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Molecular Diversity of Alveolates Associated with Neritic North Atlantic Radiolarians

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Seven species of polycystine radiolarians and one phaeodarian species were investigated in order to determine the diversity of their associate organisms and their species specificity. Twelve partial 18S ribosomal DNA (rDNA) sequences were obtained showing a high diversity of associates, both within spumellarian and nassellarian radiolarians and among species. Two of the sequences obtained are highly similar to *Scrippsiella*, a dinoflagellate genus already reported as a symbiont of polycystine radiolarians. Nine of the new 18S rDNA sequences group with various alveolates. Some of these groups include parasites, such as the lethal endoparasite *Amoebophrya*, while others consist of non-annotated novel organisms found worldwide in various types of marine environments. We also obtained a sequence from a bacillariophyten highly similar to the 18S rDNA of the diatom species *Diatoma tenue*, which may derive from radiolarian food. Additionally, this is the first study to report on a phaeodarian associate.

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Introduction

Polycystine radiolarians (including spumellarians and nassellarians) and phaeodarians are single celled, heterotrophic planktonic protists that thrive in marine water masses. They are widely distributed in the ocean and are found throughout the entire water column. Both polycystine radiolarians and phaeodarians are characterized by having a central capsule that divides the protoplasm into an

endoplasm and ectoplasm (Anderson 1983a; De Wever et al. 2001), and this was until recently one of the most important criteria for grouping them together under Radiolaria. However, 18S ribosomal DNA (rDNA) studies have shown that the phaeodarians in fact belong to Cercozoa, a diverse clade lacking distinctive morphological or behavioral characters (Polet et al. 2004; Yuasa et al. 2006).

While phaeodarians have never been observed to harbor other living microorganisms (Kling and Boltovskoy 1999), this is quite common for the

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polycystines (Anderson 1983a). Various types of dinoflagellate, prymnesiophyte and prasinophyte associations have been observed (though not at the same time), with the dinoflagellates being most common (Anderson 1976, 1983a; Anderson et al. 1983).

When hosting phototrophic algae, these are mainly kept within the polycystine extracapsulum and held within the rhizopodial network surrounding the central cell body (Anderson 1983b), though a few associates have also been observed within the central capsule (Anderson and Matsuoka 1992). The endoplasmatic algae are often distributed in the outer cell periphery during daylight, and repositioned around the capsular wall during darkness (Anderson 1983a). Experiments show that radiolarians containing phototrophic algae survive longer in nutrient-sparse water than those without. These radiolarian associates are therefore assumed to be symbionts that play a nutritive role for the radiolarians (Anderson 1983a).

However, some radiolarian-associate interactions are not mutually favorable. Various types of parasites have been found infecting the radiolarians intracellularly or even intranuclearly. Heterotrophic dinoflagellate species of *Merodinium* and *Solenodinium*, for example, have been observed within the spumellarian species *Collozoum inerme* and *Thalassicolla* sp., respectively (Anderson 1983a). After invasion, they begin dividing profusely, causing the death of the radiolarian specimen. *Amoebophrya*, another well known, lethal endoparasitic dinoflagellate, infects a large variety of marine planktonic organisms including acantharians and *Sticholonche* spp. (both groups recently defined as radiolarians; Adl et al. 2005), ciliates, chaetognaths, siphonophores, as well as other dinoflagellates (Cachon 1964; Cachon and Cachon 1987). Of the seven thus far recognized species of *Amoebophrya*, some show a high degree of host specificity, while others are known for their broad host range (Park et al. 2004). However, the *Amoebophrya* species concept is still rather vague and there are strong indications that the number of species will rise as molecular studies continue (Gunderson et al. 2002). This may also change our view on their host specificity.

Firm taxonomic identification of radiolarian symbionts and parasites based on microscopic studies are often difficult due to the lack (or modification) of characteristic and reliable taxonomic features (such as thecal plates, scales and flagella). Therefore, molecular diagnostics are useful due to the continuous increase of DNA sequence data being added into publicly available

databases. Analyses of these data have already shown some interesting results regarding radiolarian associates. When studying dinoflagellate symbionts in six different spumellarian species from the Sargasso Sea (*Collozoum caudatum*, *Thalassicolla nucleata*, *Spongostaurus* sp. plus three unidentified species), Gast and Caron (1996) found that all symbionts had identical 18S rDNA belonging to the phototrophic dinoflagellate species *Scrippsiella nutricula*. Other 18S rDNA studies in the Sargasso Sea showed the presence of a phototrophic prasinophyte (closely related to *Scherffelia* and *Tetraselmis*) within *Spongostaurus* sp. (Gast et al. 2000) and a non-pigmented, possibly parasitic, dinoflagellate within *Thalassicolla nucleata* (Gast 2006).

These molecular analyses demonstrate that even taxonomically divergent radiolarians can contain the same type of symbiotic dinoflagellate (i.e., *Scrippsiella nutricula*) and that the symbiont may not be host-specific. They also confirm ultrastructural studies that radiolarians may harbor different algal symbionts and that they can be infected by parasites.

Given the diversity of associates found in subtropical spumellarians, we wished to investigate if this diversity is also present in high-latitude cold water spumellarians. Additionally, we wanted to add data for the other major polycystine group, the nassellarians. Five species of spumellarians (cf. *Actinomma* sp., *Hexacantium giganteum*, *H. pachydermum*, *Phorticium pylonium* and *Streblacantha circumtexta*) and two species of nassellarians (*Androcyclas gamphonyca* and *Ceratospyris hyperborea*) were collected in the Sogndalsfjord, on the west coast of Norway, in order to determine their associate diversity and specificity, both from the standpoint of aquatic geography. Furthermore, we included one phaeodarian species (*Challengeron diodon*) to look for possible associates, even though these have not been observed earlier (Kling and Boltovskoy 1999).

Results and Discussion

The 12 different partial 18S rDNA sequences obtained from our analysis of 5 species of solitary spumellarians (cf. *Actinomma* sp., *H. giganteum*, *H. pachydermum*, *P. pylonium*, and *Streblacantha circumtexta*), two species of nassellarians (*Androcyclas gamphonyca* and *Ceratospyris hyperborea*) and one phaeodarian species (*Challengeron diodon*) were between 1538 and 1708 nucleotides

Table 1. The analyzed polycystine radiolarian and phaeodarian species with authors and publication year, number of specimens analyzed (No.), sample collection date, base pair numbers (Bp), accession numbers in the NCBI GenBank ([®]), closest hits using MegaBLAST ([®] search performed 9 February 2006) and references to Figure 2.

Species	Authors	Type	No.	Associate	Collected	Bp	Accession ^a	Closest hits ^b	Fig. 2
cf. <i>Actinomma</i> sp.		Spumellaria	1	29	25.05.2004	1653	DQ916407	AY256246 – Uncultured eukaryote	A–D
<i>Phorticum pylonium</i>	Haeckel (1887)	Spumellaria	1	23B	24.05.2004	1622	DQ916400	AB183677 – <i>Scrippsella</i> sp.	R
<i>Phorticum pylonium</i>	Haeckel (1887)	Spumellaria	2	62/66	29.06.2004	1595	DQ916401	AB183677 – <i>Scrippsella</i> sp.	S
<i>Hexacantium giganteum</i>	Cortese and Bjørklund (1998)	Spumellaria	1	31	25.05.2004	1641	DQ916402	AY129041 – Uncultured marine eukaryote	F–G
<i>Hexacantium pachydermum</i>	Jørgensen (1900)	Spumellaria	1	58A	29.06.2004	1610	DQ916403	AJ535143 – <i>Diatoma tenue</i>	J–K
<i>Hexacantium pachydermum</i>	Jørgensen (1900)	Spumellaria	1	21	24.05.2004	1664	DQ916404	AY256246 – Uncultured eukaryote	H–I
<i>Hexacantium pachydermum</i>	Jørgensen (1900)	Spumellaria	7	68	29.06.2004	1538	DQ916405	AF290078 – Uncultured marine alveolate Group I	E
<i>Streblacantha circumtexta</i>	Jørgensen (1900)	Spumellaria	1	11	20.06.2003	1707	DQ916406	AF290078 – Uncultured marine alveolate Group I	Q
<i>Ceratospyrus hyperborea</i>	Jørgensen (1905)	Nassellaria	1	8	24.05.2004	1640	DQ916399	Uncultured marine alveolate Group I DQ116022 – <i>Dinophyceae</i> sp. ex <i>Thalassicola nucleata</i>	L–M
<i>Androcyclos gamphonyca</i>	(Jørgensen 1900)	Nassellaria	1	37	25.05.2004	1633	DQ916408	AB191417 – Uncultured eukaryote	N–O
<i>Androcyclos gamphonyca</i>	(Jørgensen 1900)	Nassellaria	1	5	20.06.2003	1708	DQ916409	DQ116022 – <i>Dinophyceae</i> sp. ex <i>Thalassicola nucleata</i>	T
<i>Challengeron didon</i>	Haeckel (1887)	Phaeodaria	1	65	29.06.2004	1612	DQ916410	AF290078 – Uncultured marine alveolate Group I	P

long (Table 1). Performing BLAST searches in GenBank identified 11 of the sequences as belonging to alveolates, while one was a diatom classified under Fragilariophyceae. Only the alveolates were analyzed further. A phylogenetic analysis based on the 18S rDNA sequences of 227 alveolates (Fig. 1; see also Supplementary Material) placed our 11 sequences among at least three supported, distantly related lineages, indicating a large associate diversity. We found no evidence for the presence of chimeric sequences representing the three different primer-pair amplicons for each sample; when analyzed again as separate fragments, each grouped within the same lineages reported above (results not shown). Additionally, our phylogenetic tree (Fig. 1; Supplementary Material) is largely congruent with previously published trees of alveolates (e.g., Saldarriaga et al. 2004). Some nodes are only poorly supported or appear only in the strict consensus tree, but this lack of resolution is also characteristic of earlier studies. A more detailed description and discussion of each of our eleven alveolate sequences follows.

The Alveolate Sequences

The two dinoflagellate sequences obtained from the spumellarian species *P. pylonium* are very similar to each other and group with a jackknife support value of 99. When searching for similar sequences using NCBI MegaBLAST (Zhang et al. 2000), both show closest alignment to the marine, photosynthetic dinoflagellate species *Scrippsiella*. The relationship to *Scrippsiella* is also indicated in our phylogenetic tree (Fig. 1), although it is not statistically supported. If our sequences belong to *Scrippsiella*, this confirms an earlier observation by Gast and Caron (1996), who described *Scrippsiella* (more specifically, *S. nutricula*) in symbiosis with six taxonomically diverse spumellarian species collected in the Sargasso Sea. A comparison between the *Scrippsiella nutricula* sequence (U52357; Gast and Caron 1996) and our associated sequences from *P. pylonium* show an identity of 96%, although they appear to belong to different lineages of *Scrippsiella*. If our assumptions are correct that these two new sequences represent *Scrippsiella*, this is the first report, to our knowledge, that *Scrippsiella* has been observed associated with neritic North Atlantic radiolarians. It also suggests that *Scrippsiella* may not only be restricted to surface dwelling spumellarians, but perhaps also hosted in spumellarians living at subsurface depths (50–250 m). Whether *Scripp-*

siella is present in nassellarians as well remains to be investigated.

Two of our ex nassellarian sequences¹ (obtained from *Androcyclas gamphonyca* and *Ceratospyrus hyperborea*) are also highly similar (identity of 97%). The MegaBLAST searches show closest alignments with two non-annotated sequences of Dinophyceae (DQ116021 and DQ116022) described from the spumellarian species *Thalassicola nucleata* (Gast 2006). The close sequence similarity is reflected in our phylogenetic analysis, which groups the four sequences together with a jackknife support value of 100 (Fig. 1). Based on cellular observations in which no pigments were seen, Gast (2006) interpreted the *Thalassicola nucleata* associates to be non-phototrophic, putative dinoflagellate parasites. However, these associates do not group with earlier described *Thalassicola* parasites such as *Solenodinium* (member of Syndinales) or *Caryotoma* (member of Blastodinales), nor any other known 18S rDNA alveolate sequences. Our two new ex nassellarian sequences do not provide any additional information on their identity, since no ultrastructural studies were performed. We are not aware of any reports of nassellarian parasites from ultrastructural studies that could provide an indication of which taxonomic group of dinoflagellates these sequences may belong to. It is therefore, as Gast (2006) also pointed out, not possible to suggest a name for these dinoflagellates other than Dinophyceae (incertae sedis). In any case, our data support Gast's (2006) assumption that these radiolarian associates live intracellularly and are not a result of sample contaminations. Furthermore, our findings reveal that these organisms have a broad host range due to the fact that they are found in both spumellarian and nassellarian Radiolaria. They also seem highly tolerant of different water characteristics, being present in both subtropical water masses of the Sargasso Sea and cold temperate waters within the Norwegian fjords.

Seven of our 18S rDNA sequences (derived from the solitary spumellarians cf. *Actinomma* sp., *H. giganteum*, *H. pachydermum* (two specimens) and *Streblacantha circumtexta*, the nassellarian species *A. gamphonyca*, and the phaeodarian species *Challengeron diodon*) group together (Fig. 1) with two recently discovered, ubiquitous

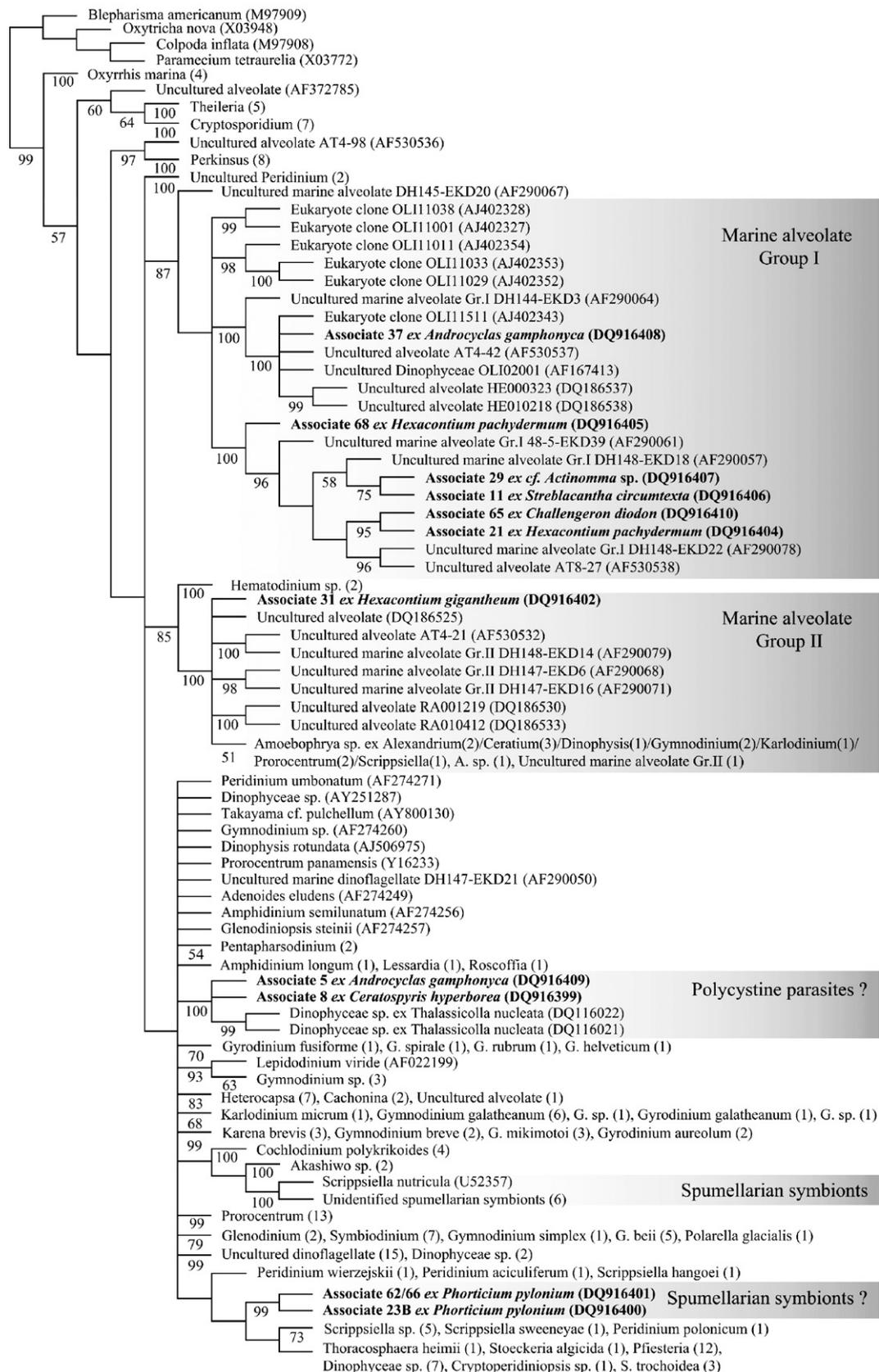
¹Associates found in various radiolarians/phaeodarians are referred to throughout the text as “associate-type ex radiolarian/phaeodarian species” (e.g., “*Scrippsiella* sp. ex *Phorticum pylonium*”).

groups of marine alveolates (López-García et al. 2001; Moon-van der Staay et al. 2001). These novel microorganisms were first described from deep-sea plankton in the Antarctic (López-García et al. 2001), but have also been observed in plankton of the Pacific (Guillou et al. 1999; Moon-van der Staay et al. 2001), the Atlantic, and the Mediterranean (Díez et al. 2001), in anoxic sediments around fumaroles in the Kagoshima Bay, Japan (Takishita et al. 2005), and in hydrothermal sediments at the Mid-Atlantic ridge (López-García et al. 2003). López-García et al. (2001) named them the “Marine alveolate Group-I and -II”, and we have adopted these names here although our corresponding clades include more accessions. Six of our ex radiolarian/phaeodarian sequences fall within the “Marine alveolate Group I”. Most of the “Marine alveolate Group I” sequences are from uncultured environmental samples, for which no further identifications have been made. It is still unknown whether these represent heterotrophic organisms with an extreme tolerance and adaptability to all marine environments, or if they primarily live in water masses and are deposited in the sediments, for example, as cysts. It is also not clear whether they are solely free-living or if they can be found in associations with other organisms. Several phylogenetic analyses have placed the “Marine alveolate Group I” between two groups of parasites, the amoebophryans and the perkinsozoans (López-García et al. 2003; Moon-van der Staay et al. 2001; Takishita et al. 2005), lending to the suggestion that they may be parasites (Moon-van der Staay et al. 2001). Although there is still no solid evidence for such a conclusion (Moreira and López-García 2002), it is tempting to suggest that a parasitic origin of this group would fit well with our observations. Irrespective of the conclusion, to our knowledge, our work represents the first report of a phaeodarian associate.

One of our sequences (Associate 31 ex *H. giganteum*) groups with the “Marine alveolate Group II” (Fig. 1). This highly supported clade (jackknife support value of 100) includes, in addition to several non-annotated alveolate samples from the Antarctic plankton (López-García et al. 2001) and sediments of the Mid-Atlantic ridge (López-García et al. 2003), sequences of the heterotrophic dinoflagellate *Amoebophrya*. The latter is a well-known endoparasite reported from a large variety of marine planktonic organisms. It is therefore reasonable to hypothesize that the 18S rDNA sequence derived from this spumellarian specimen is also from a parasite, possibly

Amoebophrya itself. Direct parasitism by *Amoebophrya*-like parasites in polycystine radiolarians has, to our knowledge, not been reported earlier, although it seems reasonable as *Amoebophrya* has already been found to infect other radiolarians such as acantharians and *Sticholonche* spp. No molecular studies have so far been performed on the *Amoebophrya*-type organisms found associated with these two groups, so it remains unknown whether our 18S rDNA sequences may be most similar to the *A. acanthometrae* or *A. sticholonchae* reported from acantharians and *Sticholonche* (respectively), or to a different species that may be specific to polycystine radiolarians. Additional ultrastructural- and molecular studies are needed to answer these questions.

Another explanation of these findings is that we have observed a case of secondary parasitism, i.e., that a parasite (e.g., *Amoebophrya*) is in fact infecting radiolarian symbionts instead of the radiolarian directly. This idea is supported by the fact that several of the *Amoebophrya*-like sequences which group in our “Marine alveolate Group II” have also been obtained from various dinoflagellates (e.g., *Scrippsiella*, *Gymnodinium*, *Ceratium* and *Prorocentrum*), and that a previous study (Gast and Caron 1996) as well as our present results suggest that radiolarians live in symbiosis with dinoflagellates such as *Scrippsiella*. All of the *Amoebophrya*-sequences ex other dinoflagellates in our analysis group with a low jackknife support (value of 51). This clade could represent *A. ceratii*, since morphological studies have shown that all *Amoebophrya* infecting other dinoflagellates can be ascribed to this species (Gunderson et al. 2002). However, molecular studies have indicated that the “*A. ceratii*” species concept is more complex than earlier assumed, and that multiple species may be involved (Gunderson et al. 2002; Janson et al. 2000; Salomon et al. 2003). If our sequences represent secondary parasitism, where *Amoebophrya* is infecting a dinoflagellate symbiont hosted by a radiolarian, one would assume them to be of the *A. ceratii*-type, i.e., that they would group with the other ex dinoflagellate sequences. Since this is not the case, our results may suggest that our potentially parasitic *Amoebophrya*-like dinoflagellates could be infecting a non-dinoflagellate radiolarian symbiont, or perhaps more likely, that the relationship represent direct parasitism (as described above) by a close relative or different species of *Amoebophrya* than *A. ceratii*.



The Diatom Sequence

The last sequence obtained in this study is from the spumellarian species *H. pachydermum*. This sequence is identical (except for 1 bp and 11 indels) to the pennate diatom *D. tenue* (GenBank accession AJ535143; Medlin and Kaczmarek 2004) classified under Fragilariophycidae. *D. tenue* is known to occur both in plankton and benthos, usually in freshwater, although Cleve-Euler (1912) described a variety that was restricted to brackish conditions (Kelly et al. 2005). Our suggestion is that this diatom specimen was transported to the Sogndalsfjord via a river (or that it lived near-shore in the brackish surface water), and from there had been taken by fjord currents to deeper waters where it settled through the water column and was captured as food and consumed by the radiolarian.

There is another more speculative, but possible explanation for finding a diatom within a radiolarian specimen. Different benthic and planktonic species of Foraminifera, recently classified with Radiolaria in the super-group Rhizaria (Adl et al. 2005), have been reported to host small, endosymbiotic, pennate diatoms of a variety of taxonomically distantly related genera (Lee and Anderson 1991; Lee et al. 1995; Mayama et al. 2000). Interestingly, one of these, i.e., *Fragilaria*, also belongs to the Fragilariophycidae. Whether radiolarians contain symbiotic diatoms in addition to the already described dinoflagellates, prymnesiophytes and prasinophytes cannot be confirmed by the present study. Further investigations of *H. pachydermum* specimens (and other polycystine radiolarians), with possible isolation and cultivation of tentative diatom symbionts, are needed.

Conclusions

Twelve diverse, partial 18S rDNA sequences analyzed in this study were retrieved from seven species of polycystine radiolarians and one phaeodarian species. Based on our methodology we are confident that the analyzed sequences come from inside the radiolarian/phaeodarian cells, and are not a result of external contamina-

tion. BLAST searches and phylogenetic analyses show that our sequences group within the Alveolata and the Bacillariophyta. They place eleven of the sequences among three lineages of alveolates, and close relationships with other described symbiotic and parasitic organisms support our conclusion that our sequences originate from living radiolarian/phaeodarian associates and not food. One sequence, however, from Bacillariophyta, originates most likely from radiolarian food. To verify our various interpretations, cellular examinations (TEM-studies) will be required.

Not only has our study demonstrated a large diversity of tentative symbionts and parasites within polycystine radiolarians/phaeodarians, it has also shown that the associate type may vary within the same species/genus. For example, the two sequences ex *Androcyclas gamphonyca* group in two very distantly related alveolate clades, and the four sequences from *Hexacontium* group within two separate, well-supported alveolate clades, as well as a diatom relative. Furthermore, this is the first study to report on a phaeodarian associate. Phaeodarian associates (either symbionts or parasites) have not been reported on earlier.

Methods

Geological and hydrographical setting: One of the world's longest fjords, the Sognefjord, lies on the west coast of Norway in the heart of the Norwegian fjord country. It extends more than 205 km inland and is at its deepest point 1308 m. This fjord is connected to the Norwegian Sea via a shallow area in the outer region, and is characterized by its many tributary fjords, carved out in the bedrock through glacial erosion. The tributary fjords are submerged valleys, some with a sill, others without. The Sogndalsfjord is a tributary system on the north side of the Sognefjord with a sill depth of only about 26 m off Nornes. This fjord basin is 11 km long and is deepest off the biological station at Skjæret, with a 260 m water depth.

Figure 1. A phylogenetic analysis of 227 alveolate 18S rDNA GenBank sequences including 11 samples new to this study (the latter marked with bold text). Four sequences of ciliates were used as outgroups. For graphic purposes, this representation of the strict consensus tree is a subset of sequences from a larger analysis (see Methods and Supplementary Material). Where accessions from the latter tree have been excluded, the total number of sequences at that node is indicated in parenthesis after the taxon name. Jackknife values above 50% are shown below branches.

Profiles of temperature, salinity and oxygen in the Sognefjord and its tributaries are typical for a poorly mixed fjord environment (Swanberg and Bjørklund 1987). The fjord system is characterized by a low saline (brackish) surface layer overlaying fully marine intermediate and bottom water masses (Swanberg and Bjørklund 1987). This strongly developed stratification is caused by a high input of fresh water from land runoff (rivers and snowmelt) and precipitation.

The influx of oceanic (North Atlantic) water is not a yearly, but rather an eight year event (Hermansen 1974). It is therefore obvious that the radiolarian and phaeodarian faunas are of a local neritic nature. However, when an influx of North Atlantic water does occur, the local faunas are recruited by new faunal elements of an oceanic origin.

Plankton sampling: Radiolarians (including their intracellular associates) were collected in the Sogndalsfjord at its deepest point (61° 12' 30" N, 07° 06' 24" E), just off the biological field station at Skjæret, during May/June in 2003 and 2004. A Juday-net with a closing mechanism and 63 µm mesh size was used. The net was towed obliquely at different depths (between 240–50 m) for about 15–30 min. The upper water masses were rich in phytoplankton, so the plankton net was closed at 50 m to avoid high concentrations of phytoplankton in the samples, which would mask the radiolarians and make it difficult to separate them. The samples were stored in glass jars containing filtered seawater from the field station's seawater inlet (brought up from about 100 m water depths, holding a temperature of ca. 8 °C and a salinity of ca 33 psu). In the laboratory the plankton jars were stored in an aquarium to optimize temperature conditions.

Sample analysis and molecular methods: The radiolarians/phaeodarians were identified and isolated from other plankton, cleaned in 0.2 µm Millipore-filtered seawater, and frozen. Each radiolarian/phaeodarian specimen was photographed (Fig. 2, Table 1) and visible foreign external material removed. As all our analyzed specimens possess a silica test, we did no attempt to separate the endoplasm from the ectoplasm. Each individual was washed three times in distilled water before being lysed with proteinase K. In most cases we analyzed single specimens, except for two cases where we pooled two individuals of *Phorticum pylonium* (Associate 62/66) and seven specimens of *Hexacantium pachydermum* (Associate 68) respectively. Each sample was analyzed directly, without first being cultured, and no replicate runs were performed.

PCR amplifications were performed in a 50 µL reaction volume using TaKaRa LA Taq-kit (TAKARA BIO Inc., Japan) using two universal eukaryotic primers (Table 2), i.e., the forward primer NSF83 (Hendriks et al. 1989) and the reverse primer B (Medlin et al. 1988), and the following PCR cycling profile: 95 °C 3 min, (95 °C 1 min, 55 °C 2 min, 72 °C 3 min) × 35. We performed a second PCR amplifications in a 25 µL reaction volume using 2 µL of the first PCR product as DNA template, the AmpliTaq DNA Polymerase buffer II kit (Applied Biosystems Foster City, California, USA), 1 mmol/L of a dNTP blend, 0.04% bovine serum albumen (BSA), 0.01 mmol/L tetramethylammonium chloride (TMACl), and 0.8 µmol/L of each primer. The 18S rDNA region was this time amplified using three pairs of universal eukaryotic primers (Table 2), i.e. NSF83/NSR581 (Hendriks et al. 1989), NSF573/NSR1147 (Hendriks et al. 1989) and NSF963/NSR1787 (Hendriks et al. 1989), producing an overlap of ca. 10 and 200 bp respectively, for the three primer pair regions. The following PCR cycling profile was used: 94 °C 3 min, (94 °C 50 s, 47 °C 50 s, 72 °C 1 min) × 35, 72 °C 10 min. The PCR products were cleaned using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) or exoSAP-IT (Amersham Biosciences, Piscataway, USA) and directly sequenced using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) using the same primers as in the PCR reaction. The products were analyzed using an ABI 3100 automated sequencer (Applied Biosystems) and the obtained sequences were edited and assembled using the Sequencher ver. 4.1 software (GenCodes Corporation, Ann Arbor, Michigan, USA).

Two samples (Associate 5 and Associate 11) were processed at Tokyo Gakugei University, and for these samples the first amplified PCR products were purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences), ligated into pGEM-T Easy Vector System (Promega, Madison, USA) and cloned in *Escherichia coli* JM109 Competent cells (Promega) before sequenced using a Shimadzu DSQ2000L with a Thermo Sequences Fluorescent Labeled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Biosciences). Three clones from each sample were sequenced, using internal primers NSF573, NSF1179, NSR581, NSR1147, pGEM-Forward and pGEM-Reverse (Table 2), giving three identical sequences.

The 12 analyzed radiolarian/phaeodarian samples did not yield any 18S rDNA from the host only

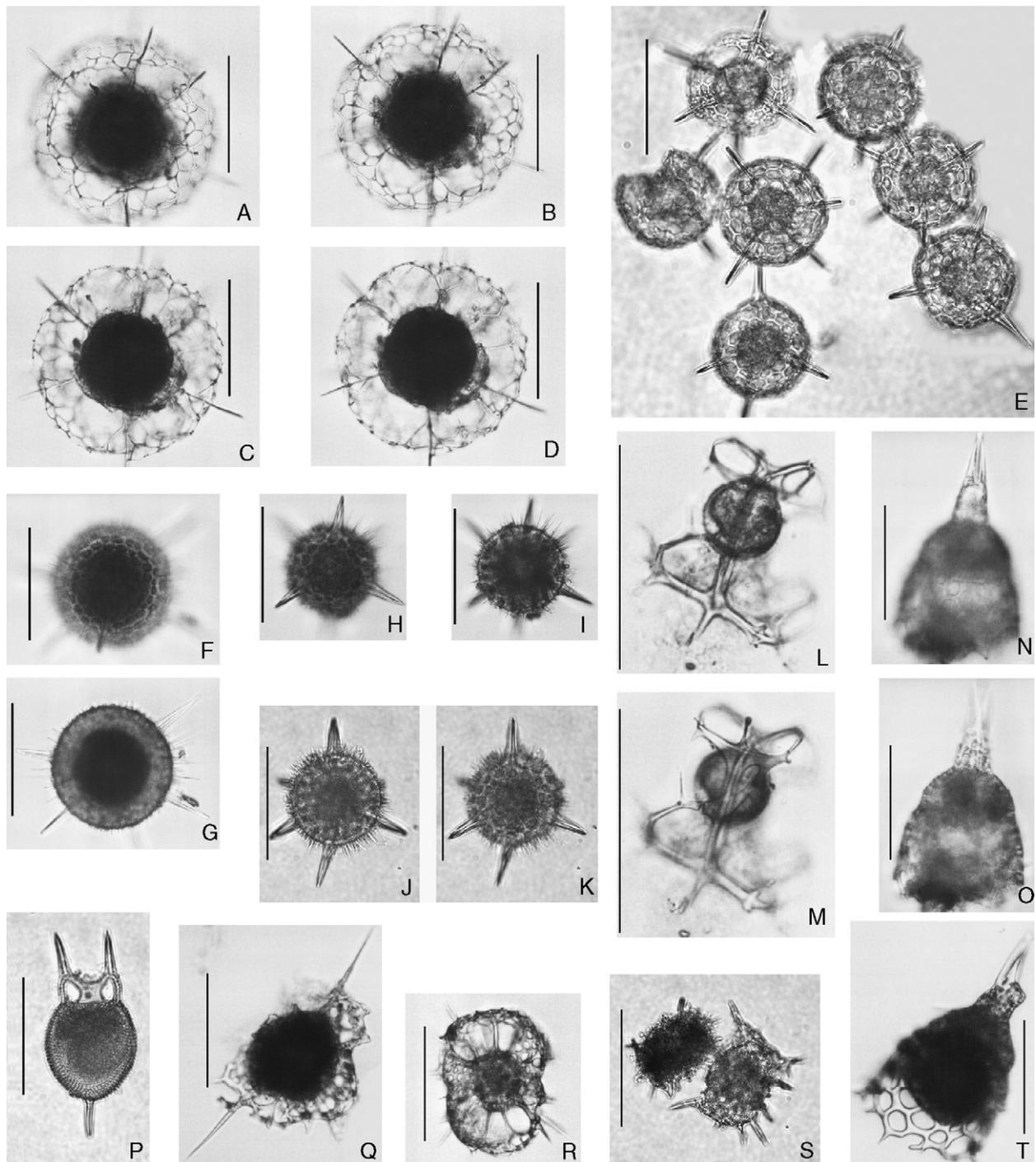


Figure 2. Light micrographs of the radiolarians and the phaeodarian used in this study. **A–D.** cf. *Actinomma* sp. (same individual, different focal planes). **E.** *Hexacontium pachydermum*. **F–G.** *Hexacontium giganteum* (same individual, different focal planes). **H–I.** *H. pachydermum* (same individual, different focal planes). **J–K.** *H. pachydermum* (same individual, different focal planes). **L–M.** *Ceratospyrus hyperborea* (same individual, different focal planes). **N–O.** *Androcyclas gamphonyca* (same individual, different focal planes). **P.** *Challengeron diodon*. **Q.** *Streblacantha circumtexta*. **R.** *Phorticium pylonium*. **S.** *P. pylonium*. **T.** *A. gamphonyca*. Scale bars = 100 μm .

Table 2. 18S rDNA primer sequences used in this study.

Primer	Sequence	Length	Reference
NSF83	5'- GAAACTGCGAATGGCTCATT -3'	20 nucleotides	Hendriks et al. (1989)
NSF573	5'- CGCGGTAATTCCAGCTCCA -3'	19 nucleotides	Hendriks et al. (1989)
NSR581	5'- ATTACCGCGGCTGCTGGC -3'	18 nucleotides	Hendriks et al. (1989)
NSF963	5'- TTRATCAAGAACGAAAGT -3'	18 nucleotides	Hendriks et al. (1989)
NSR1147	5'- CCGTCAATYYTTTTRAGTTT -3'	20 nucleotides	Hendriks et al. (1989)
NSF1179	5'-AATTTGACTCAACACGGG -3'	18 nucleotides	Hendriks et al. (1989)
NSR1787	5'- CYGCAGGTTACCTACRG -3'	18 nucleotides	Hendriks et al. (1989)
B	5'-CCTTCTGCAGGTTACCTAC -3'	20 nucleotides	Medlin et al. (1988)
pGEM-F	5'-ACTCACTATAGGGCGAATTGG-3'	21 nucleotides	T. Yuasa (unpubl.)
pGEM-R	5'- TCAAGCTATGCATCCAACG-3'	19 nucleotides	T. Yuasa (unpubl.)

their associates. The nucleotide data from all 12 sequences have been entered the NCBI GenBank under accession numbers DQ916399- DQ916410 (see Table 1).

Phylogenetic analyses: BLAST sequence similarity searches (Altschul et al. 1990; Zhang et al. 2000) of the 12 retrieved sequences against the non-redundant (nr) NCBI database (<http://www.ncbi.nlm.nih.gov>) confirmed that all sequences were eukaryotic. One sequence (Associate 58A) was identified as a diatom and was excluded from the phylogenetic analyses. The top 100 alveolate hits with the highest expectation values from individual BLAST searches of each of the 11 sequences were chosen and used to create a data matrix. Redundant sequences were deleted and additional sequences of relevance were added to this matrix, e.g., additional *Amoebophrya* sequences, some eukaryotic environmental samples earlier found to be close relatives of the dinoflagellate lineage (Moon-van der Staay et al. 2001), and *Oxyrrhis*, suggested to be a derived dinoflagellate (Cavalier-Smith and Chao 2004). Four sequences of ciliates were obtained from the European Ribosomal RNA database (<http://www.psb.ugent.be/rRNA/>) and used as outgroups. An alignment consisting of 231 18S rDNA sequences was constructed with ClustalW ver. 1.83 (Thompson et al. 1994) and manually adjusted using the program BioEdit ver. 7.0.2 (Hall 1999). Highly ambiguous positions in the alignment were excluded from our analyses, resulting in a total of 1766 nucleotide characters. The data set is available on request from the corresponding author.

The matrix was subjected to parsimony analysis using the program TNT (Goloboff et al. 2000) with the following settings: new technology search, stabilize consensus 2 times with factor 75 and initial addseq = 5. The 61 trees retained were

amplified further to 1000 trees with traditional search and TBR branch swapping. To estimate support for internal branches, parsimony jackknifing (Farris et al. 1996) was performed using TNT. One thousand replicates were conducted, each performing TBR branch swapping with five random entry orders per replicate and a deletion frequency of 36%. A representation of the strict consensus tree using a subset of sequences from the larger analysis (see Supplementary Material) is shown in Figure 1.

To further evaluate the possibility of picking up recombinant or chimeric sequences during the second amplification, we modified our data matrix so that the nine (alveolate) samples that had not been cloned were each partitioned into three separate sequence fragments representing the three different primer pair amplicons. This second data set was analyzed using the same parameters as above. The phylogenetic positions of the three sequence fragments from each individual in the strict consensus tree (not shown) were compared to our first analysis (Fig. 1; Supplementary Material).

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.protis.2006.07.004](https://doi.org/10.1016/j.protis.2006.07.004).

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