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Phylogeny of Choanozoa, Apusozoa, and Other Protozoa and Early Eukaryote Megaevolution

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Abstract. The primary diversification of eukaryotes involved protozoa, especially zooflagellates—flagellate protozoa without plastids. Understanding the origins of the higher eukaryotic kingdoms (two purely heterotrophic, Animalia and Fungi, and two primarily photosynthetic, Plantae and Chromista) depends on clarifying evolutionary relationships among the phyla of the ancestral kingdom Protozoa. We therefore sequenced 18S rRNA genes from 10 strains from the protozoan phyla Choanozoa and Apusozoa. Eukaryote diversity is encompassed by three early-radiating, arguably monophyletic groups: Amoebozoa, opisthokonts, and bikonts. Our taxon-rich rRNA phylogeny for eukaryotes allowing for intersite rate variation strongly supports the opisthokont clade (animals, Choanozoa, Fungi). It agrees with the view that Choanozoa are sisters of or ancestral to animals and reveals a novel nonflagellate choanozoan lineage, Ministeriida, sister either to choanoflagellates, traditionally considered animal ancestors, or to animals. Maximum likelihood trees suggest that within animals Placozoa are derived from medusozoan Cnidaria (we therefore place Placozoa as a class within subphylum Medusozoa of the Cnidaria) and hexactinellid sponges evolved from demosponges. The bikont and amoebozoan radiations are both very ill resolved. Bikonts comprise the kingdoms Plantae and Chromista and three major protozoan groups: alveolates, excavates, and Rhizaria. Our analysis weakly suggests that Apusozoa, represented by *Ancyromonas* and the apusomonads (*Apusomonas* and the highly diverse and much more ancient genus *Amastigomonas*, from which it evolved), are not closely related to other Rhizaria and may be the most divergent bikont lineages. Although *Ancyromonas* and apusomonads appear deeply divergent in 18S rRNA trees, the trees neither refute nor support the monophyly of Apusozoa. The bikont phylum Cercozoa weakly but consistently appears as sister to Retaria (Foraminifera; Radiolaria), together forming a hitherto largely unrecognized major protozoan assemblage (core Rhizaria) in the eukaryote tree. Both 18S rRNA sequence trees and a rare deletion show that nonciliate haplosporidian and paramyxid parasites of shellfish (together comprising the Ascetosporea) are not two separate phyla, as often thought, but part of the Cercozoa, and may be related to the plant-parasitic plasmodiophorids and phagomyxids, which were originally the only parasites included in the Cercozoa. We discuss rRNA trees in relation to other evidence concerning the basal diversification and root of the eukaryotic tree and argue that bikonts and opisthokonts, at least, are holophyletic. Amoebozoa and bikonts may be sisters—jointly called anterokonts, as they ancestrally had an anterior cilium, not a posterior one like opisthokonts; this contrasting ciliary orientation may reflect a primary divergence in feeding mode of the first eukaryotes. Anterokonts also differ from opisthokonts in sterol biosynthesis (cycloartenol versus lanosterol pathway), major exoskeletal polymers (cellulose versus chitin), and mitochondrial cristae

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(ancestrally tubular not flat), possibly also primary divergences.

Key words: Apusozoa — *Amastigomonas* — *Ministeria* — Cercozoa — Ascetosporea — Choanozoa — Placozoa — Hexactinellid sponges — Sterol biosynthesis — Bikonts

Introduction

Discussing the consequences of the origin of "a new and peculiar line of life," Darwin (1872) pointed out that "when this adaptation had once been effected, and a few species had thus acquired a great advantage over other organisms, a comparatively short time would be needed to produce many divergent forms, which would spread rapidly and widely, throughout the world." Subsequent paleontological work amply confirmed this view, showing that for almost all major groups for which there is a good fossil record, most of the distinctive subgroups into which they are divided originate a relatively short time after the group itself (Simpson 1944). Thus the history of life is better represented as a set of many successively nested bushes, each with many parallel stems, rather than as a tree like a conifer with a central trunk and well-spaced branches. As there are sound theoretical reasons stemming from both ecology and developmental biology to expect such a pattern (Simpson 1953), it is reasonable to expect it to apply also to organisms for which the fossil record is weak or nonexistent. This expected pattern of successive early radiations poses a problem for the reconstruction of the relationship between major groups by molecular sequence trees, because most of the radiations of morphologically distinctive subgroups will be concentrated in a relatively short time span after the origin of their parental group. If a gene evolves as a stochastic clock, which was assumed in the early days of molecular evolution (Zuckerkandl and Pauling 1962) but is now know to be generally untrue (Ayala 1999; Cavalier-Smith 2002a), although it is sometimes a useful approximation, then it can be calculated, for example, that the branching order during the Cambrian explosion of animal phyla could not be robustly resolved by an 18S rRNA tree if the successive branches were more closely spaced than about 40 My (Philippe et al. 1994).

Some of the established deviations from clock-like behavior make matters even worse. Saturation effects can reduce resolution and unequal rates or modes of evolution in different lineages can introduce systematic biases that can give false topologies with high "statistical" support (Philippe and Adoutte 1998). The phenomenon of heterotachy ([Lopez et al. 2002] also known as covarion shifts—changes among lineages in which parts of a molecule vary and how rapidly) can also give incorrect branching orders if, as is usual, tree-calculating algorithms do not allow for them or, if it takes place convergently in similar ways in unrelated groups, can give spurious confidence in trees (Lockhart et al. 1998). But heterotachy, as a special case of quantum evolution (unusually dramatic change in a relatively short time span [Simpson 1944]), may also make it possible to resolve some features of the tree more reliably than expected on the molecular clock assumption. Rare molecular changes that have occurred only once or twice in the history of life and never been reversed can also help give confidence to particular groupings (Rokas and Holland 2000), but even they have to be interpreted critically (Bapteste and Philippe 2002). Despite these qualifications, the broad conclusion of molecular evolutionists that basal radiations are hard to resolve in single-gene trees is generally true and is to be expected from paleontological experience and evolutionary theory.

The single most dramatic organismal innovation in the history of life since the establishment of the first bacterial cell was the origin of phagotrophy and the eukaryotic cell (Stanier 1970; Cavalier-Smith 1987a, 1991a, 2002b). Given the powerful arguments of Darwin (1872) and Simpson (1953), one would expect a relatively rapid early radiation of major eukaryotic lineages (as postulated earlier, this could have taken less than 50 million years [Cavalier-Smith 1978]), which ought to be relatively difficult to resolve in single-gene trees. Early 18S rRNA gene trees suggested an early divergence of three amitochondrial lineages (microsporidia, diplomonads, and Parabasalia [Vossbrinck et al. 1987; Sogin et al. 1989; Leipe et al. 1993; Cavalier-Smith 1993a]) and, apparently somewhat later, three aerobic ones (myxogastrid slime molds, heterolobosean amoeboflagellates [Percolozoa], and Foraminifera [Pawlowski et al. 1996]). Despite early arguments that rRNA could not be a molecular clock (Cavalier-Smith 1980), the seriousness of the topological distortion of these trees was not realized until Philippe and Adoutte (1996, 1998) argued that all these apparently early branchings might be a long-branch artifact caused by the attraction of the long-branch archaebacterial outgroup into long-branch eukaryotes that may actually be evolutionarily derived. Very soon tubulin and other protein trees (Li et al. 1996; Roger 1996) showed that microsporidia were really Fungi and that their deep position in 18S rRNA and a few protein trees was indeed an artifact (for detailed discussion of their highly derived secondarily amitochondrial nature see Roger [1999], Keeling et al. [2000], Fast and Keeling [2001], and Arisue et al. [2002a]).

Likewise, the less deep but apparently relatively early positions of the slime molds *Physarum* and

Dictyostelium also turned out to be long-branch artifacts (Baldauf et al. 2000); these and other protein data also supported the monophyly of Mycetozoa. More recently the grouping as the secondarily amitochondrial Archamoebae of *Entamoeba* and the pelobiont *Mastigamoeba* (*Phreatamoeba*) *balamuthi*, which occupied independent relatively deep positions in early rRNA trees (Hinkle et al. 1994), and the sister relationship of Archamoebae and Mycetozoa (as the amoebozoan subphylum Conosa [Cavalier-Smith 1998a]) were strikingly confirmed by maximum likelihood trees based on 123 proteins (Bapteste et al. 2002). Though not seen in early rRNA trees (Sogin 1991), the monophyly of both Mycetozoa and Conosa was seen in the first taxonomically well-sampled 18S rRNA tree (with 150 eukaryotes), albeit with weak support (Cavalier-Smith 1993a); subsequent rRNA trees also showed the monophyly of Archamoebae with high bootstrap support (Cavalier-Smith and Chao 1997), which has recently been even more robustly confirmed by trees including many more pelobionts and entamoebids (Edgcomb et al. 2002), and some placed them high in the tree with the lobose Amoebozoa (Cavalier-Smith and Chao 1996). A combined analysis of rRNA and two elongation factor proteins confirms the monophyly of both Archamoebae and Conosa (Arisue et al. 2002b). An 18S rRNA phylogenetic analysis with very broad taxonomic sampling of Amoebozoa showed their monophyly (Bolivar et al. 2001), albeit with relatively insignificant bootstrap support compared with the multiple gene trees. This history of steadily increasing support for the Conosa and Amoebozoa illustrates the danger of drawing premature conclusions of conflict with morphological evidence from molecular trees with low taxon sampling, especially single-gene trees.

Long-branch attraction seriously affects the rooting of the eukaryote tree: both rooting by prokaryotic outgroups and rooting of paralogue trees by their sisters almost always involve very long outgroup branches; contradictory results have been obtained for different genes, none of them trustworthy (Philippe and Adoutte 1998; Embley and Hirt 1998; Philippe et al. 2000; Cavalier-Smith 2002a,b). Recently a shared derived gene fusion between dihydrofolate reductase (DHFR) and thymidylate synthase (TS) has been used to argue that the root of the eukaryote tree lies between opisthokonts (animals, Choanozoa, and fungi [Cavalier-Smith 1987b]) and bikonts (all other eukaryotes except Amoebozoa [Cavalier-Smith 2002b]). As all studied bikont groups have this apparently derived gene fusion, it is argued that unless the fusion originated in the ancestral eukaryote and was secondarily reversed in those that lack it, bikonts as a whole must be holophyletic and the eukaryote root cannot lie among them (Stechmann and Cavalier-Smith 2002). (Here we use monophyly in its correct classical sense to embrace both holophyly and paraphyly; thus we do not follow the confusing fashion of using monophyly as an ambiguous synonym for holophyly.) Bikonts include not only the plant kingdom and the chromalveolates (chromists and alveolates), both of which arose by a single symbiogenetic event, but also the protozoan infrakingdoms Excavata and Rhizaria (Cavalier-Smith 2002b). If the eukaryote tree is correctly rooted between opisthokonts and bikonts (Stechmann and Cavalier-Smith 2002), it follows that diplomonads and Parabasalia are highly derived and advanced eukaryotes (as their complex ultrastructure long suggested: they occupy a derived position within the excavates [Cavalier-Smith 2002b]) and that the rRNA tree has been as thoroughly misleading about the nature of early eukaryotes as Philippe and Adoutte (1996, 1998) suggested.

Nonetheless, despite the above serious drawbacks, single-gene rRNA trees remain the method of choice for initial explorations of protozoan molecular diversity, both because amplifying and sequencing rRNA remain technically much easier, quicker, and cheaper than for proteins and because the database into which new sequences can be fitted is immensely larger. Furthermore, any one protein suffers to varying degrees in different groups from similar evolutionary biases to rRNA. As there are a large number of genera of zooflagellate (Patterson et al. 2002a) and amoeboid protozoa (Patterson et al. 2002b) which have not confidently been placed in phyla or other higher taxa, but which may be as crucial for understanding eukaryote evolution and diversification as much more familiar groups (Cavalier-Smith 2000a), rRNA genes provide a quick way of showing whether they fall clearly within established major lineages or constitute potentially important divergent groups that will merit extensive study (Brugerolle et al. 2002). We have therefore been sequencing as many of these phylogenetically uncharacterized protists as possible to deepen our knowledge of the overall range of eukaryote diversity. In this paper we report a detailed analysis of those that have turned out to belong in the protozoan phyla Choanozoa (a member of the opisthokonts and, therefore, important for understanding animal origins) and Apusozoa, the bikont lineage that is currently the least well characterized. Some of these sequences were included in an illustrative tree in a recent review of eukaryote diversity (Cavalier-Smith 2000a), but that tree did not allow for intramolecular rate variation, which is important for obtaining more accurate trees (Bapteste et al. 2002; Moreira and Philippe 2000; van de Peer et al. 2000). The present study is the first to combine broad taxon sampling of these two phyla with a phylogenetic model allowing for such rate variation.

Table 1. Details of the protozoan strains and accession numbers of their 18S rRNA sequences[a]

Species studied	Phylum (class/order)	Source	GenBank No.
Calliacantha sp. *simplex*	Choanozoa (Choanoflagellatea)	South Africa marine, University of Cape Town, Zoology Department aquarium	AF272000
Monosiga ovata	Choanozoa (Choanoflagellatea)	A.P. Mylnikov (now ATCC 50635)	AF271999
Ministeria vibrans	Choanozoa (Cristidiscoidea)	ATCC 50519	AF271998
Ministeria vibrans	Choanozoa (Cristidiscoidea)	South Africa marine; Cape Town	AF271997
Amastigomonas sp. ?*bermudensis*	Apusozoa (Apusomonadida)	ATCC 50234	AY050178
Amastigomonas mutabilis	Apusozoa (Apusomonadida)	South Africa marine, Klein Riviersvlei, Western Cape	AY050182
Amastigomonas sp. 2	Apusozoa (Apusomonadida)	Contaminant in CCAP979/5 (marine)	AY050179
Amastisomonas sp. 1	Apusozoa (Apusomonadida)	Scotland marine, Millport	AY050181
Amastigomonas sp. 50062	Apusozoa (Apusomonadida)	ATCC 50062 (marine)	AY050180
Ancyromonas sigmoides	Apusozoa (Ancyromonadida)	ATCC 50267 (Atlantic)	AF053088

[a] The five cultures lacking an ATCC number or donor were isolated by us.

Our results draw attention to the phylogenetic importance of *Ministeria*, a tiny choanozoan protozoan with a highly distinctive phenotype of radiating microvilli, which appears to be the sister either of choanoflagellates or of animals as a whole. If the latter were true (though we consider it less likely than the former), *Ministeria* would be the closest known protozoan to animals. We also suggest that Apusozoa may be of key importance for understanding the diversification of bikonts, as our trees weakly suggest that they may be the most divergent of all bikonts. A third conclusion of broad evolutionary significance relates to the recently established phylum Cercozoa (Cavalier-Smith 1998a, b), which turns out to contain the majority of the zooflagellates of uncertain evolutionary position (Patterson et al. 2002a) that we have so far studied molecularly (Cavalier-Smith 2000a). Although the present paper does not include these new cercozoan sequences, our analysis provides new insights into the composition and evolutionary position of the Cercozoa. We have found a single-nucleotide deletion from a conserved loop that is shared by all 82 classical cercozoans and all members of the parasitic group Ascetosporea (haplosporidia and paramyxids); furthermore, ascetosporans group with reasonably good bootstrap support with the classical Cercozoa, and the paramyxid *Marteilia* is robustly nested within the haplosporidia, contradicting claims based on poorly sampled trees that did not allow for intramolecular rate variation that haplosporidia and paramyxids are unrelated (Berthe et al. 1998). Although the precise position of ascetosporans within Cercozoa remains unclear, they may be related to plasmodiophorids and phagomyxids, which parasitize plants, not animals as do the Ascetosporea. Our rRNA trees also reproducibly but weakly support a sister relationship between Cercozoa and Retaria (Foraminifera and Radiolaria), consistently with the grouping of Cercozoa and Foraminifera on actin trees (Keeling 2001).

We discuss the overall structure of the rRNA tree in relation to recent trees using multiple proteins, with which they are broadly congruent when rooted as suggested by the DHFR–TS gene fusion. We also attempt to unify the picture provided by molecular sequence trees, arguments about the evolution of protist cells, especially the evolution of cilia and the cytoskeleton (Cavalier-Smith 2002b) and the phenomena of secondary symbiogenesis (Cavalier-Smith 1999, 2002c, 2003a), both of which can help to polarize the direction of change within major parts of the tree.

Materials and Methods

Cell Cultures

Cultures were obtained from the American Type Culture Collection (ATCC), donated by colleagues, or isolated by us directly from nature into uniprotozoan culture as summarized in Table 1, which also gives accession numbers for the new sequences. Our uniprotozoan *M. vibrans* culture was purified from a mixed culture with a zooflagellate obtained by serial dilution into microtiter plates containing SES medium (9 parts autoclaved seawater:1 part SES; see the UK National Culture Collection [UKNCC] catalog) from a coastal marine sample collected near Cape Town, South Africa, 1996. *Amastigomonas* (*Am.*) *mutabilis* was cultured from an estuarine sample from Kleinriviersvlei, Western Cape, South Africa. Both these South African strains were maintained in culture for several years and successfully frozen but were lost in a freezer accident. An only tentatively identified stalked and loricate acanthoecid choanoflagellate resembling *Calliacantha simplex* was isolated by serial dilution from a seawater/sediment sample from a marine aquarium at the Department of Zoology, University Cape Town, South Africa, in 1996 and grown for a few months in seawater medium but died before it could be deposited in a collection or fully identified. As, in the absence of electron microscopy, we cannot exclude the possibility that it is an undescribed species, we designate it *Calliacantha* ?*simplex*, which is known from South African waters (Manton and Oates 1979). *Monosiga ovata* was a Russian isolate donated by AP Mylikov (Borok, Russia), which is now deposited in the ATCC, *Amastigomonas* sp. 30062 was obtained both directly from the ATCC (30062) and indirectly from the Woods Hole Oceanographic Institute culture collection labeled "*Rhynchomonas nasuta*" (but annotated in the culture list as

identified by D.J. Patterson as *Am. debruynei or Am. trahens*); however, microscopical observation shows that it is distinctly larger than these species or *Am. filosa*, which some cells resemble in having filose projections. DNA from both cultures had the same 18S rRNA sequence. *Am.* sp. CCAP was found as a contaminant in a *Cryptomonas rostrella* culture from the CCAP and purified. *Am.* sp. Millport was cultured from a marine aquarium at The Marine Biological Laboratory, Millport, Scotland.

M. vibrans, *Am. mutabilis*, and *A. sigmoides* were fixed in glutaraldehyde and osmium tetroxide in cacodylate buffers, and Epon-embedded sections were stained with uranyl and lead by standard methods.

Gene Sequencing and Phylogenetic Analyses

DNA isolation, purification, 18S rRNA gene amplification by PCR, sequencing, editing and addition to multiple alignments were as described previously (Cavalier-Smith and Chao 1995). The best-aligned and most conserved alignment positions were selected for analysis using PAUP* v. 4.0 (Swofford 1999) on a Macintosh G4. Modeltest (Posada and Crandall 1998) selected the GTR model with Γ correction for intersite rate variation and allowance for invariant sites as the best of 56 substitution models for all data sets; the appropriate parameters were calculated separately for each data set and the corresponding GTR + Γ + *I* distance matrices used for neighbor-joining trees (ties broken randomly) and for full heuristic distance searches using both the minimum evolution criterion and the least-squares (power 2) methods for the best tree, using TBR branch swapping and MULtrees but no rapid descent. Invariant sites were removed in proportion to base frequencies estimated from all sites.

The new sequences were aligned manually with our aligned database of over 450 diverse eukaryote sequences and a representative subset of 284 sequences including all protozoan phyla selected for detailed analysis. Initially two taxon samples of 279 and 227 sequences were selected, which together included all protozoan phyla and major subgroups for which sequences were available. To reduce long-branch attraction problems the 279-sequence data set excluded the ultralong-branch sequences of the paramyxid *Marteilia* and the four foraminiferan sequences. These sequences were included in the 227-sequence data set, from which the longest-branch excavate taxa (diplomonads, Parabasalia, Percolozoa, and Euglenozoa) were excluded. Minimum evolution and bootstrapped neighbor-joining trees (GTR + Γ + *I*) were calculated for both data sets using only the best-aligned 1344 nucleotide positions. All well-supported nodes showed the same branching order for both data sets (and tree-construction methods) and even the branching orders of the very weakly supported ones were mostly the same. The main differences were in the basal branching order among bikonts (especially plants and chromalveolates).

As none of the new sequences under study here grouped with the long-branch excavates and their exclusion or inclusion made no significant difference to the large-scale structure of the tree, we excluded them from the tree shown here for 100 representative sequences drawn from the 227-sequence data set (Fig. 1), to enable a larger number of nucleotide positions (1672; a PHYLIP alignment is available from GenBank, accession No.XXXXXXXXX) to be included (these extra positions are missing or very hard to align in diplomonads, Parabasalia, and Percolozoa and very divergent in Euglenozoa). The Amoebozoa are also very divergent, and the subphylum Conosa has very long branches, so a data set including only 1567 positions tailored to Amoebozoa was also analyzed. As Amoebozoa, with the sole exception of "*Mastigamoeba*" *invertens*, were monophyletic in the 1344- and 1567-position trees, we also excluded the long-branch Conosa (Mycetozoa plus Archamoebae [Cavalier-Smith 1998a; Bapteste et al. 2002]) from the smaller trees to allow 1672 alignment positions to be used reasonably. All three gave the same topology for well-supported nodes but differed in the basal branching order among bikonts (especially plants and chromalveolates). When they were included, Archamoebae (pelobionts and entamoebids) were monophyletic with moderate bootstrap support; Mycetozoa were also monophyletic, but with low bootstrap support (*Gephyramoeba* and *Filamoeba* cluster with them). It appears that *Gephyramoeba* and *Filamoeba* may actually belong in the Conosa, as they always group within them (Bolivar et al. 2001). Bootstraps used 100 resamplings for the 227- and 279-sequence trees and 500 heuristic resamplings with a time limit of 5 min for each TBR branch swapping. For the 100- and 96-sequence trees, heuristic searches used 100 random additions each with unlimited TBR branch swapping: initial trees for minimum evolution and weighted least-squares analyses were obtained by these methods, not by neighbor joining.

For 100 and 66 taxon samples and 1672 positions we also calculated maximum likelihood trees using quartet puzzling (GTR + Γ + *I*; parameters and substitution rate matrix calculated by Modeltest) with empirical base frequencies and 1000 puzzling steps.

Results and Discussion

Overall Structure of the Eukaryotic 18S rRNA Tree

Irrespective of the data set there was always a clear-cut division of eukaryotes between the opisthokonts and the bikonts/Amoebozoa. In the 279-species and 227-sequence trees based on 1344 positions, bootstrap support for this was very high (94 and 93%, respectively), in keeping with the 100% support found for this bipartition in trees using 123 proteins (Bapteste et al. 2002) and very strong support with 4 concatenated proteins (Baldauf et al. 2000). With the 100-sequence least-squares tree (Fig. 1), the bootstrap support for this bipartition was somewhat lower (66%) than in our previous studies using neighbor joining and no Γ correction (typically in the range of 73–79% [Cavalier-Smith 1993a, 2002b; Cavalier-Smith and Chao 1996], despite variations in the number of included positions from 1515 up to the entire molecule; only once was support as low as 53% [Cavalier-Smith 1995]). An early Γ-corrected tree for 79 eukaryotes including long-branch excavates and archaebacterial outgroups and only 1260 positions gave 98% support for the opisthokont clade, while the parsimony tree for the same data set had 89% support (Cavalier-Smith and Chao 1996). Yet a recent Γ-corrected maximum likelihood tree also had only 20% support for opisthokonts as well as only 49% for animals (but 66% for Choanozoa! [Brugerolle et al. 2002]), possibly partly because some of the most distinctive opisthokont signatures (see below) were excluded because of the incompleteness of the *Diphylleia* sequence (only 1305 positions used). Despite variations in the bootstrap support with method, taxon, and positional sampling, the strength and reproducibility of the bipartition between opisthokonts and the rest contrast markedly with the

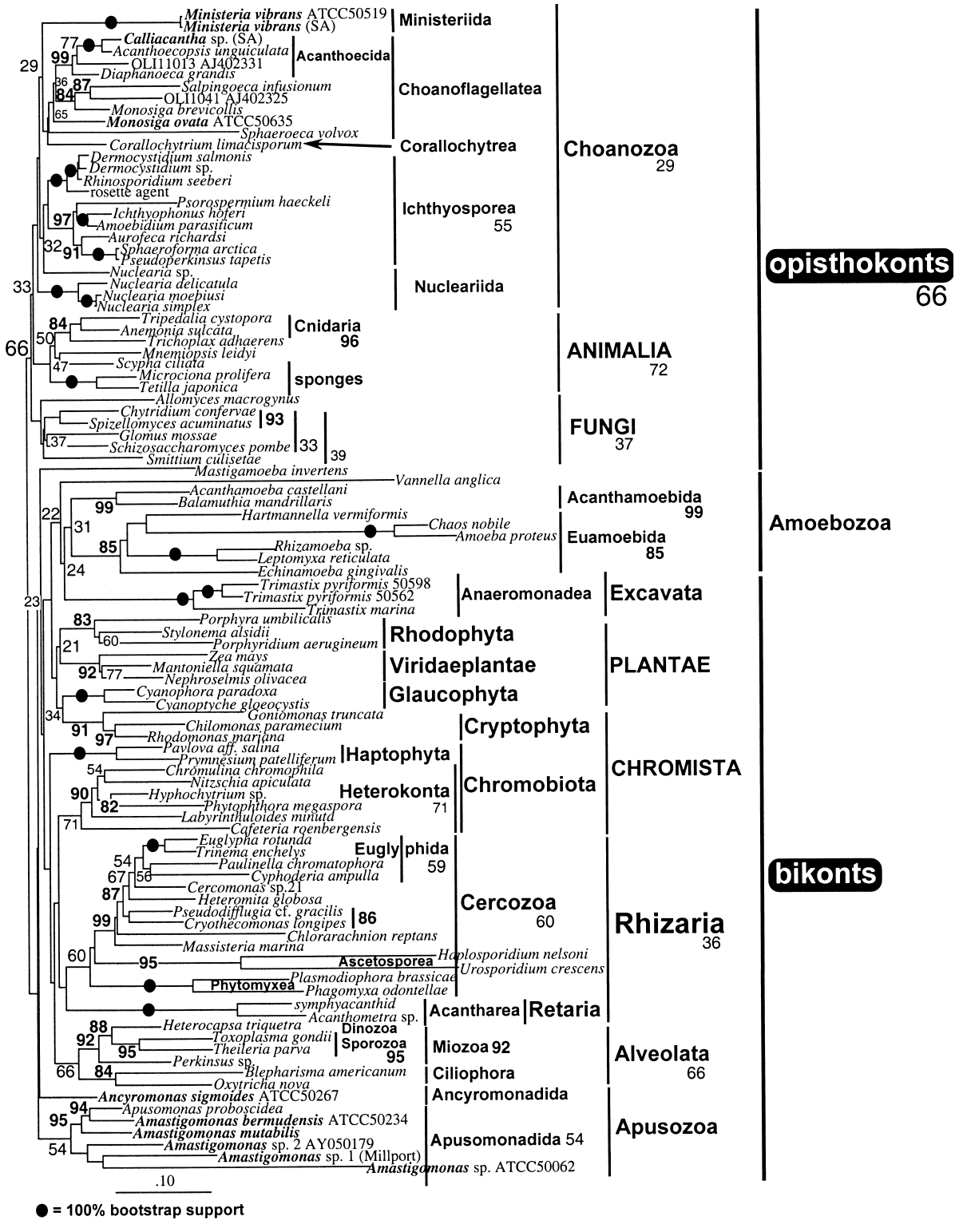

Fig. 1. Distance tree of 100 18S rRNAs using 1672 positions (weighted least squares, power 2; GTR + Γ + *I* model, α = 0.62018, *i* = 0.26839). The figures next to the nodes (or, if insufficient space, after the group names) are bootstrap percentages over 20% (boldface if 80% or more). All sequences outside the four derived kingdoms (animals, fungi, plants, chromists) belong to the basal kingdom Protozoa.

absence of significant bootstrap support anywhere in the backbone of the bikont part of the tree—the highest support is the 23% for the Apusozoa being the most divergent branch; no other deep node exceeds 20%. This striking contrast is broadly consistent with numerous previous trees (e.g., van de Peer et al. 2000; Atkins et al. 2000; Silberman et al. 2002).

Figure 1 was rooted between the opisthokonts and the bikonts because the derived gene fusion between DHFR and TS indicates that the root cannot lie

within the bikonts (Stechmann and Cavalier-Smith 2002) and indels in EF1α and enolase (Baldauf and Palmer 1993; Baldauf 1999) show that the root cannot lie within the opisthokonts.

In contrast to the consistent recovery of an opisthokont clade, the monophyly of Amoebozoa and the bikonts is not observed. In Fig. 1 Amoebozoa appear to branch within the bikonts and do not form a clade. In the 279- and 227-taxon trees that also include the conosan Amoebozoa, unlike Fig. 1, all Amoebozoa excluding *M. invertens* do form a very weakly supported clade; we have calculated many other Γ distance trees with different taxon sampling (in the range of 100–250 sequences), different numbers of positions included, and a variety of methods. In some of them *M. invertens* groups weakly with other Amoebozoa and in some it does not. The precise position of Amoebozoa varies, as does that of the two apusozoan groups Apusomonadida and Ancyromonadida. In Fig. 1 Apusozoa are the outgroups to all other bikonts plus the Amoebozoa, but with only 23% support. This topology is similar to our earlier non-Γ-corrected distance tree (Cavalier-Smith 2000a), where Ancyromonadida and Apusomonadida appeared as the topologically closest groups to opisthokonts (but in reverse order compared to Fig. 1). However, a recent maximum likelihood analysis placed Amoebozoa within a paraphyletic Apusozoa as sisters to other bikonts (assuming that the root is between opisthokonts and bikonts/Amoebozoa) (Cavalier-Smith 2002b). With some taxon samples Ancyromonadida and Apusomonadida appear weakly as a holophyletic group, but with others they do not even stay together as a paraphyletic group. None of these positions has good bootstrap support, and (as Atkins et al. [2000] rightly indicate) it is unlikely that the relative branching order of Amoebozoa and Apusozoa and other bikonts or the basal branching within bikonts will ever be reliably resolved by 18S rRNA trees. Nor can they establish whether or not Amoebozoa, Apusozoa, or bikonts are monophyletic, as morphological evidence suggests for them all (Cavalier-Smith 2000a, b). Therefore we are now gathering protein sequence data for both Apusozoa and Amoebozoa to clarify this issue and attempt to get molecular evidence for or against the monophyly of both groups.

Ministeria Is a Distinctive Choanozoan at Least as Closely Related to Animals as Are Any Other Organisms

Choanozoa is a protozoan phylum of unicellular or colonial heterotrophs with characteristically flat, typically nondiscoid, mitochondrial cristae (Cavalier-Smith 1981, 2002b). It now comprises four distinct

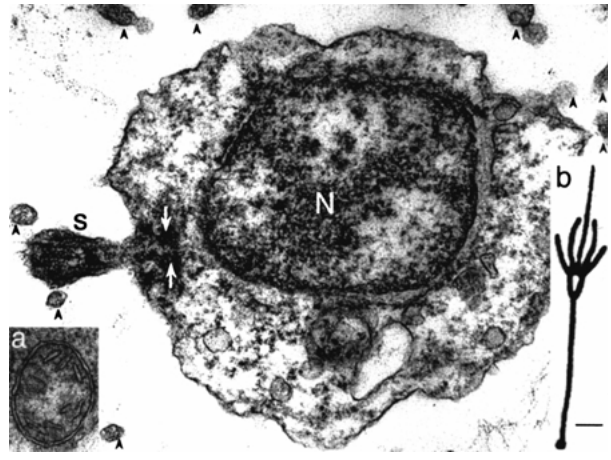

Fig. 2. Electron micrograph of an ultrathin section of the South African *M. vibrans* (courtesy of B. Oates). The nucleus (N) has fibrous material in the lumen of its envelope. As the stalk (s) has structures resembling doublet microtubules and a dense truncated basal body, it might be a modified cilium; the densities around the basal body somewhat resemble pericentriolar satellites (*arrows*). The microvilli (mostly in cross section; *arrowheads*) contain indistinct microfilaments like the similarly slender actin-containing tentacles of choanoflagellates but, unlike heliozoan axopodia, lack a microtubule skeleton. **Inset a:** A mitochondrion with flat, somewhat discoid, cristae. **Inset b:** A sketch of the stalked, loricate acanthoecid choanoflagellate, *Calliacantha ?simplex* in Fig. 1 (scale bar, 10 μm).

classes (Choanoflagellatea, Ichthyosporea, Corallochytrea, and Cristidiscoidea [Cavalier-Smith 2000a]), of which choanoflagellates have traditionally been regarded as closely related to animals because they resemble the collar cells (choanocytes) of sponges (James-Clark 1868). In all trees Choanozoa were placed closer to animals than to fungi, but with low bootstrap support. This animal/choanozoan grouping is stabler than in earlier trees assuming intersite rate uniformity, agreeing with protein evidence (Snell et al. 2001; King and Carroll 2001). Of particular interest is the position of the apparently nonflagellate marine protozoan *Ministeria vibrans* (Tong 1998). *M. vibrans* has radiating microvilli similar to those of choanoflagellates but not forming a collar; it sticks to substrates by a vibratile stalk, which electron microscopy (Fig. 2; also T.C.-S. and B. Oates, in preparation) suggests is a degenerate cilium. *Ministeria* is clearly a choanozoan, like the filose amoebae *Nuclearia* with which it is classified in the Cristidiscoidea (Cavalier-Smith 2000a). However, it appears closer to choanoflagellates and *Corallochytrium* than to either *Nuclearia* clade (Fig. 1).

Within choanoflagellates the purely marine order Acanthoecida, characterized by extracellular loricas built from siliceous strips (Cavalier-Smith 1998b), is robustly holophyletic (99% support). However, the branching order within Choanozoa is unstable; to clarify their relationships further, we also analyzed an alignment including many more animals and fungi

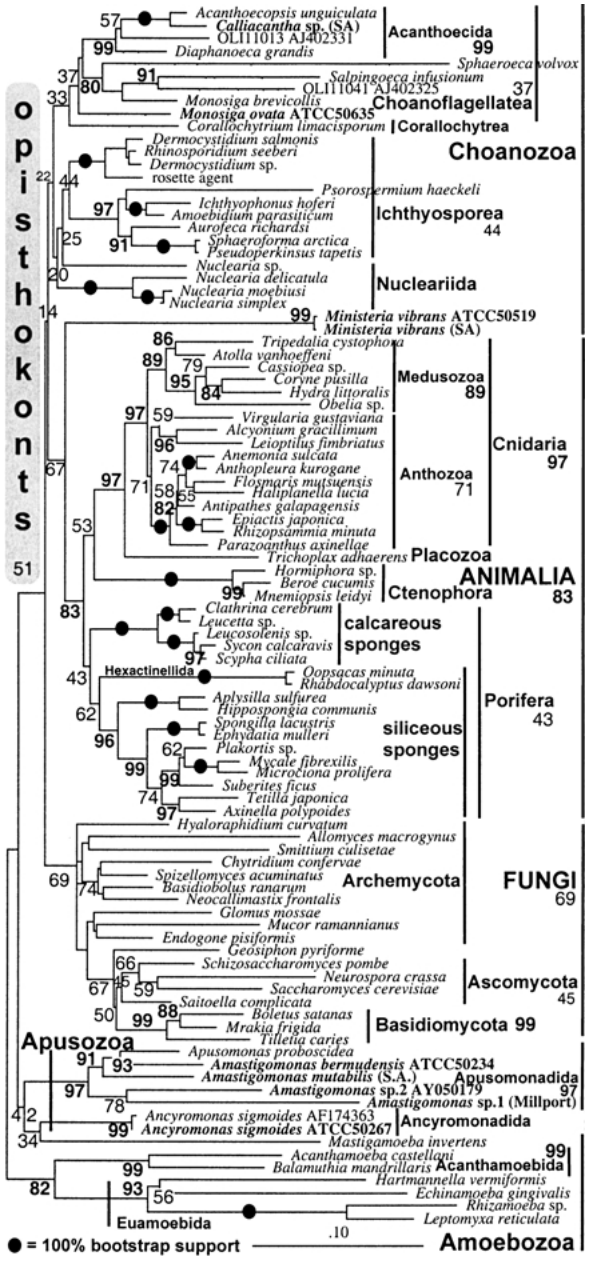

Fig. 3. Weighted least-squares (power 2) distance tree of 96 opisthokont, amoebozoan, and apusozoan 18S rRNAs using a GTR + Γ + *I* model (α = 0.6492, *i* = 0.32511). In the consensus bootstrap tree choanoflagellates without siliceous lorica (Craspedida) were holophyletic with low support.

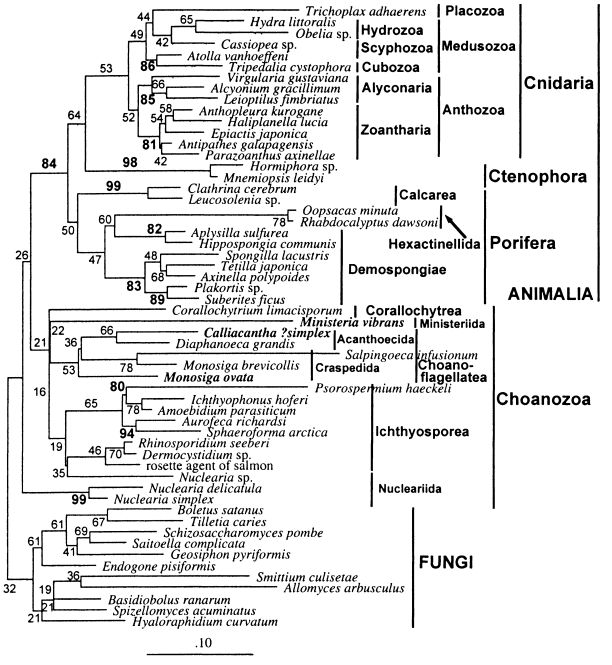

Fig. 4. Quartet puzzling maximum likelihood tree for 56 opisthokonts using the GTR+ Γ + *I* model (α = 0.530781, *i* = 0.364794, and the rate matrix given by Modeltest) for 1672 positions. Puzzling support values are shown.

but only Apusozoa and Amoebozoa (often the closest taxa to opisthokonts) as outgroups (Fig. 3). Using this and other taxon samples and distance and maximum likelihood algorithms, choanoflagellates are always closer to animals than to fungi. *Ministeria*, though grouping with choanoflagellates plus *Corallochytrium* in Fig. 1, or with choanoflagellates alone in some trees, often groups with animals instead (Fig. 3), depending on the taxon sample and phylogenetic algorithm. In the quartet puzzling tree with data and parameters identical to those in Fig. 1, *Ministeria* even went within the sponges with 29% support, but this tree is untrustworthy, as it had a few other peculiarities, notably fungi not being monophyletic and muddled up among Amoebozoa (with low puzzling support)—it appears that for the poorly supported parts of the tree Γ-corrected quartet puzzling copes less well with large data sets having very unequal branches than identically Γ-corrected distance methods. This high sensitivity to long-branch problems (Ranwez and Gascuel 2001) makes quartet puzzling unsuited to this data set, but for this many taxa a full maximum likelihood was too computationally intensive.

Trees using a more conservative set of only 1581 positions failed clearly to establish the position of *Ministeria*. A minimum evolution distance analysis (GTR + Γ + *I*) including only opisthokonts, Apusozoa, and Amoebozoa placed *Ministeria* as sisters to animals when the long-branch choanoflagellate *Sphaeroeca* was included, but at the base of the choanoflagellate/*Corallochytrium* clade when *Sphaeroeca* was excluded. However, a minimum evolution distance analysis (GTR + Γ + *I*) of 81 opisthokont sequences alone showed *Ministeria* as sister to animals with 42% bootstrap support. It was also sister to animals, with low support in the 227- and 279-sequence minimum evolution trees using only 1344 positions. In the Γ-corrected maximum likelihood tree for opisthokonts alone, from which the longest-branch taxa from Fig. 1 were deliberately excluded (Fig. 4), which a priori should be the most

reliable, *Ministeria* was sister to choanoflagellates (with low bootstrap support). Clearly with these contradictory results and low bootstrap support we cannot say whether *Ministeria* is really the closest known protozoan to animals (Fig. 3) or a closer relative to choanoflagellates (Figs. 1 and 4). Its long branch with no close relatives hinders its accurate placement.

Sequencing the complete 18S rRNA genes of our South African isolate and Tong's type strain from Europe shows that they differ in only six nucleotides in the most variable regions. Though the internal transcribed spacer (ITS) regions differ very substantially (not shown), we regard them as strains of the same morphospecies. Ultrastructure indicates that, like Tong's (1998), our strain has flat mitochondrial cristae (Fig. 2). Occurring in South Africa, Australia (Tong, personal communication), and Europe (Tong 1998), *M. vibrans* is probably a cosmopolitan coastal marine protozoan, previously overlooked through its minuteness. Compared with established protozoan groups, *Ministeria* have a novel phenotype; they are not flagellates or amoebae or actinopod, ciliate, or sporozoan in form. They are heterotrophs that feed by phagocytosing bacteria (in which our cultures abound). Tong's strain does not vibrate when in uniprotozoan culture; around each cell is a zone of bacterial depletion of the same diameter as the filopodial array, suggesting that (like those of choanoflagellates, sometimes referred to as "tentacles" [Leadbeater 1983], some of which aggregate to form their distinctive periciliary collar) they help to trap bacteria before phagocytosis. Our strain differs physiologically by vibrating actively in uniprotozoan culture, which would allow it to harvest bacteria more widely. Because of its distinctive morphology and possible close relationship with animals, *Ministeria* deserves thorough molecular investigation. The only other known species, *M. marisola* (Patterson et al. 1993), is not in culture.

Corallochytrium groups with or within the recently recognized parasitic Ichthyosporea (Cavalier-Smith 1998b) in some trees. As *Corallochytrium* has a wall and no cilium, like some ichthyosporeans, this is slightly more comprehensible than its more common position as sister to choanoflagellates. The parasitic *Nuclearia* sp. almost invariably groups with Ichthyosporea (in two quartet puzzling trees it grouped with choanoflagellates), but the free-living *N. simplex* cluster only sometimes does. The extremely divergent position of the *N. simplex* cluster as the deepest-branching choanozoan lineage in Figs. 1 and 4 (also noted by Amaral Zettler et al. 2001) might be a long-branch artifact; the movement, in the taxonomically more restricted Fig. 3, of *N. simplex* to become sister to the Ichthyosporea/*Nuclearia* sp. clade is consistent with this. Whether Nucleariida are really holophyletic sisters to Ichthyosporea or polyphyletic is unclear. However, multiple protein sequences are required to establish the branching order of the five choanozoan groups and whether Choanozoa are paraphyletic (as in Figs. 3 and 4) or holophyletic (as in Fig. 1); bootstrap support for choanozoan holophyly is too low to exclude the possibility that Ichthyosporea/*Nuclearia* are closer to fungi than to animals/choanoflagellates/*Ministeria*. Studies of wall chemistry and protein sequences in ichthyosporeans and *Corallochytrium* are needed to investigate the possibility that one or both might actually be the sister group to fungi.

The assertion that the animal kingdom "has its roots within the Order Choanoflagellida," implying that choanoflagellates and Choanozoa are both paraphyletic (Patterson 2002), is premature at best. No molecular trees clearly show animals branching within the choanoflagellates. It is thus likely that crown choanoflagellates are holophyletic, as Figs. 1, 3, and 4 show with low bootstrap support, and that animals evolved from a stem choanozoan with choanoflagellate-like morphology (Cavalier-Smith 1998b). (Crown and stem are cladistic terms invented by Jefferies [1979] but misused by Knoll [1992], an error unwittingly copied by many molecular evolutionists: "crown eukaryotes" properly means *all* descendants of the eukaryote cenancestor, whereas stem eukaryotes would be those diverging earlier whose descendants are all extinct; thus *all* extant eukaryotes are crown eukaryotes.) Irrespective of whether *Ministeria* is the sister to animals or to choanoflagellates, the topology of our trees makes it likely that the ancestral choanozoan possessed actin-based filopodia, as these are present in *Ministeria*, *Nuclearia*, and choanoflagellates. They were probably lost in *Corallochytrium* and Ichthyosporea (conceivably in a common ancestor, if the rare trees grouping them together are correct).

Placozoa May Be Derived from Cnidaria, and Hexactinellida from Demosponges

It has long been controversial whether *Trichoplax* is a primitively simple metazoan "missing link" or became simplified from a more complex ancestor by degeneration (Schuchert 1993). Most previous trees showed the placozoan *Trichoplax* as sister to Cnidaria plus Bilateria (e.g., Cavalier-Smith et al. 1996; Collins 1998). This position made it unlikely that *Trichoplax* is an early-diverging animal, since in that case it should have been sister to all other animals, and more likely that it originated by degeneration from a more complex radiate animal. Although our Γ-corrected distance trees usually place it as sister to Cnidaria, in some large taxon samples including only opisthokonts it appeared within the Cnidaria as sister

to Medusozoa with 52% bootstrap support (in Fig. 4 this has only 44% support), the Cnidaria/Placozoa clade having 90% support (53% in Fig. 4). As the Γ-corrected maximum likelihood tree actually places it within Medusozoa with low puzzling support, in agreement with the parsimony tree of Bridge et al. (1995), we suggest that its usual exclusion from Cnidaria may be a long-branch artifact, especially in trees that also include Bilateria, which are inordinately long branches (Cavalier-Smith et al. 1996; Collins 1998). We suggest that *Trichoplax* evolved from a scyphozoan ancestor and represents a neotenous planula larva, as others have repeatedly speculated (see review by Collins 1998). Such degeneration is consistent with the evidence that it has only one Hox gene, more related to the more numerous ones of Scyphozoa and Hydrozoa than to those of Bilateria (Schierwater and Kuhn 1998). Positive antibody staining for a neuropeptide (Schuchert 1993) is consistent with a derivation from an ancestor with a nervous system. If *Trichoplax* is a degenerate medusozoan, it should not be excluded from Cnidaria as a separate phylum (Cavalier-Smith 1998a). We therefore place it as a fourth class within subphylum Medusozoa; compared with Cavalier-Smith (1998a), this usefully reduces the number of animal phyla to 22.

Although our distance trees placed the syncytial hexactinellid sponges as sisters to demosponges, our maximum likelihood analysis puts them within demosponges as sisters to the *Aplysilla*/*Hippospongia* clade with moderate bootstrap support (Fig. 4). As they have a very long bare branch, we suspect that their usual exclusion from demosponges may be a long-branch artifact. We suggest that Demospongiae are ancestral to them and thus paraphyletic (but, nonetheless, should be retained as a class). The distinctive syncytial organization and spicules of Hexactinellida are sufficient reason to maintain them as a distinct class. However, their probable derivation from demosponges makes it even less desirable than before to treat them as a separate subphylum; it is better to include both classes in the subphylum Hyalospongiae Vossmaer 1886 (siliceous sponges) (Cavalier-Smith 1998a). Distinctive unsaturated fatty acids also strongly support the unity of Hyalospongiae (Thiel et al. 2002). Although our trees mostly do not support earlier hints that sponges may be paraphyletic (Cavalier-Smith et al. 1996; Collins 1998), they do not strongly exclude this possibility, which is biologically attractive.

The Great Genetic Depth and Probable Antiquity of Apusozoa

Apusozoa are a small protozoan phylum of zooflagellates (Cavalier-Smith 2002b) (formerly subphylum [Cavalier-Smith 2000a]) comprising only the class Thecomonadea, with three orders, of which sequence data are available for only two biciliate ones: Apusomonadida (*Apusomonas* and *Amastigomonas*) and Ancyromonadida (*Ancyromonas*). The very same strain of *Ancyromonas sigmoides* was independently sequenced by Atkins et al. (2000), who reached similar conclusions on its deep divergence from *Apusomonas* and its evolutionary position as in our preliminary tree (Cavalier-Smith 2000a). As the sequence of Atkins et al. (2000) has seven single nucleotides missing in highly conserved regions where we found no deletions (our sequences are otherwise identical), our sequence is probably slightly more accurate, so only ours is included in Fig. 1. For comparison, their sequence, with its slightly longer branch, is also included in Fig. 3. The apusomonad clade is often strongly supported, with *Apusomonas* nesting shallowly within and undoubtedly derived from *Amastigomonas*. Although the *Am.* sp. 50062 lineage has evolved several times faster than most others, even the short-branch part of the *Amastigomonas* subtree shows remarkable phyletic depth comparable with that of fungi or radiate animals. Unless the whole apusomonad clade has evolved unusually rapidly, for which there is no internal evidence from the tree, the genus *Amastigomonas* has probably persisted with similar morphology for several hundred million years—thus it may be an unusually conservative phenotype (Fig. 5). The length of its 18S rRNA is pretty average: the most rapidly evolving 18S rRNAs are usually substantially longer (Euglenozoa, Archamoebae) or shorter (diplomonads, Microsporidia, Parabasalia, Percolozoa) than usual. Our tree (Fig. 1) shows five highly divergent lineages: as most *Amastigomonas* are very similar morphologically (Molina and Nerad 1991), we were able to identify only one of the three that we isolated (*Am. mutabilis*) with confidence. The seven described species (Molina and Nerad 1991) probably greatly underrepresent their diversity; there are certainly more than the four recognized by the lumper's perspective (Patterson and Lee 2000; Lee and Patterson 2000). We suspect that there are many undescribed species of very similar appearance; our light microscopic examination of *Am.* sp. ATCC 50062 suggests that it cannot be assigned to a known species and that numerous cultures must be studied to establish species boundaries.

Sometimes the other biciliate apusozoan, *Ancyromonas* (Mylnikov 1990), weakly groups with apusomonads, but often it does not; neither has consistent close relatives. Although their shared subplasma membrane dense layer (Fig. 5) is unlikely to be convergent, they probably mutually diverged very early in eukaryotic evolution, consistent with the tubular mitochondrial cristae of apusomonads and flat cristae and different body form of *Ancyromonas*.

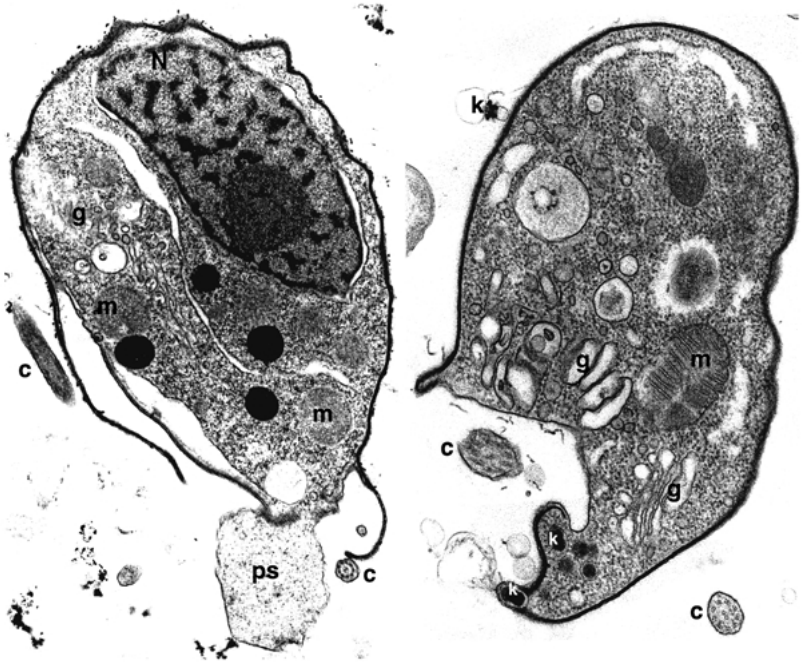

Fig. 5. Electron micrographs of the apusozoans *Amastigomonas mutabilis* (**left;** magnification, ×10,855) and *Ancyromonas sigmoides* (**right;** magnification, ×22,255). Note that the dense layer (“theca”) underlying the plasma membrane is only on the dorsal surface; the ventral surface from which the cilia (c) and, in *Am. mutabilis,* pseudopods (ps) emanate is a plain plasma membrane. The mitochondria of *Am. mutabilis* have tubular cristae, and those *of Ancyromonas* flat cristae. *Ancyromonas* has kinetocyst (k) extrusomes, unlike *Amastigomonas*. The *Am. mutabilis* nucleus (N) has much condensed chromatin as in *Am. bermudensis* (Molina and Nerad 1991), which is very unusual for protozoa.

Most eukaryotes (e.g. animals, plants, ciliates, heterokonts)	GAGAG
Cryptomonads, many plants, Acantharea	GAAAG
82 classical Cercozoa	GA--AG
Ascetosporea:	
Haplosporidium costale and *Urosporidium crescens*	GA--AG
Haplosoridium nelsoni	CA--AG
Haplosporidium louisiana	AA--AG
Marteilia refringens (paramyxid)	GA--AT
Minchinia teredinis (haplosporidian)	GA--TG
Miozoa, some animals, Polycystinea, some Foraminifera	GATAG
Other Foraminifera	GCTAG

Fig. 6. Alignment showing the central part of the terminal loop of the V6 region of 18S rRNA where all 82 classical Cercozoa and all Ascetosporea share a single nucleotide deletion.

Paramyxids Are Derived from Haplosporidia and Probably Evolved from Cercozoan Flagellates

Cercozoa is the only eukaryote phylum established primarily as a result of molecular phylogenetic discoveries (Bhattacharya et al. 1995; Cavalier-Smith and Chao 1997; Cavalier-Smith 1997, 1998a, b). Until recently the phylum comprised five primarily biciliate and soft-surfaced zooflagellate classes, the uniciliate algal class Chlorarachnea, the nonciliate filose testate amoebae, and the Phytomyxea, a group of plasmodial plant parasites with laterally biciliate zoospores (plasmodiophorids and phagomyxids) (Cavalier-Smith 2000a). Not only do these cercozoan taxa group together with high bootstrap support in rRNA trees (Cavalier-Smith 2000a; Kuhn et al. 2000; Vickerman et al. 2002; Wylezich et al. 2002; Brugerolle et al. 2002), but we have noticed that all 82 established cercozoan sequences in our database have deleted the central nucleotide N from the sequence GANAG that forms the end of the single-stranded loop at the end of helix 37 of variable region V6 (Neefs et al. 1993) in their 18S rRNA (Fig. 6). For short-branch eukaryotic clades N is usually G, A, or T and tends to be conserved within clades (e.g., G or T in alveolates, G or T in opisthokonts, G or A in plants, G in most chromobiotes, T in Polycystinea and Foraminifera, A in all Acantharea and cryptomonads). Apart from Cercozoa, the only other eukaryotes in which N is missing are all hard-to-place long-branch taxa (all Haplosporidia, a few euglenoids, two long-branch heterokonts, and possibly all Parabasalia, most Percolozoa, and *Ministeria*—as the latter three also have one or more substitutions here, their alignment has an ambiguity, making it uncertain which nucleotide was deleted).

Ascetosporea, an economically important group of nonciliate plasmodial parasites of shellfish comprising the orders Haplosporida and Paramyxida, were omitted from an earlier otherwise rather comprehensive eukaryote tree chosen to illustrate the position of the major flagellate groups (Cavalier-Smith 2000a) because they are not flagellates and from a recent maximum likelihood analysis (Cavalier-Smith 2002b) and rate-corrected distance analysis of short-branch eukaryotes (van de Peer et al. 2000) because they are rather long branches and hard to align, so their affinity with Cercozoa was not previously suspected. When included in our Γ-corrected trees, however, paramyxids and haplosporidia unexpectedly branch reproducibly with the classical Cercozoa (Figs. 1 and 7). Although bootstrap support is only 60% when only haplosporidia are included (Fig. 1), and even less when the excessively long-branch paramyxid *Marteilia* is added, this position is consistent

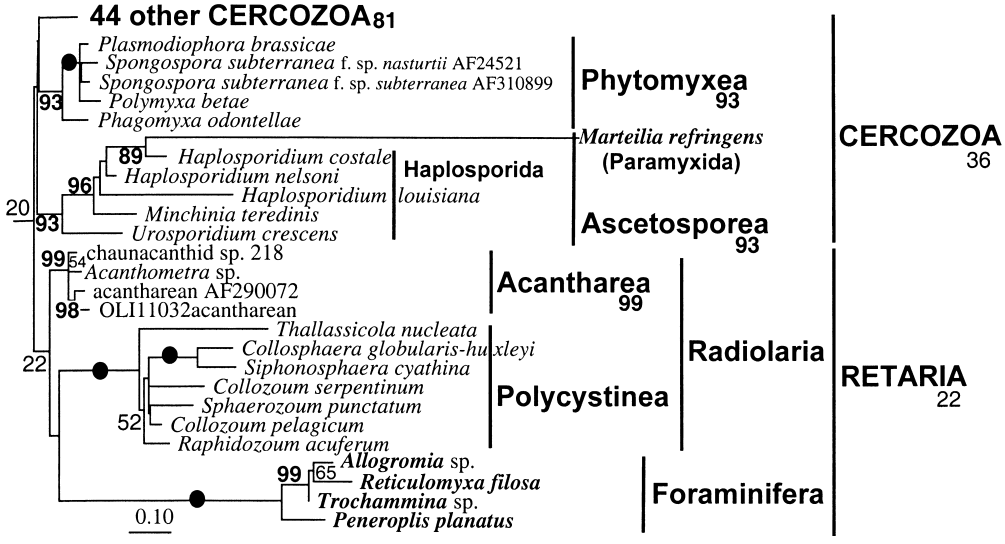

Fig. 7. The Retaria/Cercozoa clade (including key long-branch taxa omitted from Fig. 1) from a minimum evolution GTR + Γ + *I* distance analysis with exhaustive heuristic search (TBR; starting tree by neighbor joining), 1344 positions and 227 taxa (including 7 Mycetozoa and 5 long-branch Archamoebae, which formed a conosan clade—sister to *Vannella*) (α = 0.65376, *I* = 0.085255; bootstrap values are for neighbor joining with 100 resamplings—not all below 80% are shown).

with the shared deletion. While all 82 classical cercozoan sequences have the sequence GAAG at the end of the loop, only two of the Ascetosporea have exactly this sequence; the four others have a different single-base substitution (Fig. 6). The moderate and low values of bootstrap support are probably attributable to the long branches of the ascetosporans. Sometimes Ascetosporea group weakly with the plasmodial phytomyxan Cercozoa, plant parasites that include the important disease agent (*Plasmodiophora*) of club-root of cabbages, but the branching order of these two taxa varies. In the trees using 1344 positions Ascetosporea were sisters to the classical Cercozoa with low bootstrap support (both in the 279-sequence tree when *Marteilia* was excluded and in the 227-sequence tree when it was included: Fig. 7); in the 1672-position tree haplosporidia were sisters to all classical Cercozoa except Phytomyxea (Fig. 1). Because of these results Ascetoporea have now been placed in the phylum Cercozoa and grouped with the Phytomyxea as the new cercozoan subphylum Endomyxa comprising plasmodial parasites of both plants and animals (Cavalier-Smith 2002b). We suggest that the cored vesicles of plasmodiophorids (Dylewski 1990) are homologous with the structurally similar ascetosporan haplosporosomes (Perkins 1990; Desportes and Perkins 1990) and thus a synapomorphy for Endomyxa (Ascetosporea plus Phytomyxea); if so, these unique organelles offer potential targets for chemical control of both parasitic groups.

Based on taxonomically poorly sampled rRNA trees ignoring intersite rate variation, the two ascetosporan groups were previously claimed to be mutually unrelated and put in separate phyla (Berthe et al. 2000). On the contrary, the paramyxid *Marteilia* nests well within haplosporidia with three very high bootstrap percentages but has an exceptionally long branch (Fig. 7) that unsurprisingly misled earlier analyses. They are simply cercozoan orders (Cavalier-Smith 2002b), not distinct phyla.

Monophyly of Retaria (Radiolaria and Foraminifera), Probable Sisters of Cercozoa

Our trees also support (albeit weakly, as for Fungi!) the sisterhood of Cercozoa and Retaria, a phylum recently established (Cavalier-Smith 1999, 2002b) for the two ecologically important groups of giant marine protozoa with net-like pseudopods (foraminifera and radiolaria). Figure 1 includes only the short-branch Acantharea, but our large trees with over 200 taxa confirm that Acantharea and the longer-branch Polycystinea together constitute a monophyletic Radiolaria (López-Garcia et al. 2002) and are not unrelated as claimed earlier (Amaral Zettler et al. 1997). Recent actin trees suggested that foraminifera might branch within Cercozoa (Keeling 2001). But as Retaria lack the characteristic cercozoan deletion discussed above, a sister relationship is somewhat more likely. As their rRNA is so rapidly evolving, we cannot exclude the possibility that their T in that position is a secondarily derived insertion and that foraminifera are really derived from Cercozoa. However, this is not supported by the fact that when we put the exceptionally long-branch foraminifera in the tree, they are sisters to Polycystinea, which also have a T here, making Radiolaria paraphyletic (Fig.

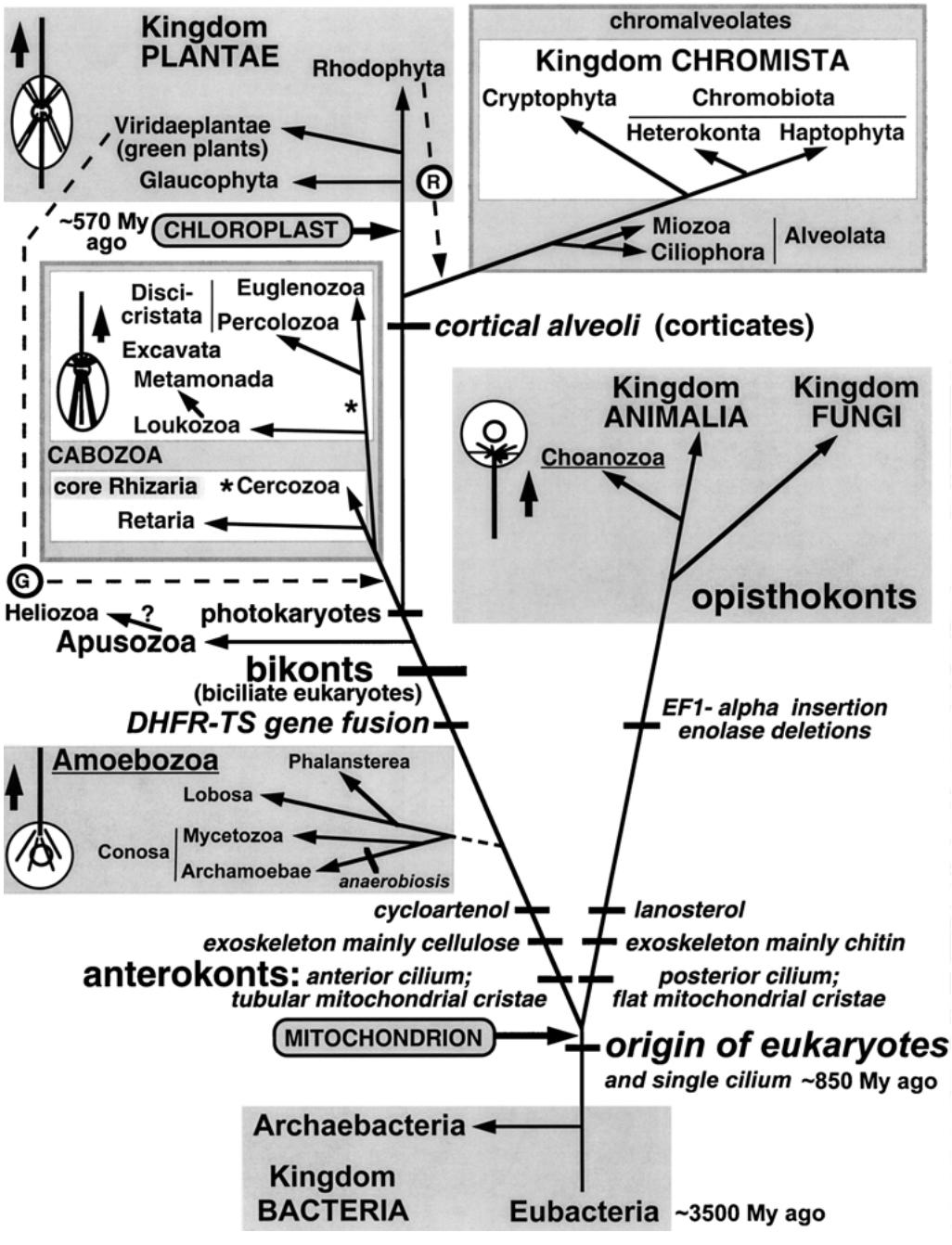

Fig. 8. Schematic interpretation of eukaryote phylogeny. The tree is not scaled by time or degree of difference: the relative branching order of the four main bikont groups remains uncertain and their vertical separation is exaggerated for clarity; probably all mutually diverged early in bikont evolution. Eukaryotes are sisters to archaebacteria; their neomuran common ancestor (Cavalier-Smith 2002a,b) is very much younger than eubacteria, the first cells, which arose about 3.5 Gy ago. After eukaryotes originated about 850 My ago by the origin of endomembranes, cytoskeleton, and phagotrophy, allowing the symbiotic origin of mitochondria (Cavalier-Smith 2002a), the ancestral uniciliate aerobic eukaryote probably split into two lineages: stem Choanozoa, with flat cristae and a posterior cilium, and stem Amoebozoa, with tubular cristae and an anterior cilium. Roughly 600 My ago the biciliate condition and ciliary transformation originated, forming bikonts in which plant chloroplasts originated from cyanobacteria and were soon laterally transferred (Cavalier-Smith 1999, 2002b,c, 2003a) from a red alga to form chromalveolates ® and from a green alga © to form euglenoid and chlorarachnean algae; whether this green-algal lateral transfer occurred once only to create an ancestrally photosynthetic clade cabozoa, as shown, or whether there were two separate lateral transfers (asterisks) into excavates and Cercozoa remains uncertain. At approximately the same time animals and fungi evolved from stem Choanozoa. Whether Amoebozoa are holophyletic or paraphyletic is unclear, as is their precise position relative to the root (Stechmann and Cavalier-Smith 2002). The *thick arrows* adjacent to the vignettes showing ciliary root patterns indicate the direction of swimming. The two *underlined* taxa comprise the Sarcomastigota, which, together with Rhizaria, Excavata, and Alveolata, constitute the paraphyletic kingdom Protozoa. Miozoa comprise Dinozoa, Protalveolata, and Sporozoa (=Apicomplexa), Retaria comprise Radiolaria and Foraminifera, and Metamonada comprise Anaeromonadea, Parabasalia, and Eopharyngia (diplomonads, retortamonads). Loukozoa include Jakobea, *Malawimonas,* and Diphylleida.

5), but Retaria are holophyletic sisters of Cercozoa (bootstrap support is weak). The Γ-corrected tree of Milyutina et al. (2001) was the first to show a holophyletic Retaria. In the 279-species tree omitting Foraminifera the two radiolarian groups (Acantharea and Polycystinea) formed a clade that was sister to Cercozoa. Thus in all three trees Retaria were sisters to Cercozoa. We designate these two phyla the "core rhizaria" as they form the core of the recently established protozoan infrakingdom Rhizaria (Cavalier-Smith 2000b) and share the propensity to form both reticulopodia and filopodia.

The Major Clades of Eukaryotes and the Root of the Tree

Figure 8 is a phylogenetic interpretation of eukaryote diversification that attempts to synthesize conclusions from molecular sequence trees, morphology, biochemistry, and discrete genetic characters such as indels and gene fusions that can be treated cladistically. It recognizes five major clades or supergroups.

1. Opisthokonts ((animals, Choanozoa) and Fungi)
2. Kingdom Plantae ((Viridaeplantae, Rhodophyta) and Glaucophyta)
3. Chromalveolates (kingdom Chromista and protozoan infrakingdom Alveolata)
4. Protozoan infrakingdom Excavata (Discicristata, Metamonada, Loukozoa)
5. Core Rhizaria (Cercozoa, Retaria)

In addition, there are two phyla, Apusozoa and Amoebozoa, which may be relatively early-branching compared with the others and for which it is unclear whether they are paraphyletic or holophyletic. A third phylum, Heliozoa, which is not early-branching, is of uncertain position (Cavalier-Smith and Chao 2003). We briefly discuss the status of the five main clades before outlining the evidence that excavates and core Rhizaria may constitute an ancestrally photosynthetic superclade, the cabozoa (Cavalier-Smith 1999), and then discuss the evidence that plants, chromalveolates, cabozoa, Heliozoa, and Apusozoa together constitute a superclade of biciliate eukaryotes: the bikonts (Cavalier-Smith 2002b).

Opisthokonts

The term opisthokont, signifying "posterior cilium," was applied to animals, Choanozoa, and Fungi because all three groups ancestrally had a single posterior cilium (Cavalier-Smith 1987b). They were argued to be a clade because they also were characterized (uniquely at the time) by flat, nondiscoid mitochondrial cristae that were not irregularly inflated like the flat cristae of Plantae (Cavalier-Smith 1987b). Four other characters also suggested that animals and fungi were more closely related to each other than plants (chitinous exoskeletons; storage of glycogen, not starch; absence of chloroplasts; and UGA coding for tryptophane, not chain termination). However, the first three were probably ancestral states for eukaryotes and the last convergent, so the ciliary and cristal morphology were stronger indications. Although early rRNA trees did not group animals and fungi together, the opisthokonts are now consistently supported by all well-sampled rRNA trees and trees using several or many proteins, as discussed above. Moreover a derived 12-amino acid insertion in translation elongation factor 1α and three small gaps in enolase clearly indicate that animals and fungi have a common ancestor not shared with plants (or other bikonts) or Amoebozoa (Baldauf and Palmer 1993; Baldauf 1999). Thus opisthokonts are now well accepted as a robust clade of eukaryotes (Patterson 1999).

Kingdom Plantae

Kingdom Plantae (*sensu* Cavalier-Smith 1981) was originally defined as comprising all eukaryotes with chloroplasts possessing an envelope of two membranes and mitochondria with (irregularly) flat cristae. It originally included Viridaeplantae (green algae and embryophyte or "higher" plants), Rhodophyta (red algae), and Glaucophyta (e.g., *Cyanophora, Glaucocystis*). It was argued that all three groups diverged from a single primary symbiogenetic origin of plastids (Cavalier-Smith 1982). Both the monophyly of plastids and that of Glaucophyta and Plantae long met unreasonably strong opposition because of widespread false dogma that symbiogenesis is easy and because the three taxa usually do not group together in 18S rRNA trees. Now, however, derived features of all plastids compared with cyanobacteria and numerous molecular trees have led to the acceptance of plastid monophyly (Delwiche and Palmer 1998) and to the monophyly of glaucophyte algae. Furthermore, a sister relation between red algae and Viridaeplantae is strongly supported by concatenated protein trees for nuclei (Moreira et al. 2000; Baldauf et al. 2000) and chloroplasts (Martin et al. 1998; Turmel et al. 1999). The sister relationship between them and glaucophytes is convincingly, but significantly more weakly, supported by the same trees. Thus the case of Plantae shows that arguments from morphology and evolutionary considerations of protein targeting during symbiogenesis (Cavalier-Smith 2000b) gave the correct answer much more rapidly than single-gene trees, which still do not clearly group all three taxa together. In all our trees in the present study (and the recent tree of Edgcomb et al. 2002), Rhodophyta and Viridaeplantae are sisters, but with weak support. Glaucophyta wander aimlessly from one place to another in different trees.

Chromalveolates

The chromalveolate clade (Cavalier-Smith 1999) and its constituent taxa, kingdom Chromista (Cavalier-Smith 1981) and protozoan infrakingdom Alveolata (Cavalier-Smith 1991b), were all proposed based on morphological, biochemical, and evolutionary reasoning about protein targeting before there was sequence evidence for any of them. Now all are strongly supported by such evidence. Chromalveolates comprise all algae with chlorophyll *c* (the chromophyte algae) and all their nonphotosynthetic descendants. They arose by a single symbiogenetic event in which an early unicellular red alga was phagocytosed by a biciliate host and enslaved to provide photosynthate (Cavalier-Smith 1999, 2002c, 2003a). The strongest evidence that this occurred once only in their cenancestor is the replacement of the red algal plastid glyceraldehyde phosphate dehydrogenase (GAPDH) by a duplicate of the gene for the cytosolic version of this enzyme in all four chromalveolate groups with plastids: the alveolate sporozoa and dinoflagellates and the chromist cryptomonads and chromobiotes (Fast et al. 2001). It would be incredible for such gene duplication, retargeting by acquiring bipartite targeting sequences, and loss of the original red algal gene to have occurred convergently in four groups, but it was already pretty incredible that these groups would all have evolved a similar protein-targeting system independently and all happened to enslave a red alga, evolve chlorophyll *c*, and place their plastids within the rough endoplasmic reticulum (ER) independently. Yet many assumed just this because of the false dogma that symbiogenesis is easy and the failure of all these groups to cluster in rRNA trees. For chromobiotes this retargeting of GAPDH has been demonstrated only for heterokonts—information is lacking for haptophytes. However, there are five strong synapomorphies for Chromobiota, making it highly probable that the group is holophyletic (Cavalier-Smith 1994). They share the presence of the periplastid reticulum in the periplastid space instead of a nucleomorph like cryptomonads, they uniquely make the carotenoid fucoxanthin and chlorophyll c_3, they uniquely have a single autofluorescent cilium, and they have tubular mitochondrial cristae with an intracristal filament. Five plastid genes now extremely robustly support the monophyly of both chromists and chromobiotes (Yoon et al. 2002). We are confident that comparable sequence evidence from nuclear genes will also eventually catch up with the general biological evidence for the holophyly of chromobiotes to convince even the most skeptical, who ignore or discount such valuable evidence that chromobiotes are holophyletic. They grouped together as a weakly supported clade in the Γ-corrected maximum likelihood tree with 100 taxa and 1672 positions but generally do not in distance trees.

Excavata

Excavates also were proposed on morphological grounds (Patterson et al. 1999; Simpson and Patterson 1999, 2001). They comprise ancestrally biciliate protozoa which ancestrally had a feeding groove associated with the posterior cilium and a characteristic array of ciliary roots including microtubular bands associated with the rims of the grooves and nonmicrotubular striated bands that give them additional strength, plus all their descendants. Now formally treated as a protozoan infrakingdom (Cavalier-Smith 2002b), excavates comprise the discicristates (phyla Euglenozoa and Percolozoa), Loukozoa (Cavalier-Smith 1999), Anaeromonadea, Eopharyngia (diplomonads and retortamonads [Cavalier-Smith 1993a]), and Parabasalia. Ribosomal RNA trees have established the holophyly of the Eopharyngia (Silberman et al. 2002). The holophyly of both Euglenozoa and Percolozoa (both established on morphological grounds [Cavalier-Smith 1981, 1993b]) has long been supported by numerous sequence trees. The holophyly of Discicristata, united by the presence of discoid mitochondrial cristae and parallel centrioles (not divergent ones as in other excavates), is weakly supported by concatenated protein trees (Baldauf et al. 2000) and more strongly by the shared presence of a 6-phosphogluconate dehydrogenase gene, ultimately of cyanobacterial origin, suggesting that they have a common photosynthetic origin (Andersson and Roger 2002). Eopharyngia and Parabasalia are united by ancestrally having a tetrakont ciliary apparatus with three anterior and one posterior cilium and by the shared horizontal acquisition of two genes from cyanobacteria (Stechmann and Cavalier-Smith 2002). Their sister relationship is supported by a 96% bootstrap value in our 279-species tree and comparably strongly in earlier trees not incorrectly rooted between them (Cavalier-Smith 2000a, 2002b). Anaeromonadea, Eopharyngia, and Parabasalia are now grouped together as Metamonada, which is almost certainly holophyletic (Cavalier-Smith 2003b). However, the holophyly of discicristates is weakly contradicted by rRNA trees, both our present tree and earlier ones (Cavalier-Smith 2002b), which place Metamonada within them as sisters to Percolozoa. We suspect that this position, which lacks significant bootstrap support but is consistently found, is a long-branch attraction artifact caused by convergent extreme divergence of both groups, which have much shorter 18S rRNAs than other excavates. In protein trees Metamonada also have particularly long branches and are hard to place, but Percolozoa do not, making their sister relationship to Euglenozoa, which is

congruent with the morphology, more convincing (Baldauf et al. 2000). In contrast to the other excavate groups, there is no evidence that Loukozoa are holophyletic and several reasons for thinking that they may be paraphyletic. Although all excavates were holophyletic in a recent maximum likelihood tree (Cavalier-Smith 2002b), that tree included only one loukozoan, the jakobid *Reclinomonas*. In our 279-species tree, Anaeromonadea (*Trimastix* and *Pyrsonympha*) did not group with other excavates (which were holophyletic as in the earlier tree) but were sisters to glaucophytes with insignificant support, whereas in Fig. 1 they intrude within the Amoebozoa. In the 227-species tree they are sisters to Amoebozoa other than *M. invertens*. We find that the position of Anaeromonadea varies as much with taxon sampling and method as does that of glaucophytes.

We do not regard the wanderings of glaucophytes, anaeromonads, haptophytes, and cryptomonads in 18s rRNA or other single-gene trees (e.g., the non-grouping of Loukozoa in tubulin trees [Edgcomb et al. 2001] or chaperonin trees [Archibald et al. 2002]) as significant evidence against the monophyly of Plantae, Excavata, and Chromista, all of which are founded on solid morphological/evolutionary arguments. They may simply reflect the early divergence of these taxa from other members of their parental groups and the inability of single-gene trees to resolve closely spaced branching at the base of ancient trees. Multiple protein trees will be necessary to determine whether Loukozoa are polyphyletic, holophyletic, or paraphyletic (as we suspect). It is sometimes asserted that one should not even discuss groups for which bootstrap support is low; but this is a scientifically harmful view, given the immense amount of evidence other than bootstrap values in trees that can be brought to bear on evolution.

Core Rhizaria

Rhizaria (Cavalier-Smith 2002b) comprise Cercozoa, Retaria, Heliozoa, and Apusozoa, whereas "core Rhizaria" comprise Cercozoa and Retaria alone, ancestrally biciliate with rather soft cell surfaces often with many projections, which contrasts with the ancestrally more rigid surfaces of the alveolates (caused by their cortical alveoli and microtubules) and excavates (caused by cortical microtubules and striated fibres). Both rRNA and actin trees support a core rhizarian clade moderately well. Centrohelid heliozoa are also included in Rhizaria because their hexagonally arranged axopodial axonemes resemble those of radiolaria and their kinetocyst extrusomes suggest a relationship with Cercozoa or Ancyromonadida (Apusozoa). However, convergence cannot be ruled out for either character, so we are now gathering molecular sequence data to test this. The only available molecular evidence for centrohelids shows that *Chlamydaster* has the DHFR-TS gene (Stechmann and Cavalier-Smith 2002), showing that centrohelids are not early-diverging eukaryote, but part of the bikont radiation, and must have evolved from biciliate ancestors by ciliary loss after axopodia evolved to trap prey. The most diverse rhizarians are Cercozoa, with a vast array of soft-surfaced zooflagellates, often living by gliding along surfaces, e.g., *Cercomonas* ("tailed monad"), and their disparate descendants. In addition to most of the most common soil zooflagellates, it includes the other most important predators on soil bacteria, filose testate amoebae (Euglyphida and *Pseudodifflugia,* which arose from zooflagellate ancestors by evolving chemically distinct tests and losing cilia, apparently independently [Fig. 1 and Wylezich et al. 2002]), the subtropical chlorarachnean algae (Ishida et al. 1999), plasmodial endoparasites of plants and protists (Phytomyxea) and animals (Ascetosporea), and many ecologically important aquatic zooflagellates (Cavalier-Smith 2000a).

Apusozoa

If Apusozoa are the most divergent bikont group, as Fig. 1 weakly suggests, then a Rhizaria that includes them (Cavalier-Smith 2002b) would be paraphyletic. The apusomonad *Amastigomonas* has the DHFR–TS fusion gene, but so far we have not been able to find it in *Ancyromonas* (Stechmann and Cavalier-Smith 2002), raising the possibility that Apusozoa might be paraphyletic and close to the point of origin of the fusion. As Apusozoa seem particularly important for early bikont diversification, we are seeking protein data to pinpoint their position. The key synapomorphy for the phylum and its sole class Thecomonadea is the dense thecal plates (one in Apusomonadida and Ancyromonadida, two in Hemimastigida [Foissner et al. 1988]), which underlie much of the plasma membrane and are characteristically curved at the junctions with the soft, nonthecate parts of the cell surface (Fig. 5). While the flat cristae of *Ancyromonas* (Fig. 5), contrasting with the tubular ones of Apusomonadida, seemingly contradict their relationship, we have observed tubular cristae in encysting *Ancyromonas* (T.C.-S. and B. Oates, in preparation); flat cristae and kinetosomes are characters shared by *Ancyromonas* and centrohelid heliozoa, which might be related (Cavalier-Smith and Chao 2003).

Amoebozoa

Amoebozoa are a key protozoan phylum because of the possibility that they are ancestrally uniciliate and unicentriolar (Cavalier-Smith 2000a,b); present data on the DHFR–TS gene fusion leaves open the possibility that they might be the earliest-diverging eukaryotes (Stechmann and Cavalier-Smith 2002),

but they may be evolutionarily closer to bikonts or even opisthokonts. Amoebozoa comprise two subphyla (Cavalier-Smith 1998a): Lobosa, classical aerobic amoebae with broad (''lobose'') pseudopods (including the testate Arcellinida), and Conosa (slime molds [Mycetozoa, e.g., *Dictyostelium*] and amitochondrial—often uniciliate—archamaebae [entamoebae, mastigamoebae]). Contrary to early analyses (Sogin 1991; Cavalier-Smith 1993a), there is no reason to regard Amoebozoa as polyphyletic; the defects of those classical uncorrected rRNA trees are shown by trees using 123 proteins that robustly establish the monophyly of both Archamoebae and Conosa (Bapteste et al. 2002). Unless the tree's root is within Conosa, *Dictyostelium* and *Entamoeba* must have evolved independently from aerobic flagellates by ciliary losses. A recent mitochondrial gene tree based on concatenating six different proteins grouped *Dictyostelium* with *Physarum* (99% support) and both Mycetozoa as sisters to *Acanthamoeba* (99% support), thus providing strong evidence for the monophyly of Mycetozoa and the grouping of Lobosa and Conosa as Amoebozoa (Forget et al. 2002)—the same tree also strongly supports the idea based on morphology that *Allomyces* should be excluded from Chytridiomycetes (in the separate class Allomycetes) and is phylogenetically closer to zygomycetes and higher fungi (Cavalier-Smith 1998a, 2000c). Furthermore, the derived gene fusion between two cytochrome oxidase genes, coxI and coxII (Lang et al. 1999), strongly supports the holophyly of Mycetozoa. Since Archamoebae secondarily lost mitochondria, the root cannot lie among them either—although anaerobiosis in Archamoebae is derived, it is unjustified to conclude from this that their simple ciliary root organization, which was a key reason for considering them early eukaryotes (Cavalier-Smith 1991c), is also secondarily derived (Edgcomb et al. 2002). Thus the root of the eukaryote tree cannot lie within the Conosa.

As Mycetozoa and Archamoebae have very long-branch rRNA sequences, Conosa were excluded from the analysis in Fig. 1, which includes only Lobosa. Although the monophyly of Acanthamoebida (99%) and of Euamoebida (85%) is well supported, the basal branching of the Lobosa is so poorly resolved that the monophyly of Lobosa might appear open to question. The four lobosan lineages apparently diverged early. However, in the 279- and 227-species trees, which included Conosa, anaeromonads did not intrude into the Amoebozoa as they do in Fig. 1, and Amoebozoa were monophyletic (low support) except for the exclusion of *M. invertens*. *M. invertens* is another wandering branch, which in some taxon sample/methods groups very weakly with other Amoebozoa, but more often ends up in a different place in each tree! We concur with the judgment of Milyutina et al. (2001) Edgcomb et al. (2002) that it should not be regarded as a pelobiont or Archamoeba, but as a lobosan that independently became an anaerobe with degenerate mitochondria. Its tendency to drift around the tree, coupled with its short branch, suggests that it may be a particularly early-diverging amoebozoan lineage. If so, its unicentriolar condition would give added support to the idea that Amoebozoa are ancestrally uniciliate, if it could be shown that Amoebozoa are either holophyletic or not at the base of the tree.

Most, if not all, amoebae evolved from amoeboid zooflagellates by multiple ciliary losses (Cavalier-Smith 2000a). As the uniciliate condition is widespread within Amoebozoa (Cavalier-Smith 2000a, 2002b), it may be their ancestral condition; if so, ordinary nonciliate amoebozoan amoebae arose several times independently. Evolution of amoebae from zooflagellates by ciliary loss also occurred separately in Choanozoa to produce *Nuclearia* and in several bikont groups, notably Percolozoa (heterolobosean amoebae, e.g., *Vahlkampfia*) and Cercozoa. However, we cannot currently exclude the possibility that the eukaryote tree is rooted within the lobosan Amoebozoa, in which case one of its nonciliate lineages (Euamoebida or Vanellidae) might be primitively nonciliate and the earliest-diverging eukaryotic lineage. However, as the idea that the nucleus and a single centriole and cilium coevolved in the ancestral eukaryote (Cavalier-Smith 1987a) retains its theoretical merits, we think it more likely that all Amoebozoa are derived from a uniciliate ancestor and that crown Amoebozoa are a clade.

Cabozoa

The idea of the clade cabozoa stems from the thesis that the formerly green-algal chloroplasts of euglenoids and cercozoan chlorarachnean algae were implanted into a common ancestral host in a single symbiogenetic event (Cavalier-Smith 1999). It rests on the same fundamentally sound principles of economy in the evolution of protein targeting as did the earlier theories of the monophyly of Plantae, Chromista, and chromalveolates, all of which are now well established. Cabozoa comprise all the descendants of that hypothetical common ancestor, i.e., excavates and core Rhizaria. Although the cabozoan theory currently lacks the hard phylogenetic evidence that supports the other three, there is no strong evidence against it, and it was premature to abandon it (Cavalier-Smith 2002b), especially as the 18S rRNA tree published in that paper showed cabozoa as a clade (of course with low bootstrap support). The large number of plastid losses that it implies is no reason to reject it; unweighted parsimony that equates losses and gains, which are much more onerous, is an unsound mode of reasoning about

evolution. The implications of the cabozoan theory for protein targeting and secondary symbiogenesis are discussed elsewhere (Cavalier-Smith 2002d, 2003a). Here we simply wish to emphasize the importance of testing the theory rigorously. If it is true, then it would firmly rule out any possibility that the root of the eukaryote tree lies within or between excavates and core Rhizaria. Clearly the common acquisition of the green algal plastid would be a shared derived character for all cabozoans. Figure 8 shows Heliozoa as outside the cabozoan clade; if, however, they really lie within cabozoa, one extra loss of plastids would be necessary.

A cabozoan clade was not evident in our 279-sequence tree: core Rhizaria were within the chromalveolates rather than sisters to excavates, but as expected from earlier studies, there was negligible bootstrap support for the branching order of core Rhizaria, chromalveolates, plants, and excavates. Ribosomal RNA trees do not provide a useful test of the theory. We need many protein data from numerous neglected taxa.

Corticates and Photokaryotes

In the absence of clear evidence for the basal branching order within bikonts, we have to rely mainly on morphological evidence and evolutionary reasoning to give us clues as to how the major eukaryotic clades are interrelated. Previously it was suggested that chromalveolates and Plantae were sisters, for which clade the name photokaryotes was proposed (Cavalier-Smith 1999). However, it now seems preferable to apply the term photokaryotes collectively to all three major clades that are individually putatively ancestrally photosynthetic, i.e., to Plantae, chromalveolates, and cabozoa. We adopt the name "corticates" (Cavalier-Smith 2003a) to refer specifically to Plantae plus chromalveolates, on the hypothesis that cortical alveoli originated in their common ancestor (Cavalier-Smith 2002b) and are a synapomorphy (albeit secondarily lost more than once) for the clade. Contrary to widespread misconceptions, cortical alveoli are not a synapomorphy for alveolates. Alveolates were distinguished by a unique combination of two characters, neither individually unique to the group—cortical alveoli and tubular mitochondrial cristae (Cavalier-Smith 1991b, 1993a) —but as it would be hard to argue that the cortical sacs of raphidophytes (Ishida et al. 2000) are not also cortical alveoli, that diagnosis was imperfect and probably reflected the ancestral state for chromalveolates, not just alveolates. As was long emphasized (Cavalier-Smith 1982), glaucophytes have cortical alveoli (see also Cavalier-Smith 2002b), and therefore the common ancestor of plants and alveolates probably did so also.

Bikonts and Ciliary and Centriolar Transformation

Recently Plantae, chromalveolates, cabozoa, Heliozoa, and Apusozoa, which all undoubtedly had biciliate ancestors, were grouped together as the bikonts (Cavalier-Smith 2002b). It was argued that the cenancestral bikont had one anterior and one posterior cilium and crept on surfaces like *Amastigomonas* and *Cercomonas*. Loss of one of the two cilia has clearly occurred several times within bikonts to generate secondarily uniciliate organisms. A key question for eukaryote evolution is whether phyla Choanozoa and Amoebozoa are secondarily uniciliate, as widely assumed (Moestrup 2000), or ancestrally uniciliate (Cavalier-Smith 2002b). In principle the uniciliate condition is simpler and would have been easier to evolve in early eukaryotes (Cavalier-Smith 1982, 1987a) than the biciliate state. All well-studied biciliate bikonts show developmental transformation of their ciliary and ciliary root structure. This complex developmental phenomenon divides their differentiation into two stages. When a bikont cilium is first assembled it is invariably anterior and typically has a different mode of beat and ciliary roots and often a different structure (e.g., with respect to hairs in chromists, hairs and paraxial rods in euglenoids, or lateral flanges in other excavates) from the posterior one (Moestrup 2000). In the next cell cycle, one daughter cell receives the older posterior centriole, whereas the other receives the preexisting anterior centriole that reorients to the posterior, changing both the structure and the mode of beat of the cilium that grows from it and acquiring a new, often very different pattern of ciliary roots (both microtubular and nonmicrotubular components of the cytoskeleton). Both daughters grow a new anterior cilium de novo. It is highly improbable that such a complex pattern of cell differentiation spread over two cell cycles could have evolved in the first eukaryote. We suggest that it first arose in the cenancestral bikont. Studies of ciliary development in Cercozoa and Apusozoa are needed to test this. Currently ciliary transformation has been well documented only in corticates and excavates.

Amoebozoa are argued to be ancestrally uniciliate, as the most divergent taxa are uniciliate. A biciliate condition is found only among Mycetozoa (protostelids and myxogastrids both include uniciliate and biciliate taxa): other amoebozoan taxa are all uniciliate with a single centriole, namely, *Hyperamoeba,* all mastigamoebids, "*Mastigamoeba*" *invertens* (as discussed above, not a mastigamoebid: it needs renaming), and *Phalansterium* (ultrastructure indicates it to be an amoebozoan, not a cercozoan [Cavalier-Smith 2002b]), or are multiciliate with unicentriolar kinetids, namely, *Pelomyxa* and *Multicilia*. It is likely that the biciliate condition evolved entirely independently in Mycetozoa from bikonts (Cavalier-Smith 2002b):

in *Physarum* the anterior centriole is older and the posterior one younger, entirely contrary to bikonts.

The Root of the Tree and Ciliary Evolution

If the root of the eukaryote tree is at the bifurcation between opisthokonts and bikonts as the DHFR–TS fusion strongly indicates (Stechmann and Cavalier-Smith 2002), this is consistent with the view that the bikont condition and ciliary transformation involving a younger anterior cilium are derived conditions that were not present in the eukaryote cenancestor (Cavalier-Smith 2002b). All bikont groups have a cortical microtubular skeleton of laterally adhering bands of exceptionally stable microtubules: three in Apusomonadida, two or more in Rhizaria, three in excavates, and three or (more usually) four in chromalveolates and plants (Cavalier-Smith 2002b). Opisthokonts have no bands that can be homologized with them: instead they have symmetrically radiating single microtubules. In contrast, ciliated Amoebozoa other than *Phalansterium*, which has only the usual amoebozoan cone of microtubules subtending the nucleus (Ekelund 2002), have at least one cortical band of microtubules. In pelobionts the cortical root is double, consisting of a broad band parallel to the cell surface and a narrower one alongside it but oriented orthogonally. Their mycetozoan sisters have a much more complex pattern of bands, which is particularly varied in protostelids (Spiegel 1990), associated with nonmicrotubular skeletal elements not obviously homologous with those of other eukaryotes. The presence of microtubular bands in most ciliated Amoebozoa suggests a closer relationship with bikonts than with opisthokonts. It has been suggested that those of Amoebozoa may be ancestral to those of bikonts, which increased in number at the time of ciliary doubling (Cavalier-Smith 2002b). Even though the evidence indicates that the ancestral state for Amoebozoa is a single cilium and basal body, the possibility that this ancestor was derived from a bikont ancestor by ciliary reduction cannot be totally ruled out. Indeed it could be argued that the position of Amoebozoa within the bikonts in Fig. 1 suggests such an origin. However, that would be reading too much into a poorly resolved tree. Although we should not place too much weight on our inability to find a DHFR–TS fusion gene in Amoebozoa (Stechmann and Cavalier-Smith 2002), if the TS genes we have found turn out to be unfused, that would clearly argue against their derivation from bikonts. In the 123-protein tree Amoebozoa are topologically closer to opisthokonts than to any bikonts (Bapteste et al. 2002), but that is insufficient evidence for the holophyly of bikonts, as the taxon sampling is poor: in particular, there are no protein data for Apusozoa, the one group that often, but not invariably, tends to be topologically closer to opisthokonts than Amoebozoa in unrooted trees.

In the rooted six-gene mitochondrial tree Amoebozoa, opisthokonts, and bikonts (albeit poorly sampled: only the red/green plant clade and the excavate *Reclinomonas*—significantly derived and not basal to all other eukaryotes) were all robustly holophyletic (Forget et al. 2002), but the position of Amoebozoa relative to the opisthokont bikont bifurcation differed in distance and maximum likelihood trees (both Γ corrected), testifying to the difficulty of resolving the branching order of the three major eukaryote groups, i.e., rooting the tree, even in this relatively favorable case where the bacterial outgroup is only a moderately long branch.

The idea that the root lies between the posteriorly uniciliate opisthokont ancestor and an anteriorly uniciliate amoebozoan and that this divergence reflects two alternative adaptive modes of feeding by an ancestral amoeboflagellate is a functionally appealing interpretation of both ciliary and pseudopodial diversification for the basic bifurcation of the eukaryote tree (Cavalier-Smith 2002b). According to this view, stem Choanozoa (ancestral to all opisthokonts) were attached to surfaces (like *Ministeria vibrans* and most choanoflagellates) and ancestrally fed from water currents created by a symmetrical acropetal ciliary undulation drawing bacteria from the side for entrapment by fllopodia, whereas stem Amoebozoa ancestrally crept along surfaces using broad pseudopodia, feeding by drawing water from ahead by the anterior cilium beating asymmetrically. This implies that Amoebozoa are sisters to bikonts, forming a superclade designated anterokonts (Cavalier-Smith 2002b). There is also a basic bifurcation with respect to mitochondrial ultrastructure between opisthokonts (ancestrally with flat mitochondrial cristae) and anterokonts (ancestrally with tubular cristae), which is precisely congruent with the rooting of the most recent Γ-corrected maximum likelihood tree for six mitochondrial proteins (Forget et al. 2002). To test this interpretation, possibly the best current working hypothesis for early eukaryote diversification, we need to establish the phylogenetic position of Amoebozoa and Apusozoa more precisely and determine whether they are holophyletic or paraphyletic.

Contrasting Sterol Biosynthesis Pathways and Wall Polymers in Opisthokonts and Anterokonts

It has long been known that opisthokonts make sterols via the lanosterol pathway, whereas all photosynthetic eukaryotes do so via an alternative pathway. It was suggested that the lanosterol path-

way might be the ancestral one for eukaryotes and the cycloartenol one might have come from the cyanobacterial ancestor of plastids (Cavalier-Smith 1987a). But subsequent studies do not support this, as cyanobacteria do not actually make sterols, but only the related hopanoids. The discovery that Amoebozoa (*Dictyostelium* and *Acanthamoeba*) and the percolozoan *Naegleria* use the cycloartenol pathway led to the suggestion that these amoeboid groups had an algal ancestry (Nes et al. 1990), but although this is probably true for Percolozoa (see above), there is no reason to think that Amoebozoa had a photosynthetic ancestry. It turns out that the key enzymes that make lanosterol or cycloartenol by cyclicizing squalene are orthologues, i.e., the cycloartenol synthase of plants, chromalveolates, and Amoebozoa and the lanosterol synthase of animals and fungi, so both enzymes diverged from a common eukaryotic ancestor, which probably evolved from a squalene–hopene synthase, the homologous eubacterial enzyme present in the actinobacterial ancestor of eukaryotes. *Dictyostelium* also resembles plants in having C24 alkylated sterols, absent from opisthokonts. Do these different sterol patterns between opisthokonts and anterokonts reflect a primary divergence at the base of the eukaryotic tree, or is one ancestral and the other derived?

Mycobacterium, a member of the actinobacteria, from which eukaryotes and archaebacteria probably evolved (Cavalier-Smith 2002a,b), was recently shown to make cholesterol (Lamb et al. 1998), so it is odd that no homologue of these squalene cyclases was identified in its genome. However, unlike other bacteria, it does have a sterol 14α-demethylase (Bellamine et al. 1999) of the same highly conserved cytochrome P450 family as eukaryotic lanosterol demethylase (of opisthokonts) and obtusifoliol (and other more specialized) demethylases of plants (Cabello-Hurtado et al. 1997). The mycobacterial enzyme is water-soluble but the eukaryotic enzymes are held in the ER membrane by an N-terminal stop transfer sequence that was probably added when the ER evolved after eukaryotes diverged from their archaebacterial sisters (Cavalier-Smith 2002b). The folding requirements of the C-terminal end of the molecule are distinctly more specific in the eukaryote enzyme, suggesting that this was a coevolutionary response to its novel placement in the ER (Lepesheva et al. 2001). The mycobacterial enzyme is roughly equally distinct in sequence from the opisthokont and plant versions, and is the most divergent member of the gene family, arguing clearly against the recent superficial claim that the genes for sterol synthesis moved by lateral transfer from eukaryotes to *Mycobacterium* (Gamieldien et al. 2002). This claim appears to be based purely on the false assumption that Mycobacteria and eukaryotes are not related by vertical descent. The authors seem unaware of the extensive evidence that eukaryotes and their archaebacterial sisters evolved by vertical descent from an actinobacterium, the very group to which *Mycobacterium* belongs (Cavalier-Smith 1987a; 2002a,b). Vertical descent of sterol biosynthesis from actinobacteria to eukaryotes is much the more parsimonious explanation for the similarities they observe. The mycobacterial enzyme demethylates both lanosterol and (more efficiently) obtusifoliol in vitro, but its natural substrate is unknown (Bellamine et al. 2001). As the mycobacterial enzyme prefers to bind obtusifoliol and other C24β alkylated sterols but will not bind cycloartenol, this might be used to suggest that this ancestral kind of enzyme is more like the anterokont than the opisthokont version; but caution is needed, as the wheat enzyme binds the fungal sterols lanosterol and ebricol more avidly than its natural substrate obtusifoliol (Cabello-Hurtado et al. 1999)—overall the *M. tuberculosis* demethylase has a broader specificity than the eukaryotic ones, in keeping with the idea that it is the ancestral form.

A complicating factor in understanding the evolutionary diversification of eukaryote sterol biogenesis is that parasitic oomycetes (secondarily nonphotosynthetic heterokont chromists) and trypanosomes (heterotrophic Euglenozoa, also probably secondarily nonphotosynthetic) use the lanosterol pathway, unlike all other studied anterokonts (Nes et al. 1990). We suggest that this came about simply by mutating their ancestral cycloartenol synthase to make it produce lanosterol instead. This very change in product specificity can be engineered in the laboratory by random (Wu and Griffin 2002) or directed (Meyer et al. 2002) mutagenesis.

There is an interesting contrast also in the exoskeletal/wall polymers used by opisthokonts (typically chitin) and anterokonts (typically cellulose). The chitin synthases of animals and fungi are homologous, and that of the oomycetes is also distantly related (Mort-Bontemps et al. 1997), suggesting that chitin synthesis was present in the ancestral eukaryote and was retained by both opisthokont and anterokont lineages but secondarily lost within each. Amoebozoa are not known to make chitin; *Acanthamoeba* and *Dictyostelium* definitely make cellulose. Cellulose synthase and chitin synthase constitute different families of the processive β-glycosyltransferase superfamily. The cellulose synthase of *Dictyostelium* is homologous with that of plants, cyanobacteria, and proteobacteria. While it is possible that plant cellulose synthesis came in with the cyanobacterium (Nobles et al. 2001), it is unlikely that this is true for Amoebozoa—more likely they and opisthokonts (a few true fungi and tunicates have long been known to make cellulose) got it from the α-proteobacterium that became the mitochondrion.

Thus both cellulose and chitin synthesis were present in the ancestral eukaryote, but the two halves of the tree (Fig. 8) have predominantly used a different polymer.

Mapping the Eukaryote Tree onto the Fossil Record

"Intracellular preservation is so poor that many or even all the so-called eukaryotes found in the period 1500–1700 million years ago could well be prokaryotes. We should look critically at the fossil record and consider seriously the possibility that eukaryotes evolved only about 700 million years ago and that eukaryote diversification into the various modern phyla occurred in the following 100 million years" (Cavalier-Smith 1978).

We now consider that eukaryotes arose before 800 My ago, but probably not before 900 My ago. Almost all the 48 extant eukaryotic phyla (notably excepting Bryophyta and Tracheophyta) probably arose in less than 100 My between 600 My and 500 My ago (Cavalier-Smith 2002b); the very doubtful nature of fossil evidence for eukaryotes before ~900 My ago was critically evaluated by Cavalier-Smith (2002a). The fossil evidence indicates a major radiation of bikont protists approximately contemporaneously with the Cambrian explosion of animals; it has been suggested that this rapid diversification might have been a consequence of the origin of chloroplasts in an early bikont (Cavalier-Smith 2002b), thus exemplifying Darwin's (1872) principle of relatively sudden divergence following major innovation, with which we began this paper. We consider this rapid radiation the simplest explanation of why single-gene trees are generally unable to resolve basal bikont radiations. The consistent resolution of the bipartition between opisthokonts, on the one hand, and bikonts/Amoebozoa, on the other, is what one would expect if the root of the eukaryote tree is between opisthokonts and anterokonts (Fig. 8) and this bifurcation was substantially earlier (e.g., about 850 My ago). Thus the fossil record and molecular trees are fundamentally in accord when both are critically interpreted. This synthetic interpretation of early eukaryote diversification needs rigorous testing, not only by continued molecular studies of protist phylogeny, but also by molecular cell biological studies of the evolutionary diversification of the cytoskeleton and endomembrane system and critical paleontological research to obtain more accurate dates for the origins of specific protist groups.

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