ISEP XIV

Foraminifera and Cercozoa share a common origin according to RNA polymerase II phylogenies

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Phylogenetic analysis of small and large subunits of rDNA genes suggested that Foraminifera originated early in the evolution of eukaryotes, preceding the origin of other rhizopodial protists. This view was recently challenged by the analysis of actin and ubiquitin protein sequences, which revealed a close relationship between Foraminifera and Cercozoa, an assemblage of various filose amoebae and amoeboflagellates that branch in the so-called crown of the SSU rDNA tree of eukaryotes. To further test this hypothesis, we sequenced a fragment of the largest subunit of the RNA polymerase II (RPB1) from five foraminiferans, two cercozoans and the testate filosean *Gromia oviformis*. Analysis of our data confirms a close relationship between Foraminifera and Cercozoa and points to *Gromia* as the closest relative of Foraminifera.

INTRODUCTION

Foraminifera are common marine protists characterized by granular and highly anastomosed pseudopodia (granuloreticulopodia) and, typically, an organic, agglutinated or calcareous test. They are present in all types of marine environments, but can also be found in freshwater (Pawlowski *et al.*, 1999) and terrestrial (Meisterfeld *et al.*, 2001) habitats. Granuloreticulopodia appear to be the shared character defining all Foraminifera regardless of their habitat and presence or absence of a test. Molecular phylogenetic studies that were mainly based on rDNA gene sequences confirmed the monophyly of Foraminifera, yet their relationships with other eukaryotes remain enigmatic.

Phylogenetic analysis of partial LSU rDNA sequences placed Foraminifera close to slime moulds (*Dictyostelium*, *Physarum*) and *Entamoeba* in the eukaryotic tree (Pawlowski *et al.*, 1994). According to small-subunit (SSU) rDNA sequences, Foraminifera branch near the base of the eukaryotic tree, between the amitochondriate diplomonads and Euglenozoa (Pawlowski *et al.*, 1996). Such an early origin contrasts with the relatively late appearance of Foraminifera in the fossil record (about 540 million years ago). Sogin (1997) proposed that the position of Foraminifera is biased by very rapid rates of rDNA evolution that produces an artificial grouping of Foraminifera with early protist lineages. The long-branch attraction phenomenon was suggested to be responsible for the position of Foraminifera and some other putatively ancient groups of protists in rDNA trees (Philippe & Adoutte, 1998).

The first protein data obtained from Foraminifera confirmed the artefactual character of their position in rDNA phylogenies. Analysis of actin-coding genes placed Foraminifera in a more derived position in the eukaryotic tree (Pawlowski *et al.*, 1999). A more recent study of actin genes suggested that Foraminifera are closely related to *Cercomonas* and to the chlorarachniophytes *Chlorarachnion* and *Lotharella* (Keeling, 2001), a relationship that has also been suggested based on the presence of a shared insertion in polyubiquitin genes (Archibald *et al.*, 2003). These genera are included in the Cercozoa, a heterogeneous assemblage which also comprises filosean testate amoebae (Testaceafilosia), some marine nanoflagellates, and plasmodiophorid plant pathogens (Bhattacharya *et al.*, 1995; Bulman *et al.*, 2001; Cavalier-Smith & Chao, 1996/7; Kühn *et al.*, 2000). Cercozoa are defined on a molecular level only, but their grouping is well supported by several genes, including rDNA, alpha- and beta-tubulin, and actin (Cavalier-Smith, 1998, 2000; Keeling *et al.*, 1998, 1999; Keeling, 2001; Wylezich *et al.*, 2002).

Recently, Burki *et al.* (2002) demonstrated a close relationship between Cercozoa and *Gromia oviformis*, a common marine protist characterized by a large organic test and smooth filose pseudopodia. In their SSU rDNA tree, *Gromia* is not related to other filosean testate amoebae and it branches early among Cercozoa. The taxonomic

position of *Gromia* has always been considered problematic (Hedley, 1958). Because their tests show a gross morphological similarity to that of some allogromiid Foraminifera, the genus has for a long time been considered to be a member of Foraminifera (Arnold, 1972). On the other hand, *Gromia* has also been classified among Filosea, based on the presence of filose, non-anastomosing pseudopodia (Bovee, 1985). In the most recent protist classification, *Gromia* is placed among amoebae of uncertain affinities (Patterson *et al.*, 2000).

To determine the relationships between Foraminifera, Cercozoa and *Gromia*, we sequenced a fragment of the largest subunit of the RNA polymerase II (RPB1) for eight representatives of these groups. Although the taxonomic sampling of RPB1 is still sparse, sequences are available for many of the higher-level eukaryotic taxa. RPB1 appears to be an alternative molecular marker useful for resolving some deep-level relationships between eukaryotic groups and the gene is considered to be present in a single-copy in most eukaryotic genomes (Klenk *et al.*, 1995). RPB1 was successfully used to confirm the phylogenetic relationship of Microsporidia and fungi (Hirt *et al.*, 1999). The sister relationship between red algae and green plants has been addressed using RPB1 (Stiller & Hall, 1997; Stiller *et al.*, 2001) and recently, Dacks *et al.* (2002) inferred a large-scale RPB1 phylogeny of eukaryotes. This report provides additional evidence for a close relationship of Foraminifera and Cercozoa.

METHODS

Cell culture, collection and DNA extraction. Three different Foraminifera, *Reticulomyxa filosa*, *Allogromia* sp. A and *Allogromia* sp. F were maintained in laboratory cultures, as described in Pawlowski *et al.* (1994, 1999). Other Foraminifera, *Ammonia beccarii* and *Haynesina germanica*, as well as *Gromia oviformis* originated from natural populations collected at Le Boucanet (France), Westhoek (The Netherlands) and the Arctic island of Spitzbergen, respectively. DNAs of Foraminifera and *Gromia* were isolated from approximately 100 adult specimens per species except for *Allogromia* sp. F, where the extraction was performed on a single gametogenetic specimen. In the case of *Reticulomyxa*, pelleted cells were used for DNA extraction and all DNAs were extracted using the DNeasy Plant Mini kit (Qiagen).

A culture of *Gymnochlora stellata* (CCMP 2057) was kindly provided by K. Ishida and maintained in f/2-Si medium at 20 °C under a 16-h light/8-h dark cycle. DNA was extracted from pelleted cells of *Gymnochlora* as above. DNA of *Cercomonas* sp. strain RS/22 (ATCC 50318) was generously provided by T. Cavalier-Smith and E. E. Chao.

Isolation of the RPB1 gene. We amplified and sequenced a 5′ fragment of the RPB1 gene encompassing the conserved regions A through D, according to the secondary structure model of Cramer *et al.* (2001). The sequences of the primers Afor (5′-GAITGYC-CIGGICAYTTYGG-3′) and Drev (5′-TTCATYTCRTCICCRTCRAA-RTC-3′), situated in the regions A and D, respectively, were kindly provided by J. W. Stiller. An additional primer Erev (5′-YTGYT-TNCCNGTCCAYAA-3′) was designed for the E region. Concerning Foraminifera and *Gromia oviformis*, the RBP1 fragment was amplified by a semi-nested PCR procedure with amplifications performed

using primers Afor/Erev and re-amplifications using primers Afor/Drev. Cercozoan RPB1 genes were amplified directly by using the Afor/Drev primers. All amplifications were carried out under standard profiles and all bands large enough to contain a viable RPB1 5′ amplification product were isolated, cloned and sequenced in both directions.

Phylogenetic analyses. New sequences and existing homologues retrieved from public databases were aligned using CLUSTAL W (Thompson *et al.*, 1994) and further adjusted by eye. Phylogenies were inferred from amino acid alignments using distance and protein maximum-likelihood. The RPB1 homologous sequence of an archaean, *Sulfolobus solfataricus*, was used as an outgroup. The total alignment used in this study consists of 37 taxa and 283 unambiguously aligned amino acid positions. Maximum-likelihood distances were calculated with TREE-PUZZLE 5.0 (Strimmer & von Haeseler, 1996) using the WAG substitution frequency matrix, amino acid composition estimated from the data, and site-to-site rate variability modelled on a gamma distribution where the shape parameter alpha was estimated using eight rate categories plus invariable sites. Trees were inferred from these distance matrices using weighted neighbour-joining with WEIGHBOR (Bruno *et al.*, 2000) and Fitch–Margoliash with FITCH (Felsenstein, 1993) using global rearrangements and 10 input order jumbles; 100 bootstrap replicates were carried out with each method using PUZZLEBOOT (shell script by A. J. Roger and M. Holder: http://www.tree-puzzle.de) with the alpha parameter and proportion of invariable sites taken from the original data. Protein maximum-likelihood trees were inferred using ProML (Felsenstein, 1993) using the JTT substitution frequency matrix, global rearrangements, and 10 input order jumbles. Site-to-site rate variation was modelled using the -R option and seven categories (corresponding to six rate categories plus invariable sites), with rates and frequencies estimated by TREE-PUZZLE as above.

RESULTS AND DISCUSSION

The length of the sequenced RPB1 gene fragments range from 1226 to 1271 nucleotides in Foraminifera, and is 1255 nucleotides in *Gromia oviformis*. The fragment is much longer in *Gymnochlora stellata* and *Cercomonas* ATCC 50318, for which 1393 and 1768 nucleotides were sequenced respectively. The size differences of RPB1 genes are the result of the presence of introns: two occur in *Gymnochlora* and five are present in *Cercomonas* ATCC 50318. No introns were found in the Foraminiferan and *Gromia* RPB1s. Several clones were sequenced for each species and no variability was observed. Distance trees were inferred initially using a dataset of 39 sequences that included the nucleomorph RPB1 genes from *Chlorarachnion* CCMP 621 and *Lotharella amoeboformis* (CCMP 2058) but the nucleomorph sequences were determined to be extremely divergent and offered little information (data not shown). As they were not relevant to the evolution of the host lineage, they were excluded from the analysis and a dataset of 37 sequences (shape parameter of 1·22 and proportion of invariable site of 0·11) was used for more comprehensive analysis.

Maximum-likelihood analysis of 37 RPB1 sequences reveals a clade consisting of Foraminifera, *Gromia* and Cercozoa (Fig. 1). This group is supported by bootstrap values of 84 and 72 % in weighted neighbour-joining (WNJ) and Fitch–Margoliash (FM) distance trees, respectively. Within
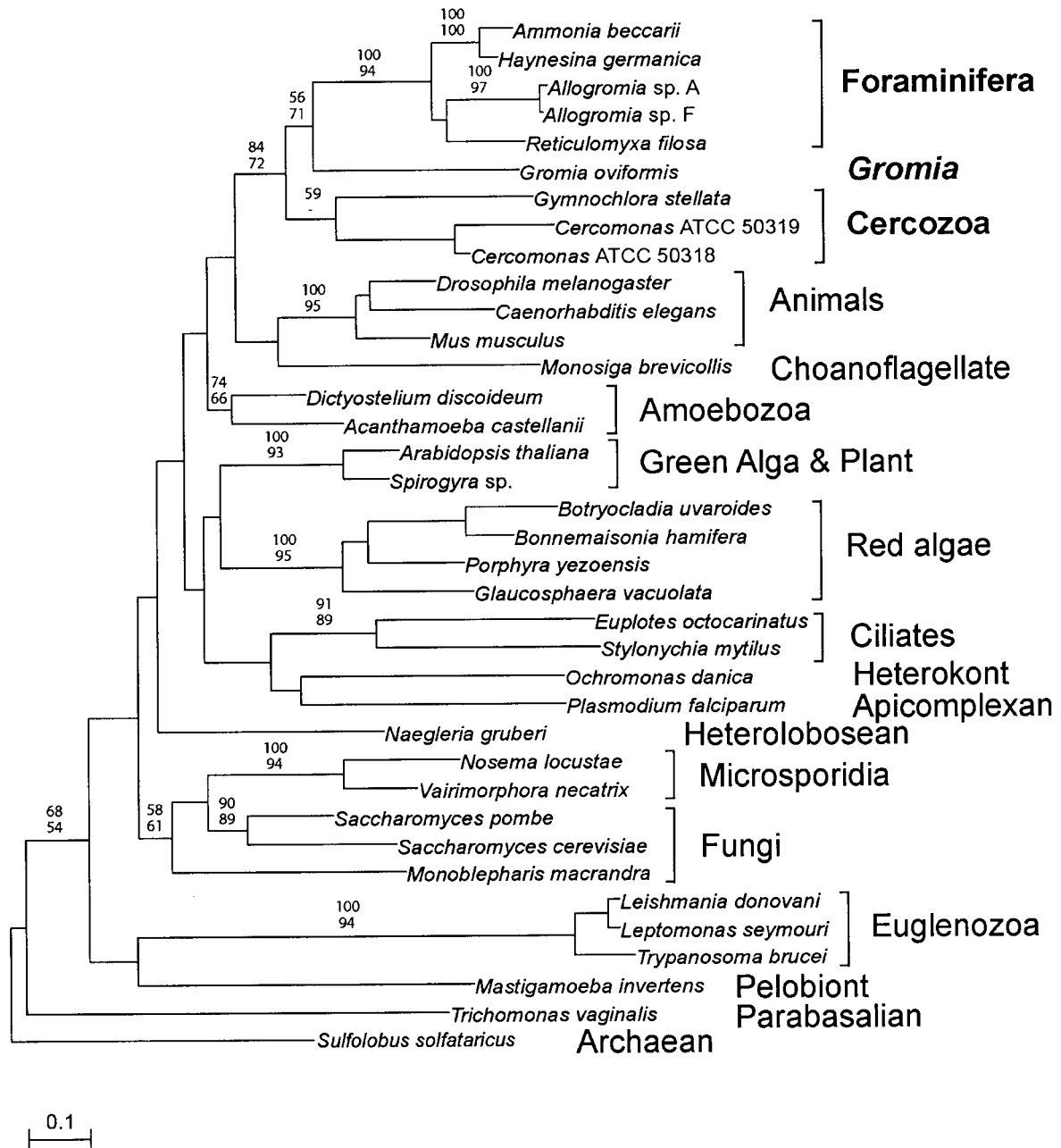
Fig. 1. Maximum-likelihood tree of eukaryotes based on partial RPB1 sequences. The tree was inferred using ProML (see text). Values at nodes indicate bootstrap support (100 replicates) greater than 50 % calculated by PUZZLEBOOT on trees constructed with WEIGHBOR (top) and Fitch–Margoliash (bottom) distance methods. Except for Foraminifera, within-group bootstrap supports are omitted for clarity. A dash on the branch leading to Cercozoa indicates that the topology shown is not supported in the Fitch–Margoliash tree. The scale bar represents 0·1 substitution per site.

this clade, Foraminifera, *Gromia* and Cercozoa form three independent lineages in maximum-likelihood (ML) and WNJ trees but the FM distance tree shows Foraminifera and *Gromia* branching within Cercozoa (data not shown). Cercozoa appeared paraphyletic in actin phylogenies (Keeling, 2001) but this topology was not supported by bootstrap. In all analyses, *Gromia* appears as the closest

relative to Foraminifera, although support for this relationship is not high.

Maximum-likelihood (Fig. 1) and distance analyses (data not shown) identified *Trichomonas*, *Mastigamoeba* and Euglenozoa as basal lineages, a position already observed in RPB1 phylogenies inferred using longer sequences (Stiller &

Hall, 2002; Dacks *et al.*, 2002). Dacks *et al.* (2002) showed that the early emergence of *Trichomonas* and *Mastigamoeba* in the RPB1 phylogeny resulted from long-branch attraction artefacts. In our trees, all other taxa emerge in a poorly resolved radiation and the branching order of the different groups varies between maximum-likelihood and distance methods. However, both methods identified the same groups of eukaryotes and in no case did Foraminifera branch among the earliest eukaryotic lineages as suggested by rDNA phylogenies (Pawlowski *et al.*, 1994, 1996).

Our analyses of RPB1 sequences support eukaryotic groups either well-recognized or observed with other molecular datasets, including ciliates, green plants, Amoebozoa (excluding *Mastigamoeba*), Euglenozoa, animals and red algae. The relationship of Microsporidia and fungi, supported by a body of evidence (for review see Keeling & Fast, 2002), is consistently recovered. Alveolates (ciliates + *Plasmodium*) and the heterokont *Ochromonas* form a clade with the heterokont falling within the alveolates. This is observed in all trees, although support is weak. The alveolate clade has already been recovered in RPB1 phylogenies (Stiller *et al.*, 2001) and Dacks *et al.* (2002) showed a relationship between *Plasmodium* and *Ochromonas*, suggesting a higher-level grouping of alveolates and heterokonts. This deep-level association is observed with protein phylogenies (Baldauf *et al.*, 2000; Fast *et al.*, 2001; Harper & Keeling 2003). Interestingly, the red and green algae were sister groups in our ML tree (Fig. 1), in contrast to the results of previous analyses of RPB1 (Stiller & Hall, 1997; Stiller *et al.*, 2001). In our ML analysis, opisthokonts do not form a monophyletic group and the clade consisting of fungi and microsporidia branches somewhat deeply, although this was not the case in distance trees (data not shown). In a recent RPB1 phylogeny, an opisthokont clade was observed but its support varied greatly (Stiller & Hall, 2002; Dacks *et al.*, 2002).

Our data confirm the close relationship between Foraminifera and Cercozoa as suggested by actin and ubiquitin sequences analysis (Keeling, 2001). The specific association of both groups is also supported by a shared insertion of one or two amino acids at the monomer–monomer junctions of polyubiquitin proteins, a feature that is unique among eukaryotes (Archibald *et al.*, 2003). Our study does not allow us to confidently resolve the relative branching order of Foraminifera and Cercozoa. However, our RPB1 phylogeny does allow us to conclude that among the examined species, *Gromia* is the closest relative of Foraminifera. The specific association of Foraminifera and *Gromia* in our study is congruent with revised analyses of SSU rDNA sequences (Berney & Pawlowski, 2003). This conclusion not only confirms the traditional view of a close relationship between *Gromia* and Foraminifera, but also supports the hypothesis of a foraminiferal origin from a filosean ancestor.

The molecular evidence for a close relationship between *Gromia* and Foraminifera is of crucial importance with regard to the study of foraminiferal evolution. Comparison of genes involved in cytoskeleton formation in *Gromia* and Foraminifera may help to identify the genetic modifications responsible for the evolution of filopodia into granuloreticulopodia. We hypothesize that additional protein data collected from *Gromia* and Foraminifera might show an even closer relationship between both lineages. If this is confirmed, *Gromia* will serve as a reliable outgroup necessary to establish higher-level relationships among Foraminifera. Up to now, comparative taxonomic studies were mostly based on rDNA genes and severely restricted by different substitution rates at higher taxonomic levels (Pawlowski *et al.*, 1997). According to the results of our preliminary study, the RPB1 gene seems to evolve at relatively stable rates in Foraminifera. Phylogenetic relationships within the examined foraminiferal species are congruent with the morphological distinction of organic-walled (*Allogromia* and *Reticulomyxa*) and calcareous taxa (*Ammonia* and *Haynesina*). Future studies will show whether these relationships hold true with a broader taxon sampling and if the RPB1 gene can be used as a molecular clock to examine the mode and tempo of foraminiferal evolution and to establish their molecular timescale.

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