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Gymnophrys cometa and *Lecythium* sp. are Core Cercozoa: Evolutionary Implications

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Summary. Recent phylogenetic analyses based on different molecular markers have revealed the existence of the Cercozoa, a group of protists including such morphologically diverse taxa as the cercomonad flagellates, the euglyphid testate filose amoebae, the chloroplast-bearing chlorarachniophytes, and the plasmodiophorid plant pathogens. Molecular data also indicate a close relationship between Cercozoa and Foraminifera (Granuloreticulosea). Little is known, however, about the origin of both groups and their phylogenetic relationships. Here we present the complete small-subunit ribosomal RNA (SSU rRNA) sequence of *Gymnophrys cometa*, formerly included in the athalamid Granuloreticulosea, as well as that of the test-bearing filose amoeba *Lecythium* sp. Our study shows that the two organisms clearly belong to the Cercozoa, and indicates that *Gymnophrys* is not closely related to Foraminifera, supporting the view that Granuloreticulosea *sensu lato* do not form a natural assemblage. Phylogenetic analyses including most available SSU rRNA sequences from Cercozoa suggest that a rigid, external cell envelope appeared several times independently during the evolution of the group. Furthermore, our results bring additional evidence for the wide morphological variety among Cercozoa, which now also include protists bearing granular pseudopodia and exhibiting mitochondria with flattened cristae.

Key words: Cercozoa, Granuloreticulosea, *Gymnophrys cometa*, *Lecythium* sp., molecular phylogeny, SSU rRNA.

Abbreviations used: ML - maximum likelihood, MP - maximum parsimony, NJ - neighbor joining, SSU rRNA - small-subunit ribosomal RNA.

INTRODUCTION

Gymnophrys cometa (Cienkowski, 1876) is a fresh-water protist, which due to its thin, grossly granular reticulopodia and absence of a test was placed by Cash (1905) in the family Reticulosa and later by De Saedeleer (1934) in the suborder Athalamia, order Granuloreticulosa (order Athalamida, class Granuloreticulosea *sensu* Bovee 1985b). The cells of *G. cometa* are solitary and move with the help of short lobose pseudopodia, which may be found in addition to the reticulopodia. Its complex life cycle includes an amoeboid stage, a cyst and a motile zoospore bearing two heterodynamic flagella lacking mastigonemes (Mikrjukov and Mylnikov 1996, 1997). Its mitochondria contain flat, plate- or ribbon-like cristae,

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and the reticulopodia contain a longitudinal bundle of 2-6 microtubules. Complex extrusive organelles (microtoxicysts) are present, which give their granular aspect to the reticulopodia. Two reduced flagella arise from a pair of conventional, almost parallel kinetosomes. Based on these particular features, Mikrjukov and Mylnikov (1997, 1998) proposed a new class Gymnophrea for *Gymnophrys* and the closely related genus *Borkovia*, but their position in the eukaryotic tree remains enigmatic. Cavalier-Smith (1998) tentatively placed *Gymnophrys* in the newly erected phylum Cercozoa (see below), whereas a recent catalogue of protists places *Gymnophrys* among heterotrophic flagellates of uncertain affinities (Patterson *et al*. 2000b), leaving open the question of its relation to Granuloreticulosea and other amoeboid protists.

Lecythium (Hertwig *et* Lesser, 1874) is a freshwater, filose amoeba which in culture can be found in groups of two to four cells. It possesses a flexible test and was placed by De Saedeleer (1934) in the suborder Testaceafilosa, order Filosa (order Gromiida, class Filosea *sensu* Bovee 1985a). Recent studies based on small-subunit ribosomal RNA (SSU rRNA) sequences revealed a close relationship between some members of the Testaceafilosa (i.e., the Euglyphida, *Pseudodifflugia* and *Gromia*) and a heterogeneous assemblage which includes the cercomonad flagellates, the chloroplast-bearing chlorarachniophytes, some marine nanoflagellates like *Cryothecomonas*, and the plasmodiophorid plant pathogens (Bhattacharya *et al*. 1995, Cavalier-Smith and Chao 1997, Atkins *et al*. 2000, Bhattacharya and Oliveira 2000, Kühn *et al*. 2000, Bulman *et al*. 2001, Burki *et al*. 2002, Vickerman *et al*. 2002). A new phylum Cercozoa was created to accommodate this assemblage (Cavalier-Smith 1998). This taxon is also supported by some protein-coding genes, including tubulin, actin and ubiquitin (Keeling *et al*. 1998, Keeling 2001, Archibald *et al*. 2003). Although the monophyly of Cercozoa is robust in most analyses, the internal relationships among cercozoan lineages are yet unclear. Notably, available data suggest the polyphyly of Testaceafilosa (Burki *et al*. 2002, Wylezich *et al*. 2002).

In order to examine the relationships between Cercozoa and other amoeboid protists, we sequenced the complete SSU rRNA gene of *G. cometa* and *Lecythium* sp. Our phylogenetic analyses clearly show that both genera belong to the Cercozoa, confirming the heterogeneous character of this group.

MATERIALS AND METHODS

Cell cultures and DNA extraction, amplification, cloning, and sequencing

The cultures of *G. cometa* and *Lecythium* sp. were taken from the culture collection of IBIW RAS (Russia). They were isolated from samples collected in waste treatment plants of Borok, Yaroslavskaya oblast, Russia. Both cultures were maintained on the artificial Pratt medium (KNO_3 0.1 ‰, K_2HPO_4 x $3H_2O$ 0.01 ‰, $MgSO_4$ x $7H_2O$ 0.01 ‰, $FeCl_3$ x $6H_2O$ 0.001 ‰; pH 6.5-7.5) with the addition of *Aerobacter aerogenes* as the source of food. DNA was extracted using the DNeasy Plant Minikit (Qiagen, Basel, Switzerland). The complete SSU rRNA gene of *G. cometa* and *Lecythium* sp. was amplified using the universal primers sA (5' ACCTGGTTGATCCTGCCAGT 3') and sB (5' TGATCCTTCTGCAGGTTCACCTAC 3'). PCR amplifications were done in a total volume of 50 µl with an amplification profile consisting of 40 cycles with 30 s. at 94 °C, 30 s. at 50 °C, and 2 min. at 72 °C, followed by 5 min. at 72 °C for the final extension. The amplified PCR products were purified using the High Pure PCR Purification Kit (Roche, Rotkreuz, Switzerland), then ligated into pGEM-T Vector System (Promega, Wallisellen, Switzerland), cloned in XL-2 Ultracompetent Cells (Stratagene, Basel, Switzerland), sequenced with the ABI-PRISM Big Dye Terminator Cycle Sequencing Kit, and analyzed with an ABI-377 DNA sequencer (Perkin-Elmer, Rotkreuz, Switzerland), all according to the manufacturer's instructions. The length of the amplified sequences of SSU rRNA of *G. cometa* and *Lecythium* sp. were 1814 and 1767 nucleotides, respectively.

Phylogenetic analyses

The complete SSU rRNA gene sequences from *G. cometa* and *Lecythium* sp. were manually aligned with sequences from diverse eukaryotes using the Genetic Data Environment software (Larsen *et al.* 1993), following the secondary structure model proposed by Neefs *et al*. (1993) and Wuyts *et al*. (2000). Preliminary analyses revealed the approximate phylogenetic position of both species (data not shown). An alignment of 43 sequences was constructed, including the two sequences obtained in this study, as well as 28 sequences from Cercozoa and 13 sequences from other eukaryotes. 1364 unambiguously aligned positions were used in the phylogenetic analyses, of which 748 were constant and 474 were parsimony informative. A second "Granuloreticulosea" dataset was designed, including sequences from *Reticulomyxa filosa* and 4 other Foraminifera, a sequence from the so-called granuloreticulosean *Diplophrys*, 8 sequences from Cercozoa and sixteen sequences from other eukaryotes. Because of the high divergence of foraminiferan SSU rRNA sequences (see, e.g., Pawlowski *et al*. 1996), only 1116 positions could be kept for phylogenetic analyses, of which 593 were constant and 404 were parsimony informative.

Phylogenetic trees were inferred using the neighbour-joining (NJ) method (Saitou and Nei 1987), the maximum parsimony (MP) method, and the maximum likelihood (ML) method (Felsenstein 1981). The reliability of internal branches was assessed using the bootstrap method (Felsenstein 1985) with 1000 replicates for NJ analyses,

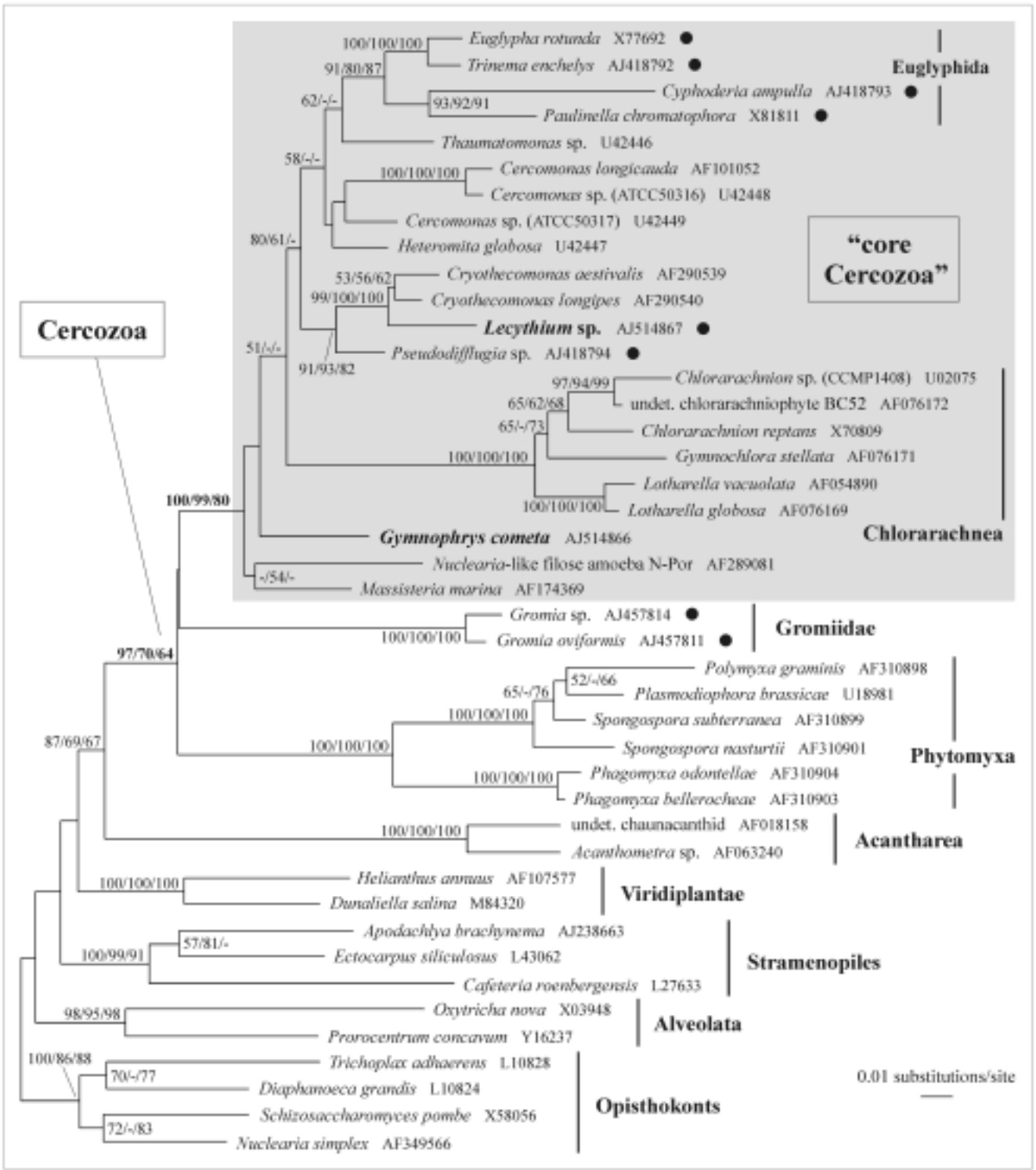

Fig. 1. Phylogenetic position of *Gymnophrys cometa* and *Lecythium* sp. among eukaryotes, inferred using the maximum likelihood method with the GTR + G + I model. Both organisms clearly belong to Cercozoa, and appear in a strongly supported clade called here "core Cercozoa" (grey box). Filose amoebae with a proteinaceous, agglutinated, or siliceous test are marked with a black circle. Numbers at nodes represent percentages of bootstrap support greater than 50% following 100 (ML), 1000 (NJ), or 500 (MP) data resamplings. All branches are drawn to scale. The tree was rooted using four sequences of opisthokonts, following a recent hypothesis on the position of the eukaryotic root (Stechmann and Cavalier-Smith 2002).

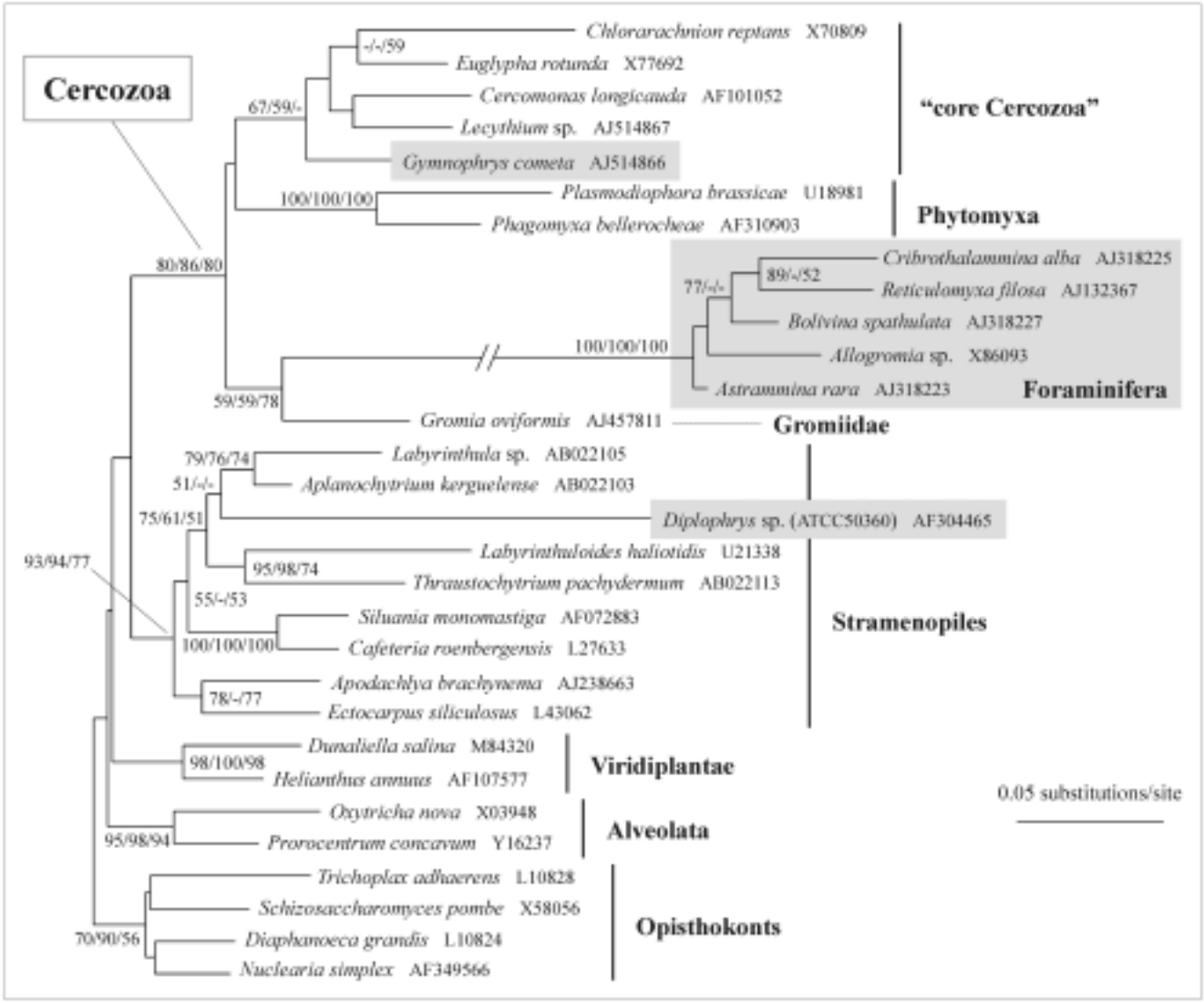

Fig. 2. Phylogenetic relationships among some eukaryotes inferred using the maximum likelihood method with the GTR + G + I model, emphasizing the polyphyly of the Granuloreticulosea *sensu lato* (grey boxes). The "athalamid" *Gymnophrys cometa* appears as a basal lineage among "core Cercozoa", while the "athalamid" *Reticulomyxa filosa* and the Foraminifera form an independent lineage branching with the marine, testate filosean *Gromia oviformis*, and the "monothalamid" *Diplophrys* sp. is closely related to the labyrinthulids and thraustochytrids (stramenopiles). Numbers at nodes represent percentages of bootstrap support greater than 50% following 100 (ML), 1000 (NJ), or 500 (MP) data resamplings. All branches are drawn to scale, except the stem branch leading to Foraminifera, which was reduced to a fourth of its actual size. The tree was rooted as in Fig. 1.

100 replicates for ML analyses, and 500 replicates for MP analyses. The PHYLO_WIN program (Galtier *et al*. 1996) was used for distance computations and NJ trees building and bootstrapping, using the HKY85 model of substitution (Hasegawa *et al*. 1985). MP and ML analyses were performed using PAUP* (Swofford 1998). The most parsimonious trees for each MP bootstrap replicate were determined using a heuristic search procedure with 20 random addition sequence replicates and tree bisection-reconnection branch-swapping. All characters were equally weighted and the transversion cost was set to twice the transition cost. ML analyses were performed using the GTR model of evolution (Lanave *et al*. 1984, Rodriguez *et al*. 1990), taking into account a proportion of invariables sites, and a gamma distribution of the rates of substitution for the variable positions, with 8 rate categories (GTR+G+I). All parameters were estimated from the dataset using Modeltest (Posada and Crandall 1998). Starting trees were obtained via 20 random addition sequence

replicates, and then swapped using the tree bisection-reconnection algorithm. In order to reduce computational time, starting trees for the ML bootstrap analysis were obtained via NJ.

RESULTS

Morphology

The morphological features of *Lecythium* sp. are characteristic for this genus. The cells are ovoid, 8 to 10 µm in length and 5 to 8 µm in width. The cell body is rigid and a flexible test is tightly adjacent to the cell surface. Flattened or narrow, needle-shaped, branched pseudopodia arise from a portion of cytoplasm extruding from the lower part of the cell, which are motile and drive the cell along the substrate. The nucleus is in the center of the cell, the contractile vacuole is lateral. Floating specimens, cysts, plasmodia, or zoospores have not been observed. *Lecythium* sp. multiplies by binary fission, and sometimes forms aggregations of three cells. The isolate we studied closely ressembles the type species, *Lecythium hyalinum* (Hertwig *et* Lesser, 1874), as illustrated by Cash and Wailes (1915; plates 49 and 51). However, the determination could not be ascertained, so we named it *Lecythium* sp., while further examination of the isolate is in progress (Mylnikov, in preparation).

The cultured strain of *G. cometa* used in this study is the same that was examined in two previous ultrastructural works (Mikrjukov and Mylnikov 1997, 1998), which provided the detailed description of the species.

Phylogenetic analyses

Analysis of our data shows that both *G. cometa* and *Lecythium* sp. clearly belong to Cercozoa, and the clade is supported by high bootstrap values (Fig. 1). Inside Cercozoa, *Gromia* and the Phytomyxea form two early diverging lineages with all methods of tree reconstruction. *G. cometa* and *Lecythium* sp. branch within a strongly supported clustering of the rest of the cercozoan taxa, called here "core Cercozoa", and which consists of the cercomonads, the chlorarachniophytes, some nanoflagellates, and some filose testate and naked amoebae. The branching order within "core Cercozoa" is not well resolved. Only three strongly supported monophyletic groups can be distinguished with all methods of tree reconstruction: the Chlorarachnea, the Euglyphida, and a group including *Lecythium* sp., *Pseudodifflugia* sp., and the nanoflagellate *Cryothecomonas*. *G. cometa* occupies a relatively basal, independent position, branching immediately after the divergence of the cercomonad *Massisteria marina* and the *Nuclearia*-like filose amoeba "N-Por", as a sister group to all other "core Cercozoa" species. NJ and MP trees are broadly congruent with the ML tree shown in Fig. 1, the main differences lying in the branching order between the most basal members of the "core Cercozoa": in the NJ tree, the Chlorarachnea are the first diverging lineage, followed by a badly supported group consisting of *G. cometa*, *M. marina* and "N-Por", whereas in the MP tree, "N-Por" is the first diverging species, followed by *M. marina*, then *G. cometa* (data not shown).

In order to test the relationships between members of the Granuloreticulosea for which SSU rRNA sequences are available, a second dataset was analysed, including *G. cometa*, *Diplophrys* sp. and five species of Foraminifera. Analysis of this dataset shows that the Granuloreticulosea *sensu lato* do not form a natural assemblage (Fig. 2). *G. cometa* branches among "core Cercozoa" with all methods of tree reconstruction, as indicated by the analysis of the larger dataset (see Fig. 1), although the bootstrap support for this clade is only moderate with a reduced number of sites. The Foraminifera branch as a sister group to the marine, testate, filose amoeba *Gromia*, whereas *Diplophrys* sp. branches among stramenopiles as a sister-group to *Labyrinthula* sp. and *Aplanochytrium kerguelense* (Fig. 2), a result confirmed by NJ and MP analyses (data not shown).

DISCUSSION

When the phylum Cercozoa was erected (Cavalier-Smith 1998), *Gymnophrys* was tentatively placed in it, and this position was later supported by a distance tree including unpublished sequences (Cavalier-Smith 2000). As a member of the Testaceafilosa, *Lecythium* could also be argued to be a member of this group. Our results allow us to refine the position of both organisms. By placing *Gymnophrys* and *Lecythium* within Cercozoa, we confirm the wide range of morphological and ultrastructural characters of this phylum (Cavalier-Smith 2000). Although the monophyly of Cercozoa is strongly supported by molecular data, no satisfying morphological definition exists for the group yet. Our study shows that such characters as the presence of a test, the form of

mitochondrial cristae or the structure of pseudopodia are poor markers for the cercozoan phylogeny.

First, our phylogenetic analyses including most available sequences from Cercozoa support the idea that a rigid, external cell envelope appeared or was lost several times independently during the evolution of the phylum. The Cercozoa include all testate, filose amoebae for which molecular data exist yet, i.e. *Gromia*, *Lecythium*, *Pseudodifflugia*, and the Euglyphida. These organisms were conveniently grouped together in the Testaceafilosa by De Saedeleer (1934), a classification scheme followed by some other authors (see, e.g., Bovee 1985a). Previous molecular data showed that the monophyletic Euglyphida (with secreted, siliceous scales) group within Cercozoa (Wylezich *et al*. 2002), but suggested the polyphyly of the Testaceafilosa as a whole, with *Gromia* and *Pseudodifflugia* appearing as independent lineages within the phylum (Burki *et al*. 2002, Wylezich *et al*. 2002). Our results are congruent with this view, and confirm that the testate, filose amoebae lacking siliceous scales (the Gromiina *sensu* Bovee 1985a) do not form a natural group. This is in agreement with the great ultrastructural differences between the tests of these organisms (see, e.g., Meisterfeld 2000), suggesting that they are not homologous features. SSU rRNA data also point out at *Cryothecomonas* to be closely related to *Lecythium* (Fig. 1). Both genera have an external covering and produce pseudopodia. In *Cryothecomonas*, however, the pseudopodia are small and produced only for the capture of prey (Thomsen *et al*. 1991), whereas the pseudopodia in *Lecythium* are bigger and present constantly, and are also used for locomotion. Besides, the covering in *Cryothecomonas* resembles an envelope outside the cell, and cannot be considered as a true test (Thomsen *et al*. 1991). Further ultrastructural studies of these two genera will be necessary to confirm their possible relationship.

By including *G. cometa* among "core Cercozoa", we also show that the shape of mitochondrial cristae cannot be used as a criterion to include or exclude a species into/from the Cercozoa. The mitochondrial cristae of *G. cometa* are flattened, whereas all other known representatives of the "core Cercozoa" have tubular cristae (Patterson 1999). At the level of the phylum Cercozoa, the only possible other exceptions are the Phytomyxa, because the plasmodiophorid plant pathogens display mitochondrial cristae of ambiguous shape, appearing either flat or sacculate (Patterson 1999). The shape of cristae in mitochondria is traditionally considered as one of the most important ultrastructural features for general protist phylogeny (Cavalier-Smith 1997; Karpov 2000; Taylor 1976, 1999), and there are only few examples of monophyletic groups, which include both tubulocristate and lamelocristate taxa (Patterson 1999). Our results indicate that the "core Cercozoa" are one of such groups.

Finally, our results show that the Cercozoa not only include protists with filose pseudopodia, but also species with granular reticulopodia, such as *Gymnophrys* (Fig. 1). Bovee (1985a) conveniently grouped all organisms presenting fine, more or less granular pseudopodia that can form more or less complex anastomosing networks in the class Granuloreticulosea, which is divided in three groups, the naked Athalamida and the testate Monothalamida and Foraminifera. Although the monophyly of Foraminifera is strongly supported by both morphological and molecular data (see, e.g., Bock *et al*. 1985, Pawlowski 2000), it is generally accepted that Athalamida and Monothalamida are heterogeneous and need taxonomic revision (see, e.g., Lee *et al*. 2000). The only previously sequenced athalamid, *Reticulomyxa filosa*, was shown to be a naked, freshwater foraminiferan (Pawlowski *et al*. 1999), confirming the profound similarities in structure and motility between the pseudopodia of *R. filosa* and foraminifers. However, our data show that this is not the case for all athalamids. Foraminifera (including *R. filosa*) are related to Cercozoa on the basis of actin (Keeling 2001), ubiquitin (Archibald *et al*. 2003) and revised SSU rRNA analysis (Berney and Pawlowski, in press), but the position of *G. cometa* within "core Cercozoa" in SSU rRNA trees is clearly distant from the position of Foraminifera, which branch with *Gromia* among the early diverging cercozoan lineages (Berney and Pawlowski, in press; see Fig. 2). This is in agreement with morphological studies that clearly distinguish the branching, rarely anastomosing filopodia of *G. cometa*, whose granular aspect is due to the presence of numerous extrusomes (Mikrjukov and Mylnikov 1998), from the "true" granuloreticulopodia of Foraminifera, which exhibit a typical bidirectional streaming of particles, are not reinforced with geometrically arrayed microtubules, and form complex anastomosing networks (Lee *et al*. 2000). The polyphyletic nature of the Granuloreticulosea *sensu* Bovee (1985a) is also confirmed by available data on another granuloreticulosean, the monothalamid *Diplophrys* sp., which is related to labyrinthulids and thraustochytrids (stramenopiles) in SSU rRNA analyses (Fig. 2), in agreement with ultrastructural studies (Patterson 1989, Patterson *et al*. 2000a). Our results support the need for

a redefinition of the Granuloreticulosea, which might ultimately be reduced to the Foraminifera alone.

According to SSU rRNA sequences, *G. cometa* has a relatively basal position within the "core Cercozoa", and appears not to be closely related to any other known member of the phylum. This is in agreement with the ultrastructural peculiarities of this genus, which prompted Mikrjukov and Mylnikov (1997, 1998) to place *Gymnophrys* in a new class Gymnophrea. Interestingly, two of the most basal "core Cercozoa", *G. cometa* and *M. marina* share some important morphological features (Patterson and Fenchel 1990, Mikrjukov and Mylnikov 1998). Both organisms are sessile and possess thin and branching filopodia, with internal bundles of microtubules, which can form chain aggregations in culture. Characteristic concentric extrusomes of similar structure, termed kinetocysts (Mylnikov 1988) are located in the filopodia and next to the body surface (Patterson and Fenchel 1990). *G. cometa* and *M. marina* have two smooth heterodynamic flagella, and an amoeboid outline of the rear part of the cell. Given the absence of bootstrap support for the branching order among basal "core Cercozoa", it is plausible that *G. cometa*, *M. marina* and possibly "N-Por" might form a monophyletic lineage. Indeed, the likelihood of the best tree where this lineage was constrained was not significantly inferior to the likelihood of the tree shown in Fig. 1 (data not shown). Alternatively, if the topology shown in Fig. 1 is correct, then the similarities between *G. cometa* and *M. marina* suggest that an amoeboid, but flagellated state might be ancestral for the "core Cercozoa". Subsequent reduction of the flagella or loss of the capacity to produce filopodia in some lineages might account for the chaotic distribution of these features along the cercozoan tree. This may explain the difficulty of finding a satisfying morphological definition for the Cercozoa, from which highly specialized lineages such as the Foraminifera might be derived. Additional protein data will be needed to test further the relationships among Cercozoa and closely related amoeboid protists.

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