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# Phylogenetic position of the small solitary phaeodarians (Radiolaria) based on 18S rDNA sequences by single cell PCR analysis

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**Abstract**

Within the holoplanktonic protists group Radiolaria, the Class Phaeodarea is today represented by several hundreds species. The phaeodarian skeletons consist of opaline silica as well as organic matter and are very fragile and vulnerable to dissolution. Their tests are therefore rarely found in the fossil records; this has caused uncertainty with regard to their phylogenetic evolution. In this study, small, solitary phaeodarian species, namely, *Protocystis xiphodon* (Haeckel), *Challengeron diodon* Haeckel and *Conchellium capsula* Borgert were examined using molecular techniques in order to clarify the phylogenetic position of the Phaeodarea. The phylogenetic trees obtained from the neighbor-joining, maximum-parsimony and maximum-likelihood methods of analysis showed that all phaeodarians formed a monophyletic group within the Phylum Cercozoa. This result contradicts Haeckel's classical taxonomy, wherein the phaeodarians were grouped along with the polycystines, i.e., nassellarians and spumellarians, and the acantharians under the common name "Radiolaria". Within the cercozoan clade, the Phaeodarea were closely related to the euglyphid and pseudodifflugid testate amoebae and the desmothoracid heliozoans. The tests and skeletons of both the phaeodarians and the euglyphid testate amoebae resemble each other in their chemical composition and construction. The similarities in the morphologic features may suggest that they are sisters as the Phylum Cercozoa in which leading from molecular methods.


*Keywords:* Phaeodarea; Radiolaria; 18S rDNA; testate amoebae; Cercozoa; molecular phylogeny

## 1. Introduction

Haeckel's taxonomic classification of Radiolaria published in 1881 and 1887, based on material collected by the H.M.S. Challenger Expedition (1873–1876), was the first comprehensive work on radiolarians. This classification was built upon similarities and differences in skeletal elements and structures of radiolarians (e.g., test geometry, number of segments or concentric spheres, and presence, number and shape of spines, etc.). Based on the structure of the central capsule,

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Haeckel (1887) classified radiolarians into four main groups, namely, Acantharia, Phaeodaria, Spumellaria and Nassellaria (the latter two are presently grouped under Polycystinea). He suggested that these four groups were monophyletic.

Haeckel's system has been the major source of information on radiolarian diversity and taxonomy and is still used as the principal taxonomic framework despite its potentially artificial character. In the last few decades, attempts at making a more natural taxonomic system have resulted in the following two approaches: (1) a paleontological classification system based on phylogenetic lineages documented by fossil evidence (e.g., Riedel, 1967; Riedel and Sanfilippo, 1986) and (2) a biological classification based on cytological and fine structural studies, particularly, with regard to the location and form of the axoplast versus the nucleus, and the distribution pattern of the axopodia (e.g., Holland and Enjumet, 1960; Cachon and Cachon, 1972a,b). Unfortunately, the paleontological approach cannot be applied to the Acantharea and the Phaeodarea, because the chemical composition and/or porous structure of their skeletons differ from those of polycystines. The acantharian and phaeodarian skeletons are less resistant to dissolution in marine sediments (e.g., Erez et al., 1982; Takahashi, 1991). Acantharians and phaeodarians are generally absent in deep-sea sediments and sedimentary rocks (e.g., Takahashi et al., 1983). This has caused much uncertainty with regard to their phylogenetic evolution. Molecular phylogenetic studies may provide important information and/or improvements regarding the taxonomy and evolutionary history of the Radiolaria.

Recent molecular studies (Amaral Zettler et al., 1997; López-García et al., 2002; Nikolaev et al., 2004; Takahashi et al., 2004; Yuasa et al., 2005) based on small-subunit ribosomal DNA (18S rDNA) sequences have resolved some of these taxonomic conflicts among the Radiolaria. These studies suggested that the Polycystinea and the Acantharea form a monophyletic group, that is, both share a common evolutionary ancestor (López-García et al., 2002; Takahashi et al., 2004). Furthermore, the Phaeodarea, which have traditionally been included in the Radiolaria, branch off within the Phylum Cercozoa (Polet et al., 2004). Therefore, the Phaeodarea do not form a monophyletic group with the Acantharea and the Polycystinea (Polet et al., 2004).

These results contradicted the widely accepted taxonomic system based on classical morphologic data and are very interesting from the point of view of the taxonomy and phylogeny of Radiolaria. However, to confirm the conclusions of the molecular studies and reinforce the reconstructed molecular phylogeny, additional information regarding the cytology, skeletal morphology and stratigraphic occurrences in continuous records of Radiolaria need to be collected.

Our effort here is to clarify the phylogenetic position of the Phaeodarea based on their 18S rDNA sequences and morphologic features. In this study, we examined three small, solitary species of the Phaeodarea using the single-cell PCR method and compared them with the sequences from the other eukaryotes.

## 2. Materials and methods

### 2.1. Sampling

Samples were collected from the following three sites (Fig. 1). Site 990528 (26°37′N, 127°47′E) is located in the East China Sea, approximately 5 km northwest of Okinawa Island, southernmost Japan. Samples were collected from the surface seawater (up to 3 m depth) at this site on May 29 and September 17, 2001, using a plankton net (net opening, 60 cm; mesh size, 37 μm). Site 031008 (38°05′N, 138°10′E) is located in the Japan Sea, approximately 6 km west of Tassha, Sado City, Sado Island, central Japan. Samples were collected from this site on November 8, 2003. Subsurface tows with a 100 μm mesh net of the Marukawa type with an opening of 30 cm diameter were carried out. Site 031027 (61°12′32″N, 7°05′55″E) is located at Sogndalsfjord, a tributary fjord of Sognefjord, southwestern Norway. Plankton samples were collected on October 27, 2003. The site is located in the deepest part of Sogndalsfjord, just off the biological field station at Skjæret. The water depth at this site is 270 m. A Nansen plankton net with an opening of 30 cm diameter and a mesh size of 45 μm was used. The net was lowered to 210 m and slowly pulled towards the surface. A messenger was dropped to 110 m and the net was closed at 100 m.

At all sites, the collected plankton samples were put in jars, placed in buckets containing ice and brought to the laboratory. Radiolarians were then separated using an inverted microscope and a binocular stereomicroscope. The radiolarians were isolated, identified to the species level and transferred to culture dishes containing filtered seawater until they were processed for 18S rDNA region amplifications.

### 2.2. Materials

In this paper, we examined three solitary phaeodarian species: *Protocystis xiphodon* (Haeckel) from the East

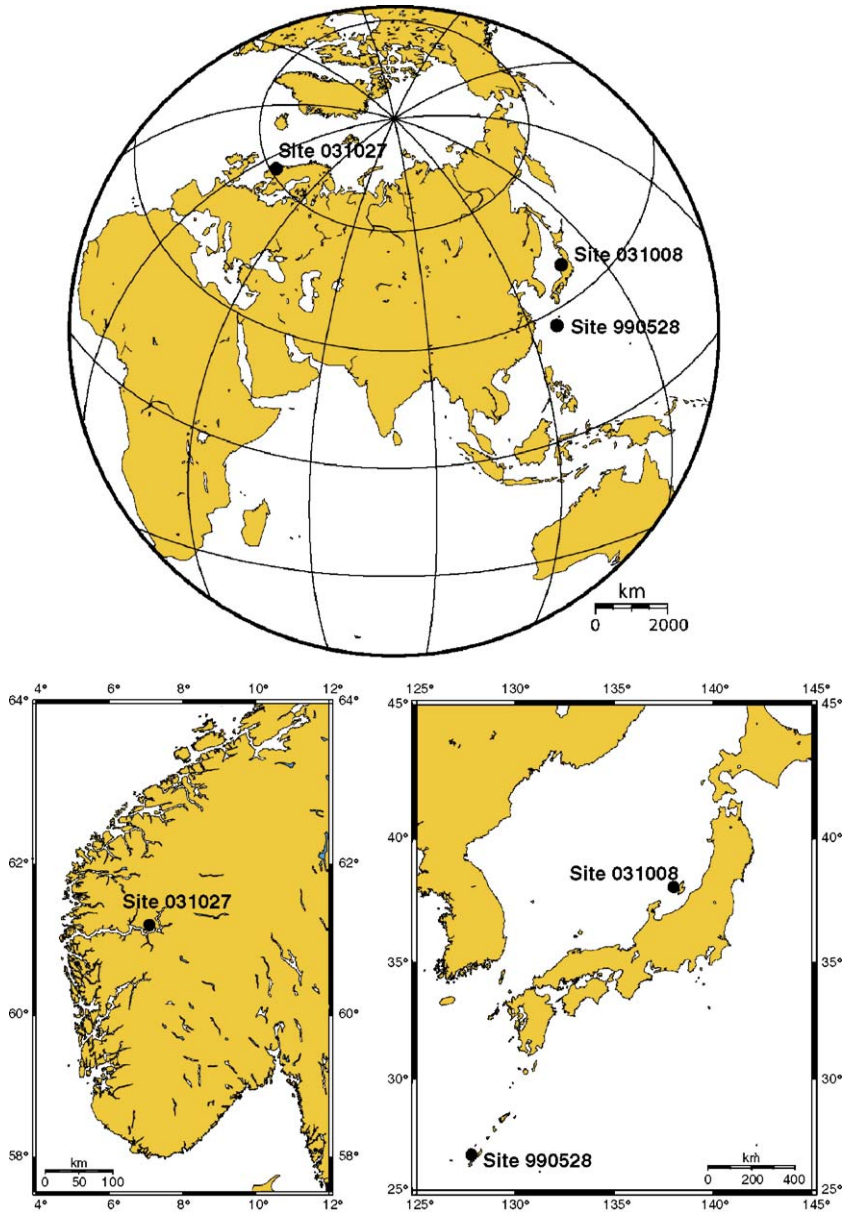


Fig. 1. Map showing location of the Site 990528 (26°37′N, 127°47′E) in the East China Sea, Site 031008 (38°05′N, 138°10′E) in the Japan Sea and Site 031027 (61°20′N, 7°00′E) at Sogndalsfjord, Norway.

China Sea, *Challengeron diodon* Haeckel from Sogndalsfjord in Norway and *Conchellium capsula* Borgert from the Japan Sea.

*P. xiphodon* (Haeckel) (Family Challengeriidae, Order Phaeogromida) was collected in Okinawa, the East China Sea. It has an elongated, laterally compressed test (Fig. 2a). The height of the test is approximately 100 μm (not including the peristome) and the diameter is slightly larger, tapering towards the peristome. The peristome is located on the dorsal side of the oral opening and has a single, three-sided vertical tooth approximately 50 μm long. The margin of the oral opening is enforced with a smooth collar-like rim that is connected to the peristome. The test has a fairly smooth surface, covered with small pores with a hexagonal frame, approximately four pores are contained within a length of 10 μm. This species is rare and found along the west coast of Norway (Jørgensen, 1900; Bjørklund, 1974), from the tropical part of the Atlantic to the north coast of Iceland (Cleve, 1899). It is also reported to be

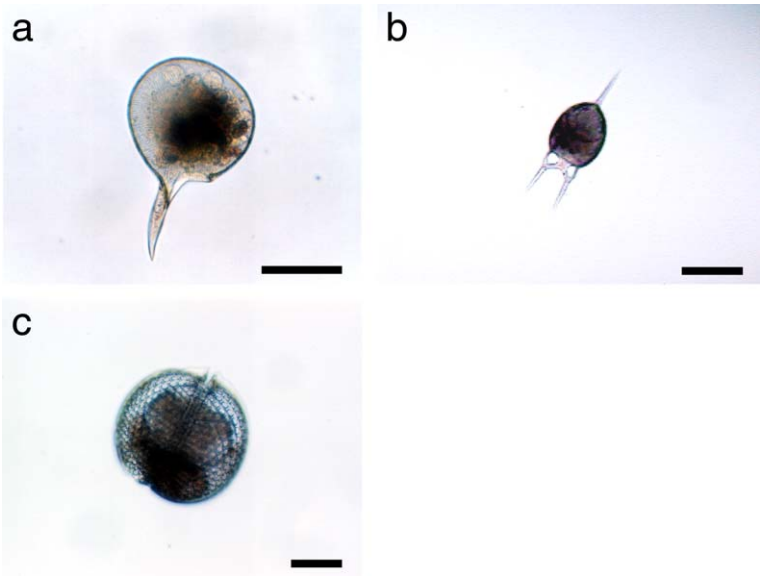


Fig. 2. Light micrographs (LM) of the phaeodarians used in this study. Scale bars indicate 50 μm. (a) *Protocystis xiphodon* (Haeckel) from the East China Sea. (b) *Challengeron diodon* Haeckel from the Sogndalsfjord, Norway. (c) *Conchellium capsula* Borgert from the Japan Sea.

found in the North Sea, the West and East Greenland Current, the Labrador Current, and in the Mediterranean (Borgert, 1901) and Japan Sea. This species has a wide distribution, probably cosmopolitan.

*C. diodon* Haeckel (Family Challengeriidae, Order Phaeogromida) was collected from Sogndalsfjord, western Norway. It has an ovate or egg-shaped, slightly compressed test, approximately 83 μm long and 74 μm in breadth (Fig. 2b). At the oral pole on the dorsal side, there is a short, broad, collar-shaped peristome, approximately twice as broad as long. The peristome is one fourth of the test's diameter, inclined obliquely over the large oral opening. On either side of the peristome, two large chambered loops are present. Each loop, about 15 μm high, is furnished with an about 40 μm non-chambered needle shaped spine. At the proximal end, there is a large (ca. 40 μm) non-chambered, needle-shaped apical spine. Occasionally, two or more smaller (ca. 10 μm), needle-shaped secondary spines are located at the apical end on the surface of the oval (egg-shaped) sphere. *C. diodon* is common in the plankton found in the photic zone of the fjords (Bjørklund, 1974; Swanberg and Bjørklund, 1987). Jørgensen (1900) reported this species to be frequent, but always sparse along the western coast of Norway. It is also reported to be found in the North Sea, the Labrador Sea, the southern part of the Atlantic and the Mediterranean Seas (Borgert, 1901), and in deep waters of a part of the Pacific Sea (Haeckel, 1887).

*C. capsula* Borgert (Family Conchariidae, Order Phaeoconchida) was collected from Niigata, in the Japan Sea. It has a latticed, nearly spherical test approximately 220 μm in diameter (Fig. 2c). The test is composed of two thick, hemispherical perforated valves of almost the same size and with aboral hinges. The pores on the surface are circular and regularly arranged. The rims of the both valves have conical dentate edges with an oral split between them. On each side of the valves, there are 10–15 conical teeth of the same length.

### 2.3. DNA extraction and amplification

Individual organisms were transferred through three filtered seawater washes and the central capsule was physically separated from the ectocytoplasm by a sterilized razor blade in order to eliminate prey items (food and waste vacuoles that constitute the phaeodium). The central capsule was rinsed twice in distilled water and then it was then incubated with 0.2 μg/μl Proteinase K solution at 37 °C for 30 min. The solution was used for the PCR reaction. The PCR amplification was carried out using the eukaryotic specific forward primer 90F (Hendriks et al., 1989), 5′-GAAACTGC-GAATGGCTCATT-3′, and the reverse primer B (Medlin et al., 1988), 5′-CCTTCTGCAGGTTCACC-TAC-3′, using the following protocol: a 3 min at 95 °C denaturing step, followed by 35 cycles consisting of 1 min denaturing at 95 °C, 2 min annealing at 55 °C and 3 min extension at 72 °C. The amplified PCR products

Table 1
Eukaryotic 18S rDNA sequences with accession numbers (including Acantharea, Phaeodarea, Polycystinea and environmental samples) used in this study

| | Taxon | Accession number |
|---|---|---|
| Acantharea | Arthracanthid 206 | AF063239 |
| | Chaunacanthid sp. 218 | AF018158 |
| | *Haliommatidium* sp. | AF018159 |
| Alveolata | *Blepharisma americanum* | M97909 |
| | *Oxytricha nova* | X03948 |
| | *Symbidionium* sp. | M88509 |
| | *Theileria annulata* | M64243 |
| Amoebozoa | *Acanthamoeba castellanii* | AF251938 |
| | *Glaeseria mira* | AY294146 |
| | *Hartmannella cantabrigiensis* | M95168 |
| Apusozoa | *Amastigomonas bermudensis* | AY050178 |
| | *Amastigomonas mutabilis* | AY050182 |
| | *Athelia bombacina* | M55638 |
| Cercozoa | *Chlorarachnion reptans* | X70809 |
| | *Euglypha rotunda* | X77692 |
| | *Gromia oviformis* | AJ457811 |
| | *Hedriocystis reticulata* | AY305010 |
| | *Pseudodifflugia* cf. *gracilis* | AJ418794 |
| Environmental samples | DH145-HA2 | AF382824 |
| | DH145-KW16 | AF382825 |
| | DH147-EKD17 | AF290072 |
| Glaucophyta | *Cyanophora paradoxa* | X68483 |
| | *Guillardia theta* | X57162 |
| Haplosporidia | *Urosporidium crescens* | U47852 |
| Haptophyta | *Emiliania huxleyi* | M87327 |
| | *Pleurochrysis carterae* | AJ246263 |
| Heliozoa | *Chlamydaster sterni* | AY268042 |
| | *Pterocystis* sp. JJP-2003 | AY268043 |
| | *Raphidiophrys ambigua* | AY305008 |
| | *Sticholonche zanclea* | AY268045 |
| | Undetermined centrohelid JJP-2003 | AY268041 |
| Phaeodarea | *Aulacantha scolymantha* | AY266294 |
| | *Aulosphaera trigonopa* | AY266292 |
| | *Challengeron diodon* | AB218765 |
| | *Coelodendrum ramosissimum* | AY266293 |
| | *Conchellium capsula* | AB218766 |
| | *Protocystis xiphodon* | AB218767 |
| Polycystinea | *Collozoum inerme* | AY266295 |
| | *Collozoum pelagicum* | AF091146 |
| | *Dictyocoryne profunda* | AB101540 |
| | *Dictyocoryne truncatum* | AB101541 |
| | *Eucyrtidium hexagonatum* | AB179735 |
| | *Pterocorys zancleus* | AB179736 |
| | *Thalassicolla nucleata* | AY266297 |
| Rhodophyta | *Compsopogon coeruleus* | AF087128 |
| | *Porphyridium aerugineum* | AF168623 |
| | *Stylonema alsidii* | AF168633 |
| Stramenopiles | *Cafeteria roenbergensis* | L27633 |
| | *Fucus distichus* | M97959 |
| | *Labyrinthuloides minuta* | L27634 |
| | *Ochromonas danica* | M32704 |
| Viridiplantae | *Chloromonas actinochloris* | AJ410445 |
| | *Mesostigma viride* | AJ250108 |
| | *Volvox carteri* f. *nagariensis* | X53904 |

were purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). These were then ligated into pGEM-T Easy Vector System (Promega) and cloned in *Escherichia coli* JM109 Competent Cells (Promega). Sequencing was performed using a Shimadzu DSQ2000L (Shimadzu) with Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Biosciences).

### 2.4. Alignment

The three obtained sequences were aligned with 54 other sequences of polycystine, acantharian, phaeodarian and other eukaryotes that were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) using ClustalW ver. 1.81 (Thompson et al., 1994). The accession numbers of all the sequences used in our analyses are given in Table 1. The alignment was manually refined using the nucleotide sequence editor Se-Al ver. 1.0a1 (Andrew, 1995). Regions with gaps were excluded from the analyses, resulting in an alignment with 1257 positions. The complete sequence alignment file of this study is available with the authors.

### 2.5. Phylogenetic analyses

Phylogenetic trees were generated using the neighbor-joining (NJ) method (Saitou and Nei, 1987), maximum-parsimony (MP) method (Eck and Dayhoff, 1966) and maximum-likelihood (ML) method (Felsenstein, 1981) with a PAUP∗ version 4.0b10 (Swofford, 2002). The NJ analyses were applied to distances that were corrected for unequal transition and transversion rates using the Hasegawa, Kishino and Yano (HKY) model (Hasegawa et al., 1985). The MP analyses were conducted using heuristic analyses (100 replicates), the tree bisection-reconnection (TBR) algorithm and random orders of sequence addition. The ML analyses were performed using the HKY model of nucleotide substitution. We initially obtained a topology by the NJ method, used it as the starting tree and ran heuristic searches with TBR branch-swapping for more detailed searching of the ML topology. Relative levels of support for nodes of the NJ, MP and ML trees were assessed by calculating full heuristic bootstrap proportion (BP) values (Felsenstein, 1985) based on 1000 replicates in NJ analyses and 100 replicates each in the MP and ML analyses.

## 3. Results

The 18S rDNA sequences for the following three phaeodarian species have been determined: *C. diodon*,

*C. capsula* and *P. xiphodon*. These have been deposited in GenBank under accession numbers AB218765, AB218766 and AB218767, respectively. The length in base pairs and G+C% (G+C content) of the 18S rDNA used in this study were as follows: *C. diodon* 1859 bp, 44.4% GC; *C. capsula*, 1745 bp, 43.5% GC; *P. xiphodon*, 1758 bp, 44.6% GC.

Fig. 3a, b and c show the NJ, MP and ML reconstructed trees of major eukaryotic groups including those of the sequences determined in our study (for a total of 54 sequences). Bootstrap values of only >50% are shown. In our phylogenetic trees, the phaeodarian sequences that we obtained were shown as a monophyletic group along with the other large phaeodarian

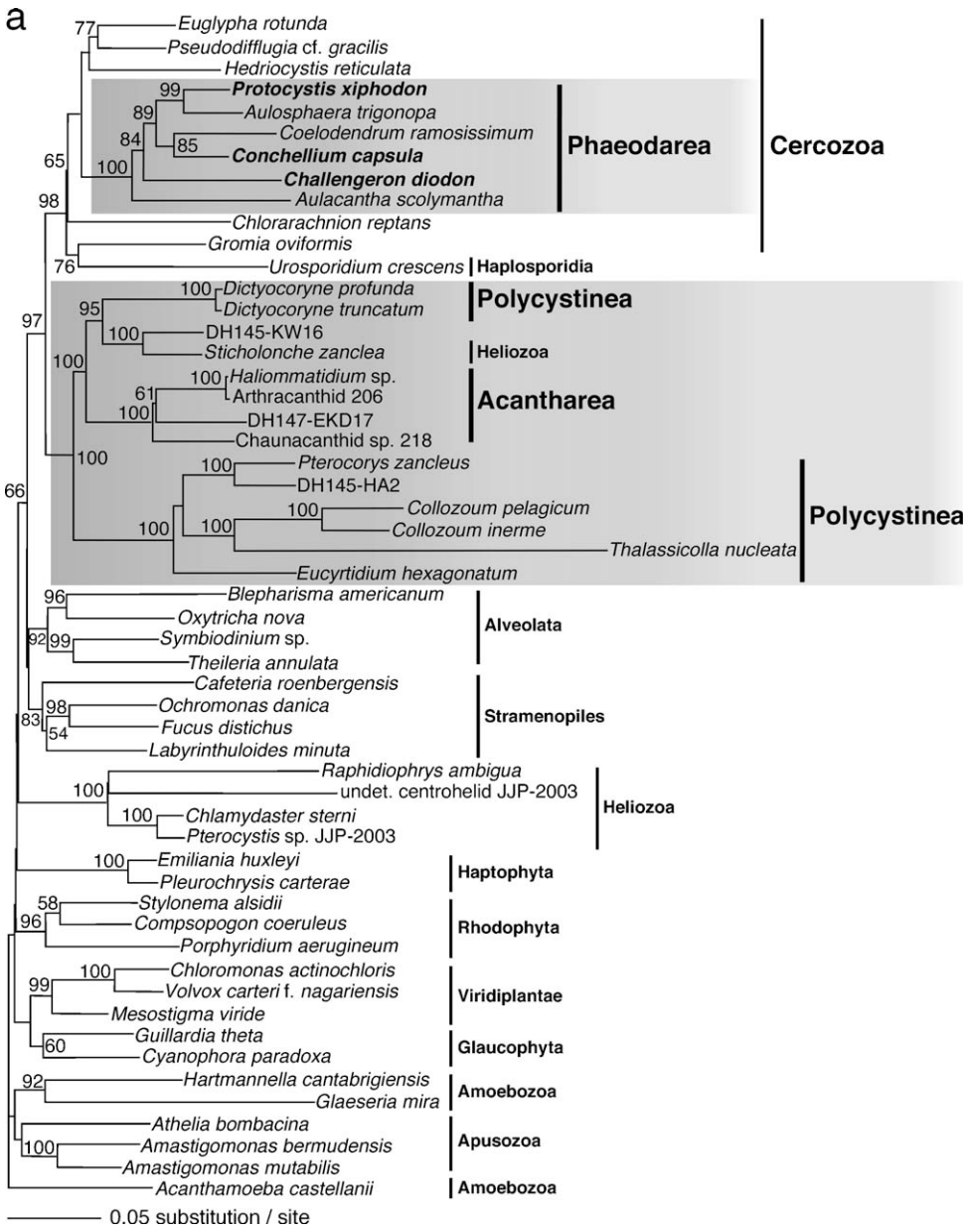


Fig. 3. Phylogenetic trees constructed from analyses of 18S rDNA sequences (54 taxa, 1257 nucleotide sites). Other information is already mentioned in the methods section. Bootstrap values greater than 50% are shown. (a) Neighbor-joining (NJ) tree constructed from the distances estimated by the HKY model (ts/tv ratio=1.229426). (b) Maximum-parsimony (MP) tree (consensus of three trees). (c) Maximum-likelihood (ML) tree (log-likelihood=−25,667.46808) on the HKY model (ts/tv ratio=1.229426).

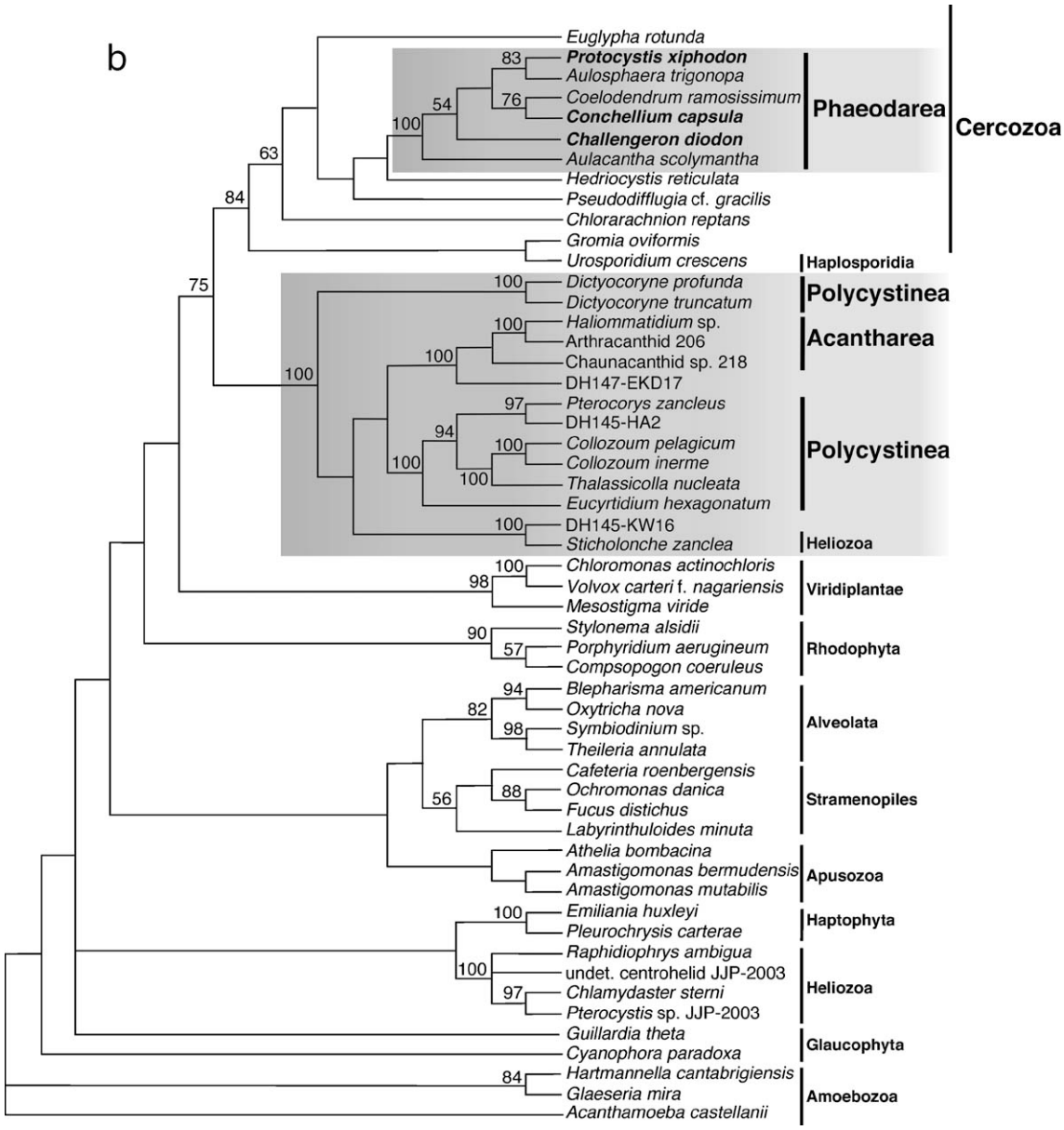


Fig. 3 (*continued*).

species of *Aulacantha scolymantha*, *Aulosphaera trigonopa* and *Coelodendrum ramosissimum* reported by Polet et al. (2004). The monophyly was supported with 100% bootstrap values in the NJ, MP and ML analyses. Furthermore, the phaeodarian group branched among the Phylum Cercozoa and formed sister groups to *Euglypha rotunda* (euglyphid testate amoebae), *Pseudodifflugia* cf. *gracilis* (pseudodifflugid testate amoebae) and *Hedriocystis reticulata* (heliozoans). The branching point for the Phylum Cercozoa including the phaeodarians was supported by 98%, 84% and 97% bootstrap values in the NJ, MP and ML analyses, respectively.

The other radiolarians, the Polycystinea and the Acantharea, formed monophyletic group with *Sticholonche zanclea* (taxopodid Heliozoa), and the clade emerged as sister to the cercozoan clade, supported by bootstrap values of 97%, 75% and 85% in the NJ, MP and ML methods, respectively. However, the branching pattern of the Polycystinea, the Acantharea and the

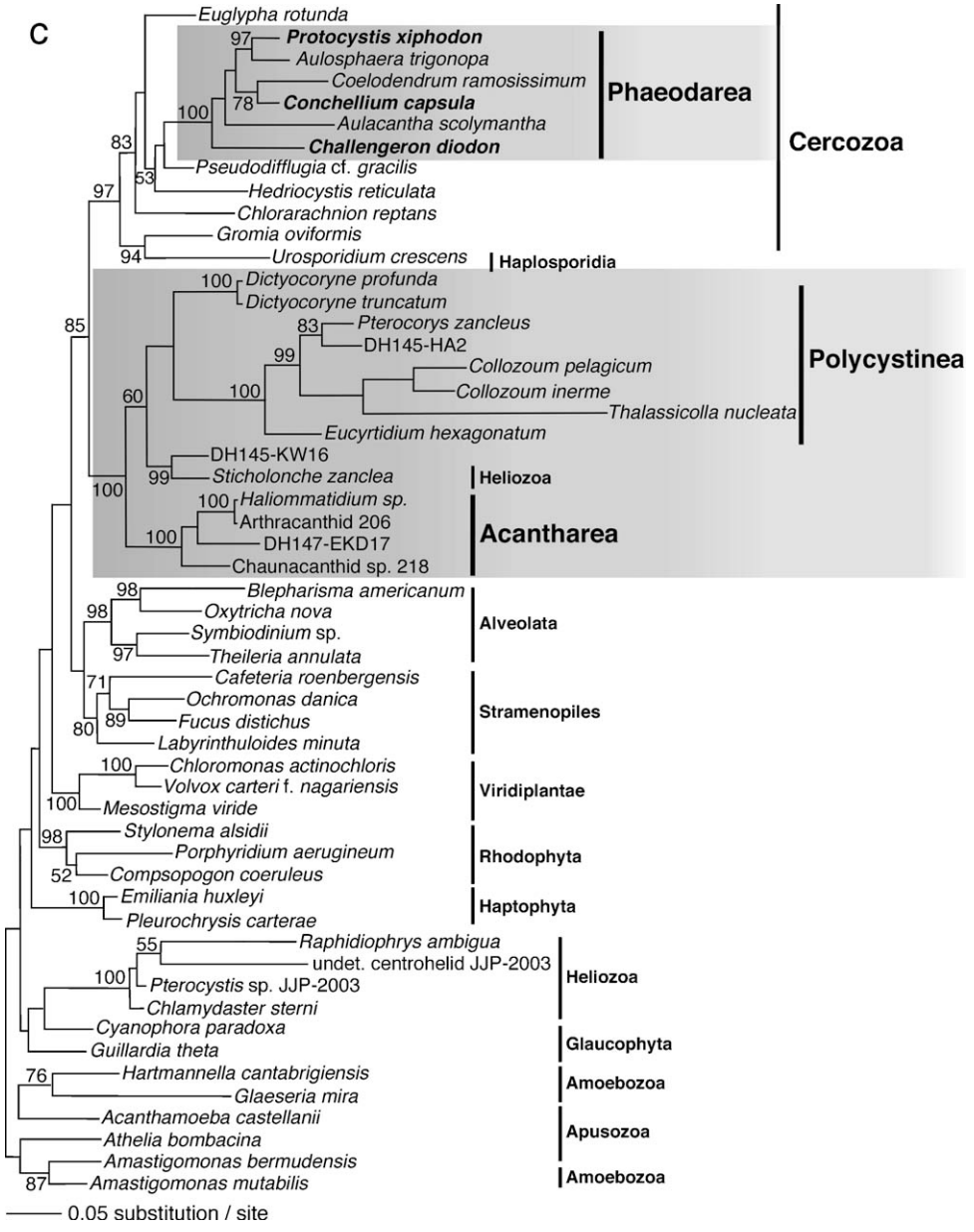


Fig. 3 (*continued*).

taxopodid Heliozoa obtained from these different phylogenetic analyses did not show a common topology.

## 4. Discussion

At present, the polycystines, the acantharians and the phaeodarians are usually classified under Actinopoda (axopod-bearing protozoa) along with the heliozoans due to radiating axopods (and filopods) that are common to all (e.g., Levine et al., 1980; Anderson, 1983; De Wever et al., 2001). The three former groups are distinguished from the heliozoans in having a central capsule that divides the cell into an intracapsulum (containing the endocytoplasm with nuclei, axoplast, mitochondria, Golgi bodies, vacuoles, etc.) and an extracapsulum (containing the ectocytoplasm with a variety of cytoplasmic structures, and symbionts, if present). The central capsule wall has perforations and/or intervening fissures that permit communication between the endocytoplasm and the ectocytoplasm (Anderson, 1983; Cachon et al., 1990).

The Phaeodarea can be separated from the two other two radiolarian groups by the following characteristics: (1) the presence of a central capsule with only three apertures. There is no evidence of thin slits penetrating the capsular wall as in the Polycystinea (Swanberg et al., 1986); (2) the incapacity to secrete strontium sulfate. The Acantharea possess the ability of fixing strontium sulfate in their tests (Anderson, 1983), while the polycystine tests have only traces of $SrSO_4$ (Stanley, 1973); (3) the lack of symbionts (e.g., Takahashi and Anderson, 2002). Many Acantharea and Polycystinea harbor algal symbionts–the so-called Zooxanthella–within their bodies (Anderson, 1983); (4) the absence of cross bridges between the microtubules of their axopodia (Cachon and Cachon, 1973); (5) the presence of digestive vacuoles (phaeodium) within the specialized vacuolar regions of the intracapsulum of the Phaeodarea, which differs from the arrangement in the polycystine Radiolaria (Swanberg et al., 1986).

The present results of the NJ, MP and ML analyses of the 18S rDNA sequences strongly supported the phylogenetic position of the Phaeodarea within the Phylum Cercozoa, separated from the Acantharea and the Polycystinea. This result is in agreement with the data of Polet et al. (2004); however, it contradicts the current taxonomy of "Radiolaria". The above-mentioned five cytoplasmic features of the Phaeodarea possibly reinforce the arguments for the taxonomic separation of the Phaeodarea from the Acantharea and the Polycystinea supported by molecular data.

The protozoan Phylum Cercozoa was established as a result of a molecular phylogenetic study by Cavalier-Smith (1998). This study embraces numerous ancestrally biciliate zooflagellates, euglyphid and other filose testate amoebae, Heliozoa, chlorarachnean algae, phytomyxean plant parasites, the animal-parasitic Ascetosporea and *Gromia* (Bhattacharya et al., 1995; Wylezich et al., 2002; Cavalier-Smith and Chao, 2003; Nikolaev et al., 2004). Within the cercozoan clade, the Phaeodarea were closely related to the euglyphid and pseudodifflugid testate amoebae and the desmothoracid heliozoans (see Fig. 3a, b and c).

Several genera from both the lineages have mineralized tests. The euglyphid testate amoebae are, for example, characterized by overlapping (imbricate) silica scales, which are usually arranged in longitudinal rows (Meisterfeld, 2000). These silica scales vary in shape, size and arrangement from species to species. For example, the test of the recent species *Cyphoderia ampulla* is composed of circular or oval, flattened silica scales, which are arranged in diagonal rows (e.g., Meisterfeld, 2000), while the Precambrian euglyphid testate amoebae, *Melicerion poikilon*, have a porous test with regularly distributed honeycomb-like holes that vary in size (Porter et al., 2003).

The phaeodarians are also characterized by having a delicate, silica test with different characteristics. This may be expressed in amphorae (amphora-shaped pores possessing honeycomb features), alveolated surfaces and tubular configurations. Furthermore, the morphological characteristics of the phaeodarian test, for example, the general shapes, the direction of the oral openings and the teeth-like oral equipment, are also very similar to the euglyphid testate amoebae. Some taxa of the testate amoebae also have funnel-shaped apertures or large apertural collars (e.g., psammonobiotid testate amoebae) and denticulate mouth plates (e.g., euglyphid testate amoebae) as in the Phaeodarea. Thus, the tests and also the skeletons of both the phaeodarians and the euglyphid testate amoebae resemble each other in their chemical composition and construction. The similarities of the morphologic features between the Phaeodarea and the euglyphid and the other testate amoebae may suggest that they are sisters as the Phylum Cercozoa in which leading from molecular methods.

Haeckel's use of skeletal elements and characteristic feature of the central capsule in the classification of radiolarians may have given an unnatural and non-hierarchical system. Moreover, as long suspected by most protistologists, the presence or absence of a silica test is less fundamental than many other cellular properties, namely, a silica-secreting ability that appeared independently at various phylogenetic positions (e.g., Danelian and Moreira, 2004). In the absence of an understanding of their biology, we have often accepted practical but sometimes inappropriate criteria for classifying the Radiolaria. Further information from ultrastructural and the molecular biological studies may help in correctly grouping the taxa. A more thorough examination of their protein-coding genes will be needed.

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