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Small-Subunit Ribosomal RNA Gene Sequences of Phaeodarea Challenge the Monophyly of Haeckel's Radiolaria

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In his grand monograph of Radiolaria, Ernst Haeckel originally included Phaeodarea together with Acantharea and Polycystinea, all three taxa characterized by the presence of a central capsule and the possession of axopodia. Cytological and ultrastructural studies, however, questioned the monophyly of Radiolaria, suggesting an independent evolutionary origin of the three taxa, and the first molecular data on Acantharea and Polycystinea brought controversial results. To test further the monophyly of Radiolaria, we sequenced the complete small subunit ribosomal RNA gene of three phaeodarians and three polycystines. Our analyses reveal that phaeodarians clearly branch among the recently described phylum Cercozoa, separately from Acantharea and Polycystinea. This result enhances the morphological variability within the phylum Cercozoa, which already contains very heterogeneous groups of protists. Our study also confirms the common origin of Acantharea and Polycystinea, which form a sister-group to the Cercozoa, and allows a phylogenetic reinterpretation of the morphological features of the three radiolarian groups.

Introduction

The radiolarians collected during the four-year cruise of HMS Challenger were classified by Ernst Haeckel (1887) into three groups: Acantharea, Phaeodarea, and Polycystinea (including Spumellarida and Nassellarida). The principal characteristics shared by these three groups are (1) the presence of a central capsule, delimited by a perforated layer described as the central capsular wall, and (2) the possession of axopodia. Axopodia are a feature that

Radiolaria sensu Haeckel and Heliozoa have in common, and for this reason they have traditionally been grouped together in the phylum Actinopoda (Hartmann 1913; Margulis et al. 1990; Melhorn and Rütthemann 1992; Penard 1904; Puytorac et al. 1987). However, several recent studies suggest that

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Nonstandard abbreviations: BV, bootstrap values; K-H, Kishino-Hasegawa; LBA, long branch attraction; ME, minimum evolution; ML, maximum likelihood; MP, maximum parsimony; S-H, Shimodaira-Hasegawa; SrSO₄, strontium sulphate; SSU rRNA, small subunit ribosomal RNA; TBR, tree-bisection-reconnection.

this phylum represents an artificial grouping of unrelated amoeboid protists (see, e.g., Febvre et al. 2000; Patterson 1999).

Haeckel's system of Radiolaria was challenged by cytological and ultrastructural studies. Schewiakoff (1926) separated Acantharea from other Radiolaria, due principally to differences in the composition of the skeleton of these groups, which was considered as a key taxonomic character. The skeleton of Acantharea is made out of strontium sulfate (SrSO_4) only, while, when present, it is siliceous in the case of Polycystinea (Anderson et al. 2000; Cachon et al. 1990) or composed of mixed silica and organic matter with traces of magnesium, calcium and copper in Phaeodarea (Cachon et al. 1990; Reschetjnak 1966; Takahashi and Anderson 2000). The three groups also differ by their central capsule. The capsular wall of phaeodarians, which is thick compared to other radiolaria, is generally perforated by only three pores (Anderson 1983; Cachon and Cachon 1973; Takahashi and Anderson 2000), while it contains numerous pores in acantharians and polycystines, through which the axopodia pass (Anderson et al. 2000; Cachon et al. 1990; Febvre et al. 2000). The pores in the central capsule of phaeodarians consist of one astropylum and usually two parapylae, situated at the opposite pole. The astropylum is the major perforation of the capsular wall and forms a cone-like opening, which connects the intracapsulum and the extracapsulum (the regions inside and outside the central capsule, respectively). Preys are captured by the axopodia and filopodia, and are digested either outside or inside the central capsule. For digestion within the intracapsulum, ingestion is made through the astropylum, which represents a complicated cytopharynx that is present neither in Acantharea, nor in Polycystinea (Cachon et al. 1990). Another characteristic feature of phaeodarians is the presence of a phaeodium, which consists of balls of darkly pigmented waste products, usually in the region of the astropylum (Anderson 1983; Cachon et al. 1990; Takahashi and Anderson 2000). These differences led some authors to separate the members of Radiolaria *sensu* Haeckel into three distinct groups (Lee et al. 2000; Riedel 1967; Takahashi and Anderson 2000).

The polyphyly of Radiolaria was suggested by the first analyses of molecular data, showing Acantharea near the base of the so-called eukaryotic crown, while Polycystinea branched below it (Amaral Zettler et al. 1997). This result was challenged by a recent study, which suggested that the position of Polycystinea in the analyses published by

Amaral Zettler et al. (1997) was artificial, due to the long branch attraction (LBA) phenomenon (López-García et al. 2002). The authors used two environmental small subunit ribosomal RNA (SSU rRNA) gene sequences to break the long stem branches of Polycystinea and Acantharea and to infer their common origin. The same result was also recovered in some other recent eukaryotic phylogenies based on maximum likelihood (ML) analysis of SSU rRNA gene sequences, although without statistical support (see, e.g., Fahrni et al. 2003; Pawlowski et al. 1999).

To test further the monophyly of Radiolaria, we obtained complete SSU rRNA gene sequences from three phaeodarians (*Aulacantha scolymantha*, *Aulosphaera trigonopa*, and *Coelodendrum ramosissimum*) belonging to three different orders: Phaeocystida, Phaeosphaerida, and Phaeodendrida, respectively. We compared them to other eukaryotic SSU rRNA gene sequences, including previously published sequences of Acantharea and Polycystinea, as well as four environmental sequences obtained by Moon-van der Staay et al. (2001), López-García et al. (2001), and López-García et al. (2002), and three new sequences of polycystines. Phylogenetic analyses allow us (1) to establish the position of Phaeodarea, which strongly branch among the recently described phylum Cercozoa, separately from Acantharea and Polycystinea, and (2) to confirm the common origin of acantharians and polycystines, which form a sister-group to the Cercozoa.

Results

We obtained six new complete SSU rRNA gene sequences for three phaeodarians (*A. scolymantha*, *A. trigonopa*, and *C. ramosissimum*) and three polycystines (*Collozoum inerme*, *Thalassicolla pellucida*, and *Thalassophysa pelagica*). The length of the obtained sequences ranges from 1779 (*T. pellucida*), 1784 (*T. pelagica*), and 1790 nucleotides (*C. inerme*) in the three polycystines to 1813 (*C. ramosissimum*), 1834 (*A. scolymantha*), and 1853 nucleotides (*A. trigonopa*) in the three phaeodarians. The G + C content of the SSU rRNA gene sequences of the three phaeodarians sequences averages 44%, while it is much lower in the polycystines, ranging from 36% to 39%. Our three new phaeodarean and three new polycystinean sequences were added to an alignment of 54 SSU rRNA gene sequences from diverse eukaryotes, including previously available sequences of Acantharea and Polycystinea and four environmental sequences. A total of 1147 unam-

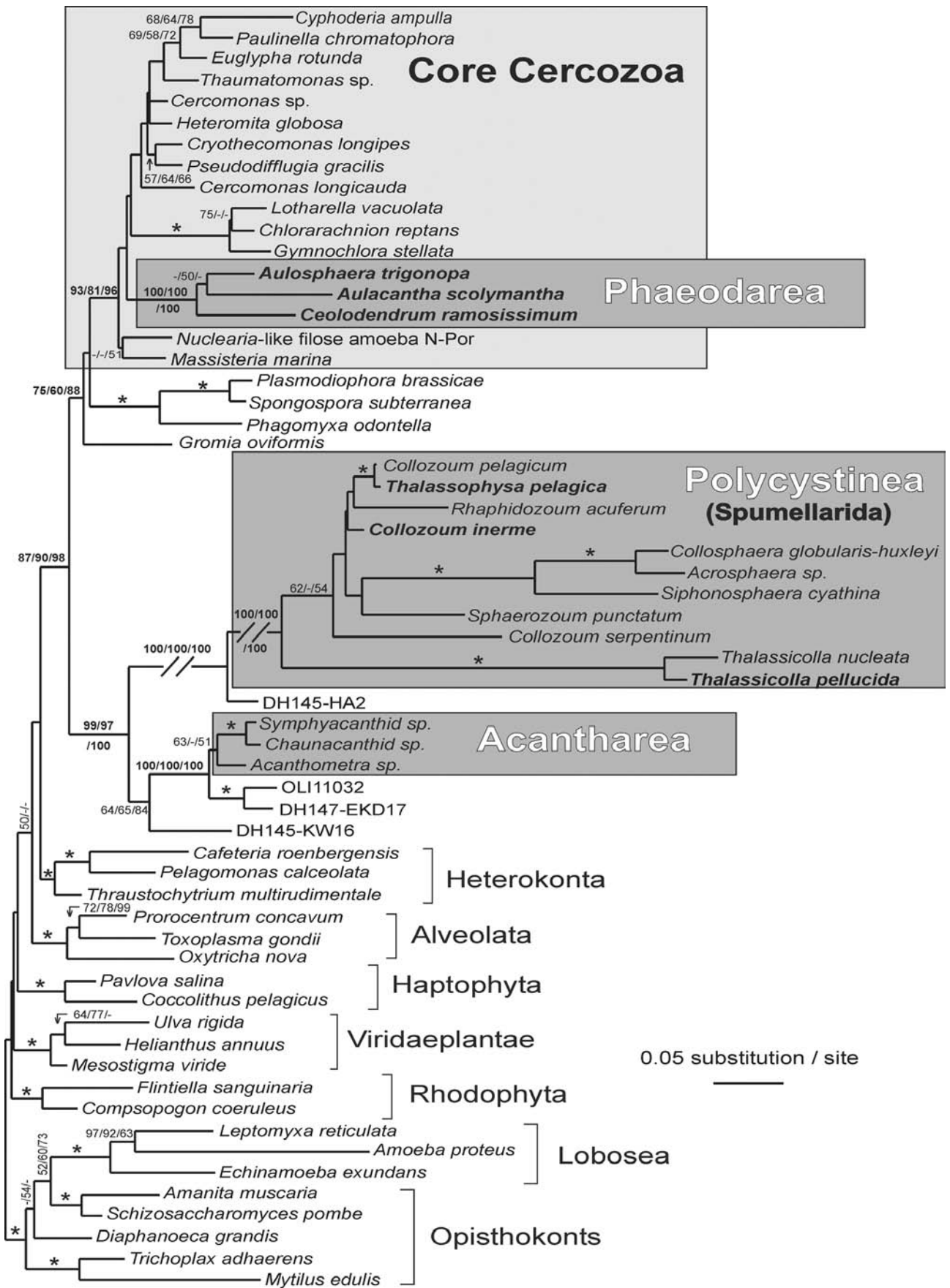
biguously aligned positions was selected for phylogenetic analyses, of which 429 were constant and 580 were parsimony-informative. A Chi-square test performed on our data set reveals a high homogeneity of base frequencies across taxa, except for Polycystinea. A relative rate test shows that the polycystinean sequences evolve significantly faster than the other taxa ($p = 10^{-7}$), while Acantharea and Phaeodarea do not show any significant rate variation.

Analysis of our data set shows that the monophyly of Phaeodarea is strongly supported, with 100% bootstrap values (BV) for all methods of tree reconstruction (Fig. 1). Phaeodarians branch among the phylum Cercozoa, separately from Acantharea and Polycystinea. The monophyly of Cercozoa, including Phaeodarea, is supported by BV of 75%, 60% and 88% for ML, maximum parsimony (MP) and minimum evolution (ME) analyses, respectively. Our analyses also show a close relationship between Acantharea, Polycystinea, and four environmental sequences (OLI11032, DH147-EKD17, DH145-KW16, and DH145-HA2), supported by BV of 99%, 97% and 100% for ML, MP, and ME analyses, respectively. Finally, all methods of tree reconstruction placed the Acantharea + Polycystinea clade as a sister-group to the Cercozoa, with relatively good support (BV of 87%, 90%, and 98% for ML, MP, and ME analyses, respectively).

The topology shown in Figure 1 is the result of an ML analysis using the general-time-reversible (GTR) model of substitution, taking into account a gamma-shaped distribution of the rates of substitution among variable sites, with 8 rate categories ($\alpha = 0.4088$). MP and ME analyses led to similar tree topologies, except for some changes in the branching order of Alveolata, Haptophyta, Heterokonta, Rhodophyta, and Viridiaeplantae (data not shown). Two environmental sequences (DH145-KW16 and DH145-HA2) are slightly shorter at the 3' end of the gene, and opisthokonts appear paraphyletic in Figure 1 because sites including specific opisthokont substitutions are lost. However, analysis of the complete gene after exclusion of these two incomplete sequences restores the monophyly of opisthokonts and, interestingly, shows that Acantharea and Polycystinea remain sister-groups with BV of 82%, 57%, and 92% for ML, MP and ME analyses, respectively (data not shown). To check the influence of the heterogeneous nucleotide composition of Polycystinea, a Logdet distance analysis was also performed. The resulting tree topology is similar to the one obtained with the GTR + G model (data not shown).

Because our analyses show sister-group relation between Cercozoa, including Phaeodarea, and the Acantharea + Polycystinea clade, and because polycystines evolve significantly faster than the other groups, it could not be excluded that Radiolaria might be monophyletic, and the topology shown in Figure 1 is the result of a LBA artifact. Therefore, we decided to test further the polyphyly of Radiolaria. First, a ML search was performed using a constrained tree topology imposing the monophyly of Acantharea, Polycystinea and Phaeodarea. The resulting best tree was tested against the non-forced topology shown in Figure 1 using the Kishino-Hasegawa (K-H) and the Shimodaira-Hasegawa (S-H) tests. Both tests revealed that the tree shown in Figure 1 has a significantly better likelihood than a tree constraining the monophyly of all Radiolaria ($p = 0.008$ for both tests). Then, we performed a ML analysis on our data set after exclusion of all polycystine sequences. The respective position of Phaeodarea and Acantharea in this ML tree did not change (data not shown), indicating that the tree topology in Figure 1 was not biased by LBA artifacts. Moreover, screening of our sequence alignment reveals that the three phaeodarean sequences share a cercozoan-specific deletion of one nucleotide in the terminal loop of helix 37 that is absent in all other eukaryotes, including acanthareans and polycystines (Cavalier-Smith and Chao 2003).

Phaeodareans clearly branch within the main lineage of Cercozoa (core Cercozoa), with BV of 93%, 81%, and 96% ML, MP, and ME analyses, respectively (Fig. 1). However, resolution within this clade is poor. To clarify the phylogenetic position of Phaeodarea inside core Cercozoa, a second data set composed only of the 21 cercozoan sequences included in our first alignment was designed. This allowed the use of 1439 unambiguously aligned positions, of which 863 were constant and 369 were parsimony informative. Analysis of this second data set led to a better resolution among the clade (Fig. 2). *Massisteria*, *Nuclearia*-like and chlorarachniophytes form the most basal branches of core Cercozoa, while Phaeodarea, together with the remaining 9 core cercozoan sequences, form a group supported by BV of 77%, 64%, and 64% for ML, MP, and ME analyses, respectively. Inside this group, Phaeodarea branch with the filose, testate amoeba *Pseudodiffugia* and the nanoflagellate *Cryothecomonas*, although their relation is not well supported (BV of 73%, 41%, and 89% for ML, MP, and ME analyses, respectively).



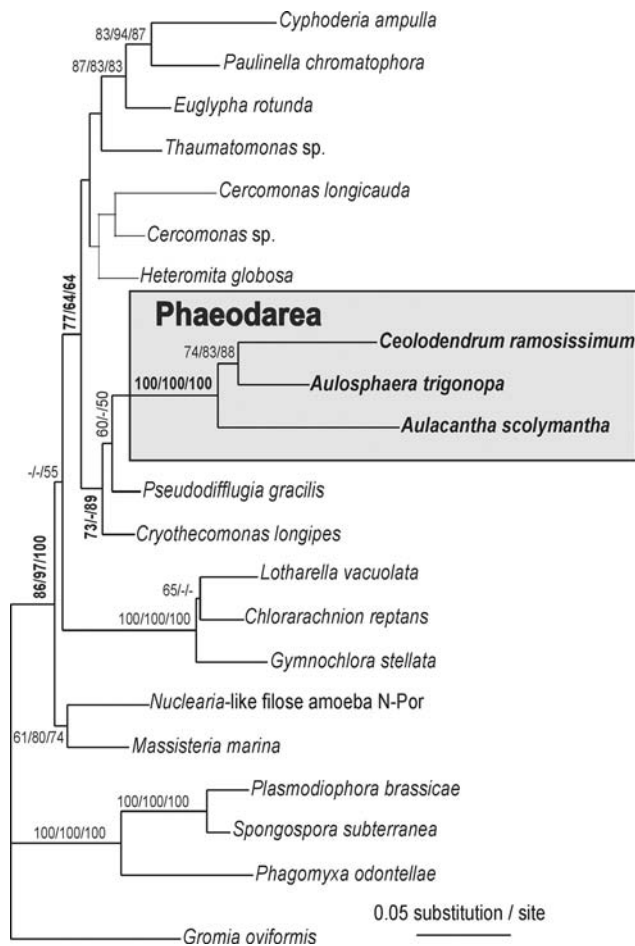


Figure 2. Phylogenetic relationships among Cercozoa inferred using the ML method with the GTR + G model; $\log(L) = -8833.3628$. The light grey box delimits Phaeodarea. Numbers at nodes represent percentages of bootstrap support greater than 50% following 100 (ML), 500 (MP), or 1000 (ME) data resamplings. The scale bar represents a distance of 0.05 substitution per site.

◀ **Figure 1.** Phylogenetic position of Phaeodarea among eukaryotes inferred using the ML method with the GTR + G model; $\log(L) = -18,034.49138$. The three lineages comprising Radiolaria *sensu* Haeckel are highlighted by dark grey boxes, while the light grey box delimits core Cercozoa. The tree was rooted on Opisthokonts + Lobosea, in agreement with a recent study (Stechmann and Cavalier-Smith 2003). Numbers at nodes represent percentages of bootstrap support greater than 50%, following 100 (ML), 1000 (MP), or 1000 (ME) data resamplings. Asterisks (*) indicate nodes supported by bootstrap values higher than 80% for all methods. The scale bar represents a distance of 0.05 substitutions per site. For more clarity, the stem branches leading to Polycystinea were reduced by half.

Discussion

Since Haeckel's monograph (1887), the monophyly of Radiolaria has been widely discussed on cytological, ultrastructural and molecular grounds (Amaral Zettler et al. 1997; Anderson 1983; Cachon et al. 1990; López-García et al. 2002; Schewiakoff 1926; Takahashi and Anderson 2000). By presenting the first molecular data on Phaeodarea, our study brings an important contribution to this discussion. Phylogenetic analyses based on the SSU rRNA gene show that our three sequences of phaeodareans clearly branch together within core Cercozoa, separately from Acantharea and Polycystinea (Fig. 1).

We assume that our phaeodarean sequences are authentic based on the fact that they group together in a broad agreement with classical taxonomy. Of course, this is not an independent evidence for the authenticity of the obtained sequences, especially in the case of uncultivated taxa such as Phaeodarea, for which molecular data did not exist previously. Ideally, the verification should be done by using *in situ* hybridization on electron microscope preparations, with sufficient resolution to identify cell organelles of the taxon in question. On the other hand, *in situ* hybridizations at optical microscopy level are not always reliable, because parasitic or endosymbiotic eukaryotes can be easily misidentified for their hosts. An example of such a misidentification was the publication of the first foraminiferan SSU rRNA gene sequence, including an "in situ verification" (Wray et al. 1995), shown later to be the sequence of some apicomplexan parasite (see, e.g., López-García et al. 2003). By using the phylogenetic approach to verify the sequences from new higher taxa of uncultivable protists, we assume that their potential parasites or endosymbionts would not follow the same phylogenetic relations as their hosts and they will not group within other well-known eukaryotic taxa.

To include Phaeodarea into Cercozoa (Fig. 1) enhances the morphological variability of this phylum, which notably includes the cercoconad flagellates, the chlorarachniophyte green algae, several filose testate amoebae, and the plasmodiophorid plant pathogens (Atkins et al. 2000; Bhattacharya and Oliveira 2000; Bhattacharya et al. 1995; Bulman et al. 2001; Burki et al. 2002; Cavalier-Smith and Chao 1997; Kühn et al. 2000; Vickerman et al. 2002). Interestingly, the observation of 40 specimens of *Aulacantha* and *Coelodendrum* undergoing sporogenesis revealed that the swarmers released during the reproduction of Phaeodarea form hundreds of plasmodial spheres, each of which is a polynucle-

ated, amoeboid cell, developing two flagella after division by schizogony (Cachon-Enjumet 1964). This feature is congruent with the widespread “cercozoan ability” to form amoeboid and/or flagellate cells at some or all stages of the life cycle (see, e.g., Nikolaev et al. 2003). Given the available SSU rRNA data, our results point to the filose, testate amoeba *Pseudodifflugia* and the nanoflagellate *Cryothecomonas* as the closest relatives of Phaeodarea (Fig. 2).

Our results also confirm that Polycystinea and Acantharea are closely related, as demonstrated by López-García et al. (2002). The hypothesis that Polycystinea could represent a fast-evolving lineage has already been advanced (Cavalier-Smith 1999). A relative rate test performed on our data set confirmed that the polycystinean sequences evolve significantly faster than the other sequences used in our analyses. As pointed out in several studies (Embley and Hirt 1998; Philippe and Adoutte 1998; Philippe et al. 2000), long-branched eukaryotes placed below the so-called eukaryotic crown in phylogenies rooted with distant outgroup sequences are probably highly derived groups, artifactually misplaced because of the LBA phenomenon. López-García et al. (2002) showed that by addition of environmental sequences, which break the long stem branches of Polycystinea and Acantharea, it is possible to retrieve a well-supported monophyletic origin of both groups. Our results show that in the absence of other long-branched taxa, the common origin of Acantharea and Polycystinea is recovered with good bootstrap support with (Fig. 1) and without (data not shown) these environmental sequences.

As expected, the three new sequences of Polycystinea we obtained cluster with already published sequences of the group, and two well-supported lineages emerge among polycystines in Figure 1: the family Collosphaeridae (*Acrosphaera*, *Collosphaera*, *Siphonosphaera*) and the family Thalassicollidae (*Thalassicola*). The family Sphaerozoidae (*Sphaerozoum*, *Collozoum*, *Raphidozoum*, and *Thalassophysa*) does not form a monophyletic unit, but the position of *Collozoum serpentinum* and *Sphaerozoum punctatum* changes depending on the method of tree reconstruction. MP analyses led to a monophyletic Sphaerozoidae (albeit with weak support), and the addition of a small number of sites in the analyses also re-establishes the monophyly of the family with ML and ME (data not shown). The lack of statistical support for the monophyly of Sphaerozoidae (Amaral Zettler et al. 1999) might be due to rate variations between the different sphaerozoid species. Interestingly, the po-

sition of *Thalassophysa pelagica*, which SSU rRNA gene sequence is very close to that of *Collozoum pelagicum*, seems to confirm an early hypothesis (Brandt 1902) that was recently pointed out (Amaral Zettler et al. 1999), considering the species *Thalassophysa sanguinolenta* as the solitary stage of *C. pelagicum*. Our results suggest that *Thalassophysa* might be the general name for a solitary stage of *Collozoum*. However, the genus *Collozoum*, including a new *Collozoum inerme* sequence, does not form a monophyletic unit in our trees, as previously shown (Amaral Zettler et al. 1999), and the taxonomic status of the species currently assigned to the genus *Collozoum* need re-examination, as suggested by Anderson et al. (1999).

By determining the respective position of Phaeodarea, Acantharea and Polycystinea, our study allows a reinterpretation of the morphological features of the three lineages. Interestingly, a characteristic that seems to be a reliable phylogenetic marker regarding the distinction of the three groups is the ability to secrete SrSO₄. Acantharians build their entire skeleton of SrSO₄ (Febvre 1990; Febvre et al. 2000), while adult vegetative polycystines belonging to the order Spumellarida as well as their swimmers contain individual crystals of SrSO₄ (Hollande and Martoja 1974). In contrast, neither adult phaeodarians nor their released swimmers can secrete SrSO₄ (Cachon et al. 1990). The presence of a sophisticated central capsule in Phaeodarea is also a distinctive characteristic that is not shared by Acantharea and Polycystinea. Furthermore, phaeodarians lack endosymbionts, while acantharians and polycystines can bear algal symbionts, providing photosynthetic products that are ingested as a nutritional source (Anderson 1983, Anderson et al. 2000). Another important feature of Phaeodarea that is not shared with Acantharea and Polycystinea is the absence of cross-bridges between the microtubules of their axopodia (Cachon and Cachon 1973). This axopodial structure, unique among the whole group of Actinopoda, resembles the system of granuloreticulopodia in the Foraminifera (Cachon et al. 1990), a group that has also been shown to be closely related to Cercozoa (Archibald et al. 2003; Berney and Pawlowski 2003; Keeling 2001; Longet et al. 2003). This suggests that the ability to secrete SrSO₄, to bear symbionts, and the organisation of microtubules inside axopodia are more reliable phylogenetic markers for radiolarians than the possession of a central capsule and the simple presence of axopodia, two features that apparently evolved more than once.

Methods

Materials: Living specimens of Phaeodarea and Polycystinea were collected at Villefranche-sur-Mer (Mediterranean Sea) in November-December 2002, using a planktonic net. Polycystine specimens included *Collozoum inerme*, *Thalassophysa pelagica*, and *Thalassicolla pellucida*, all of them belonging to the order Spumellarida (Amaral Zettler et al. 1999; Anderson et al. 2000; Cachon et al. 1990; Hollande and Enjumeat 1960). Phaeodarean specimens belong to three different orders: *Aulacantha scolymantha* to Phaeocystida, *Aulosphaera trigonopa* to Phaeosphaerida, and *Coelodendrum ramosissimum* to Phaeodendrida (Takahashi and Anderson 2000). Three colonies of *C. inerme* and several individual central capsules of all phaeodareans ($n = 3-150$), *T. pelagica* ($n = 3$), and *T. pellucida* ($n = 3$) were isolated immediately after collection and identification. All of them were carefully washed individually in order to avoid any contamination prior to processing.

DNA extraction, amplification, cloning, and sequencing: DNA was extracted using the DNeasy Plant Minikit (Qiagen, Basel, Switzerland). Two μ l of DNA extract was added to each PCR reaction. PCR amplifications were done in a total volume of 25 or 50 μ l with an amplification profile consisting of 35 cycles with 30 s at 95 °C, 1 min at 50 °C, and 2 min at 72 °C, followed by 5 min at 72 °C for the final extension. The amplified PCR products were purified using High Pure PCR Purification Kit (Roche, Rotkreuz, Switzerland), then ligated into pGEM-T Vector System (Promega, Wallisellen, Switzerland), cloned in XL-2 Ultracompetent Cells (Stratagene, Basel, Switzerland), sequenced with the ABI-PRISM

Big Dye Terminator Cycle Sequencing Kit, and analyzed with an ABI-377 DNA sequencer (Perkin-Elmer, Rotkreuz, Switzerland), all according to each manufacturer's instructions. The complete SSU rRNA genes of the phaeodarians and polycystines were amplified in two overlapping fragments, using combinations of the primers described in Table 1, and for each PCR product, three to five clones were sequenced. The new sequences reported in this paper have been deposited in the GenBank database (accession numbers AY266292 through to AY266297).

Phylogenetic analyses: Our six new complete SSU rRNA gene sequences were manually aligned with sequences from 54 diverse eukaryotes using the Genetic Data Environment software (Larsen et al. 1993), following the secondary structure models proposed by Neefs et al. (1993) and Wuyts et al. (2000). Sequences were chosen so that the main groups of eukaryotes were represented, avoiding particularly fast evolving taxa. Species names, taxonomic position, and GenBank accession number of all the sequences used in our analyses are given in Table 2. Sequence alignments used in this study are available on request from the corresponding author. Phylogenetic trees were inferred using the ML method (Felsenstein 1981), the MP method, and the ME method. The reliability of internal nodes was assessed using the bootstrap method (Felsenstein 1985), with 100, 1000, and 1,000 replicates for ML, MP, and ME analyses, respectively. All analyses were performed using PAUP* version 4.0b10 (Swofford 1998). ML analyses were performed using the GTR model of substitution (Lanave et al. 1984; Rodriguez et al. 1990), taking into account a gamma distribution of the rates of substitution among vari-

Table 1. Sequences of all the primers used in this study.

Primer name	Sequence	Specificity	Direction	Position ^a
sA	5' - GGT TGA TYC TGC CAG W - 3'	universal	forward	6
s12pc	5' - AGR TAT TAR TAT TTT RKC G - 3'	Polycystinea	forward	874
s12.2	5' - GAT YAG ATA CCG TCG TAG TC - 3'	universal	forward	994
s12.2pcr	5' - CCT AGC TGA TAT AGT TTA CAG - 3'	<i>C. inerme</i>	reverse	1038
s13.1ph	5' - ACC CAG ACG CTT ACG GTT TCC - 3'	<i>A. scolymantha</i>	reverse	1097
s13.2ph	5' - CCG GAA CYT AAA AGC TTA CGA - 3'	<i>A. trigonopa</i> + <i>C. ramosissimum</i>	reverse	1102
s15.1pc	5' - CAK CCA CAA AAT CAG GAC AGG - 3'	<i>T. pellucida</i>	reverse	1261
s17.1pc	5' - AAT GTA CTA TTT AGT AAG TGA TAG - 3'	<i>T. pelagica</i>	reverse	1349
sB	5' - TGA TCC TTC TGC AGG TTC ACC TAC - 3'	universal	reverse	1792

^aBeginning of the primer based on the sequence of *Collozoum pelagicum* (GenBank accession number AF091146).

Table 2. List of the SSU rRNA gene sequences analysed in this study.

Species Name	Taxonomic Position	Accession Numbers
<i>Aulacantha scolymantha</i> ^a	Phaeodarea	AY266294
<i>Aulosphaera trigonopa</i>	Phaeodarea	AY266292
<i>Coelodendrum ramosissimum</i>	Phaeodarea	AY266293
<i>Acanthometra</i> sp.	Acantharea	AF063240
<i>Chaunacanthid</i> sp.	Acantharea	AF018158
<i>Symphycanthid</i> sp.	Acantharea	AF063242
DH145-HA2	Environmental	AF382824
OLI11032	Environmental	AJ402342
DH147-EKD17	Environmental	AF290072
DH145-KW16	Environmental	AF382825
<i>Collozoum inerme</i>	Polycystinea	AY266295
<i>Collozoum pelagicum</i>	Polycystinea	AF091146
<i>Collozoum serpentinum</i>	Polycystinea	AF018162
<i>Raphidozoum acuferum</i>	Polycystinea	AF091147
<i>Sphaerozoum punctatum</i>	Polycystinea	AF018161
<i>Thalassophysa pelagica</i>	Polycystinea	AY266296
<i>Acrosphaera</i> sp.	Polycystinea	AF091148
<i>Collosphaera globularis-huxleyi</i>	Polycystinea	AF018163
<i>Siphonosphaera cyathina</i>	Polycystinea	AF091145
<i>Thalassicolla nucleata</i>	Polycystinea	AF057744
<i>Thalassicolla pellucida</i>	Polycystinea	AY266297
<i>Oxytricha nova</i>	Alveolata	X03948
<i>Prorocentrum concavum</i>	Alveolata	Y16237
<i>Toxoplasma gondii</i>	Alveolata	U12138
<i>Phagomyxa odontellae</i>	Cercozoa	AF310904
<i>Plasmodiophora brassicae</i>	Cercozoa	U18981
<i>Spongospora subterranea</i>	Cercozoa	AF310899
<i>Gromia oviformis</i>	Cercozoa	AJ457811
<i>Massisteria marina</i>	Cercozoa	AF174369
<i>Nuclearia</i> -like filose amoeba N-Por	Cercozoa	AF289081
<i>Chlorarachnion reptans</i>	Cercozoa	U03477
<i>Gymnochlora stellata</i>	Cercozoa	AF076171
<i>Lotharella vacuolata</i>	Cercozoa	AF054890
<i>Pseudodifflugia gracilis</i>	Cercozoa	AJ418794
<i>Cryothecomonas longipes</i>	Cercozoa	AF290540
<i>Cercomonas longicauda</i>	Cercozoa	AF101052
<i>Cercomonas</i> sp.	Cercozoa	U42449
<i>Heteromita globosa</i>	Cercozoa	U42447
<i>Thaumatomonas</i> sp.	Cercozoa	U42446
<i>Euglypha rotunda</i>	Cercozoa	X77692
<i>Paulinella chromatophora</i>	Cercozoa	X81811
<i>Cyphoderia ampulla</i>	Cercozoa	AJ418793
<i>Coccolithus pelagicus</i>	Haptophyta	AJ246261
<i>Pavlova salina</i>	Haptophyta	L34669
<i>Cafeteria roenbergensis</i>	Heterokonta	AF174364
<i>Pelagomonas calceolata</i>	Heterokonta	U14389
<i>Thraustochytrium multirudimentale</i>	Heterokonta	AB022111
<i>Amoeba proteus</i>	Lobosea	AJ314604
<i>Echinamoeba exundans</i>	Lobosea	AF293895
<i>Leptomyxa reticulata</i>	Lobosea	AF293898
<i>Amanita muscaria</i>	Opisthokonts	AF026631
<i>Schizosaccharomyces pombe</i>	Opisthokonts	X58056
<i>Diaphanoeca grandis</i>	Opisthokonts	L10824
<i>Trichoplax adhaerans</i>	Opisthokonts	L10828
<i>Mytilus edulis</i>	Opisthokonts	L33448
<i>Flintiella sanguinaria</i>	Rhodophyta	AF168621
<i>Compsopogon coeruleus</i>	Rhodophyta	AF087128
<i>Helianthus annuus</i>	Viridiaeplantae	AF107577
<i>Mesostigma viride</i>	Viridiaeplantae	AJ250108
<i>Ulva rigida</i>	Viridiaeplantae	AJ005414

^a Sequences in bold were obtained in this study.

able sites, with 8 rate categories. All necessary parameters were estimated from the data set using MODELTEST (Posada and Crandall 1998). Starting trees of ML searches were obtained via BioNJ (Gascul 1997), and then swapped using the tree-bisection-reconnection (TBR) algorithm. The most parsimonious trees for MP analyses were determined using a heuristic search procedure with 20 random-addition-sequence replicates and TBR branch-swapping. All characters were equally weighted and the transition-transversion ratio was set to 3:2. ME analyses were performed with the same model and parameters values as ML analyses, using the BioNJ algorithm. A constrained tree topology was designed using TreeView (Page 1996). K-H (Kishino and Hasegawa 1989) and S-H (Shimodaira and Hasegawa 1999) tests were performed with PAUP*. The homogeneity of base frequencies across sequences was evaluated using TREE-PUZZLE (Strimmer and von Haeseler 1996). Relative rate tests were performed with the program RRtree (Robinson-Rechavi and Huchon 2000) using Kimura's 2 parameters model of substitution (Kimura 1980).

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