*Glugea* and possibly *Vavraia*). In *Pleistophora*, there is a series of divisions whereby the plasmodium separates into smaller and smaller segments, ending in uninucleate sporoblasts, plasmotomy.

Little is known of the ploidy of the nuclei in genera such as *Pleistophora*, *Vavraia*, and *Glugea* where the nuclei remain isolated throughout the life cycle or in genera such as *Nosema* or *Anncaliia* where the nuclei remain as diplokarya throughout their life cycle. However, in the genera *Thelohania*, *Amblyospora*, *Parathelohania*, and *Polydispyrenia*, which have nuclei in diplokaryon arrangement at the onset of sporogony, and in *Gurleya*, which has unikaryotic sporonts, structures interpreted as synaptonemal complexes have been observed in the young sporont nuclei. These have been taken to indicate meiosis (Loubès 1979).

The original demonstration of karyogamy is that of Hazard and Brookbank (1984) in *Amblyospora* sp. in *Culex salinarius*. They found that fusion of the two haploid nuclei of the diplokaryon occurred in presporonts only when they had entered cells of the fat body after a phase in hemocytes. They further reported that synapsis of homologous chromosomes occurred not after this fusion but later, after chromosome replication and restoration of the diplokaryon. These new diplokaryotic nuclei were diploid. There was some uncertainty about the events of meiosis, which according to Hazard and Brookbank (1984) are "dramatically unlike classical meiosis." Subsequently, Flegel and Pasharawipas (1995) clarified and corrected the conclusions of Hazard and Brookbank (1984) by reanalyzing their data into " two developmental

sequences, one abortive and the other for typical meiosis, a better fit was obtained between cytological stages and micro photometric measurement of DNA content," thus demonstrating that microsporidial meiosis is in fact typical for eukaryotes.

The number of diplokarya in the early sporont of the genera that undergo meiosis determines the number of haploid nuclei and, therefore, the number of sporoblasts arising from sporont division: eight sporoblasts are derived from sporonts with one diplokaryon in *Amblyospora*; 32 and 64 from sporonts with four and eight diplokarya, respectively, in *Polydispyrenia*. In *Gurleya*, where the sporont has a single nucleus, four haploid sporoblasts are produced in the sporophorous vesicle. In *Janacekia* the diplokaryotic nuclei characteristic of the meronts, separate in preparation for sporogony, and cytoplasmic division between them gives uninucleate sporonts as in *Gurleya. Janacekia* differs from *Gurleya* in that meiosis is followed by several mitoses, giving rise to 16 to 32 haploid nuclei and a corresponding number of sporoblasts. In this genus (as in other genera of the family Tuzetiidae), as the sporoblasts separate, the sporophorous vesicle accompanies the division, and each sporoblast becomes individually enclosed in a vesicle. According to genus, sporophorous vesicles can thus contain 1, 2, 4, 8, 16, or a variable number of sporoblasts, depending on the number of nuclei in the sporogonial plasmodium.

(b) Development Without Sporophorous Vesicles (formerly apansporoblastic)

In the remaining genera no envelope of parasitic origin is formed to isolate the stages of sporogony from the host cell cytoplasm, and the laying down of the electron-dense surface coat is the only sign of the onset of sporogony (Figs. 18 and 20). An exception is the genus *Enterocytozoon* (see below).

Sporogony of this type is often referred to as apansporoblastic. A diversity of division processes also distinguishes genera in this group. Sporonts may be diplokaryotic with disporoblastic development (Fig.18), *Nosema*, *Ichthyosporidium*, and *Mrazekia*, or with polysporoblastic development, *Golbergia*. Alternatively, sporonts may have isolated nuclei and disporoblastic development, *Encephalitozoon* (in a host-derived parasitophorous vacuole) (Figs.13, 19) and *Unikaryon*; tetrasporoblastic development, *Tetramicra*; or polysporoblastic development, *Perezia*,

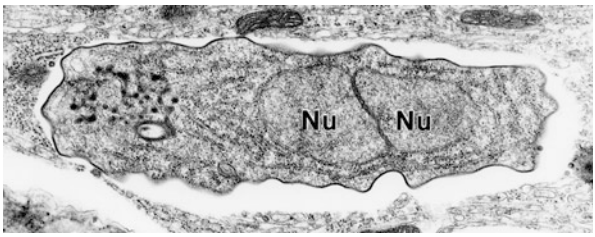

Fig. 20 *Nosema bombycis* late sporoblast, undergoing the morphogenic process for spore formation. This is indicated by the presence of the vesicular Golgi and associated tubular cross sections of polar filament. Additionally, the "thick" cell limiting membrane will become the exospore coat (Reprinted from Cali 1970)

Ameson, and *Nosemoides*. In some genera sporonts produce variable numbers of sporoblasts, *Culicospora* and *Orthosomella*.

Polymorphism Polymorphism, whereby a single species is able to express sporogonic and even proliferative development of entirely different types, each type formerly considered to be characteristic at the generic and even subordinal level, is being more and more commonly demonstrated among Microsporidia. Sometimes one sporogonic sequence takes place in a sporophorous vesicle giving uninucleate spores, while another gives rise to binucleate spores free in the cytoplasm, *Parathelohania*, *Amblyospora*, *Vairimorpha*, and *Burenella*. There are probably others of this type, in which the sequence producing spores in sporophorous vesicles closely resembles that of the foregoing genera, *Polydispyrenia*. In *Amblyospora* spp., the free spores are produced in adult females and are responsible for transovarial transmission to the next generation. This has been shown for a number of *Amblyospora* species, e.g., in the mosquito *Aedes cantator* (Andreadis 1983). The uninucleate spores in sporophorous vesicles produced in larvae are not infective to mosquitoes; Hazard and Brookbank (1984) found that these spores are haploid. In a species of *Amblyospora* in *Culex annulirostris*, Sweeney *et al*. (1985) demonstrated that the uninucleate spores are infective to the copepod *Mesocyclops albicans*, in which a further cycle of development takes place, producing a third type of spore infective to mosquitoes. Similar results were obtained by Andreadis (1985) who transmitted a species of *Amblyospora* from the mosquito, *Aedes cantator*, to the copepod, *Acanthocyclops vernalis*. These findings – that meiosis, polymorphism, and an alternation of hosts can occur in some microsporidial life cycles – are important discoveries in microsporidial biology.

In other types of dimorphism, both spore types are free. In *Hazardia*, binucleate and uninucleate spores are formed: one sequence is *Nosema*-like; the other produces sporonts with 1, 2, 4, 8, or 16 isolated nuclei and gives rise to corresponding numbers of uninucleate sporoblasts. In the genus *Spraguea* one sequence is *Nosema*-like while the other is *Nosemoides*-like, i.e., multinucleate with isolated nuclei (Loubes et al. 1979b). Therefore, there are features common to *Hazardia* and *Spraguea*, but by reason of other aspects of their development they are considered distinct genera. In *Nosema helminthorum*, a hyperparasite of cestode worms, unikaryotic and diplokaryotic stages have been observed (Canning and Gunn 1984). These engage in disporoblastic sporogony, which results in two types of free spores. This may be yet another type of dimorphism with stages resembling *Unikaryon* and *Nosema*.

The Sporoblast After the last cell division of sporont cells, the resulting cells will undergo a metamorphosis into spores. These morphogenic cells are the sporoblasts (Fig. 20). Young sporoblasts usually have an increased complement of endoplasmic reticulum compared with earlier developmental stages. Their surface characteristics, including the electron-dense coat deposited previously at the surface of the sporont, often leaves them with crenated outlines when observed by electron microscopy. As the sporoblasts mature, the organelles characteristic of the spore can be seen at various

stages of development. Sometimes the differentiation begins even before the division of the sporont is complete. The polar filament develops, at least in part, from a vesicular mass, described as Golgi-like and demonstrated to be a true Golgi apparatus histochemically in *Glugea stephani* (Takvorian and Cali 1994, 1996). Polar filament morphogenesis has been most completely described by Vinckier (1975) for *Nosemoides vivieri*. In this species, an anterior anchoring disc (polar sac) develops from a vesicle that lies between the nucleus and the Golgi vesicles. The Golgi vesicles coalesce to form the core and sleeve of the polar filament. The polar sac then migrates to the anterior end of the spore, while the nucleus and Golgi vesicles move to the posterior end. In the final stages, after the entire polar filament has been elaborated the Golgi vesicles may coalesce to form the posterior vacuole (posterosome). This structure is not, however, present in the spores of all species. In *Anncaliia algerae*, the Golgi has been observed as a vesicular mass that remains as such in the mature spore and becomes apparent in the activated spores (Cali et al. 2002).

The polaroplast, an organelle associated with the extrusion of the polar filament, develops as a series of flattened sacs and vesicles around the manubroid part of the polar filament (Takvorian and Cali 1986; Takvorian et al. 2006). Spore morphogenesis is completed by the deposition of the electron lucent chitinous endospore, which appears as "intermingled fibrils" when prepared by freeze-fracture (Bigliardi et al. 1996) between the plasma membrane and the electron dense exospore. Some studies also indicate that the endospore wall contains several novel glycosylated proteins that may have a role in host invasion (Hayman et al. 2005; Li et al. 2009; Southern et al. 2007).

Enterocytozoon bieneusi, the most common human-infecting microsporidium, is an exception to this developmental process. The multinucleate sporogonial plasmodium forms many polar filament structures prior to the last cell division process. Each developing polar filament forms in association with and surrounding a nucleus (Fig. 21). Subsequently, cell division occurs, isolating each nucleus/polar filament complex into individual late sporoblasts cells which then secrete the electron lucent endospore wall thus becoming mature spores (Cali and Owen 1990).

The Spore. Microsporidial spores range in size and shape from the 1 μm-diameter spherical spores of *Chytridiopsis aquaticus* and *Enterocytozoon bieneusi* to cylindrical spores, more than 20 μm long, of *Mrazekia argoisi*. Most Microsporidia have ovoid or ellipsoid spores measuring about 4 μm in length. A variety of more complex shapes is exhibited and has been used to distinguish between genera which otherwise follow similar patterns of development. Spores, such as those of *Caudospora*, *Jirovecia*, and *Inodosporus*, may be adorned with appendages, and there are abundant fine filaments like a covering of hairs on spores of *Ameson* and *Hirsutusporos* (Batson 1983). These appendages appear on developmental stages and persist, during spore morphogenesis, to become extensions of the exospore layer (Vavra and Larsson 2014).

In general, the microsporidial spore contents are encased in a resistant structure consisting of the exospore (electron dense secretions that first appear at the onset of sporogony, in most species) and the endospore (the electron lucent region forming last in spore maturation). Internally, it is lined by a membrane (formerly the cell limiting membrane, now the sporoplasm isolation membrane) which forms

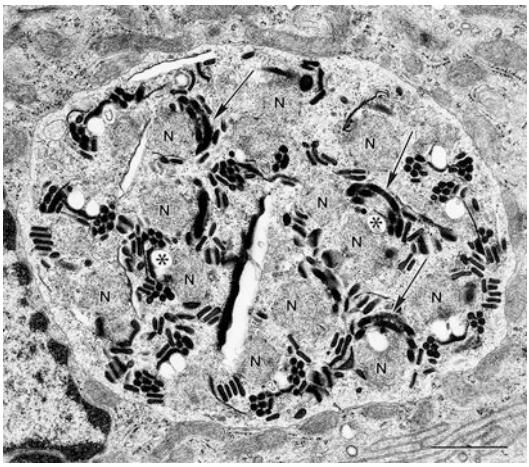

Fig. 21 *Enterocytozoon bieneusi* plasmodium with multiple developing polar filaments and many nuclei. The sporogonial plasmodium contains at least 12 nuclei (N) in a single plane of section. The round nuclei are each associated with electron dense disc complexes and electron lucent inclusions (*). Electron dense discs fuse into arcs forming polar filament coils (*arrows*). Despite the advanced maturation and organelle separation associated with each nucleus, there is no evidence of cytokinesis or plasmalemmal thickening which will occur after polar filament formation is complete. This developmental sequence is unique to the *Enterocytozoonidae* (Reprinted from Cali and Owen 1990)

invaginations around the developed polar filament. The plasmalemma, formerly thought to be just an envelope lining the endospore, has recently been demonstrated to be much more elaborate with infoldings surrounding the polar filament and thus isolating the sporoplasm from the extrusion apparatus (Cali et al. 2002).

The spore houses the infective agent (sporoplasm) and an array of structures comprising the extrusion apparatus (Vavra and Larsson 2014; Cali and Takvorian 2014). At the anterior end is an anchoring disc shaped rather like the cap of a mushroom, with the base of the polar filament inserted like its stalk (the anterior attachment complex). The polar filament runs a straight course (manubroid portion) diagonally posteriad and is posteriorly coiled in the peripheral layers of cytoplasm, later becoming isolated by the membrane infoldings (described above). The coils may be of uniform diameter (isofilar) or show a sudden change of diameter along its length (anisofilar). Surrounding the manubroid part of the filament is the polaroplast, usually a system of flattened membranes but sometimes described as vesicular, granular, or septate (Takvorian and Cali 1986; Takvorian et al. 2006). The posterior end of the spore may contain a vacuole or posterosome. Whether or not the posterior vacuole is membrane bound has not been definitively resolved (Figs. 4, 12).

In *Anncaliia algerae*, an additional organelle, the MIN (multilayered interlaced network) has been observed (Cali et al. 2002). It has been demonstrated to be associated with the end of the polar filament and surrounds the sporoplasm during the extrusion process and Golgi-like in activity (Takvorian et al. 2013). The

remaining spore contents (the sporoplasm) is the cytoplasm containing rough endoplasmic reticulum and free ribosomes surrounding one or two nuclei which is located centrally within the coiled region of the polar tube (Figs. 4 and 12a).

During spore activation, pressure attributed to swelling of the polaroplast membranes and posterosome builds up inside the spore and causes the polar filament to evert at the anterior end, breaking through the anchoring disc and the spore wall at its thinnest point (Vavra and Larsson 2014; Cali et al. 2002). This process proceeds in a fraction of a second and results in the polar filament becoming a tube as it everts with great speed and force, enabling it to inject the sporoplasm into a host cell (Figs. 5 and 6).

There have been several theories as to the mechanism of this germination process. In general, the germination requirements of Microsporidia are consistent for each species but vary among the different species. Jaronski concluded that "the spores responded to one or more stimuli: pH, ion concentration, osmolarity, digestive enzymes, redox potential and digestive products. Several lines of evidence suggest that sodium and potassium ions, within a limited pH range, act as primary germination stimuli for *Anncaliia* (*Nosema*) *algerae*." (Jaronski 1979). Weidner and Byrd (1982) found that the swelling of the polaroplast was accompanied by the displacement of internal calcium from the polaroplast membranes, possibly into the matrix between the membranes. They also found, as had Ishihara (1967), that external calcium blocked the polar tube discharge. During studies on germination in *Encephalitozoon hellem*, removal of calcium ions from the germination solution resulted in a decrease in polar filament extrusion (Leitch et al. 1995).

The pH effect on spore extrusion has been related to "priming" the spores. With *Ameson* (*Nosema*) *michaelis* a pH 10 has been reported (Weidner 1972). While *Glugea hertwigi* (Scarborough-Bull and Weidner 1985) and *E. hellem* respond to a pH 9.0 (Leitch et al. 1995), some organisms, e.g., *Vavraia culicis*, require a neutral or acidic pH to activate germination (Undeen 1983).

Undeen and Vander Meer (1994), using *Anncaliia algerae*, were able to link the role of the ions to activation or release of an enzyme trehalase which cleaves the disaccharide trehalose into smaller molecules. The rapid increase in solute concentration that results from this enzymatic action is believed to increase the intrasporal hydrostatic pressure providing the force for germination.

More recently, the infectious process of these organisms has been linked to the rapid influx of water into spores, presumably via aquaporins (AQPs), transmembrane channels that facilitate osmosis (Frixione et al. 1997). An AQP-like sequence of the microsporidium *Encephalitozoon cuniculi* (EcAQP) was cloned and expressed in oocytes of *Xenopus laevis*, which rendered these oocytes highly permeable to water (Fadiel et al. 2009; Ghosh et al. 2006).

Several small groups of Microsporidia are recognized which do not have the typical spore organelles described above. These belong to the class Rudimicrosporea, order Metchnikovellida, exclusively hyperparasitic in gregarines (Apicomplexa, Gregarinida), in which the polaroplast is absent and the polar tube consists only of a short thick tube, usually described as manubroid, terminating in a funnel. In four families, Chytridiopsidae, Hesseidae, Burkeidae, and Buxtehudeidae of the class Microsporea, the coiled polar tube is present but the polaroplast is absent (Larsson 2014).

Classification

Phylum Microsporidia Balbiani 1882, *stat. nov.*, Weiser 1977.

According to Levine et al. 1980 (in "The newly revised classification of the Protozoa") "the responsibility for the name of the high-level taxon is that of the person who established its actual level and its concept." Although Balbiani 1882 is credited with the name Microsporidies, he did not specify a taxonomic level of phylum. It was not established as a phylum until 1977, when two separate publications elevated the group (Sprague 1977; Weiser 1977) with different names. Technically, the publication of Sprague preceded that of Weiser by a few months and has priority; however, since Sprague and Becnel (1998) elected to accept Microsporidia as the correct phylum name and its usage has prevailed, the accepted form is Phylum Microsporidia.

History: A Linnaean classification of the microsporidia was first developed by Stempell in 1909, establishing three families in the Order Microsporidia. Subsequently, Leger and Hesse (1922) produced a system based only on spore morphology which was adopted by Kudo in the first major monograph on the microsporidia (Kudo 1924). Two attempts to produce a more encompassing classification to replace the previously used system were published independently in the same year by Sprague (1977) and Weiser (1977). While their classifications varied, they both elevated the microsporidia to phylum status where it has remained.

Two discoveries in microsporidial biology have had a profound effect on our concept of generic relationships. These discoveries, which apply to some genera, are the occurrence of meiosis at the onset of sporogony and the expression of dimorphic sequences of sporogony to which reference has been made above. The full significance of dimorphism was appreciated when it was shown that the two sequences could occur alongside one another; in the genus *Vairimorpha* the two sequences occur together at temperatures of 20°C and below. Sprague's system of separating the classical forms (order Microsporida) into two suborders, Pansporoblastina and Apansporoblastina, based on the presence or absence of sporophorous vesicles (pansporoblast membranes) in sporogony, is hardly tenable when both types of sporogony can occur in the life cycle of one species.

Weiser (1977) chose to separate the classical forms into two orders, Pleistophoridida and Nosematidida, based on the nuclear arrangement (whether isolated or abutting on one another as diplokarya) during sporogony and in the spores. This system does not distinguish between microsporidia that have isolated nuclei throughout development and may never undergo karyogamy and meiosis, and those that exhibit diplokarya at some stage in their life cycle – these nuclei separating and undergoing meiosis to produce the isolated haploid nuclei of the sporoblasts. The genus *Nosema* and some other genera are diplokaryotic in merogony and sporogony and, no synaptonemal complexes or karyogamy having been seen, it is presumed that meiosis is not a regular part of their development.

The importance of nuclear phenomena as a basis for indicating relationships in a classification system has been demonstrated by the splitting of the genus *Polydispyrenia* from the genus *Pleistophora* (Canning and Hazard 1982), and in the

splitting of the genus *Tuzetia* into four with the creation of the genera *Nelliemelba*, *Alfvenia*, and *Janacekia* (Larsson 1983).

Subsequent major classifications have been published including Sprague 1982; Issi 1986; Sprague et al. 1992; Voronin 1999; and Vossbrinck and Debrunner-Vossbrinck 2005 (with a rebuttal by Larsson 2005). Many molecular biologists have regrouped clusters of microsporidial genera with no indication as to how they fit into a higher scheme of the classification, and several new genera have been published without higher taxonomic placement. Sprague 1977, seems to have been the most widely used classification scheme and it can accommodate most current descriptions. We have chosen to use much of his basic scheme, omitting the suborders "Pansporoblastina" and "Apansporoblastina" which are based on the presence or absence of sporophorous vesicles. Additionally, new family names have been added to accommodate the subsequently described microsporidia as perceived by Voronin (1999) and those published since 1999. It should be noted that researchers disagree with the lines of division between the groupings and or their validity. Currently, the classification of the Phylum Microsporidia is still in major flux as demonstrated by the following published comments:

"None of the five different classification systems for microsporidia (Issi 1986; Sprague 1977; Sprague et al.1992; Vossbrinck and Debrunner-Vossbrinck 2005; Weiser 1977) satisfies the requirement of harmonizing structural data conventionally used in microsporidia classification with molecular phylogeny relationships. This is the general situation in microsporidia, where synapomorphic structural data are not well defined. The best example is *Senoma globulifera*, a mosquito parasite, which, although phylogenetically the closest relative of *Binucleata daphniae*, is structurally so dissimilar to *Binucleata*, that any conventional taxonomist would assign them at least into different families." (Refardt et al. 2008)

Currently, microsporidian sequence data can be accessed at MicrosporidiaDB (http://microsporidiadb.org). It is an NIH funded eukaryotic pathogen database resource that is continuously updated.

The following is in no way a validation or commitment to a classification but rather a work in progress. Further, as a reflection of the variations in the classification schemes, a checklist of available generic names for the Microsporidia including their type species and type hosts is presented here as has become custom (Canning and Lom 1986; Larsson 1999; Sprague and Becnel 1999; Becnel et al. 2014).

In the absence of a comprehensive revision, the higher classification proposed by Sprague and Vavra (1977) and Sprague (1982) is outlined below with the addition of new families.

•MICROSPORIDIA Balbiani 1882, *stat. nov.*, Weiser 1977
  ••Rudimicrosporea Sprague 1977
    •••Metchnikovellida Vivier, 1975
      ••••Metchnikovellidae Caullery and Mesnil, 1914
  ••Microsporea Corliss and Levine, 1963
    •••Chytridiopsida Weiser, 1974
      ••••Chytridiopsidae Sprague, Ormières and Manier, 1972
      ••••Hesseidae Ormières and Sprague, 1973

- ••••Burkeidae Sprague 1977
- ••••Buxtehudeidae Larsson, 1980
- •••Microsporida Balbiani 1882
- ••••Nosematidae Labbé, 1899
- ••••Glugeidae Thélohan, 1892
- ••••pleistophoridae Doflein 1901
- ••••Telomyxidae Léger and Hesse, 1910
- ••••Mrazekiidae Léger and Hesse, 1922
- ••••Cougourdellidae Poisson, 1953
- ••••Caudosporidae Weiser, 1958
- ••••Thelohaniidae Hazard and Oldacre, 1975
- ••••Spraguidae Weissenberg, 1976
- ••••Amblyosporidae Weiser 1977
- ••••Culicosporidae Weiser 1977
- ••••Duboscqiidae Sprague, 1977
- ••••Gurleyidae Sprague 1977
- ••••Pereziidae Loubès, Maurand, Comps and Campillo, 1977
- ••••Pseudopleistophoridae Sprague 1977
- ••••Tuzetiidae Sprague, Tuzet and Maurand, 1977
- ••••Unikaryonidae Sprague 1977
- ••••Burenellidae Jouvenaz and Hazard, 1978
- ••••Tetramicridae Matthews and Matthews, 1980
- ••••cylindrosporidae Issi and Voronin, 1986
- ••••golbergiidae Issi 1986
- ••••striatosporidae Issi and Voronin, 1986
- ••••abelsporidae Azevedo, 1987
- ••••encephalitozoonidae Voronin, 1989
- ••••enterocytozoonidae Cali and Owen 1990
- ••••culicosporellidae Becnel and Fukuda, 1991
- ••••janacekiidae Vedmed, Krylova and Issi, 1991
- ••••ichthyosporidiidae Sprague, Becnel, Hazard, 1992
- ••••microfilidae Sprague, Becnel, Hazard, 1992
- ••••ovavesiculidae Sprague, Becnel and Hazard 1992
- ••••neonosemoidiidae Faye, Toguebaye, Bouix, 1996
- ••••ordosporidae Larsson et al., 1997
- ••••flabelliformidae Voronin
- ••••glugoididae Voronin 1999
- ••••neopereziidae Voronin 1999
- ••••rectisporidae Voronin 1999
- ••••tubulinosematidae Franzen et al. 2005

The following is a checklist of available generic names for Microsporidia with type species and type hosts. This list includes the generic names that are deemed to have met the criteria of availability as defined by the Code of Zoological Nomenclature.

1. *Abelspora* Azevedo, 1987. Type species *Abelspora portucalensis* Azevedo, 1987. Type host *Carcinus maenas* (L.) Leach, 1814 (Decapoda, Portunidae).
2. *Acarispora* Redek, Kariton, Dabert and Alberti, 2015. Type species: *Acarispora falculifera* Redek, Kariton, Dabert and Alberti, 2015. Type host: *Falculifer rostratus* (Astigmata: Pterolichoidea).
3. *Aedispora* Kilochitskii, 1997. Type species *Aedispora dorsalis* Kilochitskii, 1997. Type host *Aedes* (*Ochlerotatus*) *caspius dorsalis* (Meigen) (Diptera, Culicidae).
4. *Agglomerata* Larsson and Yan, 1988. Type species *Agglomerata sidae* (Jírovec, 1942) Larsson and Yan, 1988. Type host *Sida crystallina* (O. F. Mueller, 1785) (Cladocera, Sididae).
5. *Agmasoma* Hazard and Oldacre, 1975. Type species *Agmasoma penaei* (Sprague, 1950) Hazard and Oldacre, 1975. Type host *Penaeus setiferus* (L.) (Decapoda, Penaeidae).
6. *Alfvenia* Larsson 1983. Type species *Alfvenia nuda* Larsson 1983. Type host *Acanthocyclops vernalis* Fisher (Copepoda, Cyclopidae).
7. *Alloglugea* Paperna and Lainson, 1995. Type species *Alloglugea bufonis* Paperna and Lainson, 1995. Type host *Bufo marinus* L. (Anura, Bufonidae).
8. *Amazonspora* Azevedo and Matos 2003. Type species *Amazonspora hassar* Azevedo and Matos 2003. Type host *Hassar orestis* (Steindachner, 1875) (Teleostei, Doradidae).
9. *Amblyospora* Hazard and Oldacre, 1975. Type species *Amblyospora californica* (Kellen & Lipa, 1960) Hazard and Oldacre, 1975. Type definitive host *Culex tarsalis* Coquillett (Diptera, Culicidae). Type intermediate host *Mesocyclops leukarti* (Claus, 1875) (Copepoda, Cyclopidae).
10. *Ameson* Sprague 1977. Type species *Ameson michaelis* (Sprague, 1970) Sprague 1977. Type host *Callinectes sapidus* (Rathbun, 1896) (Decapoda, Portunidae).
11. *Amphiacantha* Caullery and Mesnil, 1914. Type species *Amphiacantha longa* Caullery and Mesnil, 1914. Type host *Ophioidina elongata* Ming. "or related species" (Gregarinida) parasite of *Lumbriconereis tingens* (Polychaeta, Eunicidae).
12. *Amphiamblys* Caullery and Mesnil, 1914. Type species *Amphiamblys capitellides* (Caullery & Mesnil, 1897) Caullery and Mesnil, 1914. Type host *Ancora* sp. (Gregarinida) parasite of *Capitellides giardi* (Polychaeta).
13. *Andreanna* Simakova, Vossbrinck, and Andreadis, 2008. Type species *Andreanna caspii* Simakova, Vossbrinck, and Andreadis, 2008. Type host *Aedes* (*Ochlerotatus) caspius* (Pallas) (Diptera, Culicidae).
14. *Anisofilariata* Tokarev, Voronin, Seliverstova, Dolgikh, Pavlova, Ignatieva, and Issi, 2010. Type species *Anisofilariata chironomi* Tokarev, Voronin, Seliverstova, Dolgikh, Pavlova, Ignatieva, and Issi, 2010. Type host *Chironomus plumosus* L. (Diptera, Chironomidae).
15. *Anncaliia* Issi, Krylova, and Nicolaeva, 1993. Type species *Anncaliia meligethi* (Issi & Radishcheva, 1979) Issi, Krylova, and Nicolaeva, 1993. Type host *Meligethes aeneus* (Coleoptera, Nitidulidae).

16. *Anostracospora* Rode, Landes, Lievens, Flaven, Segard, Jabbour-Zahab, Michalakis, Agnew, Vivarès, and Lenormand, 2013. Type species *Anostracospora rigaudi* Rode, Landes, Lievens, Flaven, Segard, Jabbour-Zahab, Michalakis, Agnew, Vivares, and Lenormand, 2013. Type hosts *Artemia franciscana* Kellogg, 1906, and *A. parthenoge- netica* Bowen and Sterling, 1978 (Anostraca, Artemiidae).
17. *Antonospora* Fries, Paxton, Tengo, Slemenda, da Silva, and Pieniazek, 1999. Type species *Antonospora scoticae* Fries, Paxton, Tengo, Slemenda, da Silva, and Pieniazek, 1999. Type host *Andrena scotica* Perkins, 1916 (Hymenoptera, Andrenidae).
18. *Areospora* Stentiford, Bateman, Feist, Oyarzún, Uribe, Palacios, and Stone, 2014. Type species *Areospora rohanae* Stentiford, Bateman, Feist, Oyarzún, Uribe, Palacios, and Stone, 2014. Type host *Lithodes santolla* Molina, 1782 (Arthropoda, Lithodidae).
19. *Auraspora* Weiser and Purrini, 1980. Type species *Auraspora canningae* Weiser and Purrini, 1980. Type host *Lepidocyrtus lignorum* Fabricius, 1781 (Collembola, Entomobryidae).
20. *Bacillidium* Janda, 1928. Type species *Bacillidium criodrili* Janda, 1928. Type host *Criodrilus lacuum* Hoffm. (Haplotaxida, Criodrilidae).
21. *Baculea* Loubes and Akbarieh, 1978. Type species *Baculea daphniae* Loubes and Akbarieh, 1978. Type host *Daphnia pulex* (de Geer, 1778) (Cladocera, Daphniidae).
22. *Becnelia* Tonka and Weiser, 2000. Type species *Becnelia sigarae* Tonka and Weiser, 2000. Type host water boatmen, *Sigara lateralis* Leach, 1817 (Heteroptera, Corixidae).
23. *Berwaldia* Larsson, 1981. Type species *Berwaldia singularis* Larsson, 1981. Type host *Daphnia pulex* (de Geer, 1778) (Cladocera, Daphniidae).
24. *Binucleata* Refardt, Decaestecker, Johnson, and Vávra, 2008. Type species *Binucleata daphniae* Refardt, Decaestecker, Johnson, and Vávra, 2008. Type host *Daphnia magna* Straus (Cladocera, Daphniidae).
25. *Binucleospora* Bronnvall and Larsson, 1995. Type species *Binucleospora elongata* Bronnvall and Larsson, 1985. Type host *Candona* sp. (Ostracoda, Cyprididae).
26. *Bohuslavia* Larsson, 1985. Type species *Bohuslavia asterias* (Weiser, 1963) Larsson, 1985. Type host *Endochironomus* sp. (Diptera, Chironomidae).
27. *Brachiola* Cali, Takvorian, and Weiss, 1998. Type species *Brachiola vesicularum* Cali, Takvorian, and Weiss, 1998. Type host *Homo sapiens* L. (Primates, Hominidae).
28. *Bryonosema* Canning, Refardt, Vossbrinck, Okamura, and Curry, 2002. Type species *Bryonosema plumatellae* Canning, Refardt, Vossbrinck, Okamura, and Curry, 2002. Type host *Plumatella nitens* Wood, 1996 (Plumatellida, Plumatellidae).
29. *Burenella* Jouvenaz and Hazard, 1978. Type species *Burenella dimorpha* Jouvenaz and Hazard, 1978. Type host *Solenopsis geminata* (Fabricius) (Hymenoptera, Formicidae).

30. *Burkea* Sprague 1977. Type species *Burkea gatesi* (Puytorac and Tourret, 1963) Sprague 1977. Type host *Pheretima hawayana* (Oligochaeta, Megascolecidae) selected here from two hosts mentioned.
31. *Buxtehudea* Larsson, 1980. Type species *Buxtehudea scaniae* Larsson, 1980. Type host *Petrobius brevistylis* Carpenter, 1913 (Thysanura, Machilidae).
32. *Campanulospora* Issi, Radischcheva, and Dolzhenko, 1983. Type species *Campanulospora denticulata* Issi, Radischcheva, and Dolzhenko, 1983. Type host *Delia floralis* Fall. (Diptera, Muscidae).
33. *Canningia* Weiser, Wegensteiner, and Zizka, 1995. Type species *Canningia spinidentis* Weiser, Wegensteiner, and Zizka, 1995. Type host *Pityokteines spinidens* Rtt. (Coleoptera, Scolytidae).
34. *Caudospora* Weiser, 1946. Type species *Caudospora simulii* Weiser, 1946. Type host *Simulium hirtipes* (Fries, 1824) (Diptera, Simuliidae).
35. *Caulleryetta* Dogiel, 1922. Type species *Caulleryetta mesnili* Dogiel, 1922. Type host *Selenidium* sp. (Gregarinida, Schizocystidae) parasite of *Travisia forbesii* (Polychaeta).
36. *Chapmanium* Hazard and Oldacre, 1975. Type species *Chapmanium cirritus* Hazard and Oldacre, 1975. Type host *Corethrella brakeleyi* (Coquillett) (Diptera, Chaoboridae).
37. *Chytridioides* Tregouboff, 1913. Type species *Chytridioides schizophylli* Tregouboff, 1913. Type host *Schizophyllum mediterraneum* Latzel = *Ommatoiulus rutilans* (Koch, 1847) (Julida, Julidae).
38. *Chytridiopsis* Schneider, 1884. Type species *Chytridiopsis socius* Schneider, 1884. Type host *Blaps mortisaga* L. (Coleoptera, Tenebrionidae).
39. *Ciliatosporidium* Foissner and Foissner, 1995. Type species *Ciliatosporidium platyophryae* Foissner and Foissner, 1995. Type host *Platyophrya terricola* (Foissner, 1987) Foissner and Foissner, 1995 (Ciliophora, Colpodea).
40. *Coccospora* Kudo, 1925. Replacement name for *Cocconema* Léger and Hesse, 1921, preoccupied. Type species *Coccospora micrococcus* (Léger & Hesse, 1921) Kudo, 1925. Type host *Tanypus setiger* Kieffer (Diptera, Chironomidae).
41. *Cougourdella* Hesse, 1935. Type species *Cougourdella magna* Hesse, 1935. Type host *Megacyclops viridis* Jurine (Copepoda, Cyclopidae).
42. *Crepidulospora* Simakova, Pankova, and Issi, 2004. Type species *Crepidulospora beklemishevi* (Simakova et al. 2003) Simakova, Pankova, and Issi, 2004. Type host *Anopheles beklemishevi* (Diptera, Culicidae).
43. *Crispospora* Tokarev, Voronin, Seliverstova, Pavlova, and Issi, 2010. Type species *Crispospora chironomi* Tokarev, Voronin, Seliverstova, Pavlova, and Issi, 2010. Type host *Chironomus plumosus* L. (Diptera, Chironomidae).
44. *Cristulospora* Khodzhaeva and Issi, 1989. Type species *Cristulospora sherbani* Khodzhaeva and Issi, 1989. Type host *Culex modestus* (Diptera, Culicidae).
45. *Cryptosporina* Hazard and Oldacre, 1975. Type species *Cryptosporina brachyfila* Hazard and Oldacre, 1975. Type host *Piona* sp. (Arachnida, Hygrobatinae).
46. *Cucumispora* Ovcharenko, Bacela, Wilkinson, Ironside, Rigaud, and Wattier, 2010. Type species *Cucumispora dikerogammari* (Ovcharenko and Kurandina,

1987) Ovcharenko, Bacela, Wilkinson, Ironside, Rigaud, and Wattier, 2010. Type host *Dikerogammarus villosus* (Sowinsky, 1894) (Amphipoda, Gammaridae).

47. *Culicospora* Weiser 1977. Type species *Culicospora magna* (Kudo, 1920) Weiser 1977. Type host *Culex pipiens* L. (Diptera, Culicidae).
48. *Culicosporella* Weiser 1977. Type species *Culicosporella lunata* (Hazard & Savage, 1970) Weiser 1977. Type host *Culex pilosus* (Dyar & Knab, 1906) (Diptera, Culicidae).
49. *Cylindrospora* Issi and Voronin, 1986. Type species *Cylindrospora chironomi* Issi and Voronin, 1986, in Issi 1986. Type host *Chironomus plumosus* L. (Diptera, Chironomidae).
50. *Cystosporogenes* Canning, Barker, Nicholas, and Page, 1985. Type species *Cystosporogenes operophterae* (Canning, 1960) Canning, Barker, Nicholas, and Page, 1985. Type host *Operophtera brumata* (L.) (Lepidoptera, Geometridae).
51. *Dasyatispora* Diamant, Goren, Yokeş, Galil, Klopman, Huchon, Szitenberg, and Karhan, 2010. Type species *Dasyatispora levantinae* Diamant, Goren, Yokes, Galil, Klopman, Huchon, Szitenberg, and Karhan, 2010. Type host *Dasyatis pastinaca* (L.) (Myliobatiformes, Dasyatidae).
52. *Desmozoon* Freeman and Sommerville 2009. Type species *Desmozoon lepeophtherii* Freeman and Sommerville 2009. Type host *Lepeophtheirus salmonis* (Krøyer) (Copepoda, Caligidae).
53. *Desportesia* Issi and Voronin, 1986. Type species *Desportesia laubieri* (Desportes & Theodorides, 1979) Issi and Voronin, 1986, in Issi 1986. Type host *Lecudina* sp. (Gregarinida, Lecudinidae) parasite of unidentified marine annelid (Echiurida). (Considered by Larsson 2014, to be a junior synonym of *Amphiamblys*)
54. *Dimeiospora* Simakova, Pankova, and Issi, 2003. Type species *Dimeiospora palustris* Simakova, Pankova, and Issi, 2003. Type host *Aedes (Ochlerotatus) punctor* Kirby (Diptera, Culicidae).
55. *Duboscqia* Pérez, 1908. Type species *Duboscqia legeri* Pérez, 1908. Type host *Termes lucifugus* = *Reticulitermes lucifugus* (Rossi) (Isoptera, Rhinotermitidae).
56. *Edhazardia* Becnel, Sprague, and Fukuda, 1989. Type species *Edhazardia aedis* (Kudo, 1930) Becnel, Sprague, and Fukuda, 1989, in Becnel, Sprague, Fukuda, and Hazard, 1989. Type host *Aedes aegypti* (L.) (Diptera, Culicidae).
57. *Encephalitozoon* Levaditi, Nicolau, and Schoen, 1923. Type species *Encephalitozoon cuniculi* Levaditi, Nicolau, and Schoen, 1923. Type host "Rabbit" (Lagomorpha, Leporidae).
58. *Endoreticulatus* Brooks, Becnel, and Kennedy, 1988. Type species *Endoreticulatus fidelis* (Hostounsky & Weiser, 1975) Brooks, Becnel, and Kennedy, 1988. Type host *Leptinotarsa undecimlineata* Stal (Coleoptera, Chrysomelidae).
59. *Enterocytospora* Rode, Landes, Lievens, Flaven, Segard, Jabbour-Zahab, Michalakis, Agnew, Vivarès, and Lenormand, 2013. Type species *Enterocytospora artemiae* Rode, Landes, Lievens, Flaven, Segard, Jabbour-Zahab, Michalakis, Agnew, Vivares, and Lenormand, 2013. Type hosts *Artemia*

franciscana Kellogg, 1906; A. *franciscana monica* Verrill, 1869; and *A. parthenogenetica* Bowen and Sterling, 1978 (Anostraca, Artemiidae).

60. *Enterocytozoon* Desportes, Le Charpentier, Galian, Bernard, Cochand-Priollet, Lavergne, Ravisse, and Modigliani, 1985. Type species *Enterocytozoon bieneusi* Desportes, Le Charpentier, Galian, Bernard, Cochand-Priollet, Lavergne, Ravisse, and Modigliani, 1985. Type host *Homo sapiens* L. (Primates, Hominidae).
61. *Enterospora* Stentiford, Bateman, Longshaw, and Feist, 2007. Type species *Enterospora canceri* Stentiford, Bateman, Longshaw, and Feist, 2007. Type host *Cancer pagurus* L. (Decapoda, Cancridae).
62. *Episeptum* Larsson 1986. Type species *Episeptum inversum* Larsson 1986. Type host *Holocentropus picicornis* (Stevens, 1836) (Trichoptera, Polycentropidae).
63. *Euplotespora* Fokin et al. Fokin et al. 2008. Type species *Euplotespora binucleata* Fokin et al. 2008. Type host *Euplotes woodruffi* (Hypotrichida, Euplotidae).
64. *Evlachovaia* Voronin and Issi 1986. Type species *Evlachovaia chironomi* Voronin and Issi 1986, in Issi 1986. Type host *Chironomus plumosus* (Diptera, Chironomidae).
65. *Facilispora* Jones, Prosperi-Porta, and Kim, 2012. Type species *Facilispora margolisi* Jones, Prosperi-Porta, and Kim, 2012. Type host *Lepeophtheirus salmonis* Krøyer (Siphonostomatoida, Caligidae).
66. *Fibrillanosema* Galbreath, Smith, Terry, Becnel, and Dunn, 2004. Type species *Fibrillanosema crangonycis* Galbreath, Smith, Terry, Becnel, and Dunn, 2004. Type host *Crangonyx pseudogracilis* (Amphipoda, Crangonyctidae).
67. *Flabelliforma* Canning, Killick-Kendrick, and Killick-Kendrick, 1991. Type species *Flabelliforma montana* Canning, Killick-Kendrick, and Killick-Kendrick, 1991. Type host *Phlebotomus ariasi* Tonnoir, 1921 (Diptera, Psychodidae).
68. *Geusia* Rühl and Korn, 1979. Type species *Geusia gamocysti* Rühl and Korn, 1979. Type host *Gamocystis ephemerae* Frantzius, 1848 (Gregarinida, Gregarinidae), parasite of *Ephemera danica* (Ephemeroptera, Ephemeridae).
69. *Globulispora* Vávra et al. Vavra et al. 2016. Type species: *Globulispora mitoportans* Vávra et al. Vavra et al. 2016. Type host: *Daphnia pulex* (Leydig, 1860).
70. *Glugea* Thélohan, 1891. Type species *Glugea anomala* (Moniez, 1887) Gurley, 1893. Type host *Gasterosteus aculea- tus* L. (Gasterosteiformes, Gasterosteidae).
71. *Glugoides* Larsson, Ebert, Vávra, and Voronin, 1996. Type species *Glugoides intestinalis* (Chatton, 1907) Larsson, Ebert, Vávra, and Voronin, 1996. Type host *Daphnia magna* Straus, 1820 (Cladocera, Daphniidae), selected here from two hosts mentioned.
72. *Golbergia* Weiser 1977. Type species *Golbergia spinosa* (Golberg, 1971) Weiser 1977. Type host *Culex pipiens* L. (Diptera, Culicidae).
73. *Gurleya* Doflein, 1898. Type species *Gurleya tetraspora* Doflein, 1898. Type host *Daphnia maxima* (Cladocera, Daphniidae).

74. G*urleyides* Voronin, 1986. Type species *Gurleyides biformis* Voronin, 1986. Type host *Ceriodaphnia reticulata* Jurine (Cladocera, Daphniidae).
75. *Hamiltosporidium* Haag, Larsson, Refardt, and Ebert, 2010. Type species *Hamiltosporidium tvaerminnensis* Haag, Larsson, Refardt, and Ebert, 2010. Type host *Daphnia magna* Straus, 1820 (Cladocera, Daphniidae).
76. *Hazardia* Weiser 1977. Type species *Hazardia milleri* (Hazard & Fukuda, 1974) Weiser 1977. Type host *Culex pipiens quinquefasciatus* Say, 1823 (Diptera, Culicidae).
77. *Helmichia* Larsson, 1982. Type species *Helmichia aggregata* Larsson, 1982. Type host *Endochironomus* sp. (Diptera, Chironomidae).
78. *Hepatospora* Stentiford, Bateman, Dubuffet, Chambers, and Stone, 2011. Type species *Hepatospora eriocheir* (Wang & Chen, 2007) Stentiford, Bateman, Dubuffet, Chambers, and Stone, 2011. Type host *Eriocheir sinensis* H. Milne Edwards, 1853 (Decapoda, Varunidae).
79. *Hessea* Ormières and Sprague, 1973. Type species *Hessea squamosa* Ormières and Sprague, 1973. Type host *Sciara* sp. (Diptera, Lycoriidae).
80. *Heterosporis* Schubert 1969. Type species *Heterosporis finki* Schubert 1969. Type host *Pterophyllum scalare* (Curs & Valens, 1831) (Perciformes, Cichlidae).
81. *Heterovesicula* Lange, Macvean, Henry, and Streett, 1995. Type species *Heterovesicula cowani* Lange, Macvean, Henry, and Streett, 1995. Type host *Anabrus simplex* Haldeman, 1852 (Orthoptera, Tettigoniidae).
82. *Hirsutusporos* Batson 1983. Type species *Hirsutusporos austrosimulii* Batson 1983. Type host *Austrosimulium* sp. (Diptera, Simuliidae).
83. *Holobispora* Voronin, 1986. Type species *Holobispora thermocyclopis* Voronin, 1986. Type host *Thermocyclops ortho- noides* (Sars) (Copepoda, Cyclopidae).
84. *Hrabyeia* Lom and Dykova, 1990. Type species *Hrabyeia xerkophora* Lom and Dykova, 1990. Type host *Nais christi- nae* Kasparzak, 1973 (Oligochaeta, Naididae).
85. *Hyalinocysta* Hazard and Oldacre, 1975. Type species *Hyalinocysta chapmani* Hazard and Oldacre, 1975. Type host *Culiseta melanura* Coquillett, 1902 (Diptera, Culicidae).
86. *Ichthyosporidium* Caullery and Mesnil, 1905. Type species *Ichthyosporidium giganteum* (Thélohan, 1895) Swarczewsky, 1914. Type host *Crenilabrus melops* L. (Perciformes, Labridae).
87. *Inodosporus* Overstreet and Weidner, 1974. Type species *Inodosporus spraguei* Overstreet and Weidner, 1974. Type host *Palaemonetes pugio* Holthius, 1949 (Decapoda, Palaemonidae).
88. *Intexta* Larsson, Steiner, and Bjørnson, 1997. Type species *Intexta acarivora* Larsson, Steiner, and Bjørnson, 1997. Type host *Tyrophagus putrescentiae* (Acari, Acaridae).
89. *Intrapredatorus* Chen, Kuo, and Wu, 1998. Type species *Intrapredatorus trinus* (Becnel & Sweeney, 1990) Chen, Kuo, and Wu, 1998. Type host *Culex fuscanus* Wiedemann (Diptera, Culicidae).
90. *Issia* Weiser 1977. Type species *Issia trichopterae* (Weiser, 1946) Weiser 1977. Type host *Plectrocnemia geniculata* (Trichoptera, Polycentropodidae).

91. *Janacekia* Larsson 1983. Type species *Janacekia debaisieuxi* (Jírovec, 1943) Larsson 1983. Type host *Simulium maculatum* Meig. (Diptera, Simuliidae).
92. *Jirovecia* Weiser 1977. Type species *Jirovecia caudata* (Léger & Hesse, 1916) Weiser 1977. Type host *Tubifex tubifex* Mueller (Oligochaeta, Tubificidae).
93. *Jiroveciana* Larsson, 1980. Type species *Jiroveciana limnodrili* (Jírovec, 1940) Larsson, 1980. Type host *Limnodrilus missionicus* (Oligochaeta, Tubificidae).
94. *Johenrea* Lange, Becnel, Razafindratiana, Przybyszewski, and Razafindrafara, 1996. Type species *Johenrea locustae* Lange, Becnel, Razafindratiana, Przybyszewski, and Razafindrafara, 1996. Type host *Locusta migratoria capito* (Saussure, 1884) (Orthoptera, Acrididae).
95. *Kabatana* Lom, Dyková, and Tonguthai, 2000. Type species *Kabatana arthuri* (Lom et al. 1999) Lom et al. 2000. Type host *Pangasius sutchi* (Siluriformes, Pangasiidae).
96. *Kinorhynchospora* Adrianov and Rybakov, 1991. Type species *Kinorhynchospora japonica* Adrianov and Rybakov, 1991. Type host *Kinorhynchus yushini* (Echinodera, Pycnophyidae).
97. *Kneallhazia* Sokolova and Fuxa 2008. Type species *Kneallhazia solenopsae* (Knell, Allen, & Hazard, 1977) Sokolova and Fuxa 2008. Type host *Solenopsis invicta* Buren (Hymenoptera, Formicidae).
98. *Krishtalia* Kilochitskii, 1997. Type species *Krishtalia pipiens* Kilochitskii, 1997. Type host *Culex pipiens pipiens* L. (Diptera, Culicidae).
99. *Lanatospora* Voronin, 1986. Type species *Lanatospora macrocyclopis* (Voronin, 1977) Voronin, 1986. Type host *Macrocyclops albidus* Jurine (Copepoda, Cyclopidae).
100. *Larssonia* Vidtmann and Sokolova, 1994. Type species *Larssonia obtusa* (Moniez, 1887) Vidtmann and Sokolova, 1994. Type host *Daphnia pulex* De Geer (Cladocera, Daphniidae).
101. *Larssoniella* Weiser and David, 1997. Type species *Larssoniella resinellae* Weiser and David, 1997. Type host *Petrova resinella* (L.) (Lepidoptera, Tortricidae).
102. *Liebermannia* Sokolova, Lange, and Fuxa, 2006. Type species *Liebermannia patagonica* Sokolova, Lange, and Fuxa, 2006. Type host *Tristira magellanica* Bruner, 1900 (Orthoptera, Tristiridae).
103. *Loma* Morrison and Sprague 1981. Type species *Loma morhua* Morrison and Sprague 1981. Type host *Gadus morhua* L. (Gadiformes, Gadidae).
104. *Mariona* Stempell 1909. Type species *Mariona marionis* (Thélohan, 1895) Stempell 1909. Type host *Ceratomyxa coris* Georgevitch, 1916 (Bivalvulida, Ceratomyxidae), parasite of *Coris julis* L. (Pisces).
105. *Marssoniella* Lemmermann, 1900. Type species *Marssoniella elegans* Lemmermann, 1900. Type host *Cyclops stren- uus* Fischer, 1851 (Copepoda, Cyclopidae).
106. *Merocinta* Pell and Canning, 1993. Type species *Merocinta davidii* Pell and Canning, 1993. Type host *Mansonia africana* (Theobald) (Diptera, Culicidae).

107. *Metchnikovella* Caullery and Mesnil, 1897. Type species *Metchnikovella spionis* Caullery and Mesnil, 1897. Type host *Polyrhabdina brasili* Caullery and Mesnil (Gregarinida, Lecudinidae) parasite of *Spio martinensis* Mesnil (Polychaeta, Spionidae).
108. *Microfilum* Faye, Toguebaye, and Bouix, 1991. Type species *Microfilum lutjani* Faye, Toguebaye, and Bouix, 1991. Type host *Lutjanus fulgens* (Valenciennes, 1830) (Perciformes, Lutjanidae).
109. *Microgemma* Ralphs and Matthews, 1986. Type species *Microgemma hepaticus* Ralphs and Matthews, 1986. Type host *Chelon labrosus* (Risso) (Mugiliformes, Mugilidae).
110. *Microsporidium* Balbiani 1884. Not an available name *sensu stricto* but used under the provisions of the code (see Glossary, p. 257) as the legitimate name of a collective group. Useful as a provisional generic name if an author desires to record an unidentified species or to form a binomen and establish a new species while there is indecision about the genus.
111. *Microsporidyopsis* Chereschewsky, 1925. Type species *Microsporidyopsis nereidis* Schereschewsky, 1925. Type host *Doliocystis* sp. (Gregarinida) parasite of *Nereis parallelogramma* Claparede (Polychaeta, Nereidae).
112. *Mitoplistophora* Codreanu, 1966. Type species *Mitoplistophora angularis* Codreanu, 1966. Type host *Ephemera danica* (Ephemeroptera, Ephemeridae).
113. *Mitosporidium* Haag, Karen L., Timothy Y. James, Jean-François Pombert, Ronny Larsson, Tobias M. M. Schaer, Dominik Refardt, and Dieter Ebert. 2014. Type species: *Mitosporidium daphnia* Haag, Karen L., Timothy Y. James, Jean-François Pombert, Ronny Larsson, Tobias M. M. Schaer, Dominik Refardt, and Dieter Ebert. 2014. Type host: *Daphnia magna* (Crustacea: Cladocera).
114. *Mockfordia* Sokolova, Sokolov, and Carlton, 2010. Type species *Mockfordia xanthocaeciliae* Sokolova, Sokolov, and Carlton, 2010. Type host *Xanthocaecilius sommermanae* Mockford, 1955 (Psocoptera, Caeciliusidae).
115. *Mrazekia* Léger and Hesse, 1916. Type species *Mrazekia argoisi* Léger and Hesse, 1916. Type host *Asellus aquaticus* L. (Isopoda, Asellidae).
116. *Multilamina* Becnel, Scheffrahn, Vossbrinck, and Bahder, 2013. Type species *Multilamina teevani* Becnel, Scheffrahn, Vossbrinck, and Bahder, 2013. Type host *Uncitermes teevani* (Isoptera, Termitidae, Syntermitinae).
117. *Myospora* Stentiford, Bateman, Small, Moss, Shields, Reece, and Tuck, 2010. Type species *Myospora metane- phrops* Stentiford, Bateman, Small, Moss, Shields, Reece, and Tuck, 2010. Type host *Metanephrops challengeri* Balss, 1914 (Decapoda, Nephropidae).
118. *Myosporidium* Baquero, Rubio, Moura, Pieniazek, and Jordana, 2005. Type species *Myosporidium merluccius* Baquero, Rubio, Moura, Pieniazek, and Jordana, 2005. Type host *Merluccius capensis*/*paradoxus* complex (Gadiformes, Merlucciidae).
119. *Myrmecomorba* Plowes et al. 2015. Type species *Myrmecomorba nylanderiae* Plowes et al. 2015. Type host *Nylanderia fulva* (Formicidae, Hymenoptera).

120. *Myxocystis* Mrazek, 1897. Type species *Myxocystis ciliata* Mrazek, 1897. Type host *Limnodrilus claparedianus* Ratzel (Oligochaeta, Tubificidae).
121. *Nadelspora* Olson, Tiekotter, and Reno, 1994. Type species *Nadelspora canceri* Olson, Tiekotter, and Reno, 1994. Type host *Cancer magister* Dana, 1852 (Decapoda, Cancridae).
122. *Napamichum* Larsson, 1990. Type species *Napamichum dispersus* (Larsson, 1984) Larsson, 1990. Type host *Endochironomus* sp. (Diptera, Chironomidae).
123. *Nelliemelba* Larsson 1983. Type species *Nelliemelba boeckella* (Milner & Mayer, 1982) Larsson 1983. Type host *Boeckella triarticulata* (Thomson) (Copepoda, Calanoidea).
124. *Nematocenator* Sapir et al. 2014. Type species: *Nematocenator marisprofundi* Sapir et al. 2014.Type host: *Desmodora marci* (Superfamily Desmodoroidea, Desmodoridae).
125. *Nematocida* Troemel, Félix, Whiteman, Barrière, and Ausubel, 2008. Type species *Nematocida parisii* Troemel, Félix, Whiteman, Barrière, and Ausubel, 2008. Type host *Caenorhabditis elegans* (Rhabditida, habditidae).
126. *Neoflabelliforma* Morris and Freeman, 2010. Type species *Neoflabelliforma aurantiae* Morris and Freeman, 2010. Type host *Tubifex tubifex* (Oligochaeta, Tubificidae).
127. *Neoperezia* Issi and Voronin, 1979. Type species *Neoperezia chironomi* Issi and Voronin, 1979. Type host *Chironomus plumosus* L. (Diptera, Chironomidae).
128. *Neonosemoides* Faye, Toguebaye, and Bouix, 1996. Type species *Neonosemoides tilapiae* Faye, Toguebaye, and Bouix, 1996. Type host *Tilapia guineensis* (Perciformes, Cichlidae).
129. *Nolleria* Beard et al. 1990. Type species *Nolleria pulicis* Beard et al. 1990. Type host *Ctenocephalides felis* (Boche, 1833) (Siphonaptera, Pulicidae).
130. *Norlevinea* Vávra, 1984. Type species *Norlevinea daphniae* Vávra, 1984. Type host *Daphnia longispina* O. F. Mueller (Cladocera, Daphniidae).
131. *Nosema* Naegeli 1857. Type species *Nosema bombycis* Naegeli 1857. Type host *Bombyx mori* L. (Lepidoptera, Bombycidae).
132. *Nosemoides* Vinckier 1975. Type species *Nosemoides vivieri* (Vinckier, Devauchelle, and Prensier, 1970) Vinckier 1975. Type host *Lecudina linei* Vinckier 1975 (Gregarinida, Monocystidae), parasite of *Lineus viridis* (Fabricius) (Heteronemertea, Lineidae).
133. *Novothelohania* Andreadis, Simakova, Vossbrinck, Shepard, and Yurchenko, 2012. Type species *Novothelohania ovalae* Andreadis, Simakova, Vossbrinck, Shepard, and Yurchenko, 2012. Type host *Aedes* (*Ochlerotatus*) *caspius* (Pallas) (Diptera, Culicidae).
134. *Nucleospora* Docker, Kent, Hervio, Khattra, Weiss, Cali, and Devlin, 1997. Type species *Nucleospora salmonis* (Chilmonczyk, Cox, & Hedrick, 1991) Docker, Kent, Hervio, Khattra, Weiss, Cali, and Devlin, 1997. Type host *Oncorhynchus tshawytscha* (Walbaum) (Salmoniformes, Salmonidae).

135. *Nudispora* Larsson, 1990. Type species *Nudispora biformis* Larsson, 1990. Type host *Coenagrion hastulatum* Charpentier, 1925 (Odonata, Coenagrionidae).
136. *Obruspora* Diamant, Rothman, Goren, Galil, Yokes, Szitenberg and Huchon 2014. Type species *Obruspora papernae* Diamant, Rothman, Goren, Galil, Yokes, Szitenberg and Huchon 2014. Type host *Callionymus filamentosus*(Teleostei: Callionymidae).
137. *Octosporea* Flu, 1911. Type species *Octosporea muscaedomesticae* Flu, 1911. Type host *Musca domestica* L. (Diptera, Muscidae).
138. *Octotetraspora* Issi, Kadyrova, Pushkar, Khodzhaeva, and Krylova, 1990. Type species *Octotetraspora* paradoxa. Type host *Wilhelmia mediterranea* (Diptera, Simuliidae).
139. *Oligosporidium* Codreanu-Bălcescu, Codreanu, and Traciuc, 1981. Type species *Oligosporidium arachnicolum* (Codreanu-Bălcescu, Codreanu, & Traciuc, 1978) Codreanu-Bălcescu, Codreanu, and Traciuc, 1981. Type host *Xysticus cambridgei* (Araneae, Thomisidae).
140. *Ordospora* Larsson, Ebert, and Vávra, 1997. Type species *Ordospora colligate* Larsson, Ebert, and Vávra, 1997. Type species *Daphnia magna* (Cladocera, Daphniidae).
141. *Ormieresia* Vivares, Bouix, and Manier, 1977. Type species *Ormieresia carcini* Vivares, Bouix, and Manier, 1977. Type host *Carcinus mediterraneus* Czerniavsky, 1884 (Decapoda, Portunidae).
142. *Orthosomella* Canning, Wigley, and Barker, 1991. Type species *Orthosomella operophterae* (Canning, 1960) Canning, Wigley, and Barker, 1991. Type host *Operophtera brumata* (L.) (Lepidoptera, Geometridae).
143. *Orthothelohania* Codreanu and Balcescu-Codreanu, 1974. Type species *Orthothelohania octospora* (Henneguy, 1892, *sensu* Pixel-Goodrich, 1920) Codreanu and Balcescu-Codreanu, 1974. Type host *Palaemon serratus* (Pennant, 1777) (Decapoda, Palaemonidae).
144. *Ovavesicula* Andreadis and Hanula, 1987. Type species *Ovavesicula popilliae* Andreadis and Hanula, 1987. Type host *Popillia japonica* Newman (Coleoptera, Scarabaeidae).
145. *Ovipleistophora* Pekkarinen et al. 2002. Type species *Ovipleistophora mirandellae* (Vaney & Conte, 1901) Pekkarinen et al. 2002. Type hosts *Gymnocephalus cernuus* (L.) (Perciformes, Percidae) and *Rutilus rutilus* (L.) (Cypriniformes, Cyprinidae), single type host not identified.
146. *Pankovaia* Simakova, Tokarev, and Issi, 2009. Type species *Pankovaia semitubulata* Simakova, Tokarev, and Issi, 2009. Type host *Cloeon dipterum* (L.) (Ephemeroptera, Baetidae).
147. *Paradoxium* Stentiford et al. 2015. Type species *Paradoxium irvingi* Stentiford et al. 2015. Type host *Pandalus montagui* (Decapoda, Pandalidae).
148. *Paraepiseptum* Hyliš, Oborník, Nebesářová, and Vávra, 2007. Type species *Paraepiseptum polycentropi* (Weiser, 1965) Hyliš, Oborník, Nebesářová, and Vávra, 2007. Type host *Polycentropus flavomaculatus* (Polycentropodidae).

149. *Paranosema* Sokolova, Dolgikh, Morzhina, Nassonova, Issi, Terry, Ironside, Smith, and Vossbrinck, 2003. Type species *Paranosema grylli* (Sokolova, Seleznev, Dolgikh, & Issi, 1994) Sokolova, Dolgikh, Morzhina, Nassonova, Issi, Terry, Ironside, Smith, and Vossbrinck, 2003. Type host *Gryllus bimaculatus* Deg. (Orthoptera, Gryllidae).
150. *Paramicrosporidium* Corsaro, D., J. Walochnik, D. Venditti, KD. Muller, B. Hauroder, and R. Michel. 2014. Type species: *Paramicrosporidium saccamoebae* Corsaro, D., J. Walochnik, D. Venditti, KD. Muller, B. Hauroder, and R. Michel. 2014. Type host: *Saccamoeba.* sp (Amoebozoa, Tubulinea, Euamoebida). (Authors of the genus considered it to be a member of the Rozellomycota related to microsporidia but not a genus of it - a sister group.)
151. *arapleistophora* Issi, Kadyrova, Pushkar, Khodzhaeva, and Krylova, 1990. Type species *Parapleistophora ectos- pora* Issi, Kadyrova, Pushkar, Khodzhaeva, and Krylova, 1990. Type host *Tetisimulium desertorum* (Diptera, Simuliidae).
152. *Parastempellia* Issi, Kadyrova, Pushkar, Khodzhaeva and Krylova, 1990. Type species *Parastempellia odagmiae* Issi, Kadyrova, Pushkar, Khodzhaeva and Krylova, 1990. Type host *Odagmia ferganica* (Diptera, Simuliidae).
153. *Parathelohania* Codreanu, 1966. Type species *Parathelohania legeri* (Hesse, 1904) Codreanu, 1966. Type host *Anopheles maculipennis* Meigen, 1818 (Diptera, Culicidae).
154. *Paratuzetia* Poddubnaya, Tokarev, and Issi, 2006. Type species *Paratuzetia kupermani* Poddubnaya, Tokarev, and Issi, 2006. Type host *Khawia armeniaca* Cholodkovsky, 1915 (Cestoda, Lytocestidae) from oligochaete *Potamothrix paravanicus.*
155. *Pegmatheca* Hazard and Oldacre, 1975. Type species *Pegmatheca simulii* Hazard and Oldacre, 1975. Type host *Simulium tuberosum* (Lindstrom, 1911) (Diptera, Simuliidae).
156. *Perezia* Léger and Duboscq, 1909. Type species *Perezia lankesteriae* Léger and Duboscq, 1909. Type host *Lankesteria ascidiae* (Lankester, 1872) (Gregarinida, Diplocystidae) parasite of *Ciona intestinalis* (L.) (Dictyobranchia, Ascidiidae).
157. *Pernicivesicula* Bylen and Larsson, 1994. Type species *Pernicivesicula gracilis* Bylen and Larsson, 1994. Type host *Pentaneurella* sp. Fittkau and Murray, 1983 (Diptera, Chironomidae).
158. *Pilosporella* Hazard and Oldacre, 1975. Type species *Pilosporella fishi* Hazard and Oldacre, 1975. Type host *Wyeomyia vanduzeei* Dyar and Knab, 1906 (Diptera, Culicidae).
159. *Pleistophora* Gurley, 1893.Type species *Pleistophora typicalis* Gurley, 1893. Type host *Cottus scorpius* = *Myoxocephalus scorpius* (L.) Perciformes, Cottidae).
160. *Pleistophoridium* Codreanu-Bălcescu and Codreanu, 1982. Type species *Pleistophoridium hyperparasiticum* (Codreanu-Bălcescu & Codreanu, 1976)

Codreanu-Bălcescu and Codreanu, 1982. Type host *Enterocystis rhithrogenae* M. Codreanu, 1940 (Gregarinida, Monocystidae), parasite of *Rhithrogena semicolorata* (Curt, 1834) (Ephemeroptera).

161. *Polydispyrenia* Canning and Hazard 1982. Type species *Polydispyrenia simulii* (Lutz & Splendore, 1908) Canning and Hazard 1982. Type host *Simulium venustum* Say = *Simulium pertinax* Kollar (Diptera, Simuliidae).
162. *Potaspora* Casal et al. 2008. Type species *Potaspora morhaphis* Casal et al. 2008. Type host *Potamorhaphis guianensis* (Beloniformes, Belonidae).
163. *Pseudoloma* Matthews, Brown, Larison, Bishop-Stewart, Rogers, and Kent, 2001. Type species *Pseudoloma neu- rophilia* Matthews, Brown, Larison, Bishop-Stewart, Rogers, and Kent, 2001. Type host *Danio rerio* (Hamilton & Buchanan, 1822) (Cypriniformes, Cyprinidae).
164. *Pseudonosema* Canning, Refardt, Vossbrinck, Okamura, and Curry, 2002. Type species *Pseudonosema cristatel- lae* (Canning, Okamura, & Curry, 1997) Canning, Refardt, Vossbrinck, Okamura, and Curry, 2002. Type host *Cristatella mucedo* Cuvier, 1798 (Plumatellidae, Cristatellidae).
165. *Pseudopleistophora* Sprague 1977. Type species *Pseudopleistophora szollosii* Sprague 1977. Type host *Armandia brevis* (Polychaeta, Opheliidae).
166. *Pulcispora* Vedmed, Krylova, and Issi, 1991. Type species *Pulcispora xenopsyllae* Vedmed, Krylova, and Issi, 1991. Type host *Xenopsylla hirtipes* (Siphonaptera, Pulicidae).
167. *Pyrotheca* Hesse, 1935. Type species *Pyrotheca cyclopis* (Leblanc, 1930) Poisson, 1953. Type host *Cyclops albidus* Jurine, 1820 (Copepoda, Cyclopidae).
168. *Rectispora* Larsson, 1990c. Type species *Rectispora reticulata* Larsson, 1990c. Type host *Pomatothrix hammoniensis* (Michaelson, 1901) (Oligochaeta, Tubificidae).
169. *Resiomeria* Larsson 1986b. Type species *Resiomeria odonatae* Larsson 1986b. Type host *Aeshna grandis* (Odonata, Aeshnidae).
170. *Ringueletium* Garcia, 1990. Type species *Ringueletium pillosa* Garcia, 1990. Type host *Gigantodox rufidulum* Wigodzinsky and Coscaron (Diptera, Simuliidae).
171. *Schroedera* Morris and Adams, 2002. Type species *Schroedera plumatellae* Morris & Adams, 2002. Type host *Plumatella fungosa* Pallas (Plumatellida, Plumatellidae).
172. *Scipionospora* Bylen and Larsson, 1996. Type species *Scipionospora tetraspora* (Léger & Hesse, 1922) Bylen and Larsson, 1996. Type host *Tanytarsus* sp. Léger and Hesse, 1922 (Diptera, Chironomidae).
173. *Semenovaia* Voronin and Issi 1986. Type species *Semenovaia chironomi* Voronin and Issi 1986, in Issi 1986. Type host *Chironomus plumosus* (Diptera, Chironomidae).
174. *Senoma* Simakova, Pankova, Tokarev, and Issi, 2005. Type species *Senoma globulifera* (Issi & Pankova, 1983) Simakova, Pankova, Tokarev, and Issi, 2005. Type host *Anopheles messeae* Fall. (Diptera, Culicidae).

175. *Septata* Cali, Kotler, and Orenstein, 1993. Type species *Septata intestinalis* Cali, Kotler, and Orenstein, 1993. Type host *Homo sapiens* L. (Primates, Hominidae).
176. *Sheriffia* Larsson 2014. Type species *Sheriffia brachynema* (Richards and Sheffield 1971) Larsson 2014. Type host *Biomphalaria glabrata* (Say, 1818) (Mollusca, Gastropoda).
177. *Simuliospora* Khodzhaeva, Krylova, and Issi, 1990. Type species *Simuliospora uzbekistanica* Khodzhaeva, Krylova, and Issi, 1990, in Issi et al. 1990. Type host *Tetisimulium alajense* (Diptera, Simuliidae).
178. *Spherospora* Garcia, 1991. Type species *Spherospora andinae* Garcia, 1991. Type host *Gigantodox chilense* (Philippi) (Diptera, Simuliidae).
179. *Spiroglugea* Léger and Hesse, 1924. Type species *Spiroglugea octospora* (Léger & Hesse, 1922) Léger and Hesse 1924. Type host *Ceratopogon* sp. (Diptera, Ceratopogonidae).
180. *Sporanauta* Ardila-Garcia and Fast, 2012. Type species *Sporanauta perivermis* Ardila-Garcia and Fast, 2012. Type host *Odontophora rectangula* (Axonolaimidae).
181. *Spraguea* Weissenberg, 1976. Type species *Spraguea lophii* (Doflein, 1898) Weissenberg, 1976. Type host *Lophius piscatorius* (Lophiiformes, Lophiidae).
182. *Steinhausia* Sprague, Ormieres, and Manier, 1972. Type species *Steinhausia mytilovum* (Field, 1924) Sprague, Ormieres, and Manier, 1972. Type host *Mytilus edulis* L. (Pelecypoda, Mytilidae).
183. *Stempellia* Léger and Hesse, 1910. Type species *Stempellia mutabilis* Léger and Hesse, 1910. Type host *Ephemera vulgata* L. (Ephemeroptera, Ephemeridae).
184. *Striatospora* Issi and Voronin, 1986. Type species *Striatospora chironomi* Issi and Voronin, 1986, in Issi 1986. Type host *Chironomus plumosus* (Diptera, Chironomidae).
185. *Systenostrema* Hazard and Oldacre, 1975. Type species *Systenostrema tabani* Hazard and Oldacre, 1975. Type host *Tabanus lineola* Fabricius (Diptera, Tabanidae).
186. *Takaokaspora* Andreadis, Takaoka, Otsuka, and Vossbrinck, 2013. Type species *Takaokaspora nipponicus* Andreadis, Takaoka, Otsuka, and Vossbrinck, 2013. Type host *Ochlerotatus japonicus japonicus* (Theobald) (Diptera, Culicidae).
187. *Tardivesicula* Larsson and Bylen, 1992. Type species *Tardivesicula duplicata* Larsson and Bylen, 1992. Type host *Limnephilus centralis* (Curtis, 1884) (Trichoptera, Limnephilidae).
188. *Telomyxa* Léger and Hesse, 1910. Type species *Telomyxa glugeiformis* Léger and Hesse, 1910. Type host *Ephemera vulgata* L. (Ephemeroptera, Ephemeridae).
189. *Tetramicra* Matthews and Matthews, 1980. Type species *Tetramicra brevifilum* Matthews and Matthews, 1980. Type host *Scophthalmus maximus* (L.) (Pleuronectiformes, Bothidae).

190. *Thelohania* Henneguy, 1892. Type species *Thelohania giardi* Henneguy, 1892, in Henneguy and Thélohan, 1892. Type host *Crangon vulgaris* (Decapoda, Crangonidae).
191. *Toxoglugea* Léger and Hesse, 1924. Type species *Toxoglugea vibrio* (Léger & *Hesse, 1922*) Léger and Hesse, 1924. Type host *Ceratopogon sp. (Di*ptera, Ceratopogonidae).
192. *Toxospora* Voronin, 1993. Type *species Toxospora volgae*Voronin, 1993. Type host *Corynoneura* sp. (Diptera, Chironomidae).
193. *Trachipleistophora* Hollister, Canning, Weidner, Field, Kench, and Marriott, 1996. Type species *Trachipleistophora hominis* Hollister, Canning, Weidner, Field, Kench, and Marriott, 1996. Type host *Homo sapiens* L. (Primates, Hominidae).
194. *Trichoctosporea* Larsson, 1994. Type species *Trichoctosporea pygopellita* Larsson, 1994. Type host *Aedes vexans* (Meig.) (Diptera, Culicidae).
195. *Trichoduboscqia* Léger, 1926. Type species *Trichoduboscqia epeori* Léger, 1926. Type host *Epeorus torrentium* Eat. (Ephemeroptera, Heptageniidae).
196. *Trichonosema* Canning, Refardt, Vossbrinck, Okamura, and Curry, 2002. Type species *Trichonosema pectinatel- lae* Canning, Refardt, Vossbrinck, Okamura, and Curry, 2002. Type host *Pectinatella magnifica* (Leidy, 1851) (Plumatellida, Pectinatellidae).
197. *Trichotuzetia* Vávra, Larsson, and Baker, 1997. Type species *Trichotuzetia guttata* Vávra, Larsson, and Baker, 1997. Type host *Cyclops vicinus* Uljanin, 1875 (Copepoda, Cyclopidae).
198. *Tricornia* Pell and Canning, 1992. Type species *Tricornia muhezae* Pell and Canning, 1992. Type host *Mansonia africana* (Theobald) (Diptera, Culicidae).
199. *Triwangia* Nai, Hsu, and Lo, 2013. Type species *Triwangia caridinae* Nai, Hsu, and Lo, 2013, in Wang et al., 2013.Type host *Caridina formosae* (Decapoda, Atyidae).
200. *Tubulinosema* Franzen, Fischer, Schroeder, Scholmerich, and Schneuwly, 2005. Type species *Tubulinosema ratis- bonensis* Franzen, Fischer, Schroeder, Scholmerich, and Schneuwly, 2005. Type host *Drosophila melanogaster* (Diptera, Drosophilidae).
201. *Tuzetia* Maurand, Fize, Fenwick, and Michel, 1971. Type species *Tuzetia infirma* (Kudo, 1921) Maurand, Fize, Fenwick, and Michel, 1971. Type host *Cyclops albidus* (Jurine, 1820) (Copepoda, Cyclopidae).
202. *Unikaryon* Canning, Lai, and Lie, 1974. Type species *Unikaryon piriformis* Canning, Lai, and Lie, 1974. Type host *Echinostoma audyi* Umathevy, 1975 (Digenea, Echinostomatidae).
203. *Vairimorpha* Pilley, 1976. Type species *Vairimorpha necatrix* (Kramer, 1965) Pilley, 1976. Type host *Pseudaletia unipuncta* (Haworth) (Lepidoptera, Noctuidae).
204. *Vavraia* Weiser 1977. Type species *Vavraia culicis* (Weiser, 1947) Weiser 1977. Type host *Culex pipiens* L. (Diptera, Culicidae).
205. *Vittaforma* Silveira and Canning, 1995. Type species *Vittaforma corneae* (Shadduck, Meccoli, Davis, & Font, 1990) Silveira and Canning, 1995. Type host *Homo sapiens* L. (Primates, Hominidae).

206. *Weiseria* Doby and Saguez, 1964. Type species *Weiseria laurenti* Doby and Saguez, 1964. Type host *Prosimulium inflatum* (Davies) (Diptera, Simuliidae).
207. *Wittmannia* Czaker, 1997. Type species *Wittmannia antarctica* Czaker, 1997. Type host *Kantharella antarctica* Czaker, 1997 (Mesozoa, Kantharellidae), parasite of *Pareledone turqueti* Joubin, 1905 (Cephalopoda).
208. *Zelenkaia* Hyliš, Oborník, Nebesářová, and Vávra 2013. Type species *Zelenkaia trichopterae* Hyliš, Oborník, Nebesářová, and Vávra 2013 Type host *Halesus digitatus* (Shrank, 1781) (Trichoptera, Limnephilidae).

Current notes on Classification: In a 2009 editorial from the International Society of Protistologists, it was proposed that the classification of Adl et al. 2005 be considered and the "elements of a description of a new taxon" used for the description of any new unicellular taxa (Lynn and Simpson 2009). However, the microsporidiologists, as a group, at "The first United Workshop on Microsporidia from invertebrate and vertebrate hosts" recommended that the Microsporidia be considered closest to the fungi but not in any fungal group and consequently "that the International Code of Zoological Nomenclature should continue to be applied for taxonomic descriptions of the Microsporidia" (Weiss 2005). This policy was made official in 2009 (Redhead et al. 2009).

Maintenance and Cultivation

Microsporidia are normally found by direct examination of natural populations of their hosts. Methods developed for the collection, maintenance, identification, processing, and storage of microsporidia have been covered in reviews by Maddox and Solter (1996), Undeen (1997), and Visvesvara et al. (1999), to which reference should be made for details. Extrusion of polar tubes is useful for confirmation of the microsporidian nature and for infection of cultures. Techniques employed for activation of the spores (depending on species) by various means, including the simple process of drying and rehydration, may be found in publications such as (Undeen 1990; Undeen and Epsky 1990; Leitch and Ceballos 2008).

Many species can be maintained in the laboratory in their natural hosts, other hosts, or in cell culture. Spores should be extracted from their hosts by homogenization and repeated washing. Excellent purification can be achieved on Percoll or Ludox gradients. Spores can be administered with the diet of terrestrial hosts or added to the water of aquatic hosts. Some of the dimorphic microsporidia, e.g., *Amblyospora* in mosquitoes, can only be transmitted horizontally in the mosquito population via a copepod intermediate host (Fig. 9).

It is often convenient to propagate a parasite of a small, natural host in a larger, more conveniently reared, laboratory host. This can sometimes be achieved in animals which are not normally susceptible by bypassing the gut and inoculating the spores into the body cavity. Large lepidopteran larvae have been used in this way to great effect for large-scale production of Microsporidia.

Several techniques have been used to infect cells. The first cultures were established by adding hemolymph, removed from silkworms previously infected with *Nosema bombycis*, to cultured silkworm ovarian cells. Infections have arisen in primary cultures of cells derived from infected animals and the most widely used technique is to harvest the spores in a sterile condition from an infected host and to add them to primary cultures or established cell lines (Visvesvara et al. 1999).

A prerequisite for successful infection of cultured cells is that the spores should germinate in contact with the cells and inoculate the sporoplasms through the membrane. Cells can be in suspension or in attached monolayers. Some microsporidia germinate easily and spontaneously when added to the cell culture medium. Cultures of *E. cuniculi* have repeatedly and independently been set up by adding spore-contaminated rabbit urine, mouse peritoneal exudate, or spores harvested and resuspended in balanced saline to cell cultures.

The cultured cells need not be derived from the natural host animals. Microsporidia of invertebrate origin have been grown in cells from other invertebrates and from vertebrates. *Anncaliia algerae*, derived from mosquitoes, has been grown in lepidopteran cell lines, in numerous types of mammalian cells, and in amphibian cells (Undeen and Maddox 1973; Undeen 1975). The upper temperature limit for survival of the invertebrate microsporidium was thought to be 35°C for many years; however, it has subsequently been shown that *A. algerae* will grow at 37°C but at a much slower rate (Lowman et al. 2000). *Encephalitozoon cuniculi* has almost always been grown in mammalian cells at 37°C, but success has also been achieved in chick fibroblasts. Surprisingly, its development has also been completed, including spore production, in fat head minnow cells, even at 18°C, but there are no records of development in invertebrate cells.

Cell types vary considerably in their ability to support growth of Microsporidia. RK13 and MDCK cell lines from the American Type Culture Collection support prolific growth of *E. cuniculi*. Spores harvested from cultures, either from the medium or by disruption of the cells, show no loss of viability. Cell culture of microsporidia provides a convenient means of studying most aspects of development.

The most convenient method of storage of microsporidial spores is in aqueous suspension with added antibiotics to inhibit bacterial growth. Stored in this way at 4°C, many species will survive for months or years. A few species can be stored dry, e.g., *Nosema whitei*. Considerable success has been achieved with storage in liquid nitrogen; several species derived from terrestrial insects have proved viable after removal from liquid nitrogen, but *Anncaliia algerae* derived from mosquito larvae did not survive. However, *Nosema eurytremae* in trematodes in aquatic snails survives well, so no general conclusion can be drawn about species from aquatic hosts. A few microsporidia, e.g., *Nosema apis*, *Nosema pyrausta*, and *Vairimorpha necatrix*, have also been lyophilized with little loss of viability (Maddox and Solter 1996).

Evolutionary History

Since their discovery, classification of the microsporidia and establishing their relationship to other eukaryotic organisms can perhaps best be characterized as one of tentative assignments. This has been primarily due to a number of unique characteristics making it difficult to unite the microsporidia into a specific phylogenetic position. The first described species of Microsporidia, *Nosema bombycis* Naegeli 1857, was considered to be related to the yeast and placed with the Schizomycetes but moved by Balbiani (1882) to the Sporozoa. For much of their history, the microsporidia have been considered to be within the phylum Protozoa, but in 1977 they were elevated to phylum status in two new classifications (Weiser 1977; Sprague 1977). Levine et al. 1980 elevated the Protozoa to subkingdom status containing seven phyla, one of which was the Microsporidia. Questions lingered about whether microsporidia should be considered protozoa which resulted in a new assignment into the group Archezoa containing ancient eukaryotes primarily based on the absence of mitochondria (Cavalier-Smith 1987), a placement soon supported by the first rRNA phylogenetic reconstruction placing them as a deep branch of eukaryotes (Vossbrinck and Woese 1986). By the mid-1990s evidence was accumulating that microsporidia were not primitively amitochondriate as they possessed mitochondrial derived genes as well as vestigial mitochondria (reviewed by Keeling et al. 2014). At about the same time, new gene trees were consistently indicating that Microsporidia were related to fungi (reviewed in Vávra and Lukeš 2013; Keeling et al. 2014 and Corradi 2015). Various studies placed them with different groups within the fungi, but other studies placed them at the base of the fungal trees raising the question as to whether the microsporidia are fungi or a sister group to the fungi, protista (James et al. 2006). The link with the newly created phylum Cryptomycota was established with the publication of the genome of *Rozella allomycis* (James et al. 2013) with this group containing the aphelids, rozellids, and the microsporidia. Relationships between members of the Cryptomyocota continue to evolve with the newly described species of *Paramicrosporidium*, parasites of amobozoans (Corsaro et al. 2014a,b) and *Mitosporidium daphnia* that emerge at the root of the microsporidian tree with spores that contain a polar filament but retains a mitochondrial genome (Haag et al. 2014). Descriptions of new species of rozellids and aphelids are adding additional information to help clarify relationships within this group and provide new insights on the origins of the microsporidia (Corsaro et al. 2016; Karpov et al. 2016). Genetic information on the primitive microsporidian groups Metchnikovellidea and Chytridopsida (Larsson 2014) have yet to be obtained but should make a significant contribution to clarifying the relationships of microsporidia to other groups within the Cryptomycota.

The use of genomics and molecular information has clearly elucidated the fact that the microsporidia are not primitive. We look forward to this technology continuing to provide information to aid in the understanding of the microsporidia and their place in the biological hierarchy in the coming years. The information gleaned since 1960, when electron microscopy was first applied to microsporidia, is akin to

our expectations of the future with the application of genomics and proteomics to the understanding of this enigmatic group of organisms.

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Apusomonadida

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Aaron A. Heiss, Matthew W. Brown, and Alastair G. B. Simpson

Abstract

Apusomonadida is a small group of free-living heterotrophic flagellates. Apusomonads are small (~5–20 μm long) gliding aerobes with two flagella. The dorsal cell membrane is underlain by a pellicle, which also supports a "skirt" of folded membrane that extends laterally/ventrally. The anterior flagellum is enclosed by a sleeve-like extension of the skirt system, forming a flexible proboscis. *Apusomonas* itself is a rounded cell with an anterior extension, the mastigophore, that contains the flagellar apparatus. All other apusomonads (usually now assigned to the genera *Amastigomonas, Chelonemonas*, *Manchomonas, Multimonas*, *Podomonas*, and *Thecamonas*) are elongated and plastic and may form ventral pseudopodia. *Apusomonas* is a soil flagellate. Most other apusomonads that have been cultured to date are marine. Apusomonads are closely related to opisthokonts (e.g., animals and fungi), making them an important group for examining, for example, the origins of multicellularity. The genome of *Thecamonas trahens* encodes several proteins and pathways previously considered specific to animals, including much of the integrin system, which functions in cell-cell communication and adhesion in metazoa. This chapter also briefly reviews breviates and ancyromonads, two groups of surface-associating flagellates that are (or may be) closely related to apusomonads and are of similar evolutionary significance. Breviates comprise

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three genera of small (~10–15 μm long) anaerobic cells that produce fine pseudopodia. Ancyromonads (synonym planomonads) comprise four genera of tiny (~5 μm long) flattened cells with an inflexible pellicle underlying most of the cell membrane and a battery of extrusomes in a lateral rostrum.

Keywords

Aerobe • Anaerobe • Ancyromonad • Apusomonad • Bacterivore • Breviate • Flagellate • Integrin • Opisthokonts • Protozoa • *Thecamonas*

Contents

Summary Classification

●**Apusomonadida**
●●**Apusomonadidae**
●●●**Apusomonadinae** (*Apusomonas*, *Manchomonas*)
●●●**Thecamonadinae** (*Thecamonas*, *Chelonemonas*)
●●●***Amastigomonas***
●●●***Multimonas***
●●●***Podomonas***
[Other Apusomonadida: "*Thecamonas*" *oxoniensis*]

●**Breviatea** (*Breviata*, *Subulatomonas*, *Pygsuia, Lenisia*)

●**Ancyromonadida (= Planomonadida)**
●●**Ancyromonadidae** (*Ancyromonas*, *Nutomonas*)
●●***Planomonas***
●●***Fabomonas***

Introduction

General Characteristics

Apusomonadida is a group of small free-living heterotrophic flagellates that glide on surfaces. All known apusomonads have two flagella, with the anterior flagellum surrounded by a membranous "sleeve" that extends from the main cell body. The combined flagellum-sleeve apparatus forms a highly mobile proboscis, which is a primary characteristic of the group (Karpov and Myl'nikov 1989). The posterior flagellum runs underneath the cell venter (ventral face), on the left side of the cell. Pseudopodia, which are used for feeding, are produced from the ventral region of the cell in some members of the group. The dorsal cell membrane is underlain by a pellicle, which continues into a ventrally projecting "skirt" on the sides of the cell, and which is continuous with the proboscis sleeve (Fig. 1).

Apusomonads are currently divided into at least five main phylogenetic groups, based on molecular and morphological data of cultured strains (Cavalier-Smith and Chao 2010; Heiss et al. 2015): (i) Apusomonadinae, containing the genera *Apusomonas* and *Manchomonas*; (ii) the genus *Podomonas*; (iii) the genus *Multimonas*; (iv) Thecamonadinae, including the genus *Chelonemonas* and the

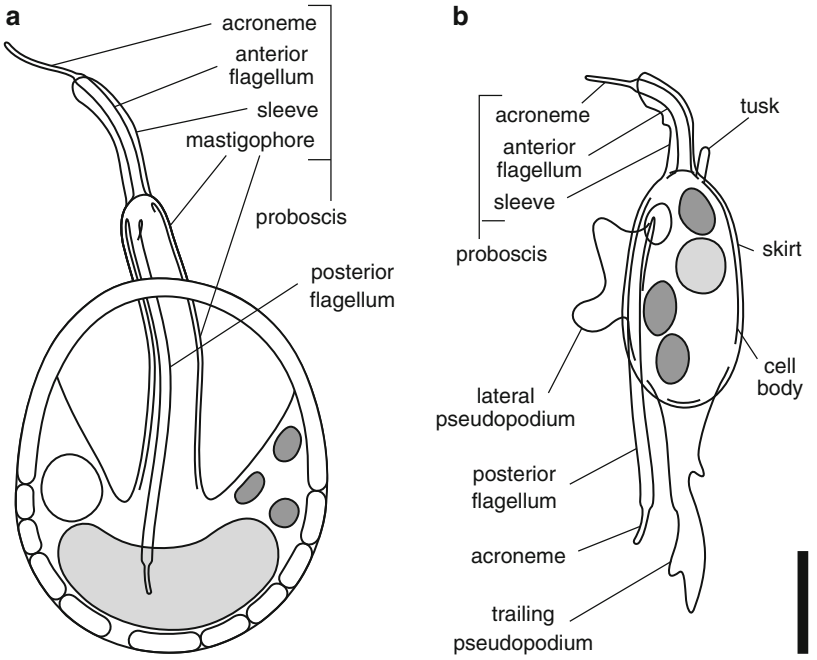

Fig. 1 Appearance by light microscopy of living apusomonads. (**a**) *Apusomonas proboscidea*; (**b**) *Thecamonas trahens*. Nuclei are *light grey*; mitochondria are *dark grey*. Scale bar in (**b**) = 2 μm for both drawings

majority of members of the genus *Thecamonas*; and (v) the single freshwater species "*Thecamonas*" *oxoniensis* (Figs. 1 and 2; Table 1). Another genus, *Amastigomonas* sensu stricto (see below), is of uncertain position relative to other apusomonads. The most distinctive genus is *Apusomonas*, which has an inflexible, rounded body and an extended "mastigophore" that contains both the proboscis and the flagellar apparatus (Karpov and Myl'nikov 1989; Vickerman et al. 1974; Figs. 1a and 2d). The other genera contain more elongate, flexible cells, with the flagellar apparatus positioned within the anterior end of the main cell body. The morphological differences between them are often subtle, and until recently all apusomonads other than *Apusomonas* were assigned to the genus *Amastigomonas* (a practice continued by some authors: Karpov 2011; Myl'nikov and Myl'nikova 2012).

Apusomonads have an important phylogenetic position within the eukaryote tree of life. They are amongst the closest relatives of Opisthokonta, the "supergroup" that includes both animals and fungi (Brown et al. 2013; Burki et al. 2016; Cavalier-Smith and Chao 1995; Cavalier-Smith et al. 2014; Derelle and Lang 2012; He et al. 2014; Kim et al. 2006; Paps et al. 2013; Torruella et al. 2012, 2015). This suggests that apusomonads are important for understanding the origins of multicellularity in animals and fungi. In particular, the genome of the apusomonad *Thecamonas trahens* encodes most components of the integrin machinery critical to cell adhesion in animals (Sebé-Pedrós et al. 2010). *Thecamonas* also has a more complex flagellar apparatus cytoskeleton than that seen in opisthokonts, and this sheds light on the deep-level evolution of the cytoskeletal architecture in extant eukaryotes (Heiss et al. 2013b).

Occurrence

The majority of known apusomonads are marine; however, *Apusomonas* occurs in soil and "*Thecamonas*" *oxoniensis* was isolated from the surface of a terrestrial plant, both being essentially freshwater organisms (Cavalier-Smith and Chao 2010). The original account of *Amastigomonas* (see below) was also of a freshwater organism (de Saedeleer 1931). Apusomonads are one of the most frequently encountered groups of heterotrophic flagellates in microscopy studies of marine sediments (Patterson and Lee 2000), though almost always at low cell numbers.

Literature and History of Knowledge

The scientific history of apusomonads extends back a century, although the group was united less than three decades ago. The first described apusomonad was originally called *Rhynchomonas mutabilis* (Griessmann 1913), although it was not recognized as an apusomonad until almost 80 years later (Larsen and Patterson 1990; true *Rhynchomonas* organisms are kinetoplastids – see ▶ Kinetoplastea). *Apusomonas* itself was known from an unpublished account in 1917 (Vickerman et al. 1974) and was formally described a few years later (Aléxéieff 1924). Shortly after

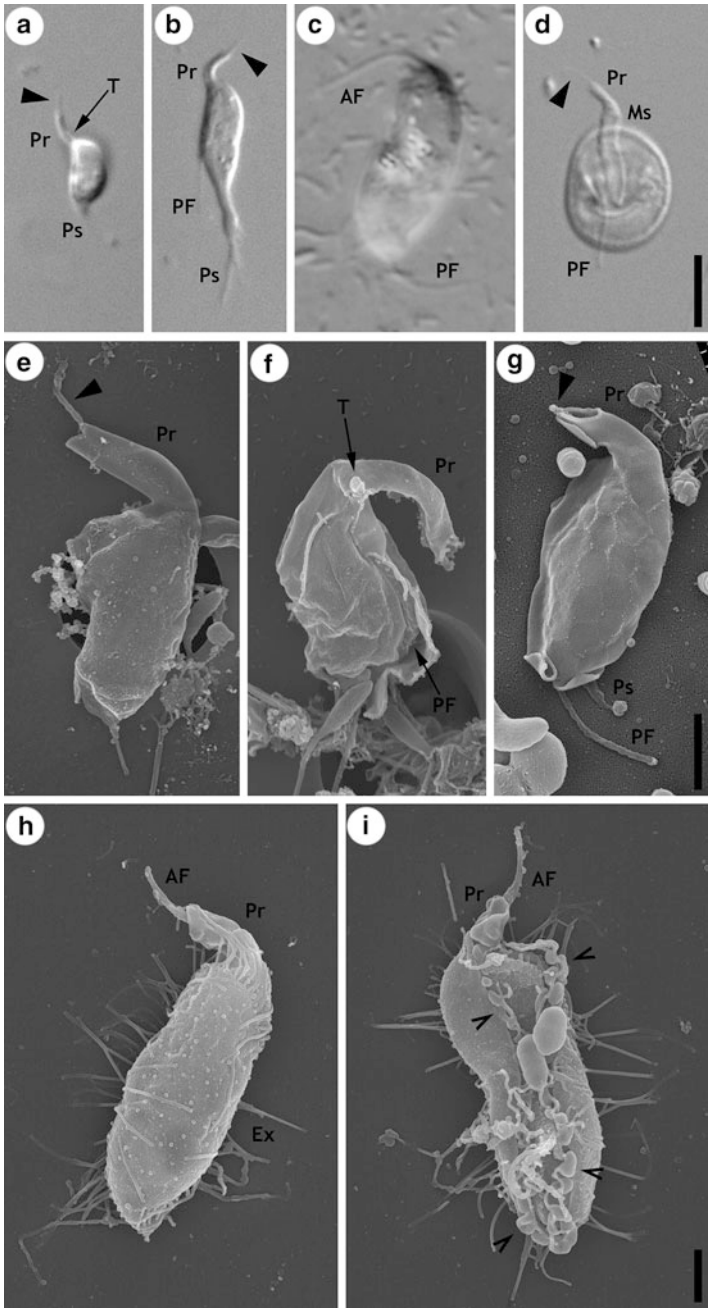

Fig. 2 Light (**a**–**d**) and scanning electron (**e**–**i**) micrographs of apusomonads. Panels (**a**–**d**) are differential interference contrast images of living cells: (**a**) *Thecamonas trahens* without prominent pseudopodia but with visible "tusk" (T); (**b**) *Thecamonas trahens* with prominent trailing

this, the genus *Amastigomonas* was established (de Saedeleer 1931) for *Amastigomonas debrunyei*, a gliding organism with a prominent proboscis but no visible flagella (hence the name), although the cells were probably biflagellated in reality (see below). Apusomonads remained little studied for the next 40 years, until the redescription of *Apusomonas proboscidea* by Vickerman et al. (1974). Additional new species were assigned to *Amastigomonas* from the 1970s onwards (Ekelund and Patterson 1997; Hamar 1979; Larsen and Patterson 1990; Myl'nikov 1999; Myl'nikov and Myl'nikova 2012; Zhukov 1975). Ultrastructural studies in the 1980s led to the recognition that *Apusomonas* was related to the various organisms described as *Amastigomonas* species, and to the proposal of the taxon Apusomonadida, containing both types of organisms (Karpov and Myl'nikov 1989). The monophyly of Apusomonadida has since been confirmed by SSU rRNA gene phylogenies (e.g., Cavalier-Smith and Chao 2003, 2010; Cavalier-Smith et al. 2004, 2008; Heiss et al. 2015; Nikolaev et al. 2006; Walker et al. 2006).

Recently there has been a considerable expansion in the number of described genera and species of apusomonads. Larsen and Patterson (1990) introduced the new genus *Thecamonas* for certain small apusomonads. It was soon recognized, however, that these organisms were very similar to those previously known as *Amastigomonas*, and *Thecamonas* was temporarily considered a junior synonym (Molina and Nerad 1991). Until 2010, additional information on *Amastigomonas*-like apusomonads continued to be referred to the genus *Amastigomonas* (Cavalier-Smith 2002; Ekelund and Patterson 1997; Lee 2002; Lee et al. 2005; Molina and Nerad 1991; Patterson and Simpson 1996; Tikhonenkov et al. 2006; Vørs 1993). In 2010, however, the first broad molecular phylogenetic study of apusomonads demonstrated that the "amastigomonad"-type apusomonads were genetically diverse and represented a paraphyletic group within apusomonads (Cavalier-Smith and Chao 2010). As a consequence, these were divided into several genera, including the reestablished genus *Thecamonas* and the new genera *Manchomonas*, *Multimonas*, and *Podomonas* (Cavalier-Smith and Chao 2010), with an additional

Fig. 2 (continued) pseudopodium; (**c**) *Podomonas magna*; (**d**) *Apusomonas* sp. Panels (**e**–**i**) are scanning electron micrographs of cells fixed with osmium tetroxide: (**e**) *Thecamonas trahens*, dorsal view, showing continuity of proboscis sleeve (*Pr*) with dorsal cell covering; (**f**) *Thecamonas trahens*, ventral view, showing different texture between "skirt" (sides of cell) and ventral surface (center), "tusk" (*T*) protruding from near origin of proboscis (*Pr*), and posterior flagellum (*PF*) tucked between cell body and "skirt"; (**g**) *Chelonemonas geobuk*, dorsal view, showing hexagonal "tortoise-shell" patterning on dorsum; (**h**) *Multimonas media*, dorsal view, showing numerous discharged extrusomes (*Ex*) and reduced proboscis (*Pr*) with exposed anterior flagellum (*AF*); (**i**) *Multimonas media*, ventral view, showing "frilled" margin of cell "skirt" (*open arrowheads*). *Closed arrowheads* – acroneme at end of anterior flagellum; *open arrowheads* – "frilled" margin of "skirt"; *AF* anterior flagellum, *Ex* extrusomes, *Ms* mastigophore, *PF* posterior flagellum, *Pr* proboscis, *Ps* pseudopodium, *T* "tusk." Scale bar in (**d**) = 5 μm for (**a**–**d**); scale bar in (**g**) = 1 μm in (**e**–**g**); scale bar in (**i**) = 1 μm in (**h**) and (**i**) (Images **a** and **b** reproduced from originals used for Heiss et al. 2013b; image **c** by AAH; image **d** courtesy of Yana Eglit (Dalhousie University); images **e**–**i** reproduced from originals used for Heiss et al. 2015)

Table 1 Summary of published species/strains of apusomonads. Type species is listed first for each genus

Taxon	Species names	Synonym(s)	Authority	Deposited Cultures	Molecular data (selected)	SEM	TEM	Comments [v]
Apusomonadinae	*Apusomonas proboscidea*		Aléxéieff 1924	CCAP 1905/1	SSU rDNA, some proteins	*	* [y]	from soil; has mastigophore
	Apusomonas australiensis		Ekelund and Patterson 1997					from soil; has mastigophore
	Manchomonas bermudensis	*Amastigomonas bermudensis*	(Molina and Nerad 1991), Cavalier-Smith 2010 [w]	ATCC 50234	SSU rDNA; ESTs [x]	*	*	
Podomonas	*Podomonas magna*		Cavalier-Smith 2010 [w]	CCAP 1901/4	SSU rDNA			
	Podomonas capensis		Cavalier-Smith 2010 [w]	prev. in ATCC	SSU rDNA	*	*	
	Podomonas gigantea	*Am. gigantea*	(Myl'nikov 1999), Cavalier-Smith 2010 [w]					
	Podomonas griebenis	*Am. griebenis*	(Myl'nikov 1999), Cavalier-Smith 2010 [w]					
	Podomonas klosteris	*Am. klosteris*	(Myl'nikov 1999), Cavalier-Smith 2010 [w]					
Multimonas	*Multimonas media*		Cavalier-Smith 2010 [w]	CCAP 1901/3	SSU rDNA; 454 "ESTs"			forms syncytia
	Multimonas marina	*Cercomonas marina, Am. marina*	(Myl'nikov 1989a), Cavalier-Smith 2010 [w]					
	Multimonas koreensis		Heiss et al. 2015		SSU rDNA	*		

(*continued*)

Table 1 (continued)

Taxon	Species names	Synonym(s)	Authority	Deposited Cultures	Molecular data (selected)	SEM	TEM	Comments [v]
Thecamonadinae	*Thecamonas trahens*	*Am. trahens*	Larsen and Patterson 1990	ATCC 50062	Genome	*	* [z]	
	Thecamonas filosa		Larsen and Patterson 1990					
	Thecamonas muscula	*Am. muscula*	(Myl'nikov 1999), Cavalier-Smith 2010 [w]					
	Thecamonas mutabilis	*Rhynchomonas mutabilis*, *Am. mutabilis*	(Griessmann 1913), Larsen and Patterson 1990					
	Chelonemonas geobuk		Heiss et al. 2015		SSU rDNA	*		
	Chelonemonas masanensis		Heiss et al. 2015		SSU rDNA	*		
"Thecamonas" oxoniensis	*"Thecamonas" oxoniensis*		Cavalier-Smith 2010 [w]	CCAP 1901/2	SSU rDNA; 454 "ESTs"			freshwater; forms cysts
Amastigomonas	*Amastigomonas debruynei*		de Saedeleer 1931					freshwater
	Amastigomonas borokensis		Hamar 1979					freshwater
	Amastigomonas caudata		Zhukov 1975					freshwater
	Amastigomonas marisrubri		Myl'nikov and Myl'nikova 2012				*	

[v]: Unless otherwise specified, all strains are marine
[w]: Description in Cavalier-Smith and Chao (2010)
[x]: Unpublished (B. Franz Lang)
[y]: Including 3D reconstuction of the flagellar apparatus
[z]: Including 3D reconstuction of the entire cell

"amastigomonad"-like genus, *Chelonemonas*, being described recently (Heiss et al. 2015). Under this scheme, the genus *Amastigomonas* has been retained to encompass only freshwater organisms closely resembling the original account of *Amastigomonas debruynei* (de Saedeleer 1931). As mentioned above, however, this scheme has not been universally accepted, with some authorities continuing to use *Amastigomonas* for all non-*Apusomonas*-type apusomonads (Karpov 2011; Myl'nikov and Myl'nikova 2012). Regardless, there are neither molecular nor ultrastructural data for *Amastigomonas* sensu stricto at present, and consequently its identity as a relative of other apusomonads is in some doubt (Cavalier-Smith and Chao 2010).

There are several cursory accounts of the ultrastructure of apusomonads (Cavalier-Smith and Chao 2010; Karpov and Myl'nikov 1989; Karpov and Zhukov 1984, 1986; Molina and Nerad 1991; Myl'nikov 1989b), with detailed reconstructions of the flagellar apparatus completed for *Apusomonas proboscidea* (Karpov 2007) and *Thecamonas trahens* (Heiss et al. 2013b). At the time of writing, most sequence data is from *Thecamonas trahens*, for which there is a genome project (Ruiz-Trillo et al. 2007).

Practical Importance

All known apusomonads are free-living. One species, "*Thecamonas" oxoniensis*, was isolated from the surface of a leaf of English ivy, but it has not been established whether this species is a true epibiont (Cavalier-Smith and Chao 2010). The ecological importance of apusomonads is essentially unknown (see below). They have not been exploited commercially. Their primary scientific relevance at present is their importance for understanding the deep evolutionary history of eukaryotes and the evolution of multicellularity (see below).

Habitats and Ecology

All known apusomonads are gliding organisms, and thus primarily surface-associated. All are heterotrophic, and primarily or exclusively bacterivorous (Karpov and Zhukov 1984; Cavalier-Smith and Chao 2010). Apusomonads appear to be ubiquitous: apusomonad cells or SSU rRNA sequences have been detected in samples from fresh water (Lee et al. 2005; Scheckenbach et al. 2006), marine material (Larsen and Patterson 1990; Lee and Patterson 2000; Massana et al. 2011; Myl'nikov and Myl'nikova 2012; al-Qassab et al. 2002; Tong 1997; Tong et al. 1998; Vørs 1993), soil samples (Ekelund and Patterson 1997; Vickerman et al. 1974), and from at least moderately hypersaline environments (Patterson and Simpson 1996). They have been recovered from surface waters (Massana and Pedrós-Alió 2008; Scheckenbach et al. 2005), littoral sediments (al-Qassab et al. 2002; Massana et al. 2015; Tikhonenkov et al. 2006), and the deep sea (López-García et al. 2003; Scheckenbach et al. 2005; Takishita et al. 2007, 2010). The marine apusomonads

(currently understood as the genera *Thecamonas* sensu stricto, *Chelonemonas*, *Multimonas*, *Podomonas*, and *Manchomonas*) are collectively among the 20 most-encountered varieties of heterotrophic flagellates in microscopy studies of marine sediment samples (Patterson and Lee 2000), although always in low densities (Arndt et al. 2000). Freshwater apusomonads ("*Thecamonas*" *oxoniensis* and *Amastigomonas* sensu stricto) have only rarely been encountered (Cavalier-Smith and Chao 2010). Soil-dwelling apusomonads are widely distributed and can be abundant (Ekelund and Patterson 1997; Foissner 1991).

It is important to note that all apusomonads other than *Apusomonas* appear rather similar and that their recognition as different genera was a recent proposal, which has not been adopted universally (see above and below). Consequently, these apusomonads have been recorded as one member or another of the genus *Amastigomonas* in almost every ecological survey published to date. Because of this, the true distribution across habitats is unknown for all genus-level taxa of apusomonads other than *Apusomonas*.

Characterization and Recognition

General Appearance

Apusomonads are all small, usually 5–10 μm in length, though some species may approach 20 μm. Most have an ovoid main cell body (though the main cell body of *Apusomonas* has a subcircular profile), with a characteristic highly mobile anterior proboscis that includes the anterior flagellum (reported cell lengths generally refer to the main cell body, without the proboscis). The proboscis has a smooth anterosinistral motion and often curves along its length as it moves (Cavalier-Smith and Chao 2010; Heiss et al. 2013b; Karpov and Myl'nikov 1989; Vickerman et al. 1974). An acroneme (from the anterior flagellum) may emerge from the tip of the proboscis, and may be distinguishable by light microscopy. The posterior flagellum runs under the cell body, along its left side, and (depending on the taxon) may extend beyond the cell outline to trail behind the cell (Figs. 1 and 2). The cell is generally two to three times as long as it is wide, though most taxa are relatively flexible. Most genera produce pseudopodia, which emerge from the cell venter and may extend in any direction, though rarely more than half the cell length (Figs. 1b, 2a, b, e, f, and 3b; Cavalier-Smith and Chao 2010; Heiss et al. 2013b; Karpov and Myl'nikov 1989), except in the case of the trailing pseudopodium (see below).

All genera except for *Apusomonas* (see below) appear quite similar under the light microscope. *Podomonas* is larger than other apusomonads (12–20 μm) and has lines of refractile granules running in parallel to the posterior flagellum, a reduced proboscis sleeve, and more-prominent pseudopodia (Fig. 2c). *Multimonas* occasionally forms syncytia. *Multimonas*, *Thecamonas*, and *Chelonemonas* will often have a prominent trailing pseudopodium that may be up to twice the length of the cell body (Fig. 2b). "*Thecamonas*" *oxoniensis* is somewhat leaf-shaped, though the cell body

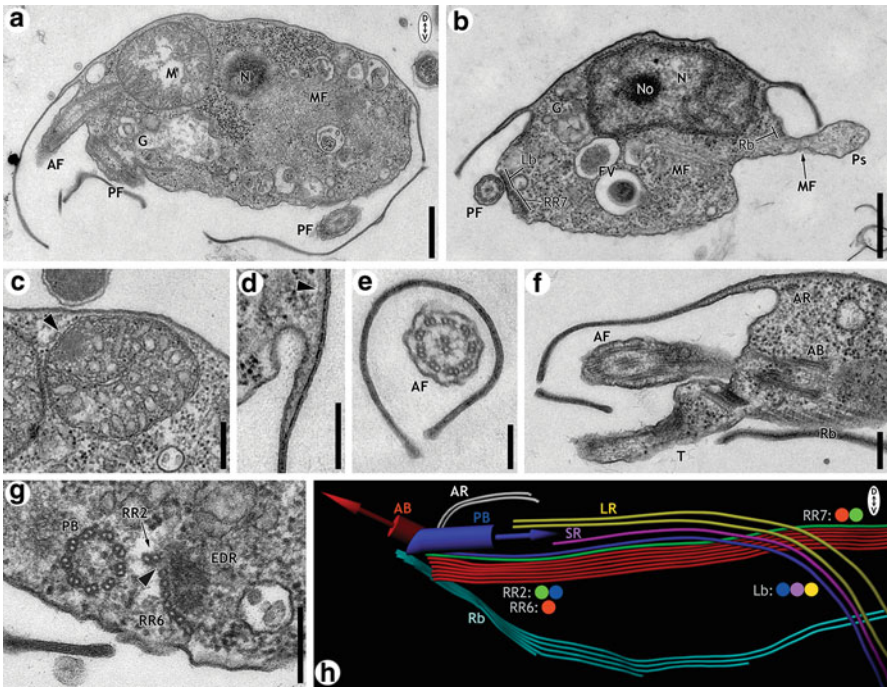

Fig. 3 Intracellular features of the apusomonad *Thecamonas trahens*. Panels (**a**–**h**) are transmission electron micrographs: (**a**) longitudinal section of whole cell, showing general cell features; (**b**) cross section roughly one third through whole cell, showing principal organelles as well as pseudopodium (*Ps*) and components of posterior flagellar apparatus; (**c**) mitochondrion, showing tubular cristae and nucleoid (*arrowhead*); (**d**) cross section of cell showing pellicle (dark layer under plasma membrane) and subpellicular layer (*arrowhead*); (**e**) cross section of proboscis, showing anterior flagellum (*AR*) surrounded by double layer of cell membrane; (**f**) internal structure of 'tusk' (*T*); (**g**) arrangement of posterior roots near distal end of posterior basal body (*PB*), with split (*arrowhead*) in right root between 2-membered (*RR2*) and 6-membered (*RR6*) subparts, and associated electron-dense rod (*EDR*). (**h**) Three-dimensional reconstruction of anterior (proximal) flagellar apparatus, with basal bodies represented as large cylinders (*arrows* begin at transition zone and indicate direction of flagellum) and individual microtubules as small cylinders. *AB* anterior basal body; *AF* anterior flagellum; *AR* anterior root; *FV* food vacuoles; *G* Golgi apparatus; *LR* left posterior root; *M* mitochondrion; *MF* bundles of microfibrils; *N* nucleus; *No* nucleolus; *PB* posterior basal body; *PF* posterior flagellum; *Ps* pseudopodium; *Rb* right band of microtubules; *RR* right posterior root; *RR2* 2-membered part of right root; *RR6* 6-membered part of right root; *SR* singlet root; *T* 'tusk'. Scale bars in (**a**) & (**b**) = 500 nm; scale bars in (**c**–**g**) = 200 nm (All micrographs and reconstruction reproduced from originals used for Heiss at al. (2013b))

can "fold" longitudinally and has tiny refractile granules and a contractile vacuole (consistent with its being a freshwater organism). *Manchomonas* has neither acronemes nor conspicuous pseudopodia and a more leftward than anteriorly oriented proboscis (Cavalier-Smith and Chao 2010; Heiss et al. 2015; Molina and Nerad 1991). *Amastigomonas* itself has a contractile vacuole and supposedly lacks a visible posterior flagellum (Cavalier-Smith and Chao 2010).

Apusomonas differs in having a relatively inflexible main cell body, from which emerges an anterior extension called the mastigophore. The mastigophore forms the proximal part of the proboscis (Figs. 1a and 2d). It contains the basal bodies of both flagella as well as the proximal portions of the posterior flagellum and of the flagellar roots (see below). It is therefore significantly thicker than the distal flagellum-and-sleeve portion of the proboscis. The proboscis of *Apusomonas* thus has a tripartite appearance (from proximal to distal): mastigophore, anterior flagellum and sleeve, and acroneme (Karpov 2007; Karpov and Myl'nikov 1989; Karpov and Zhukov 1984, 1986).

Ultrastructure

Apusomonads have characteristic folds that emerge from the lateral edges of the cell body and extend ventrally, forming a "skirt" about the cell body that is most easily resolved using scanning or transmission electron microscopy (SEM; TEM). The "skirt" is continuous with the sleeve that extends around the anterior flagellum to form the proboscis (Figs. 1 and 2; Heiss et al. 2013b; Vickerman et al. 1974). The posterior flagellum runs between the left lip of the skirt and the main cell body for at least half of the cell length (Figs. 1, 2, and 3a, b). The dorsal cell membrane is underlain by a thin pellicle (sometimes called a "theca," a term that usually denotes an extracellular covering) that extends into and supports the skirt. As a consequence, the dorsal cell membrane is smoother than the unsupported ventral cell membrane when viewed by SEM or TEM (Figs. 2e–i and 3a, b). The pellicle has a polygonal substructure in *Chelonemonas* that is visible on the dorsal surface of the cell in SEM preparations (Heiss et al. 2015; Fig. 2g). Scanning electron microscopy images of the dorsal surface of *Multimonas* strains have shown small knobs and elongate strands that may represent undischarged and discharged extrusomes, respectively (Heiss et al. 2015; Fig. 2h). Some apusomonads have an anterior projection of the cell body, the "tusk," which is rigid, and contains complex supporting material (Figs. 1b, 2a, f, and 3f; Heiss et al. 2013b). It is known to be present in at least some strains of *Thecamonas*, *Chelonemonas*, and *Podomonas* and to be absent in *Manchomonas* and *Apusomonas*. The tusk is under 1 μm long in *Thecamonas* and is just barely visible using light microscopy under optimal conditions (Fig. 2a; Heiss et al. 2013b, 2015).

Internally, apusomonads have a dorsally positioned nucleus with a distinct central nucleolus. The nucleus is usually but not always circular; in *Apusomonas*, it is strongly reniform (Figs. 1a and 2d). The single Golgi body is found near the anterior of the cell. The multiple mitochondria have tubular cristae (Fig. 2c). At least some taxa have conspicuous, densely-staining microbodies; in *Thecamonas trahens*, there is usually one per cell (Heiss et al. 2013b). Food vacuoles are most often found in the ventral half to two-thirds of the cell (Fig. 3a, b; Cavalier-Smith and Chao 2010; Heiss et al. 2013b; Karpov 2007).

The flagellar apparatus has been reconstructed in detail for *Apusomonas* (Karpov 2007) and *Thecamonas trahens* (Fig. 3h; Heiss et al. 2013b). The flagellar apparatus

comprises the two basal bodies, which are joined by at least two (probably three) fibrous connectives, plus three posterior microtubular roots, one anterior microtubular root, a "ribbon" of microtubules associated with the anterior basal body, and a number of nonmicrotubular accessory structures (Fig. 3b, f–h). Apusomonads appear to show the typical eukaryotic pattern of flagellar transformation during the cell cycle, with the anterior basal body younger and the posterior elder (Cavalier-Smith and Chao 2010). The posterior right root ("RR"; equivalent to R2 in the universal terminology of Moestrup 2000) comprises several microtubules (eight to sixteen have been reported: Heiss et al. 2013b; Karpov 2007; Karpov and Myl'nikov 1989; Molina and Nerad 1991); the leftmost two of which split off from the remainder (Fig. 3g, h). The posterior left root ("LR," equivalent to R1) generally contains two microtubules, and a singlet root arises between the other posterior roots. The anterior microtubular root ("AR," equivalent to R3) is a doublet that runs across the dorsal cell surface posteriorly and to the left (Fig. 3f, h). The "ribbon" has sometimes been identified as a flagellar microtubular root (Cavalier-Smith and Chao 2010; Karpov 2007; Molina and Nerad 1991) but may instead be homologous to the systems of secondary peripheral microutubules in other eukaryotes (Heiss et al. 2013b). It originates alongside the anterior basal body in association with a non-microtubular sheet. In the posterior half of *T. trahens* at least, the posterior roots reorganize into two structures, both on the left side of the cell; (i) a dorsally displaced root comprising most of the right root microtubules ("RR7") and (ii) a ventral "left band" made of the left root, the singlet root, and one microtubule from the right root (Fig. 3b, h; Heiss et al. 2013b). The left band extends to the posterior end of the cell, likely into the base of the trailing pseudopodium. The right root runs opposite the left lip of the skirt, and in a similar fashion, the ribbon runs opposite the right lip of the skirt, likely reinforcing the cell outline.

Life Cycle

Only a single cell type has been observed in the majority of apusomonads, that of gliding flagellates. Cells divide by mitotic binary fission; sex has not been observed (Karpov and Myl'nikov 1989). Cysts are known for "*Thecamonas*" *oxoniensis* (Cavalier-Smith and Chao 2010). *Apusomonas* can recover following seasonal desiccation, but a true cyst form appears to be absent (Cavalier-Smith and Chao 2010; Karpov and Myl'nikov 1989). Vickerman et al. (1974) reported a wall-less cryptobiotic stage in *Apusomonas* but also indicated that this form does not survive total desiccation.

Systematics

The formal taxon for apusomonads is the family Apusomonadidae Karpov and Myl'nikov 1989, the sole member of order Apusomonadida Karpov and Myl'nikov 1989. The clade comprising *Apusomonas* and *Manchomonas* has been recognized as

the subfamily Apusomonadinae Karpov and Myl'nikov 1989) (sensu Cavalier-Smith and Chao 2010) and that comprising *Chelonemonas* and marine *Thecamonas* as Thecamonadinae Larsen and Patterson 1990 (sensu Heiss et al. 2015).

Maintenance and Cultivation

Members of five apusomonad lineages have been maintained in monoprotistan but not axenic laboratory culture (see Table 1). Such cultures were generally established through serial dilution (e.g., Cavalier-Smith and Chao 2010), although cell migration has also been used (Heiss et al. 2015; Molina and Nerad 1991). They have been grown in standard laboratory media, generally water of appropriate salinity (distilled water, sterilized bottled mineral water, or artificial or natural seawater, often diluted) with a plant-based carbon source (either a sterile cereal grain or an infusion of such material, e.g., Cerophyl). Live prey bacteria (e.g., *Pseudomonas*) can be added instead of the carbon source. When grown in standard culture tubes or tissue culture flasks, cultures of most strains can last for >2 months but are always sparse; cultures generally last longer and grow to higher density in tissue culture flasks than in tubes (AAH, pers. obs.). When grown in Petri plates, the same strains can form visible plaques of very high density within days of inoculation, but die within ~2 weeks (AAH, pers. obs.). Cultures can be maintained at 14 °C (e.g., Cavalier-Smith and Chao 2010) but often are more robust at 16–21 °C or room temperature (AAH, pers. obs.).

Evolutionary History

Internal Relationships

As discussed above, five clades of apusomonads with cultured representatives have been delimited using phylogenies of SSU rRNA genes (Cavalier-Smith and Chao 2010; Heiss et al. 2015). Two of these are Apusomonadinae (comprising the genera *Apusomonas* and *Manchomonas*) and Thecamonadinae (comprising the genus *Chelonemonas* and the marine members of the genus *Thecamonas*). Another two lineages are represented by individual genera (*Podomonas* and *Multimonas*). The final lineage comprises the single freshwater species "*Thecamonas*" *oxoniensis*, a species with no specific relationship to the marine members of the genus *Thecamonas* (Cavalier-Smith et al. 2014; Heiss et al. 2015). No stable relationships between the five lineages have been established to date (Cavalier-Smith and Chao 2010; Heiss et al. 2015). At least four additional lineages are known from environmental sequences only; nothing is known about the biology of the organisms corresponding to those sequences.

Overall Phylogenetic Position

The first molecular phylogenetic study to include an apusomonad identified the group as a possible relative of opisthokonts (Cavalier-Smith and Chao 1995). Some of the first multigene phylogenies also weakly supported this relationship (Kim et al. 2006). Recent multigene analyses (Katz et al. 2011; Paps et al. 2013) and phylogenomic studies (Brown et al. 2013; Burki et al. 2016; Cavalier-Smith et al. 2014; Derelle and Lang 2012; Torruella et al. 2012, 2015; Zhao et al. 2013) have lent increasing support to the placement of apusomonads as a sister group to opisthokonts.

Some studies have also demonstrated that another enigmatic group of organisms, the breviates, (see Coda and Fig. 4a) is most closely related to apusomonads and opisthokonts, and this relationship has been formalized by the erection of the taxon Obazoa, which encompasses all three groups (Brown et al. 2013). However, it is not clearly resolved whether apusomonads alone represent the closest relatives to opisthokonts or whether it is an apusomonad-breviate clade that represents the sister group to opisthokonts. The most detailed phylogenomic analysis to date, with relatively limited taxon sampling, found that the preferred phylogeny depended on the evolutionary model used for phylogenetic inference (Brown et al. 2013): more complex evolutionary models incorporating among-site model heterogeneity, such as CAT-GTR (Le et al. 2008), favor an apusomonad-opisthokont clade, to which breviates are the sister group (Brown et al. 2013). Recent phylogenomic studies with expanded taxon sampling have not clearly resolved between these hypotheses (Cavalier-Smith et al. 2014); it is hoped that additional taxon and gene sampling data will more precisely resolve the position of the apusomonads in the near future.

Meanwhile, Cavalier-Smith (2002) proposed that the pellicle of apusomonads was homologus to that of another "non-supergroup" lineage, the ancyromonads (see Coda and Fig. 4b), and suggested a common evolutionary history for the two groups. Interestingly, some early SSU rRNA phylogenies including ancyromonads suggested a close relationship with opisthokonts (Atkins et al. 2000b; Cavalier-Smith and Chao 2003), similarly to the early SSU rRNA phylogenies of apusomonads (see above). This arrangement has been loosely supported by recent multigene phylogenetic and phlyogenomic analyses, although it is unclear at present whether ancyromonads fall within Obazoa like apusomonads, or are a sister to Obazoa, or are more distantly related (Cavalier-Smith et al. 2014; Katz et al. 2011; Paps et al. 2013).

Implications for Eukaryote Evolution

Apusomonads are important for our understanding of eukaryote evolution for at least two reasons. One of these concerns the evolution of the flagellar apparatus

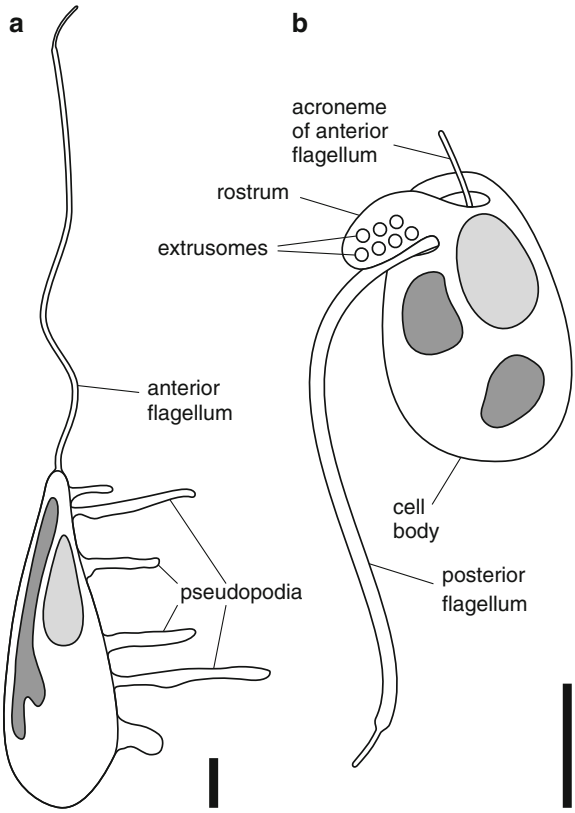

Fig. 4 Appearance by light microscopy of living breviates and ancyromonads. (**a**) *Breviata anathema*; (**b**) *Ancyromonas sigmoides*. Nuclei are *light grey*; mitochondria (*Ancyromonas*) or mitochondrion-related organelles (*Breviata*) are *dark grey*. Scale bars = 2 μm for each drawing

cytoskeleton at the "supergroup" level. Apusomonads possess a complex flagellar apparatus with multiple posterior microtubular roots, including an R2 root that splits into two parts and a "supernumerary" singlet microtubular root, as well as a posteriorly directed array of secondary microtubules (the ribbon). These structures are also found together in ancyromonads and breviates (Heiss et al. 2011, 2013a), in "typical excavates" (Simpson 2003), and in other taxa such as some stramenopiles (Moestrup and Thomsen 1976; Yubuki et al. 2010), suggesting that these specific features may have been ancestral to the majority of major eukaryote lineages (Cavalier-Smith 2013; Heiss et al. 2013b; Leander and Yubuki 2013). Since apusomonads are most closely related to opisthokonts and (less so) to amoebozoans, this suggests that the simple flagellar apparatus cytoskeletons seen in opisthokonts and many flagellated amoebozoans (e.g., pelobionts) are not primitive ancestral systems but in fact could represent independent secondary simplifications from a complex ancestral form.

The other area of importance concerns the evolution of multicellularity. The supergroup Opisthokonta includes two substantial lineages that have evolved multicellularity independently of one another: animals and fungi. Each lineage has

established key systems associated with multicellularity, including cell-cell communication and adhesion (Grosberg and Strathmann 2007). Interestingly, elements of some of these systems are encoded in the genome of *Thecamonas trahens*, indicating that such pathways were present in the common ancestor of apusomonads and opisthokonts, and substantially predate the evolution of multicellularity in both animals and fungi. For example, the *Thecamonas* genome encodes most components of the integrin system (Sebé-Pedrós et al. 2010). In animals, integrins span the cell membrane, connecting to the actin cytoskeleton on the cytoplasmic side (via a series of associated proteins, mostly present in *Thecamonas*) and binding to the laminin and collagen of the extracellular matrix. Sodium-channel (Cai 2012) and calcium-signalling (Cai and Clapham 2012) genes involved in cell communication in animals, and absent from fungi, are also present in the *Thecamonas* genome. The cyclin dependent kinase 4/6 and cyclin D subfamilies are also thought to play a part in the development of animal-type multicellularity and are also found in *Thecamonas*, as well as in amoebozoans (Cao et al. 2014).

Coda: Breviates and Ancyromonads

Apusomonads are not the only organisms to have been suggested to have a close relationship to opisthokonts. Molecular phylogenetic evidence indicates that several more obscure lineages of small protozoa, mostly heterotrophic flagellates, may also be closely related to opisthokonts and/or apusomonads. The best known of these understudied groups are breviates and ancyromonads.

Breviates (Cavalier-Smith et al. 2004) are a group of anaerobic or microaerophilic amoeboid flagellates with an apical anterior flagellum and either a posterior flagellum or a nonflagellated posterior basal body (Figs. 4a and 5). There are four described genera, each with a single species: the freshwater *Breviata anathema* and the marine *Subulatomonas tetraspora*, *Pygsuia biforma* and *Lenisia limosa*. They are surface-associated gliding organisms, although a distinct swimming stage is also known in *Pygsuia* (Figs. 5a, c; Brown et al. 2013). Breviates produce fine pseudopodia that typically form at near-regular intervals from a point at the anterior end of the cell, thus forming a series down the cell as it glides forward (Figs. 4a and 5b). The cells engulf bacteria with these pseudopodia (Heiss et al. 2013a). The cells have a moderately complex cytoskeleton including several flagellar microtubular roots, and some unusual non-microtubular elements (Figs. 5g-k; see Heiss et al. 2013a for details). At least some breviates are reported to form cysts, though this is not well documented (Katz et al. 2011; Walker et al. 2006). No sexual stages have been observed. Cultures and/or SSU rRNA sequences have been obtained from environmental samples taken from Europe, North America, and Japan (Katz et al. 2011; Brown et al. 2013).

All investigated breviates have a large mitochondrion-related organelle (MRO; Fig. 5e), which in *Breviata* has occasionally been found to contain a few tubular cristae (Fig. 5f; Heiss et al. 2013a). However, all cultured breviates are maintained exclusively under anaerobic or suboxic conditions (Brown et al. 2013; Heiss

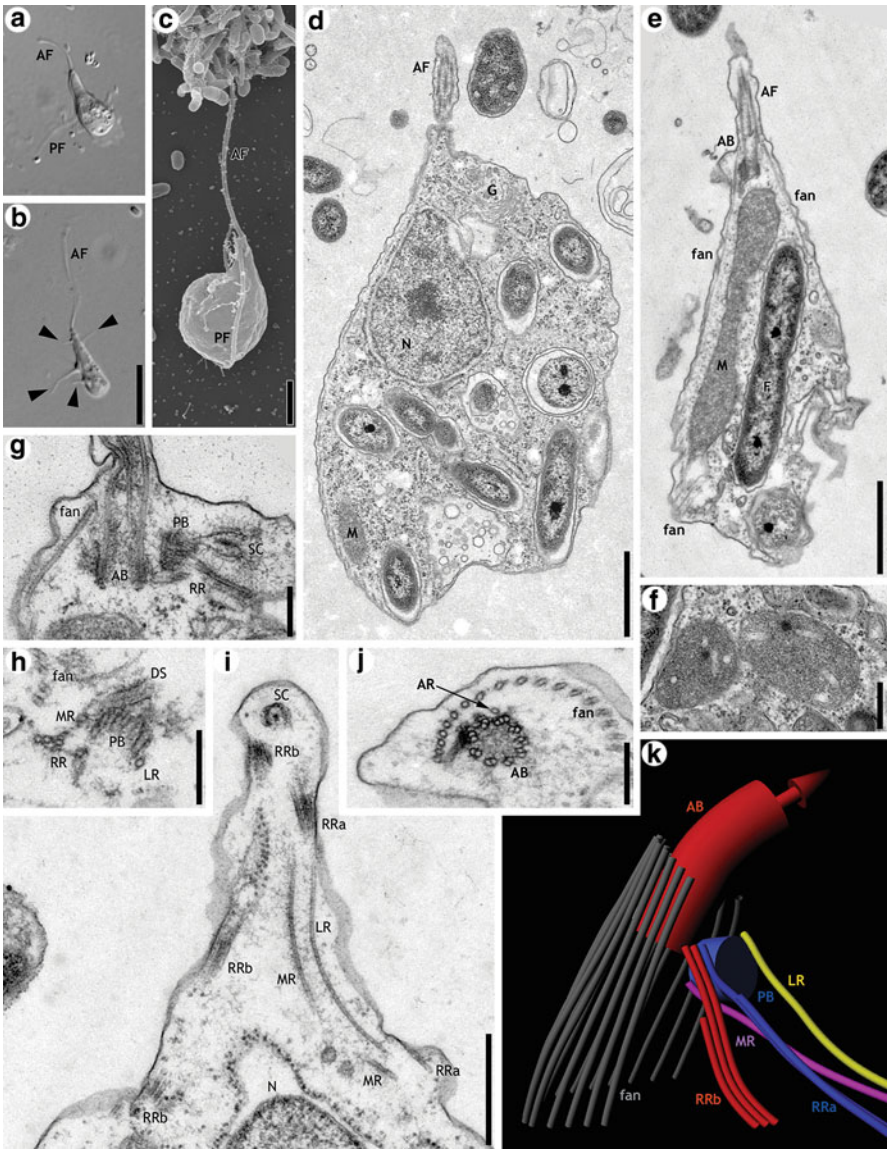

Fig. 5 Images of breviates. Panels (**a** and **b**) are differential interference contrast images of live cells: (**a**) *Pygsuia biforma*, showing both flagella; (**b**) *Breviata anathema*, showing its single flagellum. Panel (**c**) is a scanning electron micrograph of *Pygsuia biforma*. Panels (**d**–**j**) are transmission electron micrographs of *Breviata anathema*: (**d**) longitudinal section of whole cell showing general cell features; (**e**) longitudinal section of whole cell showing size of mitochondrion-related organelle (*M*) and its proximity to anterior basal body (*AB*); (**f**) mitochondrion-related organelle with tubular cristae; (**g**) longitudinal section through flagellar apparatus showing relationship between flagellated anterior (*AB*) and nonflagellated posterior (*PB*) basal bodies, as well as longitudinal section through "semicone" structure (*SC*); (**h**) section through posterior basal body

et al. 2013a; Katz et al. 2011; Walker et al. 2006). The biochemical capacity of these MROs has been inferred primarily from transcriptome data from *Pygsuia* (Stairs et al. 2014), with some additional information from *Breviata* (Minge et al. 2009). The MRO does not produce energy using classical oxidative phosphorylation (electron transport chain complexes I, III–V are absent) but instead acts as a hydrogenosome that generates ATP anaerobically via substrate-level phosphorylation (Stairs et al. 2014). The *Pygsuia* MRO has several highly unusual features, the most notable being an archaeal-related "SUF" system for Fe-S cluster assembly, which appears to have replaced the "ISC" system that is found in virtually all other mitochondria and MROs across eukaryotes (Stairs et al. 2014).

The first known breviate (*Breviata*) was originally identified as a member of the pelobiont genus *Mastigamoeba* (see ▶ Archamoebae), a situation that led to *Mastigamoeba* appearing to be polyphyletic when molecular phylogenies included multiple organisms attributed to it (Edgcomb et al. 2002; Stiller and Hall 1999). Resolution of this misidentification led to the recognition of a new lineage of eukaryotes (Cavalier-Smith et al. 2004) and the new genus *Breviata* for this single species (Walker et al. 2006). The first phylogenomic analysis including (relatively sparse) data from this strain suggested that breviates were basal to or branched within Amoebozoa (Minge et al. 2009). However, several environmental SSU rRNA sequences have been identified as belonging to breviates (summarized in Katz et al. 2011), and strains from additional lineages have now been cultivated in the laboratory, including the recently described *Subulatomonas tetraspora* (Katz et al. 2011), *Pygsuia biforma* (Brown et al. 2013) and *Lenisia limosa* (Hamann et al. 2016). Recent SSU rRNA gene trees, multigene phylogenies, and phylogenomic analyses that include more breviate species show that breviates are actually most closely related to apusomonads and/or opisthokonts (Brown et al. 2013; Burki et al. 2016; Cavalier-Smith et al. 2014; Katz et al. 2011; see above). Interestingly, as with the apusomonad *Thecamonas trahens*, a large complement of genes encoding integrin complex proteins is present in the breviate *Pygsuia* (Brown et al. 2013).

Fig. 5 (continued) (*PB*) showing roots near point of origin; (**i**) longitudinal section through anterior end of cell showing paths of posterior roots, as well as cross-section of "semicone" (*SC*); (**j**) section through anterior basal body (*AB*) showing short anterior root (*AR*) and dorsal fan. (**k**) Three-dimensional reconstruction of flagellar apparatus, with basal bodies represented by large cylinders (*arrow* begins at transition zone and points in direction of flagellum) and individual microtubules by small cylinders. *AB* anterior basal body, *AF* anterior flagellum, *AR* anterior root, *DS* "double sandwich" structure between basal bodies, *F* food (bacteria), *fan* dorsal microtubular fan, *G* Golgi apparatus, *LR* left posterior root, *M* mitochondrion-related organelle, *MR* middle posterior root, *N* nucleus, *PF* posterior flagellum, *Ps* pseudopodium, *RR* right posterior root, *RRa* left part of right root, *RRb* right part of right root, *SC* "semicone" structure. Scale bar in (**b**) = 10 μm for (**a** and **b**); scale bar in (**c**) = 2 μm; scale bars in (**d** and **e**) = 1 μm; scale bars in (**f**, **g**, **i**) = 500 nm; scale bars in (**h**, **j**) = 200 nm (Micrograph in (**a**) by MWB; micrograph in (**b**) by AAH; scanning electron micrograph in (**c**) reproduced from original used for Brown et al. (2013); transmission electron micrographs and reconstruction in (**d**–**k**) reproduced from originals used for Heiss et al. (2013a))

Ancyromonads (Atkins et al. 2000b; Cavalier-Smith 1997; also called planomonads: see Cavalier-Smith et al. 2008; Heiss et al. 2010) are a molecularly diverse but morphologically conservative group of small bacterivorous flagellates (Figs. 4b and 6). There are about 15 nominal species in four genera. Ancyromonads have a rounded cell body, typically 4–6 μm long, which is dorsoventrally compressed and essentially inflexible (Figs. 4b and 6a–c). The anterior-left portion of the cell forms a laterally/posteriorly directed rostrum that contains extrusomes (Figs. 4b and 6e, f). The anterior flagellum is generally short and often either terminates at the cell outline or is almost entirely an acroneme (Figs. 4b and 6c, d) and thus difficult to detect by light microscopy. The posterior flagellum is about two to three times the length of the cell (4B, 6A, 6C). The cell adheres to the substrate using the distal portion of the posterior flagellum and either glides or tethers to one location. The cell body nods rapidly due to flexure of the proximal part of the flagellum (Glücksman et al. 2013; Heiss et al. 2010). Like apusomonads, ancyromonads have a pellicle (Fig. 6h), in this case underlying almost all of the cell surface. Unlike apusomonads, ancyromonads have flat mitochondrial cristae (Fig. 6d, j). The microtubular cytoskeleton is complex, with five distinct flagellar microtubular roots (Figs. 6i-k; see Heiss et al. 2011 for details). Neither sexual stages nor cysts are known. Ancyromonads are as widely distributed as apusomonads, though generally more locally abundant (Atkins et al. 2000a; Chen et al. 2008; Ekelund and Patterson 1997; Hänel 1979; Larsen and Patterson 1990; Lee 2002; Lee and Patterson 2000; Lee et al. 2005; Patterson and Simpson 1996; Patterson and Zölffel 1991; al-Qassab et al. 2002; Scheckenbach et al. 2005; 2006; Stock et al. 2009; Tikhonenkov et al. 2006; Tong 1997; Tong et al. 1997, 1998; Vørs 1993), and have been cultured under the same conditions (Cavalier-Smith et al. 2008; Glücksman et al. 2013; Heiss et al. 2010; Myl'nikov 1990).

Ancyromonads were first identified over 130 years ago (Saville Kent 1882) but received little mention until phylogenetic analysis identified them as an independent lineage with opisthokont affinities (Atkins et al. 2000b; Cavalier-Smith 1997). The most recent taxonomic scheme for ancyromonads (Glücksman et al. 2013) is based on SSU rRNA gene phylogenies, which distinguish five clades. Three of these are marine and correspond to the genera *Ancyromonas*, *Planomonas*, and *Fabomonas*. The other two clades are known from fresh water and have both been placed in the genus *Nutomonas*, as they are sister taxa; they have been separated into the subgenera *Striomonas* (containing *N. longa*) and *Nutomonas* (containing all remaining species in the genus). *Ancyromonas* and *Nutomonas* are sisters in published phylogenies (Cavalier-Smith et al. 2014; Glücksman et al. 2013), comprising a clade that has been given the name Ancyromonadidae (Glücksman et al. 2013). The other genera (*Planomonas* and *Fabomonas*) may or may not be a clade; the name Planomonadidae has been proposed for such a grouping. Another fresh water genus, *Phyllomonas* (Klebs 1893), has been regarded as an ancyromonad by some researchers (Lemmermann 1914; Patterson and Simpson 1996; Tong et al. 1998) but not by others (Cavalier-Smith et al. 2008; Hänel 1979; Patterson and Zölffel 1991; Patterson et al. 2000); its actual status awaits a modern study (Cavalier-Smith et al. 2008; Heiss et al. 2010).

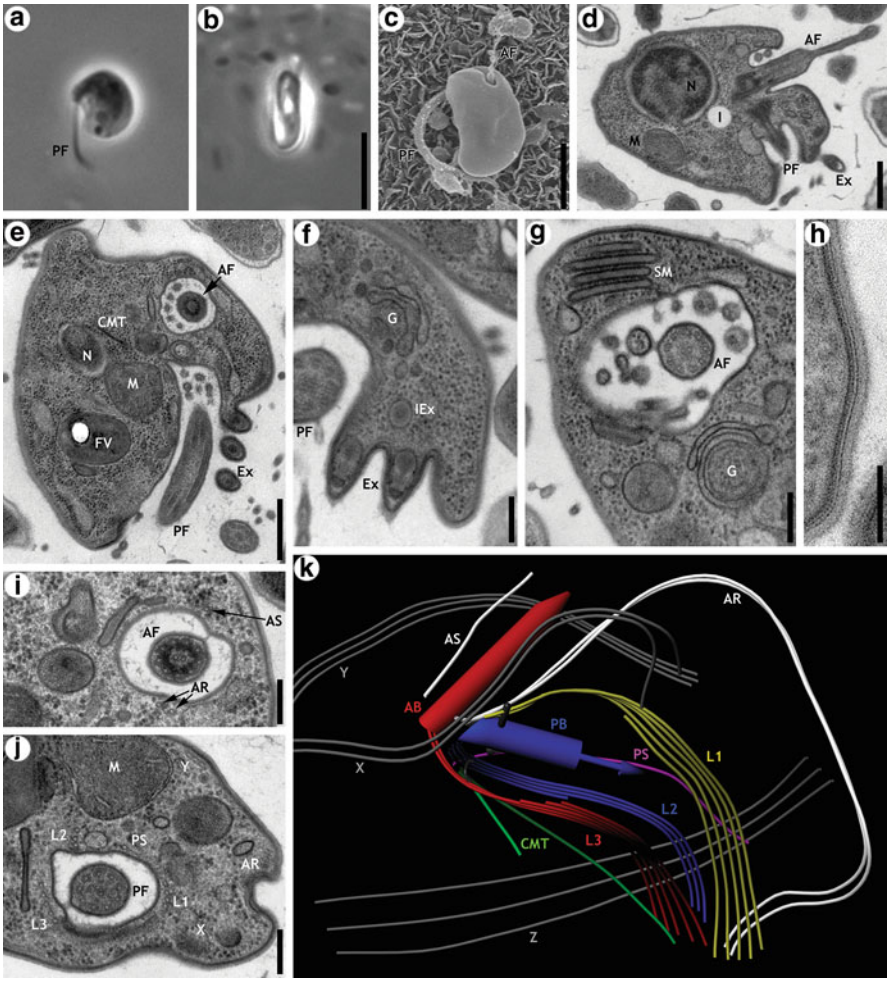

Fig. 6 Images of *Ancyromonas sigmoides*. Panels (**a** and **b**) are phase-contrast micrographs of living cells, viewed from dorsal (**a**) and lateral (**b**) aspects. Panel (**c**) is a scanning electron micrograph of a fixed cell. Panels (**d**–**j**) are transmission electron micrographs: (**d**) section through both basal bodies, showing full extent of anterior flagellum (*AF*); (**e**) longitudinal section through cell, with cross-section of extrusomes (*Ex*); (**f**) longitudinal section through both Golgi apparatus (*G*) and extrusomes (*Ex*), with immature extrusome material (*IEx*) in transition from Golgi apparatus to "firing" position; (**g**) cross section through Golgi apparatus (*G*) and section through stacked membrane structures (*SM*); (**h**) closeup of pellicle; (**i**) anterior root (*AR*) and anterior singlet (*AS*) on either side of anterior flagellum (*AF*) in flagellar pocket; (**j**) arrangement of roots around posterior flagellum (*PF*) in flagellar pocket, as well as mitochondrion (*M*) with flat cristae. (k) Three-dimensional reconstruction of flagellar apparatus, including full extent of anterior basal body (*AB*) and flagellum, as well as various peripheral microtubular structures (*X, Y, Z*; the latter two are possible homologues to the dorsal fan of breviates and the right band of apusomonads). *AB* anterior basal body, *AF* anterior flagellum, *AR* anterior root, *AS* anterior singlet, *CMT* crescent microtubules (part of posterior left root), *Ex* extrusome, *G* Golgi apparatus, *I* electron-lucent inclusion, *IEx* immature extrusome material, *L1* posterior left root, *L2*, *L3* parts of posterior

As stated above, the phylogenetic positions of breviates and ancyromonads are not fully resolved (the latter especially). However, both groups have complex flagellar apparatus cytoskeletons with most of the potentially ancestral features for eukaryotes discussed above for apusomonads (Figs. 3h, 5k, and 6k; Heiss et al. 2011, 2013a, b). Irrespective of the precise phylogenetic positions of ancyromonads and breviates, this reinforces the notion that the ancestors of the opisthokonts and Amoebozoa each had complex cytoskeletons.

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Fig. 6 (continued) right root, *M* mitochondrion, *N* nucleus, *PB* posterior basal body, *PF* posterior flagellum, *PS* posterior singlet root, *SM* stacked membrane structure, *X, Y, Z* peripheral microtubular systems "X," "Y," "Z." Scale bars in (**b**) = 5 μm for (**a** and **b**); scale bar in (**c**) = 2 μm; scale bars in (**d** and **e**) = 500 nm; scale bars in (**f**, **g**, **i**, **j**) = 200 nm; scale bar in (**h**) = 50 nm (Micrographs in **a** and **b** reproduced from originals used for Heiss et al. (2010). Micrograph in **c** courtesy of Won Je Lee (Kyungnam University, South Korea). All transmission electron micrographs and reconstruction reproduced from originals used for Heiss et al. (2011))

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