Evolution of the microtubular cytoskeleton (flagellar apparatus) in parasitic protists

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ARTICLE INFO

ABSTRACT

The microtubular cytoskeleton of most single-celled eukaryotes radiates from an organizing center called the flagellar apparatus, which is essential for locomotion, feeding and reproduction. The structure of the flagellar apparatus tends to be conserved within diverse clades of eukaryotes, and modifications of this overall structure distinguish different clades from each other. Understanding the unity and diversity of the flagellar apparatus provides important insights into the evolutionary history of the eukaryotic cell. Diversification of the flagellar apparatus is particularly apparent during the multiple independent transitions to parasitic lifestyles from free-living ancestors. However, our understanding of these evolutionary transitions is hampered by the lack of detailed comparisons of the microtubular root systems in different lineages of parasitic microbial eukaryotes and those of their closest free-living relatives. Here we help to establish this comparative context by examining the unity and diversity of the flagellar apparatus in six major clades containing both free-living lineages and endobiotic (parasitic and symbiotic) microbial eukaryotes: stramenopiles (e.g., *Phytophthora*), fornicates (e.g., *Giardia*), parabasalids (e.g., *Trichomonas*), preaxostylids (e.g., *Monocercomonoides*), kinetoplastids (e.g., *Trypanosoma*), and apicomplexans (e.g., *Plasmodium*). These comparisons enabled us to address some broader patterns associated with the evolution of parasitism, including a general trend toward a more streamlined flagellar apparatus.

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1. Introduction

The cytoskeleton of eukaryotic cells consists of an array of microtubular and fibrous roots that stem from the basal bodies of the flagellar apparatus [36]. The flagellar apparatus is a fundamental component of eukaryotic cells and is, therefore, among the only ultrastructural systems that can be compared across the entire tree of eukaryotes. The overall structure of the flagellar apparatus is relatively conserved but variable enough to identify homologous traits between very distantly related lineages [36,68]. The high level of conservation in the flagellar apparatus reflects its vital functions in all eukaryotic cells, including division, shape, internal organization, motility and feeding [35,53].

A comprehensive comparison of the flagellar apparatus across the tree of eukaryotes has shown that the last eukaryotic common ancestor (LECA) already had a complex flagellar apparatus that was most similar to the system found in free-living extant excavates [68]. This suggests that the absence of specific traits, such as different flagellar roots, in many different groups of eukaryotes reflects independent streamlining (i.e., trait losses) from a more complex ancestral condition. This punctate pattern of trait loss is also observed within less inclusive taxonomic groups. For instance, the flagellar apparatus in early branching prasinophycean green algae (e.g., *Pterosperma* and *Pyramimonas*) is complex, whereas more recently diverged core chlorophycean green algae (e.g., *Chlamydomonas* and *Micromonas*) have a much simpler flagellar apparatus reflecting trait losses [33,52].

A large and diverse body of evidence supports the inference that the LECA was a free-living, single-celled bacteriovore living in a marine habitat. Free-living microbial lineages represent most of eukaryotic diversity; endobiotic lineages (i.e., parasites, commensals and mutualists) are distributed in many different positions across the tree of eukaryotes. Each major group of single-celled eukaryotic parasites has emerged independently within a clade of free-living lineages in order to exploit endobiotic lifestyles within several different kinds of hosts (e.g., animals and plants). The tran-
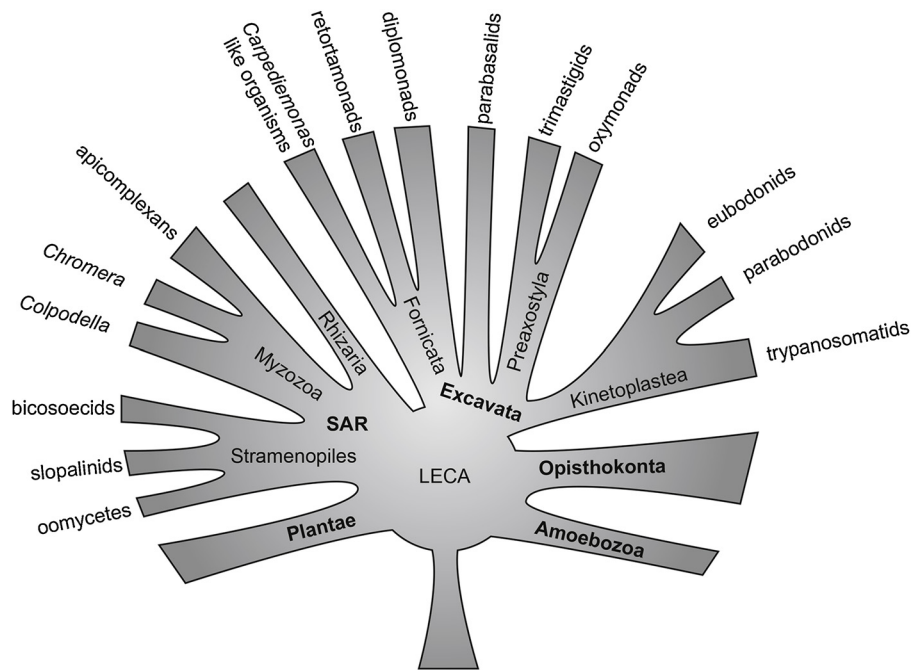
Fig. 1. Illustration showing the general molecular phylogenetic relationship of the specific lineages of eukaryotes addressed in this review. Lineages of mostly free-living eukaryotes that are not relevant to this review have been trimmed for clarity (e.g., ciliates, dinoflagellates, brown algae, euglenids). The names in bold denote the major groups of eukaryotes.

sition to a parasitic lifestyle from free-living ancestors involved substantial changes at the genomic, ultrastructural and behavioral levels. In this review, we examine the evolutionary diversification of the microtubular root systems of flagellar apparatus in six different groups of parasitic protists within the context of their nearest free-living relatives: stramenopiles, fornicates, kinetoplastids, parabasalids, preaxostylids and apicomplexans (Figs. 1 and 2).

These six lineages are nested within different eukaryotic supergroups. Stramenoples and apicomplexans (Myzozoa, Alveolata) both fall within a more inclusive group called SAR (Stramenopiles, Alveolata and Rhizaria), which consists of many different lineages with diverse in morphologies; SAR has been established on the basis of molecular phylogenetic analyses rather than shared morphological features [13]. Fornicates, kinetoplastids, parabasalids and preaxostylids are nested within the Excavata, which also includes photosynthetic lineages (e.g., *Euglena*) and free-living heterotrophic flagellates (e.g., *Jakoba*). The best synapomorphy for excavates is a conspicuous feeding groove on the ventral side of the cell, which is supported by several microtubular roots stemming from the flagellar apparatus [58]. Broad-level comparisons of the flagellar apparatus across the tree of eukaryotes enabled us to identify some reoccurring trends associated with the independent evolution of parasitic lineages in microbial eukaryotes. The focus on microtubules more than fibrous structures is intentional. While fibers in the flagellar apparatus undoubtedly play an important role as a cellular component in eukaryotes, their homology across different lineages is usually elusive because of diverse morphologies. Comparisons of these ultrastructural data are also affected by the application of different methodologies, such as fixation and staining protocols.

2. The flagellar apparatus of parasitic protists

Biologists working within the context of different groups of parasites have inadvertently applied different terms for homologous traits, making comparisons of cytoskeletal systems in different groups of parasites unnecessarily difficult. In order to avoid con-

fusion when comparing homologous traits associated with the flagellar apparatus, we will use terminology that can be applied to all eukaryotes as advocated previously [36,68].

The flagellar apparatus in most microbial eukaryotes usually consists of two basal bodies that anchor the flagellar axonemes and a system of microtubular and fiberous roots (Fig. 3). Each basal body is usually associated with two different microtubular roots, producing a total of four roots within the cell. The oldest (most posterior) basal body is labeled "1" (i.e., basal body 1 or B1), and the youngest (most anterior) basal body is labeled "2" (i.e., basal body 2 or B2). The two microtubular roots associated with basal body 1 are labeled "root 1" (i.e., R1) and "root 2" (i.e., R2); the two microtubular roots associated with basal body 2 are labeled "root 3" (i.e., R3) and "root 4" (i.e., R4) in a clockwise orientation when viewed from the tip of the flagellum [36]. A single microtubular root (i.e., SR) also originates from B1 and is located between R1 and R2. This SR is well characterized in excavates, ancyromonads and apusomonads and has also been observed in some of the earliest diverging lineages of stramenopiles [23,69]. The SR consists of only a single microtubule and is, therefore, easily overlooked, so the true distribution of this root might be much broader than the currently available descriptions indicate.

3. The Stramenopiles

The Stramenopiles is a diverse clade of mostly microbial eukaryotes living in marine, freshwater, terrestrial, and endobiotic environments [39]. Trophic modes within the group include photoautotrophy (e.g., brown algae and diatoms), phagotrophy (e.g., bicosoecids *Cafeteria*), saprotrophy (e.g., Labyrinthulomycetes), and parasitism (e.g., Oomycetes and *Blastocystis*). Molecular phylogenetic evidence demonstrates that the most recent ancestor of stramenopiles was likely a free-living bacterivorous biflagellate [55]. Parasitic stramenopiles have a polyphyletic distribution within the group and therefore evolved independently several times [14]. Oomycetes (or Peronosporomycetes), for instance, are fungus-like rhizoidal organisms with a flagellated zoospore
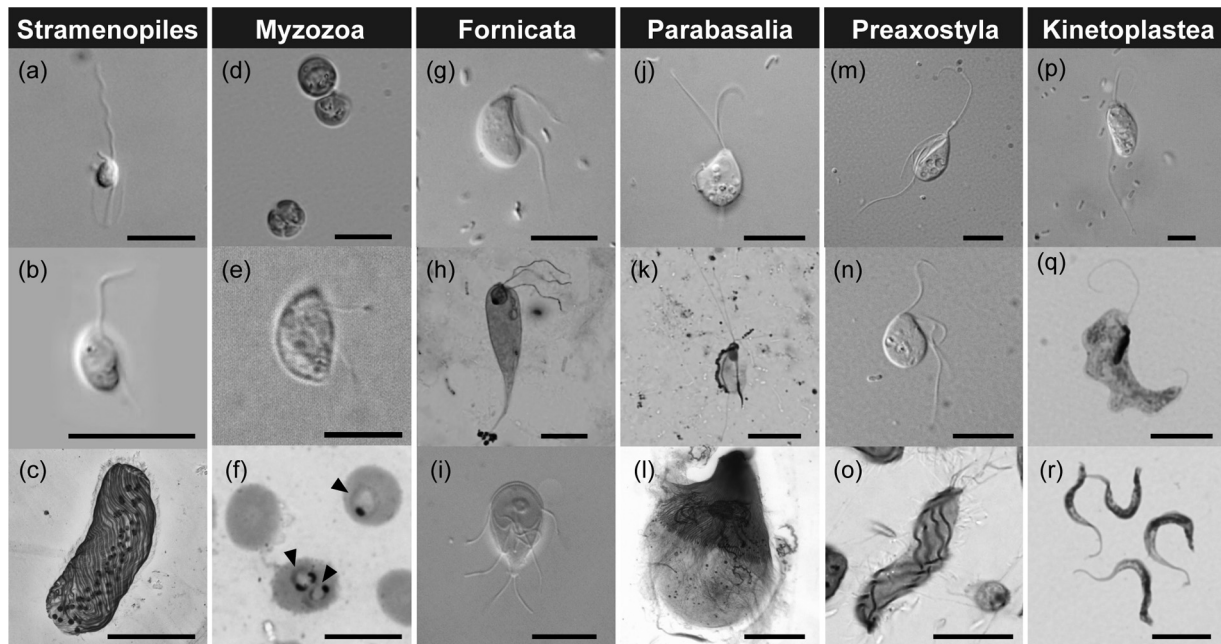
Fig. 2. Light micrographs representing the major eukaryotic groups including both free-living and parasitic lineages. All scale bars are 10 μm except for 100 μm in (c) and (l). (a) Stramenopile, *Bicosoeca* (Bicosoecida); (b) Stramenopile, *Ulkenia* (Labyrinthulomycetes), taken with permission from Ref. [66]; (c) Stramenopile, *Cepedea* (Slopalinida); (d) Myzozoa, *Chromera* (Chromerida); (e) Myzozoa, *Colpodella* (Colpodellida), taken with permission from Ref. [32]; (f) Myzozoa, *Plasmodium* (arrowheads) (Apicomplexa); (g) Fornicata, *Kipferlia* (CLO); (h) Fornicata, *Chilomastix* (Retortamonadida); (i) Fornicata, *Giardia* (Diplomonadida); (j) Parabasalia, *Pseudotrichomonas* (Trichomonadida); (k) Parabasalia, *Tetratrichomonas* (Trichomonadida); (l) Parabasalia, *Trichonympha* (Trichonymphida); (m) Preaxostyla, *Trimastix* (Trimastigida); (n) Preaxostyla, *Monocercomonoides* (Oxymonadida); (o) Preaxostyla, *Pyrsonympha* (Oxymonadida); (p) Kinetoplastida, *Bodo* (Eubodonida); (q) Kinetoplastida, *Trypanoplasma* (Parabodonida). (r) Kinetoplastida, *Trypanosoma* (Trypanosomatida).

stage (e.g., the infamous potato pathogen *Phytophthora infestans*). *Blastocystis* is an anaerobic parasite that inhabits the lower gastrointestinal tract of humans and a wide range of other animals; it is the only stramenopile known to commonly infect humans [54]. Molecular phylogenetic analyses show that *Blastocystis* is closely related to slopalinids, which are anaerobic intestinal commensals of animals, especially anuran amphibians. Slopalinids are either small biflagellated cells (around 15 μm) with one nucleus or large multiflagellated cells (up to several millimeter) with multiple nuclei. *Blastocystis* plus the slopalinids form one of the earliest branching lineages of the stramenopiles along with the free-living Bicosoecida, the free-living Placididea, and the saprotrophic Labyrinthulomycetes [14]; the branching order among those lineages still remains unclear.

The flagellar apparatus is relatively uniform within members of the Stramenopiles, despite the very high level of diversity in morphology, behavior and modes of nutrition, except for the modifications found in slopalinids and *Blastocystis*. Stramenopiles typically possess four microtubular roots that radiate from two basal bodies. The free-living *Cafeteria* (Bicosoecida), however, has two roots, R1 and R2, associated with the posterior B1 and only one root, R3, associated with the anterior B2; this lineage has lost R4. Cytoplasmic microtubules extend from R3 to support the dorsal side of the cell (Fig. 4a). R2 divides into two subroots that reinforce a feeding pocket used in bacterivory [45]. The flagellar apparatus in the zoospore of the parasitic *Phytophthora* (Fig. 4b) is very similar to the flagellar apparatus in *Cafeteria,* the zoospores of labyrinthulids (e.g., *Thraustochytrium*) and most photosynthetic stramenopiles. R1 and R2 originate from the posterior B1 and extend posteriorly to the left and right sides of a ventral furrow, respectively [1]. The zoospores of *Thraustochytrium* do not perform phagotrophy and do not have a cytostome; however, the zoospores still possess an R2 that is divided into two subroots [1]. An anteriorly directed R3 from the anterior B2 supports an array of cytoskeletal microtubules, which is a common configuration in stramenopiles.

In slopalinids, *Proteromonas* with two flagella and *Karotomorpha* with four flagella represent the early branching lineages in this group; members with multiple flagella and multiple nuclei, such as *Opalina* and *Protoopalina*, are more recently derived [28]. Slopalinids have lost the basic flagellar root system and the cytostome found in free-living lineages of stramenopiles. The cone shaped anterior end of *Proteromonas* is supported by microtubules extending from two anterior basal bodies [9] (Fig. 4c); the folded cell surface of *Protoopalina* is supported by rows of basal bodies and fibrous connectives) [46]; *Blastocystis* has completely lost basal bodies and a microtubular cytoskeleton beneath the cell surface.

4. The Myzozoa (Alveolata)

The Myzozoa consists of two major subgroups – dinoflagellates and apicomplexans – plus several other lineages of parasites, symbionts and eukaryovorous flagellates (e.g., *Perkinsus*, *Chromera* and *Colpodella*). Apicomplexans are obligate intracellular parasites, some of which cause major diseases in livestock and humans world-wide. For example, *Plasmodium* spp. (the causative agent of malaria), *Cryptosporidium* spp. and *Toxoplasma gondii* are significant human pathogens. The feature that distinguishes this group from other eukaryotes is the "apical complex", which functions in host cell invasion. The apical complex contains structural elements, such as a polar ring that organizes the subpellicular microtubules, and secretory organelles, called rhoptries and micronemes, that release enzymes during the cell invasion process. The closest free-living relatives of the obligately parasitic apicomplexans are photosynthetic symbionts of corals (e.g., *Chromera*) and predators (e.g., *Colpodella*) [29,37,43]. *Chromera* possesses a functional chloroplast. *Colpodella* is a predatory biflagellate that feeds by sucking out the cell contents from prey cells (i.e., myzocytosis) with a cytoskeletal/secretory apparatus that is homologous to the apical complex in apicomplexans [31,59].
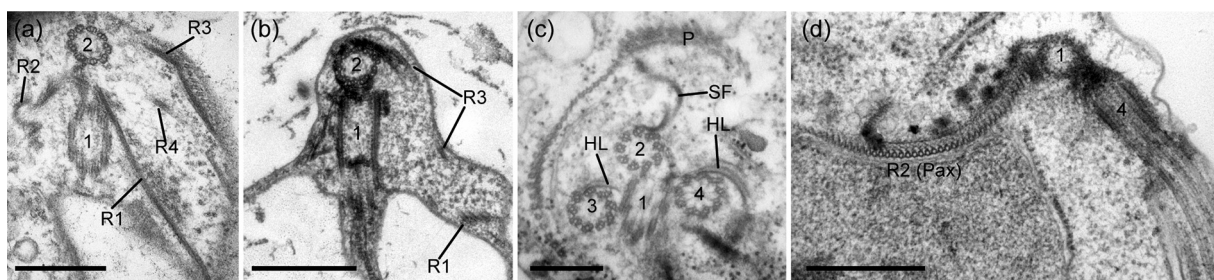
Fig. 3. Transmission electron micrographs showing the basal bodies, major microtubular roots and fibers of the flagellar apparatus in representative lineages of eukaryotes. Scale bars, 500 nm. (a) The Stramenopiles, *Apoikia* (Chrysophyta). (b) The Fornicata, *Kipferlia* (CLO). (c) The Parabasalia, *Pseudotrichomonas* (Trichomonadida). HL, SF and P refer "hooked lamina", "sigmoidal fiber" and "pelta" microtubular rows, respectively. (d) The Preaxostyla, *Monocercomonoides* (Oxymonadida). Pax refers to "preaxostyle".

The diversity and evolution of the cytoskeleton in apicomplexans and their free-living relatives was reviewed recently [43,44]. Except from some microgametes, apicomplexans have essentially lost the flagellar root system altogether [40]. The ultrastructure of microgametes in *Toxoplasma* shows two possible root structures: (1) a posterior root with five microtubules extending from the area between two basal bodies and (2) a row of 15 short microtubules positioned in the anterior end of the cell [47]. The position of the posterior root with five microtubules is most similar to R1 in *Colpodella* and other myzozoan flagellates. There are three microtubular roots in *Colpodella*: a "bypassing" microtubular root, R1 from the posterior B1, and R4 from the anterior B2. The bypassing root connects with the dorsal side of B1. Although this root extends toward the anterior end of the cell, it does not merge with the apical complex. R1 is relatively small and short, and R4 extends to the apical complex [7]. The flagellar apparatuses of *Chromera* and *Colpodella* are similar, but *Chromera* has a short R2 positioned between the two basal bodies. The bypassing root in *Chromera* terminates at the anterior end of the apical complex [50].

Apicomplexans also have a fiber that connects the apical complex to the centrioles and consists of "striated fiber assembly" (SFA) proteins that are essential for cytokinesis and the inheritance of cytoskeletal structures into the daughter cells [20]. SFA proteins were originally described in green algae (i.e., *Chlamydomonas*) as an organizing element connecting the flagellar roots to the basal bodies [73]. However, because apicomplexans with the exception of the microgamates have lost the flagellar apparatus altogether [20], speculated that the ancestral apicomplexan retained the SFA fiber from their free-living biflagellated relatives to organize cell division. Because homologous SFA proteins have been detected in two very distantly related lineages of eukaryotes (i.e., green algae and apicomplexans), these proteins were either inherited from their most recent common ancestor or acquired by a more recent lateral gene transfer event. The SFA fiber in apicomplexans is probably homologous with R4 in *Colpodella* and *Chromera*, so it would be interesting to demonstrate whether R4 and any associated fibers in these lineages contain SFA proteins.

5. The Fornicata (Excavata)

The Fornicata represents one of the major lineages within the much more inclusive Excavata. Fornicates are organized within three assemblages: *Carpediemonas*-like organisms (CLOs), the Retortamonadida (e.g., *Retortamonas*) and the Diplomonadida (e.g., *Giardia*). CLOs represent a paraphyletic group of free-living flagellates that encompasses the most recent ancestor of all fornicates. Retortamonads also represent a paraphyletic/polyphyletic group within fornicates [15,27,63]. Except for one free-living species (i.e., *Chilomastix cuspidata*), retortamonads are either obligate commensals or parasites within the guts of animals. Some members of the Diplomonadida are the causative agents of important diseases

such as the human parasite *Giardia intestinalis* and the fish parasites *Spironucleus* spp.; some diplomonads, however, have become free-living organisms presumably from parasitic ancestors (e.g., *Trepomonas agilis*) [56]. Members of the Diplomonadida have either a single flagellar apparatus and an associated nucleus (single karyomastigont) or, more often, two flagellar apparatuses (usually with eight flagella in total) and two associated nuclei (double karyomastigont). Because both single- and double-karyomastigont organisms are paraphyletic/polyphyletic groups in molecular phylogenetic analyses, the evolution of the karyomastigont cell system is still unresolved [26].

The free-living CLOs have a typical excavate flagellar apparatus with two flagella, which is one of the most complex flagellar apparatuses known and is inferred to approximate the flagellar apparatus in the LECA [68]. The main microtubular roots originate from the posterior B1 and reinforce the ventral feeding groove: R1, R2 and SR (i.e., the singlet root) (Fig. 4d). The cytopharynx at the base of the ventral groove is mainly supported by split R2 roots. The anterior B2 anchors a relatively small R3 that extends to the anterior part of the cell. In retortamonads, four flagella are grouped into two pairs, but the flagellar root system is very similar to the one of free-living CLOs described above [3,6]. The cytoskeletal structure in retortamonads also includes the interlinked microtubular corset positioned underneath the cell membrane [5]. The flagellar apparatus of diplomonads is much simpler and contains a smaller microtubular root system than those in CLOs and retortamonads. R1, R2 and R3 have traditionally been referred to as the "infranuclear fiber" (Inf), "cytostomal fiber (or funis)" (Cf), and "supranuclear fiber" (Snf), respectively. In diplomonads wherein the flagellar apparatus has doubled to form two "kinetids" (e.g., *Hexamita*), the R1's of each kinetid cross in the middle of the cell and the R3's of each kinetid cross in the anterior region of the cell (Fig. 4e) [11]. The evolution of the flagellar apparatus in the Fornicata appears to be associated with the evolution of a feeding groove on the ventral side of the cell; prominent microtubular roots, R1 and R2 are associated with the large feeding grooves in CLOs and retortamonads, and simpler roots are associated with either feeding pockets (e.g., *Trepomonas*) or feeding tubes (e.g. *Hexamita*) or the absence of a feeding structure (e.g., *Giardia*) in diplomonads.

Giardia intestinalis is a parasitic diplomonad that causes intestinal disease in humans. *Giardia* attaches to the epithelium of the small intestine using a specialized microtubule structure called a "ventral disc" (Fig. 3f). This adhesive disc is supported by a spiral array of parallel microtubules (vdMts) associated with the "median body" (MB), the "funis" and the "supernumerary microtubule array" (snMTs) [18,24] (Fig. 3f). The MB is a semi-organized microtubule array that is positioned on the ventral side of the cells. Although its function is unknown, researches have suggested several different possibilities such as the storage of prepolymerized tubulin for the ventral disc assembly for the next generation or participation in caudal tail flexion [48]. The funis is a sheet of microtubules asso-
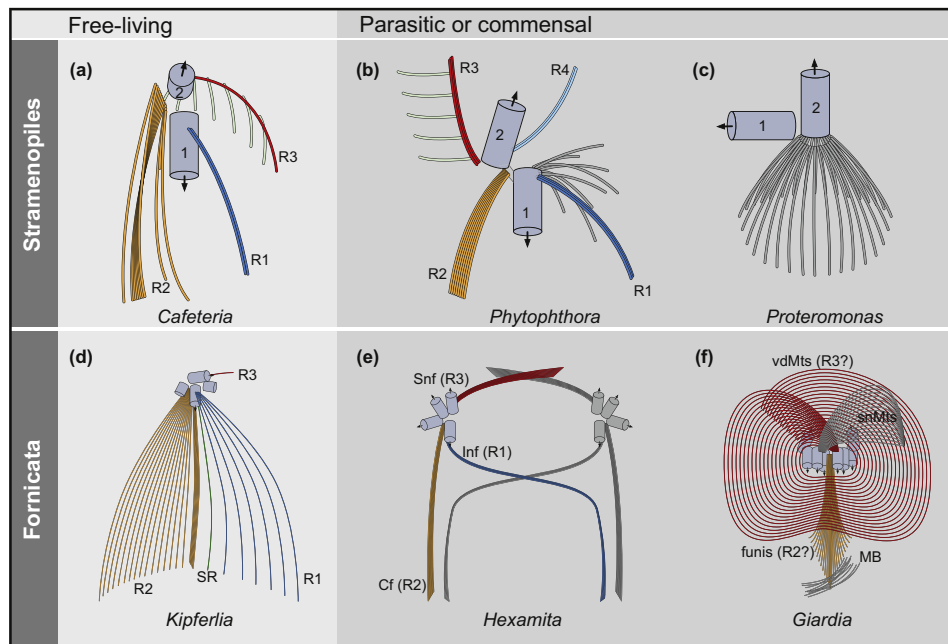
Fig. 4. Illustration of the flagellar apparatus of Stramenopiles (a–c) and Fornicata (d–f). The flagellar roots in the same colors correspond each other. Arrows indicate the direction of flagella. (a) Free-living *Cafeteria roenbergensis* (Bicosoecida), redrawn based on the data in Ref. [45]. (b) Parasitic *Phytophthora parasitica* (oomycetes), redrawn based on the data in Ref. [1]. (c) Endobiotic *Proteromonas lacertae* (Slopalinida), redrawn based on the data in Ref. [9]. (d) Free-living *Kipferlia bialata* ('*Carpediemonas*-like organism', CLO), redrawn based on the data in Ref. [71]. (e) Parasitic *Hexamita* (Hexamitinae, Diplomonadida), redrawn based on the data in Ref. [6]. The Inf, Cf and Snf refer to infranuclear fiber, cytostomal fiber, and supranuclear fiber, respectively. (f) Parasitic *Giardia* (Diplomonadida), redrawn based on the data in Refs. [2,12]. The vdMTs, snMTs and MB refer to ventral disc microtubule array, supernumerary microtubule array and median body, respectively.

ciated with a caudal axoneme and is located on the posterior side of the cell. The snMTs form a partial, left-handed, spiral array that is positioned against the microtubular array that forms the ventral disc (vdMTs). The relationships of these roots to each other and the basal bodies suggest that the funis and the vdMTs are homologous with R2 and R3, respectively, in other diplomonads (and eukaryotes as a whole) (Fig. 4f).

6. The Parabasalia (Excavata)

The Parabasalia contains a morphologically diverse assemblage of mostly parasites/commensals of insects and vertebrates; for instance, trichomonads are relatively small cells ($\sim$10–20 μm) with six or fewer flagella and hypermastigids are very large cells ($\sim$200 μm) with up to thousands of flagella [11]. Hypermastigids play an important role in the digestion of cellulose within the guts of termites and wood eating cockroaches. Some trichomonads are important parasites of animals, including humans (e.g., *Trichomonas vaginalis* and *Tritrichomonas foetus*). The Parabasalia contains very few free-living representatives, and these lineages, have a punctate distribution among parasitic species in molecular phylogenetic analyses [70]; this suggests that the free-living lineages of trichomonads evolved from parasitic ancestors several times independently. Moreover, molecular phylogenetic data also suggest that the hypermastigid cell type evolved multiple times independently from simpler ancestors. Therefore, "trichomonads" and "hypermastigids" represent morphotypes within parabasalids rather than clades [16,41].

The Parabasalia are distinguished from other eukaryotes by the presence of hydrogenosomes, and a "parabasal apparatus" consisting of striated fibers connecting the Golgi body to the flagellar apparatus and the absence of a cytostome. Although some species are aflagellated (e.g., *Dientamoeba*) and some are multiflagellated, the typical arrangement of the parabasalid flagellar apparatus consists of four basal bodies: one directed posteriorly (B1) and three

directed anteriorly (B2–B4). The anteriorly directed basal bodies have distinctive fibrous appendages: B2 has a sigmoidal fiber; B3 and B4 have a hooked lamina (Fig. 3b). The primary cytoskeletal feature composed of microtubules, called the "pelta-axostyle complex", has been inferred to be homologous with the peripheral microtubules of other eukaryotic groups [58]. The pelta is a microtubular array that covers the anterior part of the cell. The axostyle is a sheet of microtubules arranged as either a hollow tube or a cone and reinforces the longitudinal axis of the cell. Many trichomonads have a conspicuous striated fiber, called the "costa" that extends posteriorly from the region containing the basal bodies. The costa is inferred to be homologous with the "C fiber" associated with R1. The C fiber is a thick structure with a multilayered appearance divided by distinct sheets and is commonly found in other excavates, such as jakobids and the free-living *Kipferlia* [60,71]. As reviewed elsewhere, various modifications of this basic cytoskeletal arrangement are found in different lineages of parabasalians [8,11,16].

7. The Preaxostyla (Excavata)

The Preaxostyla is a relatively small group of excavates that contains two subgroups of heterotrophic flagellates living in low oxygen environments: the Oxymonadida and the Trimastigida [58]. The Oxymonadida is a group of either obligate symbionts that live mostly in the hindguts of termites and wood eating cockroaches. Members include the streamlined and relatively tiny (ca. 10 μm) *Monocercomonoides* with four flagella and the relatively large (ca. 200 μm) and multinucleated *Barroella* with numerous axostyles throughout the cell body. Oxymonads are among the most problematic and interesting groups of microbial eukaryotes from an evolutionary perspective. Oxymonads are nested within a paraphyletic assemblage of free-living trimastigids, which brings the Preaxostyla firmly within the Excavata [72].

The free-living *Trimastix* has four basal bodies associated with four flagella. Trimastigids have most of the cytoskeletal traits
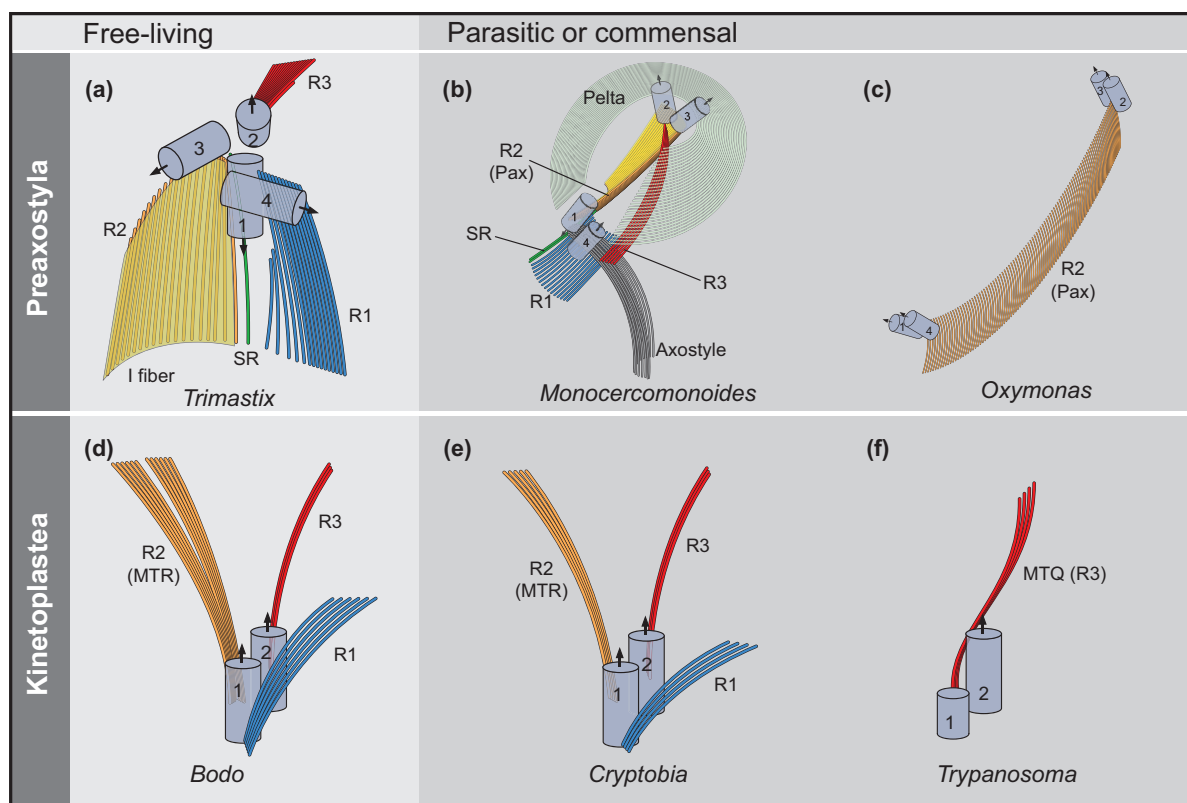
Fig. 5. Illustration of the flagellar apparatus of Preaxostyla (a–c) and Kinetoplastea (d–f). The flagellar roots in the same colors correspond each other. Arrows indicate the direction of flagella. The number of microtubule shown in each cross section for microtubular corset is not significant here. (a) Free-living *Trimastix marina* (Trimastigida), redrawn based on the data in Ref. [62]. (b) Commensal *Monocercomonoides hausmanni* (Oxymonadida), redrawn based on the data in Refs. [51,62,58]. The Pax refers to preaxostyle. (c) Parasitic *Oxymonas* (Oxymonadida), redrawn based on the data in Ref. [10] (d) Free-living *Bodo* spp. (Eubodonida), redrawn based on the data in Refs. [4,55]. The MTR refers to the reinforced microtubular band. (e) Parasitic *Cryptobia iubilans* (Parabodonida), redrawn based on the data in Ref. [42]. (f) Parasitic *Trypanosoma brucei* (Trypanosomatida), redrawn based on the data in Refs. [19,30]. The MTQ refers to the microtubule quartet.

associated with the typical excavate flagellar apparatus like that found in *Kipferlia* and retortamonads (Fornicata): three microtubular roots (R1, R2 and SR) from the posterior B1 and an R3 from the anterior B2 (Fig. 5a). The cytopharynx at the base of the ventral groove in *Trimastix* is mainly supported by two bands of microtubules stemmed from R2. The ventral (or inner) face of R2 near the anterior end of the cell is associated with lattice-like "I fiber" [58].

Oxymonads have a distinctive cytoskeletal organization composed of four flagella organized as two pairs of basal bodies separated by a striated structure called the "preaxostyle". Oxymonads (e.g., *Pyrsonympha*) also have a dynamic "axostyle" composed of an array of parallel and interlinked microtubules that run along the longitudinal axis of the cell. An anterior array of microtubules, called the "pelta", supports the anterior part of cell. Detailed examination of the flagellar apparatus in the oxymonad *Monocercomonoides hausmanni* confirmed that the cytoskeletal organization is very similar to *Trimastix* and other excavates [51,62]. The most posterior basal body (B1) is associated with two microtubular roots: R1 runs posteriorly beneath the posterior flagella (Fig. 5b). Simpson et al. [62] suggested that the preaxostyle complex in *Monocercomonoides* and other oxymonads is homologous to R2 and the "I fiber" in *Trimastix*. The most anterior basal body (previously called B4) is actually B2 and is associated with the posteriorly directed R3 (previously called R2) from which the microtubules of the pelta extend [58].

Some termite symbionts (e.g., *Oxymonas* and *Pyrsonympha*) attach to the intestinal wall of their hosts by a holdfast structure consisting of "microfibrils". *Oxymonas*, for instance, has a long anterior "rostellum" that is supported by microtubules and terminates

in the holdfast [10]. This ribbon of microtubules runs though the entire cell body from the anterior holdfast to the posterior end of the cell. The flagellar apparatus of *Oxymonas* is therefore very divergent; the only remnant of the excavate configuration of microtubular roots in this genus is a potential homolog of R2, namely the preaxostyle that separates the two pairs of basal bodies (Fig. 4c).

8. The Kinetoplastea (Excavata)

The Kinetoplastea is one of four major subgroups within the Euglenozoa (Excavata), all of which share three distinguishing cytoskeletal traits: paraflagellar rods within the flagella, tubular extrusomes and a conserved flagellar root system [57]. Most lineages in the Euglenozoa are free-living (e.g., euglenids, symbiontids and diplonemids), but parasitic lifestyles evolved several times independently within the Kinetoplastea [34,61]. A shared feature of kinetoplastids is a distinctive mitochondrial inclusion called a "kinetoplast", which is a condensed mass of DNA (i.e., kDNA). Although many lineages of kinetoplastids are parasites of animals, the most recent ancestor of all kinetoplastids was clearly free-living, like the vast majority of other euglenozoans. However, prokinetoplastids infect a wide range of marine and freshwater fish and represent some of the earliest diverging lineages within the Kinetoplastea in molecular phylogenetic trees. The Eubodonida, Neobodonida and Parabodonida are mainly free-living flagellates that play important roles in aquatic ecosystems as consumers of bacteria and detritus. Many members of *Bodo* are common, abundant and cosmopolitan. A few lineages of bodonids, such as *Cryptobia* and *Trypanoplasma*, parasitize commercially important fish [65]. Trypanosomatids, by contrast, are obligate parasites of

animals, including humans. *Trypanosoma brucei*, for example, is among the best-studied kinetoplastids because it is the causative agent of African sleeping sickness.

The general arrangement of the flagellar apparatus in the Euglenozoa is remarkably similar, consisting of two parallel basal bodies and three asymmetrically distributed microtubular roots. Many taxa have a microtubular corset that supports the cell surface. Previous studies of euglenozoan ultrastructure named the three microtubular roots according to their position in the cell; for example, the microtubular roots on the dorsal side, ventral side and middle of the cell were called the "dorsal root", "ventral root" and "intermediate root", respectively. It is more consistent to instead apply the universal terminology used for the eukaryotic flagellar apparatus to the euglenozoan root system as recommended by Yubuki et al. [71]: the "intermediate root" is R1, the "ventral root" is R2, and the "dorsal root" is R3.

The free-living biflagellate *Bodo* (eubodonid) and the fish parasite *Cryptobia* (parabodonid) share the typical microtubular root system of euglenozoans [12,42] (Fig. 5d and e). The R2 microtubules in kinetoplastids support the feeding apparatus (if present) and form a bundle called the "reinforced microtubular band" (MTR). The parasitic trypanosomatids have a significantly reduced flagellar apparatus consisting of only one flagellum stemming from the anterior B2. The highly reduced posterior B1 is called the "protobasal body". All of the cytoskeletal elements, which are normally associated with the posterior B1 are absent in trypanosomatids. Like in other eukaryotes, R3 is associated with B2 in trypanosomatids, but it forms a bundle of only four microtubules, called the "microtubule quartet" (MTQ), which is a key feature of trypanosomatid cells and the only remnant of R3 [19,30] (Fig. 5f). *T. brucei*, in particular, was shown to have a distinctive hairpin-shaped "bilobe" structure that is associated with the MTQ (R3), contains a specific isoform of centrin (TbMORN) and wraps around the flagellar pocket [19,38]. A similar cytoskeletal element, called the "pellicle microtubule organizing center" (pMTOC), was inferred to play a critical role in the organization and inheritance of the complex system of pellicle strips in euglenids [67], which are close relatives to kinetoplastids. It is possible that the proposed pMTOC is homologous with the bilobe structure in trypanosomatids and was therefore present in the most recent ancestor of all euglenozoans. This hypothesis can be tested by showing that the same isoform of centrin, found in the bilobe structure of *T. brucei*, is also present in a similar structure in euglenids (i.e., a pMTOC).

9. Independent streamlining and complexity in parasitic protists

Major modifications of the microtubule root system in parasitic protists reflect adaptations associated with different mode of motility, attachment and nutrition within different host compartments. On one hand, some parasitic protists have independently evolved complex structures to survive within different host environments (e.g., the ventral disc in *Giardia*, the rostellum in *Oxymonas*, and the apical complex in *Toxoplasma*). On the other hand, the evolutionary transition from free-living lifestyles to parasitic lifestyles in different lineages of protists has involved the progressive reduction of the ancestral feeding apparatus and associated microtubular roots. Typical traits found in free-living predatory flagellates, such as a split R2 that reinforces a ventral feeding groove, is generally lost in parasitic lineages (except retortamonads); however, remnants of the R2 remain in many parasitic lineages such as in oomycetes, diplomonads and kinetoplastids. Free-living protists usually have an R3 that functions as the organizing center (MTOC) for the microtubules that support the dorsal surface of the cell, called a "corset", a "dorsal fan" or "cytoplasmic microtubules". Many par-

asitic protists have lost R3 but still retain an array of cytoplasmic microtubules under the cell surface (e.g., kinetoplastids, opalinids and retortamonads). Therefore, a general trend in the evolution of the microtubular cytoskeleton in different groups of parasitic protists is the independent loss of traits (i.e., streamlining) present in their free-living ancestors.

Free-living, kinetoplastids, for instance, have three microtubular roots (R1, R2 and R3) and two flagella associated with two parallel basal bodies (B1 and B2), while trypanosomatids only have R3 and a single anterior directed flagellum associated with one mature basal body (B2). Although the zoospores of oomycetes have a flagellar apparatus that is similar to free-living stramenopiles (e.g., bicosoecids), symbiotic slopalinids and the parasitic *Blastocystis* have lost the stramonopile-type microtubular root system altogether. Moreover, aside from some reduced microgametes, apicomplexans have essentially lost the flagellar apparatus from their free-living (myzocytosis-feeding) ancestors.

By contrast, endobiotic retortamonads (e.g., *Retortamonas*) and some parasitic diplomonads (e.g., *Hexamita* and *Spironucleus*) have retained the excavate-type microtubular root system found in their free-living ancestors. Other parasitic diplomonads (e.g., *Giardia*) have substantially increased the complexity of microtubular structures with new innovations that facilitate their parasitic lifestyles. This added complexity is built upon homologs of the ancestral roots (e.g., R2 and R3) [6,58], but genomic data suggest that certain traits (e.g., the ventral disc in *Giardia*) are built from proteins that do not have homologs with known cytoskeletal proteins [22]. Other very different lineages of parasites, such as hypermastigid parabasalids, oxymonads and opalinids, thrive within animal guts and have increased cytoskeletal complexity by multiplying a flagellar mastigont system. These lineages have much larger cells than their free-living ancestors and have up to thousands of flagella that generate currents to bring nutrients across the surface of their cells.

The most recent common ancestor of eukaryotes had a complex (excavate-type) flagellar apparatus that was independently streamlined in several different ways across the tree of eukaryotes [68]. This general pattern of streamlining is consistent with the independent evolutionary transitions to parasitic lifestyles from free-living ancestors representing very distantly related groups of eukaryotes. These patterns of ultrastrutural streamlining are also consistent with the reoccurring loss of functional redundancy in the genome of several different groups of parasitic organisms, such as in microsporidians, *Ichthyophthirius*, *Helicosporidium* and *Mycoplasma* [17,21,25,49,64]. Nonetheless, detailed comparisons of the flagellar apparatus in microbial eukaryotes remain difficult because the number of organisms that have been adequately described at the ultrastructural level is relatively small compared to the total number of known species. Improvements in our overall understanding of evolutionary parasitology will depend on continued efforts to discover and characterize both parasitic species and their nearest free-living relatives using both culture-dependent and culture-independent methods.

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