

## A GLORIOUS HALF-CENTURY OF MICROTUBULES

# Evolution of microtubule organizing centers across the tree of eukaryotes

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## SUMMARY

The architecture of eukaryotic cells is underpinned by complex arrays of microtubules that stem from an organizing center, referred to as the MTOC. With few exceptions, MTOCs consist of two basal bodies that anchor flagellar axonemes and different configurations of microtubular roots. Variations in the structure of this cytoskeletal system, also referred to as the 'flagellar apparatus', reflect phylogenetic relationships and provide compelling evidence for inferring the overall tree of eukaryotes. However, reconstructions and subsequent comparisons of the flagellar apparatus are challenging, because these studies require sophisticated microscopy, spatial reasoning and detailed terminology. In an attempt to understand the unifying features of MTOCs and broad patterns of cytoskeletal homology across the tree of eukaryotes, we present a comprehensive overview of the eukaryotic flagellar apparatus within a modern molecular phylogenetic context. Specifically, we used the known cytoskeletal diversity within major groups of eukaryotes to infer the unifying features (ancestral states) for the flagellar apparatus in the Plantae, Opisthokonta, Amoebozoa, Stramenopiles, Alveolata, Rhizaria, Excavata, Cryptophyta, Haptophyta, Apusozoa, *Breviata* and Collodictyonidae. We then mapped these data onto the tree of eukaryotes in order to trace broad patterns of trait changes during the evolutionary history of the flagellar apparatus. This synthesis suggests that: (i) the most recent ancestor of all eukaryotes already had a complex flagellar apparatus, (ii) homologous traits associated with the flagellar apparatus have a punctate distribution across the tree of eukaryotes, and (iii) streamlining (trait losses) of the ancestral flagellar apparatus occurred several times independently in eukaryotes.

**Keywords:** biodiversity, cytoskeleton, evolution, excavates, flagellar apparatus, protists.

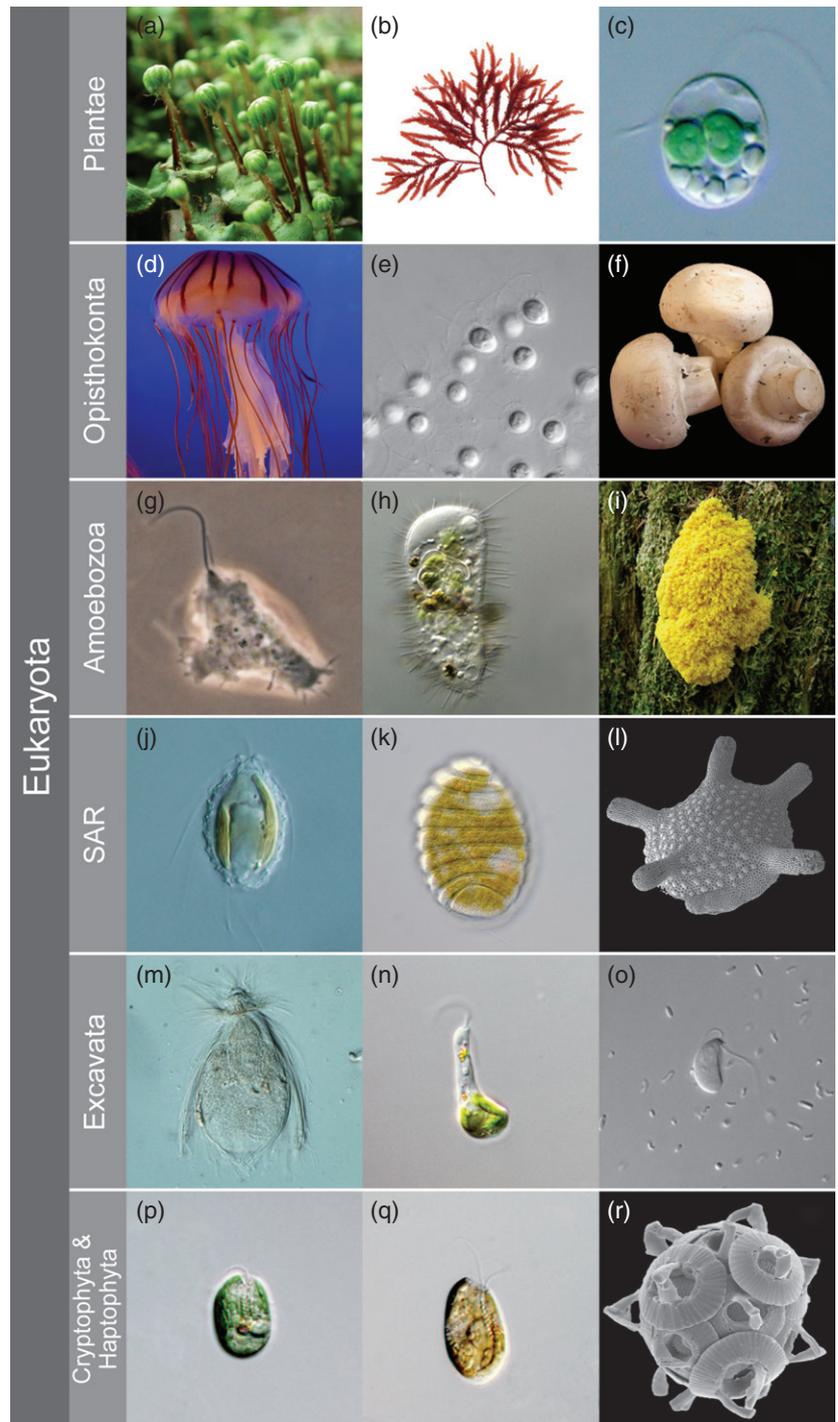
## A BRIEF OVERVIEW OF EUKARYOTIC DIVERSITY

Eukaryotes that are not plants, animals or fungi, the so-called 'protists', are much more diverse than is usually appreciated, particularly in regard to total species numbers and the ultrastructural and genomic complexity of their cells (Ishida *et al.*, 2010; Parfrey *et al.*, 2010; Adl *et al.*, 2012). A comprehensive tree of eukaryotes with resolved phylogenetic relationships among dozens of very different lineages is beginning to emerge through the rapid accumulation and comparative analysis of genomic data (e.g., Parfrey *et al.*, 2010; Burki *et al.*, 2012; Brown *et al.*, 2012). This phylogenetic context provides the foundation for inferences about the evolution of the eukaryotic cytoskeleton. The most widely accepted tree of eukaryotes currently consists of several minor groups, without a clear sister lineage (the

Apusozoa, *Breviata*, Collodictyonidae, Cryptophyta and Haptophyta) and five major clades or 'supergroups' [the Plantae, Opisthokonta, Amoebozoa, SAR (Stramenopiles, Alveolata and Rhizaria) and Excavata] (Kim *et al.*, 2006; Burki *et al.*, 2009; Roger and Simpson, 2009; Ishida *et al.*, 2010; Parfrey *et al.*, 2010; Heiss *et al.*, 2011; Adl *et al.*, 2012; Zhao *et al.*, 2012).

Land plants, animals and fungi are each most closely related to different lineages of single-celled eukaryotes, and are nested within different supergroups. For instance, land plants are nested within the Plantae, which also includes red algae, green algae and glaucophytes (Graham and Wilcox, 2000; Archibald and Keeling, 2004) (Figures 1a–c). Members of this supergroup possess plastids that were derived directly from a cyanobacterium via (primary) endosymbiosis; glaucophytes still possess a cyanobacterial

**Figure 1.** Images representing major lineages of eukaryotes. Some photos are modified with permission from Micro\*scope (c, g, h, k):  
 (a) Marchantiophyta, *Conocephalum*;  
 (b) Rhodophyta, *Phacelocarpus*;  
 (c) Glaucophyta, *Cyanophora*;  
 (d) Scyphozoa, *Chrysaora*;  
 (e) Choanoflagellata  
 (f) Fungi, *Agaricus*;  
 (g) Myxogastria, *Didymium* ('*Hyperamoeba*');  
 (h) Archeamoeba, *Mastigina*;  
 (i) Myxogastria, *Physarum*;  
 (j) Stramenopile, *Mallomonas*;  
 (k) Dinoflagellate, *Polykrikos*;  
 (l) Rhizaria, *Calcarina*;  
 (m) Metamonada, *Trichonympha*;  
 (n) Discoba, *Rapaza*.  
 (o) Metamonada, *Kipferlia*;  
 (p) Cryptophyta, *Chroomonas*;  
 (q) Cryptophyta, *Cryptomonas*;  
 (r) Haptophyta, *Gephyrocapsa*.



cell wall around their plastids (Aitken and Stanier, 1979; Scott *et al.*, 1984). Animals and fungi are both nested within the Opisthokonta, which also includes choanoflagellates and chytrids, among other lineages (Lang *et al.*, 2002) (Figures 1d–f). Most species in this supergroup possess a posterior flagellum that undulates to push the cell forwards (Cavalier-Smith and Chao, 2003a). The nearest sister group to opisthokonts is the Amoebozoa, which con-

tains a diverse assemblage of amoeboflagellates and slime molds (Watkins and Gray, 2008; Shadwick *et al.*, 2009) (Figures 1g–i). The Apusozoa branches as the nearest sister group to a clade consisting of the Opisthokonta and Amoebozoa (a clade that was once recognized as ‘unikonts’; Kim *et al.*, 2006; Cavalier-Smith and Chao, 2010).

Most of the supergroups also contain representatives of what are arguably the three most dissimilar modes of

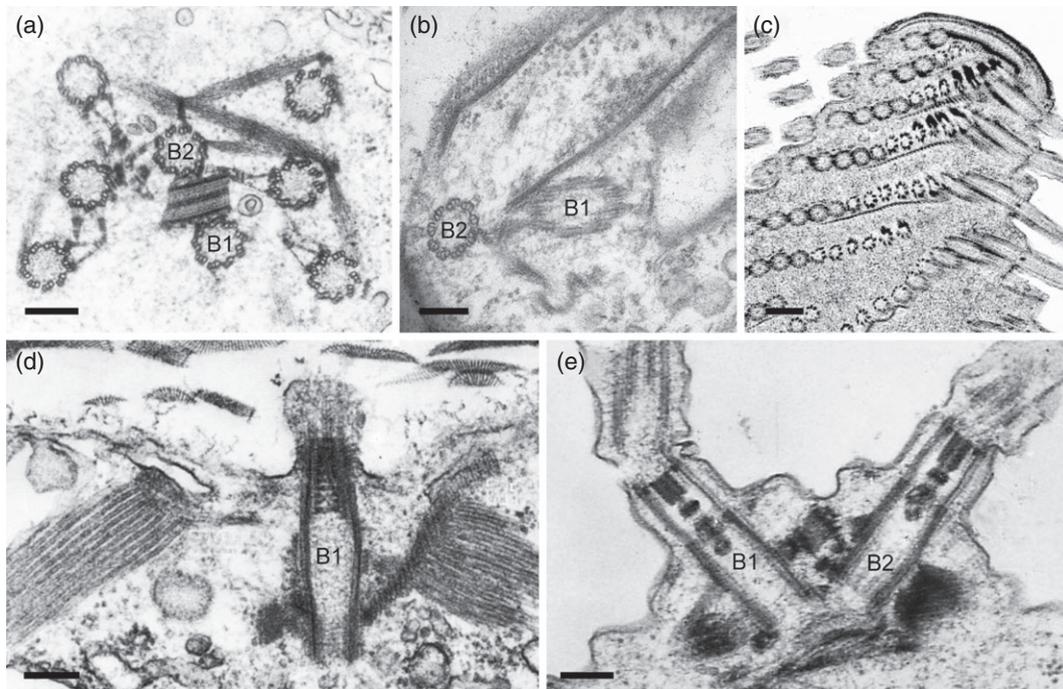
(eukaryotic) life on the planet: photoautotrophs, predators and parasites. For instance, the SAR clade contains plant-like kelps and diatoms, saprophytic water molds (oomycetes), filter-feeding radiolarians, predatory ciliates and the notorious blood-born parasite *Plasmodium* (Patterson, 1989; Cavalier-Smith and Chao, 2003b; Leander and Keeling, 2003; Roger and Simpson, 2009; Burki *et al.*, 2010; Parfrey *et al.*, 2010) (Figures 1j–l). The Excavata contains photosynthetic *Euglena* and relatives, the diarrhea-causing parasite *Giardia*, and several other different lineages of parasites and free-living phagotrophs (Figures 1m–o). There is still debate as to whether or not the Excavata is monophyletic (a group that consists of an ancestor and all of its descendants), an inference that happens to be crucial for understanding the origin and evolution of the eukaryotic cytoskeleton (Simpson and Roger, 2004; Simpson *et al.*, 2006). The synthesis of cytoskeletal traits we provide below suggests that the Excavata is not monophyletic and instead represents a (paraphyletic) stem group from which all other eukaryotes evolved.

#### FUNDAMENTAL FEATURES OF MTOCS: THE FLAGELLAR APPARATUS

Comparative morphology of the microtubular cytoskeleton in eukaryotes started at about the same time the first

images of eukaryotic cells were taken with a transmission electron microscope in the 1960s. The most recent review of the eukaryotic flagellar apparatus in microalgae was published more than a decade ago (Moestrup, 2000); however, a lot has changed since then, especially knowledge about new lineages of eukaryotic cells and where they fit into the overall tree of eukaryotes. In an attempt to understand broad patterns of cytoskeletal homology in eukaryotes, we present a relatively comprehensive overview of the eukaryotic flagellar apparatus within a modern molecular phylogenetic context. We address the known cytoskeletal diversity within the major groups of eukaryotes introduced above in order to infer ancestral traits and subsequent trait changes during the evolutionary history of the flagellar apparatus.

The eukaryotic flagellar apparatus is almost always composed of two (or more) basal bodies that anchor the axonemes, if present, and different microtubular roots; the system functions as the MTOC for the entire eukaryotic cell (Moestrup, 1982; Sleight, 1988; Andersen *et al.*, 1991; Beech *et al.*, 1991; Moestrup, 2000) (Figure 2). The flagellar apparatus is therefore a complex ultrastructural system in almost every eukaryotic cell, and plays vital roles in a variety of cell functions, such as locomotion, feeding



**Figure 2.** Transmission electron microscopy (TEM) sections showing the diversity and major components of the flagellar apparatus in five representative lineages of eukaryotes:

- (a) Plantae, *Pyramimonas* ('Prasinophyta'), modified with permission from Hori and Moestrup (1987);  
 (b) Stramenopile, *Apoikia* (Stramenopile);  
 (c) Excavata, *Uteronympha* (Metamonada), modified with permission from Brugerolle (2006b);  
 (d) Haptophyta, *Pleurochrysis*, modified with permission from Inouye and Pienaar (1985);  
 (e) Collodictyonidae, *Collodictyon*, modified with permission from Brugerolle *et al.* (2002). Scale bars: (a, b, d, e) 200 nm; (c) 400 nm.

(phagocytosis), and the formation of microtubular arrays for structural support, vesicle transport and cell division (Moestrup, 1982). The overall architecture of the flagellar apparatus is relatively stable within major taxonomic groups, where only subtle differences in structure tend to exist between closely related lineages (e.g. 'family level' and 'genus level'; Moestrup, 2000; Figures 3–6). The flagellar apparatus represents one of the only ultrastructural systems in eukaryotic cells that is essentially universally distributed, conserved enough to make confident homology statements over large phylogenetic distances and variable enough to discriminate different lineages from one another.

Any attempt to reconstruct the evolutionary history of the flagellar apparatus requires confident homology statements about the relevant traits being compared in different eukaryotes. Therefore, it is important to identify which basal bodies, flagella and microtubular roots are comparable between the species of interest. This is not always straightforward. In *Chlamydomonas*, for instance, two flagella of the same length are inserted in the apical region of the cell; based on gross morphology alone it is impossible to distinguish the two flagella in the right or left orientation of the cell. However, the two basal bodies (and associated flagella and microtubular roots) in the cell have different generational origins (Gely and Wright, 1986; Beech *et al.*, 1988, 1991; Moestrup, 2000). One basal body is younger (basal body 2 or B2), and is formed anew during the most recent round of cell division; the other basal body is older (basal body 1 or B1), and is formed during an earlier round of cell division and is inherited from the parent cell. Therefore, the basal bodies and associated flagella in each cell reflect different generations (or cell division events), and are inherited in a semi-conservative pattern much like DNA replication. If we know which of the two basal bodies is oldest (B1), then we can determine a right or left orientation of the cell and identify the four different microtubular roots (R1, R2, R3 and R4) associated with the basal bodies (Figure 3a). R1 and R2 are associated with the older B1; R3 and R4 are associated with the younger B2. The microtubular roots are also distinguished from one another using their relative positions in the cell, their point of insertion onto the basal bodies, and their affiliations with other cell features. In green algae, for instance, the positions of the eyespot and the mating structure are closely associated with R4 and R2, respectively (Beech *et al.*, 1988, 1991) (Figure 3a). This approach allows us to identify homologous structures in the eukaryotic flagellar apparatus that can be compared across the tree of eukaryotes; however, reconstructions and subsequent comparisons of the flagellar apparatus are challenging, because these studies require sophisticated microscopy, spatial reasoning and the consistent usage of detailed terminology.

## THE DIVERSITY OF MTOCS REFLECTS THE MAJOR GROUPS OF EUKARYOTES

Comparisons of MTOCs in different groups of eukaryotes is complicated by the fact that researchers working in different fields (e.g. phycology versus protozoology versus zoology versus botany) have applied different terms and notation to describe components of the flagellar apparatus in their organisms of interest. In order to keep our homology statements as clear as possible, we have applied the terminology recommended by Moestrup (2000) to describe the flagellar apparatus of all the eukaryotic lineages highlighted below.

### Plantae (green algae, land plants and relatives)

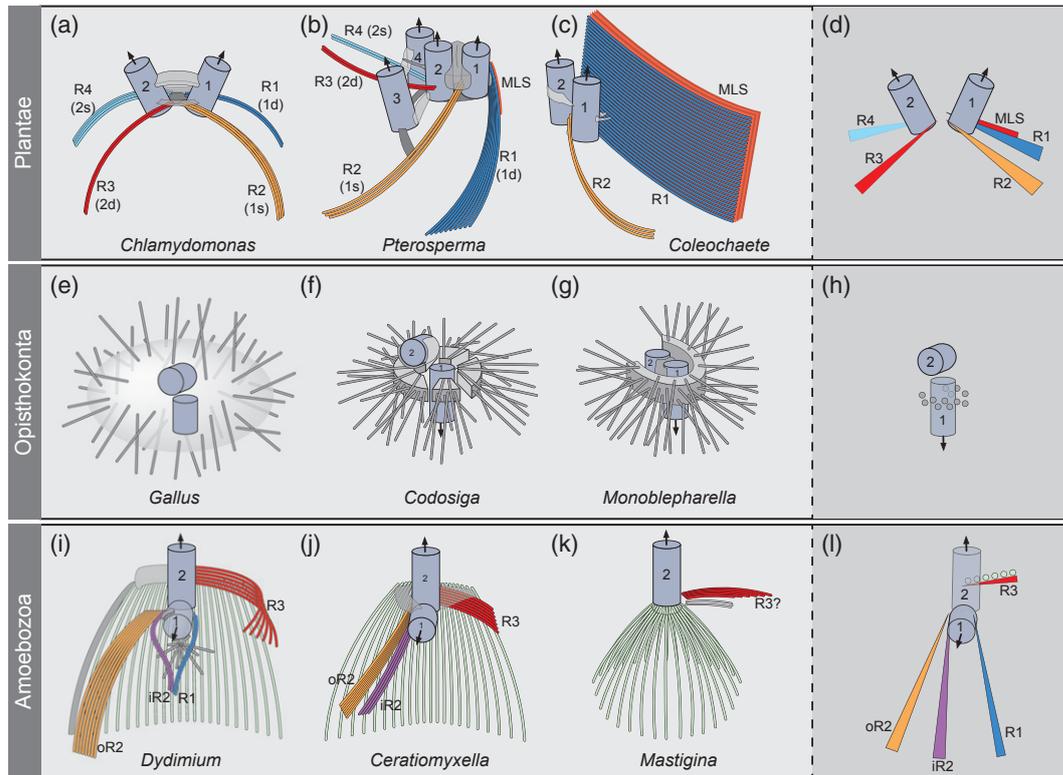
With a few exceptions, members of this clade have a cruciate flagellar apparatus, or some modification thereof (Figures 3a–d). The basic apparatus consists of two basal bodies (B1 and B2), often in a V-like orientation, and four microtubular roots (R1–R4). The traditional notations for the flagellar apparatus in green algae (1d, 1s, 2d, 2s or X-2-X-2) are revised here to R1, R2, R3 and R4, respectively. B2 and its associated R3 and R4 form a unit that is developmentally equivalent to B1 and its associated R1 and R2, but with a 180° rotation (Figure 3a). A distinctive multilayered structure (MLS) of variable size is connected to R1 in certain 'prasinophytes', in the zoospores and sperm of streptophyte algae, and in the sperm of most non-flowering land plants (Moestrup, 1978; Stewart and Mattox, 1978; Melkonian, 1980, 1982; O'Kelly and Floyd, 1983; Vouilloud *et al.*, 2005) (Figures 3b–d). Red algae lack a flagellar apparatus altogether, which is highly unusual for eukaryotes, and knowledge of the glaucophyte flagellar apparatus is currently incomplete (Mignot *et al.*, 1969; Kies, 1979, 1989).

### Opisthokonta (animals, fungi and relatives)

With a few exceptions, members of this clade possess MTOCs consisting of two orthogonal basal bodies (syn., centrioles) within an amorphous matrix (i.e. the centrosome) and a system of radiating microtubules (Figures 3e–h). The centrioles within the centrosome of animal cells are structurally homologous to basal bodies, but no longer organize flagellar axonemes. There are no conspicuous microtubular roots present in this lineage (Hibberd, 1975; Barr, 1981; Karpov and Leadbeater, 1998).

### Amoebozoa

With a few exceptions, members of this clade have three or four robust microtubular roots linked to two basal bodies. B1 anchors R1 and R2, the latter of which is split into an inner ribbon (iR2) and an outer ribbon (oR2). R3 is connected to B2 and functions to anchor a dorsal array of superficial microtubules (Figures 3i–l). Some amoebozoa



**Figure 3.** Illustrations of the flagellar apparatus in the Plantae, Opisthokonta and Amoebozoa. The diversity of the flagellar apparatus within each group of eukaryotes is represented by a row of three different genera and a simpler depiction of the inferred ancestral traits for the group (right-hand column, with darker background). The labels in parentheses (1d, 1s, 2d and 2s) in (a) and (b) refer to the traditional terminology in the Plantae. Arrows indicate the directions of the flagella. (a) Chlorophyta, *Chlamydomonas reinhardtii*, redrawn based on the data in Ringo (1967) and Geimer (2004). (b) 'Prasinophyta', *Pterosperma cristatum*, redrawn based on the data in Inouye *et al.* (1990). (c) Streptophyta, *Coleochaete pulvinata*, redrawn based on the data in Sluiman (1983). (d) The inferred ancestral traits for the flagellar apparatus in the Plantae. (e) Vertebrate interphase cell of *Gallus*, redrawn based on the data in Doxsey (2001). (f) Choanoflagellate, *Codosiga botrytis*, redrawn based on the data in Hibberd (1975). (g) Fungus, *Monoblepharella*, redrawn based on the data in Barr (1978, 1981). (h) The inferred ancestral traits for the flagellar apparatus in the Opisthokonta. (i) Myxogastria, *Didymium dachnaya*, redrawn based on the data in Walker *et al.* (2003). (j) Schizoplasmodiida, *Ceratiomyxella*, redrawn based on the data in Spiegel (1981). (k) Archamoebae, *Mastigina hylae*, redrawn based on the data in Brugerolle (1991). (l) The inferred ancestral traits for the flagellar apparatus in the Amoebozoa.

ans, like most mastigamoebids and uniflagellate myxogastriid slime molds, have lost B1 and its associated microtubular roots (Brugerolle, 1991; Karpov and Myl'nikov, 1997; Walker *et al.*, 2003) (Figure 3k).

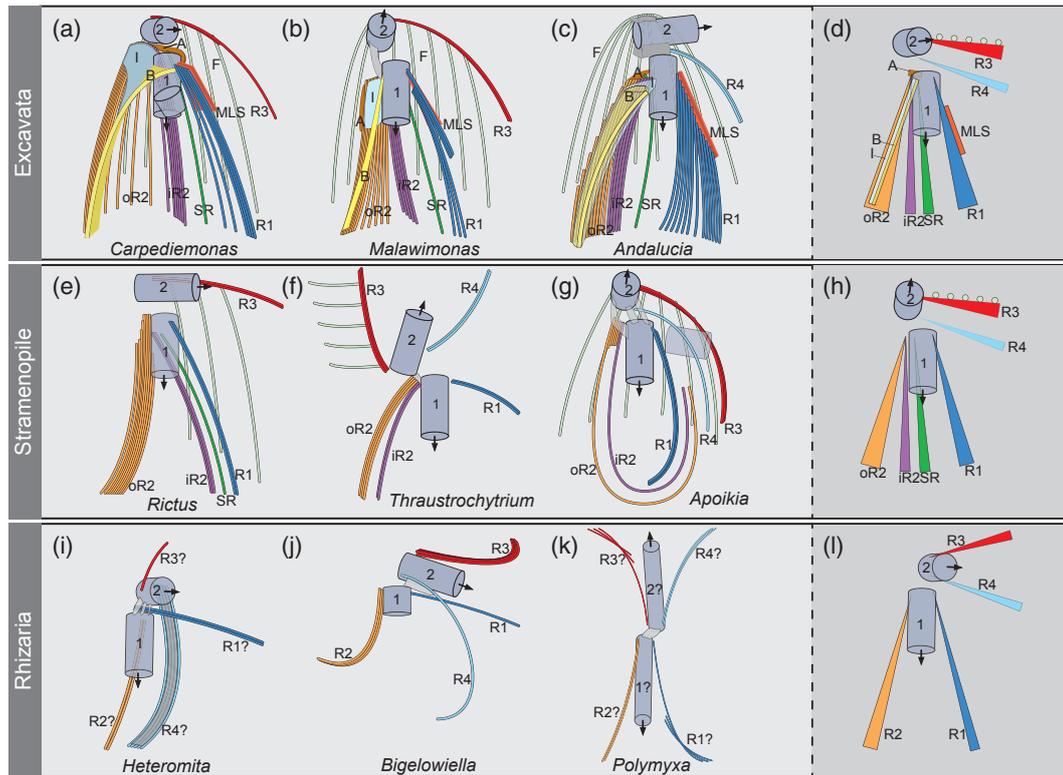
### Excavata

The most complicated flagellar apparatus known is common in a variety of tiny (<10  $\mu\text{m}$ ) bacterivorous excavates (Figures 1o and 4a–d). B1 not only anchors R1, SR and R2, split into iR2 and oR2, but also four distinct fibers: A, B, C and I fibers. B2 anchors R3, which supports a dorsal fan of superficial microtubules (Figures 4a,b), and in some taxa (e.g. *Andalucia*), R4 extends from the posterior side of B2 within the space between B1 and B2 (Figures 4c,d). The C fiber is attached to the dorsal side of R1 and is therefore equiv-

alent to what we have already recognized as the MLS in other taxa (e.g. the Plantae, Figures 3b–d). The I fiber has a cross-hatched appearance and is ventrally located on the concave side of R2. The separation of R2 into iR2 and oR2 ribbons supports the two sides of a ventral feeding groove (Simpson and Patterson, 2001; Simpson, 2003; Simpson and Roger, 2004; Yubuki *et al.*, 2007, 2013a) (Figures 1o and 4a–d).

### Stramenopiles

Members of this clade are extremely diverse in morphology and size (ranging from a few  $\mu\text{m}$  in some flagellates to over 50 m in kelps), and have diverse modes of nutrition, including phototrophy, mixotrophy, phagotrophy, osmotrophy and parasitism. Despite this diversity, the structure of the flagellar apparatus has remained remark-



**Figure 4.** Illustrations of the flagellar apparatus in the Excavata, Stramenopiles and Rhizaria. The diversity of the flagellar apparatus within each group of eukaryotes is represented by a row of three different genera and a simpler depiction of the inferred ancestral traits for the group (right-hand column, with darker background). Arrows indicate the directions of flagella.

- (a) Metamonada, *CarpEDIEMONAS membranifera*, redrawn based on the data in Simpson and Patterson (1999).  
 (b) *MalawIMONAS jakobiformis*, redrawn based on the data in O'Kelly and Nerad (1999).  
 (c) Discoba, *ANDALUCIA (Jakoba) incarcerata*, redrawn based on the data in Simpson and Patterson (2001).  
 (d) The inferred ancestral traits for the flagellar apparatus in the Excavata.  
 (e) Bicosoecida, *RICTUS lutensis*, redrawn based on the data in Yubuki *et al.* (2010).  
 (f) Labyrinthulomycetes, *ThraUSTROCHYTRIUM aureum*, redrawn based on the data in Barr and Allan (1985).  
 (g) Chrysophyceae, *APOIKIA lindahlII*, redrawn based on the data in Kim *et al.* (2010).  
 (h) The inferred ancestral traits for the flagellar apparatus in the Stramenopile.  
 (i) *Heteromita* sp., redrawn based on the data in Karpov (1997).  
 (j) Chlorarachniophyta, *BIGELOWIELLA natans*, redrawn based on the data in Moestrup and Sengco (2001).  
 (k) Endomyxa, *POLYMYXA graminis*, redrawn based on the data in Barr and Allan (1982).  
 (l) The inferred ancestral traits for the flagellar apparatus in the Rhizaria.

ably uniform throughout the clade (Patterson, 1989; Andersen, 1991, 2004; Moestrup and Andersen, 1991; Moestrup, 2000). B1 anchors R1 and R2, and as in excavates, the separation of R2 into iR2 and oR2 ribbons supports the two sides of a ventral feeding apparatus (Figures 4e–h). In early-diverging stramenopiles, like the bicosoecid *Rictus*, B1 also anchors a distinctive root formed from a single microtubule (SR) that is positioned between R1 and R2 (Moestrup and Thomsen, 1976; Karpov *et al.*, 2001; Yubuki *et al.*, 2010) (Figure 4e). B2 anchors R3 and R4, the former of which supports an array of superficial microtubules (Andersen, 1991; Kim *et al.*, 2010).

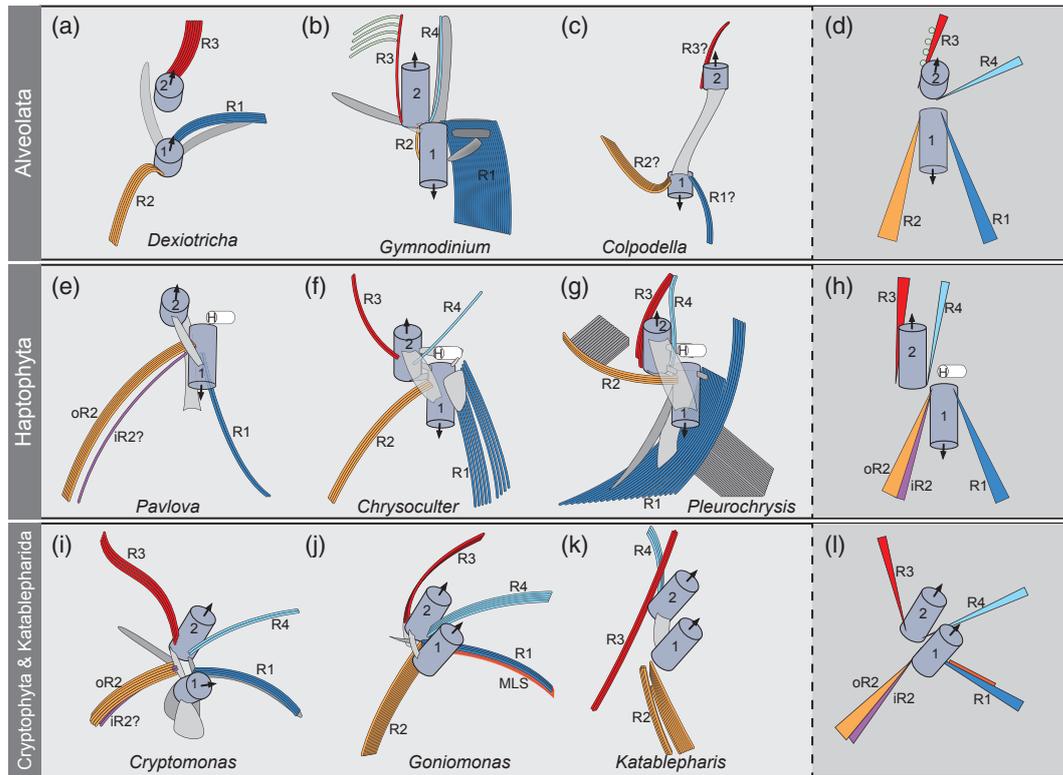
### Rhizaria

This clade consists of many different lineages that are extremely diverse in morphology (e.g. radiolarians, forami-

niferans, chlorarachniophytes, testate amoebae and several kinds of predatory amoeboflagellates), and has therefore been established on the basis of comparative genomic data rather than shared morphological traits. Nonetheless, flagellated members of this clade possess the basic elements of the eukaryotic flagellar apparatus: B1 anchoring R1 and R2, and B2 anchoring R3 and R4 (Karpov, 1997; Moestrup and Sengco, 2001; Cavalier-Smith and Chao, 2003b). R2 is not split into two ribbons, and no SR, MLS, or a superficial array of microtubules associated with R3 is present (Figures 4i–l).

### Alveolata

Members of this clade share several ultrastructural traits (e.g. an arrangement of alveoli beneath the plasma membrane and distinctive micropores), despite the very high



**Figure 5.** Illustrations of the flagellar apparatus in the Alveolata, Haptophyta and Cryptophyta. The diversity of the flagellar apparatus within each group of eukaryotes is represented by a row of three different genera and a simpler depiction of the inferred ancestral traits for the group (right-hand column, with darker background). Arrows indicate the directions of flagella.

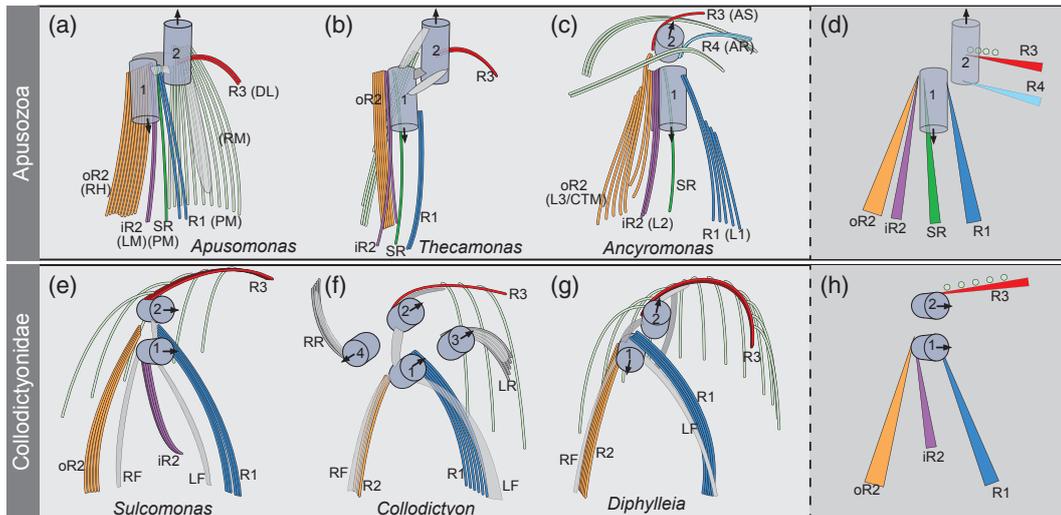
- (a) Ciliate, *Dextiotricha* redrawn based on the data in Peck (1977).  
 (b) Dinoflagellate, *Gymnodinium chlorophorum*, redrawn based on the data in Hansen and Moestrup (2005).  
 (c) Colpodelida, *Colpodella vorax*, redrawn based on the data in Brugerolle (2002b).  
 (d) The inferred ancestral traits for the flagellar apparatus in the Alveolata.  
 (e) Pavlovophyceae, *Pavlova pinguis*, redrawn based on the data in Green (1980).  
 (f) Prymnesiophyceae, *Chrysoculter rhomboideus*, redrawn based on the data in Nakayama *et al.* (2005).  
 (g) Prymnesiophyceae, *Pleurochrysis* sp. (coccolithophorid), redrawn based on the data in Inouye and Pienaar (1985).  
 (h) The inferred ancestral traits for the flagellar apparatus in the Haptophyta.  
 (i) Cryptophyceae, *Cryptomonas ovata*, redrawn based on the data in Perasso *et al.* (1992).  
 (j) Goniomonadea, *Goniomonas avonlea*, redrawn based on the data in Kim and Archibald (2013).  
 (k) Katablepharida, *Katablepharis* sp., redrawn based on the data in Lee *et al.* (1992).  
 (l) The inferred ancestral traits for the flagellar apparatus in the Cryptophyta.

levels of diversity represented in the three major subclades: dinoflagellates, ciliates and apicomplexans. The structure of the flagellar apparatus in these groups and in flagellates such as *Colpodella* and perkinsid zoospores is also very diverse, which makes it difficult to infer the unifying traits of the alveolate MTOC (Figures 5a–d). For instance, apicomplexans are obligate intracellular parasites that have essentially lost the flagellar apparatus altogether (except in some microgametes), whereas ciliates have duplicated the flagellar apparatus to extreme levels, often organizing hundreds of copies over the cell surface. Nonetheless, all four roots, R1–R4, associated with two basal bodies, B1 and B2, are present in at least some members of the Alveolata (Figures 5a–d). Moreover, an array of superficial microtubules extends from R3 in some alveolates, especially in dinoflagellates (Roberts, 1991;

Roberts and Roberts, 1991; Brugerolle, 2002a,b; Myl'nikova and Myl'nikov, 2010).

### Haptophyta

This group of microalgae is unified by a novel cell extension called the haptonema (H), and consists of two main subclades: the Prymnesiophyceae (including coccolithophorids) and Pavlovophyceae (Figure 1r). In prymnesiophyceans (e.g. *Chrysochromulina*, *Chrysoculter* and *Pleurochrysis*), each basal body usually possesses two microtubular roots. In coccolithophorids (*Pleurochrysis*), R1 and R2 organize robust crystalline arrays of microtubules (Figure 5g). B2 anchors R3 and R4, which consist of only a few microtubules (Beech *et al.*, 1988; Birkhead and Pienaar, 1995; Inouye and Pienaar, 1985; Kawachi and Inouye, 1994; Yoshida *et al.*, 2006) (Figures 5f–g). In pavlovo-



**Figure 6.** Illustrations of the flagellar apparatus in the Apusozoa and Collodictyonidae. The diversity of the flagellar apparatus within each group of eukaryotes is represented by a row of three different genera and a simpler depiction of the inferred ancestral traits for the group (right-hand column, with darker background). Arrows indicate the directions of flagella.

(a) Apusomonada, *Apusomonas proboscidea*, redrawn based on the data in Karpov (2007).

(b) Apusomonada, *Thecamonas trahens*, redrawn based on the data in Heiss *et al.* (2013).

(c) Ancyromonada, *Ancyromonas sigmoides*, redrawn based on the data in Heiss *et al.* (2011). Note that Heiss *et al.* (2011) designated the anterior singlet (AS) and the anterior root (AR) of *Ancyromonas* as R4 and R3, which should be reversed as R3 and R4, respectively, based on the locations and directions of their origin on the anterior basal body.

(d) The inferred ancestral traits for the flagellar apparatus in the Apusozoa.

(e) Collodictyonidae, *Sulcomonas lacustris*, redrawn based on the data in Brugerolle (2006a).

(f) Collodictyonidae, *Collodictyon triciliatum*, redrawn based on the data in Brugerolle *et al.* (2002).

(g) Collodictyonidae, *Diphyllia rotans* (= *Aulacomonas submarina*), redrawn based on the data in Brugerolle and Patterson (1990).

(h) The inferred ancestral traits for the flagellar apparatus in the Collodictyonidae.

phyceans, B1 anchors R1 and R2, the latter of which is separated into two ribbons; B2 lacks microtubular roots (R3 and R4) altogether (Green, 1980) (Figures 5e–h).

### Cryptophyta and Katablepharida

Members of these groups (Figures 1p–q) constitute a relatively unified clade of microalgae that possess a flagellar apparatus with two basal bodies, B1 and B2, and four microtubular roots, R1–R4. The previously recognized striated root-associated microtubules (SRm) are inserted on the dorsal side of B1, and are therefore recognized here as R1 (Figures 5i–l). The previously recognized ‘rhizostyle’ is a bundle of microtubules running longitudinally from the ventral side of B1, and is therefore recognized here as R2 (Moestrup, 2000). The band of microtubules that inserts on the dorsal side of B2 is recognized here as R3; the band of microtubules that inserts on the ventral side of B2 is recognized here as R4 (Roberts, 1984; Perasso *et al.*, 1992; Kim and Archibald, 2013) (Figure 5i–l). In early diverging cryptomonads, like *Goniomonas*, and katablepharids, the equivalent of the MLS described in other taxa is present (Kim and Archibald, 2013; Lee *et al.*, 1992) (Figure 5k).

### Apusozoa

The monophyly of apusomonads and ancyromonads is still somewhat controversial, but we consider ancyromonads

members of the Apusozoa based on phylogenetic inferences derived from small subunit rRNA gene sequences and the presence of a theca (Cavalier-Smith and Chao, 2003a). The overall architecture of the flagellar apparatus is complicated, and is most similar to the flagellar apparatus in excavates (e.g. *Carpodimonas* and *Malawimonas*) and stramenopiles (e.g. *Rictus* and *Apoikia*). B1 anchors R1, SR and R2, and the separation of R2 into the iR2 and oR2 ribbons supports the two sides of a ventral feeding apparatus (Figures 6a–d). An MLS attached to R1 is not present. B2 anchors R3 and sometimes an R4 (e.g. *Ancyromonas*), the former of which supports an array of superficial microtubules (Figures 6c,d).

### Collodictyonidae

This is a small but distinctive group of microbial eukaryotes with flagellar apparatuses that are reminiscent of those found in excavates, apusomonads and stramenopiles. B1 anchors R1 and R2, and the separation of R2 into the iR2 and oR2 ribbons supports the two sides of a ventral feeding apparatus (Figures 6e–h). An MLS attached to R1 is not present. B2 anchors R3, which supports an array of superficial microtubules. There is no R4 or SR associated with B2 (Brugerolle, 2006a; Brugerolle and Patterson, 1990; Brugerolle *et al.*, 2002; Cavalier-Smith and Chao, 2010). The so-called left and right fibers (LF and RF) are

associated with R1 and R2, respectively, and support the left and right margins of the ventral feeding groove (Figures 6e–h). Two extra basal bodies, B3 and B4, are present in the tetraflagellate *Collodictyon*; B3 and B4 anchor the left root (LR) and the right root (RR), respectively, both of which are the developmental equivalent of R2 in the next generation.

### **Breviata**

This genus includes only one species, *Breviata anathema*, with an unresolved phylogenetic position within the tree of eukaryotes. These cells have four robust microtubular roots linked to two basal bodies. B1 anchors R1 and R2, the latter of which is split into an inner ribbon (iR2) and an outer ribbon (oR2). A distinctive singlet root (SR) is positioned between R1 and R2. R3 is connected to B2 and functions to anchor a dorsal array of superficial microtubules (Walker *et al.*, 2006; Minge *et al.*, 2009).

### **HOMOLOGOUS ELEMENTS IN DIFFERENT MTOCS**

Previous reconstructions and comparisons of the flagellar apparatus were accomplished with a relatively limited comparative context, and accordingly these studies established different descriptive terms for the MTOCs in different groups of eukaryotes. Detailed information about the excavate flagellar apparatus, for instance, was not available until relatively recently (Yubuki *et al.*, 2013a). Our synthesis suggests that the basic architecture of the eukaryotic flagellar apparatus consists of two basal bodies (B1 and B2), four main microtubular roots (R1–R4) and a singlet root originating from B1 (Moestrup, 2000).

### **R1 and associated MLS**

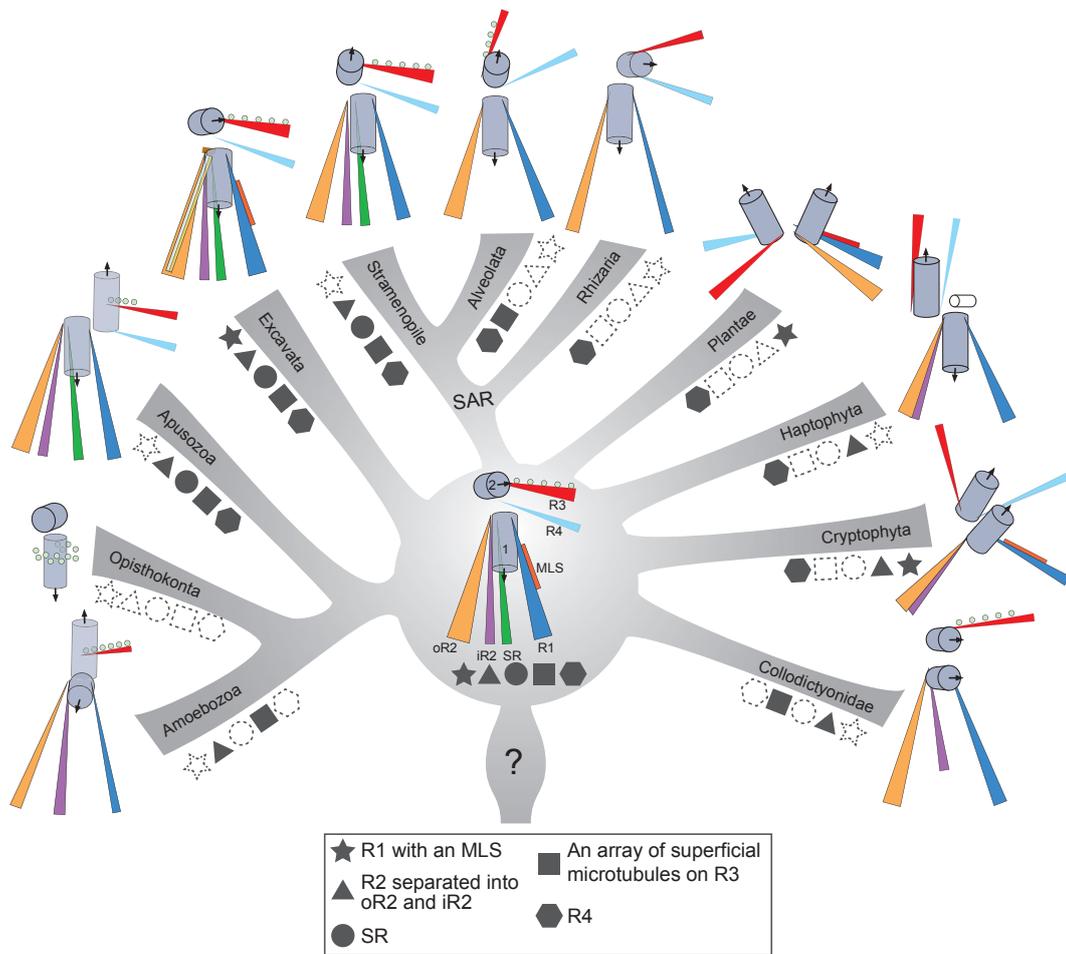
When present, R1 originates from the left side of B1 and extends towards the left side of the cell. R1 is associated with an MLS in the flagellated stages of streptophyte life-cycles (*Coleochaete*, *Chara*, *Klebsormidium* and the sperm of liverworts, mosses, ferns, *Ginkgo* and cycads), and in some prasinophytes (e.g. *Cymbomonas*, *Halosphaera* and *Pterosperma*), which are inferred to have retained many ancestral states for the Plantae as a whole (Carothers and Kreitner, 1967; Moestrup, 1978; Melkonian, 1980; Li *et al.*, 1989; Graham and Wilcox, 2000; Vouilloud *et al.*, 2005; Archibald, 2009). Interestingly, an early diverging streptophyte, *Mesostigma viride*, has two MLSs, one associated with R1 and one associated with R3 (Rogers *et al.*, 1981). R1 and R3 are developmentally equivalent after flagellar transformation, whereby the R3 on B2 in the parent cell transforms into the R1 on B1 in one of the daughter cells after division (Moestrup, 2000). The presence of an MLS on R3 in *M. viride* is inferred to reflect heterochrony, namely a change in developmental timing so that the MLS–R1 association develops prior to

cell division, and the complete transformation of B2 and its roots to B1 and its roots. Nonetheless, the presence of an MLS is a trait shared by chlorophytes, streptophytes and almost certainly the most recent ancestor of green plants (i.e. the Viridiplantae; Lewis and McCourt, 2004; McCourt *et al.*, 2004).

The MLSs are also more broadly distributed across the tree of eukaryotes, such as in excavates, cryptophytes, katablepharids, *Palpitomonas* and some dinoflagellates (Wilcox, 1989; Roberts and Roberts, 1991; Lee *et al.*, 1992; Yubuki *et al.*, 2010; Kim and Archibald, 2013) (Figure 7). The MLS has been labeled as the C fiber in excavates. The C fiber in some excavates, like jakobids, is very well developed, and the potential homology of the C fiber with the MLS in green plants has been discussed previously (O’Kelly, 1993). Furthermore, a remnant of the B fiber in some excavates, like *Dysnectes* and *Kipferlia*, forms a thin sheet-like structure on the ventral side of R1 (Yubuki *et al.*, 2007, 2013a). This structure in excavates is very similar in form and position to the ‘plate-like structure’ described in the prasinophyte *Crustomastix* and the ‘keels’ described in the prasinophyte *Mesostigma* (Melkonian, 1989; Nakayama *et al.*, 2000). A green algal-like MLS has also been reported in other excavates like the euglenozoan *Eutreptiella* (Moestrup, 1978, 1982). Altogether, these data provide evidence that R1 and an associated MLS is homologous in plants and excavates, as well as in cryptophytes and katablepharids, suggesting that these structures were already present in the most recent common ancestor of eukaryotes (Figure 7).

### **R2 facilitates phagotrophy**

The microtubules originating from R2 form a feeding apparatus on the ventral side of the cell in several different groups of protists (Patterson, 1989; Andersen, 1989, 1991; Simpson, 2003; Simpson and Roger, 2004; Heiss *et al.*, 2011, 2013; Yubuki *et al.*, 2009, 2013b). In ‘typical’ excavates, phagotrophy is accomplished between two separate ribbons of microtubules derived from R2: oR2 and iR2 (an outer ribbon and an inner ribbon; Bernard *et al.*, 1997; Simpson *et al.*, 2000; Yubuki *et al.*, 2007, 2013a). Euglenozoans, by contrast, are a very diverse group of atypical excavates with a different kind of feeding apparatus consisting of two robust feeding rods that function together with a system of four vanes in euglenids (Leander *et al.*, 2007). Euglenozoans lack many conserved traits associated with the excavate flagellar apparatus and feeding groove, but the group is strongly affiliated with the excavate concept through their robust molecular phylogenetic relationship with jakobids (Simpson, 2003; Simpson and Roger, 2004). Even though the feeding apparatus in euglenozoans seems fundamentally different from that in typical excavates, the microtubules that support the feeding rods still originate from R2 (synonymous with the ‘ventral root’ in



**Figure 7.** Illustrations of the inferred ancestral traits for the flagellar apparatus in all 11 eukaryotic groups mapped onto a modern molecular phylogenetic context. The five traits mapped onto the tree are represented by different shapes: microtubule root 1 (R1) with a multilayered structure (MLS), R2 involved with phagotrophy, R3 with an array of superficial microtubules, a singlet root (SR) that helps support the ventral feeding groove and R4. Solid shapes refer to the presence of a trait; dashed outlines of shapes refer to the loss of a trait. The inferred ancestral flagellar apparatus for the most recent ancestor of eukaryotes is best represented by the traits found in typical excavates. The most recent common ancestor of the Apusozoa, Opisthokonta and Amoebozoa lost an MLS on R1; R4 was lost in the most recent common ancestor of the Opisthokonta and Amoebozoa. An MLS on R1 was also lost in the most recent common ancestor of the SAR (Stramenopiles, Alveolata and Rhizaria) clade.

euglenozoan notation; Attias *et al.*, 1996; Leander, 2004; Yubuki *et al.*, 2013b).

The functional relationship of R2 microtubules with feeding structures extends to several other groups of eukaryotes, such as stramenopiles, amoebozoans, apusozoans and collodictyonids. Detailed investigations of stramenopile feeding behavior in chrysophycean algae (e.g. *Epipyxis* and *Apoikia*) and deep-branching heterotrophic bicosoecids (e.g. *Rictus*) demonstrate that prey particles (i.e. bacteria) are engulfed via a ‘cytostome’ that is formed by inner and outer microtubules derived from R2 (Andersen and Wetherbee, 1992; Wetherbee and Andersen, 1992; Kim *et al.*, 2010; Yubuki *et al.*, 2010). The separation of R2 into two ribbons, oR2 and iR2, is widely distributed in stramenopiles, and is considered a shared feature for the entire clade (Andersen, 1991; Moestrup and Andersen, 1991). It is worth noting that cross-hatched fibers on the

ventral face of oR2 in some stramenopiles is essentially identical to the position and appearance of the I fiber, present in different lineages of excavates, and a fibrous structure associated with oR2 in apusomonads (Karpov, 2007; Heiss *et al.*, 2011; Yubuki *et al.*, 2010).

Collodictyonids (*Collodictyon*, *Diphyllia* and *Sulcomonas*) also have a robust ventral groove that runs down the longitudinal axis of the cell that functions to phagocytize relatively large food particles. This groove is supported by microtubules derived from both R1 and R2, and highly resembles the feeding grooves of excavates and stramenopiles (Brugerolle and Patterson, 1990; Brugerolle *et al.*, 2002; Brugerolle, 2006a). The flagellar apparatus of excavates and collodictyonids has been compared in detail, and the so-called right ventral root (rvR) and Golgi nucleus root (gnR) of *Sulcomonas* (Brugerolle, 2006a) is inferred to be homologous with the oR2 and iR2 in exca-

vates (Cavalier-Smith and Chao, 2010). The iR2 (synonymous with gnR) in collodictyonids extends towards the interior of the cell, instead of supporting the superficial feeding groove itself (as in excavates and stramenopiles), which facilitates the engulfment of larger prey particles such as other eukaryotic cells (Cavalier-Smith and Chao, 2010).

The R2 in amoebozoans (e.g. *Didymium*), haptophytes (e.g. *Pavlova* and *Diacronema*) and the cryptomonad *Goniomonas* is also separated into an oR2 and iR2; however, whether or not these microtubules function in feeding is unknown (Green and Hibberd, 1977; Green, 1980; Walker *et al.*, 2003). The phagotrophic alveolate *Colponema loxodes* possesses a ventral feeding groove that is supported by two microtubular roots that correspond with R2, a configuration that resembles the MTOCs in excavates and collodictyonids (Mignot and Brugerolle, 1975; Myl'nikova and Myl'nikov, 2010). However, ultrastructural studies of this flagellate are incomplete, so compelling homology statements about the MTOC in *Colponema* would be premature. Although it is possible that similarities in the form and function of R2 microtubules could reflect convergent evolution, there is no supporting evidence for this. The most parsimonious interpretation of current data is that the separation of R2 microtubules into oR2 and iR2 ribbons that support a feeding apparatus represents deep homology that evolved in the most recent common ancestor of excavates, stramenopiles, apusozoans, amoebozoans, collodictyonids, haptophytes, cryptophytes (e.g. *Goniomonas*) and probably alveolates.

### The singlet root

This 'root' is formed by a single microtubule with a distinctive orientation within the overall context of the eukaryotic flagellar apparatus. Although it is identifiable in many different groups of eukaryotes, it is inconspicuous and easily overlooked. Nonetheless, the singlet root (SR) is relatively well studied in excavates, and is considered an important trait that unifies the entire group (Simpson, 2003; Simpson and Roger, 2004). In excavates, stramenopiles (e.g. bicosoecids) and apusozoans, the SR originates on B1 and extends towards the posterior end of the cell, between R1 and R2 microtubules, to support the feeding apparatus (Moestrup and Thomsen, 1976; Karpov *et al.*, 2001; Yubuki *et al.*, 2010; Heiss *et al.*, 2011, 2013). Although the SR has not been fully investigated in all major groups of eukaryotes, the presence of this root in excavates, apusozoans and stramenopiles suggests that it is a homologous trait derived from the most recent common eukaryotic ancestor.

### R3 and arrays of superficial microtubules

Several major groups of eukaryotes have an R3 that originates from basal B2 and curves clockwise towards the anterior end of the cell. R3 functions as the MTOC for an

array of superficial microtubules that shape the cell surface. In excavates, this array of superficial microtubules is called the 'dorsal fan' (O'Kelly, 1993; O'Kelly and Nerad, 1999; Simpson and Patterson, 1999; Simpson, 2003; Simpson and Roger, 2004). R3 in euglenids (corresponding to the 'dorsal root') organizes an array of microtubules, called the 'dorsal band', that ultimately supports the complex and highly distinctive system of pellicle strips (Owens *et al.*, 1988; Yubuki *et al.*, 2009; Yubuki and Leander, 2012). This suggests that the novel flagellar apparatus in englenozoans contains several homologous traits with the basic flagellar apparatus found in typical excavates.

In stramenopiles, R3 (confusingly corresponding to R1 in earlier literature) curves clockwise towards the anterior end of the cell and anchors the superficial microtubules that support the cell shape: this configuration is a unifying feature of the group as a whole, ranging from a diverse assemblage of photosynthetic species to tiny bacteriophage flagellates (Andersen, 1987, 1991; Moestrup and Andersen, 1991; Karpov *et al.*, 2001; Kim *et al.*, 2010; Yubuki *et al.*, 2010). A very similar configuration of R3 microtubules has been described in dinoflagellates (Alveolata), apusozoans, amoebozoans and collodictyonids (Farmer and Roberts, 1989; Brugerolle and Patterson, 1990; Roberts, 1991; Roberts and Roberts, 1991; Spiegel, 1991; Brugerolle *et al.*, 2002; Walker *et al.*, 2003; Karpov, 2007).

### R4

The presence of R4 is widely distributed across the tree of eukaryotes, absent only in opisthokonts, amoebozoans and collodictyonids. This root forms a band of only a few microtubules (less than five) that originate from the ventral side of B2 and extend towards the left side of the cell. Although R4 is common in eukaryotes, its function is not well understood. In stramenopiles, the microtubules of R4 support the left margin of the feeding groove (Kim *et al.*, 2010; Yubuki *et al.*, 2010). It is important to realize that R4 is developmentally equivalent to R2 (on B1); during cell division, B2 with R4 (and R3) in the parent cell transforms into B1 with R2 (and R1) in one of the daughter cells (Moestrup, 2000; Yubuki and Leander, 2012; Yubuki *et al.*, 2013a). Therefore, the presence of R4 microtubules facilitates the future development of the critical R2 microtubules that ultimately support the ventral feeding apparatus in many groups of eukaryotes.

### RECONSTRUCTING THE ANCESTRAL MTOC

The flagellar apparatus of typical excavates has all of the traits present in various combinations in other major groups of eukaryotes: R1 with an MLS; R2 involved with phagotrophy; R3 with an array of superficial microtubules; SR that helps support the ventral feeding groove; and R4. In other words, the excavate flagellar apparatus is a sum-

mation of all of the major components of the flagellar apparatus found in different lineages across the tree of eukaryotes (Figure 7). Several of the phylogenetic relationships between the major clades of eukaryotes (i.e. the deepest nodes in the tree) are still uncertain, and this context is ultimately needed to trace the origins of cellular traits back to the most recent ancestor of all eukaryotes (Figure 7). Nonetheless, the integration of molecular phylogenetic data with comparative ultrastructural data from diverse groups of protists has elucidated many events in the evolutionary history of the eukaryotic cell. As explained more below, the comparative analysis of the flagellar apparatus presented here suggests that the flagellar apparatus found in several living excavates are fantastic examples of morphostasis that approximate the flagellar apparatus present in the most recent ancestor of all eukaryotes (Figure 7).

The monophyly of the Excavata has so far not been supported in molecular phylogenetic analyses (Simpson, 2003; Berney *et al.*, 2004; Simpson and Roger, 2004; Parfrey *et al.*, 2006; Simpson *et al.*, 2006; Ishida *et al.*, 2010). The composition of this putative clade is grounded squarely on comparative ultrastructure and the punctate phylogenetic distribution of tiny flagellates with a remarkably complex and uniform flagellar apparatus and feeding groove (i.e. the typical excavate cytoskeletal configuration). The Excavata consists of three major lineages that are otherwise very different from one another at both the ultrastructural and genomic levels, yet each contain the typical excavate flagellar apparatus: (i) Discoba (Jakobida, Heterolobosea and Euglenozoa), (ii) *Malawimonas*, and (iii) Metamonada (*Trimastix*, *Preaxostyla*, *Parabasalia* and *Fornicata*) (Simpson, 2003; Simpson and Roger, 2004; Simpson *et al.*, 2006; Kolisko *et al.*, 2010). The high level of homology between the typical excavates in each of these otherwise very different lineages provides the primary basis for the hypothesis that they are all part of a monophyletic group: the Excavata.

As described above, however, the flagellar apparatus of typical excavates is very similar to the flagellar apparatus in stramenopiles, apusozoans and collodictyonids (Figure 7). In all of these groups the microtubules of R1 and the two ribbons of R2 (oR2 and iR2) originate from B1 and reinforce the feeding groove; R3 originates from the dorsal side of B2 and supports an array of superficial microtubules on the dorsal side of the cell. This typical excavate architecture is much more similar to stramenopiles, apusozoans and collodictyonids than it is to highly diversified 'excavate' lineages like euglenids and parabasalids. Molecular phylogenetic data are essentially silent on this issue, but indicate that the Apusozoa and Collodictyonidae are only very distantly related to the other eukaryotic supergroups (Kim *et al.*, 2006; Zhao *et al.*, 2012). This suggests that the excavate-like flagellar apparatus is more widely

distributed across the tree of eukaryotes than is currently appreciated (Figure 7).

In fact, the major groups of eukaryotes all have a flagellar apparatus with a combination of components that can be traced back to the excavate configuration. This suggests that the five main components of the flagellar apparatus, when present, are homologous across the tree of eukaryotes, and ultimately descended from an 'excavate-like' common ancestor (Figure 7). This hypothesis: (i) postulates that excavates are not monophyletic unless you synonymize the group with the Eukarya, and (ii) leaves open a very intriguing question – how did the complicated flagellar apparatus of typical excavates evolve in the first place?

### BROAD PATTERNS OF MTOC EVOLUTION: INDEPENDENT STREAMLINING

The diversity of the flagellar apparatus across the tree of eukaryotes is best explained by several independent losses of ancestral, excavate-like traits (Figure 7). For instance, the most recent ancestor of opisthokonts (including animals and fungi) appears to have lost every microtubular root present in the flagellar apparatus of the ancestral eukaryote, retaining only basal bodies B1 and B2 (Roger and Simpson, 2009) (Figure 7).

The most recent ancestor of the Plantae, however, shares R1–MLS and iR4 with excavates, but lost the SR, a branched R2 (oR2 and iR2) and an array of superficial microtubules from R3. The loss of SR, the branched R2 and the associated feeding apparatus in the Plantae is almost certainly correlated with the origin of photosynthesis via primary endosymbiosis; this event changed the mode of nutrition dramatically, which led to new selection pressures that ultimately shaped the evolution of a more streamlined cytoskeleton. By contrast, the most recent ancestor of stramenopiles shares every component of the flagellar apparatus with excavates, except for the loss of an MLS on R1. Although many stramenopiles are photosynthetic via secondary endosymbiosis (e.g. diatoms and brown algae), the most recent ancestor of the clade was probably a tiny bacterivore with a mode of nutrition that is nearly identical to typical excavates. This helps explain why the overall flagellar apparatus is so similar in excavates and stramenopiles, an interpretation that applies to the relatively similar flagellar apparatus found in apusomonads and collodictyonids as well. As illustrated in Figure 7, the most recent ancestor for all of the major lineages of eukaryotes is inferred to have lost some combination of components found in the typical excavate cytoskeletal configuration.

### CONCLUSIONS

The flagellar apparatus is a complex ultrastructural system that is fundamental to the vast majority of eukaryotic cells, conserved enough to infer homology over large phylogenetic distances, and variable enough to distinguish different

clades of eukaryotes from one another. We anticipate that continued effort to discover new species of protists and characterize new configurations of the flagellar apparatus will play an important role in understanding the evolutionary history of eukaryotes, despite the challenges associated with the collection and comparison of these data. It has been more than a decade since the last review of the eukaryotic cytoskeleton was published (Moestrup, 2000), and a lot of new knowledge about the diversity and phylogenetic relationships of eukaryotes has accumulated since. The comprehensive overview of the flagellar apparatus presented here enabled us to postulate that the most recent ancestor of all eukaryotes had a complex flagellar apparatus consisting of the components found in typical excavates: R1 with an MLS, R2 involved with phagotrophy, R3 with an array of superficial microtubules, SR that helps support the ventral feeding groove and R4. This hypothesis is supported by the punctate distribution of these traits across the tree of eukaryotes. Although the evolutionary history that gave rise to the ancestral flagellar apparatus remains elusive, once established, this ancestral flagellar apparatus was independently streamlined several times through the loss of different traits in different lineages of eukaryotes.

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