Molecular phylogeny of Amoebozoa and the evolutionary significance of the unikont *Phalansterium*

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Abstract

The taxonomic position of the uniciliate, unicentriolar zooflagellate *Phalansterium* is problematic; its distinctive ultrastructure with a pericentriolar microtubular cone placed it in its own order and suggested phenotypic closeness to the eukaryote cenancestor. We sequenced the 18S rRNA of a unicellular *Phalansterium*. Phylogenetic analysis shows that it belongs to Amoebozoa, decisively rejecting a postulated relationship with the cercozoan *Spongomonas*; *Phalansterium* groups with Varipodida ord. nov. (*Gephyramoeba/Filamoeba*) or occasionally Centramoebida emend. (Acanthamoebidae/Balamuthiidae fam. nov.), centrosomes of the latter suggesting flagellate ancestors. We also studied *Phalansterium solitarium* cyst ultrastructure; unlike previously studied *P. solitarium*, this strain has pentagonally symmetric walls like *P. consociatum*. We also sequenced 18S rRNA genes of further isolates of *Hyperamoeba*, an aerobic unicentriolar amoeboflagellate with conical microtubular skeleton; both group strongly with myxogastrid Mycetozoa. However, the four *Hyperamoeba* strains do not group together, suggesting that *Hyperamoeba* are polyphyletic derivatives of myxogastrids that lost fruiting bodies independently. We revise amoebozoan higher-level classification into seven classes, establishing Stelamoebea cl. nov. for Protosteliida emend. plus Dictyosteliida (biciliate former 'protostelids' comprise Parastelida ord. nov. within Myxogastrea), and new subphylum Protamoebae to embrace Variosea cl. nov. (Centramoebida, Phalansteriida, Varipodida), Lobosea emend., Breviatea cl. nov. for '*Mastigamoeba invertens*' and relatives, and Discosea cl. nov. comprising Glycostylida ord. nov. (vannellids, vexilliferids, paramoebids, *Multicilia*), Dermamoebida ord. nov. (Thecamoebidae) and Himatismenida. We argue that the ancestral amoebozoan was probably unikont and that the cenancestral eukaryote may have been also.
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Introduction

Amoebozoa is one of the most important protozoan phyla (Cavalier-Smith, 2002), but a clear picture of its circumscription and evolutionary affinities is only now emerging; Amoebozoa typically have non-filopodial pseudopodia and branched tubular mitochondrial cristae and include the classical lobose amoebae (naked and testate), pelobionts, and most slime moulds (Cavalier-Smith, 1998a). Although the name Amoebozoa is substantially older (Lühe, 1913), the present concept of the phylum dates from relatively recent major revisions of protozoan classification that take account of ultra-structural data and molecular sequence trees in addition to the classical light microscope evidence (Cavalier-Smith 1996/7, 1998a). As now constituted Amoebozoa excludes all 'amoebae' with true filopodia (ones able to pull the cell forwards: Page, 1988), most of which belong

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instead in the phylum Cercozoa with a variety of ancestrally biciliate flagellates and the chlorarachnean algae (Cavalier-Smith and Chao, 1996/7, 2003c)—though the filopodial Nucleariidae are Choanozoa, which otherwise comprise choanoflagellates, ichthyosporean parasites, *Corallochytrium* and *Ministeria* (Cavalier-Smith and Chao, 2003a). The parasitic class Aphelidea (Gromov, 2000), placed by Karpov (2001) in an undoubtedly polyphyletic assemblage of 'Rhizopods', almost certainly also belongs to Choanozoa, not Amoebozoa, in view of their posteriorly uniciliate zoospores and flat cristae; they should be a third order of Ichthyosporea (Cavalier-Smith, 1998b). It is clear that Heterolobosea, which comprise amoebae or amoeboflagellates with typically eruptive pseudopods and flat mitochondrial cristae (not tubular as in Amoebozoa (Page and Blanton, 1985)) are not closely related to Amoebozoa (Hinkle and Sogin, 1993; Roger et al., 1996; Andersson and Roger, 2002) but belong in the probably ancestrally quadriciliate excavate phylum Percolozoa, together with the lyromonads and *Percolomonas*, and probably *Stephanopogon* (Cavalier-Smith, 1993a, b).

Amoebozoa comprise two subphyla: Lobosa, which are aerobic amoebae with or without tests that typically lack fruiting bodies and typically have broad pseudopodia (lobes or sheets) and are but rarely ciliate; and Conosa, which comprise the Mycetozoa and the secondarily amitochondrial Archamoebae, both commonly uniciliate. The uniciliate amoeba *Hyperamoeba* is related to myxogastrid Mycetozoa (Cavalier-Smith and Chao, 1999; Zaman et al., 1999; Walker et al., 2003), whereas the multiciliate amoeba *Multicilia* is probably a lobosan (Cavalier-Smith, 2000). We show here that the uniciliate and unicentriolar condition is much more fundamental to amoebozoan evolution than generally realised by demonstrating that the non-amoeboid unikont flagellate *Phalansterium* is a non-conosan amoebozoan, as recently proposed (Cavalier-Smith, 2002).

The monophyly of the ancestrally unikont Archamoebae (*Pelomyxa*, mastigamoebids and entamoebids) and their relationship to Mycetozoa were slow at being accepted because early rRNA distance trees did not group these taxa together (Hinkle et al., 1994), and often did not even show Mycetozoa as monophyletic (Sogin, 1991) or did so only weakly (Cavalier-Smith, 1993a). As maximum likelihood methods were increasingly used for tree reconstruction, the monophyly of Mycetozoa proved somewhat easier to recover on rRNA trees (Cavalier-Smith and Chao, 1996/7) and was supported by the EF1-α protein tree (Baldauf and Doolittle, 1997). Maximum likelihood (ML) rRNA trees were also the first to provide support for the monophyly of Archamoebae (Cavalier-Smith, 1995b), which is now well supported also by much better taxonomically sampled rRNA distance trees, when gamma correction for intersite variation is used (Bolivar et al., 2001; Milyutina

et al., 2001). These two studies also strongly suggested that *Mastigamoeba invertens*, which never groups with the other amitochondrial amoebae, is neither a *Mastigamoeba* nor an archamoeba, a conclusion confirmed by Edgcomb et al. (2002) with additional mastigamoebid sequences. Even more decisive evidence for the monophyly of Archamoebae and the first compelling molecular evidence for monophyly of Conosa came from trees combining sequences from 123 different proteins (Bapteste et al., 2002). The unique presence of neo-inositol polyphosphates in *Entamoeba* and *Phreatamoeba* (Martin et al., 2000) suggests that they may be a synapomorphy for Archamoebae. It now seems clear that the difficulty of finding Archamoebae and Conosa as clades on early rRNA trees was an artefact of the very long branches of most of these taxa (Philippe and Adoutte, 1998; Philippe, 2000; Philippe and Germot, 2000; Philippe et al., 2000; Van de Peer et al., 2000) and their marked tendency to intermingle with the often even longer branches of many excavate protozoa (Cavalier-Smith and Chao, 2003a).

Lobosa also differ among themselves considerably in 18S rRNA tree branch lengths, but some of them have rather short branch lengths that can cause them to be artefactually excluded from the artificial clusters of long-branch eukaryotes that for a period were mistakenly regarded as early diverging. Several studies of lobosan amoebae seemed to suggest that they were polyphyletic (Sogin et al., 1996; Silberman et al., 1998; Peglar et al., 2003). Only with methods allowing for intersite rate variation and large taxon sampling is evidence growing for the monophyly of Amoebozoa (Bolivar et al., 2001; Milyutina et al., 2001), but bootstrap support for this is very weak indeed, probably because of the combination of deep closely spaced radiations coupled with the great disparity in rRNA evolutionary rates within the group that causes long-branch attraction (Cavalier-Smith and Chao, 2003a). Peglar et al. (2003) noted that gymnamoebae do not all come together on their tree because of the presence within them of the bikont Cercozoa and haptophytes. But that was purely an artefact of the arbitrary and incorrect rooting of their tree between gymnamoebae and myxogastrids; had it been rooted between Amoebozoa and the bikont outgroups, which the gene fusion evidence indicates is correct (Stechmann and Cavalier Smith, 2003a) and cytoskeletal evolution also fits (Cavalier-Smith, 2002), Amoebozoa and gymnamoebae would both have been monophyletic (holophyletic and paraphyletic, respectively); that tree also sampled outgroups (and to a lesser extent Amoebozoa) too sparsely to test amoebozoan monophyly critically.

Concatenated mitochondrial trees currently suffer from very limited taxon sampling, but unambiguously strongly establish a relationship between *Acanthamoeba* (order Centramoebida within the subphylum Lobosa: Appendix A) and the mycetozoan *Dictyostelium*

(Lang et al., 2002); the same clade is seen on RNA polymerase II trees (Dacks et al., 2002). The necessarily derived character of the mitochondrial gene fusion between the cytochrome oxidase 1 and 2 genes proves that this amoebozoan clade is holophyletic (Cavalier-Smith, 2000). In conjunction with the protein evidence that Archamoebae are phylogenetically sisters to *Dictyostelium* (Bapteste et al., 2002) and from rRNA trees closer to *Dictyostelium* than Centramoebida (Bolivar et al., 2001; Cavalier-Smith, 2002), it follows that Centramoebida and *Dictyostelium* must be descended from ciliated ancestors by the independent loss of cilia. A ciliated ancestry had already been suspected, because Centramoebida have distinctive lamellate centrosomes (not centrioles: Sawyer and Griffin, 1971), as does *Dictyostelium*. It has been proposed that Conosa are ancestrally uniciliate with a single centriole and that Lobosa, in which the flagellate *Multicilia* is currently placed, may be also (Cavalier-Smith, 2000). We now provide the first molecular evidence that the non-amoeboid, uniciliate zooflagellate *Phalansterium* is a member of the Amoebozoa. As it does not branch within Conosa but within Lobosa, this suggests that the amoebozoan common ancestor of Conosa and *Phalansterium* had only a single centriole and cilium.

Phalansterium, though long known (Cienkowski, 1870), has been hard to place satisfactorily in a higher taxon (Patterson and Zölfell, 1991). Hibberd's (1983) pioneering ultrastructural study showed that *P. digitatum* was unique at the time in its combination of characters: a single cilium and centriole, attached by a diverging cone of microtubules to the nucleus; well developed mitochondria with unbranched tubular cristae; a collar around the base of the cilium consisting of a continuous, not subdivided fold of cytoplasm. In all three respects *Phalansterium* differed from choanoflagellates (which have two centrioles, flat cristae and a collar of microvilli), with which it had occasionally been grouped (Starmach, 1985). Accordingly Hibberd (1983) created a new order, Phalansteriida for it. The term unikont was introduced by Cavalier-Smith (1995a) with respect to the Mastigamoebida, and defined as the state of having just a single centriole as well as a single cilium per kinetid (Cavalier-Smith, 2002). Unikonty with only one cilium per cell has been argued to be the ancestral state for all ciliated eukaryotes (Cavalier-Smith, 1982, 1987a, 1992, 2000, 2002) and was also suggested as the ancestral state for Amoebozoa (Cavalier-Smith, 2002). More recently *Phalansterium* was suggested as a more suitable prototype than mastigamoebids for the ancestral eukaryote (Cavalier-Smith, 2000, 2002), making its phylogenetic position of considerable evolutionary significance. Recently it has been inferred that Amoebozoa are the sister group to opisthokonts (animals, Choanozoa, Fungi) and that the common ancestor of both was probably unikont (Stechmann and Cavalier

Smith, 2003a, b); Amoebozoa plus opisthokonts are therefore collectively designated unikonts (Stechmann and Cavalier Smith, 2003a, b) to contrast them with the other branch of the eukaryote tree, the ancestrally biciliate bikonts (Cavalier-Smith, 2002).

Karpov (1990) thought that *Phalansterium* was related to the biciliate zooflagellate *Spongomonas*, for which Hibberd (1983) established the separate order Spongomonadida, and placed both genera in the Spongomonadida. However, the fundamentally bikont character of *Spongomonas* and its ciliary roots compared with the unikont character of *Phalansterium* makes it improbable that they are closely related (Cavalier-Smith, 2000, 2002). Their distinctiveness led Cavalier-Smith (2000) to create separate classes for each. Spongomonadida is now established as a member of the large ancestrally biciliate phylum Cercozoa (Cavalier-Smith, 2000; Cavalier-Smith and Chao, 2003c). However, although *Phalansterium* has received further ultrastructural study (Ekelund, 2002), its phylogenetic position has hitherto remained unclear. Preliminary trees including the sequence analysed here in detail were ambiguous; parsimony trees weakly suggested a relationship with Lobosa, while distance trees weakly suggested one with the zooflagellate Apusomonadida (Cavalier-Smith, 2000). Therefore *Phalansterium* was conservatively left among the zooflagellates in the phylum Neomonada, which is now known to be polyphyletic (Stechmann and Cavalier-Smith, 2002; Cavalier-Smith and Chao, 2003a; Stechmann and Cavalier Smith, 2003a, b) and was therefore recently abandoned and split into the separate phyla Choanozoa and Apusozoa (Cavalier-Smith, 2002). Our present analyses are based on much richer taxon samples for both Amoebozoa (51 sequences) and Apusozoa and also allow for intramolecular evolutionary rate variation for the first time for data sets including *Phalansterium*. All methods used agree in showing that *Phalansterium* is not sister to *Spongomonas*, Choanozoa or Apusomonadida, but invariably branches within Amoebozoa, although its precise position as sister to Centramoebida or to *Filamoeba/Gephyramoeba* is sensitive to the method and taxon sampling. A position within Amoebozoa is consistent with the evidence that the same *Phalansterium* strain probably lacks the dihydrofolate reductase/thymidylate synthetase (DHFR/TS) derived gene fusion found in Apusozoa, Cercozoa and other bikonts (Stechmann and Cavalier-Smith, 2002) but not in the two protozoan unikont phyla Choanozoa and Amoebozoa (Stechmann and Cavalier Smith, 2003a). We have also studied ultrastructurally the *Phalansterium solitarium* strain that we sequenced, and find that it is substantially different from another strain also identified as *P. solitarium* (Ekelund, 2002), suggesting that unicellular *Phalansterium* may be much more diverse than hitherto supposed. For comparison we carried out

electron microscopy on *Spongomonas minima* UT1, for which 18S rRNA was recently sequenced (Cavalier-Smith and Chao, 2003c).

Hyperamoeba is an aerobic uniciliate tubulicristate amoeba that has a paired centriole attached to the nucleus and cell surface respectively by two nested cones of microtubules (Karpov and Mylnikov, 1997; Walker et al., 2003), in contrast to the single centriole and single nuclear-associated cones of *Mastigamoeba* and *Phalansterium*. A preliminary analysis of one *Hyperamoeba* strain (Cavalier-Smith and Chao, 1999; Cavalier-Smith, 2000) indicated a close relationship to the myxogastrid slime moulds, which have a similar ultrastructure, as did a full analysis of a second *Hyperamoeba* strain (Zaman et al., 1999). We have now sequenced the 18S rRNA gene of a third *Hyperamoeba* strain, and a fourth is now available from Genbank. Our phylogenetic analysis, which now includes a broader taxon sampling of myxogastrids, shows that all four *Hyperamoeba* strains group very robustly with the myxogastrids, three being interspersed among them. None of the *Hyperamoeba* strains group more closely with each other than with true myxogastrids. This supports the finding of Walker et al. (2003) that the genus *Hyperamoeba* is polyphyletic and shows that the four studied isolates have in fact evolved from myxogastrid ancestors by losing fruiting bodies. While supporting the inclusion of both *Dictyostelium* and myxogastrids/*Hyperamoeba* within Amoebozoa, our analysis leaves the question of the precise ancestry of both groups and the monophyly or polyphyly of the Mycetozoa open.

Our new findings increase the growing evidence that all Amoebozoa had uniciliate ancestors, and require a revision of the higher-level classification of Amoebozoa, in which we establish a new class Variosea to embrace Phalansteriida, Centramoebida emend. and *Gephyramoeba*/*Filamoeba* here grouped into the new order Varipodida. We make the class Lobosea and its major order Euamoebida both much more uniform by segregating Variosea and a second novel class Discosea. Vannellidae and Paramoebidae are structurally closer to *Multicilia* and Vexilliferidae than to Lobosea sensu stricto or Variosea, and we group them all together as a new order Glycostylida, which we combine with Himatismenida and Thecamoebidae as the class Discosea, comprising fundamentally discoid amoebae with a lamellipodium rather than lobose pseudopods. We group Lobosea, Variosea, and Discosea together as the core of a new amoebozoan subphylum Protamoebae, which also contains the new class Breviatea for the aberrant anaerobic '*Mastigamoeba invertens*' (Stiller and Hall, 1999; Edgcomb et al., 2002), which we show for the first time has other putatively anaerobic close relatives at present only known from environmental DNA sequencing (Dawson and Pace, 2002). We discuss the reasons for these and additional lower-level

systematic changes and the phylogeny of the major lineages of Amoebozoa, including the nature of the ancestral protamoeba and amoebozoan, and the sister relationship between Amoebozoa and opisthokonts.

Materials and methods

Cell cultures

Cultures were obtained from the American Type Culture Collection (*Phalansterium solitarium* ATCC50327, *Spongomonas minima* UT1 ATCC50405) or donated by A.P. Mylnikov (*Hyperamoeba flagellata*). *Hyperamoeba* sp. (strain EJC) DNA was donated by T. Nerad.

Gene sequencing and phylogenetic analyses

DNA isolation, purification, 18S rRNA gene amplification by PCR, sequencing, editing and addition to multiple alignments were as previously described (Cavalier-Smith et al., 1995). The new sequences (Genbank accession numbers: AF280078; AF411289; AF411290) were aligned manually with over 500 diverse eukaryote sequences from Genbank and a representative subset of 142 sequences including all protozoan phyla selected for analysis. We tried to include all available nearly complete amoebozoan sequences except multiple closely related ones of *Acanthamoeba*, *Entamoeba* and *Hartmannella vermiformis*; the sequence of 'Hartmannellidae sp. LOS7N/I' was excluded as it differed by only 9 nucleotides, 8 in highly conserved regions that seem likely to be sequencing errors, from *Saccamoeba limax*, so this amoeba from salmon is almost certainly also *Saccamoeba limax*.

The best aligned and most conserved 1549 alignment positions were selected for analysis using PAUP* v. 4.0b10 (Swofford, 1999) on a Macintosh G4. Using Modeltest v. 3.06 (Posada and Crandall, 1998) PAUP selected the general time reversible (GTR) model with gamma correction for intersite rate variation and allowance for invariant sites as the best of 56 substitution models for all datasets; the appropriate parameters were calculated separately for each dataset and the corresponding GTR distance matrices used for neighbor joining (NJ) trees (ties broken randomly) and for heuristic distance searches using both the minimum evolution (ME) criterion and the weighted least squares (WLS: power 2) methods for the best tree using TBR branch swapping, but no rapid descent. For heuristic searches initial trees were by random addition and jumbles as well as NJ to increase the chances of finding the best one. Invariant sites were removed in proportion to base frequencies estimated from all sites. For the partial sequences BOLA187 and 366 (see below) the

missing nucleotides were replaced by Ns prior to the analyses and analyses were also run omitting these sequences to check that their presence did not distort the rest of the tree. We also calculated maximum likelihood trees by PAUP (GTR + Γ + I; parameters and substitution rate matrix calculated by modeltest; four gamma rate categories) with empirical base frequencies and by MrBayes v. 2 (Huelsenbeck and Ronquist, 2001) using eight rate categories and 500,000 steps, otherwise as in Richards et al. (2003), plus unweighted parsimony trees using 100 or more random additions and unlimited TBR branch swapping. Bootstrap analysis used 100–1000 pseudoreplicates.

Electron microscopy

Cells were fixed for 1 h in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2, washed three times in the buffer and postfixed for 1 h in 1% osmium tetroxide in the same buffer, dehydrated, embedded in Spurr's resin, and thin sections stained in uranyl acetate and lead citrate.

Results

Structure of *Phalansterium solitarium* ATCC50327

This strain differs from *P. digitatum* (Hibberd, 1983) in being non-colonial. Whether it is correctly identified as *P. solitarium* (Sandon, 1924, 1927) is uncertain, as no collar is evident under phase contrast when cells are transferred onto a slide; the collar appears only transiently when feeding. Sandon implied that the collar was always present. Otherwise, this strain closely resembles that of Ekelund (2002) under phase and interference contrast. Ekelund (2002) noted that in his *P. solitarium* strain the collar was often hard to see and that cells often lost the cilium and became amoeboid when put on slides. The ATTC strain catches bacteria by adhesion to the cilium and moves them down steadily to the base, exactly as in Ekelund's (2002) strain; like him and Sandon (1924) we did not observe actual ingestion, but the presence of bacteria there seems to induce the temporary extension of the collar. Like Sandon, but unlike Ekelund, we observed no contractile vacuoles.

Electron microscopy shows the presence of bacteria within the collar (Fig. 1a and d) and in large food vacuoles. Thus this strain is a phagotroph, like Ekelund's and probably ingests bacteria at the base of the collar after surrounding them by it. Sandon's conclusion that *P. solitarium* is a saprotroph was probably mistaken because ingestion is hard to observe. The general ultrastructure of trophic cells agrees closely with that of *P. solitarium* of Ekelund (2002), except that

the Golgi often appears larger (Fig. 1c) and more peripheral in trophic cells (Fig. 1c and d), and will not be described in detail. As he observed, the numerous mitochondria have branched tubular cristae and the nucleus has a single nucleolus (Fig. 1a) and numerous clumps of dense chromatin, relatively unusual for protists, but also a feature of Apusozoa (Cavalier-Smith and Chao, 2003a). There seem to be more rough endoplasmic reticulum cisternae than in the Ekelund strain, many closely underlying the cell surface (Fig. 1a and b); the cell surface is less regular, with numerous structures resembling tiny pseudopodia (Fig. 1a).

Sandon never observed cysts; they are frequent in the ATCC strain but quite different in structure from in Ekelund's. Fig. 1e shows a developing cyst, which is pentagonal in cross section like the cysts of *P. consociatum*, which were elongated ovoids with five longitudinal ridges (Cienkowski, 1870), in contrast to the simple spheres with a uniform thick wall in Ekelund's strain. The five-fold symmetry is produced by five huge endoplasmic reticulum (RER) cisternae closely underlying the plasma membrane, which have become inflated outwards; at this stage the external wall is much thinner than in his strain. The underlying cytoplasm has numerous RER cisternae with exceptionally dense thick plates largely filling their lumen. Similar plates are seen within the five inflated cisternae adhering to the membrane adjacent to the underlying cytoplasm; they are mostly thinner than in the internal non-inflated cisternae and have numerous fragmented pieces on their outer surface. It appears as if their material is dissolving and/or becoming hydrated; this may exert a swelling force that could generate the five segment shapes in the cell cortex. Though this arrangement superficially resembles the cortical alveoli of alveolates, the inflated membranes differ in that large numbers of ribosomes remain attached to their cytosolic face adjacent to the cell interior; the outer surface of these cisternae that faces the narrow layer of cortical cytoplasm is largely smooth, though a few ribosomes appear to be trapped in this region. Nearer the Golgi there are also large amounts of normal RER without internal dense plates. There are two types of large vacuoles; ones with heterogeneous contents, possibly autophagic or residual digestive vacuoles, and others with a clear lumen. There are also small heterogeneous clear patches in the cytoplasm, reminiscent of unstained glycogen granules. The thin cortical cytoplasmic layer contacts the cell interior in five places at some of which microtubules or largely smooth membranes may be present.

Phylogenetic analysis

Fig. 2 is a distance analysis for *Phalansterium* and the two *Hyperamoeba* sequences plus 139 other eukaryotes
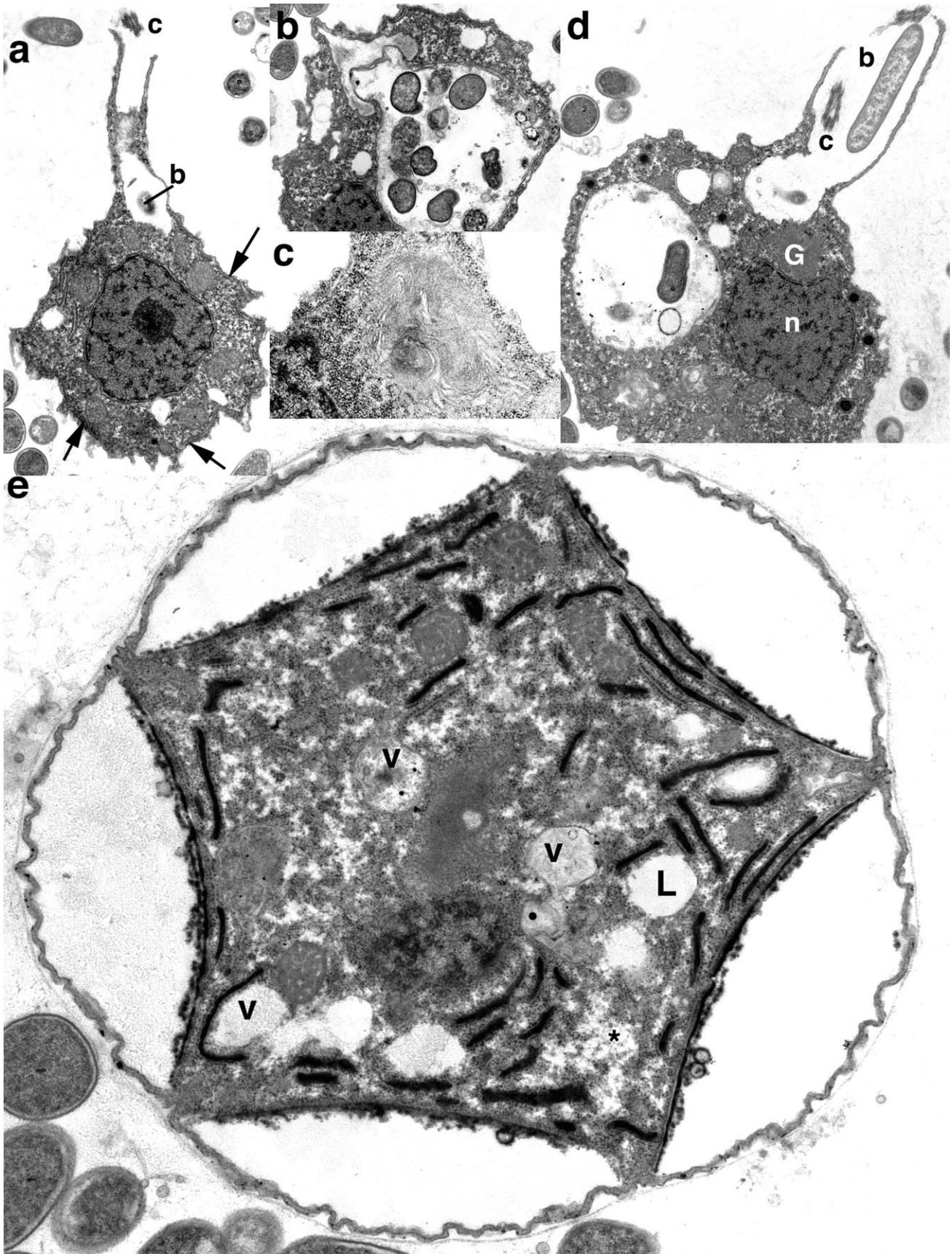
including representatives of all 12 protozoan phyla (Cavalier-Smith, 2002; Cavalier-Smith and Chao, 2003a, b). *Hyperamoeba flagellata* is sister to the myxogastrids plus the other three *Hyperamoeba* strains.

Phalansterium is an amoebozoan

Phalansterium is sister, with weak bootstrap support (48% NJ, 46% WLS; 39% ME), to the clade comprising *Acanthamoeba*, *Comandonia* and *Balamuthia*. This analysis indicates that *Phalansterium* does not group with *Spongomonas*, contrary to the classification of Karpov (1990). *Spongomonas minima* UT1 is firmly within the bikont phylum Cercozoa, with high bootstrap support (87–97% for being in Cercozoa, 93–99% for being in its subphylum Filosa, 78–80% for being in superclass Monadofilosa). An earlier preliminary tree (Cavalier-Smith, 2000) concluded that *Spongomonas* was a cercozoan; however, it now turns out that the sequence labelled *Spongomonas* in that tree (and *Spongomonas* sp. 7A in Cavalier-Smith and Chao (2003c)) is from another cercozoan culture, not from the *Spongomonas* 7A strain, the structure of which was illustrated by Cavalier-Smith and Chao (2003c); until we have clarified its source (it is not a *Spongomonas*) we label that sequence only by its genbank accession number (AF411282); thus our present conclusion is based only on the position of *Spongomonas minima* UT1, but we have unpublished sequence evidence that UT1 is sister to a strain that is unambiguously a *Spongomonas* morphologically (Bass and Cavalier-Smith, submitted).

The conclusion that *Phalansterium* is an amoebozoan is insensitive to the number of positions included in the analysis: when only 1127 relatively more conservative positions were used in an alignment of 190 eukaryote sequences, Amoebozoa was still holophyletic, except for '*M. invertens*', which grouped instead with *Ancryomonas* (and this clade plus apusomonads was sister to the rest of the Amoebozoa). In that gamma plus invariant BioNJ tree (not shown) *Phalansterium* was also sister to the *Acanthamoeba/Balamuthia* clade (39% bootstrap support), and the general structure of the tree was very similar to Fig. 2, including the main clades within Amoebozoa; however, that tree also recovered a mycetozoan clade (*Dictyostelium* plus myxogastrids), while *Filamoeba* and *Gephyramoeba* also formed a clade that was its sister.

Ultrastructure of *Spongomonas minima* UT1

As electron micrographs of this strain have never been published, Fig. 3 includes some representative ones. Both cilia emerge from a shallow pocket at the rim of which microtubules are found, sometimes apparently two as in *Spongomonas* sp. 7A; though medial sections were not found, the angle of obliquely sectioned cilia/centrioles suggests that the two basal bodies may be less parallel than in *Spongomonas* 7A, *S. uvella* and *Rhipidodendron*. Traces of a pericentriolar cup (Fig. 3b and c) suggest a specific relationship to other Spongomonadida (Cavalier-Smith and Chao, 2003c). The general cell structure, with a Golgi with an exceptionally large number of cisternae adjacent to the centrioles and nucleus, the saccate mitochondrial cristae, and a very large elongate microbody attached both to the nucleus and to a mitochondrion are also similar to those of other Spongomonadida, but less diagnostic as they are also found in *Cryothecomonas*, with which UT1 weakly branches on well-sampled cercozoan trees (Cavalier-Smith and Chao, 2003c). The ciliary transition region has two transverse plates; above the more distal one there is a space lacking the centre pair—both features are present in Spongomonadida and Cryomonadida (Cavalier-Smith and Chao, 2003c) as are numerous muciferous bodies (Fig. 3f). The cyst wall is three-layered (Fig. 3d); unlike *Phalansterium* (Fig. 1e) there are no inflated cortical cisternae bearing dense plates.

Two amoebozoan clades

Fig. 2 is superior to some recent much more taxonomically sparsely sampled trees in that there is clear-cut separation between the major amoebozoan clade and Excavata. In sparsely sampled trees that include the longest branch excavates, the long-branch excavates and the longest branch Amoebozoa, notably the myxogastrids, seem to be attracted towards each other (Edgcomb et al., 2002; Silberman et al., 2002). Our large data set that uses somewhat shorter branch excavates to represent the four phyla has largely circumvented this artefactual long-branch attraction problem. However, although Amoebozoa and Excavata are not artefactually intermingled and their longest branches have not clustered together, both appear as two separate clades. All Amoebozoa except the '*Mastigamoeba invertens*' clade form a single large

Fig. 1. *Phalansterium solitarium*, transmission electron micrographs. (a) Oblique longitudinal section showing the periciliary collar with a bacterium (b) near its base and a section of the cilium (c) near its apex; numerous RER cisternae (arrows) underlie the cell surface. (b) Portion of another cell showing a large food vacuole containing numerous Gram-negative bacteria and cortical RER cisternae. (c) The Golgi is large with numerous cisternae, often whorled. (d) Oblique section through the periciliary collar, showing an internal rod-shaped Gram-negative bacterium (b), ciliary profile (c) and a basal pocket. A separate food vacuole contained a bacterium and membrane vesicles. Part of the Golgi (G) is between the nucleus (n) and the basal pocket. (e) Developing cyst showing pentagonal symmetry with five inflated cortical RER cisternae, digestive/autophagic vacuoles (V), lucent vesicles (L), possible storage material (*); see text for details.

● = 100% support in WLS and ME

amoebozoan clade, in which the centramoebid/*Phalansterium* clade is sister to three other major clades corresponding to the Conosa (plus Varipodida), Lobosea and Discosea as defined in the revised classification of Appendix A. '*M. invertens*' is robustly sister to two unidentified sequences from anaerobic environments: BOLA187 and 366 (Dawson and Pace, 2002). This implies that there is a whole group of anaerobic Amoebozoa more closely related to '*M. invertens*' than to Archamoebae. This putatively anaerobic breviate clade appears as sister to one of the two excavate clades (Jakobea/Euglenozoa), but the bootstrap support for this grouping or its exclusion from the main amoebozoan clade is negligible.

The second excavate clade comprises the anaerobic phylum Metamonada (Cavalier-Smith (2003a), i.e., Anaeromonadea/*Carpediemonas*/retortamonads) plus the loukozoan *Malawimonas*; bootstrap support for its grouping with the Apusomonadida rather than the Jakobea/Euglenozoa is also negligible. The basal resolution of the rRNA tree is so poor, and so lacking in bootstrap support that the presence of two apparently distinct amoebozoan clades on Fig. 2 (and two excavate clades) is no reason to question the monophyly of Amoebozoa (or excavates), especially as the kingdoms Plantae and Chromista are also not recovered but well established by other evidence (see, discussion in Cavalier-Smith and Chao (2003a)). It is well known that very long-branch taxa can misleadingly alter the topology of trees as can too distant outgroups. Therefore more restricted data sets were studied that excluded the longest branches and/or more distant outgroups. Fig. 4 is a Bayesian analysis including all major groups of eukaryotes but with the longest branches from Fig. 2 excluded. The overall topology is not substantially different, but several features of this tree are more consistent with other evidence than is Fig. 2. The breviates still do not group with the major amoebozoan clade. However, this major amoebozoan clade (within which *Phalansterium* is nested) has 100% a posteriori support, as does the position of *Phalansterium* as sister to *Gephyramoeba*/*Filamoeba*, which clade itself has 100% support. The Archamoebae, Euamoebida, Discosea, and Vannellidae each have 100% support as do all but one of the clades within them. However, the basal branching order within Amoebozoa is poorly supported, as is its position within the whole tree. Although Bayesian support values are well known to be somewhat inflated compared with bootstrap support values (cf. the only 84% distance and 97% ML bootstrap support for

the *Gephyramoeba*/*Filamoeba* clade found by Amaral Zettler et al. (2000) and the lower distance support values for several amoebozoan clades on Fig. 4), there seems little doubt that *Phalansterium* does belong in Amoebozoa and is related either to Centramoebida (Fig. 2) or *Gephyramoeba*/*Filamoeba* (Fig. 4). The other main differences between Figs. 2 and 4 are all in basal branches poorly supported by both. In neither Fig. 2 nor Fig. 4 can the tree be rooted precisely between unikonts and bikonts (where gene fusions indicate the root really lies (Stechmann and Cavalier Smith, 2003a), and rooted Hsp90 trees show it (Stechmann and Cavalier-Smith, 2003b), because bikont and amoebozoan lineages are intermixed. In Fig. 2 the two amoebozoan clades are well within the bikonts. In Fig. 4 the unikont and bikont lineages are more nearly separated: only Loukozoa and Apusomonadida intrude within the unikonts.

The position of *Phalansterium* within the Amoebozoa

To examine the position of *Phalansterium* within the Amoebozoa more thoroughly and try to distinguish between slightly different positions shown on Figs. 2 and 4, a separate analysis was carried out using only amoebozoan sequences plus those of opisthokonts, now known to be sister group to Amoebozoa (Stechmann and Cavalier Smith, 2003a, b) (i.e., excluding all bikont sequences). On minimum evolution and weighted least squares distance trees (Fig. 5) the amoebozoan tree topology is largely unaltered by the exclusion of the bikonts. However there are a few differences among the three distance methods; *Phalansterium* is sister to a Mycetozoa/*Gephyramoeba*/*Filamoeba* clade with weighted least squares, to Centramoebida with 60% support with BioNJ tree and to both clades jointly with ME. In all trees the four *Hyperamoeba* strains, no two of which cluster together, are in precisely the same positions as in Fig. 2.

To see if this conflict among methods could be resolved, a still smaller data set confined only to the Amoebozoa was analysed by maximum likelihood and the three distance methods (Fig. 6). In maximum likelihood *Phalansterium* is sister to *Gephyramoeba*, *Filamoeba*, and Myxogastrea and this clade is sister to Centramoebida, while *Dictyostelium* is sister to Archamoebae. This topology is identical to the Bayesian analysis that excluded myxogastrids. However in weighted least-squares *Dictyostelium* was sister to myxogastrids with low support (49%) (this clade was also present in the bootstrapped consensus tree with

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Fig. 2. Distance tree of 142 eukaryote 18S rRNAs using 1549 positions (BioNJ: GTR Γ + I model: $\alpha = 0.628375$; $i = 0.139134$). The 3 new sequences are in bold; all others were from Genbank. The *Phalansterium* and *Spongomonas* sequences are arrowed. Bootstrap percentages (530 pseudoreplicates) for separate least squares (left/upper) and ME (right/lower) analyses (using the same substitution model) are given for amoebozoan clades if above 35% and for major outgroup clades only, for clarity mostly by their names rather than on the tree (bold if 80% or more). A single black blob marks clades with 100% support by both least squares and ME.
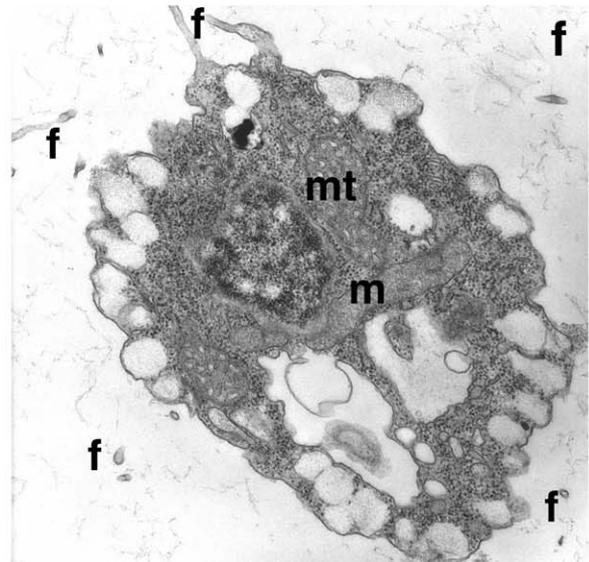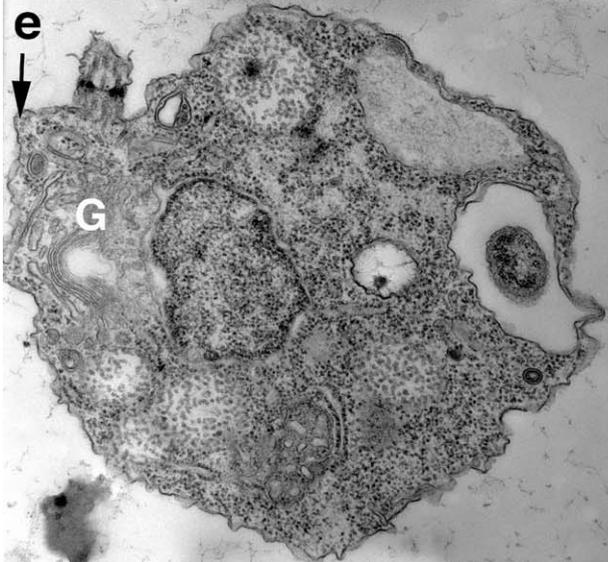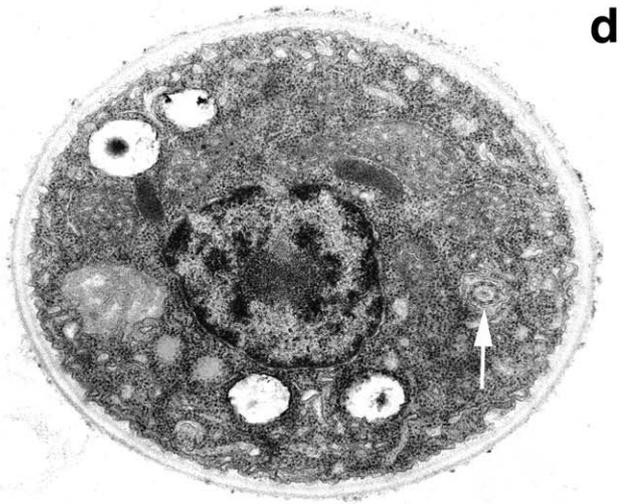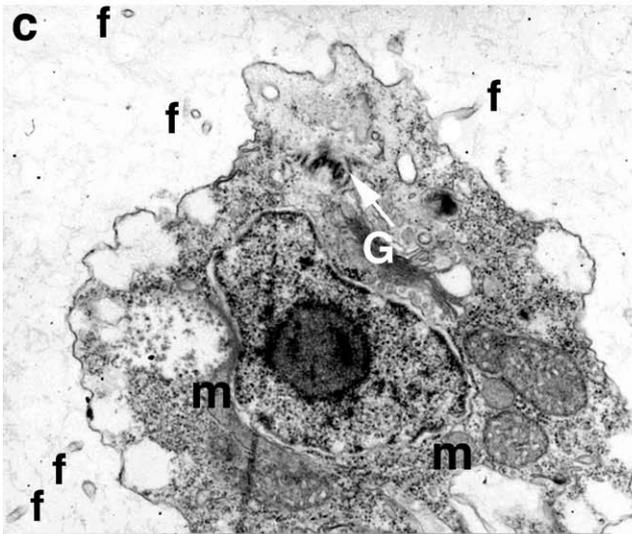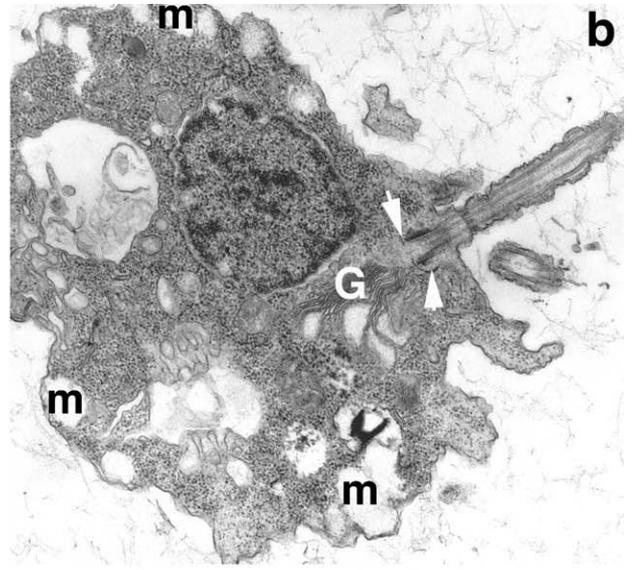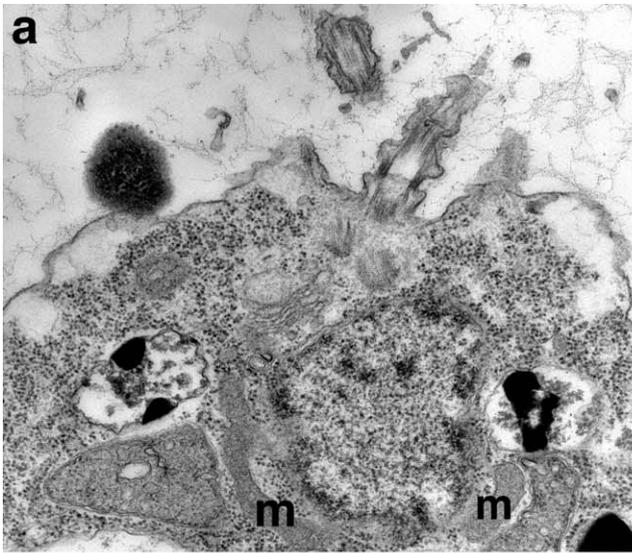
55% support). To see if these groupings were affected by the presence or absence of the longest branches, another analysis was done that omitted all the longest branch Amoebozoa (Fig. 7). Gamma-corrected distance methods (both minimum evolution and weighted least squares) and ML agreed in showing exactly the same grouping comprising Centramoebida, *Phalansterium*, *Filamoeba* and *Gephyramoeba* (here grouped as new class Variosea: Appendix A) with precisely the same topology as in the large Bayesian tree (Fig. 4). Lobosea and Breviatea had strong bootstrap support, but the support for the position of *Dictyostelium* as sister to Archamoebae was weak with ML but very strong with distance methods. The branching order of the five main non-breviate clades was not robust. *Phalansterium* was sister to *Gephyramoeba/Filamoeba* in all three trees with reasonable support by ML and weak support with distance. The branching order was the same or almost the same within the major clades. In most trees *Hartmannella* was sister to Echinamoebidae as in the less taxon-rich NJ tree of Bolivar et al. (2001) and the far more sparsely sampled trees of Amaral Zettler et al. (2000), but occasionally it was sister of (or even within) the leptomyxid/amoebid clade, making Hartmannellidae appear less deeply paraphyletic.

A significant difference between the analysis restricted to Amoebozoa (Fig. 6) and those with outgroups (Figs. 2 and 5) is that *Hyperamoeba flagellata* branches within myxogastrids like the other three *Hyperamoeba* species (with ML and distance), and is not the sister to myxogastrids plus the other three. The latter position in Figs. 2 and 5 is likely to be artefactual.

Discussion

Phalansterium belongs to Amoebozoa

Our analysis clearly refutes the idea that *Phalansterium* and *Spongomonas* are closely related (Karpov, 1990), and places *Phalansterium* with reasonable confidence within the Amoebozoa. Although bootstrap support for the monophyly of Amoebozoa, even when 'Mastigamoeba invertens' groups with the rest of the Amoebozoa, as it occasionally does (Bolivar et al., 2001), is abysmally low. Bayesian a posteriori support for the major amoebozoan clade, excluding breviates is 100%, as is it is for the position of *Phalansterium* as sister to *Gephyramoeba/Filamoeba*. Therefore, there is much stronger support for *Phalansterium* being within the Amoebozoa than there is for the more distinctly pseudopodial *Mastigamoeba invertens*, and just as much support as there is for the inclusion of Centramoebida and Discosea in the same phylum as Lobosea and Mycetozoa. The present study thus supports the parsimony tree, rather than the distance tree from our earlier preliminary analysis that did not allow for intersite rate variation (Cavalier-Smith, 2000). With the present improved taxonomic representation for many protist groups, especially Amoebozoa, and consequently improved alignment, there was no tendency for *Phalansterium* to group with the apusomonads as observed on that early distance tree. The Kimura 2-parameter distance tree, not allowing for intersite rate variation (the method used by Cavalier-Smith (2000)) for the data set of Fig. 2, placed *Phalansterium* within the Amoebozoa weakly as sister to a clade comprising *Acanthamoeba/Balamuthia* plus Vexilliferidae/Paramoebidae (i.e., Paramoeboidea: Appendix A). On NJ, ME and WLS distance trees that allow for intersite rate variation the same grouping with *Acanthamoeba/Balamuthia* is seen on the most taxon-inclusive trees, where vast differences in rate among branches probably do not allow proper distance estimates, but for the taxonomically narrow/shorter branch data set, which should allow sounder phylogenetic reconstruction, these gamma-corrected distance methods agree with ML in indicating a close relationship between *Phalansterium* and *Gephyramoeba/Filamoeba* and that this clade (not *Phalansterium* alone) is sister to *Acanthamoeba/Balamuthia*.

There is no ultrastructural reason to connect *Phalansterium* with either *Spongomonas* or apusomonads; both these other taxa are biciliate cells with a microtubular skeleton of discrete microtubular bands (three in the former, two in the latter) like other bikonts (Cavalier-Smith, 2002). As mentioned above the complex ciliary transition region of Spongomonadida is very similar to that of Cryomonadida, with which *S. minima* groups on the rRNA tree, and very different from the much simpler one of *Phalansterium*, which like all Amoebozoa and the vast majority of protists lacks double transition region plates. The unicentriolar kinetid, rooted by a

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Fig. 3. Ultrastructure of *Spongomonas minima* UT1. (a) Oblique section through both cilia and nuclear region showing paranuclear microbody (m) and mitochondria with tubular cristae. (b) Oblique section through both cilia and nuclear region showing traces of pericentriolar cup (arrows), adjacent Golgi apparatus (G) and mucocysts (m). (c) Another cell showing traces of the pericentriolar cup (arrow), many filopodia (f) and microbody (m). (d) Developing cyst showing multilayered wall and intracellular ciliary axoneme (arrow). (e) Cell with ciliary base showing the edge of the transverse partition and microtubules (arrow) at the edge of the periciliary depression. (f) Cell with numerous filopodia (f), mucocysts, large microbody (m) and mitochondria with unbranched tubular cristae (mt).

Gephyramoeba sp.
Filamoeba nolandi | **Varipodida**
74
Phalansterium ?solitarium | **Phalansteriida**
Acanthamoeba castellani | **Centramoebida**
Balamuthia mandrillaris
57
Rhizamoeba sp.
Paraflabellula reniformis
Leptomyxa reticulata | **Leptomyxoidea**
86
-/53
Amoeba leningradensis
Chaos nobilis
Chaos carolinensis | **Amoebidae**
Amoeba proteus
66
60
Saccamoeba limax
91
Hartmannella vermiformis | **Hartmannellidae**
98
Echinamoeba exundans | **Echinamoebidae**
50
59
95
Phreatamoeba balamuthi
98
Mastigella commutans
Mastigamoeba sp.
Mastigamoeba simplex
71
Dictyostelium discoideum | **Dictyosteliida**
Vexillifera armata
Korotnevella stella | **Vexilliferidae**
99
Neoparamoeba pemaquidensis | **Paramoebidae**
86
Lingulamoeba leei
Vannella miroides
96
Platyamoeba plurinucleolus | **Vannellidae**
99
Vannella anglica

Variosea | **Amoebozoa**
Euamoebida | **Lobosea**
Archamoebae | **Conosa**
Glycostylida | **Discosea**

73
97
BOLA187
'*Mastigamoeba invertens*' | **Breviatea**
90
Jakoba libera
Reclinomonas americana | **Loukozoa** | **Excavata**
Jakoba incarcerata
Malawimonas jakobiformis

Nuclearia simplex
Nuclearia delicatula
74
Nuclearia sp.
98
Dermocystidium salmonis
57
Amoebidium parasiticum
Aurofeca richardsi
97
Corallochytrium confervae
Monosiga ovata
Monosiga brevicollis
Diaphanoeca grandis
71
Ministeria vibrans | **Choanozoa**
50
Anemonia sulcata
97
96
Trichoplax adhaerens
Mnemiopsis leidyi
Scypha ciliata | **ANIMALIA**
Tetilla japonica
Chytridium confervae
Smittium culisetae
Glomus mossae | **FUNGI**
71
Schizosaccharomyces pombe
Amastigomonas bermudensis
Apusomonas proboscidea
Amastigomonas mutabilis
Amastigomonas sp. Millport | **Apusozoa**
Amastigomonas sp. CCAP

opisthokonts

Goniomonas truncata
Chilomonas paramecium | **Cryptomonada** | **CHROMISTA**
98
Rhodomonas mariana
Trimastix marina | **Anaeromonadea** | **Excavata**
Trimastix pyriformis
Cyanophora paradoxa
Cyanoptyche gloeocystis | **Glaucophyta**
Zea mays
Nephroselmis olivacea | **Viridaeplantae** | **PLANTAE**
Mantoniella squamata
82
87
Porphyra umbilicalis
Stylonema alsidii | **Rhodophyta**
Porphyridium aerugineum
59
unidentified microheliozoan
Raphidiophrys ambigua | **Heliozoa**
78
Chlamydaster sterni
Heterophrys marina
Prymnesium parvum
Pavlova aff. *salina* | **Haptophyta**
Nitzschia apiculata
91
Bolidomonas pacifica
Fucus distichum
Chromulina chromophila
Developayella elegans
Phytophthora megasperma | **Heterokonta**
Hyphochytrium sp.
Siluania monomastigina
97
Cafeteria roenbergensis
Wobblia lunata
Labyrinthuloides minuta

CHROMISTA | **bikonts**

Euglypha rotunda X77692
Thaumatomonas sp. KV
Cercomonas sp. 21
99
Proleptomonas faecicola
Cryothecomonas aestivale
Pseudodifflugia cf. *gracilis*
Gymnophrys cometa
AF411282
Chlorarachnion reptans | **Cercozoa** | **Rhizaria**
92
Massisteria marina ATCC50266
npor filose amoeba
Plasmodiophora brassicae
Phagomyxa odontellae
Gromia oviformis AJ457811
chaunacanthid sp. 218
arthracanthid AF063239 | **Retaria**
Heterocapsa triquetra
Perkinsus sp.
Toxoplasma gondii | **Miozoa**
Theileria parva
Babesia bigemina
Blepharisma americanum | **Alveolata**
75
Oxytricha nova
Colpoda inflata | **Ciliophora**
Paramecium tetraaurelia
57
Ancyromonas sigmoides **Apusozoa**

● = 100% support

Fig. 4. Bayesian tree of 114 eukaryote 18S rRNAs omitting longer branch taxa, using 1549 positions. A posteriori support values (left or above) and bootstrap percentages (right or below for a separate distance analysis by weighted least squares, power 2: GTR $\Gamma + I$ model: $\alpha = 0.698758$; $i = 0.255129$, included for Amoebozoa only) are shown (bold if 80% or more). On the consensus bootstrapped distance tree *Phalansterium* moved slightly within the Variosea, being sister to the Centramoebida with 52% support.
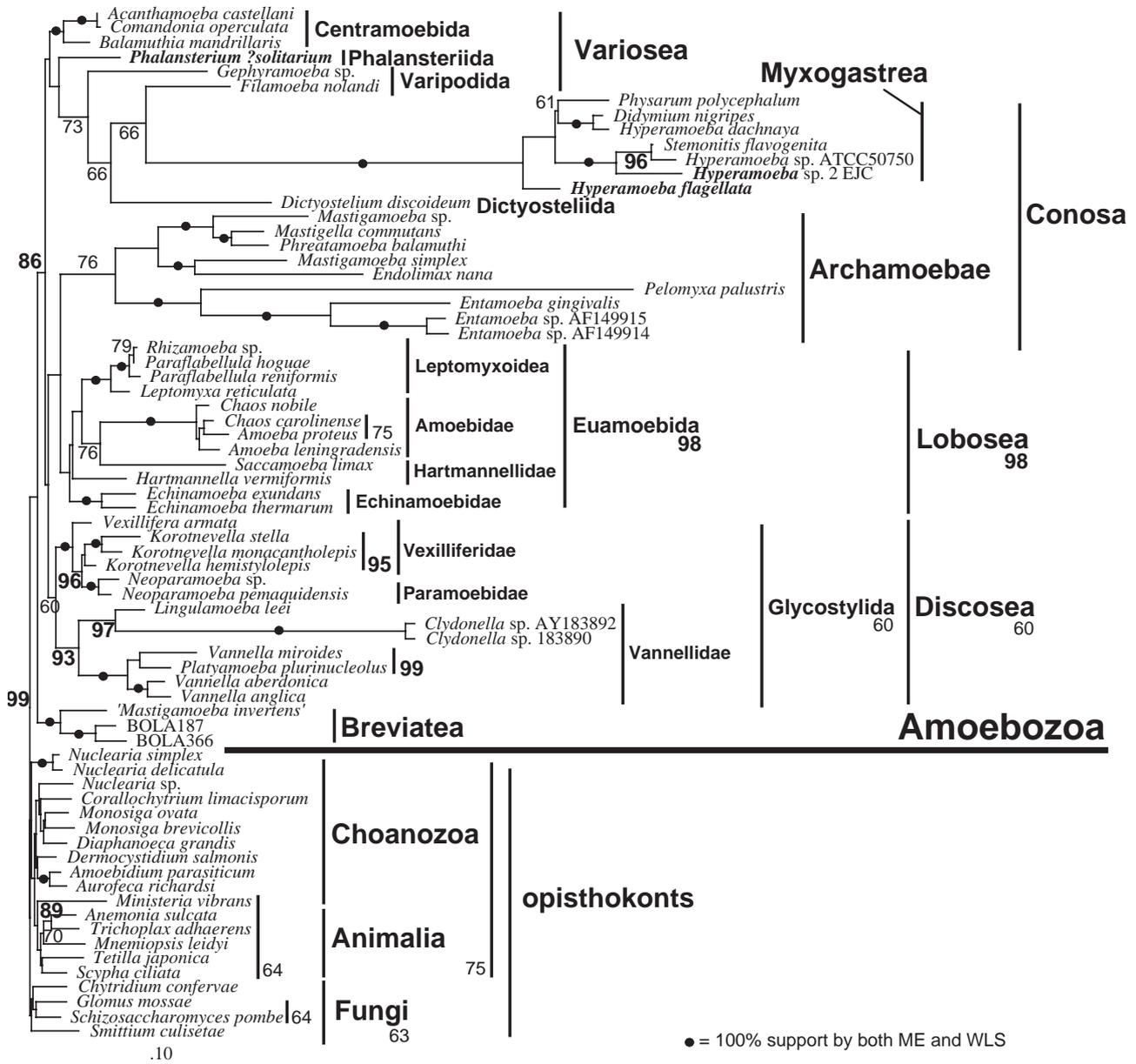
Fig. 5. Distance tree of 71 unikont 18S rRNAs using 1549 positions (weighted least squares, power 2: GTR Γ + I model: $\alpha =$ 0.681673; $i = 0.185819$). The new sequences are in bold. Bootstrap percentages of 60% or more (using the same model; 1000 pseudoreplicates) are for clarity sometimes by their names rather than on the tree (bold if 80% or more: those with 100% support also had 100% support by ME). On the corresponding BioNJ tree *Dictyostelium* was sister to Archamoebae.

microtubular cone in *Phalansterium* is closely similar to that in the ciliate Archamoebae, the main difference being that the latter have in addition a transverse microtubular band attached to the centriole (Walker et al., 2001). Several protostelid genera (e.g., *Cavostelium*, *Protoplanostelium*) also have only a single centriole and microtubular cone, but have two separate transverse microtubular bands (Spiegel, 1981, 1990). The similarity of the conical part of the cytoskeleton of protostelids, myxogastrids and archamoebae led to the establishment of the subphylum Conosa; the predicted

relationship between dictyostelids and archamoebae is now firmly supported by 123 protein trees (Bapteste et al., 2002). The present molecular evidence that *Phalansterium* belongs to Amoebozoa and is more closely related to Conosa than is one uniciliate amoebozoan group (Breviatea) is thus fully congruent with their cytoskeletal architecture, which is unknown for non-amoeboid zooflagellates from other phyla but widespread in Amoebozoa. Shortened cones that diverge apically not basally, as in most Amoebozoa, are present in the aerobic *Multicilia* (Mikrjukov and
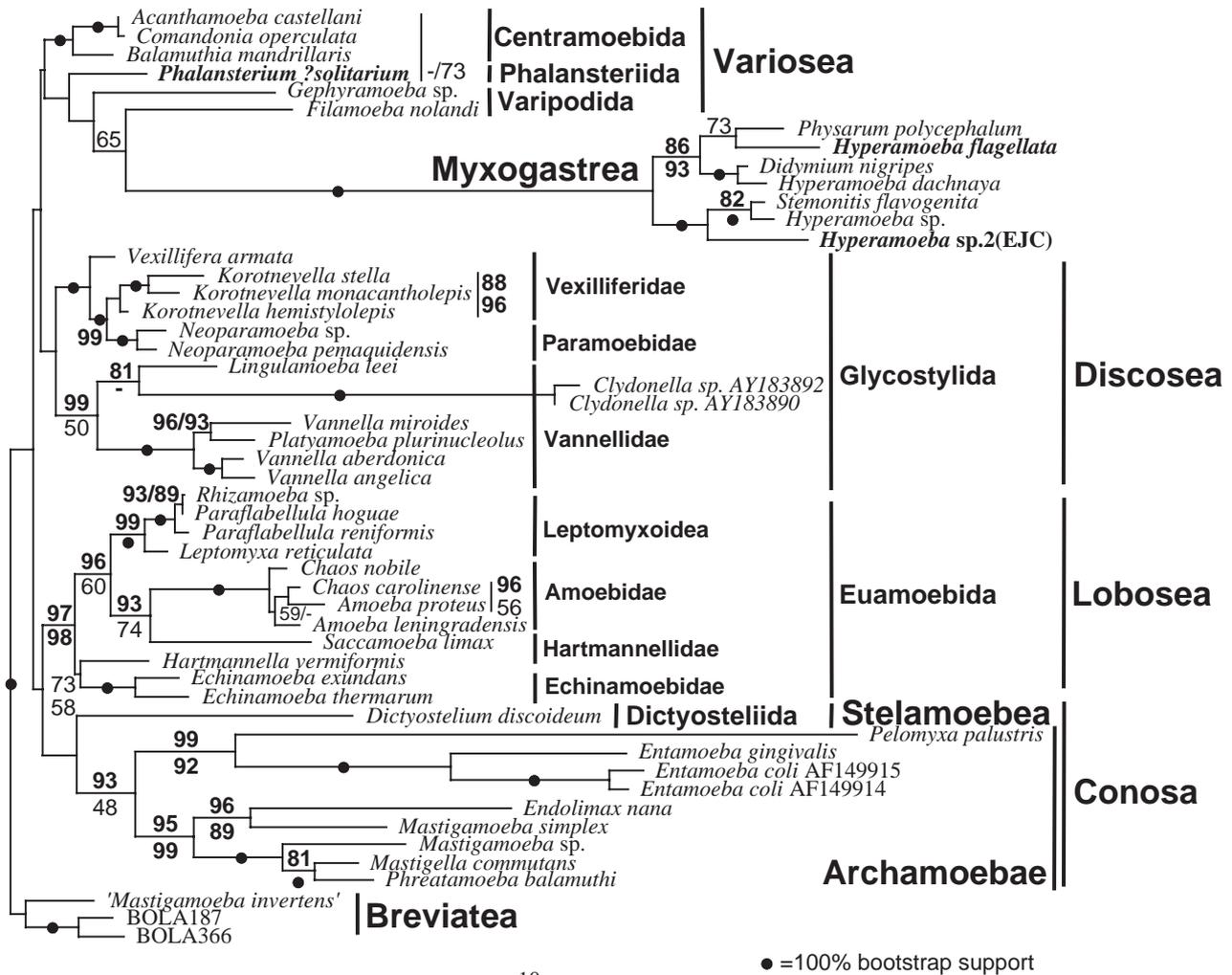
Fig. 6. ML tree of 51 amoebozoan 18S rRNAs using 1549 positions (Γ + I model: $\alpha = 0.55084$; $i = 0.26839$). This tree had the highest log likelihood (−25487.62) of those yielded by 11 independent random additions of taxa. New sequences in bold. The figures are bootstrap percentages (bold if 80% or more) using the same maximum likelihood model (left or above) or using ME (right or below).

Mylnikov, 1996, 1998), here placed within the new order Glycostylida with the Vannellidae and Vexilliferidae on account of shared surface glycostyles (Appendix A). Even 'M. invertens' has a pericentriolar cone, though possibly laterally incomplete (O'Kelly, pers. comm.). As 'M. invertens' appears to be the most divergent of all Amoebozoa, a uniciliate and unicentriolar amoeba with a pericentriolar microtubular cone was probably the ancestral state for all Amoebozoa, not just for Conosa.

Given recent reasonably strong evidence that the root of the eukaryote tree is between the bikonts and unikonts (= Amoebozoa plus opisthokonts: Stechmann and Cavalier Smith, 2003a), a position of *Phalansterium* within the Amoebozoa is as far away phylogenetically from that of *Spongomonas* or apusomonads as a eukaryote can possibly be. They lie on opposite sides of the basal eukaryote split into unikonts and bikonts. Such similarities as there are between *Phalansterium* and

Spongomonas (Karpov, 1990) must be ancestral for all eukaryotes (probably true for most, e.g., tubular mitochondrial cristae) or convergent. The inability to detect the DHFR/TS fusion gene that is a derived state characterising bikonts in *Phalansterium* supports its inclusion in Amoebozoa, not Cercozoa, which have the fusion gene (Stechmann and Cavalier-Smith, 2002). Although positive proof that the DHFR and TS genes are separate (the ancestral state) exists only for one amoebozoan (*Hartmannella cantabrigiensis*: Stechmann and Cavalier Smith, 2003a) it is likely to be true for all, including *Phalansterium*. The only other protozoan phylum with separate DHFR and TS genes is the Choanozoa (Stechmann and Cavalier Smith, 2003a). Although *Phalansterium* has sometimes been classified as a choanoflagellate (Starmach, 1985), ultrastructural differences in the collar (continuous, not microvillar) and mitochondrial cristae (tubular not flat) firmly
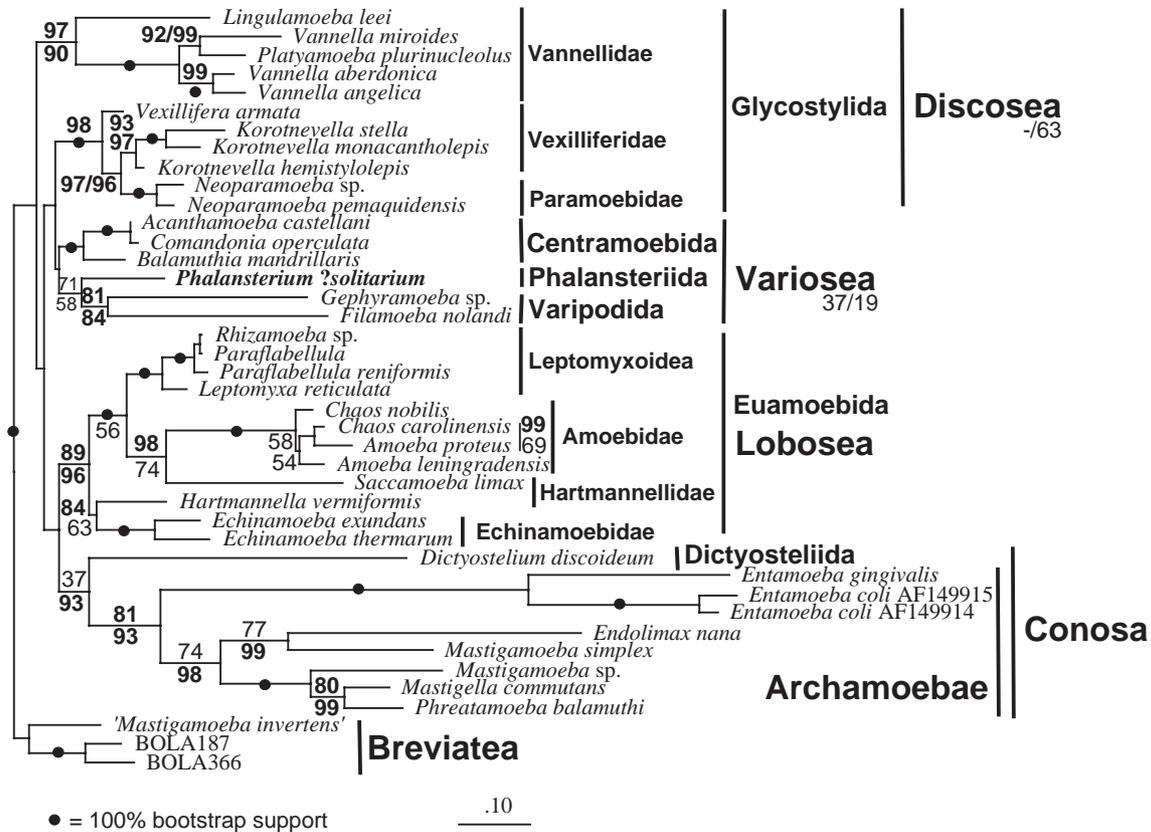
Fig. 7. Maximum likelihood tree of 41 amoebozoan 18S rRNAs using 1549 positions ($\Gamma + I$ model: $\alpha = 0.52619$; $i = 0.250947$). New sequence in bold. The figures are bootstrap percentages (bold if 80% or more) using the same model (ML on left or above: WLS on right or below).

refuted this assignment (Hibberd, 1983). Our molecular trees fully support this, as there is never any tendency for *Phalansterium* to group with opisthokonts to the exclusion of Amoebozoa. Nonetheless it is interesting that according to the present rooting of the eukaryote tree (Stechmann and Cavalier Smith, 2003a), *Phalansterium* and other Amoebozoa are more closely related to the choanoflagellates and other Choanozoa than they are to any zooflagellates in other protozoan or chromistan phyla. The phyla Amoebozoa and Choanozoa now constitute the higher taxon Sarcomastigota: Cavalier-Smith (2003b) recently raised it to subkingdom (its original rank: Cavalier-Smith, 1983a) rather than just infrakingdom (Cavalier-Smith, 2002). The predominantly radial symmetry of both sarcomastigote phyla probably stems directly from that of their common ancestor, and arguably also from the ancestor of all eukaryotes (Cavalier-Smith, 2000, 2002; Cavalier-Smith and Chao, 2003a).

Hyperamoeba is polyphyletic: are Mycetozoa also?

All *Hyperamoeba* strains branch well within the Myxogastrea in Fig. 6; three are clearly more closely related to a different genus of slime moulds (*Didymium*, *Physarum* and *Stemonitis*) than they are to other *Hyperamoeba*. Clearly all four *Hyperamoeba* strains evolved from myxogastrid slime mould ancestors by independent losses of the fruiting bodies. Thus *Hyperamoeba* is a convenient form genus rather that a true taxon.

It has long been debated whether Mycetozoa and Protostelea are monophyletic or polyphyletic. Spiegel (1990) argued that the similarity between the kinetids of Protostelea and Myxogastrea argues for monophyly. However most features shared by them all are also found in Archamoebae (notably excepting the second transverse microtubular band) and some, notably the outer microtubular cone, are probably ancestral for all Amoebozoa. Therefore they do not preclude separate origins for unikont protostelids and for bicentriolar protostelid/myxogastrids within Amoebozoa. The widespread presence of taxa with single centrioles in Amoebozoa does not support the view that unikont protostelids are derived from bikont ancestors (Spiegel, 1981, 1991). Although that possibility cannot be excluded, it is quite likely that unikont protostelids were ancestral to the bikont ones or that Protostelea are polyphyletic. Because of the marked differences between

the unikont and bikont protostelids, we have abandoned the class Protostelea and transferred the bicentriolar species to a new order Parastelida (Appendix A), placed within the Myxogastrea (often biciliate) as they typically have microplasmodia. Protosteliida sensu stricto (the unikont/non-plasmodial taxa) are grouped with Dictyosteliida as the new class Stelamoebea. Most of our trees suggest that Mycetozoa may be polyphyletic but a few showed a mycetozoan clade; the presence of two microtubular bands per centriole uniquely among Amoebozoa argues for the monophyly of all protostelids plus myxogastrids, but says nothing about whether Dictyostelium is part of the same clade as it lacks cilia. The inner cone and its nucleating centre of myxogastrids and some 'protostelids' (e.g., *Planoprotostelium aurantium* and "*Echinostelium apophysatum*") and myxogastrids suggest a derived common ancestry for these taxa and might also have given rise to the centrosome/microtubular cytoskeleton of dictyostelids (Guhl and Roos, 1994) when their ancestor lost centriole(s) and the attached outer cone.

Although the EF-1α tree clearly shows that all three mycetozoan taxa are relatively closely related (Baldauf and Doolittle, 1997), no other aerobic Amoebozoa were included, so some of them could be closer to either *Dictyostelium* or *Physarum* than they are to each other on the EF-1α tree. *Dictyostelium* and *Physarum* group together on tubulin (Keeling and Doolittle, 1996) and actin (Drouin et al., 1995) trees, but sampling of other Amoebozoa is relatively limited. The rRNA tree is still of limited value because of the absence of any protostelids and the representation of dictyostelids by only one species. Although myxogastrids and *Dictyostelium* both branch consistently within Amoebozoa on our trees, the position of the long-branch myxogastrid clade varies with taxon sampling and method. In most trees it is sister to *Filamoeba* not *Dictyostelium* suggesting that Mycetozoa may be polyphyletic. The fact that myxogastrid spores develop endogenously from within the plasmodium, whereas in dictyostelids and typical unikont protostelids they are exospores, essentially like the cysts of ordinary amoebae, also calls into question the homology of the fruiting bodies of Myxogastrea and Stelamoebea.

Most of our trees suggest that *Filamoeba* is the sister group to myxogastrids only, not to Mycetozoa as a whole as in the rate-corrected tree of Bolivar et al. (2001). Either position would be supported by the fact that *Filamoeba* is a flattened cell with thin pointed pseudopods like those of Mycetozoa. By contrast *Gephyramoeba*, which usually appears as sister to Myxogastrea plus *Filamoeba*, is a branched flattened amoeba with broad pseudopods and lacks obvious specific structural affinity to any major amoebozoan group. The branching of this part of the tree is often somewhat perturbed by the very long branch of myxogastrids and changes when they are added or subtracted. When they are omitted *Gephyramoeba* and *Filamoeba* form a clade, which on most trees is joined by *Phalansterium*.

Our trees do not support the suggestion that dictyostelids are more closely related to the acanthamoebids than to other Amoebozoa (Patterson, 1999; Walker et al., 2001). Support for a *Dictyostelium*/Archamoebae clade (Conosa) that excludes both Centramoebida and *Phalansterium* is usually relatively high (95% on the Bayesian tree), when the long-branch myxogastrids are excluded. But when the long-branch myxogastrids are included the trees become unstable: *Dictyostelium* may either group with or within the *Filamoeba*/myxogastrid clade or with the Archamoebae or with both, but it is never sister to Centramoebida. Even non-ciliate Stelamoebea are characterised by an elaborate cytoplasmic microtubule skeleton (Guhl and Roos, 1994), whereas Centramoebida (like Lobosea) lack interphase cytoplasmic microtubules, implying that they are not closely related. Protostelid, parastelid and additional dictyostelid rRNA sequences are badly needed to test our inferences; they may also stabilise the trees and decide whether myxogastrids are close to *Dictyostelium* and really belong in Conosa or are sisters to *Filamoeba* and evolved from Variosea (Fig. 8). If the latter turns out to be correct, as we suspect it to be, Myxogastrea should be transferred from Conosa to Protamoebae. Even though there is uncertainty about the monophyly of Mycetozoa, the rRNA trees consistently show that both myxogastrids and dictyostelids belong within the Amoebozoa; like the actin and tubulin trees (Baldauf et al., 2000) and the Hsp90 tree (Stechmann and Cavalier-Smith, 2003b) they do not support the suggestion that myxogastrids are related to cercomonads (Karpov, 1997).

The new class Breviatea

For the first time we have identified sequences related to those of the problematic '*Mastigamoeba invertens*'. '*M. invertens*' is so divergent on rRNA (and RNA polymerase: Dacks et al., 2002) trees and in structure from other Amoebozoa that we have established the new class Breviatea to accommodate it and the two putatively anaerobic clones to which it is robustly related on our trees. We agree with Milyutina et al. (2001) and Edgcomb et al. (2002) that '*M. invertens*' is not an archamoeba; as it has a cilium shorter than the body (Edgcomb et al., 2002), in contrast to the true *M. invertens* (Lemmermann, 1914), this strain is a probably still undescribed genus and species misidentified as '*M. invertens*'. The suggestion that '*M. invertens*' fails to group with other Archamoebae because of long-branch problems (Stiller and Hall, 1999) seems unlikely.
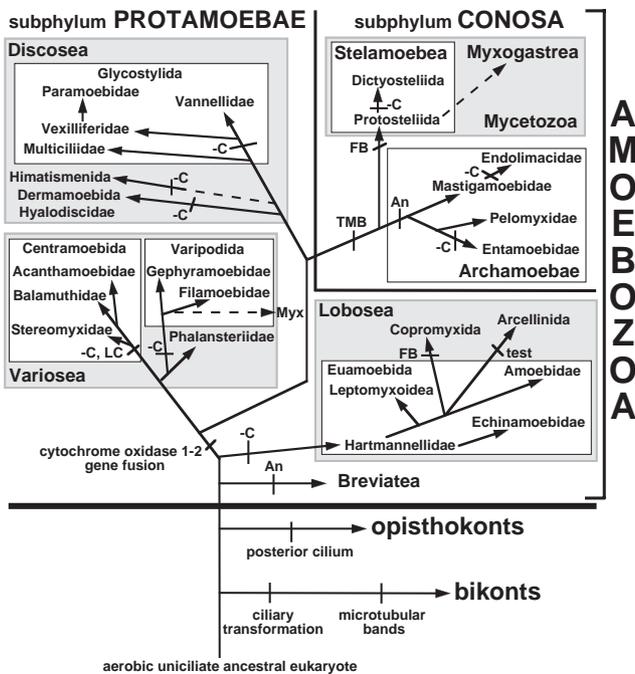
Fig. 8. Hypothetical reconstruction of major features of amoebozoan evolution. The topology shown involves 9 ciliary losses (−C); a different branching order within Discosea could allow fewer losses. Mitochondria were converted into mitosomes or lost in two separate secondarily anaerobic lineages (An). LC = lamellate centrosome. FB = fruiting body. TMB = transverse microtubular band. Because of the uncertainty in the position of myxogastrids (Myx) alternatives are shown with dashed lines.

Overall classification and phylogeny of Amoebozoa

Unlike the classical Sarcodina (Levine et al., 1980), Amoebozoa as emended by Cavalier-Smith (1998a) is not a polyphyletic assemblage of structurally highly disparate amoeboid eukaryotes. With the possible (but doubtful) exception of the poorly studied breviates, it is probably monophyletic and a sound taxon. The revised diagnosis (Appendix A) emphasises the ancestral characters of unikont kinetid, branched tubular mitochondrial cristae, and non-filopodial amoeboid motion that together uniquely distinguish the phylum from all other protists. The slow recognition of the phylum has been because of the multiple losses within the group of some of these characters, increasing its phenotypic diversity, and the grossly non-clock like character of rRNA evolution within the group, causing it falsely to appear polyphyletic on early trees. As discussed below, we now consider that the unikont conose kinetid that prompted the name Conosa was ancestral for all Amoebozoa; the synapomorphy for Conosa was the combined presence of the pericentriolar microtubular cone and at least one transverse microtubular band. The cone (modified to a cylinder in *Mastigamoeba simplex*) plus single microtubular band has been shown to characterize all ciliated Archamoebae yet examined ultrastructurally (Walker et al., 2001), with the possible exception of *Pelomyxa*, where though the cone is obvious a transverse fibre was not noticed (Griffin, 1988). *Multicilia* has transverse microtubular bands interconnecting the cones of its numerous unikont kinetids. As the archamoeba *Pelomyxa* is multiciliate, as often are the unikont protostelids *Cavostelium apophysatum* and *Planoprotostelium aurantium* (Spiegel, 1990), the common ancestor of these four genera may have been multiciliate and the transverse band of Conosa a relic of the *Multicilia*-like interconnecting bands. The transverse microtubular band is absent in *Phalansterium* (though it has a similarly located dense amorphous root: Ekelund, 2002); we predict that it will prove to be absent also in breviates. Thus the cone was present in the ancestral amoebozoan, whereas the transverse band evolved only in the ancestral conosan. Either a second microtubular band evolved in the putative common ancestor of myxogastrids and parastelids when centriole doubling first occurred (Cavalier-Smith, 2002) — or both bands evolved then if parastelids/myxogastrids are not conosans.

The class Lobosea of recent higher-level protozoan classifications (Cavalier-Smith, 1993a; Corliss, 1994; Karpov, 2001) is actually structurally quite heterogeneous (Page, 1988; Smirnov and Goodkov, 1999), more so than is desirable for a single class. Though Lobosea was abandoned altogether in a recent treatment of protozoan diversity that eschewed many useful high-level taxa (Lee et al., 2002), it is better to include these

As 'M. invertens' and its sister clade of two uncultured sequences (BOLA187 and BOLA366: Dawson and Pace, 2002) are the shortest branch of all within the Amoebozoa, their apparent position as outgroup to all other Amoebozoa is probably correct. If so, they probably became anaerobic entirely independently of Archamoebae long before the latter separated from their aerobic dictyostelid sisters. Conosa have several derived signatures compared with Protamoebae; for the 'M. invertens'/BOLA187 clade really to be sisters to Archamoebae, they would have to have lost these signatures (inconsistent with their sequence being the most conservative in the phylum) or these signatures must be unparsimoniously assumed to be convergent between *Dictyostelium* and Archamoebae. The presence of a single cilium and microtubular cone in both Archamoebae and 'M. invertens' is not evidence for a specific relationship, as this was almost certainly ancestral for all Amoebozoa (the deep branching position of 'M. invertens' itself strengthens this conclusion). The failure of 'M. invertens' to group with *Dictyostelium* and *Acanthamoeba* on RNA polymerase trees may, however, be a long-branch problem (Dacks et al., 2002).

taxa within a single subphylum subdivided into a small number of structurally distinctive classes. Accordingly Lobosea was raised in rank to a subphylum (Lobosa) to allow this (Cavalier-Smith, 1996/7, 1998a). However many of the constituent taxa of the subphylum are not well described by the term lobose, the pseudopodial shape varying from the classical cylindrical lobose pseudopods of the Amoebidae and Hartmannellidae through the lamellipodia of the Vannellidae to the acanthopodia of Acanthamoebidae. We have therefore retained the name Lobosea only for a class, now restricted to the amoebae with classical cylindrical lobose pseudopods (new superfamily Amoeboidea) plus the leptomyxoids (families Leptomyxidae and Flabellulidae) and Echinamoebidae, which are more flattened, but clearly related to them according to the molecular trees (Bolivar et al., 2001). We introduce the new name Protamoebae for the former subphylum Lobosa, appropriately described by a name meaning 'first amoebae' since it seems clear that such a subphylum is paraphyletic, with Conosa nested within it; Lobosa is not descriptively apposite for the Protamoebae now placed in the new classes Discosea and Variosea. In fact, the only Amoebozoa that have the classical cylindrical lobose pseudopods are Amoeboidea, Copromyxida and Arcellinida. Fig. 8 indicates the postulated relationships among the major amoebozoan taxa, in which these three taxa are shown as a single small clade.

The Euamoebida (here much narrowed in scope: equivalent to 'Gymnamoebia sensu stricto' of Bolivar et al. (2001)) is the only one of the three orders in the revised Lobosea for which sequences are available. The monophyly of the Euamoebida is very robust, except for some uncertainty in the position of *Hartmannella*. Our trees confirm earlier evidence (Amaral Zettler et al., 2001; Bolivar et al., 2001) that *Filamoeba* is not related specifically to Echinamoebidae, so we establish a new family Filamoebidae for it and group it with Gephyramoebidae as the new order Varipodida, in accordance with the topology of Figs. 4 and 7 and earlier evidence of Amaral Zettler et al. (2001). In addition to removing Acanthamoebidae and *Balamuthia* from Lobosea to join *Phalansterium* and Varipodida as the new class Variosea, the other former Lobosea are now grouped in a new class Discosea, comprising highly flattened, often discoid amoebae that move slowly by a leading lamellipodium (Appendix A).

Discosea are subdivided into three orders based on contrasting structural differentiations of their surface coat. The discovery of glycostyles in *Multicilia* (Mikrjukov and Mylnikov, 1996) makes it unnecessary to retain a separate class for it, so we have grouped Multiciliidae with the other two glycostyle-containing families (Vannellidae, Vexilliferidae), plus the scale-bearing Paramoebidae, as the order Glycostylida. The other discose groups are the Cochliopodidae (order Himatismenida)

with a dorsal tectum of organic scales and the Thecamoebidae (new order Thecamoebida) with a very thick amorphous coat tending to develop longitudinal folds. Even though the glycostyles of vexilliferids are hexagonal and those of *Vannella* are pentagonal, and neither appears to be structurally homologous to the paramoebid or himatismenid scales, Discosea seem to have a much greater propensity than the other six amoebozoan classes to evolve morphologically complex discrete surface structures, suggesting that this reflects common shared characteristics of their secretory machinery. The scales of *Cochliopodium* are very variable in structure (Kudryavtsev, 1999); the dumbbell-shaped scales of *C. gulosum* (Kudryavtsev, 2000) have broad bases and a columnar structure somewhat like glycostyles and might be distantly related, even though differing considerably in detail. Molecular data are unavailable for dermamoebids, himatismenids or *Multicilia*, but Paramoebidae always group within Vexilliferidae and Vannellidae and Vexilliferidae/Paramoebidae group together on some but not all of our trees; neither shows any tendency to group with Euamoebida (Lobosea). Thus molecular evidence is reasonably congruent with the present division of Protamoebae into three structurally discrete classes: Lobosea, Variosea and Discosea. Their branching order with respect to each other and the Conosa cannot be determined from our rRNA trees, though earlier trees weakly suggested that vannellids might be sister to Conosa (Bolivar et al., 2001; Milyutina et al., 2001). As first noted by Peglar et al. (2003), the two discosean clades are sister on some trees but not on others. If myxogastrids are actually sisters of *Filamoeba*, not stelamoebids, Variosea will be paraphyletic not holophyletic.

The grouping of Stereomyxidae with Acanthamoebidae in Centramoebida by Rogerson and Patterson (2002) may be correct, but these branched or reticulate marine forms lack cysts, and the presence of a centrosome in most of them is weak evidence for an affinity with acanthamoebids as the presence of a centriole/centrosome was the ancestral state for all Amoebozoa. The name Centramoebida was published earlier still without a diagnosis or rank but with a circumscription that also included Dictyosteliidae (Patterson, 1994), and the informal name centramoebae was used later for this group characterised by the combination of lamellate centrosomes and branching tubular cristae (Patterson, 1999). Lamellate centrosomes seem to be polyphyletic inventions following the loss of cilia, occurring in many centrohelid Heliozoa as well as some Amoebozoa. As the rRNA trees do not support a sister relationship between *Dictyostelium* and Centramoebida, it is likely that such centrosomes arose at least twice within Amoebozoa. The presence of branched mitochondrial cristae in Stereomyxidae is insufficient evidence that they are Amoebozoa; branched cristae

are present also in several Cercozoa, e.g., *Gromia*, cercozoan filose testate amoebae (themselves polyphyletic: Cavalier-Smith and Chao, 2003c), *Dimorpha*, *Tetradimorpha*, and in *Commation*, so are also polyphyletic. Thus the informal group 'ramicristates' (Patterson, 1999) is not a clade but a polyphyletic assemblage of all aerobic Amoebozoa (i.e., not the amitochondrial Archamoebae and breviates) plus most secondarily non-ciliate Cercozoa. However, the broad pseudopods of Stereomyxidae (quite different from the true filopodia and axopodia of cercozoan rhizopods) show them to be Amoebozoa, not Cercozoa. As branching and blunt-ended non-eruptive non-cylindrical pseudopods are both characteristic also of *Balamuthia* and *Gephyramoeba*, but not found together in other Amoebozoa, a position within Variosea is reasonable. We have to place Stereomyxidae *incertae sedis* within Variosea until molecular data clarify their position. However, we also considering retaining them in Centramoebida as there is no obviously better place based on morphology alone. Grell (1991) advocated a relationship with myxogastrids because of their shared propensity for membrane fusion, but their pseudopodial morphology is very different from myxogastrids (unlike *Filamoeba*), so does not support this.

The 18S rRNA tree of Amoebozoa simultaneously suffers from two severe problems that impede accurate reconstruction: very weak resolution at the base, as in most higher taxa such as phyla, classes and orders, where it may reflect rapid radiation following the origin of a novel body plan, and grossly disparate rates of evolution among lineages. As similar problems may apply to most single-gene trees, it is important to seek other types of evidence that can be used to define groupings more robustly. One such is the derived mitochondrial gene fusion between the cytochrome oxidase 1 and 2 genes shared by *Dictyostelium* and *Acanthamoeba* (Cavalier-Smith, 2000); as this is absent from other phyla and also apparently *Hartmannella* (Gray, pers. comm.), a survey of the presence or absence of this character across the Amoebozoa could be used to partition them cleanly into an ancestral and a derived group. The main limitation is that it cannot be used for Archamoebae or '*M. invertens*' as they lack mitochondrial genomes. However we can already use this to suggest that the weakly closer association of Conosa with Euamoebida than with Centramoebida on many trees is incorrect, and may be an artefact of their longer branches compared with Centramoebida; the gene fusion suggests that Euamoebida should be the outgroup to Conosa plus Variosea. The weak association of Discosea with either Variosea or Conosa on our trees suggests that all three taxa may turn out to constitute a clade that would be sister to Lobosea. We intend to test this prediction using this gene fusion.

Ciliary and mitochondrial losses within Amoebozoa

Of the seven amoebozoan classes recognised here only Lobosea is totally devoid of cilia. If Amoebozoa are holophyletic, then the ancestor of Lobosea must have lost its cilium. There must also have been at least six other ciliary losses, at least once within Discosea and Stelamoebea and at least twice within Archamoebae and Variosea. Even if Amoebozoa are paraphyletic this could be true, but it need not be. If they were paraphyletic and the root of the tree lay within one of the non-ciliate subgroups, then only six ciliary losses need currently be invoked. The presence of a derived triple gene fusion involving the first three genes of pyrimidine biosynthesis in *Dictyostelium* and opisthokonts, but no bikonts or bacteria, indicates that the root of the eukaryote tree cannot lie within the clade that includes them, i.e. opisthokonts plus Conosa (Stechmann and Cavalier Smith, 2003a). This means that all the non-ciliate or amitochondrial Conosa must have lost these organelles. The cytochrome oxidase 1 and 2 gene fusion mentioned above means that Centramoebida at least must also be part of this clade, and therefore must also have lost cilia after diverging from Conosa and *Phalansterium*. We predict that all Amoebozoa, as the phylum is presently circumscribed (Appendix A), will be found to have the triple gene fusion, which would show that the root of the eukaryote tree lies entirely outside Amoebozoa and that all Amoebozoa had a ciliated ancestry. This would also show that breviates had aerobic ancestors. It would also clearly show that Amoebozoa, including breviates, are monophyletic, not polyphyletic, but not establish that they are holophyletic, since the possibility that they are paraphyletic ancestors of opisthokonts would not be ruled out. However, we predict that one or more clearly derived characters will eventually be discovered that demonstrate unambiguously that Amoebozoa are holophyletic.

The Gymnamoebia of Haeckel (1866) has long been known to be polyphyletic; even in the restricted sense of Page (1976) following the exclusion of Heterolobosea (Page and Blanton, 1985), the several ciliary losses within Amoebozoa mean that his subclass Gymnamoebia is polyphyletic. Therefore the taxon is abandoned in the present classification, which partitions its former members among the three protamoeban classes (Appendix A). It will be useful to retain the term gymnamoebae as an informal term for a grade of organisation: for aerobic Amoebozoa lacking cilia, fruiting bodies or tests.

In five of the six amoebozoan classes that contain at least one ciliated taxon, individual kinetids are invariably monokinetids with a single centriole, even though some taxa (*Multicilia*, *Pelomyxa*, Cavosteliidae) have numerous kinetids. Since the ciliated Archamoebae,

Phalansterium, and breviates are all clearly outgroups to Myxogastrea, which alone is bicentriolar, we can confidently infer that the unicentriolar state was ancestral for Amoebozoa and that the bicentriolar and more rarely biciliate state of Myxogastrea is secondarily derived, as postulated earlier (Cavalier-Smith, 2000, 2002). If *Multicilia* is indeed related to vannellids it would be a fifth, separately branching unicentriolar amoebozoan outgroup to Myxogastrea. Very likely, therefore, the biciliate state of myxogastrids, where the posterior cilium is younger (Wright et al., 1980), evolved independently of that in bikonts, where it is invariably older (Cavalier-Smith, 2000, 2002). If ontogeny recapitulates phylogeny in both cases, myxogastrids added a second cilium by accelerated development of the new young centriole and then reorienting it backwards (as *Physarum*'s non-ciliated amoeba's second centriole does when cilia grow), retaining the ancestrally anterior amoebozoan cilium unmodified. In contrast, bikonts evolved a posterior cilium by redirecting the pre-existing anterior centriole backwards in its second cell cycle and growing a new anterior one. Thus ciliary transformation is not developmentally homologous in myxogastrids and bikonts.

Only one doubling of centriole/ciliary number is needed on our amoebozoan tree — in the ancestor of myxogastrids and *Hyperamoeba*. The alternative of five independent reversions to the unikont state in a single phylum (breviates, *Phalansterium*, *Multicilia*, Archamoebae, protostelids sensu stricto) is too unlikely to be taken seriously. Since the ancestrally unicentriolar Amoebozoa are the outgroup to opisthokonts, which were ancestrally uniciliate, the common ancestor of all Sarcomastigota was almost certainly uniciliate, and arguably also unicentriolar, i.e. unikont (Cavalier-Smith, 2002; Stechmann and Cavalier Smith, 2003a, b). The frequent second centriole in opisthokonts is probably a secondary adaptation (Cavalier-Smith, 2000, 2002), not a relic of a biciliate ancestry as was often assumed in the past. We consider, therefore, that the unikont state of these five uniciliate taxa is the primitive one not only for Amoebozoa, but also for eukaryotes. Centriolar evolution must be considered in relation to the cell cycle, which differs in Amoebozoa from most other eukaryotes in lacking a G1 phase (Mitchison, 1971); as animal centrioles duplicate at the onset of S phase, they have four centrioles during S and G2, not two as in myxogastrid flagellate phases.

A simultaneous origin of the centriole, cilium and nucleus to yield initially a monokinetid with a cone of microtubules subtending the nucleus, as in Amoebozoa, was postulated on theoretical grounds to explain how efficient DNA segregation evolved in the cenancestral eukaryote (Cavalier-Smith, 1987b). The kinetid of *Phalansterium* is the least altered from this postulated ancestral state as it lacks the transverse microtubular root of Conosa. The main difference from that earlier discussion in our present picture of early eukaryote evolution is that the cenancestral eukaryote was a unikont mitochondrial aerobe like *Phalansterium*, not a unikont amitochondrial anaerobe like *Mastigamoeba* or breviates. However, until breviates are thoroughly studied molecularly and ultrastructurally we cannot totally eliminate the possibility that they are relics of a premitochondrial phase of evolution (Cavalier-Smith, 1983b). Unambiguously establishing the monophyly of Amoebozoa and whether breviates are secondarily or primarily anaerobic is important for consolidating this new interpretation of the nature of the ancestral eukaryote.

From a previous study it was unclear whether or not *Entamoeba* and *Endolimax* had a non-ciliate common ancestor (Edgcomb et al., 2002). Our trees strongly and consistently indicate independent losses by the two genera. *Entamoeba* is strongly sister to *Pelomyxa*, whereas *Endolimax* is nested within the Mastigamoebidae with strong bootstrap support. The trees of Edgcomb et al. (2002) probably sampled too few taxa and sites to demonstrate this. There is therefore no longer justification for retaining a purely non-ciliate taxon embracing both *Entamoeba* and *Endolimax*, which would be polyphyletic. Nor, as ciliary loss by both taxa is firmly established, is there any reason to place them in a separate class from the mastigamoebids and *Pelomyxa* (Cavalier-Smith, 1993a), which would rank ciliary loss too highly. Our trees confirm the monophyly of all Archamoebae for a much larger taxon sample than when it was first shown (Cavalier-Smith, 1995b; Cavalier-Smith and Chao, 1996/7); bootstrap support for archamoeba monophyly is relatively high in the present study and those of Bolivar et al. (2001) and Milyutina et al. (2001) compared with Fig. 1 of Edgcomb et al. (2002), for which the number of sites analysed is not stated.

As Archamoebae was the first taxon established to group mastigamoebids, *Pelomyxa* and *Entamoeba* (Cavalier-Smith, 1983a, 1987b), it is a more appropriate name (with appropriately modified suffix) for a single class that embraces them all than would be any of the five class names that have been applied to only one or several members of the Archamoebae (Caryoblastea (Margulis, 1974), Pelobiontea (Page, 1976) stat. nov. et em. (Cavalier-Smith, 1987a, b), Mastigamoebea (Cavalier-Smith, 1987a, b), Entamoebea (Cavalier-Smith, 1991), Peloflagellata (Goodkov and Seravin, 1991)). Caryoblastea was proposed as a phylum for *Pelomyxa* alone on the erroneous assumption that it lacks mitosis (Margulis, 1974); except for Page (1988) who downranked it to class, it has seldom been used by others and never for anything other than *Pelomyxa*. Pelobiontida was first proposed as an order by Page (1976) for *Pelomyxa* alone and raised to a class to include

Entamoeba by Cavalier-Smith (1987b) on the assumption that *Pelomyxa* also was non-ciliate, which was disproved by Griffin (1988). Peloflagellata was proposed for *Pelomyxa* alone by Goodkov and Seravin (1991) who thought it to be related to 'flagellates' but not to 'Rhizopoda' or other Archamoebae and that *Pelomyxa* has Golgi dictyosomes. However, identification of the *Pelomyxa* smooth membranes as Golgi complexes (Seravin and Goodkov, 1987) is questionable (Walker et al., 2001), and while it might be argued that the *Pelomyxa* branch is so long that its grouping within the Archamoebae might be an artefact (a shared deletion of a C at position 2308/9 specific for Conosa plus *Filamoeba* argues against this) one can hardly suppose any longer that *Pelomyxa* lies outside the Amoebozoa, so their primary reason for replacing the name Pelobiontea by one emphasising their 'flagellate' nature no longer applies. Our trees and those of Edgcomb et al. (2002) show that the distinction between Mastigamoebidae and Pelomyxidae is much deeper phylogenetically than those between Mastigamoebidae and *Endolimax* or between *Pelomyxa* and *Entamoeba*. Therefore the distinction made by Cavalier-Smith (1987b) between Pelobiontea and Mastigamoebea was cladistically sounder than the subsequent closer grouping of the ciliated taxa to each other than to *Entamoeba* (Cavalier-Smith, 1991). As the groupings of this 1987 archamoeba classification have been entirely supported by the molecular trees, we have retained it by simply reducing the ranks of the two classes to orders (Pelobiontida and Mastigamoebida). The main innovation is to establish a new family, Endolimacidae, for *Endolimax* and *Endamoeba*, placed within Mastigamoebida in accordance with the trees. Both archamoeba orders, thus circumscribed, are holophyletic with strong bootstrap support.

As *Phreatamoeba balamuthi* is firmly nested within Mastigamoebida, and its ultrastructure turns out to be basically similar (Brugerolle, 1991), we agree with Walker et al. (2001) that it no longer deserves a separate order (Cavalier-Smith, 1991). However, the trees show *Phreatamoeba* as sister to *Mastigella* not to either *Mastigamoeba* species. For this reason and because alone among the well-studied mastigamoebids *P. balamuthi* is predominantly non-ciliate for much of its life cycle (Chavez et al., 1986), there is merit in retaining the genus *Phreatamoeba* separate from *Mastigamoeba*, so we do not accept its renaming as *M. balamuthi* (Simpson et al., 1997). The characters that *Mastigamoeba* and *Phreatamoeba* share are almost certainly ancestral for all Archamoebae, not derived. The marked difference in the ciliary transition region and root structure of *M. simplex* (where the cone uniquely is almost cylindrical) compared with *M. punctachlora* and *M. schizophrenia* (which both have an unusual dense column in the transitional region) (Walker et al., 2001) would probably justify their placement in two separate genera. But until the type species *M. aspera* and many other named species are described ultrastructurally and their rRNA genes sequenced, it will not be possible to decide on a sensible circumscription of the genus *Mastigamoeba*.

It is intriguing that *Pelomyxa* and Mastigamoebida (Milyutina et al., 2001; Edgcomb et al., 2002) have the longest known eukaryote 18S rRNA genes (3.7 and 2.5–2.7 kb, respectively), whereas those of *Entamoeba* are only slightly longer than average. Was expansion independent in Mastigamoebida or *Pelomyxa* (the longest of all) or are those of *Entamoeba* secondarily shortened?

As Walker et al. (2001) point out, Pelobiontida move by amoeboid locomotion, whereas Mastigamoebidae are primarily swimming flagellates, like *Phalansterium*. As Endolimacidae are derived from Mastigamoebidae, their ancestor probably evolved eruptive lobopodia then, independently of the flattened eruptive pseudopodia of leptomyxoids, which according to the molecular trees evolved independently and are most closely related to the cylindrical non-eruptive pseudopodia of Amoeboidea. The semi-eruptive pseudopods of *Entamoeba* are distinctly different from the fountain streaming of their sister *Pelomyxa*.

Concluding remarks

The diversity of pseudopodial movement among Amoebozoa might in part reflect an independent origin of purely amoeboid motion in the various subgroups following the multiple losses of cilia or their restriction to brief dispersal stages in many Mycetozoa. If this is so, the ancestral amoebozoan may have been a soft-surfaced flagellate like *Phalansterium* or Mastigamoebidae; however, there is no denying the marked propensity to lose cilia and become amoebae within the phylum, analogous to but even more striking than in the bikont phyla Cercozoa (Cavalier-Smith and Chao, 2003c) and Percolozoa (Cavalier-Smith, 1993b), which since their inception have been accepted as ancestrally flagellate. This now appears likely to be true also of Amoebozoa. Our perception of the Amoebozoa has been over-influenced by the phenotype of the purely non-ciliate superfamily Amoeboidea. However, since Bütschli (1885) it has been suspected that amoebae evolved from amoeboflagellates by multiple ciliary losses. We now know he was right. It is highly probable that Haeckel's (1866) earlier view that the first eukaryote was a non-ciliate amoeba was mistaken, though we shall not be sure until we either establish the holophyly rather than paraphyly of Amoebozoa or in some other way eliminate the now remote possibility that the root of the eukaryote tree is within or adjacent to the Lobosea or Discosea. This will establish Amoebozoa as an

ancestrally uniciliate phylum that radiated into many niches by losing cilia and evolving a great variety of pseudopodial and plasmodial morphotypes (Smirnov and Goodkov, 1999), cell surface structures, and dormant cysts or spore-bearing fruiting bodies. It is possible that Mycetozoa are polyphyletic and Myxogastrea are actually closer to Variosea than to the rest of the Conosa; if verified they should be transferred from Conosa to Protamoebae.

Acknowledgements

Appendix A. Revised classification of phylum Amoebozoa into 7 classes and 20 orders

Phylum Amoebozoa Lühe 1913 stat. nov. Corliss 1984 emend. Cavalier-Smith 1998a, b.

Revised diagnosis: Ancestrally unikont and aerobic eukaryotes, typically with a single kinetid per cell with only one centriole associated with the apex of a cone of microtubules or (usually) secondarily non-ciliate and non-centriolar; rarely (*Multicilia*, *Pelomyxa*, some protostelids) multiciliate with monokinetids; rarely secondarily biciliate with plasmodial as well as amoeba and flagellate stages (Myxogastrea); non-ciliate forms and some ciliated forms undergo amoeboid motion, using pseudopods that are typically lobose or lamellipodial (with or without pointed or blunt subpseudopodia); true filopodia (i.e. ones able to attach to surfaces and draw the cell forwards) absent; mitochondria with usually branched tubular cristae, or reduced to tiny non-cristate mitosomes; stacked Golgi dictyosomes obvious in aerobic, but not in most anaerobic species.

Subphylum 1. Protamoebae[1] Cavalier-Smith subphyl. nov. Diagnosis: amoebae with Golgi dictyosomes; fruiting bodies typically absent, when rarely present (Copromyxida) without stalk tube; typically aerobic (except for breviates); mitochondria with branched tubular cristae.

Class 1. Breviatea Cavalier-Smith cl. nov. Diagnosis: uniciliate anaerobic amoebae distinguished from Archamoebae by cilium being shorter than body and lacking their rRNA signatures. Etymology: *L. brevis*, short,

referring to the short cilium, to the unusually short branches of this taxon on rRNA trees compared with other Amoebozoa, and to the fact that its rRNA is the shortest of any Amoebozoa, in contrast to ciliated Archamoebae, which have the longest. The vernacular term 'breviates' is appropriate for the group; I am aware that 'breviate' also means 'a summary' or 'lawyers brief', but it is virtually obsolete in both senses, so no confusion should arise from this novel usage.

Order Breviatida Cavalier-Smith ord. nov. Diagnosis as for the class (e.g., '*Mastigamoeba invertens*')

Class 2. Lobosea Carpenter 1861 emend. Diagnosis: aerobic uninucleate amoebae, lacking glycostyles or scales; locomotion by monopodial or polypodial lobose pseudopods that were ancestrally cylindrical and non-eruptive, but are sometimes flattened or eruptive; lack cilia or centrosomes; often with very thick glycocalyx; pointed protoplasmic projections absent, or if present (*Echinamoeba* only) much sparser than in acanthamoebids, *Filamoeba* or Mycetozoa; open mitosis.

Order 1. Euamoebida Lepşi emend. Lobosea without tests or fruiting bodies
Superfamily 1. Amoeboidea Ehrenberg stat. nov. Type genus *Amoeba*.
Diagnosis: aerobic amoebae with non-eruptive cylindrical lobose pseudopods
Family 1. Amoebidae Ehrenberg 1838 (e.g., *Amoeba*, *Chaos*, *Saccamoeba*, *Polychaos*)
Family 2. Hartmannellidae Volkonsky 1931 (e.g., *Hartmannella*, *Cashia*, *Glaeseria*)
Superfamily 2. Echinamoeboidea Page stat. nov.
Diagnosis: flattened irregular aerobic amoebae often with sparse short spine-like subpseudopodia; cysts with single-layered walls, with or without pores closed by opercula. Type genus *Echinamoeba*.
Family Echinamoebidae Page 1975 (*Echinamoeba*)
Superfamily 3. Leptomyxoidea Pussard and Pons 1976 stat. nov.
Family 1. Flabellulidae Bovee 1970 (e.g., *Paraflabellula*)
Family 2. Leptomyxidae Pussard and Pons 1976 (*Leptomyxa*, *Rhizamoeba*)

Order 2. Copromyxida Cavalier-Smith 1993a, b (with fruiting bodies)
Family 1. Copromyxidae Olive and Stoianovitch 1975 (*Copromyxa*, *Copromyxella*)

Order 3. Arcellinida Kent 1880 (testate: see Meisterfeld, 2002)
Suborder 1. Arcellinina: Haeckel 1884 (3 families)

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[1] Probably paraphyletic.

Suborder 2. Difflugiina Meisterfeld 2002 (13 families)

Suborder 3. Phryganellina Bovee 1985 (2 families)

Class 3. Discosea Cavalier-Smith cl. nov. Diagnosis: strongly flattened amoebae with leading lamellipodium with or without subpseudopodia; non-eruptive movement; usually with glycostyles, organic scales or very thick amorphous coat; mostly monopodial and uninucleate. Etymology: L. *discus* a disc; a meaningless euphonious suffix as in Lobosea and Conosa.

Order 1. Glycostylida Cavalier-Smith ord. nov. Diagnosis: aerobic uninucleate amoebae ancestrally with surface coat of hexagonal glycostyles (vexilliferids) or other hexagonal filamentous glycocalyx components in *Platyamoeba,* putatively modified to pentagonal glycostyles in *Vannella* or fenestrated scales in many paramoebids; non-ciliate with no centrosome or (rarely) with numerous cilia with unicentriolar kinetid

Superfamily 1. Vannelloidea Bovee stat. nov. Diagnosis: ancestrally with typically pentagonal glycostyles and cilia (often lost); lamellipodium without dactylopodia; parasomes absent. Type genus *Vannella*.

Family 1. Vannellidae Bovee 1970 (*Vannella, Platyamoeba, Lingulamoeba, Clydonella*)

Family 2. Multiciliidae Poche 1913. Revised diagnosis: weakly amoeboid multiciliate cells with unicentriolar kinetids over the whole cell surface; mitochondria with tubular cristae; surface glycostyles; centrioles surrounded by inverted truncated microtubular cones interconnected by cortical microtubular bands. (*Multicilia*)

Superfamily 2. Paramoeboidea Poche 1913 stat. nov.

Diagnosis; non-ciliate; with dactylopodia and parasomes. Type genus *Paramoeba*

Family 1. Vexilliferidae Page 1987 (*Vexillifera, Neoparamoeba*)

Family 2. Paramoebidae Poche 1913 (e.g., *Paramoeba, Mayorella, Korotnevella*)

Order 2. Himatismenida Page 1987 (dorsal organic scales)

Family Cochliopodiidae De Saedeleer 1934 (*Cochliopodium, Gocevia, Paragocevia*)

Order 3. Dermamoebida Cavalier-Smith ord. nov.

Diagnosis: aerobic uninucleate, or sometimes bi- or multinucleate, amoebae with thick, usually amorphous glycocalyx lacking scales or glycostyles; antero-posteriorly elongated discs moving by a broad crescent-shaped lamellipodium

Family Thecamoebidae Schaeffer 1926 (e.g., *Dermamoeba, Thecamoeba*)

Discosea *incertae sedis*: Family Hyalodiscidae Poche 1913 (e.g., *Hyalodiscus*)

Class 4. Variosea Cavalier-Smith cl. nov. Diagnosis: cells ancestrally with a single centriole and cilium and resting cyst, no fruiting body or locomotory lamellipodia; typically with many subpseudopodia, pointed or broad but non-eruptive (in latter case cell appearing branched) pseudopodia or branched pseudopods; mitochondria with branched cristae; cilium usually absent, in which case a lamellate centrosome is often present. Etymology: L. *varis* diverse, various; a meaningless euphonious suffix as in Lobosea and Conosa. Emphasizes their exceptionally varied phenotype compared with the other 3 classes of Protamoebae, with some species amoebae (with or without centrosomes) and others flagellates, some with thin, pointed pseudopodia and others with broad blunt ones, some with branched pseudopods and some with anastomosing ones.

Order 1. Phalansteriida Hibberd 1983

Family Phalansteriidae Kent 1880/1 emend. (*Phalansterium*)

Order 2. Centramoebida Rogerson and Patterson 2002 emend. (the name was first suggested by Patterson (1994) without diagnosis but with a circumscription that included also Stereomyxidae and Dictyosteliida but excluded *Balamuthia*).

Emended diagnosis: cells and cyst wall tripartite; inner endocyst is undulated, in places well separated by a low density mesocyst from the exocyst, but in contact in places giving the cyst wall a ridged appearance in surface view; usually with lamellate centrosome—exception *Comandonia*.

Family 1. Acanthamoebidae Sawyer and Griffin 1971 (pointed subpseudopodia) (*Acanthamoeba, Comandonia,*[2] *Protacanthamoeba*)

Family 2. Balamuthiidae Cavalier-Smith fam. nov.

Diagnosis: lobose pseudopodia without pointed subpseudopodia (Type genus: *Balamuthia*, Visvesvara et al., 1993)

Order 3. Varipodida Cavalier-Smith ord. nov. Branched non-ciliate flattened amoebae without cilia or centrosomes and cysts with smooth single-layered

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[2] Amaral Zettler et al. (2001) place it in *Acanthamoeba*.

walls; with non-plasmodial branched cells with broad non-eruptive pseudopods

Family 1. Gephyramoebidae Pussard and Pons 1976 (*Gephyramoeba*)

Family 2. Filamoebidae Cavalier-Smith fam. nov.

Diagnosis: flattened fan-shaped aerobic free-living amoebae with numerous slender pointed filiform pseudopodia arising from the hyaline edge; fruiting bodies, cilia, centrioles, scales or glycostyles absent; ovoid cysts with smooth single-layered thick wall (type genus: *Filamoeba* Page 1967)

Variosea *incertae sedis*: Family Stereomyxidae Grell 1966 (*Stereomyxa*, *Corallomyxa*) (slender branched pseudopodia, tapering but blunt-tipped, sometimes anastomosing; marine; lamellate centrosome, no cilia or centrioles)

Subphylum 2. Conosa Cavalier-Smith 1998a, b
Infraphylum 1. Archamoebae Cavalier-Smith 1983 stat. nov. 1998
Class Archamoebea Cavalier-Smith 1983 stat. nov.

Order 1. Pelobiontida Page 1976 (with amoeboid motion; amoeboid phases dominate the life cycle)

Family 1. Pelomyxidae Schulze 1977 (*Pelomyxa*, *Mastigina*)

Family 2. Entamoebidae Chatton 1925 (*Entamoeba*)

Order 2. Mastigamoebida Frenzel 1982 em.

Family 1. Mastigamoebidae (see footnote 1) Goldschmidt 1907 (swimming flagellates typically lacking amoeboid motion) (*Mastigamoeba*, *Mastigella*, *Phreatamoeba*)

Family 2. Endolimacidae Cavalier-Smith fam. nov.

Diagnosis: flattish amitochondrial monopodial amoebae with eruptive pseudopods; intestinal commensals lacking cilia, centrioles, contractile vacuoles or intracellular crystals. (*Endolimax* Kuenen and Swellengrebel 1913 (type genus), *Endamoeba*)

Infraphylum 2. Mycetozoa[3] De Bary 1873 stat. nov. Cavalier-Smith 1998 (pseudopods usually acutely pointed, typically with stalked sorocarps; ciliate stages ancestrally with outer cone of microtubules underlying the cell surface and inner cone attached to a central fibrous centriolar root)

Class 1. Stelamoebea (see footnote 1) Cavalier-Smith cl. nov. Diagnosis: minute fruiting bodies (sorocarps)

arise from single amoebae or aggregates of amoebae; sorocarps comprise a sorus of one to many spores borne on a cellulosic stalk tube; amoebae with pointed pseudopods; with one to several unikont (unicentriolar) cilia or non-ciliate; non-ciliate amoebae with an elaborate cytoplasmic microtubular skeleton in interphase (Guhl and Roos, 1994); spores are exospores formed by migrating amoebae, not endogenously by subdivision within plasmodia as in Myxogastrea

Order 1. Protostelida (see footnote 1) Olive and Stoianovitch 1966 emend. Revised diagnosis: minute fruiting bodies (sorocarps) arise from single amoebae; ciliated phases when present with single centrioles.

Family 1. Protosteliidae Olive and Stoianovitch 1966 (e.g., *Protostelium*, *Schizoplasmodium*)

Family 2. Cavosteliidae Olive and Stoianovitch 1964 (e.g., *Cavostelium*, *Planoprotostelium*)

Order 2. Dictyosteliida Lister 1909 or Olive 1970

Family 1. Acytosteliidae Raper in Raper and Quinlan 1958 (*Acytostelium*)

Family 2. Dictyosteliidae Rostafinski 1875 (*Dictyostelium*, *Polysphondylium*, *Coenonia*)

Class 2. Myxogastrea Fries 1829 stat. nov. Cavalier-Smith 1993 emend. Diagnosis: with lamellipodial/filopodial amoeba, flagellate, and (usually) plasmodial phases; flagellate phase with two centrioles, each with two microtubular bands, biciliate or sometimes uniciliate; mitochondria typically with central rod-shaped nucleoid; with multilayered cysts or with spores (developing within the plasmodia) borne on fruiting bodies; flagellate stage with inner microtubular cone attached apically to a microtubule nucleating centre at the end of a fibrillar root emanating from the anterior centriolar base

Order 1. Parastelida Cavalier-Smith ord. nov. Diagnosis: minute fruiting bodies (sorocarps) arise from single amoebae, aggregates of amoebae or microscopic plasmodia; distinguished from other Myxogastrea by having only 1–4 spores on a very delicate stalked sorocarp.

Family 1. Ceratiomyxidae Schröter 1889 (*Ceratiomyxa*, *Ceratiomyxella*)

Order 2. Echinosteliida Keller and Brooks 1976

Family 1. Echinosteliidae Rostafinski 1873 (*Echinostelium*)

Family 2. Clastodermidae Alexopoulos and Brooks 1971 (*Barbeyella*, *Clastoderma*)

Order 3. Liceida Jahn 1928 (3 families: Listerellidae Jahn, 1928; Liceidae Rostafinski, 1873; Enteriidae Farr, 1982)

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[3] It is possible that Mycetozoa are polyphyletic and Myxogastrea are actually closer to Variosea than to the rest of the Conosa; if verified they should be transferred from Conosa to Protamoebae.

Order 4. Trichiida Macbride (1922) (2 families: Dianemidae Macbride, 1899; Trichiidae Rostafinski, 1873)

Order 5. Stemonitida Macbride 1922 (Stemonitidae Rostafinski, 1873, e.g., *Stemonitis*)

Order 6. Physarida Macbride 1922
    Family 1. Elaeomyxidae Hagelstein 1982 (*Elaeomyxa*)
    Family 2. Physaridae Rostafinski 1873 (e.g., *Physarum*, *Badhamia*, *Fuligo*)
    Family 3. Didymiidae Rostafinski 1873 (e.g., *Didymium*)

Myxogastrea incertae sedis: "*Echinostelium*" *bisporum* (ultrastructure as *Cladostelium bisporum* (Furtago and Olive, 1970)); *Hyperamoeba* spp. aggregate[4]
    Mycetozoa incertae sedis: Family Echinosteliopsidae (*Echinosteliopsis*)

The order Trichosida Möbius 1889, family Trichosidae Möbius, 1889 (*Trichosphaerium*) is here provisionally excluded from Amoebozoa on the basis of early reports of a biciliate stage (Schaudinn, 1899; Minchin, 1922); such a stage, although not mentioned by recent authors (Page, 1983), if present would suggest, together with the complex multiphasic life cycle, that this genus may belong among the bikont Rhizaria (Cavalier-Smith, 2002) rather than in the Amoebozoa. Whether its curious lobose pseudopods are related to those of Lobosea or not is unclear, though they are more suggestive of a position within Protamoebae than within Rhizaria.

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[4] *Hyperamoeba* is polyphyletic.

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