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Protist phylogeny and the high-level classification of Protozoa

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Protist large-scale phylogeny is briefly reviewed and a revised higher classification of the kingdom Protozoa into 11 phyla presented. Complementary gene fusions reveal a fundamental bifurcation among eukaryotes between two major clades: the ancestrally uniciliate (often unicentriolar) unikonts and the ancestrally biciliate bikonts, which undergo ciliary transformation by converting a younger anterior cilium into a dissimilar older posterior cilium. Unikonts comprise the ancestrally unikont protozoan phylum Amoebozoa and the opisthokonts (kingdom Animalia, phylum Choanozoa, their sisters or ancestors; and kingdom Fungi). They share a derived triple-gene fusion, absent from bikonts. Bikonts contrastingly share a derived gene fusion between dihydrofolate reductase and thymidylate synthase and include plants and all other protists, comprising the protozoan infrakingdoms Rhizaria [phyla Cercozoa and Retaria (Radiozoa, Foraminifera)] and Excavata (phyla Loukozoa, Metamonada, Euglenozoa, Percolozoa), plus the kingdom Plantae [Viridaeplantae, Rhodophyta (sisters); Glaucophyta], the chromalveolate clade, and the protozoan phylum Apusozoa (Thecomonadea, Diphylleida). Chromalveolates comprise kingdom Chromista (Cryptista, Heterokonta, Haptophyta) and the protozoan infrakingdom Alveolata [phyla Ciliophora and Miozoa (= Protalveolata, Dinozoa, Apicomplexa)], which diverged from a common ancestor that enslaved a red alga and evolved novel plastid protein-targeting machinery via the host rough ER and the enslaved algal plasma membrane (periplastid membrane). The branching order of the five bikont groups is uncertain: Plantae may be sisters of or ancestral to chromalveolates (jointly designated corticates as they share cortical alveoli); Rhizaria and Excavata (jointly cabozoa) are probably sisters if the formerly green algal plastid of euglenoids and chlorarachneans (Cercozoa) was enslaved in a single event in their common ancestor. Apusozoa may be sisters of Excavata and centrohelid heliozoa may be sisters to Haptophyta.

Key words: Protozoa; protist; classification; phylogeny; bikonts; unikonts.

Protists are polyphyletic

Eukaryotes comprise the basal kingdom Protozoa and four derived kingdoms: Animalia, Fungi, Plantae, and Chromista (Cavalier-Smith 1998). Protists (unicellular eukaryotes) can no longer be considered a taxon (e.g. a kingdom) as they are found in all five kingdoms and are undoubtedly polyphyletic. This is most strikingly shown by the recent evidence that Myxozoa, most of which are plasmodial in the trophic state, are derived from bilateral animal ancestors by the loss of gut and nervous system (Okamura et al. 2002) and that the anaerobic microsporidia evolved from aerobic filamentous zygomycotine fungi (Keeling 2003) as postulated (Cavalier-Smith 2000a). Such phylogenetically deceptive evolution by simplification and character loss has occurred not only in such para-

sites but also in free-living protists. Molecular evidence now implies that all known groups of anaerobic, apparently amitochondrial, protists evolved by the loss of mitochondrial genomes, cytochromes, and oxidative phosphorylation and the conversion of mitochondria into double-membraned organelles of different function: hydrogenosomes or mitosomes (Roger 1999; Silberman et al. 2002) – these retain their ancestral mitochondrial mechanisms of membrane heredity and protein targeting: (Cavalier-Smith 2004a; Williams et al. 2002; Embley et al. 2003; van der Giezen et al. 2003). Likewise it is almost certain that all non-ciliated protists {e.g. many amoebae (Cavalier-Smith et al. 2004), yeasts, *Corallochytrium* (Cavalier-Smith and Chao 2003a), Myxozoa, microsporidia, *Blastocystis*} evolved ultimately from ciliated ancestors by losing cilia (= eukaryotic flagella) and centrioles (= basal bodies).

A new systematic synthesis

These new insights have come not just from molecular sequence studies but by integrating them with numerous other lines of evidence, genetic, structural and biochemical. The classical view developed over two centuries that reliance on a single line of evidence or character is often very misleading for phylogeny and systematics is at last penetrating the previously over-dogmatic and over-self-confident field of molecular systematics. There really ought to be no such field, for good systematics should be fully integrative of all available evidence. 'Molecular systematics' that concentrates on trees from just one molecule and ignores other evidence is poor systematics. Although there have long been conclusive theoretical reasons for thinking that sequence trees can sometimes be profoundly misleading and all too easily misinterpreted (Felsenstein 1978) and that ribosomal rRNA is certainly not a molecular clock (Cavalier-Smith 1980), the recent spread of a more critical approach to protist molecular phylogenies owes much to the balanced and integrative perspective of André Adoutte (Baroin et al. 1988; Philippe and Adoute 1996) and critical analyses by his former collaborators (Philippe and Adoutte 1998; Philippe 2000; Philippe and Germot 2000; Philippe et al. 2000a; Philippe et al. 2000b; Lopez et al. 2002). It is now widely, but by no means universally, accepted that single-gene trees often lack

the resolution to group together taxa that really are related, sometimes group together those that are not and can be profoundly misleading about where the root of a subtree really lies.

Despite these problems, the overall evidence now allows us to group protists into a relatively small number of phyla (25, counting all four fungal phyla as including protists (Cavalier-Smith 1998), of a total of 48 eukaryotic phyla), most now established with reasonable confidence as monophyletic - mainly holophyletic, though one is certainly paraphyletic (i.e. Chlorophyta, one of the three plant phyla containing protists: Cavalier-Smith 1998) and two probably are (Archemycota, Loukozoa). The present paper summarises this evidence and outlines my current interpretation of relationships among the 11 phyla of the necessarily paraphyletic kingdom Protozoa and the four derived (holophyletic) kingdoms (Fig. 1). In this revised system for kingdom Protozoa (see appendix) the phyla Choanozoa and Amoebozoa (both I suspect holophyletic, but either or both may be paraphyletic as shown on some trees: resolution is insufficient to decide either way – Cavalier-Smith and Chao 2003a; Cavalier-Smith et al. 2004) are grouped as the undoubtedly paraphyletic subkingdom Sarcomastigota, from which animals and fungi independently evolved (Cavalier-Smith 2000a; King et al. 2003). The other nine protozoan phyla (all but Loukozoa probably holophyletic) constitute the paraphyletic subkingdom Biciliata from which the holophyletic kingdoms Plantae and Chromista evolved. Biciliata comprise three probably holophyletic infrakingdoms (Alveolata, Rhizaria, Excavata) plus the phylum Apusozoa, which may have affinities with Excavata or Rhizaria or be more deeply branching.

Bikonts, unikonts and the eukaryotic root

Plantae, Chromista, and Biciliata are all clearly ancestrally biciliate and together constitute a clade designated the bikonts (Cavalier-Smith 2002). A major shared derived character for all three groups (not yet clearly demonstrated for Rhizaria) is ciliary transformation in which the anterior cilium/centriole and its associated roots are always the first formed; in the next cell cycle they undergo often marked changes in structure and function to become the corresponding posterior organelles

bikonts (ancestrally biciliate eukaryotes)   **unikonts**

corticates

CABOZOA

chromalveolates

Kingdom CHROMISTA

Kingdom ANIMALIA   Kingdom FUNGI

Discicristata   Euglenozoa
Percolozoa

Excavata

Cryptista   Chromobiota

Choanozoa

Metamonada

Heterokonta Haptophyta

receptor tyrosine kinases

parallel centrioles

Heliozoa

tetrakonty

plastid inside RER; tubular ciliary hairs

?

opisthokonts

Malawimonadea   * Jakobea

Loukozoa

Alveolata │ Ciliophora
          │ Miozoa

EF1- alpha insertion
posterior cilium; flat mitochondrial cristae

ciliary vanes + groove

Rhizaria   Retaria
* Cercozoa

Kingdom PLANTAE

Viridaeplantae (green plants)

replacement of plastid GAPDH

Amoebozoa

amoeboid locomotion

polyubiquitin insertion

(G)   Rhodophyta

(R)

phosphofructokinase internal duplication

Glaucophyta

Apusozoa

CHLOROPLAST

Thecomonadea

Diphyllatea

cortical alveoli

triple pyrimidine biosynthesis fusion gene

?

DHFR-TS fusion gene

ciliary transformation

MITOCHONDRION ➤ tubular mitochondrial cristae
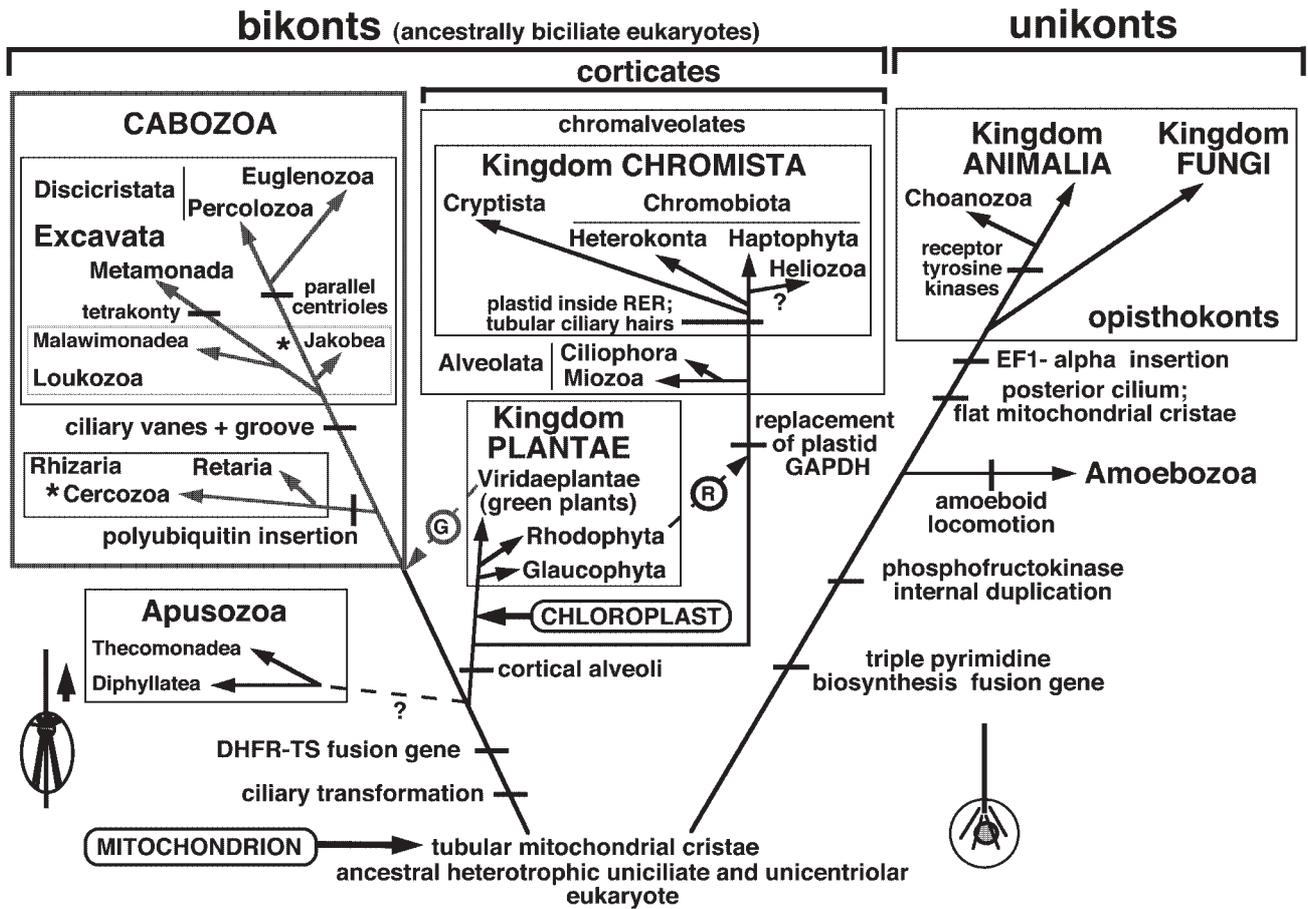ancestral heterotrophic uniciliate and unicentriolar eukaryote

Fig. 1. The eukaryotic phylogenetic tree based on a synthesis of ultrastructural, cell biological and molecular evidence, showing the four major symbiogenetic events. Taxa outside the four derived kingdoms belong to the basal kingdom Protozoa (unlabelled). The ancestral eukaryote is held to have been a phagotrophic uniciliate, unicentriolar aerobic zooflagellate that arose from a neomuran bacterial ancestor by the simultaneous origin of the cytoskeleton, endomembrane system, nucleus, and cilium, coupled with the overlapping symbiogenetic origin of mitochondria from an intracellular α-proteobacterium (Cavalier-Smith 2000b, 2002). Unikonts are ancestrally heterotrophic if chloroplasts arose symbiogenetically in an early bikont, as is almost certain. Chromalveolates are holophyletic, ancestrally photophagotrophic, and evolved by the single enslavement of a red alga ® by a bikont host to form a eukaryote-eukaryote chimaera. There are almost equally strong protein-targeting arguments for the single secondary origin of the cabozoan chloroplast (G) in a common ancestor of Cercozoa and Euglenozoa (Cavalier-Smith 1999), but the idea remains controversial as compelling sequence evidence is unavailable to disprove the alternative possibility that euglenoid and chlorarachnean plastids were separately implanted as shown by the asterisks. Whether Apusozoa are really ancestrally heterotrophic, early divergent bikonts, as shown, is also uncertain. It is unclear whether the duplication within phosphofructokinase is a synapomorphy for unikonts or its loss one for bikonts (Stechmann and Cavalier-Smith 2003a).

(Moestrup 2000). An independent shared derived character for bikonts is a fusion between the genes for thymidylate synthase (TS) and dihydrofolate reductase (DHFR) to encode a single bifunctional chimaeric protein. This fusion appears to have taken place in the common ancestor of the ancestral bikont after the two genes became inverted in order in the cenancestral eukaryote compared with the ancestral state in bacteria where they are separately translated but usually in the same operon (Stechmann and Cavalier-Smith 2002; Stechmann and Cavalier Smith 2003a). Both genes are separately translated in Sarcomastigota, animals and Fungi; these three taxa are referred to as unikonts

because it is argued that their common ancestor probably had only a single centriole and cilium per kinetid (Cavalier-Smith 2002); this unikont state (Cavalier-Smith 2002) is found in most distinct amoebozoan clades – *Phalansterium*, mastigamoebids, *Multicilia* and protostelids (sensu Cavalier-Smith et al. 2004) and possibly '*Mastigamoeba invertens*'. Unikonty is argued to be the ancestral state for Amoebozoa (Cavalier-Smith 2002; Cavalier-Smith et al. 2004) as only myxogastrids and former protostelids arguably related to them have bicentriolar kinetids. The bicentriolar state of myxogastrids and many Choanozoa is developmentally different from that of bikonts, as their anterior cilium remains anterior in successive cell cycles and does not transform into a posterior one: thus it is arguably not homologous to the bikont state with true ciliary transformation.

A second gene fusion involving the first three enzymes of pyrimidine biosynthesis (carbamoyl phosphate synthase, dihydroorotase, aspartate carbamoyltransferase) is apparently a shared derived character for unikonts, absent from bikonts and the ancestral prokaryotes (Stechmann and Cavalier-Smith 2003a). As this involved two simultaneous fusion events, it is even less likely to ever have been reversed than the DHFR/TS fusion. If none of these gene fusions has ever been reversed during evolution, then they together indicate that the root of the eukaryote tree cannot lie within unikonts or bikonts, but must lie between these two clades (Stechmann and Cavalier-Smith 2003a). Earlier structural and molecular evidence had supported the idea that Animalia, Choanozoa and Fungi together form a clade, the opisthokonts, characterised ancestrally by a single posterior cilium with a bicentriolar kinetid and flat mitochondrial cristae (Cavalier-Smith 1987). Extant Choanozoa are either a clade that is sister to Animalia or a paraphyletic group ancestral to them (Cavalier-Smith and Chao 2003a; Rokas et al. 2003); a sister relationship between animals and choanoflagellates at least is supported by the probable gene fusion that generated the receptor tyrosine kinases from a cytoplasmic kinase and calcium-binding epidermal growth factor in their common ancestor after it diverged from Amoebozoa (King and Carroll 2001). The opisthokont clade is also supported by a shared derived 11–17 amino acid insertion in protein synthesis elongation factor EF1-α absent from prokaryotes and all other eukaryotes (Baldauf 1999). This character is quite strong, though some

indels used to interpret protist phylogeny have proved unreliable, in particular deletions used to imply early branching of Parabasalia (Bapteste and Philippe 2002).

If Choanozoa and Amoebozoa are both holophyletic, the branching order for the four unikont groups in Figure 1 is almost certainly correct, with Amoebozoa being the immediate outgroup to opisthokonts. Sequence phylogenies based on over 100 protein-coding genes strongly support monophyly of Archamoebae (mastigamoebids, *Pelomyxa*, *Entamoeba* and *Endolimax*) and their relationship with the cellular slime mould *Dictyostelium* (Bapteste et al. 2002) as well as the closer relationship of choanoflagellates to animals than to Fungi (Philippe, Bapteste pers. comm.). A shared derived fusion between cytochrome oxidase 1 and 2 mitochondrial genes (Lang et al. 1999) shows that *Dictyostelium* and *Acanthamoeba* are part of an amoebozoan clade within which the eukaryote root cannot lie. These and other data indicate that dictyostelid slime moulds and acanthamoebids evolved by independent ciliary losses from ciliated Amoebozoa. The circumscription and phylogeny of both Amoebozoa and Choanozoa have been clarified by recent molecular studies. The filose amoebae *Nuclearia* and the enigmatic microvillar *Ministeria* turn out to be Choanozoa - phylogenetically distant from each other and the previously established Choanozoa (choanoflagellates, *Corallochytrium*, Ichthyosporea) (Cavalier-Smith and Chao 2003a). The not obviously amoeboid uniciliate *Phalansterium* is an amoebozoan with affinities to *Gephyramoeba/Filamoeba* and (probably more distantly) acanthamoebids (Cavalier-Smith et al. 2004). The basal branching order within bikonts is much less clear than for unikonts.

The chromalveolate clade

Mechanisms of protein targeting to organelles are remarkably conservative features of cells, as Blobel and I argued when first discussing their origin 23 years ago (Blobel 1980; Cavalier-Smith 1980). This led me to establish the kingdom Chromista for all eukaryotes with plastids located within a periplastid membrane within the lumen of the rough endoplasmic reticulum and their putative aplastidic descendants (Cavalier-Smith 1986). It is now clear that the mechanisms of protein targeting

of nuclear-coded genes of all three major chromist groups (cryptomonads, Heterokonta, Haptophyta) are indeed homologous, all using bipartite (or tripartite for intrathylakoid proteins) N-terminal targeting sequences. Increasing evidence reviewed elsewhere (Cavalier-Smith 2003b) shows that these mechanisms are also fundamentally similar in the two alveolate groups with plastids {dinoflagellates and Sporozoa (with Apicomonadea constituting Apicomplexa), now grouped with protalveolates in the phylum Miozoa (Cavalier-Smith 1999)} and that alveolates and chromists together form a clade of ancestrally photosynthetic eukaryotes created by a single ancient enslavement of a red alga. Concatenated chloroplast gene trees (Yoon et al. 2002) strongly support the monophyly of chromists and Chromobiota (Haptophyta plus Heterokonta). The shared derived gene duplication of a nuclear glyceraldehyde-3-phosphate dehydrogenase gene and the acquisition of a bipartite targeting sequence by one copy, allowing it to replace the original red algal chloroplast protein, strongly supports chromalveolate monophyly (Fast et al. 2001).

Several chromalveolate clades have lost photosynthesis while retaining leucoplasts (e.g. most Sporozoa, *Chilomonas*, some pedinellids, some diatoms, some chrysomonads), whereas others have apparently lost plastids altogether (e.g. Ciliophora, Pseudofungi, Sagenista, *Goniomonas*). The complete loss of plastids has caused some colourless chromists to be treated in the past as protozoa. The retention of cryptophyte-like ejectisomes and flat mitochondrial cristae allowed *Goniomonas* to be recognised as a chromist even before molecular evidence proved it. Such evidence is still lacking for kathablepharids, recently placed in Cryptista because of their single-scroll ejectisomes (Cavalier-Smith 2004b); their tubular cristae do not contradict such an assignment because this is the ancestral state for chromalveolates, the flat cristae of cryptomonads being secondarily derived. Many aplastidic heterokonts were recognised as such because of their tripartite (rarely bipartite) tubular hairs on the anterior younger cilium, but several heterokonts have lost such hairs (notably *Caecitellus* and Opalinata) or cilia altogether. Unless there is other ultrastructural evidence (as for *Caecitellus*) the chromist (rather than protozoan) affinity of such taxa is likely to be obscured in the absence of gene sequences. Molecular data are needed to confirm suggestions that actinophryid 'heliozoa' are related to pedinellid heterokonts. Centrohelid Heliozoa are almost certainly not directly related to actinophryids or desmothoracids: recent rRNA trees weakly suggest a relationship with Haptophyta (Cavalier-Smith and Chao 2003b). Although an alternative even weaker relationship with the thecomonad *Ancyromonas* cannot be ruled out, I now place Heliozoa (Centrohelea only) within Chromobiota (Table 1), on the hypothesis that their microtubular axonemes are related to those of the haptonema and that a common ancestor with haptophytes used long filiform microtubule-supported cell extensions to trap prey.

Table 1.   Classification of the kingdom Chromista Cavalier-Smith 1981 emend.

Subkingdom 1. Cryptista Cavalier-Smith 1989 emend. (3 classes)
    Phylum Cryptista Cavalier-Smith 1986 (Cryptophyceae, goniomonads, kathablepharids)
Subkingdom 2. Chromobiota Cavalier-Smith 1989 emend.
  Infrakingdom 1. Heterokonta Cavalier-Smith 1986 (19 classes)
    Phylum 1. Ochrophyta Cavalier-Smith 1986 (13 essentially algal classes: with plastids)
    Phylum 2. Bigyra Cavalier-Smith 1998 (heterotrophs with double transitional helix)
      Subphylum 1. Bigyromonada Cavalier-Smith 1998 (1 class: *Developayella*)
      Subphylum 2. Pseudofungi Cavalier-Smith 1986 (2 classes: Oomycetes, Hyphochytrea)
      Subphylum 3. Opalinata Wenyon 1926 stat. nov. Cavalier-Smith 1997 (3 classes)
    Phylum 3. Sagenista Cavalier-Smith 1995 (3 classes: Labyrinthulea, Bicoecea, *Wobblia* etc.)
  Infrakingdom 2. Haptista infrakingd. nov. Diagnosis: ancestrally biciliate and feeding on prey by one or more microtubular-supported filiform extensions.
    Phylum 1. Haptophyta Cavalier-Smith 1986 (2 classes: Pavlovophyceae, Prymnesiophyceae)
    Phylum 2. Heliozoa Haeckel 1866 stat. nov. Margulis 1974 emend. (class Centrohelea only)

For fuller details, including the rest of the 25 classes see Cavalier-Smith 2004b

Infrakingdom Rhizaria revised

Removing centrohelids and thecomonads from Rhizaria makes the infrakingdom more homogeneous structurally and confined to the phyla Cercozoa and Retaria (Radiozoa plus Foraminifera) plus Phaeodarea and order Desmothoracida, provisionally left incertae sedis within Rhizaria. Elsewhere I suggest that Foraminifera evolved from polycystine radiolaria (Cavalier-Smith and Chao 2003b): I think their common ancestor was reticulopodial and that the multiply porous central capsules of polycystine radiolaria and multiply porous walls of foraminifera are evolutionarily related. The triply porous central capsule of Phaeodarea is more different; unlike Polycystinea and Acantharia there is no evidence that they ever had dodecagonal axonemes or can sequester strontium sulphate, so I exclude them from a revised Radiozoa. A shared single amino acid insertion in polyubiquitin convincingly unites Cercozoa and Foraminifera (Archibald et al. 2003): I predict it will be in Radiozoa and Phaeodarea too; it is absent from centrohelids and thecomonads (Herden, Chao, Cavalier-Smith, unpublished), here excluded from Rhizaria. Cercozoa have proved to be a major protozoan phylum that probably includes the majority of zooflagellates of previously uncertain phylogenetic position, several distinct groups of filose testate rhizopods, reticulose protozoa such as gymnophryids, *Massisteria*, the parasitic plasmodiophorids and Ascetosporea (Cavalier-Smith and Chao 2003c). Unpublished studies using phylum-specific PCR primers to amplify DNA extracted directly from environmental samples suggest that Cercozoa are even more diverse than this and may be one of the most speciose protozoan phyla (Bass and Cavalier-Smith). Actin trees support the monophyly of Rhizaria (Keeling 2001) as more weakly do some rRNA trees (Cavalier-Smith and Chao 2003c).

Infrakingdom Excavata

The excavate concept originated from shared ciliary structures (lateral vanes) and ciliary root patterns that were most pronounced in members of the group possessing a ventral feeding groove (Simpson and Patterson 1999, 2001). Excavates are a good example of a polythetic taxon, i.e. one char-acterised not by one or more universally shared derived characters but by an array of characters partially overlapping in distribution but missing from many members of the group. In this respect and in the monophyly of the group often not being supported by single-gene trees excavates resemble Chromista and are encountering similar scepticism from some with naïve expectations of what single-gene trees can resolve. Multiple losses of the feeding groove and ciliary vanes have impeded understanding of the group. As presently constituted it embraces four phyla: Euglenozoa and Percolozoa (grouped together as superphylum Discicristata on account of their flat, typically discoid cristae and parallel centrioles) plus the basal biciliate Loukozoa and the highly derived, secondarily amitochondrial, ancestrally tetrakont Metamonada (Anaeromonadea, Parabasalia, Carpediemonadea, and Eopharyngia) (Cavalier-Smith 2003a).

It appears that the ancestral percolozoan and metamonad independently evolved a quadriciliate condition (Cavalier-Smith 2003a); it is likely that, as in secondarily quadriciliate green algae such as *Pyramimonas*, their ciliary transformation is spread over two cell cycles – a complex derived state. Though excavates sometimes appear holophyletic (Cavalier-Smith 2003a), their unity is obscured on many rRNA trees by dramatic variation in this molecule's evolutionary rate. This also leads the longest branch excavates to attract artefactually archaebacterial outgroup sequences and thus misleadingly show the root within Metamonada. It appears that secondary loss of mitochondria and their ribosomes often leads to dramatically increased evolutionary rates of cytosolic ribosomal rRNA and proteins, as in metamonads, microsporidia, Archamoebae and to spurious grouping on the trees, especially when intra-site rate variation is not allowed for and taxon sampling is too sparse. Metamonad unity has also been obscured by secondary losses of Golgi stacking (independently in Eopharyngia and oxymonads) and by the presence of hydrogenosomes in some but (probably) only mitosomes in others (e.g. Eopharyngia like *Giardia*). Golgi stacking was also lost in Percolozoa within Heterolobosea, the lyromonad subclade of which became anaerobic/hydrogenosomal independently of Metamonada (O'Kelly et al. 2003).

Loukozoa are probably paraphyletic; the classes Jakobea and Malawimonadea apparently diverged

very early in the group's history. Several rRNA and protein trees weakly suggest that jakobids are related to Euglenozoa, whereas *Malawimonas* may be sister to Metamonada. I earlier added Diphylleida to Loukozoa on the grounds that both are ancestrally biciliate taxa with ventral feeding grooves (Cavalier-Smith 2002), even though diphylleids lack ciliary vanes and their ciliary microtubular roots supporting the groove rims are less like those of jakobids and *Malawimonas* than the latter are to each other. More recently, to make Loukozoa more homogeneous I transferred Diphyllatea to Apusozoa as a second class (Cavalier-Smith 2003a). Numerous protein gene sequences are needed to test this relationship and establish the position of Apusozoa, as single-gene trees lack the requisite resolution. The ventral groove makes it possible that Apusozoa are sisters to excavates, but rRNA and Hsp90 trees weakly suggest they may be more distant.

The jakobid loukozoan *Reclinomonas* is notable for the most primitive mitochondrial genome known, in particular retaining proteobacterial RNA polymerase genes lost from most other eukaryotes following the acquisition from a virus of a simpler nuclear-coded RNA polymerase (Lang et al. 1999). The apparently derived position of *Reclinomonas* within bikonts on concatenated mitochondrial gene trees (Lang et al. 2002) does not support the a priori plausible suggestion based solely on this primitiveness that Loukozoa might be very early diverging eukaryotes (Cavalier-Smith 1999, 2000a). If the viral polymerase was acquired by the ancestral eukaryote, multiple independent losses of the then redundant proteobacterial polymerase are entirely plausible. Multiple independent losses of many other mitochondrial and chloroplast genes have certainly occurred.

The cabozoan hypothesis

The same considerations of economy in the origin of organelle protein-targeting machinery during symbiogenesis that led to the now widely accepted (but initially ignored or opposed) ideas of monophyly of Plantae, Chromista, Chromobiota and chromalveolates led me to suggest that chlorarachnean algae (phylum Cercozoa) and euglenoids (Euglenozoa) obtained their green chlorophyll a/b plastids by a single enslavement of a green alga by their common ancestor (Cavalier-

Smith 1999). If this were correct it would mean that Rhizaria and Excavata together form a clade (cabozoa), that their last common ancestor was a photophagotroph and that all aplastidic cabozoa have lost plastids (roughly 14 separate losses!). There are growing hints from cyanobacteria-like genes found in Percolozoa, trypanosomes and even metamonads that the common ancestor of discicristates and metamonads (presumably a loukozoan) may have had a plastid, but there is currently no comparable evidence for or against this for any aplastidic rhizarian (Cavalier-Smith 2003a, b). It is important to test whether Rhizaria and Excavata are sisters and seek plastid-related genes in aplastidic Rhizaria and Loukozoa. If the cabozoan hypothesis turns out to be correct it will greatly simplify eukaryote phylogeny: a single enslavement of a green alga by the ancestral cabozoan would add further compelling evidence that the root of the eukaryote tree cannot lie within cabozoa, any more than it can within Plantae or chromalveolates. It would also show that bikonts comprise but three major groups (Plantae, chromalveolates, cabozoa), each characterised by the ancestral enslavement of a foreign cell. If Apusozoa are sisters of Excavata or Rhizaria they also would be cabozoa – but if not they could be the only primitively non-photosynthetic bikonts.

The corticate hypothesis

If there are indeed only three or four bikont groups, what is their branching order? Long ago I postulated that cortical alveoli of Glaucophyta (kingdom Plantae) and alveolates are homologous (Cavalier-Smith 1982). If this is correct and they evolved in the common ancestor of Plantae and chromalveolates, they would be a shared derived character (albeit multiply lost) for a clade comprising both groups (hence designated corticates: Cavalier-Smith 2003b) or alternatively a reason for considering Plantae paraphyletic with chromalveolates sister to Glaucophyta alone (less likely but not impossible). If corticates and cabozoa both turn out to be clades we should have established the large-scale structure of the bikont part of the eukaryotic tree and rendered it highly probable that all bikont groups (i.e. most protozoan phyla) were ancestrally photosynthetic and that organelle loss has been even more important in protist evolution than often thought.

If the cabozoan or the corticate hypothesis (or both) proves to be mistaken, such disproof would probably in itself establish the correct branching order of the four main bikont groups: excavates, Rhizaria, Plantae and chromalveolates. We would then probably have a well-corroborated, properly rooted tree for all really major protist and eukaryotic groups.

Uncertainties

Key things to be tested more thoroughly are the cabozoan hypothesis and the positions of Apusozoa, centrohelids, Phaeodarea, and xenophyophores. Telonemea, Holosea and Schizocladea remain virgin territory for phylogenetics, and there are numerous other obscure protists of uncertain affinities that have not received the study they deserve. Eventually they will probably slot into the general scheme presented here. Some new classes will almost certainly be characterised, but our enumeration of eukaryote phyla may be close to completion, though we may still be in for a few big surprises, exemplified by the possibility that the obscure worm *Xenoturbella* represents a new animal phylum (Bourlat et al. 2003). Nonetheless, I suspect that we are entering a period of consolidation for high-level protist phylogeny and taxonomy after two revolutionary decades. There are almost certainly no undiscovered eukaryotic kingdoms; claims for novel kingdoms based on sequencing environmental DNA samples (Dawson and Pace 2002) are based on misrooted rRNA trees suffering from gross long-branch artefacts and inadequate taxon sampling.

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Appendix

Classification of kingdom Protozoa**
and its 11 phyla and 64 or 65 classes

Subkingdom SARCOMASTIGOTA** Cavalier-Smith 1993 emend.

 Phylum 1. Amoebozoa Lühe 1913 stat. nov. Corliss 1984 emend. Cavalier-Smith 1998

 Subphylum 1. Protamoebae+ Cavalier-Smith 2004

   Class 1. Breviatea+ Cavalier-Smith 2004 (e.g. 'Mastigamoeba invertens')

   Class 2. Lobosea Carpenter 1861 emend. (e.g. *Amoeba, Chaos, Saccamoeba, Hartmannella, Echinamoeba, Leptomyxa, Rhizamoeba, Copromyxa, Arcella*)

   Class 3. Discosea+ Cavalier-Smith 2004 (e.g. *Vannella, Platyamoeba, Multicilia, Vexillifera, Paramoeba, Mayorella, Cochliopodium, Thecamoeba*)

   Class 4. Variosea+ Cavalier-Smith 2004 (e.g. *Phalansterium, Acanthamoeba, Balamuthia, Gephyramoeba, Filamoeba, Stereomyxa*)

 Subphylum 2. Conosa Cavalier-Smith 1998

 Infraphylum 1. Archamoebae Cavalier-Smith 1983 stat. nov. 1998

   Class Archamoebea Cavalier-Smith 1983 stat. nov. (e.g. *Pelomyxa, Mastigina, Entamoeba, Mastigamoeba, Phreatamoeba, Endolimax*)

 Infraphylum 2. Mycetozoa De Bary stat. nov.

   Class 1. Stelamoebea*+ Cavalier-Smith 2004 (e.g. *Protostelium, Schizoplasmodium, Cavostelium, Planoprotostelium, Acytostelium, Dictyostelium*)

   Class 2. Myxogastrea Fries 1829 stat. nov. Cavalier-Smith 1993 emend. (e.g. *Hyperamoeba, Ceratiomyxa, Stemonitis, Physarum, Didymium*)

 Phylum 2. Choanozoa Cavalier-Smith 1981 emend. 1998

   Class 1. Choanoflagellatea Kent 1880 stat. nov. Cavalier-Smith 1998 (e.g. *Monosiga*)

   Class 2. Corallochytrea Cavalier-Smith 1995 (*Corallochytrium*)

   Class 3. Ichthyosporea Cavalier-Smith 1998 (e.g. *Dermocystidium, Ichthyophonus*)

   Class 4. Cristidiscoidea Page 1987 stat. nov. Cavalier-Smith 1997 (e.g. *Ministeria, Nuclearia*)

Subkingdom BICILIATA** subkingd. nov. Diagnosis: ancestrally biciliate unicellular eukaryotes in which dihydrofolate reductase and thymidylate synthase genes, if present, are fused into a single unit of translation; lacking plastids in the cytoplasm with double envelopes and lacking plastids within the rough endoplasmic reticulum or tubular ciliary hairs; mitochondrial cristae usually tubular or discoid (i.e. all bikonts excluding Plantae and Chromista)

 Infrakingdom 1. Rhizaria Cavalier-Smith 2002 emend.

 Phylum 1. Cercozoa Cavalier-Smith 1998 emend. 2002

 Subphylum 1. Filosa Cavalier-Smith 2003.

   Superclass 1. Reticulofilosa Cavalier-Smith 1997 stat. nov. 2003

   Class 1. Chlorarachnea Hibberd and Norris 1984 (e.g. *Chlorarachnion, Bigelowiella, Lotharella, Cryptochlora, Gymnochlora*)

   Class 2. Proteomyxidea* Lankester 1885 em. Cavalier-Smith 2003. (e.g. *Pseudospora, Leucodictyon, Reticulamoeba, Massisteria, Dimorpha, Gymnophrys, Borkovia*)

   Superclass 2. Monadofilosa Cavalier-Smith 1997 stat. nov. 2003

   Class 1. Sarcomonadea Cavalier-Smith 1993 emend. 2003 (e.g. *Cercomonas, Heteromita, Bodomorpha, Proleptomonas, Allantion*)

   Class 2. Thecofilosea Cavalier-Smith 2003 (e.g. *Cryptodifflugia, Cryothecomonas*)

   Class 3. Spongomonadea Cavalier-Smith 2000 (e.g. *Spongomonas, Rhipidodendron*)

   Class 4. Imbricatea Cavalier-Smith 2003 (e.g. *Thaumatomonas, Thaumatomastix, Allas, Gyromitus, Euglypha, Trinema, Paulinella*)

 Subphylum 2. Endomyxa Cavalier-Smith 2002 emend. 2003

   Class 1. Phytomyxea Engler and Prantl 1897 (e.g. *Phagomyxa, Plasmodiophora*)

   Class 2. Ascetosporea Sprague 1979 stat. nov. Cavalier-Smith 2002 (*Minchinia, Haplosporidium, Urosporidium, Bonamia, Marteilia, Paramyxa, Paramarteilia, Claustrosporidium, Microcytos*)

   Class 3. Gromiidea Cavalier-Smith 2003 (*Gromia*)

 Phylum Retaria Cavalier-Smith 1999

 Subphylum 1. Radiozoa Cavalier-Smith 1987 emend. (Ancestrally with axopodia having an open dodecagonal meshwork of microtubules and able to sequester strontium sulphate intracellularly)

   Class 1. Acantharea Haeckel 1881 stat. nov. Cavalier-Smith 1993 (e.g. *Acanthometra*)

   Class 2. Sticholonchea Poche 1913 stat. nov. Petrushevskaya 1977 (*Sticholonche*)

   Class 3. Polycystinea Ehrenberg 1838 stat. nov. Cavalier-Smith 1993 (e.g. *Collozoum*)

 Subphylum 2. Foraminifera (D'Orbigny 1826) Eichwald 1830 stat. nov. Mikhalevich 1980

   Class 1. Athalamea Haeckel 1862 (*Reticulomyxa*)

   Class 2. Polythalamea Ehrenberg 1838 stat. nov. Mikhalevich 1980 (e.g. *Allogromia, Textularia, Globigerina, Miliola*)

   Class 3. Xenophyophorea Schulze 1904 (e.g. *Psammina*) Possibly also class Schizocladea Cedhagen and Mattson 1992 (*Schizocladus*)

Rhizaria incertae sedis: Class Phaeodarea Haeckel 1879 (e.g. *Aulacantha, Castanella*)

Infrakingdom Excavata Cavalier-Smith 2002

 Phylum 1. Loukozoa* Cavalier-Smith 1999 emend. 2003

   Class 1. Jakobea Cavalier-Smith 1999 (*Reclinomonas, Histiona, Jakoba*)

   Class 2. Malawimonadea Cavalier-Smith 2003a (*Malawimonas*)

 Phylum 2. Metamonada Grassé 1952 stat. nov. emend. Cavalier-Smith 2003a

 Subphylum 1. Anaeromonada Cavalier-Smith 1996/7

   Class Anaeromonadea Cavalier-Smith 1996/7 emend. 1999 (*Trimastix*; Oxymonadida e.g. *Pyrsonympha, Dinenympha*)

 Subphylum 2. Trichozoa Cavalier-Smith 1996/7 stat. nov. emend. 2003*

   Superclass 1. Parabasalia Honigberg 1973 stat. nov. Cavalier-Smith 2003

   Class 1. Trichomonadea Kirby 1947 stat. nov. Margulis 1974 emend. Cavalier-Smith 2003 (e.g. *Trichomonas, Lophomonas, Microjoenia, Spirotrichonympha*)

   Class 2. Trichonymphea Cavalier-Smith 2003a (e.g. *Trichonympha*)

Superclass 2. Carpediemonadia Cavalier-Smith 2003a
   Class Carpediemonadea Cavalier-Smith 2003a *(Carpediemonas)*
Superclass 3. Eopharyngia Cavalier-Smith 1993 stat. nov.
   Class 1. Trepomonadea Cavalier-Smith 1993
      Subclass 1. Diplozoa Dangeard 1910 stat. nov. Cavalier-Smith 1996 (diplomonads: *Trepomonas, Hexamita, Spironucleus, Giardia, Octomitus*)
      Subclass 2. Enteromonadia Cavalier-Smith 1996 *(Enteromonas)*
   Class 2. Retortamonadea Cavalier-Smith 1993 (e.g. *Retortamonas, Chilomastix*)
Superphylum 1. Discicristata Cavalier-Smith 1993
 Phylum 1. Percolozoa Cavalier-Smith 1991 stat. nov.
      Class 1. Heterolobosea Page and Blanton 1985 (e.g. *Naegleria, Tetramitus, Vahlkampfia, Lyromonas, Psalteriomonas*)
      Class 2. Percolatea Cavalier-Smith 2003a *(Percolomonas, Stephanopogon)*
 Phylum 2. Euglenozoa Cavalier-Smith 1981
  Subphylum 1. Plicostoma Cavalier-Smith 1998
      Class 1. Euglenoidea Bütschli 1884 (e.g. *Petalomonas, Peranema, Rhabdomonas, Euglena, Astasia, Eutreptia, Trachelomonas*)
      Class 2. Diplonemea Cavalier-Smith 1993 *(Diplonema, Rhynchopus)*
  Subphylum 2. Saccostoma Cavalier-Smith 1998
      Class 1. Kinetoplastea Honigberg 1963 stat. nov. Margulis 1974 (e.g. *Bodo, Rhynchomonas, Ichthyobodo, Trypanosoma, Crithidia, Leishmania*)
      Class 2. Postgaardea Cavalier-Smith 1998 *(Postgaardi, Calkinsia)*
Infrakingdom Alveolata Cavalier-Smith 1991
 Phylum 1. Miozoa Cavalier-Smith 1987 stat. nov. 1999
  Subphylum 1. Protalveolata* Cavalier-Smith 1991 em.
      Class 1. Colponemea Cavalier-Smith 1993 *(Colponema)*
      Class 2. Perkinsea Levine 1978 *(Perkinsus)*
  Subphylum 2. Dinozoa Cavalier-Smith 1981 em.
   Infraphylum 1. Ellobiopsa infraph. nov. Diagnosis as for the class Ellobiopsea
      Class Ellobiopsea Loeblich III 1970 (e.g. *Ellobiopsis, Thalassomyces*)
   Infraphylum 2. Dinoflagellata Butschli 1885 stat. nov. Cavalier-Smith 1999
    Superclass 1. Syndina Cavalier-Smith 1993
      Class Syndinea Chatton 1920 stat. nov. Loeblich 1976 (e.g. *Amoebophrya*)
    Superclass 2. Dinokaryota Fensome et al. 1993
      Class 1. Noctilucea Haeckel 1866 stat. nov. Cavalier-Smith 1993 (e.g. *Noctiluca*)
      Class 2. Peridinea   Ehrenberg 1830 stat. nov. Wettstein (e.g. *Peridinium, Prorocentrum, Haplozoon, Dinophysis, Ceratium, Crypthecodinium, Symbiodinium, Polarella, Oxyrrhis*)
  Subphylum 3 Apicomplexa Levine 1970 emend. stat. nov.
   Infraphylum 1. Apicomonada Cavalier-Smith 1993 stat. nov.
      Class 1. Apicomonadea Cavalier-Smith 1993 (e.g. *Acrocoelus, Parvilucifera, Colpodella*)
   Infraphylum 2. Sporozoa Leuckart 1879 stat. nov. Cavalier-Smith 1999
      Class 1. Coccidea* Leuckart 1879 (e.g. *Hepatozoon, Cryptosporidium, Toxoplasma*)
      Class 2. Gregarinea Dufour 1828 (e.g. *Monocystis, Ophriocystis*)
      Class 3. Hematozoa Vivier 1982 (e.g. *Plasmodium, Babesia, Theileria*)
 Phylum 2. Ciliophora Doflein 1901 (ciliates and suctorians)
  Subphylum 1. Postciliodesmatophora Gerassimova and Seravin 1976
      Class 1. Karyorelictea Corliss 1974 (e.g. *Kentrophoros, Tracheloraphis, Loxodes*)
      Class 2. Heterotrichea Stein 1859 (e.g. *Stentor, Folliculina, Blepharisma*)
  Subphylum 2. Intramacronucleata Lynn 1996
      8 Classes: Spirotrichea (e.g. *Oxytricha, Euplotes, Tintinnus, Metopus*), Litostomatea (e.g. *Didinium, Lacrymaria, Entodinium*), Phyllopharyngea (e.g. *Dysteria, Podophrya*), Colpodea (e.g. *Colpoda*), Nassophorea (e.g. *Nassula*), Prostomatea (e.g. *Coleps*), Plagiopylea, Oligohymenophorea (e.g. *Tetrahymena, Paramecium, Vorticella*)

Biciliata incertae sedis:
   1. Phylum Apusozoa Cavalier-Smith 1996/7 stat nov. 2003 emend.
   Class 1. Diphyllatea Cavalier-Smith 2003a *(Diphylleia, Collodictyon)*
   Class 2. Thecomonadea Cavalier-Smith 1993 stat. nov. 1995 (e.g. *Amastigomonas, Apusomonas, Ancyromonas, Hemimastix, Spironema*)
   2. Class Telonemea Cavalier-Smith 1993 *(Telonema, Nephromyces)*

Protista incertae sedis (Protozoa or chromists): Class Holosea Cavalier-Smith 1993 *(Luffisphaera)*; Order Commatiida Cavalier-Smith 1996/7 *(Commation)*; Order Discocelida Cavalier-Smith 1997 *(Discocelis)*

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*Probably paraphyletic groups
**Certainly paraphyletic groups
+Diagnoses of these five new taxa are in Cavalier-Smith et al. (2004)