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Untangling the Phylogeny of Amoeboid Protists[1]

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ABSTRACT. The amoebae and amoeboid protists form a large and diverse assemblage of eukaryotes characterized by various types of pseudopodia. For convenience, the traditional morphology-based classification grouped them together in a macrotaxon named Sarcodina. Molecular phylogenies contributed to the dismantlement of this assemblage, placing the majority of sarcodinids into two new supergroups: Amoebozoa and Rhizaria. In this review, we describe the taxonomic composition of both supergroups and present their small subunit rDNA-based phylogeny. We comment on the advantages and weaknesses of these phylogenies and emphasize the necessity of taxon-rich multigene datasets to resolve phylogenetic relationships within Amoebozoa and Rhizaria. We show the importance of environmental sequencing as a way of increasing taxon sampling in these supergroups. Finally, we highlight the interest of Amoebozoa and Rhizaria for understanding eukaryotic evolution and suggest that resolving their phylogenies will be among the main challenges for future phylogenomic analyses.

Key Words. Amoebae, Amoebozoa, eukaryote, evolution, Foraminifera, Radiolaria, Rhizaria, SSU, rDNA.

FROM SARCODINA TO AMOEBOZOA AND RHIZARIA

THE amoebae and amoeboid protists form an important part of eukaryotic diversity, amounting for about 15,000 described species (Adl et al. 2007), among which are several ecologically important taxonomic groups. Lobose naked and testate amoebae are common elements of soil and freshwater microbial communities, and include species of critical medical importance (e.g. *Entamoeba histolytica*). Radiolarians are among the most abundant and diverse groups of marine holoplankton. Organic-walled and agglutinated benthic foraminiferans dominate the deep-sea meiofauna, while planktonic and large benthic calcareous species are among the main calcifying protists, contributing to almost 25% of the present-day carbonate production in the oceans (Langer 2008). Both Foraminifera and Radiolaria are major groups of microfossils, widely used in paleostratigraphic and paleoclimatic reconstructions.

For convenience, all these taxonomic groups were placed within the class or phylum Sarcodina, defined as protists possessing pseudopodia or locomotive protoplasmic flow, with flagella usually restricted to developmental stages (Levine et al. 1980). Depending on the type of pseudopodia, the Sarcodina were further subdivided into the superclass Rhizopodea comprising protists having lopobodia, filopodia, and reticulopodia and the superclass Actinopodea, composed of all axopodia-bearing protists (Lee, Hutner, and Bovee 1985; Levine et al. 1980). Although this system was vigorously criticized based on ultrastructural studies (Patterson 1994), no alternative classifications were proposed until the advent of molecular phylogenies.

The first molecular phylogenies based on the small subunit (SSU) rDNA sequences provided strong evidence for the polyphyletic origin of amoeboid protists. The independent branching of *Acanthamoeba* and *Naegleria* (Clark and Cross 1988) confirmed the ultrastructural differences between Lobosea and Heterolobosea (Page and Blanton 1985). However, the erratic distribution of other amoeboid protists in eukaryotic trees was strongly influenced by heterogeneity of the evolutionary rate in ribosomal genes. The most spectacular fast-evolving lineages, such as foraminiferans (Pawlowski et al. 1996), polycystines (Amaral Zettler, Sogin, and Caron 1997), pelobionts (Hinkle et al. 1994), entamoebids (Silberman et al. 1999), and mycetozoans, were all affected by long-branch attraction artifacts in early studies (Philippe and Adoutte 1998; Stiller and Hall 1999).

It was only after the development of probabilistic methods and the introduction of new evolutionary models correcting for among-site heterogeneity that the SSU rDNA phylogeny of amoeboid protists could be partially resolved (Bolivar et al. 2001; Milyutina et al. 2001). Complementing these results, protein-coding genes also became available for a few species (Fahrni et al. 2003; Keeling 2001; Pawlowski et al. 1999). New taxonomic entities of amoeboid protists, such as Amoebozoa and Rhizaria, started to emerge following these improvements (Cavalier-Smith 1998, 2002). Further multigene studies and better taxon sampling in SSU rDNA trees have contributed to definitely establish both major groups (Archibald et al. 2003; Bapteste et al. 2002; Burki and Pawlowski 2006; Cavalier-Smith and Chao 2003b; Cavalier-Smith, Chao, and Oates 2004; Longet et al. 2003; Nikolaev et al. 2004; Takishita et al. 2005).

Consequently, most sarcodinids were placed within either Amoebozoa or Rhizaria in the new classification of protists (Adl et al. 2005). There are in fact only four taxonomic groups, traditionally included in Sarcodina, that now branch outside these supergroups. Among them are two orders of Heliozoa (i.e. Actinophryida and Centrohelida), the class Heterolobosea, and the genus *Nuclearia*. With the notable exception of Centrohelida, the other three taxa have been confidently placed in one of the other eukaryotic supergroups. Actinophryida branch among stramenopiles in SSU rDNA trees, either as sister to Opalozoa (Cavalier-Smith and Chao 2006) or close to the ultrastructurally similar pedinellid algae (Nikolaev et al. 2004), but the support for either relationship is weak and there are currently no other genes, except for a partial sequence of *Actinosphaerium* actin (Nikolaev et al. 2004) to test these hypotheses. Heterolobosea are usually grouped with Euglenozoa in the taxon Discicristata, based on rDNA and protein sequence data (Baldauf 2003; Cavalier-Smith 2002; Keeling and Doolittle 1996), but some recent multigene phylogenies suggested that they are more closely related to jakobids (Simpson, Inagaki, and Roger 2006). *Nuclearia* branches as sister group to Fungi, as first revealed by SSU rDNA trees (Amaral Zettler et al. 2001) and later confirmed by multigene analyses (Steenkamp, Wright, and Baldauf 2006). The branch of Centrohelida is floating in current phylogenetic trees depending on the analyzed genes. In SSU rDNA trees, centrohelids appeared either as sister to haptophytes (Cavalier-Smith and Chao 2003a) or as sister to rhodophytes

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(Sakaguchi et al. 2005). A seven-gene analysis placed them as a sister group to a clade comprising Chromalveolates and Plantae, but without statistical support (Sakaguchi, Inagaki, and Hashimoto 2007).

MOLECULAR PHYLOGENY OF AMOEBOZOA

The supergroup Amoebozoa includes all naked and testate lobose amoebae, which are traditionally classified in the class Lobosea, Carpenter 1861 (Page 1987), as well as the pelobionts, entamoebids, and mycetozoans (Cavalier-Smith 1998). In addition to the amoeboid forms, Amoebozoa also comprise the uniciliate zooflagellate *Phalansterium solitarium* (Cavalier-Smith et al. 2004) and the multiciliated species *Multicilia marina* (Nikolaev et al. 2006). Finally, the group includes the class Breviatea, introduced by Cavalier-Smith (2004) for the enigmatic free-living amoeboflagellate *Mastigamoeba invertens*, redescribed as *Breviata anathema* (Walker, Dacks, and Embley 2006), and recently shown to be likely in a sister position to all other Amoebozoa in a phylogenomic analysis (Minge et al. 2008).

The taxon Amoebozoa (Lühe 1913) was emended as a phylum by Cavalier-Smith (1998). Its taxonomic composition barely changed since its creation (Adl et al. 2005). However, molecular evidence for the monophyly of all members is still quite circumstantial. The close relationship between some lobose amoebae and mycetozoans was first suggested based on similarities of *Acanthamoeba* and *Dictyostelium* mitochondrial genomes (Gray, Burger, and Lang 1999; Iwamoto et al. 1998), but these features are not found in other amoebozoan taxa (Kudryavstev, pers. commun.). The grouping of lobose amoebae together with entamoebids, pelobionts, and mycetozoans was demonstrated in SSU rDNA and actin trees (Bolivar et al. 2001; Fahrni et al. 2003; Milyutina et al. 2001), yet the support for this clade was very weak. Much stronger support was obtained in Bayesian analyses of the SSU rDNA (Nikolaev et al. 2006) or the concatenated alignment of four genes (Tekle et al. 2008). The monophyly of Amoebozoa was also suggested by the presence of a particular type of myosin II (Richards and Cavalier-Smith 2005) and confirmed by its phylogenetic analysis (Berney and Cavalier-Smith 2007). However, a recent addition of two lobosean amoebae (*Hartmannella vermiformis* and *Acanthamoeba castellanii*) to the phylogenomic analyses of ESTs from Archamoebae and Mycetozoa did not improve the support for the monophyly of all Amoebozoa (Minge et al. 2008).

The lack of strong statistical support for the larger grouping also applies to the phylogenetic relationships within Amoebozoa. Most of the taxonomic groups recognized in recent classifications (Adl et al. 2005; Smirnov et al. 2005) have been based solely on SSU rDNA phylogenies. However, very few of these groups are robustly supported and there is at present no clear evidence for the branching pattern among them. Because the composition and position of some of these groups greatly depends on the choice of sites and taxa in SSU rDNA analyses, we present here an amoebozoan phylogeny in the form of a schematized consensus tree with a basal multifurcation that reflects better the uncertainties (Fig. 1).

Six major clades can be distinguished in this tree: Tubulinea, Flabellinea, Conosea, Variosea, Thecamoebida, and Acanthopodida. The clades Tubulinea and Flabellinea comprise the majority of naked and testate lobose amoebae. Tubulinea, which include Tubulinida, Arcellinida, Leptomyxida, and incertae sedis genus *Echinamoeba* and *H. vermiformis*, appears in most of SSU rDNA and actin trees (Bolivar et al. 2001; Fahrni et al. 2003; Nikolaev et al. 2005; Smirnov et al. 2005; Tekle et al. 2008). Tubulinea are relatively well supported and also defined by tubular pseudopodia and monoaxial cytoplasmic flow (Smirnov et al. 2005). In the case of Flabellinea, phylogenetic analyses are much less consistent and the distinction of Vannellida and Dactylopodida, first shown by Peglar et al. (2003), is not well supported. This is mainly due to the rapidly evolving sequences of *Clydonella*, *Ripella*, *Pessonella*, and *Vexillifera minutissima*, which have a tendency to group together probably because of long-branch attraction (see Fig. 1). The genus *Cochliopodium*, whose position is still unresolved (Kudryavtsev et al. 2005), also seems to belong to this clade (Fig. 1).

The grouping of Archamoebae and Mycetozoa (Dictyostelia+Myxogastria) representing the class Conosea (Cavalier-Smith 1998; Smirnov et al. 2005), appears in some but not all SSU rDNA trees (Nikolaev et al. 2006). The extremely divergent sequences of myxogastrids often branch separately as a sister group to some Variosea (Cavalier-Smith et al. 2004; Tekle et al. 2008). However, the monophyly of Dictyostelia and Myxogastria is strongly supported by elongation factor (EF) 1A phylogenies (Arisue et al. 2002; Baldauf and Doolittle 1997) and by phylogenomic analyses (Bapteste et al. 2002; Minge et al. 2008). In a recent analysis of EF1A and SSU rDNA data including a large taxon sampling, this clade also comprises some Protostelida (*Ceratiomyxa*) but most protostelids branch separately (Fiore-Donno et al. unpublished). It has been proposed that Conosea also includes a group of flagellated amoebozoans (*Phalansterium*, *Multicilia*) and some lobose amoebae (*Acramoeba* = former *Gephyramoeba*, *Filamoeba*) that often branch as a paraphyletic assemblage at the base of Mycetozoa and Archamoebae (Nikolaev et al. 2006). This group partially corresponds to the class Variosea (Cavalier-Smith 2004) whose monophyly is supported by a conserved motif of eight nucleotides in the variable region V7.

Among the other clades of Amoebozoa, Acanthopodida (including *Acanthamoeba* and *Balamuthia*) are the only strongly supported group in all types of analyses. This clade, considered as the order Centramoebida, was included into the class Variosea by Cavalier-Smith (2004), but there is no support for this relationship in any SSU rDNA trees. There is also no support for the position of Thecamoebida, which appears in the most recent SSU rDNA analyses and has been confirmed by myosin II data (Berney, pers. commun.). Moreover, there is neither indication concerning the position of *Vermistella antarctica* (Moran et al. 2007) nor that of the clade *Mayorella+Dermamoeba*. Among the incertae sedis amoebozans, there are also *Trichosphaerium* spp., which branch as sister group to Myxogastria in some analyses (Tekle et al. 2008), but this is likely to be due to their extremely fast evolving SSU rDNA.

MOLECULAR PHYLOGENY OF RHIZARIA

Rhizaria are the most recently recognized supergroup of eukaryotes, commonly including organisms bearing ''root-like reticulose or filose pseudopodia'' (Cavalier-Smith 2002). It contains the majority of protists that were traditionally classified among Rhizopoda (Filosea, Granuloreticulosea) and Actinopoda (Fig. 2). However, in addition to typically amoeboid taxa, such as euglyphids, gromiids, foraminiferans, and radiolarians, Rhizaria also includes a large diversity of free-living flagellates, amoeboflagellates, and parasitic protists.

The supergroup Rhizaria has been established based exclusively on molecular data. The first presage for this grouping was a clade formed by the euglyphid testate amoebae and the photosynthetic chlorarachniophytes (Bhattacharya, Helmchen, and Melkonian 1995). This clade was later enlarged to include the zooflagellates *Cercomonas*, *Heteromita*, and *Thaumatomonas*, as well as the plasmodiophorid plant parasites (Cavalier-Smith and Chao 1996/1997), leading to the creation of the phylum Cercozoa (Cavalier-Smith 1996/1997). The next important step was the finding that Cercozoa and Foraminifera are related in actin phylogeny (Keeling 2001). This unexpected result was later confirmed by the

discovery of an amino acid insertion between the monomers of the polyubiquitin gene in Cercozoa, Foraminifera, and Plasmodiophorida (Archibald and Keeling 2004; Archibald et al. 2003), and analyses of the large subunit of RNA polymerase gene (Longet et al. 2003) and SSU rDNA (Berney and Pawlowski 2003).

The taxonomic composition of Cercozoa was progressively expanded by including various zooflagellates (Atkins, Teske, and Anderson 2000; Kühn, Lange, and Medlin 2000), gromiids (Burki, Berney, and Pawlowski 2002), testate amoebae (Wylezich et al. 2002), filose and reticulate protists (Nikolaev et al. 2003), and radiolarians (Polet et al. 2004). The position of plasmodiophorid plant pathogens among Cercozoa was confirmed by molecular studies (Bulman et al. 2001). The haplosporidian parasites were placed together with plasmodiophorids and gromiids in the subphylum Endomyxa (Cavalier-Smith 2003; Cavalier-Smith and Chao 2003b). Cercozoa were suggested to be sister group to Retaria, composed of Polycystinea, Acantharea, and Foraminifera (Cavalier-Smith 1999). Both Cercozoa and Retaria formed the new infrakingdom Rhizaria (Cavalier-Smith 2002). It was initially proposed that Rhizaria should also include Apusozoa and Centrohelida, but these lineages are in fact unrelated to Cercozoa and Retaria. A strong support for Rhizaria, composed of all previously included taxonomic groups, plus Desmothoracida and Taxopodida, was recovered in a combined analysis of actin and SSU rDNA genes (Nikolaev et al. 2004). A close relationship between Cercozoa and Foraminifera was confirmed by the analysis of three cytoskeletal proteins (Takishita et al. 2005) and more recently by phylogenomic analyses of EST data (Burki and Pawlowski 2006; Burki, Shalchian-Tabrizi, and Pawlowski 2008; Burki et al. 2006, 2007). The rhizarian supergroup is growing continuously by new inclusions, such as the marine flagellate ebriids (Hoppenrath and Leander 2006), the amoeboid *Corallomyxa* (Tekle et al. 2007), the parasitic plasmodial *Paradinium* (Skovgaard and Daugbjerg 2008), and the soil flagellate *Sainouron* (Cavalier-Smith et al. 2008).

Phylogenetic relationships within Rhizaria have been studied using SSU and LSU rDNA, actin, RNA polymerase II (RPB1), and tubulins (Cavalier-Smith and Chao 2003b; Longet et al. 2003; Moreira et al. 2007; Nikolaev et al. 2004; Takishita et al. 2005). Our schematized SSU rDNA tree represents an actual view of rhizarian phylogeny with indications of an alternative branching possibility for Foraminifera (Fig. 2). In general, we can distinguish two major clades (i.e. Cercozoa and Radiolaria) and an assemblage of six more or less diverse clades of uncertain position, which correspond to Phytomyxea, Foraminifera, Haplosporidia, and the genera *Paradinium*, *Gromia*, and *Filoreta*—a new genus comprising the misidentified *Corallomyxa tenera* (Tekle et al. 2007) and an organism previously identified tentatively as ''*Reticulamoeba*'' (Bass et al. 2008).

The clade Cercozoa (corresponding to the subphylum Filosa in Cavalier-Smith 2003, and ''core'' Cercozoa in Nikolaev et al. 2004) includes the euglyphids, phaeodarians, desmothoracids, chlorarachniophytes, ebriids, and various flagellated genera, which are often able to form filopodia. The relationships among these taxa have been extensively studied based on the SSU rDNA (Bass and Cavalier-Smith 2004; Bass et al. 2005; Cavalier-Smith and Chao 2003b), but the resolution of the cercozoan SSU rDNA trees is generally poor. The monophyly of Cercozoa is consistently recovered, although not always strongly supported (Bass and Cavalier-Smith 2004; Cavalier-Smith and Chao 2003b). A characteristic feature of this group is the insertion of two amino acids in the polyubiquitin protein, but some taxa (*Metopion*, Chlorarachnea) harbor only one amino acid insertion (Bass et al. 2005).

The grouping of Phytomyxea, Haplosporidia, Foraminifera, and the monogeneric clades of *Gromia*, *Paradinium*, and *Filoreta* is not well supported and the relationships among these different clades are not resolved. All these groups are characterized by a single amino acid insertion in the polyubiquitin gene (Bass et al. 2005) and the GA-AG deletion in SSU rDNA also present in Cercozoa (Cavalier-Smith and Chao 2003b), but which is apparently modified in Foraminifera and subject to ambiguities in the alignment. Moreover, Haplosporidia, *Paradinium*, *Gromia*, and *Filoreta tenera* (*C. tenera*) share the specific stem E23-13-1 (Tekle et al. 2007). However, this stem is absent in other species of *Filoreta* ''*Reticulamoeba*'' and its identification in highly divergent foraminiferan sequences is ambiguous.

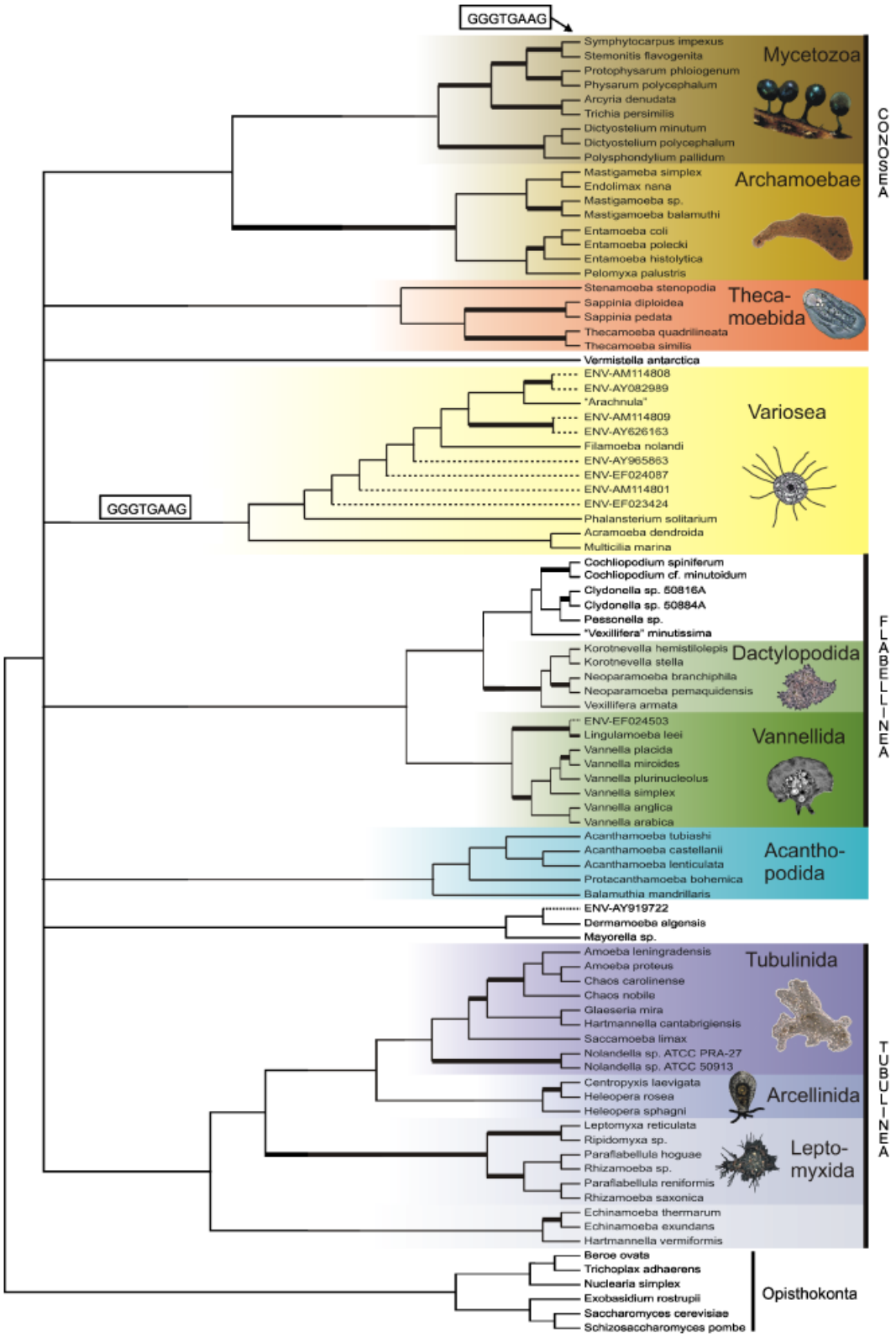
The most controversial question is the position of Foraminifera. In the SSU rDNA trees, Foraminifera were placed either close to Haplosporidia and Gromiida (Berney, Fahrni, and Pawlowski 2004; Longet et al. 2004; Nikolaev et al. 2004) or as sister group to Polycystinea (Cavalier-Smith and Chao 2003b). They appeared as sister to polycystine-like and *Sticholonche*-like clones in a combined analysis of SSU and LSU rDNA data (Moreira et al. 2007), but with very small taxon sampling. Knowing the extreme acceleration of the foraminiferan stem lineage and the relatively rapid evolution of radiolarian SSU rDNA sequences, this result could well be an artifact of long-branch attraction. The best evidence for the position of Foraminifera close to Haplosporidia, *Gromia*, and *Filoreta* clades is the presence of a polyubiquitin insertion in all these taxa and its apparent absence in Radiolaria (Bass et al. 2005). However, the grouping of one of two foraminiferan actin paralogs with the actin of Polycystinea (Tekle et al. 2007) further complicates the situation.

The clade Radiolaria (Radiozoa in Cavalier-Smith 1987) is entirely composed of various types of radiolarians, including Polycystinea (Spumellarida, Nasselarida, Collodaria), Acantharea, and Taxopodida. Phylogenetic studies of this clade are based exclusively on the SSU rDNA sequences (Amaral Zettler and Caron 2000; Amaral Zettler, Anderson, and Caron 1999; Amaral Zettler et al. 1997; Kunitomo et al. 2006; Lopez-Garcia, Rodriguez-Valera, and Moreira 2002; Polet et al. 2004; Takahashi et al. 2004; Yuasa et al. 2006). The only protein-coding genes available for Radiolaria are three sequences of actin (Nikolaev et al. 2004) and five polyubiquitin sequences (Bass et al. 2005). The relationships shown in our tree (Fig. 2) are similar to those obtained by Yuasa et al. (2006) and Kunitomo et al. (2006). We could recover the monophyly of Polycystinea (except *Larcopyle* that branches within Taxopodida); however, only the relations between Nasselarida and Collodaria are strongly supported. As in most rhizarian clades, good support is found for each taxonomic group (with the exception of Taxopodida), but the relationships among these groups remain unresolved.

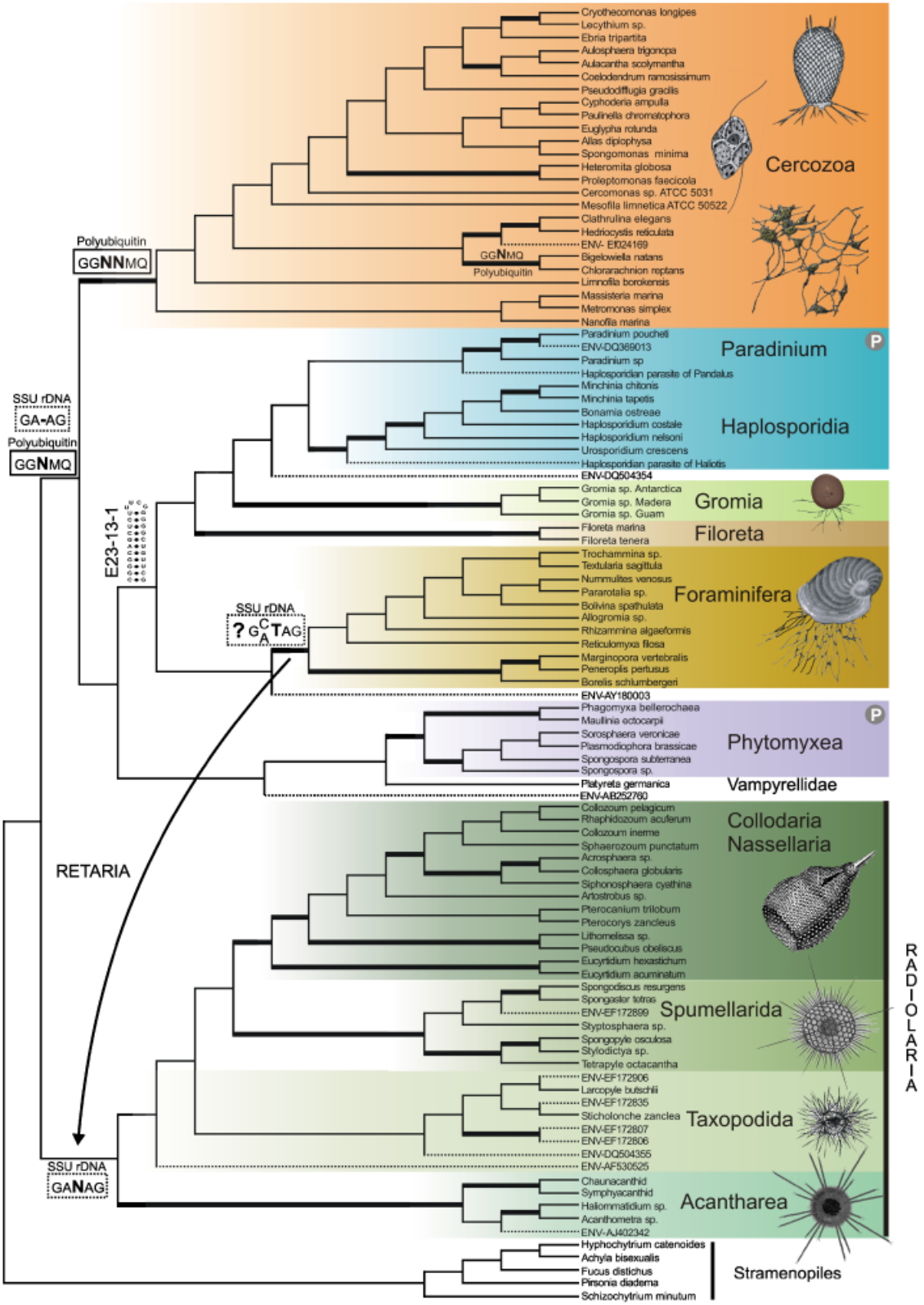
Fig. **1.** Phylogeny of Amoebozoa. Maximum likelihood small subunit rDNA tree showing the current knowledge for the evolutionary relationships between and within the main groups of Amoebozoa. Tree inferred from 1,298 aligned positions and a GTR+I+G8 model of nucleotide substitutions, obtained with the program TREEFINDER (Jobb, von Haeseler, and Strimmer 2004), and subsequently schematized by hand to better emphasize the confidences and uncertainties (see text). A RAxML (Stamatakis 2006) tree was also obtained with the same alignment and differed by the branching of Mycetozoa within Variosea (not shown). Thick branches denote bootstrap support $>90\%$. The conserved motif of eight nucleotides in the variable region V7 (GGGTGAAG) is indicated on the branches where it is found. Drawings were adapted from the following sources: Thecamoebida, Variosea, and Leptomyxida (Micro*scope, http://starcentral.mbl.edu/microscope/portal.php?pagetitle=index), Mycetozoa, Archamoebida, and Tubulinea (http://www.unige.ch/sciences/biologie/biani/msg/Amoeboids/Eukaryotes.html), Flabelinea (Alexander Kudryavtsev).

IMPORTANCE OF ENVIRONMENTAL SEQUENCING

Small subunit rDNA-based analyses of environmental DNA samples revealed an extraordinarily large and hidden diversity of protists in all types of examined habitats (reviewed in Epstein and Lopez-Garcia 2008). Dawson and Pace (2002) have proposed that some environmental sequences even represent novel eukaryotic



lineages, but a careful analysis shows that most of the sequences can be placed into one of the existing supergroups of eukaryotes (Berney et al. 2004; Cavalier-Smith 2004). Some of these sequences have been characterized as belonging to Amoebozoa or Rhizaria. Although their proportion is relatively small compared with other groups of eukaryotes, they significantly increase the



taxon sampling in some amoebozoan and rhizarian groups and help to resolve their phylogenies by filling the evolutionary gaps that exist between identified species. A good example is the case of the amoebozoan clade Variosea. Composed of flagellate and amoeboid species, this clade is considered as crucial for the placement of the root of the amoebozoan tree (Nikolaev et al. 2006). However, in most SSU rDNA analyses, Variosea appeared as a series of independent lineages branching at the base of Conosea (Nikolaev et al. 2006; Tekle et al. 2008). It was only after adding five new environmental sequences that the monophyly of Variosea was recovered (Fig. 2), in agreement with the signature in SSU rDNA present in all members of this clade.

The importance of environmental sequencing for revealing hidden diversity is particularly tangible in the case of Cercozoa, Radiolaria, and monothalamous Foraminifera. The environmental surveys of Cercozoa using specific polymerase chain reaction (PCR) primers revealed nine novel cercozoan clades and 168 distinct lineages (Bass and Cavalier-Smith 2004), some of global geographic distribution (Bass et al. 2007). The study of picoeukaryotic diversity in the Sargasso Sea revealed five new radiolarian clades, of which two are related to Taxopodida that were known until then only on the base of one described species *Sticholonche zanclea* (Not et al. 2007). Finally, the foraminiferal-specific environmental surveys revealed two new clades of freshwater species (Holzmann et al. 2003) and a large diversity of monothalamous foraminiferans (Habura et al. 2004, 2008). Strikingly, some rhizarian groups (i.e. Haplosporidia, Phytomyxea, Foraminifera) are preceded by the divergence of single environmental sequences (Fig. 2). Future isolation and characterization of the eukaryotic lineages to which these sequences belong will certainly provide important information about the ancestors of these groups and the evolutionary changes that have permitted their speciation.

The environmental sequence data, however, must be interpreted with caution. Polymerase chain reaction amplification of SSU rDNA gene frequently produces chimeric sequences that are not always easy to detect (Berney et al. 2004). Therefore, all sequences that slightly differ from well-established clades should be carefully checked for the presence of chimeras using programs such as CHECK CHIMERA (Larsen et al. 1993). On the other hand, it is well known that taxonomic composition of environmental surveys is strongly biased by PCR conditions. For example, it is rare to find amoebozoans (except Variosea) in environmental sequences and almost impossible to amplify foraminiferan SSU rDNA with typical ''universal'' eukaryotic primers. Hence, the generally low environmental diversity for some Amoebozoa and Rhizaria groups is probably artifactual, and reveals the need to search for other genomic markers (perhaps among mitochondrial genes) to obtain a better view of the diversity of these groups.

ADVANTAGES AND LIMITATIONS OF SSU rDNA PHYLOGENIES

Almost all that we know about the phylogeny of Amoebozoa and Rhizaria is based on the SSU rDNA sequences. This sum of evidence based on a single molecular character is certainly the most important handicap of amoebozoan and rhizarian phylogenies. Nevertheless, the SSU rDNA possesses several obvious advantages that make it the most commonly used phylogenetic marker. First, due to an elevated number of homogenous copies and the presence of highly conserved regions, SSU rDNA is undeniably the most easily amplified nuclear gene. This is particularly important for those amoeboid protists, like the foraminiferans and radiolarians, which can hardly be cultivated and have to be amplified from single cell extractions. This is also one of the reasons why almost all environmental surveys of protists are based solely on SSU rDNA sequences.

Another advantage of the SSU rDNA is the presence of conserved and variable regions that enable recovery of phylogenetic relationships at different taxonomic levels. Of particular interest are the conserved motifs that can be defined as phylogenetic signatures. Some of them have been used to design specific amplification primers, like the AAC insertion in foraminiferan stem 33 (Pawlowski 2000). Others are used to define larger phylogenetic groupings, for example the GA-AG deletion in Cercozoa (Cavalier-Smith and Chao 2003b) or the stem 23-13-1 defining the clade of *Gromia*+Haplosporidia+''*Corallomyxa*'' (Tekle et al. 2007) (Fig. 2). Another putatively important conserved motif of eight nucleotides (GGGTGAAG), not yet described in the literature, can be found in the variable domain V7 of all representatives of the class Variosea, including the environmental sequences, but not in other Amoebozoa, except the myxogastriid *Symphytocarpus impectus* (Fig. 1). Although this motif cannot be used in phylogenetic analyses because of the lack of homologous regions in other amoebozoans, its significance is certainly more important than the weak bootstrap support for Variosea in SSU rDNA-based trees. Further discovery of such motifs in other groups of amoeboid protists may give a yet unexploited source of phylogenetic information.

The main weakness of the SSU rDNA is the heterogeneity of substitution rates. The amoeboid protists seem particularly affected by this phenomenon. For instance, an extraordinary acceleration characterizes the stem lineage of Foraminifera (Pawlowski and Berney 2003). As a consequence, this group was for a long time excluded from phylogenetic reconstructions of eukaryotes and its position is still highly controversial (Moreira et al. 2007). Exceptional rate variations have also been observed between and within foraminiferan groups (De Vargas and Pawlowski 1998; Pawlowski et al. 1997). Fast evolving species are also common in Amoebozoa, in particular among the pelobionts, entamoebids, and myxomycetes. The most spectacular acceleration is observed within the amoebozoan genus *Trichosphaerium* (Pawlowski and Fahrni 2007; Tekle et al. 2007), which is thus not included in our analyses. In this genus, 69 out of 609 SSU rDNA sites conserved in almost all ($>95\%$) amoebozoans are modified, rendering its accurate placement practically impossible, even with the best methods and models.

Difficulties in placing the fast evolving amoebozoan and rhizarian species are only one of the drawbacks of SSU rDNA phylogenies. More generally there is a lack of overall support at

Fig. **2.** Phylogeny of Rhizaria. Maximum likelihood small subunit (SSU) rDNA tree showing the current knowledge for the evolutionary relationships between and within the main groups of Rhizaria. Tree inferred from 1,167 aligned positions and the GTRGAMMA model of nucleotide substitutions, obtained with the program RAxML (Stamatakis 2006), and subsequently schematized by hand to better emphasis the confidences and uncertainties (see text). Thick branches denote bootstrap support $>90\%$. The insertion in the polyubiquitin protein, deletion and E23-13-1 stem in the SSU rDNA gene are indicated on the branches where they are found. The letter ''P'' represents the parasitic lineages. An alternative branching pattern for Foraminifera, corresponding to the Retaria hypothesis, is represented by the arrow. Species names: *Mesofila limnetica* (formerly Dimorpha like); *Limnofila borokensis* (formerly *Gymnophrys cometa*); *Nanofila marina* (formerly N-Por) have been changed following Bass et al. (2008). Drawings were adapted from the following sources: Cercozoa (Jahn, Bovee, and Jahn 1979; Taylor 1990, John Archibald, pers. commun.), Radiolaria (Haeckel 1862), *Gromia* and Foraminifera (photos of the authors).

different phylogenetic levels, especially for deep branches of SSU rDNA trees. Among Amoebozoa, only the Acanthopodida and Myxogastria are supported by more than 95% bootstrap values (Fig. 1). The situation is slightly better among Rhizaria (Fig. 2), but in both cases the relationships between major clades remain largely unresolved. Therefore, although the SSU rDNA sequences will remain extremely valuable as first indicators of phylogenetic affinities, the inferred SSU rDNA-based phylogenies should be considered with a lot of caution.

NEW CHALLENGES FOR FUTURE PHYLOGENOMIC STUDIES

Because the SSU rDNA phylogenies cannot reliably resolve all relationships between amoeboid protists, it is absolutely necessary to search for other molecular markers. As described above the number of protein-coding genes available for Amoebozoa and Rhizaria is very limited. There are also only few genomic data available for members of both supergroups. Among Amoebozoa, the genomes of *E. histolytica* and *Dictyostelium discoideum* have been sequenced (Eichinger et al. 2005; Loftus et al. 2005), and those of some other entamoebids, dictyostelids, and *A. castellanii* are in progress. EST data are available for *Mastigamoeba balamuthi* (Bapteste et al. 2002), *H. vermiformis*, *Physarum polycephalum*, *Hyperamoeba dachnaya*, and *Hyperamoeba* sp. (Watkins and Gray 2008). Among Rhizaria, the *Bigellowiella natans* genome has been sequenced but not yet published (Archibald, pers. commun.) and a project to sequence the *Paulinella chromatophora* genome has been recently accepted (Yoon, pers. commun.). EST data are available for five rhizarians, including two foraminiferans *Reticulomyxa filosa* and *Quinqueloculina* sp., three cercozoans *Cercomonas*, *B. natans*, and *Gymnophrys* (Burki and Pawlowski 2006; Burki et al. 2007; Rodriguez-Ezpeleta et al. 2007), as well as a yet unpublished dataset for the reticulate amoeba *Filoreta* (Lewis, pers. commun.). Importantly, the recent development of high-throughput sequencing technologies, such as the 454 system, will lead to a massive increase of genomic data. Notably, several EST projects on amoebozoan and rhizarian taxa are in progress (e.g. *Gromia sphaerica*, *Plasmodiophora brassicae*, *Spongospora subterranean*, *Vannella* sp., etc.), in order to address important phylogenetic questions.

In the case of Amoebozoa, it is first essential to confirm their monophyly in a broadly sampled tree of eukaryotes, and if possible find a molecular synapomorphy for the group. The hypothetical position of the root of Amoebozoa between Conosea and other amoebae, as suggested in Nikolaev et al. (2006), needs to be tested. The monophyly of the major groups (Tubulinea, Flabellinea, Variosea) suggested by the SSU rDNA analyses should be confirmed and their relationships need to be established. The position of incertae sedis amoebozoans with fast evolving SSU sequences (e.g. *Trichosphaerium*) should be revised.

In the case of Rhizaria, the Retaria hypothesis urgently requires testing. As discussed above, this hypothesis is contradicted by some current SSU rDNA phylogenies (Fig. 2) as well as the absence of the polyubiquitin insertion in all tested radiolarian species. Furthermore, Radiolaria generally lack the cercozoan-specific SSU rDNA deletion. In Foraminifera, the site of the deletion is situated in a variable region, impeding any conclusion about its ancestral or derived character. It cannot be excluded that both Foraminifera and Radiolaria cluster together either as sister group to other rhizarians or within the rhizarian radiation. This question is particularly important given that Foraminifera and Radiolaria possess very old and well-preserved fossil records and their position is crucial to calibrate the tree of eukaryotes.

So far, phylogenomic studies have been extremely efficient in resolving the deep eukaryote phylogeny, answering important questions concerning the branching order among the supergroups (Burki et al. 2007, 2008; Hampl pers. commun.; Rodriguez-Ezpeleta et al. 2007). We expect that the coming genomic data will also be very useful for inferring intra-supergroups phylogenies. In many respects, the molecular study of Amoebozoa and Rhizaria has proven to be particularly demanding and therefore working with both groups constitutes a challenging test for the phylogenomic approach. Excitingly, despite the important advances in the phylogeny of Amoebozoa and Rhizaria reported in this review, our understanding of their evolution is still relatively poor and further progress will depend on access to much larger genomic database.

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