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Phylogenetic Position of *Gromia oviformis* Dujardin inferred from Nuclear-Encoded Small Subunit Ribosomal DNA

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***Gromia oviformis* Dujardin is a common marine protist characterised by a large, globular test and filose pseudopodia. First considered a foraminifer, *Gromia* was later placed within the Filosea and recently included among amoebae of uncertain affinities. In order to clarify the phylogenetic position of this genus, we sequenced the complete small-subunit ribosomal DNA gene of *G. oviformis* collected at five different geographic localities. The high divergence of obtained sequences suggests that *G. oviformis* is a species complex composed of several genetically distinct sibling species. Sequence analyses show *Gromia* to be a member of the Cercozoa, a heterogeneous assemblage which includes filose amoebae, the amoeboflagellate cercozoans, the chlorarachniophytes and the plasmodiophorid plant pathogens. Contrary to traditional classification, *Gromia* is not closely related to other testate filose amoebae (the Euglyphida), but seems to branch early among the Cercozoa. Our analyses also show a close relationship between the Cercozoa and the Acantharea. Because the Cercozoa are related to the Foraminifera based on other molecular data, we propose that most protists possessing filopodia, reticulopodia and axopodia have a common origin.**

Introduction

Gromia oviformis Dujardin is a common marine protist characterised by a large proteinaceous test, filose pseudopodia and a complex life cycle. Its spherical to ovoid test is rigid or slightly flexible (Loeblich and Tappan 1964), with a light brownish tinge. The test wall is radially perforated (Hedley and Bertaud 1962). On its inner surface lies a layer of multiple 'honeycomb membranes', a structure unique to this genus (Hedley and Wakefield 1969).

The test is modified at one point to form a distinctive oral capsule through which the cytoplasm extrudes in the form of transparent, non-granular, filose pseudopodia (Hedley and Bertaud 1962). The size of *G. oviformis* ranges from 0.1 to 5 mm, but may reach up to 38 mm in some other deep-sea species (Gooday et al. 2000).

G. oviformis is a cosmopolitan species, widely distributed in different marine habitats from polar shelves to tropical coral reefs. It is abundant in the intertidal zone of rocky shores, where it can be found in tufts of algae at the base of seaweeds (Arnold 1972), or in association with tunicates (Lwoff 1925) or sponges (Arnold 1951). A large population

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of *G. oviformis* has also been reported from the Antarctic shelf (Gooday et al. 1996). Another species of this genus, *G. sphaerica*, has been described from deep-sea sediment samples (Gooday et al. 2000). This latter species is considered to be an important scavenger, actively participating in the degradation of organic matter near the sediment-water interface, as evidenced by the presence of detritus and stercomata within its cytoplasm (Gooday et al. 2000).

In spite of the abundance and cosmopolitan distribution of *Gromia*, species diversity within the genus is poorly understood. Because of the paucity of morphological characters, only a few species have been described, with most of the descriptions dating back to the 19th century. The literature reports eleven different species of *Gromia*. Six of them (*G. fluvialis* Dujardin 1841, *G. terricola* Leidy 1879, *G. brunnerii* Blanc 1888, *G. nigricans* Penard 1890, *G. gemma* Penard 1899 and *G. squamosa* Penard 1899) are freshwater species. Some of them are considered foraminiferans according to the characteristics of their pseudopodia (reviewed in Holzmann and Pawlowski, in press). Among the five marine species, two (*G. dujardinii* Schultze 1854 and *G. ovoidea* Rhumbler 1904) are considered junior synonyms of *G. oviformis* Dujardin 1835 (Hedley 1958; Jepps 1926). Therefore, to our knowledge, there are only three valid marine *Gromia* species: *G. oviformis* Dujardin 1835, *G. dubia* Gruber 1884 and *G. sphaerica* Gooday et al. 2000.

The evolutionary origins and phylogenetic relationships of *Gromia* remain unclear. Until now, the systematic position of this genus was exclusively based on morphological and ultrastructural features. First considered a foraminiferan, *Gromia* was later placed with the Euglyphida in the subclass Testaceafilosia (de Saedeleer 1934) or in the order Gromiida within the class Filosea (Bovee 1985). Traditionally, the Filosea comprise two groups: the naked Aconchulinia and the testate Testaceafilosia (or Gromiida). Recent molecular studies, however, demonstrated that some members of the Aconchulinia (the nucleariid naked filose amoebae) branch within the Opisthokonta (Amaral Zettler et al. 2001), suggesting that the Filosea are polyphyletic. Molecular data also show a close relationship between some Testaceafilosia (*Euglypha rotunda* and *Paulinella chromatophora*) and a heterogeneous assemblage which includes the amoeboflagellate cercomonads, the chlorarachniophytes (green amoeboflagellate algae), some marine nanoflagellates, and the plasmodiophorid plant pathogens (Atkins et al. 2000; Bhattacharya and Oliveira 2000; Bhattacharya et al. 1995; Bulman et al. 2001; Cavalier-

Smith 1998, 1999; Cavalier-Smith and Chao 1997; Kühn et al. 2000; Vickerman et al. 2002). A new phylum Cercozoa was created to accommodate this assemblage (Cavalier-Smith 1998). This phylum is presently defined only on a molecular level, but it is well supported by several genes (Cavalier-Smith 2000; Keeling 2001; Keeling et al. 1998). Because of the lack of molecular data and a small number of shared morphological characters, *Gromia* was placed in an assemblage of amoebae of uncertain affinities in a recent classification (Patterson et al. 2000).

Until now, the only available molecular data for *Gromia* was a partial sequence of the gene coding for the large subunit of ribosomal RNA (Pawlowski et al. 1994). The analysis of this sequence showed that *Gromia* branches within the so-called eukaryotic crown. Its exact phylogenetic position, however, could not be established because of the lack of LSU rDNA gene sequences for various eukaryotic groups. In this study, we sequenced the complete small-subunit ribosomal DNA (SSU rDNA) of *Gromia* from five localities. Phylogenetic analyses allow us to establish the position of *Gromia* among other eukaryotes, as well as to understand the relationships between the Filosea, the Cercozoa and other protists.

Results

The specimens that were examined possessed a large spherical or ovoid test with a distinctive aperture typical for *G. oviformis* (Fig. 1A,B). The size of individuals from Reunion, Tunisia and Madeira reached up to 1 mm, whereas those collected in McMurdo Sound (Antarctica) measured up to 3 mm in diameter. Only the individuals from Guam (called here *Gromia* sp.) were slightly different, having much smaller (about 0.2 mm) pyriform tests (Fig. 1C). The filopodia protruding from the oral region have been observed in the specimens maintained in culture, but none of them were found surrounding the test of *Gromia* as is often the case in the Foraminifera.

The length of the SSU rDNA genes ranged from 1877 (Madeira), 1884 (Reunion) and 1891 (Antarctica) nucleotides, to 1957 and 1963 nucleotides in specimens from Tunisia and Guam, respectively. This difference was due to a size variation in the V4 domain of the secondary structure model (Neefs et al. 1993). The observed dissimilarity between the SSU sequences, compared two by two, ranged from 1.58% (for specimens from Madeira and Tunisia) to 4.04% (for specimens from Antarctica and Reunion). The sequence divergence observed

between specimens from the same locality was inferior to 1%.

Two data sets were analysed. The first one contained 38 SSU rDNA sequences, including two of *G. oviformis* (from Tunisia and Madeira) and 36 from diverse eukaryotes. The second data set contained the five sequences of *Gromia* obtained in this study, as well as 23 sequences of Cercozoa available in the database and four sequences of other eukaryotes. The number of unambiguously aligned positions used in the analyses was 1259 for the first data set and 1432 for the second data set. A Chi-square test performed on both data sets demonstrates a high homogeneity of base frequencies across taxa ($\chi^2 = 47.69$, $p = 1.00$ for the first data set; $\chi^2 = 23.79$, $p = 1.00$ for the second data set).

Analysis of the first data set shows the phylogenetic position of *Gromia* among eukaryotes (Fig. 2). The tree only contains eukaryotes belonging to the so-called crown in order to avoid long-branch attraction artefacts. Because the relationships between different crown lineages are not well established the tree is presented in an unrooted form. In all analyses, *Gromia* branches as one of three distinct lineages that collectively define the Cercozoa.

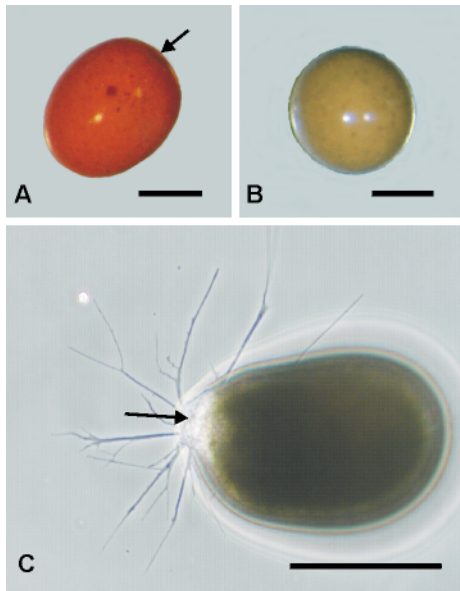


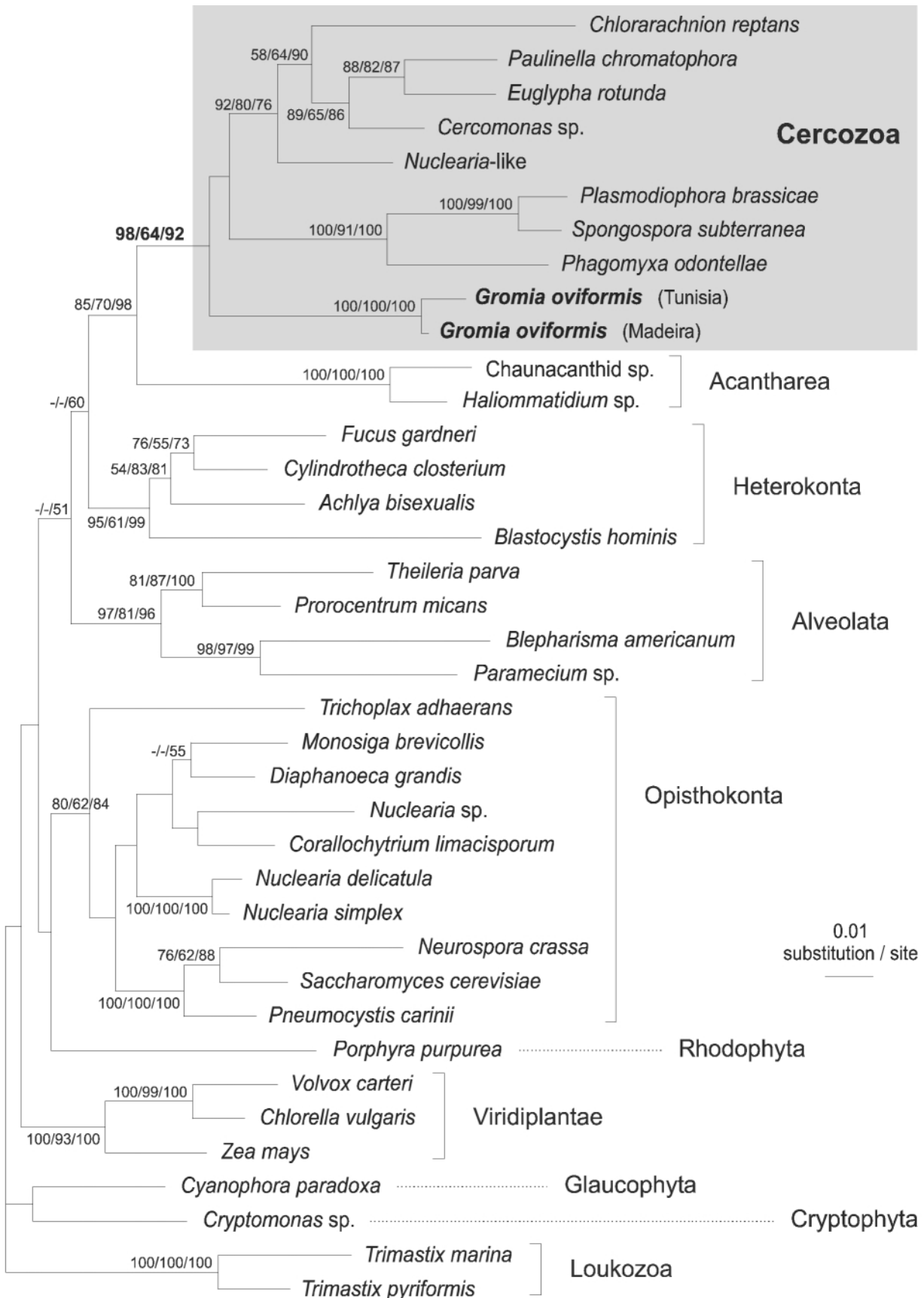
Figure 1. Photomicrographs of *Gromia oviformis* from different localities. **A.** Specimen from McMurdo Sound (Antarctica) showing the circular aperture (arrow), scale bar = 1 mm. **B.** Specimen from Madeira (Atlantic Ocean), scale bar = 0.5 mm. **C.** Specimen from Guam (Western Pacific) showing the network of filose pseudopodia extruding from the oral region (arrow), scale bar = 0.1 mm.

The monophyly of the group has good bootstrap support (98%, 92% and 64% for ML, NJ and MP analyses, respectively). The Filosea (sensu Bovee 1985) appear as a polyphyletic assemblage, with the nucleariids being placed within Opisthokonta. Interestingly, all methods of tree reconstruction placed the Acantharea as the sister-group to the Cercozoa, with robust bootstrap support (85%, 98%, 70%, for ML, NJ and MP analyses, respectively).

The second data set allows a more detailed analysis of the relationships among the Cercozoa (Fig. 3). In this analysis, one alveolate sequence (*Prorocentrum micans*) and one heterokont sequence (*Fucus gardneri*) were used as the outgroup. Within the Cercozoa, *Gromia* forms the sister-group to the Phytomyxea (sensu Cavalier-Smith 1996/97), but this relationship is not well supported (Fig. 3). The remaining cercozoan sequences form a heterogeneous cluster including the Sarcomonadea, the Chlorarachnea and some other Filosea. Within this cluster, the chlorarachniophytes form a monophyletic lineage, but sarcomonads appear as a paraphyletic group and the filose amoebae are divided in two independent groups – one containing the well-defined monophyletic Euglyphida and the other containing the *Nuclearia*-like filose amoeba (Bhattacharya and Oliveira 2000), related to the heterotrophic flagellate *Massisteria marina*. These two species are strongly related in the NJ analysis (93% bootstrap); however, the bootstrap support for this group is low (under 50%) in MP and ML analyses and its position varies depending to type of analysis.

Discussion

This is the first report of SSU rDNA sequences of one of the most common marine protists, *G. oviformis*. Comparison of *G. oviformis* sequences clearly shows that this species is genetically much more variable than suggested by its simple morphology. All specimens examined here have typical morphological characteristics of *G. oviformis*. However, each of them is characterised by a different sequence. Sequence divergence among the five *Gromia* is comparable to the divergence observed between *Spongospora subterranea* and *Polymyxa graminis*, two genera of plasmodiophorids or to the divergence between different species of the genus *Chlorarachnion*. This suggests that *G. oviformis* represents a species complex composed of several sibling species. Determination of these species requires more detailed morphological, cytological and genetic analyses (study in progress). But even based on the few sequences obtained here, we pre-



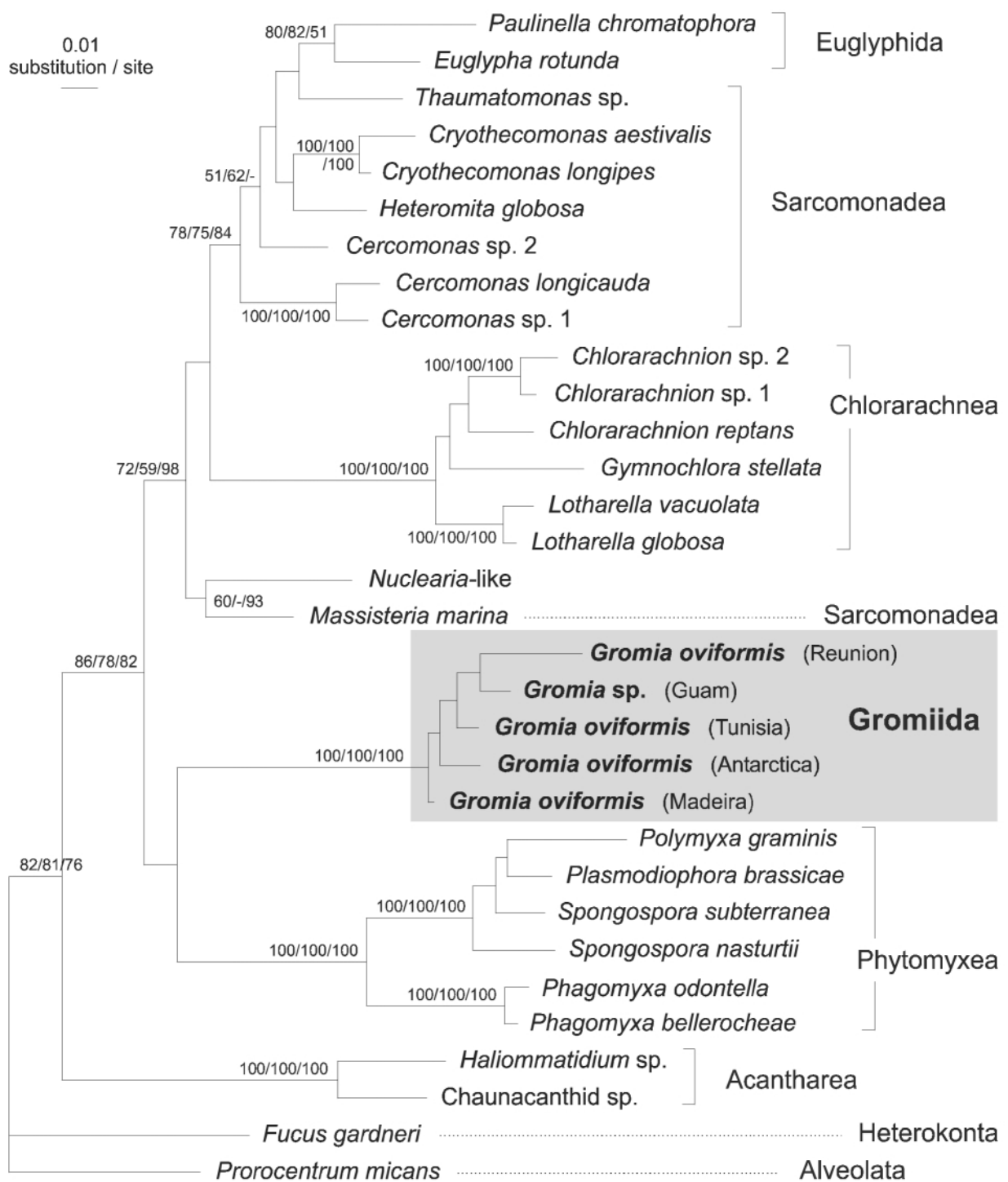


Figure 3. Phylogenetic relationships among the Cercozoa inferred using the maximum likelihood method with the HKY85 model. Numbers at nodes represent percentages of bootstrap support greater than 50% following 200 (ML), 300 (MP) or 1000 (NJ) data resampling. The scale bar represents a distance of 0.01 substitutions per site.

Figure 2. Phylogenetic position of *Gromia oviformis* among eukaryotes inferred using the maximum likelihood method with the HKY85 model. Numbers at nodes represent percentages of bootstrap support greater than 50% following 100 (ML), 200 (MP) or 1000 (NJ) data resampling. The scale bar represents a distance of 0.01 substitutions per site.

Table 1. List of the SSU rDNA sequences analyzed in this study.

Species Name	Taxonomic Position	Accession Numbers	Species Name	Taxonomic Position	Accession Numbers
<i>Gromia oviformis</i> (Madeira)^a	Cercozoa	AJ457811	chaucanthid sp.	Acantharea	AF018158
<i>Gromia oviformis</i> (Reunion)	Cercozoa	AJ457812	<i>Haliommatidium</i> sp.	Acantharea	AF018159
<i>Gromia oviformis</i> (Antarctica)	Cercozoa	AJ457813	<i>Blastocystis hominis</i>	Heterokonta	U51151
<i>Gromia</i> sp. (Guam)	Cercozoa	AJ457814	<i>Achiya bisexualis</i>	Heterokonta	M32705
<i>Gromia oviformis</i> (Tunisia)	Cercozoa	AJ457815	<i>Cylindrotheca closterium</i>	Heterokonta	M87326
<i>Plasmodiophora brassicae</i>	Cercozoa	U18981	<i>Fucus gardneri</i>	Heterokonta	X53907
<i>Polymyxa graminis</i>	Cercozoa	AF310898	<i>Blepharisma americanum</i>	Alveolata	M97909
<i>Spongospora subterranea</i>	Cercozoa	AF310899	<i>Paramecium</i> sp.	Alveolata	X03772
<i>Spongospora nasturtii</i>	Cercozoa	AF310901	<i>Procentrum.micans</i>	Alveolata	M14649
<i>Phagomyxa odontellae</i>	Cercozoa	AF310904	<i>Theileria parva</i>	Alveolata	L02366
<i>Phagomyxa bellerucheae</i>	Cercozoa	AF310903	<i>Neurospora crassa</i>	Opisthokonta	X04971
<i>Cercomonas longicauda</i>	Cercozoa	AF101052	<i>Saccharomyces cerevisiae</i>	Opisthokonta	Z75578
<i>Cercomonas</i> sp. 1	Cercozoa	U42448	<i>Pneumocystis carinii</i>	Opisthokonta	X12708
<i>Cercomonas</i> sp. 2	Cercozoa	U42450	<i>Nuclearia simplex</i>	Opisthokonta	AF349566
<i>Heteromita globosa</i>	Cercozoa	U42447	<i>Nuclearia delicatula</i>	Opisthokonta	AF349563
<i>Cryothecomonas longipes</i>	Cercozoa	AF290540	<i>Nuclearia</i> sp.	Opisthokonta	AF349564
<i>Cryothecomonas aestivalis</i>	Cercozoa	AF290539	<i>Corallochytrium limacisporum</i>	Opisthokonta	L42528
<i>Thaumatomonas</i> sp.	Cercozoa	U42446	<i>Diaphanoeca grandis</i>	Opisthokonta	L10824
<i>Euglypha rotunda</i>	Cercozoa	X77692	<i>Monosiga brevicollis</i>	Opisthokonta	AF100940
<i>Paulinella chromatophora</i>	Cercozoa	X81811	<i>Trichoplax adhaerans</i>	Opisthokonta	L10828
<i>Chlorarachnion reptans</i>	Cercozoa	U03477	<i>Porphyra purpurea</i>	Rhodophyta	L26201
<i>Chlorarachnion</i> sp. 1	Cercozoa	AF076172	<i>Cryptomonas</i> sp.	Cryptophyta	AJ007281
<i>Chlorarachnion</i> sp. 2	Cercozoa	AF054832	<i>Chlorella vulgaris</i>	Viridiaeplantae	X13688
<i>Gymnochlorella stellata</i>	Cercozoa	AF076171	<i>Volvox carteri</i>	Viridiaeplantae	X53904
<i>Lotharella vacuolata</i>	Cercozoa	AF054890	<i>Zea mays</i>	Viridiaeplantae	U42796
<i>Lotharella globosa</i>	Cercozoa	AF076169	<i>Cyanophora paradoxa</i>	Glaucochyta	X68483
<i>Massisteria marina</i>	Cercozoa	AF174369	<i>Trimastix marina</i>	Loukozoa	AF244905
<i>Nuclearia</i> -like filose amoeba	Cercozoa	AF289081	<i>Trimastix pyriformis</i>	Loukozoa	AF244903

^a Sequences in bold were obtained in this study.

dict that the concept of a single widely distributed morphospecies (Finlay's "everything everywhere") (Finlay 1998; Finlay and Clarke 1999) will not be confirmed in the case of *G. oviformis*.

Based on our data, *Gromia* belongs to the phylum Cercozoa. This phylum also includes other filoseans, with which this genus was traditionally classified (Bovee 1985), but according to the SSU rDNA sequences, *Gromia* is not closely related to any of these organisms. The five sequences of *Gromia* form an independent lineage within the Cercozoa. Although its position in our analyses is not completely resolved (see Fig. 3), it is clear that *Gromia* branches neither with the Euglyphida, nor with the naked *Nuclearia*-like amoeba.

Our study also confirms that the Filosea are polyphyletic. The polyphyly of this group was already suggested by an analysis of the genus *Nuclearia* (Amaral Zettler et al. 2001). It had been proposed, however, that the Filosea should be redefined at the exclusion of the *Nuclearia*-containing Cristidiscoidea, placed in the Choanozoa (Cavalier-Smith 2000). In this study we have integrated all available sequences from filose amoebae, including the three *Nuclearia* sequences published by Amaral Zettler et al. (2001) and the sequence of the *Nuclearia*-like filose amoeba published by Bhattacharya and Oliveira (2000). Our analyses (see Fig. 2) confirm the position of these sequences in different regions of the eukaryotic tree, indicating the polyphyletic nature of the subclass Aconchulinia. The polyphyletic origin of the Testaceafilosia is also indicated by the respective positions of *Gromia* and the Euglyphida. Therefore, the taxonomic definition of the Filosea and its subclasses Aconchulinia and Testaceafilosia should be revised and the Gromiida, represented by the genus *Gromia*, should be considered a separate taxon.

Our study shows that the SSU rDNA sequences are of limited value to infer relationships between major cercozoan groups. Most of these groups are characterised by long, unbroken basal stem branches, suggesting that they may have undergone accelerations of their rates of substitution early in their evolution. This lack of resolution may also result from incomplete taxon sampling, as suggested by the recent finding of a new *Nuclearia*-like amoeba in a red algal culture (Bhattacharya and Oliveira 2000). The relationships within the Cercozoa need thus to be examined using protein-coding genes (study in progress).

Protein data are also necessary to revise the relationships between the Cercozoa and other eukaryotes. A recent analysis of actin-coding genes of *Cercomonas* and *Chlorarachnion* showed that

these two cercozoan genera branch together with the Foraminifera (Keeling 2001). Besides, the present study reveals a close relationship between the Cercozoa and the Acantharea (Fig. 2). This relationship was not observed in previous studies, because either the sequences of acanthareans were not included to the examined set of "crown" eukaryotes (Van de Peer and De Wachter 1997; Van de Peer et al. 2000) or the branching point of the Acantharea among the crown radiation was poorly resolved (Amaral Zettler et al. 1997). If these results are confirmed by analyses of other genes, this would suggest that most organisms possessing filopodia, reticulopodia and axopodia may form a natural assemblage. This does not mean that filopodia evolved only once. The example of *Nuclearia* (Amaral Zettler et al. 2001) shows that filopodia-like structures have evolved independently in a lineage that is not closely related to the Cercozoa. The origins of filopodia, however, may not be as frequent as suggested based on ultrastructural observations (Patterson 1984). The present study contributes to growing evidence that most filopodia-bearing protists have a common origin. Additional molecular data is necessary to test further this hypothesis.

Methods

Materials: Living specimens of *G. oviformis* were collected from five localities: Madeira (Atlantic Ocean), Guam (Western Pacific), Tunisia (Mediterranean Sea), Reunion (Indian Ocean) and McMurdo Sound (Antarctica). Those from Guam and Tunisia were maintained alive for a few weeks in Modified Føyns Erdschreiber Medium, with the addition of heat-killed *Dunaliella* as the source of food. The others were processed immediately after collection.

DNA extraction, amplification, cloning and sequencing: DNA was extracted using the DNeasy Plant Minikit (Qiagen, Basel, Switzerland). Two microliters of DNA extract was added to each PCR reaction. 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 52 °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 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, Basle, Switzerland), sequenced with the ABI-PRISM Big Dye Terminator Cycle Se-

quencing Kit and analysed with an ABI-377 DNA sequencer (Perkin-Elmer, Rotkreuz, Switzerland), all according to the manufacturers' instructions.

The complete SSU rDNA genes of the five isolates of *G. oviformis* were amplified in two overlapping fragments, using the following primer pairs: RibA-GRSSU3 and S6-GRSSU1. The sequences of the primers RibA and S6 have been published elsewhere (Pawlowski et al. 1996). The primers GRSSU1 (5'-TCCAAAGTTTTACACGGATC-3') and GRSSU3 (5'-AAAGTAAACGAT(CG)AGAGTCC-3') are two new unpublished primers. To ensure the authenticity of our sequences, we amplified a fragment of rDNA between the universal SSU primer s20 (5'-TTGTACACACCGCCCGTC-3') and the *Gromia*-specific LSU primer GRLSU1 (5'-TGACATCACATT CCAATGAA-3'). The latter primer was designed according to the previously published LSU sequence of *Gromia* (Pawlowski et al. 1994) obtained by direct sequencing from total RNA extraction (Bolivar pers. comm.).

Phylogenetic analyses: The complete SSU rDNA sequences from the five isolates of *G. oviformis* 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). The alignment was submitted to the GenBank database, accession number ALIGN_000337. Species name, taxonomic position and GenBank accession numbers of all the sequences used in our analyses are given in Table 1. Phylogenetic trees were inferred using the neighbor-joining (NJ) method (Saitou and Nei 1987), the maximum parsimony (MP) method and the maximum likelihood (ML) method (Felsenstein 1981). For the first data set, the reliability of internal branches was assessed using the bootstrap method (Felsenstein 1985) with 1000 replicates for NJ analyses, 100 replicates for ML analyses and 200 replicates for MP analyses. For the second data set, 1000, 200 and 300 replicates were performed for NJ, ML and MP analyses, respectively. The PHYLO_WIN program (Galtier and Gouy 1996) was used for distance computations and NJ trees building and bootstrapping, using Kimura's two-parameter model of substitution (Kimura 1980). 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 transition-transversion ratio was set to 2:1. ML analyses were performed using the HKY85 model of evolu-

tion (Hasegawa et al. 1985). Starting trees were obtained via NJ and then swapped using the tree-bisection-reconnection (TBR) algorithm.

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