Protist, Vol. 154, 341–358, October 2003 © Urban & Fischer Verlag
http://www.urbanfischer.de/journals/protist
Published online 17 October 2003

Protist

ORIGINAL PAPER

Phylogeny and Classification of Phylum Cercozoa (Protozoa)

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Submitted February 12, 2003; Accepted June 2, 2003
Monitoring Editor: Michael Melkonian

The protozoan phylum Cercozoa embraces numerous ancestrally biciliate zooflagellates, euglyphid and other filose testate amoebae, chlorarachnean algae, phytomyxean plant parasites (e.g. *Plasmodiophora, Phagomyxa*), the animal-parasitic Ascetosporea, and *Gromia*. We report 18S rRNA sequences of 27 culturable zooflagellates, many previously of unknown taxonomic position. Phylogenetic analysis shows that all belong to Cercozoa. We revise cercozoan classification in the light of our analysis and ultrastructure, adopting two subphyla: Filosa subphyl. nov. a clade comprising Monadofilosa and Reticulofilosa, ranked as superclasses, ancestrally having the same very rare base-pair substitution as all opisthokonts; and subphylum Endomyxa emend. comprising classes Phytomyxea (Plasmodiophorida, Phagomyxida), Ascetosporea (Haplosporidia, Paramyxida, Claustrosporida ord. nov.) and Gromiidea cl. nov., which did not. Monadofilosa comprise Sarcomonadea, zooflagellates with a propensity to glide on their posterior cilium and/or generate filopodia (e.g. *Metopion*; *Cercomonas*; Heteromitidae – *Heteromita*, *Bodomorpha*, *Proleptomonas* and *Allantion*) and two new classes: Imbricatea (with silica scales: Euglyphida; Thaumatomonadida, including *Allas, Thaumatomastix*) and Thecofilosea (Cryomonadida; Tectofilosida ord. nov. – non-scaly filose amoebae, e.g. *Pseudodifflugia*). Reticulofilosa comprise classes Chlorarachnea, Spongomonadea and Proteomyxidea (e.g. *Massisteria*, *Gymnophrys*, a *Dimorpha*-like protozoan). Cercozoa, now with nine classes and 17 orders (four new), will probably include many, possibly most, other filose and reticulose amoebae and zooflagellates not yet assigned to phyla.

Introduction

The protozoan phylum Cercozoa was established (Cavalier-Smith 1998a, 1998b) as a result of molecular phylogenetic studies that showed that chlorarachnean algae (e.g. *Chlorarachnion* (Hibberd and Norris 1984), *Lotharella* (Ishida 1996b)), euglyphid filose testate amoebae (Bhattacharya et al. 1995), the zooflagellates *Cercomonas*, *Heteromita*, and *Thaumatomonas* (earlier grouped as Sarcomonadea (Cavalier-Smith 1993b)), and the plasmodiophorid plant parasites (previously often treated as fungi or slime moulds: Braselton 2002) were all mutually related (Cavalier Smith 1996/7; Cavalier-Smith and Chao 1996/7). Bulman et al. (2001) confirmed the inclusion of both Plasmodiophorida and Phagomyxida in the Cercozoa (Cavalier Smith 1996/7). Other filose testate amoebae, *Pseudodifflugia* (Wylezich et al. 2002) and *Gromia* (Burki et al. 2002), are also Cercozoa, but not directly related to each other or to euglyphids. However, the non-testate filose nucleariid amoebae are Choanozoa not Cercozoa (Amaral

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1434-4610/03/154/03–04-341 $ 15.00/0

Zettler et al. 2001; Cavalier-Smith and Chao 2003a). Recently Cercozoa was expanded (Cavalier-Smith 2002a) to include also the Ascetosporea, parasites of shellfish belonging to two distinctive orders (Haplosporida and Paramyxida) each sometimes treated as separate phyla (Berthe et al. 2000). On gamma-corrected 18S rRNA trees, however, Ascetosporea are robustly holophyletic and group with moderate bootstrap support with other Cercozoa; moreover they share an almost unique nucleotide deletion with all sequenced Cercozoa (Cavalier-Smith and Chao 2003a). In addition the haplosporosomes of Ascetosporea (Desportes and Perkins 1990; Perkins 1990) are structurally similar to the cored vesicles of plasmodiophorids (Barr and Allan 1982; Miller et al. 1983) in that they consist of two concentric membranes with very dense material within the inner one; despite the fact that the space between the two membranes is pale in plasmodiophorids but of medium density in ascetosporans, it has been proposed that these unique organelles are homologous and a synapomorphy (Cavalier-Smith and Chao 2003a) for the recently established cercozoan subphylum Endomyxa, comprising classes Ascetosporea and Phytomyxea (orders Plasmodiophorida and Phagomyxida) (Cavalier-Smith 2002a).

By contrast with the parasitic Endomyxa, the free-living cercozoan zooflagellates have been very little studied, even though some of them (e.g. *Cercomonas*, *Heteromita*) are the most ubiquitous flagellates in soil and ecologically significant predators, especially on bacteria, in virtually all aquatic habitats. Free-living zooflagellates in general are neglected compared with most other groups of protists, despite their fundamental evolutionary importance for understanding the early diversification of the eukaryotic cell (Cavalier-Smith 2000) and there are many genera that have not been convincingly placed in a phylum or any higher taxon (Patterson et al. 2002a; Patterson and Zölffel 1991), although molecular evidence now supports the classification of the zooflagellates *Cryothecomonas* (Kuhn et al. 2000) and *Proleptomonas* (Vickerman et al. 2002), and the filose zooflagellate *Massisteria* (Atkins et al. 2000) as Cercozoa (Cavalier-Smith 1998b). As part of a long-term programme to use molecular methods to elucidate the phylogeny of as many zooflagellates of uncertain phylogenetic and taxonomic position as possible, we report here the 18S rRNA sequences of 27 such zooflagellate strains (obtained from culture collections or isolated by us from the field in South Africa, Costa Rica, and Canada) that turn out to be Cercozoa. We also did electron microscopy to verify or help elucidate the identity of some strains.

Our phylogenetic analysis reveals about a dozen major clades within the phylum and provides the basis in conjunction with other data for a substantial revision of the higher-level classification of Cercozoa. We also discuss key aspects of cell evolution within the group. Cercozoa now constitute a major protozoan assemblage containing the majority of the zooflagellates of previously unclear taxonomic affinity and are of particular evolutionary and ecological importance. Their closest relatives are probably the Retaria (Foraminifera and Radiolaria: Cavalier-Smith 1999, 2002a). Molecular and other evidence indicates that the phyla Cercozoa and Retaria are sisters (Archibald et al. 2003; Burki et al. 2002; Cavalier-Smith 2002a; Cavalier-Smith and Chao 2003a; Keeling 2001); they are together informally called ‘core Rhizaria’ (Cavalier-Smith 2003), the protozoan clade in which filopodia, reticulopodia and axopodia are most widespread.

Results

Revised Classification of Cercozoa

Table 1 summarizes the major cercozoan groups recognized here. Eight new taxa are established. Two (subphylum Filosa and class Gromiidea) are very strongly supported by our phylogenetic analysis (95% and 100% bootstrap support respectively on Fig. 1). Bootstrap support is only moderate for the class Thecofilosea (58%) and very weak for the class Imbricatea, which is characterized uniquely by silica scales. We cannot yet assess the molecular support for the new orders, as three (Reticulosida, Metopiida, Tectofilosida) have only a single species on the tree (Metopiida currently has only one species anyway: its distinctness and considerable divergence from other orders is evident on the trees, as is that of the other two) and the fourth (Claustrosporida) has no molecular data available.

Figure 1. Distance tree of 116 rhizarian 18S rRNAs using 1638 positions (weighted least squares, power 2: GTR Γ+I model: α = 0.628375; i = 0.139134). The 27 newly sequenced taxa are in bold. Bootstrap percentages (using the same model) are given for major clades only, for clarity mostly by their names rather than on the tree (bold if 80% or more).

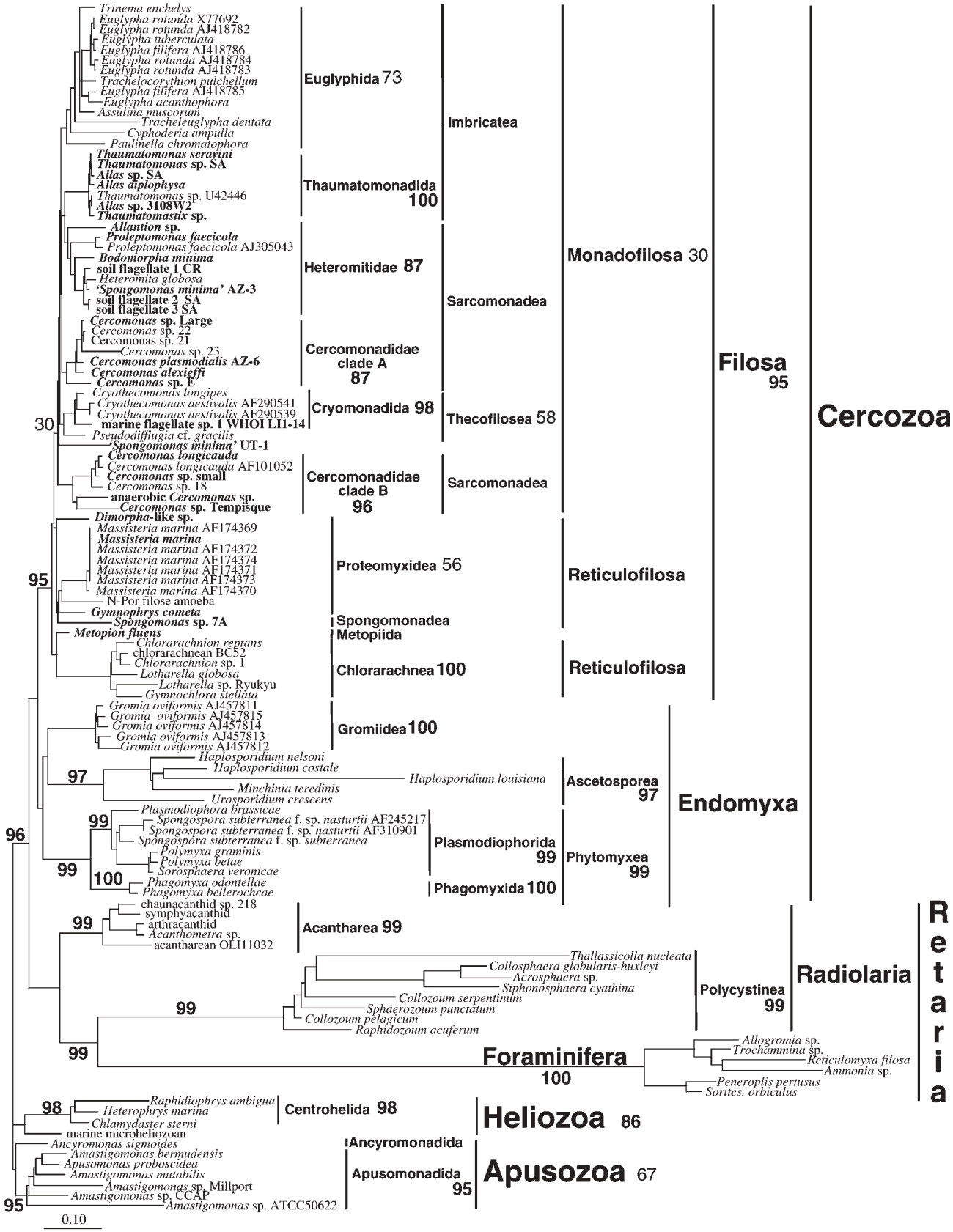

Trinema enchelys
Euglypha rotunda X77692
Euglypha rotunda AJ418782
Euglypha tuberculata
Euglypha filifera AJ418786
Euglypha rotunda AJ418784
Euglypha rotunda AJ418783
Trachelocorythion pulchellum
Euglypha filifera AJ418785
Euglypha acanthophora
Assulina muscorum
Tracheleuglypha dentata
Cyphoderia ampulla
Paulinella chromatophora
Euglyphida 73
Thaumatomonas seravini
Thaumatomonas sp. SA
Allas sp. SA
Allas diplophysa
Thaumatomonas sp. U42446
Allas sp. 3108W2
Thaumatomastix sp.
Thaumatomonadida 100
Imbricatea
Allantion sp.
Proleptomonas faecicola
Proleptomonas faecicola AJ305043
Bodomorpha minima
soil flagellate 1 CR
Heteromita globosa
'Spongomonas minima' AZ-3
soil flagellate 2 SA
soil flagellate 3 SA
Heteromitidae 87
Cercomonas sp. Large
Cercomonas sp. 22
Cercomonas sp. 21
Cercomonas sp. 23
Cercomonas plasmodialis AZ-6
Cercomonas alexieffi
Cercomonas sp. E
Cercomonadidae clade A 87
Sarcomonadea
Monadofilosa 30
Filosa 95
Cercozoa
Cryothecomonas longipes
Cryothecomonas aestivalis AF290541
Cryothecomonas aestivalis AF290539
marine flagellate sp. 1 WHOI LI1-14
Cryomonadida 98
Thecofilosea 58
30
Pseudodifflugia cf. gracilis
'Spongomonas minima' UT-1
Cercomonas longicauda
Cercomonas longicauda AF101052
Cercomonas sp. small
Cercomonas sp. 18
anaerobic Cercomonas sp.
Cercomonas sp. Tempisque
Cercomonadidae clade B 96
Sarcomonadea
Dimorpha-like sp.
Massisteria marina AF174369
Massisteria marina
Massisteria marina AF174372
Massisteria marina AF174374
Massisteria marina AF174371
Massisteria marina AF174373
Massisteria marina AF174370
95
N-Por filose amoeba
Gymnophrys cometa
Spongomonas sp. 7A
Proteomyxidea 56
Spongomonadea
Reticulofilosa
Metopion fluens
Metopiida
Chlorarachnion reptans
chlorarachnean BC52
Chlorarachnion sp. 1
Lotharella globosa
Lotharella sp. Ryukyu
Gymnochlora stellata
Chlorarachnea 100
Reticulofilosa
Gromia oviformis AJ457811
Gromia oviformis AJ457815
Gromia oviformis AJ457814
Gromia oviformis AJ457813
Gromia oviformis AJ457812
Gromiidea 100
Haplosporidium nelsoni
Haplosporidium costale
97
Haplosporidium louisiana
Minchinia teredinis
Urosporidium crescens
Ascetosporea 97
Endomyxa
Plasmodiophora brassicae
99
Spongospora subterranea f. sp. nasturtii AF245217
Spongospora subterranea f. sp. nasturtii AF310901
Spongospora subterranea f. sp. subterranea
96
Plasmodiophorida 99
Phytomyxea 99
Polymyxa graminis
Polymyxa betae
99
Sorosphaera veronicae
100
Phagomyxa odontellae
Phagomyxa bellerocheae
Phagomyxida 100
chaunacanthid sp. 218
symphyacanthid
arthracanthid
Acanthometra sp.
acantharean OLI11032
99
Acantharea 99
Thallassicolla nucleata
Collosphaera globularis-huxleyi
Acrosphaera sp.
Siphonosphaera cyathina
Collozoum serpentinum
99
Sphaerozoum punctatum
Collozoum pelagicum
Raphidozoum acuferum
Polycystinea 99
Radiolaria
Retaria
99
Foraminifera 100
Allogromia sp.
Trochammina sp.
Reticulomyxa filosa
Ammonia sp.
Peneroplis pertusus
Sorites. orbiculus
Raphidiophrys ambigua
98
Heterophrys marina
Centrohelida 98
Heliozoa 86
Chlamydaster sterni
marine microheliozoan
Ancyromonas sigmoides
Ancyromonadida
Amastigomonas bermudensis
Apusomonas proboscidea
Amastigomonas mutabilis
Amastigomonas sp. Millport
Amastigomonas sp. CCAP
Apusomonadida 95
Apusozoa 67
95
Amastigomonas sp. ATCC50622
0.10

Table 1. Classification of the 9 classes and 17 orders of phylum Cercozoa Cavalier-Smith 1998.

Subphylum 1. Filosa Cavalier-Smith subphyl. nov. Diagnosis: filose amoebae with tubular mitochondrial cristae or ancestrally biciliate (rarely uni- or multiciliate) zooflagellates or uniciliate algae with a nucleomorph and green plastid within a periplastid and epiplastid membrane; if flagellates, lacking cortical alveoli, tubular ciliary hairs or a sub-plasma membrane dense plate, but often with a strong propensity for emitting filopodia or reticulopodia, and usually with tubular (rarely flat) mitochondrial cristae; ancestrally with a substitution in helix 49 of small subunit rRNA of an AU base-pair for the normal CG (Cavalier-Smith and Chao 2003a) and lacking the adjacent heterokont substitution (Cavalier-Smith et al. 1994); extrusomes frequent, usually simple dense and near-spherical or kinetocysts, rarely long and somewhat resembling trichocysts.

Superclass 1. Reticulofilosa Cavalier-Smith 1996/7 stat. nov.

Class 1. Chlorarachnea Hibberd and Norris 1984 (With green plastid and nucleomorph within periplastid and epiplastid membranes (Cavalier-Smith 2003); often with uniciliate and/or filose or meroplasmodial stages)

Order Chlorarachnida Hibberd and Norris 1984 (e.g. *Chlorarachnion*, *Bigelowiella*, *Lotharella, Cryptochlora, Gymnochlora*)

Class 2. Spongomonadea Cavalier-Smith 2000 (Biciliates with parallel centrioles and perforated pericentriolar cups, but no pseudopodia or plastids)

Order Spongomonadida Hibberd 1983 (e.g. *Spongomonas*, *Rhipidodendron*)

Class 3. Proteomyxidea* Lankester 1885 emend. (Uninucleate feeding stage with filopodia or reticulopodia, usually two cilia, and extrusomes; chloroplasts absent)

Order 1. Pseudosporida Cavalier-Smith 1993. Family Pseudosporidae Berlese in Saccardo 1888 (e.g. *Pseudospora*)

Order 2. Leucodictyida Cavalier-Smith 1993 emend. Diagnosis: biciliate protists with branching filopodia that can fuse temporarily to form meroplasmodia; filopodia bear extrusomes, are appressed to the substratum and are supported in part by irregularly arranged microtubules; mitochondrial cristae tubular.

Family 1. Leucodictyidae Cavalier-Smith 1993 (e.g. *Leucodictyon, Reticulamoeba*)

Family 2. Massisteriidae Cavalier-Smith 1993 (*Massisteria*)

Order 3. Heliomonadida Cavalier-Smith 1993 (axopodia bear extrusomes: *Dimorpha*, *Tetradimorpha*)

Order 4. Reticulosida Cavalier-Smith ord. nov. Diagnosis: uninucleate reticulose amoebae with two centrioles, plate-like mitochondrial cristae; filopodia bear extrusomes (e.g. *Gymnophrys*, *Borkovia*; probably also *Biomyxa, Chlamydomyxa*). Sole family at present: Gymnophryidae (Mikryukov and Mylnikov 1996).

Superclass 2. Monadofilosa Cavalier-Smith 1996/7 stat. nov.

Class 1. Sarcomonadea Cavalier-Smith 1993 emend. Revised diagnosis: ancestrally biciliate zooflagellates with strongly divergent centrioles; lacking scales or external theca; often glide on posterior cilium; cell surface soft without obvious cortical filamentous or membranous skeleton; sometimes strongly amoeboid and temporarily make filopodia; cilia without scales or hairs and with a simple transition region (latter contrasting with cryomonads); mitochondrial cristae tubular; extrusomes, if present, typically small near-spherical, concentric and capped; usually with microbody (peroxisome?) attached to the nucleus.

Order 1. Metopiida Cavalier-Smith ord. nov. Diagnosis: biciliate non-pseudopodial zooflagellates that glide on posterior cilium; distinguished from heteromitids by anterolateral groove from which both cilia emerge.

Family Metopiidae Cavalier-Smith fam. nov. Diagnosis as for the order (type: *Metopion fluens* Larsen and Patterson 1990).

Order 2. Cercomonadida Poche 1913 emend. Vickerman in Honigberg 1983 Lack anterolateral groove.

Family 1. Cercomonadidae Kent 1880/1 emend. Cell surface very flexible, often prone to generate filopodia and typically drawn out posteriorly into a trailing point to which the posterior cilium normally adheres. Cell typically spindle shaped, sometimes with plasmodial phase. (*Cercomonas*)

Family 2. Heteromitidae Kent 1880/1 emend. Cell surface semi-rigid, not generating filopodia or plasmodia, cell posterior normally rounded, not extended into a point adhering to the posterior cilium. Cells typically ovoid, but sometime more elongate. Anterior cilium sometimes absent. (*Heteromita*, *Bodomorpha*, *Proleptomonas*, *Allantion*)

Class 2. Thecofilosea Cavalier-Smith cl. nov. Diagnosis: uninucleate cell surrounded by an organic flexible tectum or rigid test with one or two apertures for filopodia; with two cilia or none; mitochondrial cristae tubular.

Table 1. (Continued).

Order 1. Tectofilosida Cavalier-Smith ord. nov. Diagnosis: uninucleate cell surrounded by an organic flexible tectum or rigid test with one or two apertures for filopodia, sometimes including foreign mineral particles (agglutinated); cilia or silica scales absent; tubular mitochondrial cristae. (Families Pseudodiffflugiidae, e.g. *Cryptodifflugia*; Chlamydophryidae; Psammonobiotidae; Amphitremidae; Volutellidae)

Order 2. Cryomonadida Cavalier-Smith 1993 (e.g. *Cryothecomonas*, WHOI LI1-14)

Class 3. Imbricatea Cavalier-Smith cl. nov. Diagnosis: uninucleate cells with surface covered with imbricated silica scales except for a terminal or lateral aperture for naked filopodia; mitochondrial cristae tubular.

Order 1. Thaumatomonadida Shirkina 1987 (e.g. *Thaumatomonas*, *Thaumatomastix*, *Allas, Gyromitus*)

Order 2. Euglyphida Copeland 1956 emend. Cavalier-Smith 1997 (4 families, e.g. *Euglypha*, *Trinema*, *Corythion*, *Assulina*, *Tracheleuglypha*, *Trachelocorythion*, *Cyphoderia*, *Paulinella*)

Subphylum 2. Endomyxa Cavalier-Smith 2002 emend.

Class 1. Phytomyxea Engler and Prantl 1897

Order 1. Phagomyxida Cavalier-Smith 1993 (e.g. *Phagomyxa*)

Order 2. Plasmodiophorida Cook 1928 (e.g. *Plasmodiophora*, *Spongospora*)

Class 2. Ascetosporea Sprague 1979 stat. nov. Cavalier-Smith 2002

Order 1. Haplosporida Caullery and Mesnil 1889 orth. em. Lühe 1900 (*Minchinia*, *Haplosporidium*, *Urosporidium*, *Bonamia*, *Mikrocytos*)

Order 2. Paramyxida Chatton 1911(*Marteilia*, *Paramyxa, Paramarteilia*)

Order 3. Claustrosporida Cavalier-Smith ord. nov. Diagnosis: uninucleate sporoplasm with haplosporosomes; spore wall with no orifice and formed on sporoplasm surface, not intracellular as in Haplosporida (*Claustrosporidium*).

Class 3. Gromiidea Cavalier-Smith cl. nov. Diagnosis: multinucleated cell with organic test with a single aperture through which branching thin filopodia pass; also a biciliate stage.

Order Gromiida Claparède and Lachmann 1856 (*Gromia*)

*As pointed out by Cavalier-Smith and Chao (2003b), Desmothoracida are also likely to be Cercozoa; if so, they would belong in this class close to heliomonads.

It is unclear whether the order Commatiida Cavalier-Smith 1996/7 (*Commation*) belongs in Cercozoa or Heterokonta. Vampyrellida Starobogatov ex Krylov et al. 1980 were formerly included in Monadofilosa (Cavalier Smith 1996/7), but are now left as Protozoa *incertae sedis* pending molecular evidence.

Most Mysterious Zooflagellates are Cercozoa

Our sequencing over the past few years of 18S rRNA genes of over fifty zooflagellates currently regarded as of uncertain phylogenetic position (Patterson et al. 2002a) indicates that the majority of them belong in the phylum Cercozoa. This was established by preliminary distance trees with nearly 300 eukaryote sequences including representatives of all major eukaryote groups. Figure 1 is a gamma-corrected distance tree that includes the 27 new sequences that grouped robustly within Cercozoa on those larger trees. These include further species or strains of the established cercozoan zooflagellates *Cercomonas*, *Heteromita*, *Thaumatomonas*, *Proleptomonas, Massisteria*, and *Cryothecomonas* as well as the newly placed zooflagellate genera *Allantion*, *Allas*, *Bodomorpha*, and *Spongomonas*, plus *Gymnophrys*, a biciliate reticulose proteomyxid, and several unidentified strains. As previously observed with a smaller cercozoan sample but much broader set of outgroups (Cavalier-Smith and Chao 2003a), Cercozoa are a clade that is sister to the phylum Retaria (Radiolaria and Foraminifera: Cavalier-Smith 1999). The tree is rooted assuming that Heliozoa/Apusozoa are outgroups, in line with earlier evidence (Cavalier-Smith and Chao 2003a, b).

In this data set, restricted to Rhizaria to establish the position of the cercozoan root more reliably, the grouping of Cercozoa with Retaria as ‘core Rhizaria’ and of Heliozoa with Apusozoa is much stronger than earlier (Cavalier-Smith and Chao 2003a, b). The high bootstrap support for the bipartition between core Rhizaria and Apusozoa/Heliozoa provides stronger evidence than hitherto that Heliozoa are not specifically related to Radiolaria and for the polyphyly of Actinopoda, in which Heliozoa and Radiolaria were traditionally grouped (Calkins 1901). It also shows for the first time that Heliozoa are not specifically related either to *Gymnophrys* (despite both groups sharing two significant morphological

characters: microtubule-supported cell extensions bearing similar kinetocyst-type extrusomes and flat mitochondrial cristae) or to the *Dimorpha*-like strain (*Dimorpha* was sometimes regarded as heliozoan: Febvre-Chevalier 1990). This tree has low bootstrap support for Cercozoa themselves, because the very long branched Foraminifera sometimes move within the Cercozoa as sister to the long-branch Ascetosporea.

When long-branch Retaria are excluded and the cercozoan sequences pruned to allow maximum likelihood analysis, bootstrap support for the monophyly of Cercozoa including Ascetosporea rises dramatically (Fig. 2). Although it has often been suggested that Foraminifera may be related to *Gromia* (e.g. they were included together in a class Reticularia Carpenter 1862 by Lankester: 1890), they never grouped with the gromiids and usually group with polycystine radiolaria instead with high bootstrap support. To check that this grouping is not merely a long-branch attraction artefact we also calculated distance, parsimony and ML trees after removing polycystines but including foraminifera for a variety of taxon samples based on those of Figures 1 and 2. In these a retarian clade comprising Acantharea and Foraminifera persisted; this clade was usually sister to Cercozoa, though in some distance or parsimony trees it moved to become sister to Ascetosporea. This occasional intermingling of Cercozoa and Retaria is probably an artefactual attraction between the very long branches of Foraminifera and relatively long ones of Ascetosporea. This interpretation is supported by the absence of the derived G-deletion signature of all Cercozoa (Cavalier-Smith and Chao 2003a) from all three retarian taxa.

The Major Clades of Cercozoa

Figures 1 and 2 both show that Cercozoa comprise four major distinctly separate subclades: the parasitic Ascetosporea; the gromiid testate amoebae; the parasitic Phytomyxea; and a large free-living clade that includes the chlorarachnean algae, most filose testate amoebae (euglyphids and *Pseudodif-*

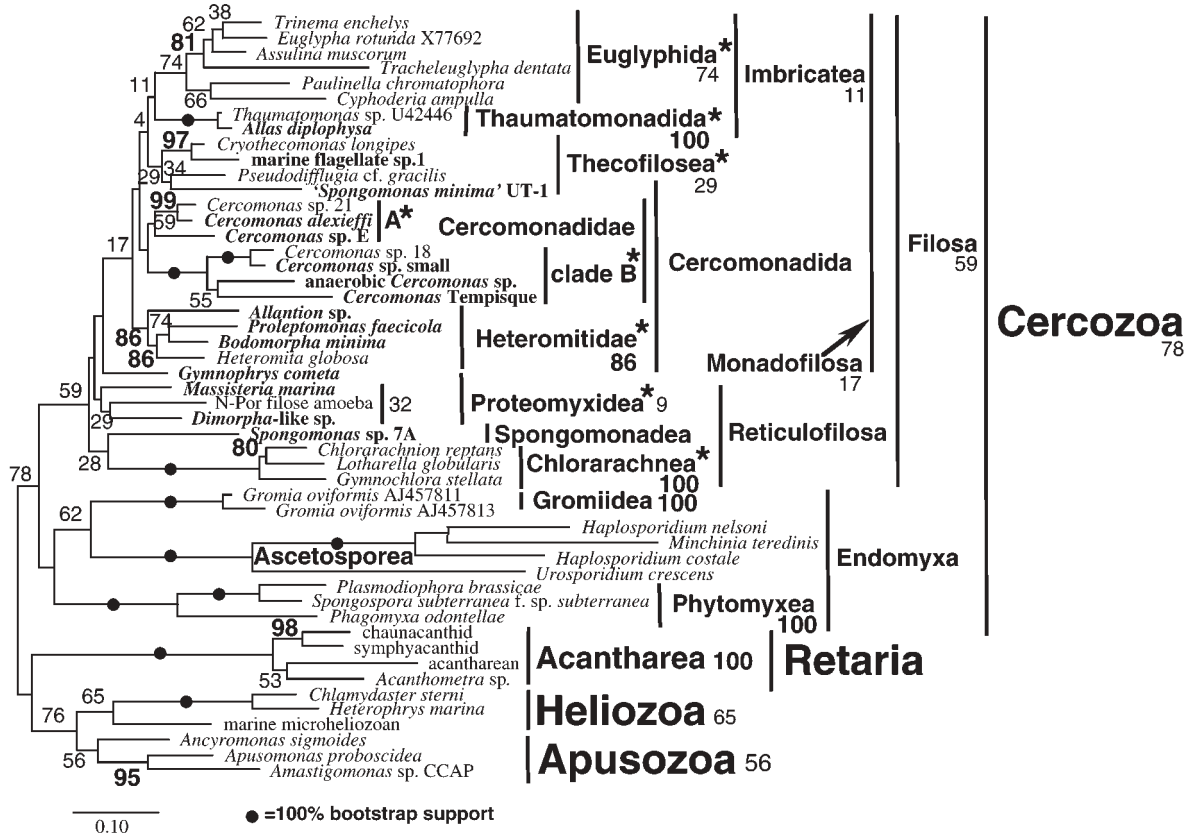


Figure 2. Maximum likelihood tree of 50 rhizarian 18S rRNAs using 1638 positions (Γ+I model: α = 0.55084; i = 0.26839). This tree had the highest log likelihood (–25487.62) of those yielded by 11 independent random additions of taxa. New sequences in bold. The figures are bootstrap percentages (bold if 80% or more) using the same maximum likelihood model.

flugia) and a huge array of zooflagellates. The latter most diverse clade, here treated as a new subphylum Filosa, consists of eight reasonably well-defined subclades, plus three single lineages (*Metopion fluens*, *Spongomonas* sp. 7A and "*Spongomonas minima*" UT-1) of inconstant position. The eight subclades (shown by asterisks on Fig. 2), are all relatively uniform internally in morphology but quite distinct from each other, except for the presence of two separate clades (A and B) for the genus *Cercomonas*. Except for the proteomyxid clade and the cryomonad/*Pseudodifflugia* clade (Thecofilosea), which have low bootstrap support, these clades are each strongly or very strongly supported. However, their relative branching order is very weakly supported and can vary with different phylogenetic analyses. Despite this, the filosan clades fall into two reproducible groups: one is a weakly supported superclade corresponding to subphylum Monadofilosa, comprising Euglyphida, Thaumatomonadida, Cercomonadida (cercomonads and heteromitids) and the cryomonad/*Pseudodifflugia* clade (Thecofilosea) plus "*Spongomonas minima*" UT-1; the other corresponds with the subphylum Reticulofilosa, and comprises the chlorarachnean algae, Proteomyxidea and *Spongomonas* 7A. Reticulofilosa are often weakly holophyletic but may sometimes appear (weakly) to be paraphyletic. The sequence of *Metopion fluens* is hard to place; as it is only partial, it was omitted from Figure 2 to avoid possible distortion of the tree. In an ML analysis restricted to Cercozoa to increase the chance of obtaining a reliable tree it was sister to other Monadofilosa (Fig. 3), suggesting that its grouping with Chlorarachnea in Figure 1 is incorrect. Parsimony and distance analyses (Fig. 4) broadly agree with the ML trees and each other, but are computationally immensely faster, allowing all new taxa to be included and exploring tree space more thoroughly.

The gromiid testate amoebae are sisters of Ascetosporea with moderate to strong support. The position of this clade is sensitive to which taxa are included as outgroups and the methods used: in most trees the ascetosporan/gromiid clade is sister to Phytomyxea (e.g. Figs 1 and 2) but in a few to Phytomyxea plus Filosa. We suspect that the latter is an artefact caused by the long ascetosporan branch, but cannot determine which position is more reliable with present data.

Filosan Signature Sequences

Almost all Filosa have a rare base-pair substitution: immediately adjacent to the analogous substitution of UA by AU, a unique but universal signature for heterokonts in helix 49 (Cavalier-Smith et al. 1994). In all Filosa, except *Cercomonas* clade B, an AU base-pair replaces the normal CG. Interestingly, all opisthokonts have precisely the same substitution, which therefore occurred at least twice in the history of life. No other major group has this opisthokont/filosan substitution, but it has occurred in a very small number of species within a few other groups in our database of over 600 eukaryotic sequences. From the phylogenies of Figures 1–4 this substitution was lost by *Cercomonas* clade B not by reversion to the ancestral CG but by convergent change to equally stable GC.

Reticulofilosan Phylogeny

A weakly supported proteomyxid clade of filose or reticulose protozoa is usually found by all methods in trees that exclude long-branch non-cercozoan outgroups; the closest relatives within this clade on distance trees (Figs 1, 4) are two marine protozoa: *Massisteria* and the filose amoeba (N-Por) which has been referred to as *Nuclearia*-like (Bhattacharya and Oliveira 2000) although it is not closely similar to *Nuclearia*, which is not a cercozoan but a choanozoan (Amaral Zettler et al. 2001; Cavalier-Smith and Chao 2003a) that lacks extrusomes (like all Choanozoa). The *Dimorpha*-like amoeboflagellate is weakly sister to them or to Npor in most trees. The reticulose amoeboflagellate *Gymnophrys* usually groups weakly with the *Massisteria*/Npor/*Dimorph*a-like clade (the much longer branch and somewhat deeper position of *Gymnophrys* on a preliminary tree (Cavalier-Smith 2000) should be ignored as that sequence proved to be a misleading chimaera).

Figure 4 shows a distance and parsimony analysis restricted to Cercozoa; here *Spongomonas* groups with chlorarachneans as with ML, and does not intrude into the proteomyxid clade, as it did in Figure 1 (but not in the corresponding bootstrap consensus tree). Even though the ciliary apparatus of chlorarachneans and *Spongomonas* is very different (see discussion in Cavalier-Smith 2002a), the presence of similar extrusomes in both groups (Fig. 5a) supports such a grouping. It is noteworthy that the strain designated *Spongomonas minima* UT-1 does not group with *Spongomonas* sp. 7A. *Spongomonas* 7A, which we isolated from garden soil in Cape Town, South Africa, is clearly a genuine *Spongomonas*, quite similar to the species recorded in soils by Sandon (1927), as it forms little cocoons of granular material around each cell with which it sticks to the culture dish – but unlike Sandon's species the 'cocoons' did not coalesce. In the light microscope one readily sees two long parallel cilia,

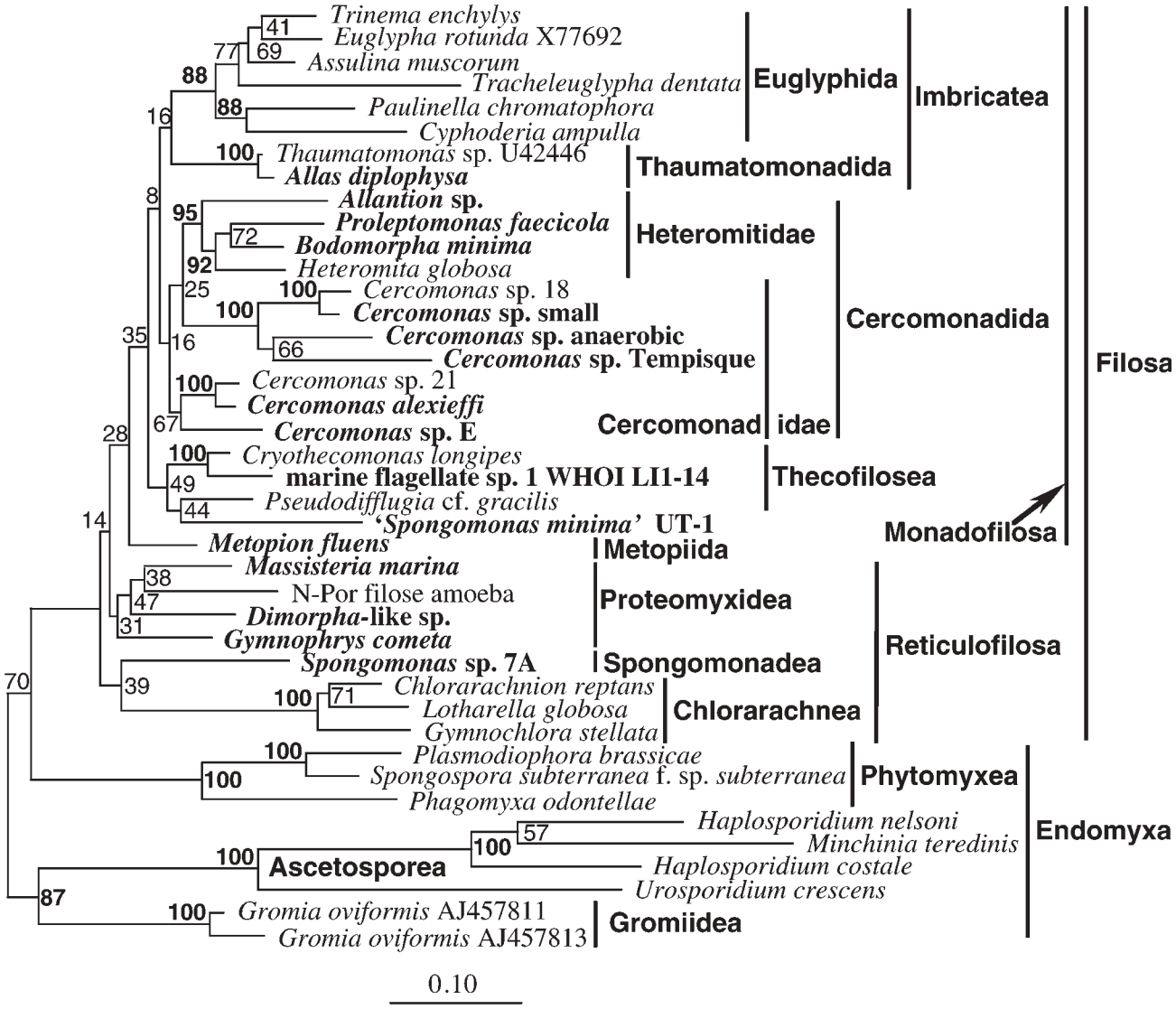
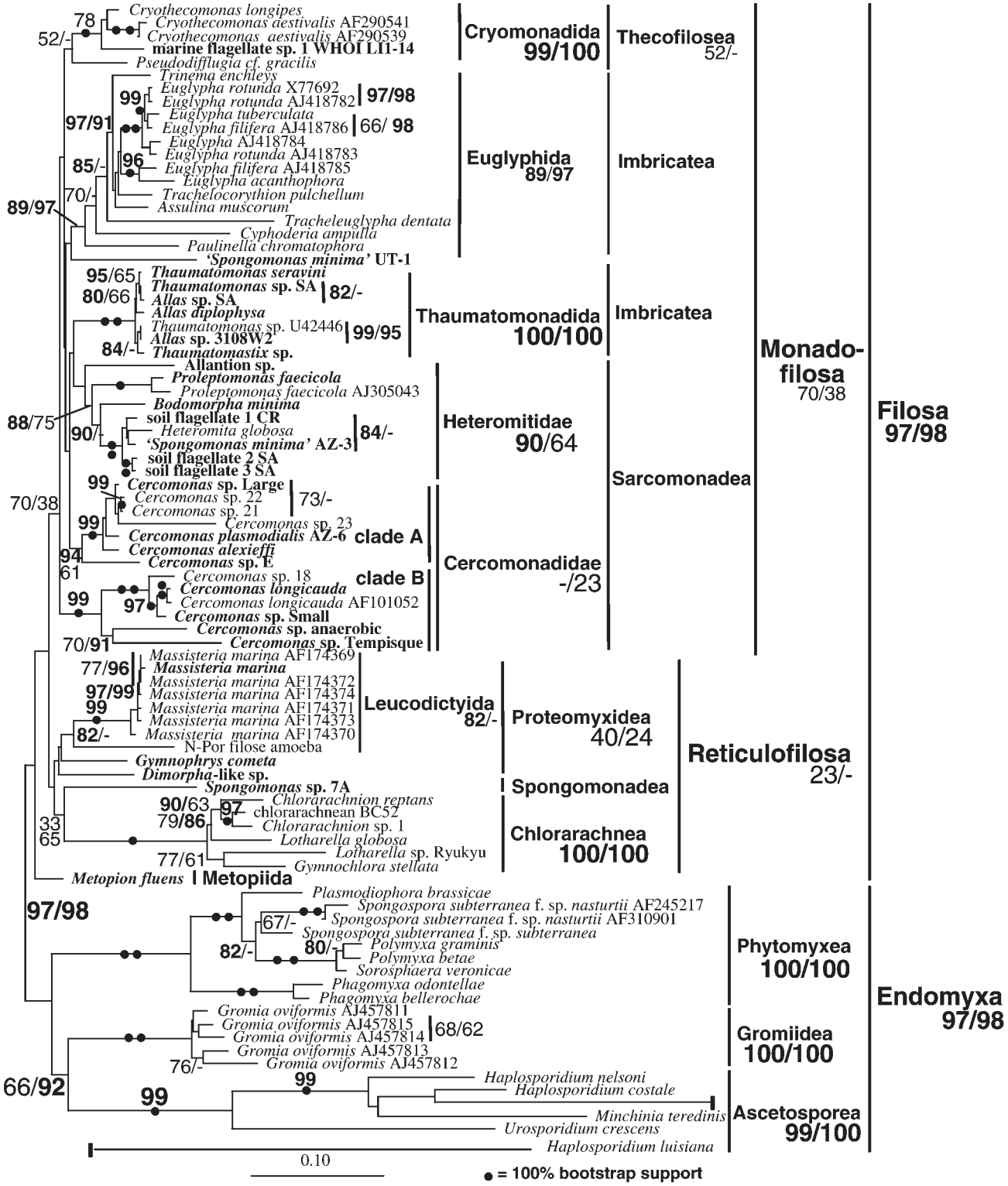


Figure 3. Maximum likelihood tree of 41 cercozoan 18S rRNAs using 1638 positions (Γ+I model: α = 0.52619; i = 0.250947). New sequences in bold. The figures are bootstrap percentages (bold if 80% or more) using the same model.

Figure 4. Distance tree of 86 cercozoan 18S rRNAs using 1638 positions (weighted least squares, power 2: GTR Γ+I model: α = 0.62018; i = 0.2739). The paramyxid *Marteilia* was omitted because of its excessively long-branch (three times as long as *Haplosporidium louisiana*, which had to be broken here to fit in): rRNA is grossly non clock-like (Cavalier-Smith 2002b). New sequences in bold. The figures are bootstrap percentages (bold if 80% or more; omitted, except for major clades, if both below 60%) – on the left (or above) for this model and on the right (or below) for the corresponding parsimony analysis. In the parsimony consensus tree UT-1 was sister to *Pseudodiffflugia* (Thecofilosea) but with only 39% support.


Cryothecomonas longipes
Cryothecomonas aestivalis AF290541
Cryothecomonas aestivalis AF290539
marine flagellate sp. 1 WHOI LI1-14
Pseudodifflugia cf. gracilis
Trinema enchleys
Euglypha rotunda X77692
Euglypha rotunda AJ418782
Euglypha tuberculata
Euglypha filifera AJ418786
Euglypha AJ418784
Euglypha rotunda AJ418783
Euglypha filifera AJ418785
Euglypha acanthophora
Trachelocorythion pulchellum
Assulina muscorum
Tracheleuglypha dentata
Cyphoderia ampulla
Paulinella chromatophora
'Spongomonas minima' UT-1
Thaumatomonas seravini
Thaumatomonas sp. SA
Allas sp. SA
Allas diplophysa
Thaumatomonas sp. U42446
Allas sp. 3108W2
Thaumatomastix sp.
Allantion sp.
Proleptomonas faecicola
Proleptomonas faecicola AJ305043
Bodomorpha minima
soil flagellate 1 CR
Heteromita globosa
'Spongomonas minima' AZ-3
soil flagellate 2 SA
soil flagellate 3 SA
Cercomonas sp. Large
Cercomonas sp. 22
Cercomonas sp. 21
Cercomonas sp. 23
Cercomonas plasmodialis AZ-6
Cercomonas alexieffi
Cercomonas sp. E
Cercomonas sp. 18
Cercomonas longicauda
Cercomonas longicauda AF101052
Cercomonas sp. Small
Cercomonas sp. anaerobic
Cercomonas sp. Tempisque
Massisteria marina AF174369
Massisteria marina
Massisteria marina AF174372
Massisteria marina AF174374
Massisteria marina AF174371
Massisteria marina AF174373
Massisteria marina AF174370
N-Por filose amoeba
Gymnophrys cometa
Dimorpha-like sp.
Spongomonas sp. 7A
Chlorarachnion reptans
chlorarachnean BC52
Chlorarachnion sp. 1
Lotharella globosa
Lotharella sp. Ryukyu
Gymnochlora stellata
Metopion fluens
Plasmodiophora brassicae
Spongospora subterranea f. sp. nasturtii AF245217
Spongospora subterranea f. sp. nasturtii AF310901
Spongospora subterranea f. sp. subterranea
Polymyxa graminis
Polymyxa betae
Sorosphaera veronicae
Phagomyxa odontellae
Phagomyxa bellerochae
Gromia oviformis AJ457811
Gromia oviformis AJ457815
Gromia oviformis AJ457814
Gromia oviformis AJ457813
Gromia oviformis AJ457812
Haplosporidium nelsoni
Haplosporidium costale
Minchinia teredinis
Urosporidium crescens
Haplosporidium luisiana
0.10
● = 100% bootstrap support
Cryomonadida 99/100
Euglyphida 89/97
Thaumatomonadida 100/100
Heteromitidae 90/64
Cercomonadidae -/23
clade A
clade B
Leucodictyida 82/-
Metopiida
Thecofilosea 52/-
Imbricatea
Imbricatea
Sarcomonadea
Proteomyxidea 40/24
Spongomonadea
Chlorarachnea 100/100
Phytomyxea 100/100
Gromiidea 100/100
Ascetosporea 99/100
Monado-filosa 70/38
Reticulofilosa 23/-
Endomyxa 97/98
Filosa 97/98

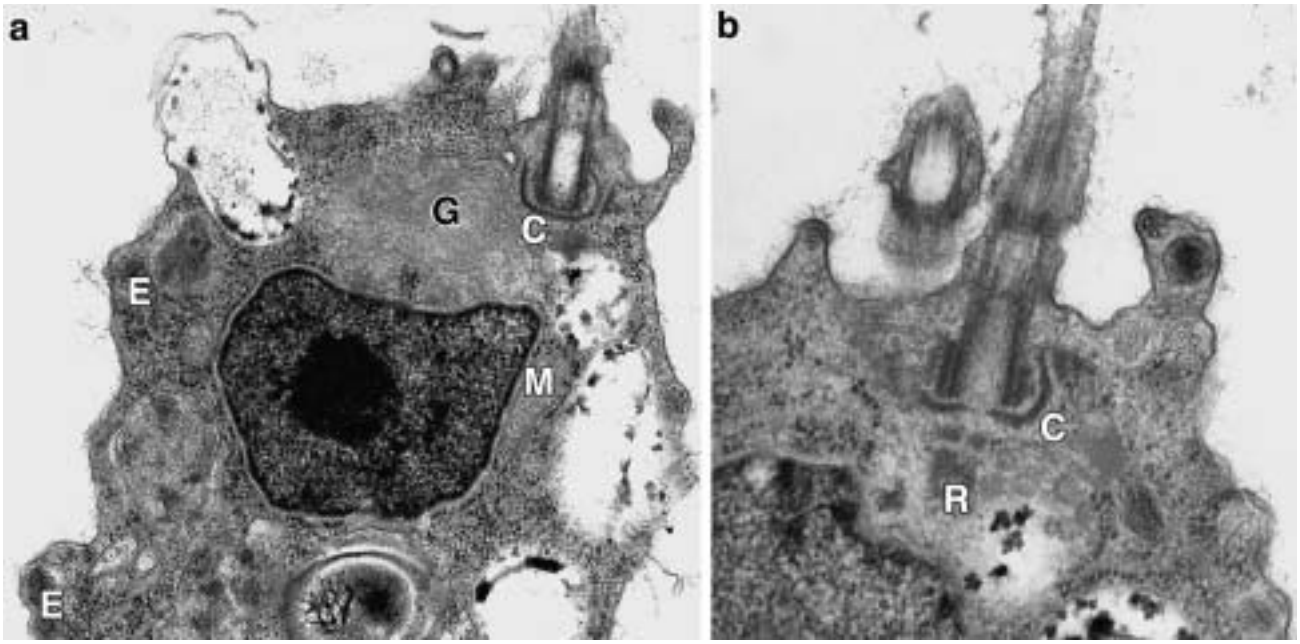


Figure 5. Electron micrographs of *Spongomonas* sp. 7A (by courtesy of Brian Oates). Extrusomes (E) are similar to those of *Chlorarachnion globosum* Ishida and Hara 1994 (Ishida 1996a Plate 6); C = centriolar cup: G = Golgi apparatus; M = microbody. **a.** 32,000×, **b.** 52,700×: this section shows only a grazing oblique view the centriolar end of the descending cross-striated root (R) that attaches the kinetid to the nucleus.

in both of which the distal half is thinner (acronematic) as in *Spongomonas uvella* (Hibberd 1976). Figure 5 shows that *Spongomonas* 7A, the individual cells of which closely resemble those of *Spongomonas uvella*, has the canonical spongomonadid ultrastructure, including a perforated pericentriolar cup, descending cross-striated root, and elongated ciliary transition region with a long zone distal to the transition plate that is devoid of central pair microtubules (Hibberd 1976, 1983). Light microscopy shows that UT-1 and A-Z flagellates are dissimilar, but neither has parallel cilia like Spongomonadida; probably both were misidentified as '*Spongomonas minima'*, which unlike them is reportedly non-ciliate (Tschermak-Woess 1950).

Monadofilosan Phylogeny

One very robust clade, comprising *Thaumatomonas*, *Thaumatomastix* and *Allas*, corresponds with the existing order Thaumatomonadida and shows for the first time that *Allas* belongs in it. The three *Allas* strains do not group together. Unlike in the other four monadofilosan clades, all thaumatomonad sequences are very closely related to each other, but with a long bare stem on the tree. ML and parsimony trees generally show thaumatomonads and euglyphids as sisters, with very weak support, this clade corresponding to the new class Imbricatea characterised by overlapping (imbricate) silica scales. However, distance trees often group thaumatomonads, equally weakly, with heteromitids.

One might expect from morphology that the two cercomonad clades should be sisters, but this is very rarely found (e.g. Fig. 2). The grouping of both with Heteromitidae on ML trees restricted to Cercozoa (Fig. 3), corresponding with the order Cercomonadida *sensu stricto*, is biologically reasonable but never has any stronger support than the topologies where either *Cercomonas* clade B (Figs 1, 4) or Heteromitidae (Fig. 2) branches as the most divergent monadofilosan clade and thaumatomonads are sisters to heteromitids (seen on most distance trees, e.g. Figs 1, 4). We suspect that the frequent deep branching of *Cercomonas* clade B on distance trees is a long-branch exclusion problem as it has substantially longer branches than clade A. The fact that clade B alone among Filosa has lost the ancestral base-pair substitution described above is consistent with its higher rate of rRNA evolution; Cercomonadida may therefore be holophyletic as indicated by some ML and parsimony trees (e.g. Fig. 3). Even if Cercomonadida are holophyletic, the apparent paraphyly of *Cercomonas* in Figure 3 might be

genuine and could be taken as evidence that Heteromitidae evolved from a *Cercomonas*-like ancestor. However, although most parsimony trees do not group clades A and B together, a few do, as do some ML trees (Fig. 2), so *Cercomonas* may be holophyletic. Protein sequence data are essential to decide between these possibilities and to give more robust evidence for the branching order of the filosan clades. However, in the absence of such evidence the ML tree of Figure 3 is morphologically reasonable. It weakly suggests that Imbricatea and Cercomonadida are sisters (as did Fig. 2) and that this joint clade is sister to Thecofilosea. The resulting superclade is apparently sister to *Metopion*, jointly corresponding to the superclass Monadofilosa, which includes all gliding cercozoan zooflagellates and all non-reticulose filose testate amoebae. However the position of *Metopion* must be regarded as less robust than other taxa as some N-terminal sequence was missing.

In contrast to thaumatomonads, which emit pseudopods from a ventral groove, and the cercomonads, which have strong pseudopodial tendencies, one sarcomonad clade comprises small monads that are not distinctly amoeboid. This clade includes *Heteromita globosa*, *Proleptomonas faecicola*, *Bodomorpha*, *Allantion*, and four unidentified nanoflagellate strains closely related to *Heteromita*. One of these strains (AZ-3) is named '*Spongomonas minima*' by ATCC, but is an anisokont biciliate entirely unlike *Spongomonas* but closely similar to *Heteromita*, and should probably be assigned to that genus; it does not resemble '*Spongomonas minima'* UT-1 microscopically and does not group with it on any trees (Figs 1, 4). A second strain 'Costa Rica soil flagellate 1′ was quite similar. Soil flagellates 2 and 3 were biciliate and uniciliate respectively, but their cultures died soon after DNA extraction and could not be further characterised. As the culture of strain 3 was regrettably not reexamined microscopically the day DNA was extracted, we cannot be totally sure that the amplified DNA was from the previously observed uniciliate and not a purely hypothetical minor biciliate contaminant overlooked when the culture was initially purified. However, at least one member (*Allantion*) of this predominantly biciliate sarcomonad clade, all here assigned to family Heteromitidae, is clearly uniciliate. Soil flagellate 2, a superficially *Metopion*-like biciliate gliding flagellate, was not examined at high magnification before it died, but was almost certainly not actually *Metopion fluens*. Our partial sequence from an authentic *Metopion fluens* never grouped within the heteromitids; its position varied a little with methods and taxon sampling; though always robustly within the Filosa, it was sister to all other sarcomonads in ML, parsimony and logDet distance trees and GTR NJ trees, but not in all GTR heuristic gamma-corrected trees.

A fourth robust sarcomonad clade comprises *Cryothecomonas* and small marine flagellate 1 (WHOI LI1-14). Electron microscopy indicates that this small flagellate lacks the thecal thickening of *Cryothecomonas*, but has similarities in its ciliary transitional region with both *Cryothecomonas* and spongomonads, notably a very dense peripheral ring at the same position in the ciliary transition region as the distal transverse plate and some central blob-like inclusions proximal to that position; it will be described in detail elsewhere and a new genus erected (TC-S and Oates in prep.). Its ciliary structure and rRNA sequence place it firmly in the order Cryomonadida (Cavalier-Smith 1993b) despite its temperate provenance. Our trees strongly confirm the finding by Wylezich et al. (2002) that *Pseudodifflugia*, which has an agglutinated test entirely unlike the euglyphid test of imbricate scales, is not directly related to euglyphids. *Pseudodifflugia* is weakly, but consistently grouped with cryomonads. A partial sequence (AJ130858) directly amplified from DNA extracted from continuously cultured lake water (van Hannen et al. 1999) branches so closely with *Pseudodifflugia* that it is almost certainly a closely related testate amoeba (not shown in the figures as its incompleteness might have distorted them). Another partial cercozoan sequence amplified from this DNA sample (van Hannen et al. 1999) was further away within the thecofilosan clade.

In ML trees (Figs 2 and 3) and parsimony trees '*Spongomonas minima'* UT-1 is weakly sister to *Pseudodifflugia*, but in distance trees it is often (even more weakly supported) a separate lineage near the base of Monadofilosa (e.g. Fig. 1) or sister to euglyphids (Fig. 4). Ultrastructurally, UT-1 lacks clear evidence of a pericentriolar cup characteristic of Spongomonadida and Pseudociliatida (*Stephanopogon*) (Cavalier-Smith and Oates in prep.). It has a very dense peripheral ring at the same position in the ciliary transition region as the distal transverse plate of *Rhipidodendron* (Hibberd 1976), *Cryothecomonas* (Thomsen et al. 1991) and marine flagellate 1, which is consistent with all three positions on the trees. We suspect that the ML and parsimony trees are more accurate and that it belongs to Thecofilosea; it lacks a theca, but has a few slender filopodia consistent with it being sister to *Pseudodifflugia*. The position of the cryomonad/*Pseudodifflugia* clade (Thecofilosea) is variable within the Monadofilosa (Figs 1–4); sometimes it is the most or most nearly divergent monadofilosan clade (Figs 1,

3), and sometimes it is sister to Euglyphida (parsimony tree with the Fig. 4 data set) or to Imbricatea (ML Fig. 2).

Discussion

The New Subphylum Filosa

A key conclusion from our present study and related ones on other groups (e.g. Cavalier-Smith and Chao 2003a) is that over half the zooflagellates of previously uncertain affinity belong in the rhizarian phylum Cercozoa, all within the new subphylum Filosa. Together with the evidence that all Ascetosporea and all studied filose testate amoebae are Cercozoa, this establishes the systematic importance of Cercozoa as one of the major protozoan phyla. All zooflagellates studied here group in either Monadofilosa or Reticulofilosa (Cavalier Smith 1996/7); although originally ranked as subphyla, our trees robustly show that these two taxa are much more closely related to each other than to the other three cercozoan clades. The shared signature sequence confirms this. We therefore have reduced their rank to superclass and grouped them together as the new subphylum Filosa (Table 1). Unlike previous usages, this taxon excludes Gromiidea (and Nucleariida) and includes numerous zooflagellates, not solely filose amoebae. However, many of the included zooflagellates have a marked propensity to make filopodia; this is true of all Proteomyxidea, Thaumatomonadida, *Cryothecomonas*, many Cercomonadida, Chlorarachnea, and UT-1; even *Metopion*, not obviously pseudopodial in the light microscope, has a very fine reticulopodial network visible ultrastructurally (Mylnikov et al. 1999). It therefore seems unnecessary to invent a new name for this major assemblage, which includes most rhizopods and flagellates that make filopodia as well as some (notably heteromitids and spongomonads) that do not.

The now-evident polyphyly of filose testate amoebae (Burki et al. 2002; Wylezich et al. 2002) makes it unjustifiable to maintain a class Filosea restricted to them and much better to group each separate clade with those zooflagellates to which it is most closely related. To effect this, we have established the new classes Imbricatea, with silica scales, to group thaumatomonad flagellates and euglyphid rhizopods, and Thecofilosea, with an organic test. As long suspected by most protistologists, the presence or absence of cilia is less fundamental than many other cellular properties, cilia having been lost many times independently.

Proteomyxid Structural Unity

Previously it was thought that *Massisteria*, a marine biciliate with branching filopodia that hug the substratum while feeding [as do the unbranched filiform projections of the *Dimorpha*-like strain], was related to the cercomonads (Patterson and Fenchel 1990), so it was placed within Sarcomonadea (Cavalier-Smith 1993a, b, 1996/7). A position among Cercozoa was confirmed by Atkins et al. (2000). The much greater cercozoan sampling of the present study, however, shows that *Massisteria* is more closely related to the marine N-Por filose amoeba (Bhattacharya and Oliveira 2000) than to Cercomonadida and that both taxa are related to the *Dimorpha*-like strain and *Gymnophrys*, although bootstrap support is weak. We have now transferred *Massisteria* to the Leucodictyida; sometimes *Massisteria* pseudopodia from different cells fuse (Patterson and Fenchel 1990) to make a temporary meroplasmodium like the much more extensive one that characterises Leucodictyidae (*Leucodictyon* and *Reticulamoeba*: Grell 1991; Grell 1994). Our trees are the first molecular evidence for a clade comprising leucodictyids, heliomonads, and reticulosids; all three share the morphology of extrusome-bearing filopodia or axopodia applied to the substratum while feeding, and which are partially or entirely supported by microtubules internally (Brugerolle and Mignot 1984a, b; Grell and Schüller 1991; Patterson and Fenchel 1990), unlike monadofilosan filopodia that are probably based just on acto-myosin.

We have applied the ancient name Proteomyxidea to this reticulofilosan clade, rather than the alternative possibility Athalamea (earlier used to embrace *Gymnophrys*: Cavalier-Smith 2000; Mikryukov and Mylnikov 1996) because the latter was recently (Lee et al. 2002) used for *Reticulomyxa*, a naked foraminiferan (phylum Retaria). The tubular cristae, simple ciliary transition region, extrusome ultrastructure and paranuclear microbody that suggested a link between *Massisteria* and Cercomonadida are likely to be ancestral characters for Filosa as a whole; such paranuclear microbodies are also found in *Rhipidodendron* (Hibberd 1976) and *Spongomonas* 7A (Fig. 5a), but apparently not *Spongomonas uvella* (Hibberd 1976). Although apparent absence of a paranuclear microbody from *Gymnophrys* was given as a reason for doubting that *Massisteria* and *Gymnophrys* were closely related (Patterson and Fenchel 1990) the probably related *Borkovia* has one (Mikrjukov 1998). The flat cristae of Reticulosida (Mikrjukov and Mylnikov 1995), in contrast to all other Cercozoa on our trees, which have tubular cristae (except for the anaerobic *Cercomonas* sp., which has dense mitochondria with

no cristae – Oates and Cavalier-Smith in prep., unlike typical *Cercomonas*: Mignot and Brugerolle 1975), indicate that flat cristae have evolved secondarily at least once within Cercozoa; but this novelty does not justify treating them as a separate class (Mikrjukov and Mylnikov 1998). It suffices to raise family Reticulosa (Cash 1905) in rank to order (Table 1). Neither Reticularia (a genus of myxogastrid Mycetozoa, i.e. Amoebozoa) nor Reticulosa (a group of hexactinellid sponges) are now appropriate names for reticulosids. We have initiated electron microscope studies to determine whether the *Dimorpha*-like strain is really a *Dimorpha* with microtubule-strengthened axopodia (Brugerolle 1984; Brugerolle and Mignot 1984a, b) or not. The filopodia of N-por (Bhattacharya and Oliveira 2000) are appressed to the substrate and granular (extrusomes?) like those of all Leucodictyida and it can reasonably be placed in that order. If the heliozoan-like Desmothoracida are actually Cercozoa, as tentatively suggested by Cavalier-Smith and Chao (2003b), their morphological characteristics (axopodia with cercozoan type extrusomes, tubular mitochondrial cristae, a biciliate stage able to form an amoeba) would clearly place them in this class close to the heliomonads.

Sarcomonad Phylogeny

Now that thaumatomonads and *Massisteria* have been removed from Sarcomonadea, the class is much more uniform, comprising only Cercomonadida and Metopiida. Its ancestral state was clearly that of biciliate zooflagellates that glide on their posterior cilium and had relatively simple extrusomes and ciliary transition region. Cercomonadida are the most abundant and widespread flagellates of soil (Sandon 1927). *Heteromita globosa* has been suggested as the commonest of all (Sandon 1927); our study reveals a cluster of five lineages closely related to it, suggesting that as for *Massisteria* there is considerable genetic diversity among morphologically similar strains. *Allantion*, another widespread gliding soil flagellate (Sandon 1927) that we are now studying ultrastructurally (Oates and Cavalier-Smith unpublished) turns out to be a heteromitid that lost its anterior cilium. Our trees clearly show that *Bodomorpha minima* is another heteromitid, genetically quite distinct from *Heteromita* itself; a relationship between *Bodomorpha* and *Heteromita* is supported by the ultrastructural similarities between *B. reniformis* (Mylnikov 1985) and *H. globosa* (Macdonald et al. 1977). Our *Proleptomonas faecicola* sequence groups tightly with that of Vickerman et al. (2002), but is significantly different. Some trees suggest that *Bodomorpha* and *Proleptomonas*, both rigid biciliates once confused with the fundamentally different bodonids, may be sisters. It is possible that other poorly characterised gliding flagellates widespread in soils or aquatic habitats also belong in the Heteromitidae.

As previously remarked (Cavalier-Smith and Chao 1996/7), the genus *Cercomonas* is phylogenetically very deep. Although many species have been described, it is hard to put names to most isolates – as Woodcock (1916) stressed long ago. However, the 12 often markedly different sequences show that their diversity is very great and that a more thorough study combining culturing, morphology and sequencing will be necessary to unravel their taxonomy. Unfortunately, we cannot yet decide whether *Cercomonas* are holophyletic or paraphyletic. The anaerobic *Cercomonas* species is of particular interest as the only anaerobe yet firmly assigned to the Cercozoa.

We have clearly established that *Allas diplophysa* is a thaumatomonad related to *Thaumatomonas* and *Thaumatomastix* (mutually very closely related). *Thaumatomonas* and *Thaumatomastix* both have silica scales (Beech and Moestrup 1986; Mylnikov and Karpov 1993). Nerad (pers. comm.) has shown that the ATCC *Allas diplophysa* also has silica scales and is ultrastructurally indistinguishable internally from *Thaumatomonas*. Our own thin section electron microscopic observations of *Allas* sp. 3108W2 confirm this (Cavalier-Smith and Oates in prep.). We doubt that the non-clustering of all three *Allas* strains is an artefact, as they occur in both the reasonably well-supported subclades. It is likely that they are independent derivatives of *Thaumatomonas* with independently shortened anterior cilium. Our sequence for *Thaumatomastix* provides the first molecular evidence that it is really very closely related to *Thaumatomonas*. It is striking how similar the rRNA sequences of the various thaumatomonad species and genera are compared with the highly divergent *Cercomonas* strains. This probably primarily reflects the much greater antiquity of the *Cercomonas* phenotype. It is not simply a consequence of the greater ease of finely dividing species morphologically when they have ultrastructurally complex scales subject to detailed variation, as in thaumatomonads, compared with a genus like *Cercomonas* where most isolates look very similar, and have probably been subdivided far too little. The euglyphids can be equally finely divided by scale characters, but apparently arose much earlier than these thaumatomonad lineages and underwent a much more temporally extended series of radiations, like *Cercomonas* and the heteromitids.

Our demonstration that a temperate-water flagellate (WHOI LI1-14) is closely related to *Cryothe-*

comonas suggests that the order Cryomonadida is not restricted to sea ice and may be widespread in the oceans. Our grouping of cryomonads with *Pseudodifflugia* as the class Thecofilosea, and the precise position of this clade need testing by protein sequencing. Mylnikov et al. (1999) suggested that *Metopion* might be related to *Cryothecomonas*. However, they do not group together on our trees and the theca of *Metopion* (Mylnikov et al. 1999) is far more delicate than in *Cryothecomonas* (Thomsen et al. 1991) and invests also the cilia, which do not protrude through pronounced ciliary collars, and a cytostomal slit is absent; the fact that WHOI LI1-14, which lacks a theca, is so strongly sister to *Cryothecomonas* also suggests that *Metopion* and *Cryothecomonas* thecae are convergent; moreover not all *Cryothecomonas* have extrusomes (Thomsen et al. 1991) so the presence of long ones in some of them as well as in *Metopion* may be convergent. Like other sarcomonads the ciliary transition region of *Metopion* is simple with only a single partition, not two as in cryomonads, and it lacks their extra transitional inclusions and helix (Thomsen et al. 1991). Although it remains unclear whether UT-1 really belongs in Thecofilosea, we suspect that most filose and reticulose rhizopod genera of uncertain affinities (Patterson et al. 2002b) will turn out to belong to Filosa, perhaps mostly to Thecofilosea or Proteomyxidea; as in Imbricatea (possible sister to Thecofilosea) thecofilosan filopodia lack extrusomes, which characterise filopodia of all Proteomyxidea on the tree. It will be interesting to see if this distinction withstands further taxon sampling.

Endomxyan Diversity

Although *Gromia* was previously shown to be a cercozoan (Burki et al. 2002) a relationship with Ascetosporea was not suspected, as the latter were not included in the earlier analysis. Even though the parasitic Ascetosporea are very different from the gromiids we do not think a separate subphylum would be justified for the gromiids, which may resemble the ancestral free-living phenotype for both Ascetosporea and Phytomyxea. The likely sisterhood of *Gromia* and Ascetosporea means that Ascetosporea and Phytomyxea probably evolved parasitism independently, which was not previously obvious (Cavalier-Smith 2002a; Cavalier-Smith and Chao 2003a).

Cercozoan Ancestry

It is evident that Testaceafilosia are polyphyletic, as previously argued (Meisterfeld 2002). At least three cercozoan groups have evolved tests independently, two of them (Euglyphida and Tectofilosida) also having lost cilia. It seems clear from our results that none of these filose testate amoebae are directly related to Foraminifera and likely that reticulopodia evolved independently in reticulosids and Retaria. It is unclear whether filopodia were present in the common ancestor of Cercozoa, as the placement of *Gromia* among the Endomyxa renders more likely, or evolved independently in the three testate groups, cercomonads, proteomyxids and *Chlorarachnion*. However molecular evidence for a sister relationship between Retaria and Cercozoa as the 'core Rhizaria' is steadily growing – both from our present and earlier rRNA trees (Cavalier-Smith and Chao 2003a) and from protein data (Archibald et al. 2003). Comparable protein sequence evidence from Radiolaria is needed to test the idea of core Rhizaria further.

According to the cabozoan theory (Cavalier-Smith 1999, 2003) core Rhizaria share a photosynthetic ancestry with excavates. Therefore, it is noteworthy that even though there are eight non-photosynthetic cercozoan classes, only four chloroplast losses would have had to occur within Cercozoa if Endomyxa, Monadofilosa and Reticulofilosa are all monophyletic, and if *Spongomonas* is sister to chlorarachneans, all of which many of our trees suggest (if *Spongomonas* were actually sister to Proteomyxidea instead, only three losses would be required). A fifth loss would be needed in the ancestral retarian, but five rhizarian plastid losses is less than can already reasonably be inferred in excavates. The better studied excavates already show evidence for at least six losses (Andersson and Roger 2002; Cavalier-Smith 2002c, 2003; Hannaert et al. 2003) of the at least seven predicted by the cabozoan theory; the precise number expected in excavates depends on their internal phylogeny, which is not fully established.

Methods

Cell cultures: Cultures were obtained from the American Type Culture Collection (ATCC), the Woods Hole Oceanographic Institution (WHOI), kindly donated by A. P. Mylnikov or isolated by us directly from nature into uniprotozoan culture. Table 2 lists their strain numbers, provenance, and sequence accession numbers.

Gene sequencing and phylogenetic analyses: DNA isolation, purification, 18S rRNA gene amplification by PCR, sequencing, editing and addition to

Table 2. Provenance and gene accession numbers of the 27 cercozoan strains sequenced.

Strain	Strain origin	Sequence accession number
Allantion sp.	ATCC 50734	AF411265
Allas diplophysa Sandon	ATCC 50365	AF411262
Allas sp. SA+	*South Africa garden soil, 7 Chippenham Road, Cape Town	AF411263
Allas sp. 3108W2+	*Canada garden compost, 3108 West 2nd Avenue, Vancouver, B.C.	AF411264
Bodomorpha minima Hollande	ATCC 50339	AF411276
Cercomonas alexieffi Lemmerman	ATCC 50395	AF411267
Cercomonas sp. E.+	Tom Nerad, ATCC	AF411269
Cercomonas longicauda Dujardin	ATCC 50344	AF411270
Cercomonas plasmodialis Mylnikov AZ-6	ATCC 50418	AF411268
Cercomonas sp. Large: strain SA-L	*South Africa garden soil, 7 Chippenham Road, Cape Town. ATCC PRA-21	AF411266
Cercomonas sp. Small: strain SA-S	*Fishhoek, Western Cape, South Africa. ATCC PRA-61	AF534712
Cercomonas sp. Tempisque+	*Costa Rica, Tempisque River	AF411271
Cercomonas sp. anaerobic	ATCC 50367	AF411272
Dimorpha-like sp.	ATCC 50522	AF411283
Gymnophrys cometa Cienkowsky	A. P. Mylnikov; now ATCC 50638	AF411284
Massisteria marina Larsen & Patterson	ATCC 50266	AF411286
marine flagellate 1+	WHOI LI1-14	AF411273
Metopion fluens Larsen & Patterson+	A. P. Mylnikov, Borok, Russia	AF411285
Proleptomonas faecicola Woodcock	ATCC 50735	AF411275
soil flagellate 1 CR+	*Costa Rica, San Jose flowerbed soil	AF411277
soil flagellate 2 SA-R+	*South Africa garden, 7 Chippenham Road, Cape Town	AF411279
soil flagellate 3 SA-M+	*South Africa garden, 7 Chippenham Road, Cape Town	AF411278
Spongomonas sp. 7A+**	*South Africa garden soil; 7 Chippenham Road, Cape Town	AF411282
AZ-3 NOT '*Spongomonas minima'*	ATCC 50404	AF411280
UT-1 '*Spongomonas minima'*	ATCC 50405	AF411281
Thaumatomonas seravini Mylnikov & Karpov	ATCC 50636	AF411259
Thaumatomonas sp. SA+	*South Africa garden soil; 7 Chippenham Road, Cape Town	AF411260
Thaumatomastix sp.	ATCC 50250	AF411261

*These cultures were isolated from nature by us by serial dilution into soil extract or cerophyll medium.
+ cultures later died and no longer available. **Note in proof: this sequence turns out not to be from strain 7A but from another non-*Spongomonas* cercozoan culture.

multiple alignments were as previously described (Cavalier-Smith and Chao 1995). The new sequences were aligned manually with our aligned database of over 450 diverse eukaryote sequences and a representative subset of 284 sequences including all protozoan phyla selected for preliminary analysis. The best aligned and most conserved 1638 alignment positions (files available on request) were selected for analysis using PAUP* v. 4.0b10 (Swofford 1999) on a Macintosh G4. Modeltest v. 3.06 (Posada and Crandall 1998) selected the 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 trees (ties broken randomly), for heuristic distance searches using both

the minimum evolution criterion and the least squares (power 2) methods for the best tree using TBR branch swapping and MULtrees, but no rapid descent. Initial trees were by random addition and 500–1000 jumbles done for heuristic trees. Invariant sites were removed in proportion to base frequencies estimated from all sites. For *Metopion* only a partial sequence was obtained as it would amplify only using an internal primer, not the usual one near the 5' end; the missing nucleotides were replaced by Ns prior to the analyses and each analysis was also run omitting *Metopion* to check that its presence did not distort the rest of the tree. The same was done for several incomplete cercozoan sequences available in Genbank from PCR studies of environmental DNA samples.

Preliminary neighbour joining trees using 284 taxa from all major eukaryote groups showed that all 27 new sequences grouped robustly within Cercozoa. More detailed analyses were carried out using as outgroups only the three other phyla of the infrakingdom Rhizaria to which Cercozoa belong (Cavalier-Smith 2002a) or subsets of them, i.e. Retaria, Heliozoa and Apusozoa. Because of their immensely long branches, sequences of the paramyxid *Marteilia* and longest branch haplosporidia were omitted from the present analyses: their firm branching among shorter branch haplosporidia included here was established previously (Cavalier-Smith and Chao 2003a). We also calculated maximum likelihood trees (GTR + Γ + I; parameters and substitution rate matrix calculated by modeltest; four gamma rate categories) with empirical base frequencies, logDet trees allowing for invariant sites (as given by modeltest), and unweighted parsimony trees using 100 or more random additions and unlimited TBR branch swapping. Bootstrap analysis used 1000 (distance and parsimony) or 100 (ML) 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 1h in 1% osmium tetroxide in the same buffer, dehydrated, embedded in Spurr's resin, and thin sections stained in uranyl acetate and lead citrate.

Acknowledgements

TCS thanks NSERC Canada and NERC UK for research grants; the Canadian Institute for Advanced Research and NERC for fellowship support. We thank T. Nerad for keeping some cultures for us while we were abroad and for unpublished information, A. P. Mylnikov and D. A. Caron for some cultures, B. Oates for electron microscopy, E. Harley and M. Berman for hospitality at the University of Cape Town, and L. Saldarriaga for help in Costa Rica.

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