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Multigene Evidence for Close Evolutionary Relations between *Gromia* and Foraminifera

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Summary. *Gromia oviformis* is a common marine rhizopod, possessing a large ovoid membraneous theca that resembles the tests of certain monothalamous (single-chambered) foraminifers. In fact, the genus *Gromia* was initially classified among the Foraminifera, but because of its non-granular, filose pseudopodia it was later included among filopodia-bearing protists (the Filosea). Recent molecular phylogenies suggested that *Gromia* branches among Cercozoa, a heterogeneous assemblage of mainly amoeboid protists, which show some affinities to Foraminifera. To test how closely related are *Gromia* and Foraminifera, we have analysed the sequences of actin, large subunit of the RNA polymerase II (RPB1) and small subunit (SSU) rRNA genes. We also analysed the structure of the polyubiquitin gene of *G. oviformis*. Our analyses show that *Gromia*'s actin is specifically related to one of the two actin genes families known in Foraminifera. In RPB1-based phylogenies, *Gromia* appears as the closest relative of Foraminifera, while in the SSU rRNA trees it branches as sister to Foraminifera and Haplosporidia. We identified also a single serine insertion in the polyubiquitin of *Gromia*, similar to that found in Foraminifera, Plasmodiophorida and some Cercozoa. Altogether, these findings support the hypothesis that the morphological resemblance between *Gromia* and Foraminifera may be due to a shared common ancestor. If further analyses of protein-coding genes including a more representative sampling of Cercozoa confirm this relationship, then the molecular study of *G. oviformis* will be of key importance for understanding the origin of Foraminifera.

Key words: actin, Cercozoa, Filosea, foraminifera, phylogeny, polyubiquitin, RNA polymerase II largest subunit, SSU rRNA.

Abbreviations: BV - bootstrap value, LBA - long branch attraction, ML - maximum likelihood, MP - maximum parsimony, NJ - neighbor-joining, PP - posterior probability, RPB1 - RNA polymerase II largest subunit, RT-PCR - reverse-transcriptase PCR, SSU - small subunit.

INTRODUCTION

Gromia oviformis is a common marine rhizopod characterized by a large ovoid membraneous test and filose pseudopodia. Morphologically, the species is very similar to some monothalamous allogromiid foraminifers, among which it was initially classified (see review in Cifelli 1990). Detailed light microscopic studies of *Gromia* revealed, however, the non-granular, filose character of its pseudopodia and prompted de Saedeleer (1934) to transfer this genus to the order Filosea. Further electron microscopic observations revealed other distinctive features that distinguished *Gromia* from allogromiid Foraminifera, such as the presence of honeycomb-like membranes on the inner aspect of the wall (Hedley and

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Bertaud 1962). Nevertheless, the gross morphological similarities between gromiid and allogromiid tests led to taxonomic confusion (Hedley 1958) and left uncertain the taxonomic status of some *Gromia*-like foraminifers, such as *Allogromia marina* (Nyholm and Gertz 1973). In some recent protozoan classifications, the genus *Gromia* was included among amoebae of uncertain affinity (Patterson *et al.* 2000).

The first molecular phylogenetic study of *G. oviformis*, based on SSU rRNA gene sequences, suggested that the species belongs to the supergroup Cercozoa (Burki *et al.* 2002). The Cercozoa comprises a heterogeneous assemblage of filose testate amoebae (Testaceafilosia), cercomonads, amoeboflagellates, chlorarachniophyte algae, as well as certain protistan parasites of plants (plasmodiophorids) and invertebrates (Haplosporidia) (Bhattacharya *et al.* 1995; Cavalier-Smith and Chao 1996/7, 2003a; Bulman *et al.* 2001; Wylezich *et al.* 2002). In a recent classification of Cercozoa based on SSU rRNA analysis, the genus *Gromia* was included in the class Gromiidea within the subphylum Endomyxea (Cavalier-Smith and Chao 2003b). These authors further showed that the Filosea *sensu* de Saedeleer (1934) are polyphyletic and evolved three times separately within the Cercozoa.

Phylogenetic analyses of actin-coding genes suggested that Foraminifera are also related to Cercozoa (Keeling 2001). This affinity was subsequently confirmed by the discovery of shared one or two amino-acid inserts at the monomer-monomer junction of polyubiquitin proteins (Archibald *et al.* 2003), and by phylogenetic analysis of sequences of the largest subunit of the RNA polymerase II (RPB1) (Longet *et al.* 2003). Revised analysis of the SSU rRNA revealed that Foraminifera branch together with *G. oviformis* as a sister group of Cercozoa (Berney and Pawlowski 2003). Recently, Archibald and Keeling (2004) showed that the Plasmodiophorida, a group of intracellular parasites of plants, weakly branch as sister to Foraminifera in actin phylogenies, suggesting a possible close evolutionary relation between both groups.

To further test the phylogenetic relationship between *Gromia* and Foraminifera, we obtained the sequences of actin and polyubiquitin genes from *Gromia* sp. and *G. oviformis*, and compared them to corresponding sequences of Foraminifera and other eukaryotes. We also re-analysed the RPB1 and the SSU rRNA gene sequences of *Gromia*. Independent analyses of these four molecular markers suggest that *Gromia* is closely related to Foraminifera, although the solid establishment of its phylogenetic position will require analysis of protein-coding genes from a broader sampling of Cercozoa.

MATERIALS AND METHODS

Specimen collection and nucleic acids extractions. Several specimens of *Gromia oviformis* living on *Corallina mediterrannea* were collected in September 2003 near Marseilles (France). After transfer to the laboratory, healthy specimens were isolated, cleaned and individually conserved in small volumes of sterile seawater. Daily observations showed that certain specimens presented characteristic cytological modifications of individuals undergoing gametogenesis as described by Arnold (1966). DNA was extracted from these gametogenetic individuals prior to gametes release with the DNeasy Plant Mini Kit (Quiagen) using a single cell per extraction.

Additionally, total RNA was extracted from one hundred *Gromia* sp. specimens collected at Svalbard during the cruise of RV Jan Mayen, in July 2001. The specimens were individually cleaned with a paintbrush and rinsed in several bath of sterile seawater prior to RNA extraction using Tri-Reagent (Molecular Research Inc.) (Chomczynski and Sacchi 1987).

Isolation of actin and polyubiquitin genes. All PCR amplifications were carried out under standard amplification protocol, and purified, cloned and sequenced in both directions as described previously (Pawlowski *et al.* 1999). The actin gene of *Gromia oviformis* was amplified from genomic DNA using the forward 5'-GGT GAY GAY GCN CCA MGA GC-3' and reverse 5'-GGW CCD GAT TCA TCR TAY TC-3' primers pair. Multiple (5) independent clones were sequenced and no variability was observed. Under the canonical GT-AG splicing sites assumption, three introns of 346, 304 and 447 base pairs were identified and removed to obtain a 1032 base pairs actin open reading frame.

The actin gene of *Gromia* sp. was also obtained from a total RNA extract by RT-PCR. This procedure allows discriminating deviant actin paralogs or pseudogenes from more slowly evolving actins and relies on the assumption that transcribed gene copies are less likely to represent deviant paralogs that gene sequences amplified directly from genomic DNA. RT-PCR was carried out with the forward 5'-AAC TGG GAY GAY ATG GA-3' and reverse 5'- RTA YTT ICK YTC IGG IGG IGC-3' primers pair and the 3' end of the molecule was obtained by 3' RACE using the 5'/3' RACE kit (Roche). The length of the obtained *Gromia* sp. actin gene sequence is of 894 base pairs.

A fragment of polyubiquitin gene of *G. oviformis* encompassing one and a half ubiquitin monomers was amplified from genomic DNA following Archibald *et al.* (2003), and cloned and sequenced as described above. An intron of 318 base pairs was identified in the ubiquitin monomer of *G. oviformis*. Removal of this intron, assuming canonical GT-AG splicing sites, yielded an ubiquitin monomer consisting of a 231 base pairs uninterrupted open reading frame. The sequences of *Gromia*'s RPB1 and SSU rRNA genes were obtained as described elsewhere (Burki *et al.* 2002, Longet *et al.* 2003).

The nucleotide sequences have been submited to the GenBank under accession numbers: AY571669-AY571670.

Protein phylogenies. Actin sequences of *Gromia oviformis* and *Gromia* sp. and their homologs representing major eukaryotic groups were aligned using Clustal X (Thompson *et al.* 1994) and further adjusted by eye. The actin alignment used consists of 59 sequences and 227 unambiguously aligned amino acid positions, and includes the recently reported actin sequences of the cercozoan-related Polycystinea (Nikolaev *et al.* 2004). The RPB1 alignment was composed of 36 taxa, representing all eukaryotic RPB1 sequences available in GenBank, and 283 unambiguously aligned amino acid positions. Both proteins were analysed identically using distance and maximum likelihood methods. To accommodate rate variations among sites, maximum likelihood distances were computed under the JTT substitution model, assuming a gamma distribution with eight rate categories plus invariable sites, using TREE-PUZZLE 5 (Strimmer and von Haeseler 1996). Fitch-Margoliash distance trees were constructed from these gamma-corrected distances with FITCH 3.6a3 (Felsenstein 1993) using the global rearrangements option. Bootstrap analyses (100 replicates) was carried out for this method with SEQBOOT 3.6a3 (Felsenstein 1993) followed by PUZZLEBOOT (www.tree-puzzle.de) under the JTT substitution matrix with the alpha parameter and proportion of invariable sites calculated by TREE-PUZZLE 5 as explained above. Maximum likelihood trees were inferred with ProML 3.6a3 (Felsenstein 1993) using the JTT substitution matrix and the global rearrangements option. Rate variations among sites were modelled with the -R option and nine categories corresponding to eight rate categories plus invariable sites with rates and their frequencies estimated with TREE-PUZZLE 5 as above. Bootstrap analyses were not performed for the maximum likelihood method due to computational limitations. In the case of actin, we further performed a Bayesian analysis using Mr Bayes 3.0B4, using a gamma distribution and 1500000 generations, with all necessary parameters estimated by the program.

SSU rRNA phylogeny. The SSU rRNA sequences were manually aligned using the Genetic Data Environment software (Larsen *et al.* 1993), following secondary structure models (Neefs *et al.* 1993, Wuyts *et al.* 2000). The sequences were selected so that major taxonomic groups of eukaryotes were represented, using an extensive sampling for Cercozoa. A total of 1115 unambiguously aligned positions were used. Evolutionary trees were inferred using the maximum likelihood (ML) method (Felsenstein 1981), the neighbour-joining (NJ) method (Saitou and Nei 1987), and the maximum parsimony (MP) method, using PAUP* (Swofford 1998). The reliability of internal branches was assessed using the bootstrap method (Felsenstein 1985), with 100, 500, and 1000 bootstrap replicates for ML, MP, and NJ analyses, respectively. ML analyses were performed with the GTR model of substitution (Lanave *et al.* 1984, Rodriguez *et al.* 1990), taking into account a proportion of invariable sites (15.92%), and a gamma-shaped distribution of the rates of substitution among variable sites, with 8 rate categories (alpha = 0.4115). All necessary parameters were estimated from the data using MODELTEST (Posada and Crandall 1998). Starting trees of ML searches were obtained via NJ and swapped with the tree-bisection-reconnection algorithm. NJ analyses were performed with ML-corrected distances using the same parameters. The most parsimonious trees for each MP bootstrap replicate were determined using a heuristic search procedure with 10 random-addition-sequence replicates and tree-bisection-reconnection branch-swapping. The transversion cost was set to twice the transition cost.

RESULTS

The actin phylogeny shows that the two *Gromia* sequences branch with one of the two foraminiferan actin paralog (type 1), while the second foraminiferan actin paralog (type 2) branches with Polycystinea (Fig. 1). This topology is congruently inferred in ML and Bayesian analyses. The distance methods support a relationship between *Gromia* and Plasmodiophorida (56% BV) and weakly recover the monophyly of two foraminiferan paralog (data not shown). A clade comprising *Gromia*, Foraminifera, Plasmodiophorida, and Polycystinea is observed in all analyses (24% BV, 0.99 PP), which support also its relation to Core Cercozoa (29% BV, 0.97 PP). Within this clade, Chlorarachniophyta and cercomonads form two basal lineages in ML and Bayesian trees but are sister groups in distance analysis. Most of the other well-recognised eukaryotic groups were recovered by actin phylogeny.

The RPB1 phylogeny placed *Gromia* sp. as a sister group of Foraminifera (Fig. 2). *Gromia* sp. and Foraminifera formed a clade supported by 75% BV, while their grouping with other Cercozoa was supported by 84% BV. The monophyly of cercomonads and chlorarachniophytes observed in ML trees (Fig. 2) was not confirmed by distance analysis where the clade represented by *Gromia* sp. and Foraminifera branched within the Cercozoa (data not shown). Compared to the consensus eukaryote phylogeny, the RPB1 sequence analysis supported most of the well established eukaryotic groups, but failed to recover the monophyly of Opisthokonts (animals, fungi and choanoflagellates) and placed a heterokont *Ochromonas* among the Alveolata.

The SSU rRNA analyses confirmed the close relation between the two *Gromia* sequences and Foraminifera, but included a group of parasitic Haplosporidia into this clade (Fig. 3). In our ML and MP analyses, the Haplosporidia branched as a sister group of Foraminifera, although with a relatively low BV (56%, 64% in ML and MP, respectively). The NJ analysis showed the monophyly of *Gromia* and Foraminifera to the exclusion of Haplosporidia but this topology was supported by a BV lower than 50%. These three groups formed a relatively well supported clade (58%, 75%, 71% BV in ML, NJ, MP trees, respectively). The Plasmodiophorida appeared next to this clade as a sister group of Core Cercozoa. All remaining cercozoan sequences clustered together in a strongly supported (92%, 83%, 93% BV in ML, NJ, MP, respectively) monophyletic group. This

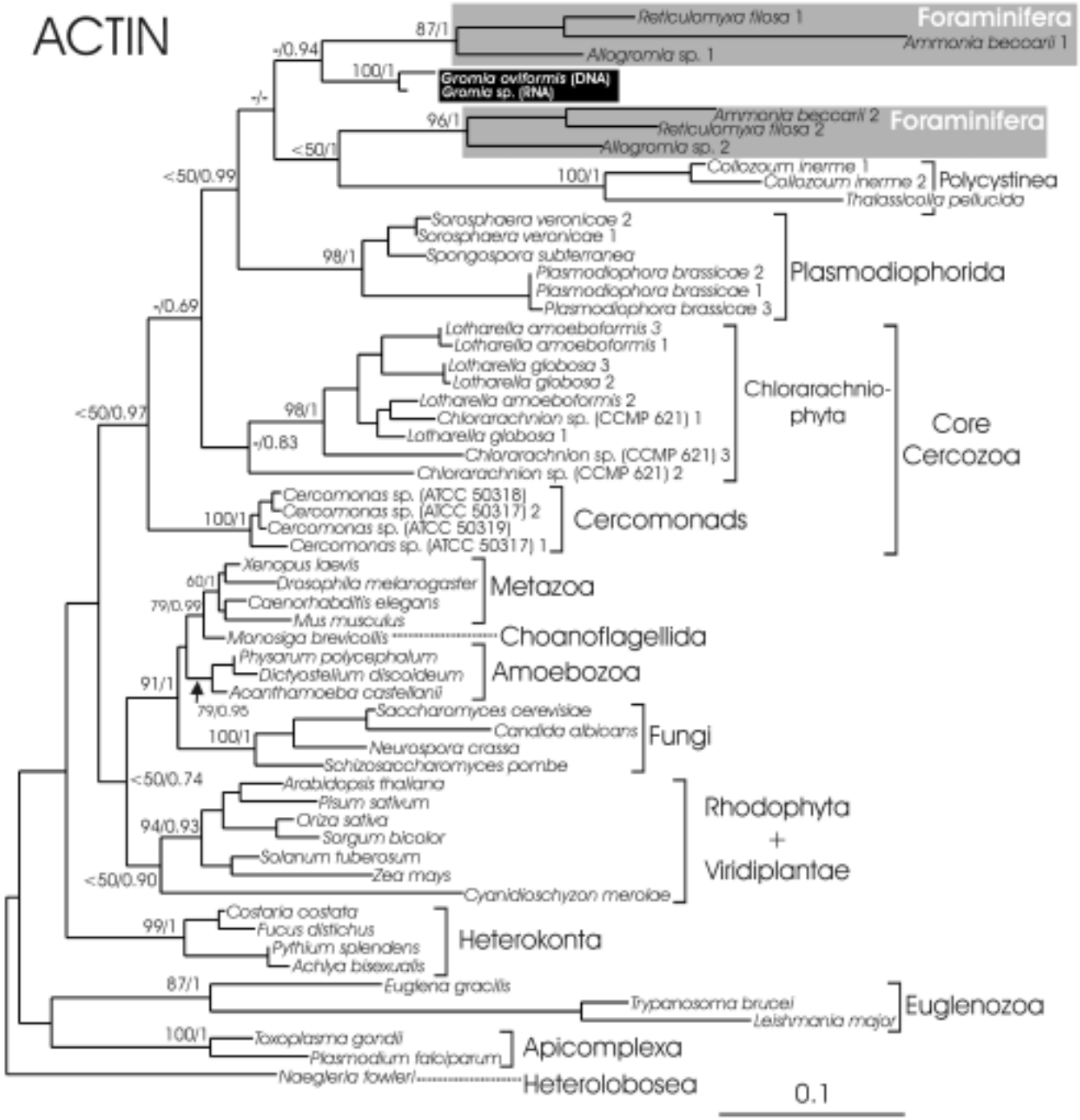

Fig. 1. Phylogenetic position of *Gromia* based on ML analysis of actin gene sequences. Values at nodes indicate bootstrap supports greater than 50% (left) calculated for a tree inferred with the Fitch-Margoliash distance method (see text) and posterior probability (right). Important nodes supported by less than 50% bootstrap valued are indicated by <50%. Except for the two foraminiferan actin families, the bootstrap supports for relations within major eukaryotic groups are omitted for clarity. For *Gromia*, the type of nucleic acid used to isolate actin sequences is indicated in brackets. Single digit numbers at the extreme right of Foraminifera, Polycystinea, Plasmodiophorida, Chlorarachniophyta, and Cercomonads species correspond to different actin gene homologs known. The scale bar represents 0.1 substitutions per site.

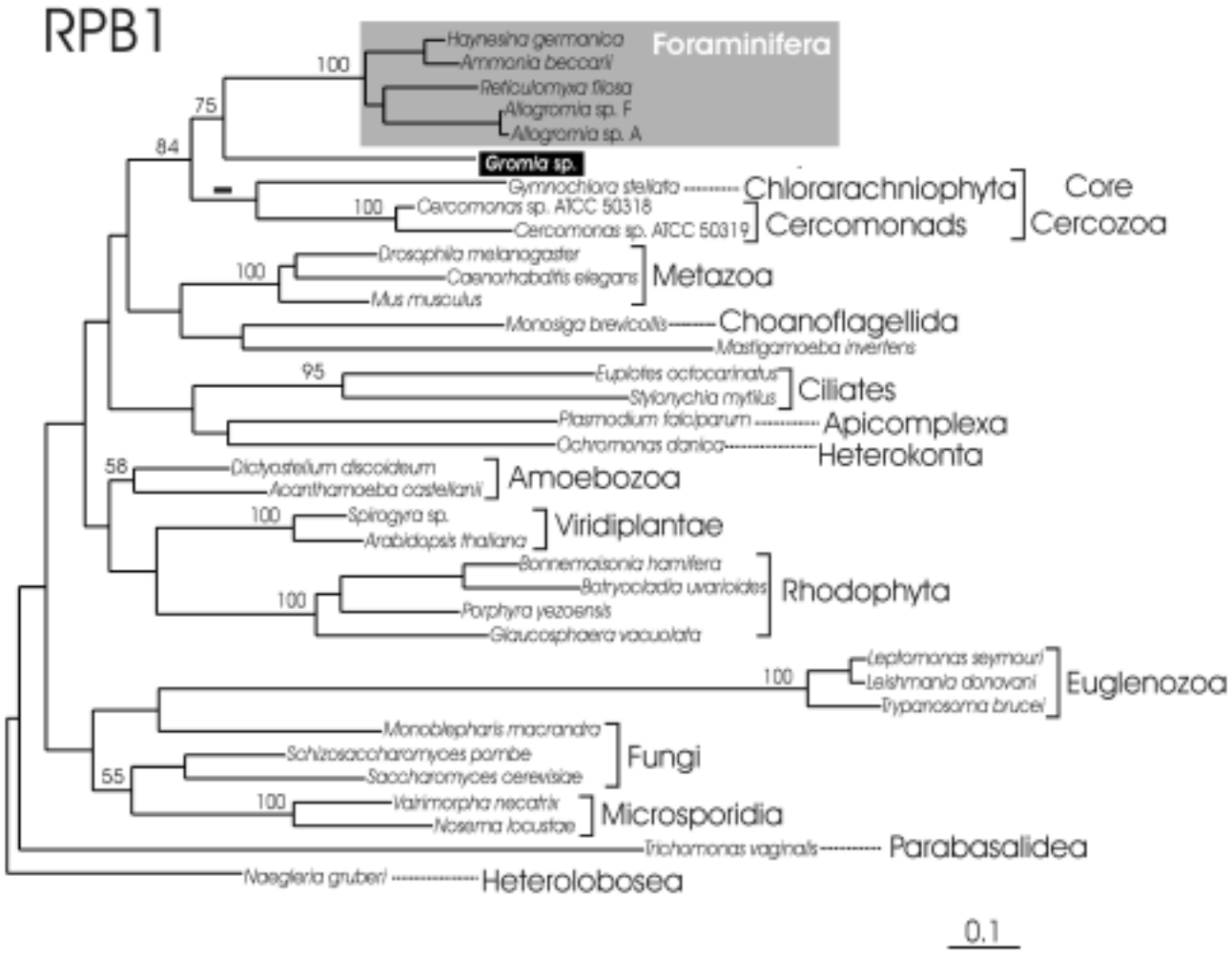

Fig. 2. Phylogenetic position of *Gromia* based on ML analysis of RPB1 sequences. Values at nodes indicate bootstrap supports greater than 50% calculated for a tree inferred with the Fitch-Margoliash distance method (see text). The bootstrap supports for relations within major eukaryotic groups are omitted for clarity. A dash on the branch leading to Core Cercozoa indicates that the topology shown is not supported in the Fitch-Margoliash distance method. The scale bar represents 0.1 substitutions per site.

clade included the chlorarachniophytes, the cercomonads, and the Euglyphida, along with the agglutinated testate amoeba *Pseudodifflugia*, the undefined filose "Nuclearia-like" strain N-por, *Massisteria*, *Cryothecomonas*, and *Thaumatomonas*. The grouping of this clade with Foraminifera, *Gromia*, Haplosporidia and Plasmodiophorida was supported by BV of 67%, 80% and 71% in ML, NJ and MP analyses, respectively. The general topology of our SSU trees was congruent with previous large-scale SSU rRNA phylogenies of eukaryotes, and all well recognised high-level taxa, including plants, red algae, alveolates, heterokonts, amoebae, animals and fungi were recovered with good statistical support.

Finally, sequencing of the monomer-monomer junction of the polyubiquitin gene of *G. oviformis* showed it being characterized by a single serine insertion. Similar insertions of one or two amino acids are observed in all Foraminifera and Cercozoa but not in any other eukaryotes (Fig. 4).

DISCUSSION

Phylogenetic analyses of three molecular markers (actin, RPB1 and SSU rRNA) support the close evolutionary relationship between *Gromia* and Foraminifera.

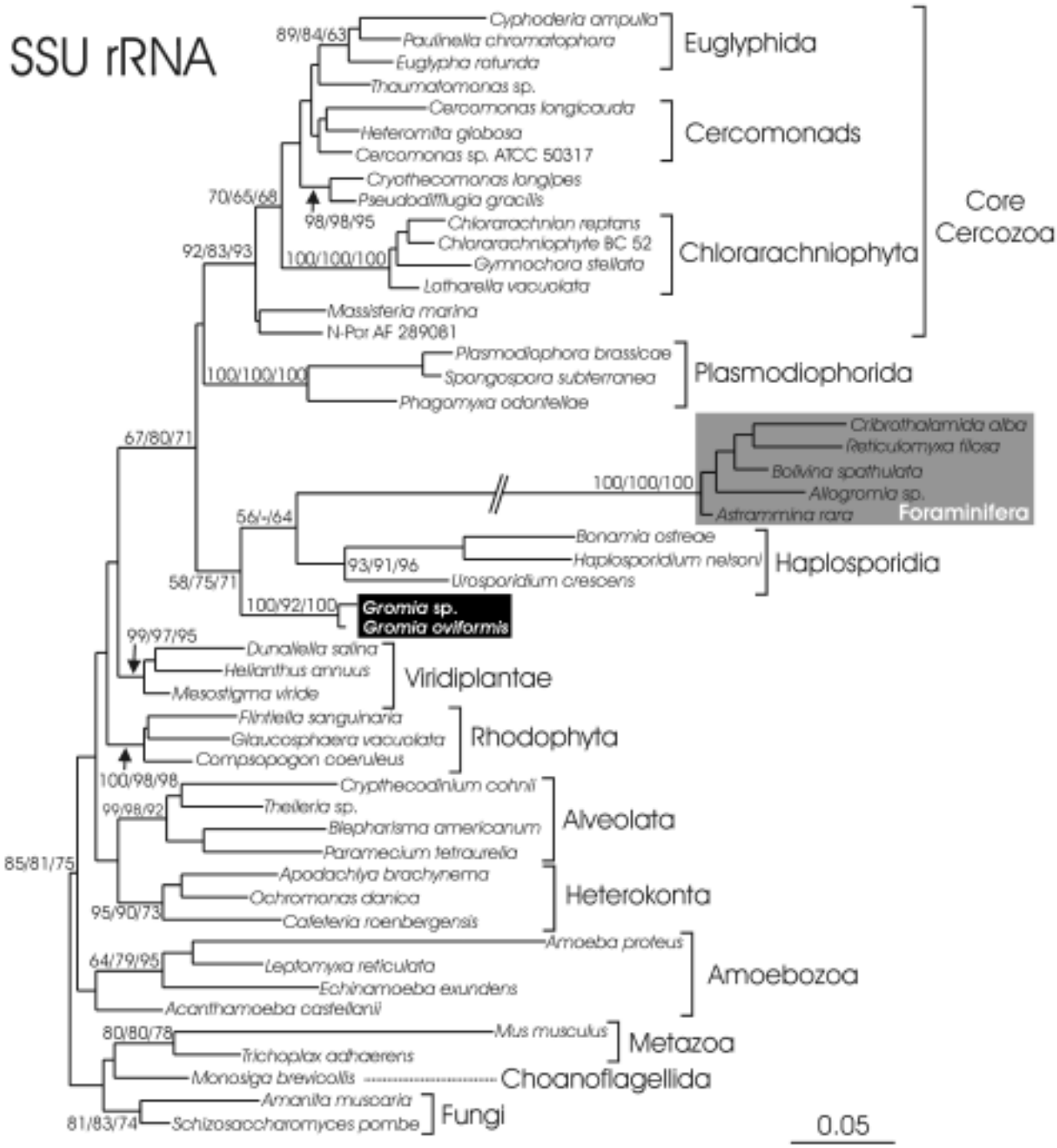

Fig. 3. Phylogenetic position of *Gromia* based on ML analysis of SSU rRNA sequences. Values at nodes indicate bootstrap supports greater than 50% calculated for the ML, NJ, and MP method, respectively. Except for Core Cercozoa, the bootstrap supports for relations within major eukaryotic groups are omitted for clarity. The branch leading to Foraminifera is not drawn to scale. The scale bar represents 0.05 substitutions per site.

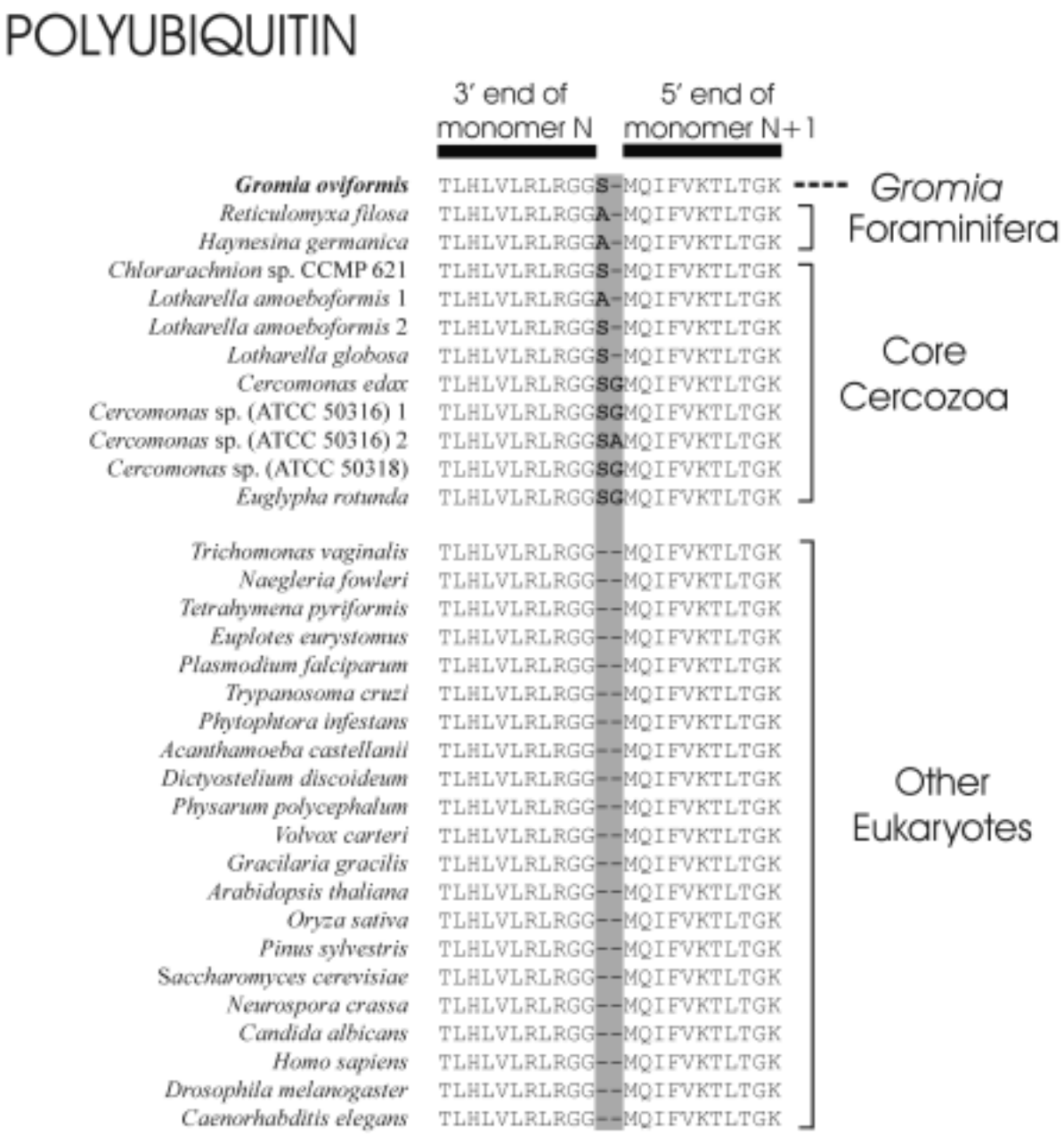

Fig. 4. Junction between two ubiquitin monomers in the polyubiquitin gene of *Gromia oviformis*, Foraminifera, Core Cercozoa and other eukaryotes. Single or double amino acid insertions are observed in *Gromia*, Foraminifera, and Cercozoa. Single digit numbers at the extreme right of chlorarachniophytes and cercomonads species correspond to different polyubiquitin gene homologs known.

However, each gene gives a slightly different image of this association.

The actin phylogeny shows two *Gromia* sequences as members of a clade comprising Foraminifera, Polycystinea, and Plasmodiophorida. Within this clade, both ML and Bayesian methods support the grouping of *Gromia* with the foraminiferan actin type 1 (Fig. 1). Polycystine radiolarians consistently branch with the type 2 and none of our analyses recovered the monophyly of the two foraminiferan paralogs, which was observed in earlier actin phylogenies (Pawlowski *et al*. 1999, Keeling 2001). The independent branching of the two foraminiferan actin paralogs in our trees likely results from discrepancies in their evolutionary rates. Indeed, a large taxonomic sampling of foraminiferan actin genes indicate higher evolutionary rates in type 2

compared to type 1 (unpublished data). Therefore, the grouping of the faster evolving type 2 with the fast-evolving polycystines can reasonably be regarded as a long branch attraction artefact.

Our study confirms the sister group relation between *Gromia* and Foraminifera suggested by earlier analysis of RPB1 gene sequences (Longet *et al.* 2003). Their close relationships are also indicated by analysis of SSU rRNA gene sequences, however, in this case Foraminifera appear more closely related to Haplosporidia than to *Gromia* (Fig. 3). The phylogenetic position of Haplosporidia as a sister group to Foraminifera in SSU trees is surprising since Haplosporidia, which are parasites of freshwater and marine invertebrates that form multinucleate plasmodia in host tissues and reproduce by spores (Perkins 2000), share no morphological features with Foraminifera. This can be explained by extreme simplification of haplosporidian cells adapted to the parasitic mode of life. Alternatively, the close relationships between both groups can be biased by the long branch attraction (LBA) phenomenon, which is very common in rRNA phylogenies (Philippe and Adoutte 1998). Close relationship between *Gromia* and Haplosporidia was earlier suggested by Cavalier-Smith and Chao (2003b), however, in the analyses performed by these authors, Foraminifera cluster with the Polycystinea, which constitutes another example of LBA phenomenon. Remarkably, in Bayesian analyses, which are much less sensitive to substitution rates heterogeneity, Foraminifera branch together with Haplosporidia and *Gromia*, while Polycystinea and other radiolarians form a separate clade (Nikolaev *et al*. 2004).

The principal difficulty in establishing the relations between *Gromia*, Foraminifera, Haplosporidia and other Cercozoa is a lack of broad taxonomic sampling of protein-coding genes. Further analyses of actin and RPB1 genes of Haplosporidia will be necessary to test their grouping with Foraminifera. Increased taxonomic sampling will also allow protein phylogenies to be constructed from concatenated datasets. Such approaches already proved successful in providing better resolution than single gene phylogenies both between and within major eukaryotic groups (e.g. Baldauf *et al.* 2000, Simpson and Rogers 2004). A generally congruent topology of actin and RPB1 trees suggests that both genes are good candidates for future concatenated phylogenies to be inferred.

The positioning of *Gromia* close to the Foraminifera in molecular phylogenies is congruent with several similarities between these organisms that go far beyond the superficial resemblance of gross test morphology and oral apparatus (Hedley and Bertaud 1962). The presence of similar laminated structures in the test wall of the single-chambered foraminifer *Allogromia laticollaris* and *Gromia oviformis* prompted Arnold (1982) to consider both species as an "isomorphic pair". Arnold (1982) also noticed the possibility of fundamental resemblance between shell formation *in G. oviformis* and the proloculus of many polythalamous Foraminifera. Moreover, *Gromia* and Foraminifera share a similar mode of life and resemble each other in having a complex life cycle with asexual and sexual phases. Arnold (1966) observed gametogenesis, fertilisation and production of amoeboid zygotes in plastogamic pairs of adult specimens of *G. oviformis* - a mode of reproduction that is strikingly reminiscent of that of some Foraminifera (Lee *et al.* 1992).

If the close relationship between *Gromia* and Foraminifera is confirmed by further phylogenetic studies, *Gromia* will become a key organism for understanding the origin of Foraminifera. It will be of great interest to compare more thoroughly the ultrastructure and the motility of their pseudopodia. Although *Gromia*'s pseudopodia are non-granular, they do anastomose and can form reticulate structures (Hedley 1962). They seem to move much slower than foraminiferan reticulopodia, but it is uncertain whether the movement is bidirectional or not (Bowser, personal comm.). The mechanisms responsible for the movement of *Gromia*'s pseudopodia are poorly known. More extensive studies of *Gromia*'s actin and tubulin genes may shed new light on genetic factors responsible for the evolution of foraminiferan granuloreticulopodia, and thus may lead to a better understanding of the exceptional success of this group.

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