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Megaphylogeny, Cell Body Plans, Adaptive Zones: Causes and Timing of Eukaryote Basal Radiations[1]

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ABSTRACT. I discuss eukaryote megaphylogeny and the timing of major innovations in the light of multigene trees and the rarity of marine/freshwater evolutionary transitions. The first eukaryotes were aerobic phagotrophs, probably substratum-associated heterotrophic amoeboflagellates. The primary eukaryote bifurcation generated unikonts (ancestrally probably unicentriolar, with a conical microtubular [MT] cytoskeleton) and bikonts (ciliary transformation from anterior cilium to ancestrally gliding posterior cilium; cytoskeleton of ventral MT bands). Unikonts diverged into Amoebozoa with anterior cilia, lost when lobosan broad pseudopods evolved for locomotion, and Choanozoa with posterior cilium and filose pseudopods that became unbranched tentacles/microvilli in holozoa and eventually the choanoflagellate/choanocyte collar. Of choanozoan ancestry, animals evolved epithelia, fibroblasts, eggs, and sperm. Fungi and Ichthyosporea evolved walls. Bikonts, ancestrally with ventral grooves, include three adaptively divergent megagroups: Rhizaria (Retaria and Cercozoa, ancestrally reticulofilose soft-surfaced gliding amoeboflagellates), and the originally planktonic Excavata, and the corticates (Plantae and chromalveolates) that suppressed pseudopodia. Excavata evolved cilia-generated feeding currents for grooval ingestion; corticates evolved cortical alveoli and ciliary hairs. Symbiogenetic origin and transfers of chloroplasts stimulated an explosive radiation of corticates—hard to resolve on multigene trees—and opisthokonts, and ensuing Cambrian explosions of animals and protists. Plantae lost phagotrophy and multiply evolved walls and macroalgae. Apusozoa, with dorsal pellicle and ventral pseudopods, are probably the most divergent bikonts or related to opisthokonts. Eukaryotes probably originated 800–850 My ago. Amoebozoa, Apusozoa, Loukozoa, and Metamonada may be the only extant eukaryote phyla pre-dating Neoproterozoic snowball earth. New subphyla are established for Choanozoa and Loukozoa; Amoebozoa are divided into three revised subphyla, with Variosea transferred into Conosa.

Key Words. Apusozoa, bikonts, Cercozoa, chromalveolates, ciliary gliding, corticates, Excavata, marine/freshwater transitions, pseudopod evolution, unikonts.

GENES and catalysts are just concerned with making the simplest building blocks of life. Lipids and skeletal molecules—proteins, peptidoglycans, and polysaccharides, not nucleic acids, really shape organisms (Cavalier-Smith 1991c, 2001, 2004b). DNA does have a central skeletal role in the cell nucleus (Cavalier-Smith 2005), but skeletal proteins and their specific attachments to membranes and to DNA give a cell its three-dimensional shape. Mutations in structural proteins must therefore be major causes of changes in protist cell body plans. My three decades of contributions to ISEP have emphasized the key importance of ciliary origins and diversification for understanding eukaryote evolution (Cavalier-Smith 1978, 1981, 1986, 1991a, 1997, 1999, 2002, 2003a). Here I paint a simple adaptive picture of the major forces in eukaryote cell diversification, arguing that basic features of eukaryote architecture evolved in cells inhabiting solid surfaces, and that cilia were initially primarily a feeding device, not mainly for locomotion. For brevity, I focus on synthesis and omit historical background; the selective references allow readers to trace the history and evidence for the main ideas and less likely alternatives, and flesh out older arguments in detail.

There are only two eukaryote supergroups: unikonts and bikonts (Cavalier-Smith 2002, 2003b; Richards and Cavalier-Smith 2005; Stechmann and Cavalier-Smith 2003) with contrasting cytoskeletal structure and ciliary development. Ancestrally both probably crawled or glided, respectively, on solid surfaces. The unikont ancestor (Fig. 1) was arguably a uniciliate aerobic amoeboflagellate with one cilium and a single centriole that acted as a focus for a cone of cytoplasmic microtubules, and slender, pointed, possibly branched, pseudopods extended by actin polymerization. It was phagotrophic, with a well-developed Golgi dictyosome; if, as is likely, it dwelt in soil or freshwater, it would also have had a contractile vacuole (CV). By contrast, the bikont ancestor had two cilia, the posterior one probably for gliding on surfaces, and a more asymmetric cytoskeleton of microtubular (MT) bands attached to the centrioles; if marine, it would have lacked a CV. For reasons explained previously (Cavalier-Smith 1982a, 1987, 1992), the first eukaryote to evolve cilia probably had a symmetric MT skeleton and only one centriole, like early unikonts. Whether the last common ancestor of extant eukaryotes was still that simple or had already evolved two centrioles and cilia is unclear, partly because of uncertainty over the phylogenetic position of Apusozoa, a little studied phylum of biciliate gliding protozoa. For recent discussions of the origins of cilia see Jékely and Arendt (2006) and Mitchell (2007); I focus here on subsequent divergences.

UNIKONT MEGAEVOLUTION: PRIMARY DIVERSIFICATION IN BENTHOS AND SOIL

The primary divergence among unikonts is between Amoebozoa and opisthokonts (Choanozoa and their descendants: animals and fungi). All major unikont groups were originally surface associated; the first animals (sponges and Anthozoa) were fixed to the sea floor, whereas most described species of Fungi and Amoebozoa inhabit soil.

Amoebozoa are here grouped in three subphyla of contrasting morphology: Conosa (Mycetozoa, Archamoebae [Cavalier-Smith 1998b], plus Variosea as emended by Smirnov et al., unpubl. observ.); Lobosa (Tubulinea; Discosea as emended by Smirnov et al., unpubl. observ.); and Protamoebae (Cavalier-Smith, Chao, and Oates 2004), now restricted to Breviatea, which multigene trees show are Amoebozoa (Minge et al. 2008), not apusomonads (Walker, Dacks, and Embley 2006). Breviates are mitochondriate amoeboflagellates with a single cilium, as ancestrally were Conosa, whereas Lobosa as now circumscribed are pure amoebae (i.e. never have a cilium). Ancestrally, Amoebozoa were probably uniciliates with irregular long, branched, pointed pseudopods. Such pseudopods now characterize *Breviata*, probably the most divergent amoebozoan, as implied by rRNA trees, and many Conosa (e.g. Varipodida, such as *Filamoeba*, *Acramoeba*

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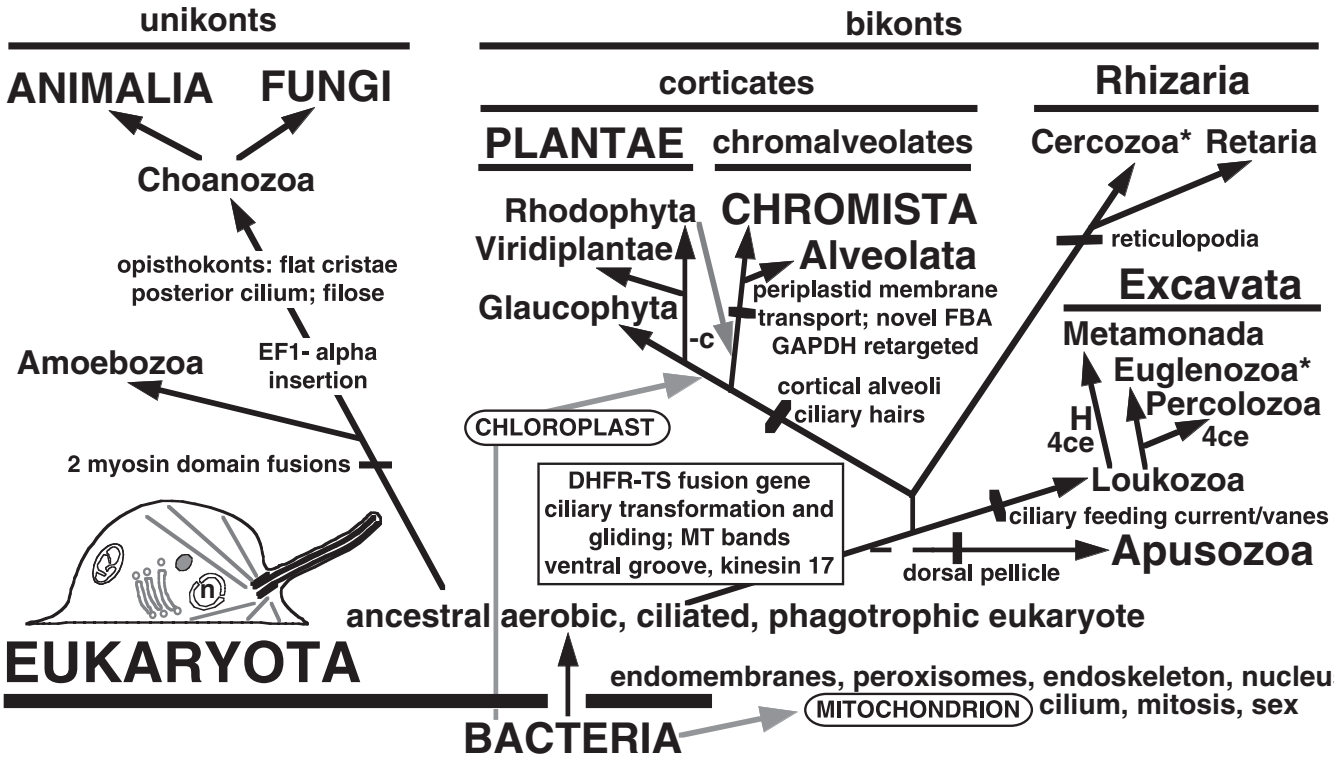

Fig. **1.** Major features of eukaryote cell megaevolution. Grey arrows show symbiogenetic events; asterisks indicate secondary symbiogenetic implantation of viridiplant plastids into ancestors of chlorarachnean Cercozoa and Euglenia (arrows omitted for clarity). Although only one secondary symbiogenesis generated all chromophyte plastids from an enslaved red alga (upper grey arrow), given probable systematic errors in multigene sequence trees, we do not know if chromalveolates and chromists are monophyletic, as shown and most likely, or are polyphyletic through plastid transfer by tertiary symbiogenesis. Some sequence trees suggest that Rhizaria might be sisters of chromalvolates not corticates (or even branch within chromalveolates). 4ce indicate independent doublings of centrioles per kinetid to four. H, conversion of mitochondria to anaerobic hydrogenosomes, some of which later became mitosomes; similar anaerobic mitochondrial modifications occurred also in Fungi (independently in Microsporidia and within Chytridiomycetes) and Amoebozoa (Archamoebae), all after the differentiation of aerobic benthic amoeboflagellates into pseudopodially crawling unikonts and ciliary gliding bikonts with two diverging centrioles and microtubular (MT) bands as ciliary root cytoskeleton. The thumbnail sketch indicates the putatively ancestral unicentriolar phenotype for unikonts. The cenancestral eukaryote was similar, although whether it had only one cilium or two is unclear, partly because of uncertainty over the monophyly and position of Apusozoa (deepest-branching bikonts or deep-branching unikonts? See text). Heliozoa are assumed to be chromists, but if this were incorrect they might be a distinct major lineage. Bacteria, Loukozoa, and Choanozoa are ancestral to other groups (paraphyletic) but none-the-worse for that (Cavalier-Smith 1998b). Protists not included in the four derived kingdoms (capitals) belong in the basal eukaryotic kingdom Protozoa.

[Smirnov, Nassonova, and Cavalier-Smith 2008], *Flamella*) and many Archamoebae; those of Mycetozoa are also thin and pointed. Phalansteriid Conosa temporarily make similar pseudopods for anchorage, but feed by ciliary undulation, drawing in bacteria that stick to the cilium and are rapidly pulled towards their collar by ciliary surface motility (perhaps mediated by dynein) for engulfment inside the periciliary pouch (Cavalier-Smith and Chao, unpubl. observ.). This static fishing was arguably the ancestral feeding mode for all eukaryotes. Blunt broad pseudopods probably evolved secondarily in the lobosan common ancestor of Tubulinea and Discosea, when it lost the cilium and focused on simultaneous unidirectional amoeboid locomotion by bulk flow of actomyosin and crawling over and simultaneously phagocytosing their food—eating on the go, in marked contrast to *Phalansterium* and Choanozoa. Breviates and most Conosa can disperse by swimming, but on losing the cilium, Lobosa evolved a ''radiosa'' floating form to drift passively.

Choanozoa are here divided into two subphyla: Cristidiscoidia subphyl. n. (diagnosis: non-ciliate amoebae with tapering pointed filopodia, sometimes branched. Class Discicristoidea: Nucleariida, Fonticulida) and Choanofila subphyl. n. (diagnosis: choanozoans with long unbranched, non-tapering tentacles supported by internal actin bundles or with cell walls in trophic phase). Choanofila comprise three classes: Choanoflagellatea, Filasterea (Shalchian-Tabrizi et al. 2008), both with tentacles; Ichthyosporea with walls, emended here by inclusion of Corallochytrida as a fourth order—see the multigene tree of Steenkamp, Wright, and Baldauf (2006). Tentacles clearly evolved before choanoflagellates and were co-opted to make their filter-feeding collar that eventually made animals (sponges) possible. Nucleariids may be sisters to Fungi (Steenkamp et al. 2006), which probably arose in soil from a common ancestor that still had a separate uniciliate phase (unlike nucleariids), by the origin of cell walls around the branched pseudopodia converting them into the branching rhizoids of the ancestral chytrid fungus (Cavalier-Smith 2000b). By contrast, Choanofila are ancestral to animals, which are sisters of Choanoflagellatea, and probably evolved from a marine stem choanoflagellate by evolving multicellular connective tissue and epithelia, plus differentiated eggs and sperm (Cavalier-Smith 1998a). Even the choanoflagellate to animal (sponge) transition was a benthic event. Thus Choanozoa were ancestrally surface associated; among the unikont protozoa (subkingdom Sarcomastigota, i.e. Amoebozoa plus Chaonozoa) only

acanthoecid choanoflagellates effectively became planktonic by evolving their remarkable silica strip loricas, allowing filter feeding while floating (Leadbeater 2008).

BIKONT MEGAEVOLUTION: FROM BENTHIC HETEROTROPHIC GLIDERS TO PLANKTONIC ALGAE

Bikonts were ancestrally biciliate with two centrioles and a MT skeleton of bands of parallel microtubules attached laterally to the centrioles, rather than a symmetrical cone of single microtubules as in most unikonts. Bikonts comprise three or four major groups, probably all clades: Apusozoa, probably the first diverging, Excavata, probably next diverging, Rhizaria, and corticates (Plantae and chromalveolates, whose constituent groups can be so intermixed on multigene trees that discrete character cladistics is probably more reliable for elucidating relationships). Rhizaria comprise the phyla Retaria (subphyla Radiozoa—probably basal (Moreira et al. 2007)—and Foraminifera) and Cercozoa (comprising two sister subphyla, Filosa and Endomyxa) (Cavalier-Smith 2002; Cavalier-Smith and Chao 2003a). All four bikont groups are ancestrally biciliate with a forward anterior cilium and posterior trailing cilium, which propels the cells by non-undulatory gliding over solid substrata in all ciliated Apusozoa (Apusomonadida, Planomonadida—previously misidentified as *Ancyromonas*; Cavalier-Smith et al. 2008a), most Filosa, many Euglenozoa, and a few chromalveolates (*Caecitellus*: Cavalier-Smith and Chao 2006). Gliding is probably based on membrane-associated kinesin molecular motors working against outer doublet microtubules. Ciliary gliding motility on surfaces and phagocytosis of mostly bacterial food is probably the ancestral adaptive zone for bikonts and much more important than swimming for all bikonts just mentioned. Ciliary gliding is essentially unknown in unikonts, but if Apusozoa are really sisters of opisthokonts, as some trees weakly suggest, not to excavates plus corticates, gliding motility and the bicentriolar condition would then appear to be even more ancient than suggested here, probably dating back to the cenancestral eukaryote.

Ciliary development also differs fundamentally from unikonts: in all four bikont groups the anterior cilium is younger and the posterior one older (Cavalier-Smith 2002; Cavalier-Smith et al. 2008a, b); at cell division one daughter receives the mature posterior cilium, while the anterior mother cilium reorients and is transformed into the posterior cilium of the second daughter; each daughter has a new young anterior cilium that grows just before division. In most groups the two cilia are structurally different and have different wave forms; in some the two centrioles are morphologically distinguishable (Karpov et al. 2006), and in most the ciliary roots attached to the anterior and posterior centrioles differ (Moestrup 2000). Thus, every cell cycle the anterior centriole, cilium, and roots transform into the posterior pattern. This complex pattern of differentiation spread over two cell cycles is the fundamental synapomorphy for bikonts, which makes their cytoskeleton distinctly more complex than that of unikonts. Some unikonts have two cilia and many have two centrioles, but I consider this secondary; the anterior one is always the older one and the unikont posterior cilium is best interpreted simply as a way of accelerating development of the anterior centriole for reasons given previously (Cavalier-Smith 2002; Cavalier-Smith et al. 2004). It does not play the fundamental role in motility and feeding that it does in bikonts.

Our studies of cercozoan kinetids (Cavalier-Smith et al. 2008b; Karpov et al. 2006) deepened understanding, modifying my earlier interpretations (Cavalier-Smith 2002). I now consider that the ancestral state for Cercozoa was two posterior dissimilar MT bands and a third anterior one, as previously suggested to be the ancestral state just for corticates and excavates (Cavalier-Smith 2002). I now think that this pattern dates back to the ancestral bikont and became established whilst evolving gliding motility as its major locomotory mechanism. In unikonts the ancestral state was probably amoeboid locomotion, later replaced by swimming with an undulating posterior cilium in most opisthokonts. Apusozoan pseudopods are branched, pointed, and rather irregular—similarly to pseudopods in varipodid and breviate Amoebozoa and many Cercozoa, so that was probably the ancestral form for all eukaryotes. The ancestral rhizarian modified them by developing anastomoses to form reticulopodia—often with extrusomes, superbly adapted for catching prey on sediment surfaces and in coarse sediment or soil interstices. Retaria and free-living Endomyxa (e.g. *Gromia*, *Arachnula*, *Filoreta*: Bass et al. 2008) all emphasized reticulopodia, and apparently lost gliding motility. Filosa often retained both filopodia and gliding motility (e.g. thaumatomonads, many cryomonads, some cercomonads), or lost one or the other becoming non-amoeboid, gliding or swimming flagellates or totally non-ciliate filose amoebae (testate euglyphids and tectofilosids or naked Granofilosea). Rhizaria mostly retain the soft amoeboid surface putatively ancestral for both bikonts and unikonts; internal mineralized skeletons evolved independently in ebriid and phaeodarian Cercozoa (silica) and in Retaria (often associated with a central capsule); all Retaria and some Cercozoa (desmothoracids, phaeodarians) also evolved MT-supported axopodia. Gliding filosan zooflagellates have two ventral MT bands; in cercomonads these are associated with a ventral groove in which the posterior gliding cilium lies, generally adhering to its ventral surface.

By contrast Apusozoa never evolved mineral skeletons or axopodia; they are dorsoventrally flattened with a soft ventral surface from which pseudopods often emerge and a semirigid dorsal pellicle strengthened by a submembrane dense proteinaceous layer, double in apusomonads and single in planomonads and the non-ciliated *Micronuclearia* (Cavalier-Smith et al. 2008a). These submembrane dorsal layers, present in all members of the phylum as presently constituted, need molecular characterization to determine whether they are homologous and evolved in a common ancestor of all Apusozoa. Inferring the ancestral state of eukaryotes is hampered by uncertainty over the evolutionary position and monophyly of Apusozoa. Sequence trees for one or a few genes show that the three apusozoan orders are not closely related to any other eukaryotes or even to each other (Cavalier-Smith et al. 2004, 2008a). Several weakly suggest that apusomonads and/or planomonads are sisters of opisthokonts (Cavalier-Smith 2000c; Cavalier-Smith and Chao 1995, 2003b; Cavalier-Smith et al. 2004; Kim, Simpson, and Graham 2006; Moreira et al. 2007); a fusion gene suggests that apusomonads are bikonts (Stechmann and Cavalier-Smith 2003) and some trees unconvincingly put one or both among bikonts (Cavalier-Smith et al. 2004; Walker et al. 2006). Gene-dense trees are needed to decide whether Apusozoa are sisters to opisthokonts as first thought, to Amoebozoa where some trees put them (Cavalier-Smith 2002), or to corticates plus excavates (as in Fig. 1, which stresses the fusion gene).

Excavates did not evolve a thick dorsal submembrane layer. Unlike Apusozoa and Rhizaria, early excavates lost pseudopods, most likely because the basal excavate phylum Loukozoa became planktonic, feeding on bacteria in suspension by vibrating a vaned posterior cilium in the feeding groove to draw in bacteria for ingestion, as in *Malawimonas* and *Jakoba*. The attached loricate *Histiona*/*Reclinomonas*-like cell, inverted with its ventral surface uppermost, is probably a derived condition. I now divide Loukozoa into two new subphyla: Vanomonada subphyl. n. (diagnosis: aerobic biciliates with at least one vaned cilium vibrating in the feeding groove to draw in bacteria for ingestion; classes Jakobea, Malawimonadea); and Diphyllatia subphyl. n. (diagnosis exactly as for its sole class Diphyllatea Cavalier-Smith, 2003a, p. 1755). That Loukozoa is ancestral to other excavates and

Choanozoa is to other opisthokonts is no reason for regarding either as ''inadmissible'' (Simpson 2003), an erroneous anti-ancestral taxon fashion (Cavalier-Smith 1998b). The double-leaf body form of diphylleids likely originated by widening the groove and extension of the rim MTs when the posterior cilium was reoriented forwards, probably soon after the first excavate became planktonic; either this occurred before vanes were lost, as they were independently by diplomonads, discicristates, and oxymonads, or before they evolved.

A shift from benthos to plankton also accounts for the key innovations of corticates: ciliary hairs to increase thrust during swimming (alternative to the similarly adaptive flanges of Loukozoa); and cortical alveoli (Gould et al. 2008) to strengthen the cell surface, allowing greater cell size and easy ingestion of larger prey, such as cyanobacteria (Cavalier-Smith 1991b). Arguably, this invasion of the planktonic photic zone led directly to the enslavement of cyanobacteria to make plastids and Plantae. After diversification to yield glaucophytes, green algae, and rhodophytes, a red alga was enslaved by another biciliate corticate in a single secondary symbiogenesis to make chromalveolate plastids (Cavalier-Smith 1999). The first diverging glaucophytes are exclusively freshwater (FW), as are most Viridiplantae—possibly ancestrally.

The red algal primary divergence is between the FW, thermophilic cyanidiophyte subphylum and the largely and probably ancestrally marine rhodellophytes (Cavalier-Smith 2007; Yoon et al. 2006). As the outgroup glaucophytes are all FW, it is most parsimonious that ancestral red algae also inhabited FW and that the primary origin of chloroplasts and Plantae occurred in a lake. Only after the ancestral rhodellophytes took the adaptive plunge into the ocean was one enslaved to form chromophytes, the dominant marine algae. I suggest that plastids evolved in bikont corticates because they were the first planktonic protists able to eat large cells. Unlike sarcomastigote unikonts, lichen fungi enslaved algae, as did corals, but neither evolved chimaeric cells.

Algal megaevolution was expounded in Cavalier-Smith (2007) so I focus on recent multigene trees that if taken at face value (seldom wise for sequence trees) might suggest that chromalveolates and/or chromists are polyphyletic (Burki, Shalchian-Tabrizi, and Pawlowski 2008; Burki et al. 2007; Hackett et al. 2007; Patron, Inagaki, and Keeling 2007). Chromists comprise three groups: Heterokonta, Haptophyta, and Cryptista. Traditionally, heterokonts and haptophyes were grouped together as Chromobiota on morphological and biochemical grounds (Cavalier-Smith 1986, 1991d). Recent multigene trees instead group Haptophyta with Cryptista, which is supported by the simplest interpretation of a shared lateral gene transfer (Rice and Palmer 2006; for reasons given by Cavalier-Smith 2007, this evidence is not totally decisive for the holophyly of Cryptista/Haptophyta), whereas heterokonts group with alveolates, as on rRNA trees. However, the simplest interpretation of this grouping of alveolates and heterokonts and of both with Rhizaria, which all have much longer branches than Cryptista/Haptophyta, is that this is a long-branch attraction (LBA) artefact. Artefactual short-branch exclusion may explain Cryptista/Haptophyta often grouping with the also short-branch Plantae, not with other chromalveolates. None of the analyses used the method of Kolaczkowski and Thornton (2008) aimed to cope with variable branch lengths. Plastid multigene trees showing monophyletic chromobiotes, Chromista, and chromalveolates (e.g. Sanchez-Puerta, Bachvaroff, and Delwiche 2007) might actually be accurately reflecting an entirely vertical transmission of chromophyte plastids, except for the clear case of tertiary symbiotic chloroplast replacement of a dinoflagellate peridinin plastid by a fucoxanthin one from haptophytes (see Patron, Waller, and Keeling 2006; Yoon et al. 2005). Leigh et al. (2008) provided evidence that some chromist host genes may have been replacement by functionally similar ones from the red algal symbiont, notably ribosomal proteins which dominate many multigene trees; if haptophytes/Cryptista had more such genes this might bias trees to group them with Plantae. Only if both artefacts–LBA and host gene replacement, and any others, can be firmly ruled out, would it be reasonable to conclude that Rhizaria are secondarily non-photosynthetic chromalveolates or to invoke tertiary symbiogenesis (which can raise more problems than they might solve).

As emphasized long ago (Cavalier-Smith, Allsopp, and Chao 1994), tertiary symbiogenesis is inherently easier than secondary symbiogenesis, as machinery for crossing extra membranes is already available, but it should not be invoked on inadequate grounds. Tertiary symbiogenesis might also in principle transfer host characters, e.g. the tubular ciliary hairs that were a key argument for the holophyly of Chromista (Cavalier-Smith 1986) and laterally transferred genes shared by chromalveolates only (Nosenko and Bhattacharya 2007), and thus be phylogenetically confusing. However, it is a weakness of multigene trees that they rely so heavily on short ribosomal proteins, the majority in many trees; such structural proteins are poorly conserved compared with some enzymes or chaperone proteins and may not be subject to sufficiently uniform stabilizing selection to be reliable markers for deep phylogeny. Interestingly, even for a taxonomically sparse chloroplast tree, Sanchez-Puerta et al. (2007) found that 24 conserved photosystem-related genes strongly supported chromobiote and chromist monophyly, whereas adding 38 faster evolving genes—mostly for ribosomal proteins made them, probably wrongly, paraphyletic because of the movement lower down of the longer heterokont branch. Yet the difference in branch length was much less than in trees constructed using nuclear genes, which are often not well-constrained proteins, unlike those of photosystems. More genes are not necessarily better for deep phylogeny. For this, molecular cladistic and morphological evidence is sometimes superior. Although tertiary symbiogenesis could in principle reconcile a single secondary symbiogenetic origin of chromophyte plastids, for which evidence is now very strong (Patron, Rogers, and Keeling 2004), with chromalveolate polyphyly, the multigene evidence against chromalveolate holophyly does not yet outweigh previous cladistic and evolutionary arguments for the holophyly of chromalveolates, chromists, and chromobiotes.

It is clear that chloroplasts and plants are monophyletic, and that all their plastids came from one cyanobacterial enslavement and one origin of the Toc/Tic import machinery and transit peptides (Cavalier-Smith 1982b, 2000a, 2007). Plantae are also holophyletic in most multigene trees (Burki et al. 2007, 2008; Hackett et al. 2007; Patron et al. 2007; Rodríguez-Ezpeleta et al. 2005). Plant cell walls evolved separately in glaucophytes, rhodophytes, and Viridiplantae after their divergence. Plants are all non-phagotrophs, except for one prasinophyte, a primitive green alga with surface scales, which unlike later evolving cell walls do not invariably prevent phagocytosis (O'Kelly 1992).

Many chromalveolates retain both phagotrophy and photosynthesis, but both have been differentially lost; the megaevolutionary consequences of these nutritional shifts for basic cell structure are discussed in detail by Cavalier-Smith (2004a). Secondary symbiogenesis also planted green algal plastids into Cercozoa (Chlorarachnea) and Euglenozoa (Euglenia). In chlorarachneans the ancestral alternating flagellate and filose amoeba phases were supplemented by a non-phagotrophic coccoid wall phase, but each of these three has been differentially lost in different lineages. Non-coccoid chlorachnean phases retain phagotrophy, but photosynthetic euglenoids lost it despite not evolving walls.

In protists generally the loss of cilia causes still more pronounced simplifications of the cell skeleton. No plants are amoeboid and

amoeboid locomotion re-evolved only rarely in chromalveolates (i.e. *Chrysamoeba*, *Chlamydomyxa*, some xanthophytes, several dinoflagellates including those with novel pallium feeding by a novel giant temporary lamellipodium compatible with their generally rigid cortex). The novel motility of the heterokont Labyrinthulea is not amoeboid, but unique. Thus corticates are largely locked in non-amoeboid rigidity.

Ancestrally rigid excavate surfaces were modified in four groups: euglenoids evolved interlocking pellicular strips that allowed active sliding and reversible elastic cortical deformations; metamonad Parabasalia internalized the MT skeleton, allowing some surface amoeboidy and even the evolution of one amoeba by ciliary loss: *Dientamoeba* (Cavalier-Smith 2003a); metamonad oxymonads evolved striking deformations by contractile internal axostyles; in Percolozoa, Heterolobosea interpolated a lobose amoeba stage into the ancestrally purely flagellate life cycle. Intriguingly myosin II, the major motor for unikont protoplasmic motility, is apparently absent in corticates, Rhizaria, and most excavates (Richards and Cavalier-Smith 2005), but a very divergent version exists in the heterolobosean amoeba *Naegleria* (Richards, pers. commun.), raising the possibility that lateral transfer from Amoebozoa could have aided the secondary origin of heterolobosean amoebae. The alternating character of their life cycle between pure amoebae and pure flagellates differs from the simultaneous amoeboflagellate character of ancestral eukaryotes. Percolozoa has five clades of secondarily non-ciliate amoebae and two derived clades of secondarily non-amoeboid zooflagellates: *Pleurostomum* and Percolatea (*Percolomonas*, *Stephanopogon*: Cavalier-Smith and Nikolaev 2008).

Many bikonts evolved novel phenotypes for catching food on rigid cell extensions, supported by actin (e.g. haptopodia of the cercozoan *Aurigamonas*: Vickerman et al. 2005) or by MTs (e.g. the haptophyte haptonema [Cavalier-Smith 1994]; axopodia of Heliozoa [centrohelids], Radiozoa, the cercozoan Phaeodaria and desmothoracids, and the heterokont pedinellids and actinophryids). It seems that the more elaborate MT skeleton of bikonts predisposed them to evolve axopodia. No unikonts ever did—actomyosin dominates, although conosan amoebae have interphase microtubules. The more rigid bikont cortical skeleton also favoured the evolution of multiciliate forms; the only multiciliate unikont protist is *Multicilia* itself (Nikolaev et al. 2006), where numerous cilia and pseudopodia uneasily coexist, neither dominating to fulfil its unique potential; thus *Multicilia* has few species. By contrast kinetids often multiplied in bikonts: to generate the multiformly adapted Ciliophora, *Stephanopogon* (Percolozoa), *Hemimastix* (probably cercozoan: Cavalier-Smith et al. 2008b), hypermastigote parabasalian metamonads (probably twice: Carpenter and Keeling 2007), and even a dinoflagellate (*Polykrikos*). Even ciliary multiplication within a kinetid is commoner in bikonts, the tetraciliate condition (with transformation across three cell cycles) evolving independently in ancestral Metamonada, early but possibly not quite ancestral Percolozoa (Cavalier-Smith and Nikolaev 2008), multiply within Chlorophytina, the cercozoan *Cholamonas* (Cavalier-Smith et al. 2008b), and the heterokont *Karotomorpha*, but never in unikonts.

DICHOTOMY BETWEEN MARINE AND FW/TERRESTRIAL PROTISTS

It is generally accepted that animals were ancestrally marine and that only a few phyla invaded land, whereas embryophytes and fungi were ancestrally terrestrial and only a few invaded the seas. Yet it is seldom discussed whether eukaryotes were ancestrally marine, FW, or terrestrial. In algae many whole classes or subclasses are exclusively either terrestrial/FW or marine. One protozoan phylum, Retaria, is almost entirely marine and probably ancestrally so, but most are widespread in both habitats. However, transitions between salty and non-marine habitats are much rarer than often supposed; in heliozoa (mostly FW: Cavalier-Smith and von der Heyden 2007), amoebae (Smirnov, Nassonova, Chao, and Cavalier-Smith 2007), and zooflagellates (Bass and Cavalier-Smith 2004; von der Heyden and Cavalier-Smith 2005; von der Heyden, Chao, and Cavalier-Smith 2004), large clades within many groups are exclusively marine or non-marine.

This sharp differentiation into marine and non-marine forms is one of the strongest features of biodiversity of all microbes, including bacteria (Lozupone and Knight 2007). For non-walled protists it is reflected in the generality of CVs in non-marine forms only, including both unikonts and bikonts. If all CVs are homologous then the ancestral eukaryote must have had them and probably evolved in soil or freshwater, with oceans being colonized secondarily. If true, it should be much more difficult for marine groups to invade FW (needing a gain of CVs) than the reverse (just loss), which should be reflected in phylogeny; marine clades should be often nested within FW ones but the reverse should be rare or absent. In several groups there are clear cases of marine subgroups nested within FW groups: heliozoa (Cavalier-Smith and von der Heyden 2007), Cercozoa (Bass et al. 2008), and bodonids (von der Heyden and Cavalier-Smith 2005). In principle it could be argued that CVs require only rather generalized eukaryotic properties: the ability to pump ions into a vesicle to allow it to swell by osmosis, to pump them out again, and to use membrane fusion proteins to add them to larger reservoirs, and then actomyosin and more membrane fusion to expel the water. Thus, a CV requires geometric topological, and temporal organization of universal eukaryotic properties and could in principle evolve polyphyletically. Nonetheless, it is still likely to be more difficult for a protist to evolve a CV than to lose it. Colonization of the sea should be easier as it requires only the excretion of sodium ions at the plasma membrane and CV suppression. Thus even if CVs are polyphyletic we expect to see phylogenies biased towards FW to marine transitions, rather than the reverse, which appears to be the case.

Although rare on an evolutionary scale there were probably over a hundred FW/marine transitions in Protozoa in 800 My. As one gets closer to the base of trees the paucity of branches makes it increasingly hard to infer the ancestral state for a potentially reversible binary character. If the very few FW/terrestrial Foraminifera have CVs, Retaria might be the best case of an ancestrally marine phylum evolving CVs. Their sister Cercozoa are arguably ancestrally marine as this is the case for the deepest branches within both subphyla. If Rhizaria are ancestrally marine their CVs may have evolved independently of those of corticates. Excavates could be argued to be ancestrally FW or marine with almost equal parsimony. Apusozoa may be ancestrally marine as this is the most parsimonious interpretation for both planomonads and apusomonads: in both groups the deepest divergences are among the more numerous marine lineages. The FW planomonads (one clade only) and apusomonads (at least three FW clades: TCS., unpubl. data) may have evolved CVs independently. Currently it is a little more parsimonious to suppose that bikonts were ancestrally marine and that unikonts had a FW ancestry, if we weigh CV losses and gains equally. In the Amoebozoa, Conosa, Variosea, and Tubulinea are largely FW groups, Discosea are mixed marine and FW, and *Breviata* FW, making a FW ancestry likely overall. Opisthokonts have a putatively ancestrally FW fungi/nucleariid clade and a putatively ancestrally marine one: holozoa (Choanofila plus animals; the only free living FW Choanofila are choanoflagellates, found only in one of the two orders: Craspedida, either FW or marine ancestrally; Ichthyosporea and Filasterea are marine or parasites of saline animal body fluids, except for *Amoebidium* that lives ectocommensally on FW arthropod cuticle). If evolving CVs were substantially harder than

losing them, it would be most parsimonious to assume a FW ancestry for all eukaryotes. Possibly molecular studies of key proteins in the CV system could distinguish between a monophyletic and polyphyletic origin, but I am not optimistic.

FOSSILS, TIMING, AND ORGANISMAL COEVOLUTION

Fossil evidence for the age of eukaryotes is problematic, but they may be no older than 800–850 My (Cavalier-Smith 2006). The oldest assured eukaryotic fossils are vase-shaped *Melanocyrillium*, probably amoeba tests, from ~ 750-My-old marine sediments (Porter and Knoll 2000; Porter, Meisterfeld, and Knoll 2003). Earlier I rejected on morphological grounds the idea that some were euglyphid Cercozoa, but accepted that others were probably arcellinids (Cavalier-Smith 2006). However, this is questionable in view of the rarity of marine to FW transitions, as all arcellinids are FW (unlike euglyphids, with a minority marine); both mostly inhabit mosses. The only marine testate amoebae are gromiids, a few divergent euglyphids, and many Tectofilosida, none of which the fossils resemble. More likely *Melanocyrillium* fossils were an ancient group of early testate amoebae—possibly neither Amoebozoa nor Cercozoa—that became extinct during the Neoproterozoic snowball earth episodes (710–635 My ago: Bodiselitsch et al. 2005). The rather short duration (~ 800–690 My ago) of such fossils (Porter and Knoll 2000) supports this interpretation; later, forams evolved to fill their vacated adaptive zone. Testate amoebae evolved convergently 4 or 5 times, so postulating a fifth/sixth extinct group is reasonable. Possibly tectofilosids are the oldest extant group, colonizing both sea and FW. Arcellinids and euglyphids appear more recent on trees and perhaps evolved ~ 400 My ago, after mosses emerged. The oldest certain fossil mosses are only Permian but they are likely almost as old as the better-fossilized tracheophytes.

The other major groups of soil protozoa (glissomonads—previously misnamed heteromitids—Howe et al. 2008), cercomonads, Conosa—both the aerobic Mycetozoa and anaerobic Archamoebae—may be of similar age and could have evolved in response to the extra carbon input into soil fuelling their bacterial prey following the spread of tracheophytes and mosses across the continents; all are distinctly younger than Amoebozoa and Conosa.

I suggest that the basal radiations of rhizaria, opisthokonts, and corticates all took place soon after snowball earth thawed ~ 635 My ago, triggered by the origin of chloroplasts then, but in time for animals to originate from stem choanoflagellates about 550 My ago. Cavalier-Smith (2000a, c) suggested that plastids originated 550–600 My ago near the lower end of the plausible range of 570–850 My ago (Cavalier-Smith 2006). I consider that the inability to resolve easily the corticate basal radiation means that plant and chromalveolate radiation was explosively fast, as expected since Darwin for such a major innovation. Likely substitutional near-saturation makes resolution harder, but I do not think the resolution difficulty can be attributed entirely to it, as multigene trees seem able to resolve excavates as diverging earlier. Possibly only excavates, Apusozoa, and Amoebozoa, among extant eukaryote groups, pre-dated snowball earth and survived it.

If Metamonada, the only ancestrally anaerobic protozoan phylum, evolved before snowball earth, they would have had an extensive anaerobic niche under the ice. Discicristates by contrast may have originated only after snowball earth melted. Microsporidia must be much younger, perhaps ~ 400 My old, losing aerobicity through parasitism, postdating their fungal ancestors and animal hosts. The third major anaerobic protist group, Archamoebae, are sisters of the strictly aerobic soil Mycetozoa, so may also have evolved in soil; both conosan groups most likely originated at the same time as the two most speciose groups of soil protozoa, glissomonads and cercomonads—both probably exclusively terrestrial or FW (Bass et al., unpubl. observ.; Howe et al. 2008). I suggest that all four groups—Archamoebae, Mycetozoa, glissomonads, and cercomonads, plus the FW planomonads and colpodid ciliates, originated during the late Silurian or Carboniferous when land plants were spreading across the continents increasing soil carbon for their bacterial food; 18S rRNA ''clock'' estimates are consistent with this for Colpodida and Glissomonadida (Howe et al. 2008; Wright and Lynn 1997). Some radiations were apparently much later, e.g. bodonids, sandonid glissomonads (Howe et al. 2008); both might have been favoured by the origin of angiosperms and grasslands with rich carbon input and ease of dispersal of cysts by dust. Several marine protist groups radiated only after the Permian mass extinction—the largest in history (e.g. dinoflagellates, diatoms, haptophytes), suggesting that they were replacements of extinguished acritarchs (possibly unknown chromalveolate group(s)), rather than invasions of never previously exploited adaptive zones as were most innovations discussed here.

The importance of the gliding flagellate phenotype has been grossly underappreciated. We estimate that the global diversity of glissomonads (Howe et al. 2008) and cercomonads (Bass et al., unpubl. observ.) alone may exceed by severalfold the previous estimate of 1,600–2,000 species for all soil protozoa (Foissner 1999) and is probably greater even than the incredible underestimate of 3,060 for all free-living protozoa (Finlay 2001); in species number, biomass, and C-recycling rates they are probably much more important than ciliates, previously considered the most speciose soil protozoa (Foissner et al. 2005).

CONCLUSIONS

Commitments to contrasting adaptive zones made early in eukaryote evolution opened and closed doors to future adaptation. The amoeboflagellate and probably benthic nature of the first eukaryotes gives a new perspective on eukaryote diversity. Nonciliate amoebae, non-amoeboid flagellates, and actinopods were all multiply derived from the amoeboflagellate ancestor by developing different aspects of its cytoskeleton. Shifts in nutrition or feeding mode and from benthos to plankton played key roles in innovations in body plan, with respect to cytoskeletal symmetry and cortical and surface structures. Ciliary gliding and use of ciliary adhesion or water currents for prey entrapment had a deeper role in eukaryote diversification than undulatory cell propulsion, which came into its own only with the secondary evolution of planktonic bikonts associated with cyanobacterial enslavement in the photic zone. This powered the Cambrian explosions and generated the modern world soon after the last near-global glaciation.

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