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Towards a molecular phylogeny of colonial spumellarian radiolaria

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Abstract

Gene sequence data from the small-subunit ribosomal RNA coding region were used to explore evolutionary relationships among the colonial spumellarian radiolaria (Polycystinea). Representatives from the two spumellarian families known to form colonies were considered including the following taxa: Sphaerozoidae: *Collozoum pelagicum*; *Collozoum serpentinum*; *Rhaphidozoum acuferum*; *Sphaerozoum punctatum*; and Collosphaeridae: *Collosphaera globularis–huxleyi*; *Acrosphaera* (*circumtexta*?); and *Siphonosphaera cyathina*. Our molecular analyses support the monophyly of the Collosphaeridae in all analyses used, but only distance analyses support the monophyly of the Sphaerozoidae. Within the Sphaerozoidae, two species of *Collozoum* (*C. serpentinum* and *C. pelagicum*) failed to branch together, indicating a more distant relationship than first suggested, a conclusion further supported by recent ultrastructural studies (see adjoining paper). Branching patterns within the Collosphaeridae indicate that *Siphonosphaera* diverged prior to the split between *Collosphaera* and *Acrosphaera*, a result which challenges evidence based on data from the radiolarian fossil record.

Keywords: *Acrosphaera*; Collosphaeridae; *Collozoum*; colonial radiolaria; *Siphonosphaera*; Sphaerozoidae

1. Introduction

Colonial spumellarian radiolaria are holoplanktonic protists (Subphylum Sarcodina, Class Polycystinea) which occur exclusively in oligotrophic open ocean environments. As in all polycystines, each cell is physically separated into the endocytoplasm and the ectocytoplasm by a porous proteinaceous capsular wall. The capsular wall, together with the cellular machinery it encloses (the nucleus, mitochondria, golgi, endoplasmic reticulum, vacuoles, and oil droplets), is referred to as the central capsule. In colonial spumellarian radiolaria, thousands of individual central capsules extend their pseudopodia into a shared gelatinous extracapsular matrix which connects the cells and also typically houses numerous symbiotic algae. Apart from isolated reports of colony formation by phaeodaria of the family Tuscaroridae (Haecker, 1908; Swanberg, 1979), the spumellaria are the only other 'radiolaria' sensu stricto (Polycystinea and Phaeodarea) which include colony-forming groups.

As 'multicellular' entities, the colonial spumel-

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laria are macroscopic and have been reported to reach lengths of up to three meters, making them very conspicuous components of tropical and subtropical pelagic marine environments (Anderson and Swanberg, 1981). Despite a visible presence in the plankton, their fragile nature and resistance to laboratory culture has left many unanswered questions regarding their biology.

Historically, the colonial spumellarian radiolaria (encompassing a wide range of morphologies varying from skeletonless forms, to those with only spicules, and others with elaborate porous shells) have been grouped together taxonomically solely on the basis of the ability to form colonies. The taxonomy of these species is based on features of the central capsules, skeletal morphology (when present) and the size and shape of the colonies. Skeletal morphotypes have been used widely in plankton biogeographic studies and in marine biostratigraphic analyses. Phylogenetic relationships among species of colonial radiolaria are poorly understood. Furthermore, ultrastructural studies, especially important for species lacking skeletal elements (e.g. *C. serpentinum*, Anderson et al., 1999), are incomplete for many taxa. This lack of understanding limits our ability to construct natural taxonomic systems and to better interpret evolutionary histories based on skeletal evidence from the sedimentary fossil record.

The colonial radiolaria are restricted to two families within the order Spumellarida: the Sphaerozoidae and the Collosphaeridae. In the Sphaerozoidae, skeletal material is either lacking or composed of silicious spicules of varying degrees of complexity. The most recent systematic treatment of the colonial spumellaria (Strelkov and Reshetnyak, 1971) divides the Sphaerozoidae into three genera, *Collozoum*, *Sphaerozoum*, and *Rhaphidozoum* [in this study, the classification scheme of Levine et al. (1980) was used for higher-level classifications and that of Strelkov and Reshetnyak (1971) for classifications at the family-level and below]. The species of the genus *Collozoum* possess either simple spines or no skeleton (Strelkov and Reshetnyak, 1971). Members of the genus *Sphaerozoum* contain characteristic paired-triradiate spines, while *Rhaphidozoum* species have both simple and radiate spines. Species designations in these genera are typically based on the structure of these spines, when present, or the morphology of the central capsular wall, as in the case of species within the genus *Collozoum*. All members of the family Collosphaeridae are characterized by siliceous, spherical latticed shells having varying degrees of ornamentation.

The cosmopolitan distributions of colonial radiolaria and their responsiveness to environmental and hydrographic factors have made them useful paleontological tools. Skeleton-bearing species, especially members of the Collosphaeridae have been used routinely in biostratigraphic analyses (Popofsky, 1917; Benson, 1966; Nigrini, 1967, 1968, 1970; Molina-Cruz, 1975; Morley, 1977; Kling, 1978; Nigrini and Moore, 1979; Lombari and Boden, 1985; Boltovskoy, 1987; Dworetzky and Morley, 1987). Classically, the phylogenetic relationships of the colonial radiolaria, as with other skeleton bearing groups, has been based on analyses of shell morphologies of specimens from plankton samples and biostratigraphically analyzed microfossils (Knoll and Johnson, 1975; Bjørklund and Goll, 1979; Riedel and Sanfilippo, 1981; Petrushevskaya and Swanberg, 1990). Morphological analyses, however, have not always been effective in resolving phylogenetic relationships among these species. Classical morphological approaches combined with modern, fine structural and molecular genetic analyses hold promise for clarifying unresolved issues in radiolarian systematics. With the application of molecular biological techniques, we now have a novel means of exploring evolution in the colonial spumellaria. To this end we sequenced the small-subunit ribosomal RNA (SSU rRNA) genes of representatives from both families of Spumellarida known to form colonies in order to examine the evolutionary relationships among the colonial spumellaria.

2. Material and methods

A simplified overview of the molecular methods used in this study is provided in Fig. 1. In summary, cells are collected, bulk DNA is extracted and rRNA genes are specifically isolated and amplified with a technique called the Polymerase Chain Reaction (PCR). PCR amplification is an enzymatic reaction which allows for many copies of a gene to be synthesized in vitro in conjunction with primers which

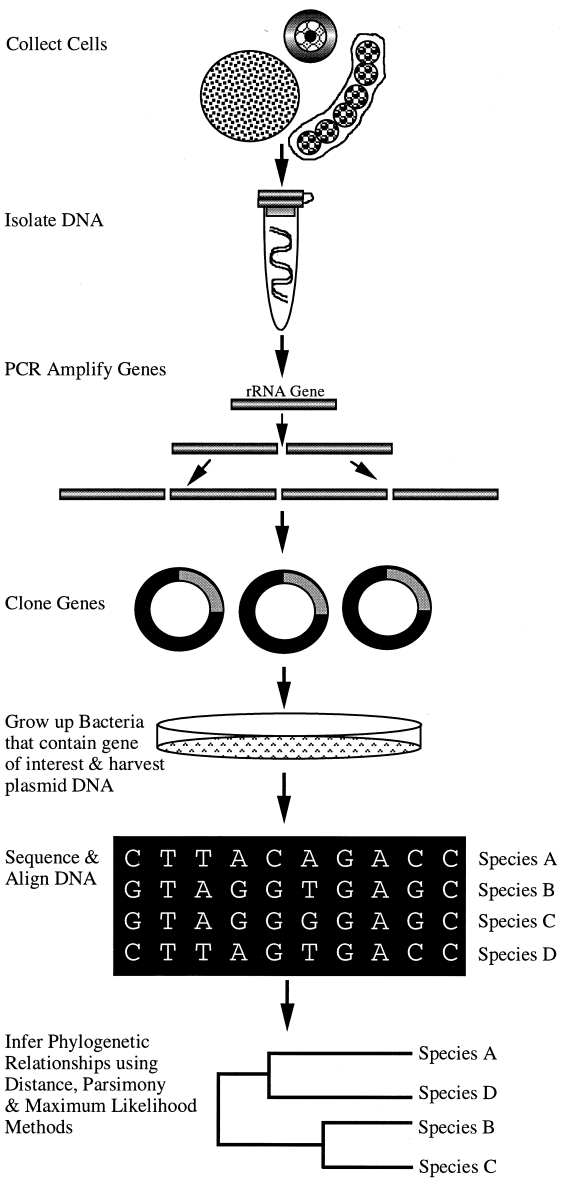


Fig. 1. Overview of molecular approach used in this study.

are specific for the ends of the gene of interest. The PCR products are then cloned by ligation into a cloning vector (plasmid) and transformation into competent bacteria that take up the foreign DNA. Bacterial clones are then cultured and the plasmid DNA (containing the rDNA of interest) is extracted and purified. This plasmid DNA is then sequenced using conventional sequencing technologies. Once the DNA sequences are obtained, the base pairs are aligned with homologous (sharing common ancestry) base pair positions from other taxa in the nucleic acid data base and then used in the construction of phylogenetic trees. There are currently three major phylogenetic approaches used in phylogenetic tree building. These include distance, maximum parsimony and maximum likelihood methods. These methods are all cited below and more detailed information about these techniques can be obtained in these citations but a simplified description follows. Distance matrix methods compute pairwise distances between taxa (some measure of the number of differences in base pairs) in a matrix form and then construct phylogenetic trees using algorithms based on these evolutionary distance values. The maximum parsimony method compares character states (what kind of nucleotide occurs at a given site) and identifies the tree that requires the smallest number of evolutionary changes. Finally, maximum likelihood approaches evaluate the net likelihood that an evolutionary model will yield the sequence data obtained and identifies a tree in which the inferred phylogenies possess the highest likelihood. One important point in considering these different methods is that the results are not absolute and the different methods do not always yield the same answer. For this reason, most studies of this kind use as many methods as possible and subject the results to statistical analyses (e.g. bootstrapping or bremer support analyses).

Colonial spumellarians were collected in glass jars by divers. All samples were collected approximately 6.4 km off the southeast coast of Bermuda on the dates listed below. Colonies were maintained in 0.22 μm Millipore-filtered seawater in glass culture tubes with brine shrimp nauplii (*Artemia salina*) as food. Samples were given individual sample designations at the time of collection and identified by microscopical examination after return to the lab. The following samples (a subset of a larger data set; Amaral Zettler, 1996) were included in this study (Appendix A). Sample designation and collection date follow the species identification: *Collozoum pelagicum* (BBSR 2, November 1993); *Rhaphidozoum acuferum* (BBSR 7, November 1993); *Collosphaera globularis–huxleyi* (BBSR 173, May 1994); *Sphaerozoum punctatum* (CR 4, May 1995); *Acrosphaera (circumtexta?)* (CR 6, May

1995); *Collozoum serpentinum* (CR 16, May 1995); *Siphonosphaera cyathina* (October 1995). The *C. pelagicum* specimen sequenced in this study consisted of a section of a vegetative (non-reproductive) colony. All other samples consisted of pooled or single central capsules from a single reproductive colony. The Genbank accession numbers for new taxa used in this study were AF091145–AF091148.

In all but the *C. pelagicum* sample, colonies were held until the early stages of swarmer production (Anderson, 1983). At that time, central capsules were physically separated by repeated micropipetting from extracapsular material which contained endosymbiotic algae. The rationale behind killing individuals at that point in their life cycle was twofold: First, a natural increase in DNA content occurs within the organism at that time as multiple copies of the genome are made in preparation for swarmer formation. Second, many species either consume or expel endocytoplasmic symbiotic algae immediately prior to swarmer formation thereby reducing the potential of enzymatically amplifying non-target DNA. Individual central capsules were pipetted through several 0.22 μm-Millipore filtered seawater rinses followed by a final MilliQ-water rinse prior to placement in a modified 1X PCR buffer solution which consisted of 50 mM KCl, 10 mM Tris, pH 8.3, 2 mM $MgCl_2$, 0.001% Gelatin, and 1.0% NP40 (Sigma; St. Louis, MO). Samples were then stored frozen at −70ºC. Samples used for molecular analyses were heated at 95ºC for 10 minutes to lyse cells and liberate DNA. An aliquot of the lysed sample (containing genomic radiolarian DNA) was used as target template in PCR amplification reactions (Saiki et al., 1988) to specifically enzymatically amplify out radiolarian rRNA genes. Sequences from *R. acuferum* and *C. globularis–huxleyi* samples were obtained from cloned products (cloning procedure described in Amaral Zettler et al., 1997). Sequence information obtained from these two samples was then used to design 'colonial spumellarian'-specific primers which were effective in amplifying SSU rRNA genes of different genera. Sequence divergence was too great to design primers that would target both the solitary *T. nucleata* and the colonial spumellaria.

Sequence data from the remaining samples were obtained from directly sequencing PCR products amplified using a combination of colonial spumellarian specific primers and Medlin primers (Medlin et al., 1988). These colonial spumellarian-specific primers were synthesized as described in Amaral Zettler et al. (1997). The nucleotide sequences are: forward primer R906, 5′-TATTAGTATTTTRTCGTT-3′; reverse primer R1451bio, 5′-TATTGTAGCCCGTGCGCT-3′ [previously used as a probe for in situ verification experiments by Amaral Zettler et al. (1997)]. See Fig. 2 for description of PCRing strategy. PCR reactions consisted of 3 minutes of denaturation at 95ºC followed by 30 amplification cycles each consisting of 94ºC for 1 minute, 42ºC for 1 minute and 72ºC for 2 minutes. Two separate 100 μl PCR reactions typically provided enough template for sequencing reactions. PCR reactions were then pooled prior to purification using the Wizard PCR Kit (Promega; Madison, WI) to obtain purified DNA for direct sequencing.

Direct sequencing of PCR products was accomplished using IR-labeled primers and reagents from the Sequitherm Long-Read Sequencing Kit (Sequitherm; Madison, WI), along with the Sequitherm Cycle sequencing protocol developed by Li-Cor (Lincoln, NE) which consisted of 5 minutes of denaturation at 95ºC prior to 30 cycles of 20 s at 95ºC (30 s for plasmid DNA), 30 s at 60ºC, and 1 minute at 70ºC using a Perkin Elmer 2400 thermo-cycler. Double stranded sequencing of the entire forward and reverse strands of the rRNA coding regions was conducted for cloned products. For directly sequenced PCR products, double-stranded reads for all but the primer-specified ends were obtained.

The 16S-like rRNA sequences of colonial spumellaria were aligned against a subset of the total eukaryotic alignment data base (Olsen et al., 1992). Sequences were aligned by eye using the Olsen Multiple Sequence Alignment Editing program with regard to primary and secondary structural conservation. The same positions were used in this analysis as were used in the data set analyzed by Amaral Zettler et al. (1997) (1368 total sites minus one site which became a gap in all taxa when some taxa from the original data set were removed). In addition to colonial spumellaria, the alignment also included the solitary spumellarian *Thalassicolla nucleata* (Amaral Zettler et al., 1997) and acantharian outgroups *Haliommatidium* sp. and Chaunacanthid 218 (Amaral Zettler et al., 1997). In reality, however, no clearly appropriate outgroups exist for the spumellaria at the

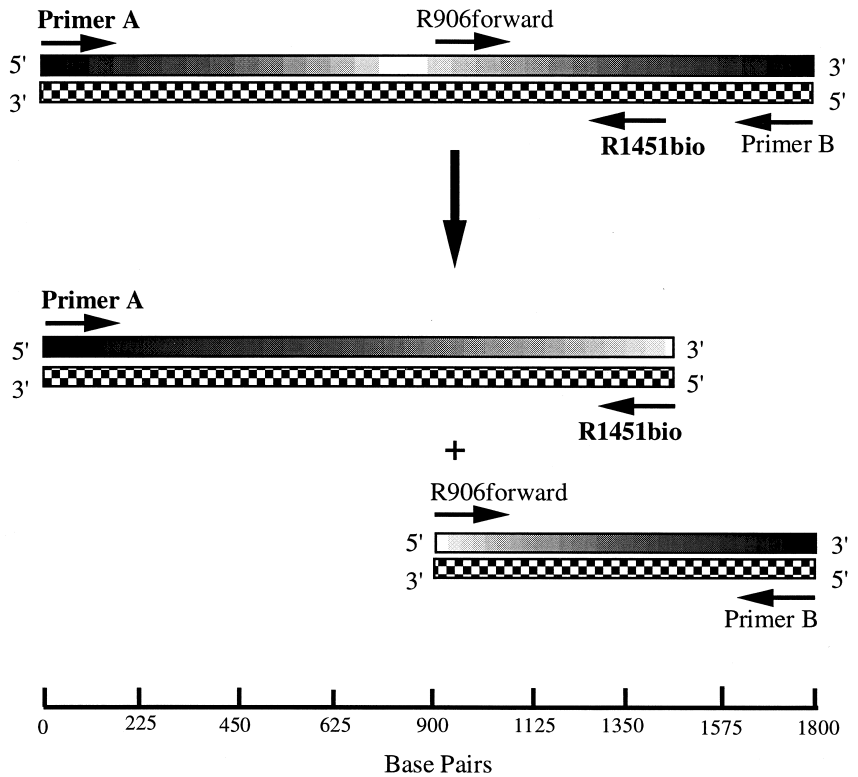


Fig. 2. The locations of colonial spumellarian-specific primers used in PCR experiments. The *R906forward* primer was used in combination with *Primer B* in PCR reactions to specifically amplify the gene fragment approximately 900 bp in length. *Primer A* was used in combination with *R1451bio* to obtain an overlapping fragment to the previous one approximately 1450 bp in length to obtain the rest of the gene.

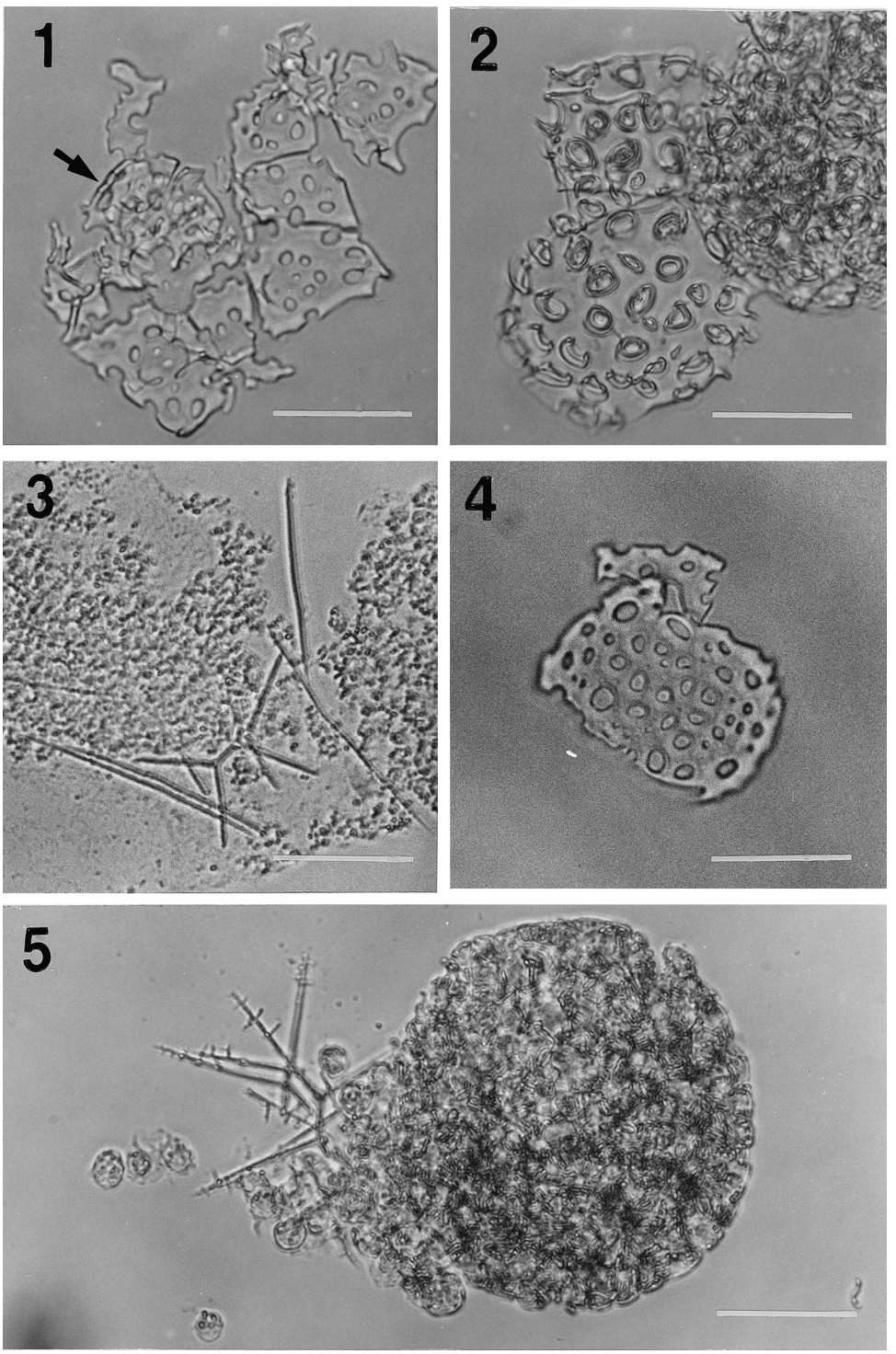
time of the writing of this manuscript since the spumellarian sequences are extremely divergent and are unrelated to any other taxa for which SSU rRNA sequence data are available.

The colonial spumellarian sequences were also analyzed independently of an outgroup (in 'unrooted' networks) in order to include more sites in the analysis (an expanded number of homologous sites which included 1635 positions). Molecular phylogenetic relationships were inferred for both data sets using distance (Olsen, 1988) (with gaps included in the distance calculation), maximum parsimony (Swofford, 1991) (with gaps treated as missing data) and maximum likelihood (Olsen et al., 1994) methods. The robustness of the tree topologies obtained were examined using 100 bootstrapping resamplings for all three methods and additionally for the maximum parsimony method using decay analyses (Bremer, 1988).

Decay analyses were accomplished by first doing an exhaustive search using PAUP 3.1.1 (Swofford, 1991) to obtain the length of the most parsimonious tree, and then sequentially adding steps to the value of the shortest tree found using the initial upper bound setting of the branch and bound search option. Resulting trees constructed at each additional step-allowance were then consensed in a strict consensus tree, and the order in which various clades 'decayed' was compared. Clades requiring relatively greater numbers of steps in order for their branches to collapse were interpreted as more robust than clades which required fewer steps to collapse.

3. Results

Photomicrographs of the skeletal structures of spicule-bearing and skeleton-bearing colonial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5

spumellaria actually used in this study are shown in Plate 1, 1–5. Species identifications were straightforward with two exceptions. Sample CR 6 (Plate 1, 1) was an *Acrosphaera*, probably *A. circumtexta*, but the species designation was difficult to ascertain. Sample BBSR 173 (Plate 1, 4) was best described as *Collosphaera globularis–huxleyi*, owing to features of the latticed shell possessed by this specimen, which appeared to exhibit qualities shared by both *C. globularis* and *C. huxleyi*. Haeckel (1887) asserted that these two species of *Collosphaera* formed intergrades. Therefore a combined species description (*globularis–huxleyi*) for this sample seemed most appropriate given the qualities of the shell morphology. The length in base pairs and % G + C content of the SSU rRNA genes of spumellaria used in this study were as follows: *Thalassicolla nucleata*, 1770 bp, 36% GC; *Rhaphidozoum acuferum*, 1813bp, 39% GC, *Sphaerozoum punctatum*, 1788 bp, 37% GC, *Collozoum pelagicum*, 1792 bp, 38% GC, *Collosphaera globularis–huxleyi*, 1797 bp, 35% GC, *Acrosphaera* (*circumtexta*?), 1803 bp, 35% GC, *Siphonosphaera cyathina*, 1791 bp, 36% GC, *Collozoum serpentinum*, 1798 bp, 38% GC.

The monophyly of the Collosphaeridae was well supported in all methods for the analyses with acantharian outgroups (Fig. 3A–C), as well as in the decay analysis wherein the node leading to the Collosphaeridae was the last to collapse, implying robust support for this clade (Fig. 4). A similar result was observed in 'unrooted' networks (based on bootstrap values of 100% in all cases, data not shown) and likewise, in decay analysis (data not shown). The branching pattern within the Collosphaeridae consistently placed *S. cyathina* branching prior to the divergence of *C. globularis–huxleyi* and *A.* (*circumtexta*?) in all methods used. The strong support for the grouping of *C. globularis–huxleyi* and *A.* (*circumtexta*?) was revealed in the decay analysis of a consensus tree (Fig. 4), in which it required an additional 45 steps before the Collosphaeridae clade completely collapsed.

The results obtained from the three different phylogenetic methods used in this study did not identify a single common tree (Fig. 3). Distance methods (Fig. 3A) failed to clearly segregate the solitary spumellarian *T. nucleata* from the colonial spumellaria as indicated by the branching of *T. nucleata* with members of the Collosphaeridae, although with insignificant bootstrap support. Maximum parsimony (Fig. 3B) was the only method which segregated the colonial spumellaria from the solitary spumellarian *T. nucleata*, however, with very low (61%) bootstrap support. Weak support for the node uniting all the colonial spumellaria was also identified in the parsimony tree by the decay analysis in which collapse of this node occurred after only three steps (Fig. 4). Maximum likelihood methods (Fig. 3C) yielded the same topology as maximum parsimony but the branching of *T. nucleata* separate from the colonial spumellaria was not well supported by bootstrapping analysis. A Kishino–Hasegawa test was conducted (data not shown) but failed to find a significant difference between the distance, maximum parsimony and maximum likelihood tree topologies.

Plate 1

Photomicrographs of voucher sections of shell-bearing and spicule bearing colonies taken of samples used in this study. Scale bar = 48 μm for all panels.

1. *Acrosphaera* (*circumtexta*?). Note the ridge-like structures often connected with thin bars (arrow). The spines, which characterize members of this genus, did not photograph well in this specimen.
2. *Siphonosphaera cyathina*. Note the cylindrical, short tube-like projections which characterize the genus. In *S. cyathina* the tube-like projections are irregularly dispersed and sometimes terminate with a folded-back distal edge.
3. *Rhaphidozoum acuferum*. This species is characterized by having both simple and radiate spines as the ones shown in this panel.
4. *Collosphaera globularis–huxleyi*. A portion of the latticed-shell of this specimen reveals smooth inner and outer surfaces which characterize members of this genus. This specimen was given a species designation of *C. globularis–huxleyi* because while most of the pore and bar dimensions matched those reported for *C. globularis* a small number of specimens possessed shapes more similar to *C. huxleyi*.
5. *Sphaerozoum punctatum*. This specimen shows the paired triradiate spicules possessed by this genus. The spines of *S. punctatum* are often barbed as seen in this photograph. Note the numerous crystal inclusions of the swarmers within the central capsule of this reproductive individual.

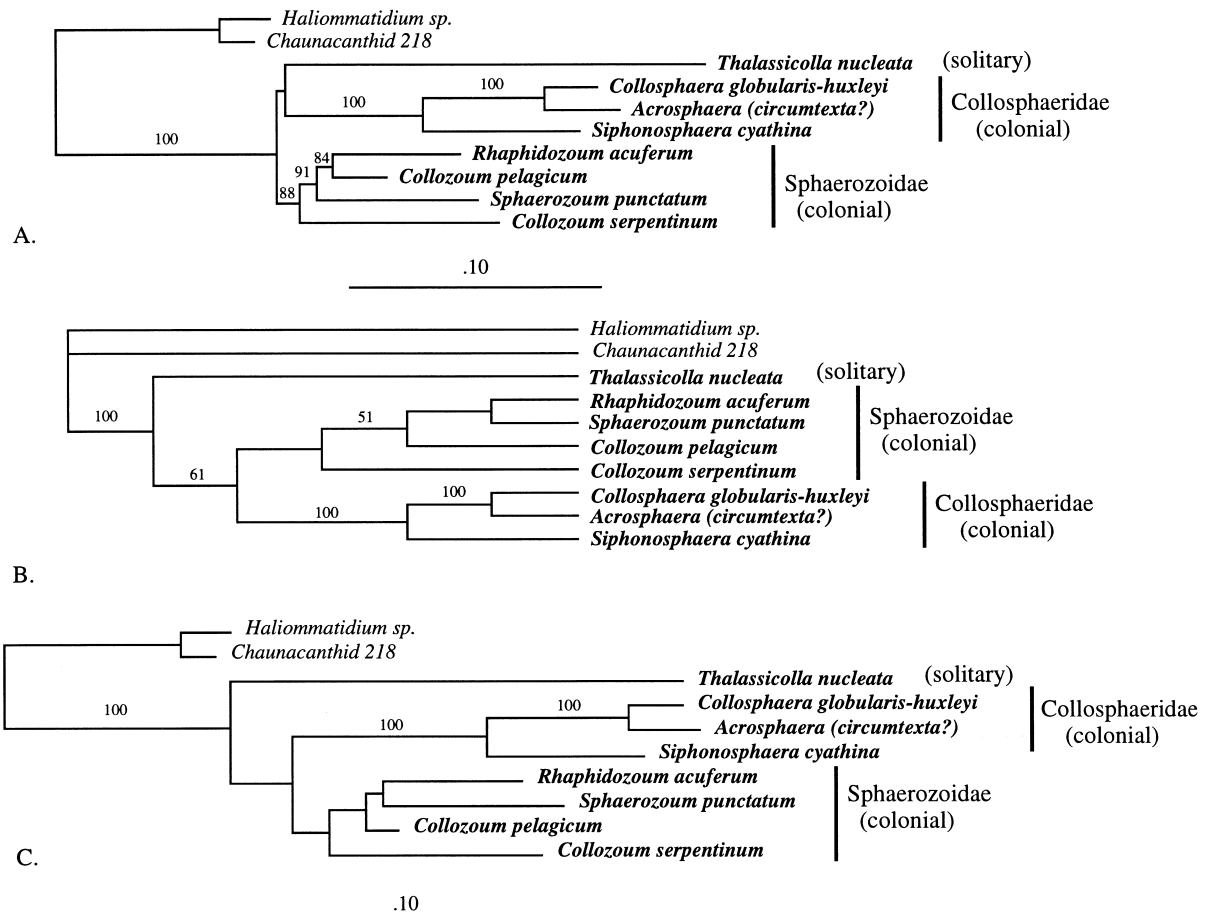

Fig. 3. Phylogenetic reconstructions for solitary and colonial spumellarians using acantharian outgroups (*Haliommatidium* sp. and Chaunacanthid 218) inferred from: (A) distance, (B) maximum parsimony and (C) maximum likelihood methods. There were 1367 positions used in the phylogenetic analyses. All bootstrap values were computed separately for 100 resamplings of the three respective data sets. Only bootstrap values greater than 50% are shown. The evolutionary distances are indicated by the bar insert (distance and maximum likelihood) which represents 10 changes per 100 nucleotides.

The separation of the Sphaerozoidae and the Thalassicollidae was not clearly supported in all cases. The bootstrap support values for separation of these two families varied dramatically in the distance analysis relative to the maximum parsimony and maximum likelihood analyses (Fig. 3A, C). The distance analysis clearly isolated the Sphaerozoidae from the Collosphaeridae and *T. nucleata* (bootstrap value of 88% on the branch leading to the Sphaerozoidae). Although the maximum parsimony and maximum likelihood methods supported a separate ancestry for the Sphaerozoidae distinct from the Collosphaeridae, the low bootstrap support for the parsimony and maximum likelihood tree topologies indicates poor support for the Sphaerozoidae as a distinct clade.

The support for branching order within the Sphaerozoidae also varied depending on the method of phylogenetic inference. In general, the branching patterns within the Sphaerozoidae were poorly resolved in the parsimony and maximum likelihood consensus trees. However, there was weak support for the grouping of *R. acuferum*, *C. pelagicum* and *S. punctatum* in the parsimony consensus tree (bootstrap value of 51%; Fig. 3B). The highest bootstrap

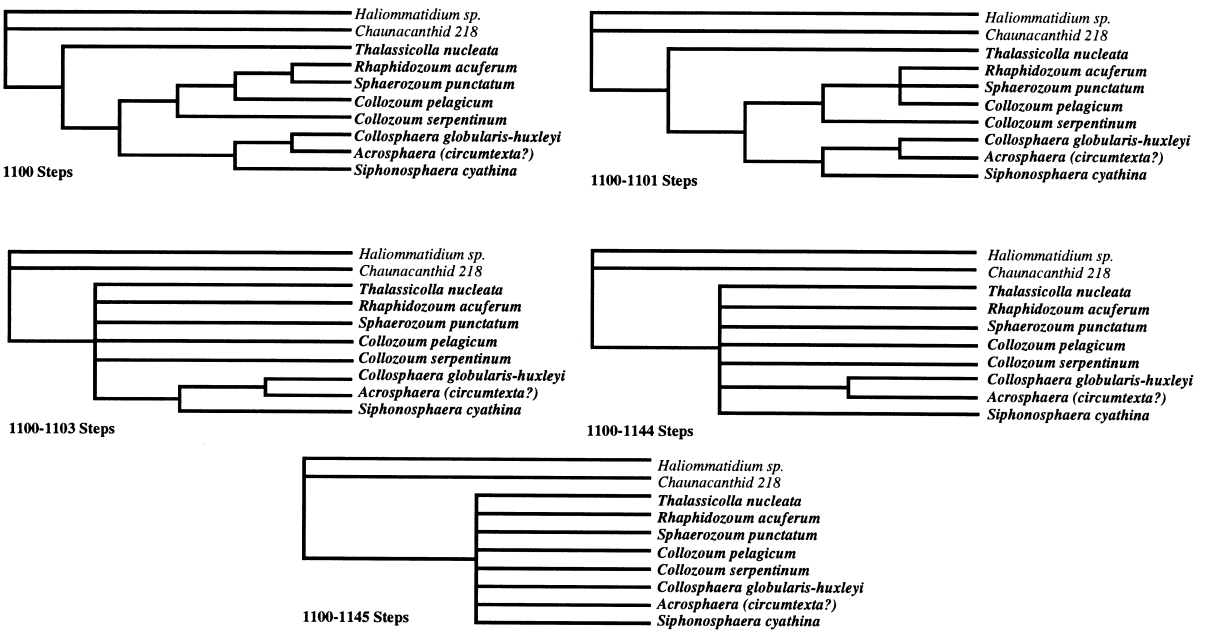

Fig. 4. Results from a decay analysis of the most parsimonious tree obtained from an exhaustive search. The number of additional steps required to produce the consensus trees with progressive degrees of collapse of major nodes is shown to the bottom left of each corresponding tree.

support values were obtained from distance analyses (Fig. 3A) and supported a branching order which separated the two species of *Collozoum*, placing one species branching early in the Sphaerozoidae and another sharing common ancestry with spicule-bearing genus *R. acuferum*. This tree also separated the two spicule-bearing species *S. punctatum* and *R. acuferum*.

Better branching support emerged from the maximum likelihood analysis from the 'unrooted networks' (data not shown) approach, which agreed with parsimony analysis, and placed the *R. acuferum* together with *S. punctatum* and *C. pelagicum* (bootstrap value of 95%).

4. Discussion and conclusions

4.1. Relationships among the Collosphaeridae

The phylogenetic reconstructions carried out in this study unanimously supported the monophyly of shell-bearing colonial spumellaria belonging to the family Collosphaeridae examined in this study. Strong support was identified by both high bootstrap values (100% in all cases) and robust Bremer (decay analysis) support. Branching patterns within the Collosphaeridae indicate that *Siphonosphaera* diverged prior to the split of *Collosphaera* and *Acrosphaera*.

The observed divergence of *Siphonosphaera* prior to *Collosphaera* and *Acrosphaera* is contrary to a hypothesis presented by Strelkov and Reshetnyak (1971). These authors speculated that members of the genus *Collosphaera* represent a more primitive line of decent and that *Acrosphaera* and *Siphonosphaera* represent more derived forms. They argued that the smooth latticed skeletons possessed by the members of the genus *Collosphaera* represent more primitive features than the more elaborate skeletons of the genus *Acrosphaera*, which have a spiny appearance or those of *Siphonosphaera* which possess tube-like projections. Anderson and Swanberg (1981) also stated that spines and tubule ornamentation are most likely more derived features. These speculations are clearly contrary to the results of our molecular analyses which consistently placed *Siphonosphaera* branching prior to *Collosphaera* and *Acrosphaera*.

Evidence from physiological and electron microscopy studies indicates that the tube-like projections present in members of the genus *Siphonosphaera* may be the result of silicification on the pore rims of the shell after the porous shell wall has been substantially deposited within the living cytoplasmic sheath known as a cytokalymma (Anderson and Swanberg, 1981; Anderson, 1983) and may involve the dynamic shaping activity of cytoplasmic flowing (Cachon and Cachon, 1972; Anderson, 1983). Observations of living spumellaria depositing spines on the shell surface clearly support the hypothesis that surface ornamentation is a product of dynamic cytoplasmic streaming during silica deposition (Anderson, 1983). If tube-like projections are deposited on the pore rims of shells subsequent to major silicification of the shell, one can imagine that these structures may have arisen any time in evolution, and possibly even more than once. Likewise, spicules may be a remnant of incomplete tube formation. This might account for the forms with spiny tube-like projections, those that are partially on the way to reduction from a tube to spicules, as well as those that are completely reduced to only one spine.

4.2. Relationships among the Sphaerozoidae

The monophyly of the Sphaerozoidae was well supported in the distance analysis (bootstrap value of 88% leading to this family, Fig. 3A), however, parsimony and maximum likelihood methods (Fig. 3B, C) generated tree topologies which were in general poorly supported by the bootstrapping method. A well-supported branching pattern was also identified within the Sphaerozoidae using distance methods (Fig. 3A). The branching pattern for the distance analysis, however, separated the two *Collozoum* species indicating a separate ancestry for the two *Collozoum* taxa. This pattern implies that a secondary loss of skeletal material (i.e. spicules) may have occurred within *C. pelagicum*.

A similar conclusion concerning secondary skeletal loss was reached by Strelkov and Reshetnyak (1971). These authors proposed that the absence of skeletal elements is a secondary phenomenon and that the common ancestor of the Sphaerozoidae was spicule-bearing. They attributed the lack of skeletal elements to a secondary phenomenon because members of the genus *Collozoum*, which are typically free of any skeletal material, are very infrequently found to possess simple spicules in their cytoplasm. It is possible, of course, that the rare occurrence of spicules in the cytoplasm of *Collozoum* may be a consequence of feeding activity rather than skeletogenesis. During our field studies, Spumellaria collected by SCUBA divers and immediately examined in the laboratory sometimes contain spicules of silicoflagellates, presumably consumed as prey. In any event, our sequence information supports the contention of Strelkov and Reshetnyak (1971) that presence of spicules may not be a reliable phylogenetic marker within the Sphaerozoidae.

The failure of the two *Collozoum* species to branch together in all analyses indicates that absence of skeletal material may be an equally unreliable phylogenetic marker. *Collozoum serpentinum* differs noticeably from *C. pelagicum*, as well as other *Collozoum* species, by the characteristics of its central capsule (see Anderson et al., 1999). The central capsule in *C. serpentinum* is elongated and often forms twisted loops whereas in *C. pelagicum* it is characterized by digitform apophyses which are often branching at the ends. Interestingly, in maximum parsimony and maximum likelihood analyses of the ‘unrooted’ network phylogenies, *C. pelagicum* was observed to branch with *R. acuferum* and *S. punctatum*, both spicule-bearing colonials. Although perhaps only coincidental, the shape of the apophyses on the central capsule of *C. pelagicum* bears a crude resemblance to the spicules of *R. acuferum* and *S. punctatum* suggesting a possible evolutionary connection between these apophyses and the radiate spicules possessed by *Rhaphidozoum* and *Sphaerozoum*.

4.3. The question of coloniality in the colonial spumellaria

The ability to form colonies is perhaps the most interesting characteristic of the colony-forming spumellaria. Many colonial spumellaria of the Sphaerozoidae family have solitary stages as part of their life cycle (Brandt, 1902; Hollande and Enjumet, 1953; Swanberg, 1979) but solitary stages appear to be absent in members of the Collosphaeridae. The genus name *Thalassophysa*, for example, is used when referring to the solitary stage of the various

members of *Collozoum*. In the taxa examined in this study, *Thalassophysa sanguinolenta* is the name given to the solitary stage of the colonial *Collozoum pelagicum* (Brandt, 1902). *Collozoum serpentinum* also has a solitary stage (Swanberg, 1979). Although solitary stages have never been observed in members of the Collosphaeridae, we cannot rule out the possibility that collosphaerids once had a solitary stage.

It is generally assumed that members of the colonial spumellaria represent a monophyletic assemblage and that the ability to form colonies has arisen only once in their evolution (Strelkov and Reshetnyak, 1971; Anderson and Swanberg, 1981). This hypothesis has been arguably difficult to address using evidence from the fossil record since only individual shells are left behind in the marine sediments. Molecular approaches, however, allow us to begin to explore this question. Our data from molecular phylogenetic analyses indicate that the ability to form colonies may have evolved more than once in the evolution of the spumellarian radiolaria but more data are needed to fully elucidate natural relationships and address this issue with greater certainty.

Molecular data did not robustly support the monophyly of colonial spumellaria. For example, the distance matrix method produced a tree topology which could not resolve the branching order of *T. nucleata*, a solitary spumellarian (incapable of colony formation), relative to the two families of colonial spumellaria examined. The maximum likelihood tree also did not show strong support for the branching order of *T. nucleata* relative to the two colonial families. Maximum parsimony analysis alone separated the colonial spumellaria from *T. nucleata*, but with a very low bootstrap value of 61% (Fig. 3B). The weak support for the monophyly of the colonial spumellaria was further echoed in the decay analysis in which the most parsimonious tree collapsed the node separating *T. nucleata* from the representatives of the Sphaerozoidae after only three steps. These results indicate that the node separating the colonial spumellaria from the solitary *T. nucleata* is not very robust.

Given that this analysis included only one solitary representative, however, it is premature to draw firm conclusions at this time. It will be necessary to examine additional solitary spumellarian taxa to test the monophyly of coloniality more fully. Furthermore, we are working under the assumption that *Thalassicolla* is derived from an ancestor that was not capable of forming colonies. The available information in the literature and personal observation indicates that the genus is strictly solitary, however, the possibility that *Thalassicolla* evolved from a colonial ancestor and has now lost its ability to form colonies cannot be excluded. For this reason, further investigation is needed to examine additional solitary species and hopefully make more conclusive statements about coloniality in spumellaria.

4.4. Phylogenetic relationships based on molecular, morphological and fossil evidence

Phylogenetic reconstructions presented here based on SSU rRNA coding regions are in disagreement with existing theories regarding the evolutionary history of the colonial spumellarian radiolaria based on morphological and fossil evidence. Using morphological criteria, Strelkov and Reshetnyak (1971) hypothesized that the skeletons of the Collosphaeridae were derived from an ancestor with spines which merged to form a skeletal structure. A similar conclusion on the possible phylogenetic relationships among the colonial spumellaria was reached by Anderson and Swanberg (1981) in their analysis of skeletal morphogenesis in representatives from the Collosphaeridae. The latter authors proposed a mechanism for shell deposition in colonial spumellaria which involved the precursory production of ‘cytokalymma’ (differentiated extracapsular cytoplasm), followed by deposition of ‘organic nucleation centers’ which served as the matrix for the developing silica shell. These authors described two methods of shell morphogenesis (bridge-growth and rim-growth) which they speculated could account for observed variations in pore characteristics and shell ornamentation such as spines and tubules. Like Strelkov and Reshetnyak (1971), Anderson and Swanberg (1981) suggested that shell-bearing forms evolved from a spicule-bearing ancestor and that lattice shells are the result of the fusion of bar-like elements.

The geological records of the colonial spumellaria yield conflicting information on their evolution history. Based on observations from the fos-

sil record, Bjørklund and Goll (1979) noted that there is no evidence for the common ancestry of the Collosphaeridae and Sphaerozoidae from the fossil record. They stated that the first occurrence of Sphaerozoidae in the fossil record is somewhat earlier (Lower Oligocene) than the Collosphaeridae (basal Miocene) and that the distributions of the Sphaerozoidae are typically high latitude whereas the Collosphaeridae originated and diversified from equatorial regions. Furthermore, the first occurrences of the Collosphaeridae in the fossil record are abrupt and characterized by fully formed lattice shells which goes against what would be expected if latticed shells were the result of fusion of spicules. Finally, fossil evidence suggests that *Acrosphaera* diverged prior to *Siphonosphaera* which is opposite to the branching patterns seen in the molecular data. This observation deserves further scrutiny using additional representatives from both of these genera.

While this molecular study has touched upon the issue of the evolution of coloniality among the spumellaria, the importance of this character in determining relationships among the Spumellarida remains very much unresolved. This analysis, while far from a complete molecular diagnosis, is the first step towards revealing potential avenues for further exploration into colonial spumellarian evolution. We believe that the colonial-specific primers that were designed during this work will greatly facilitate the speed and ease with which additional molecular sequence data are obtained. Because these primers are specific for the group, more time-consuming culturing methods are not required thereby increasing the ease with which future data collection can be accomplished. These molecular tools will hopefully also be helpful in determining the extent to which morphological variability seen in colonial spumellaria is reflected at the genetic level. Such studies as this will hopefully provide much-needed insights into the life history of these morphologically and genetically diverse protists.

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Appendix A. Taxonomic list

Thalassicolla nucleata Huxley, 1851
Rhaphidozoum acuferum Müller, 1855
Sphaerozoum punctatum Meyen, 1834
Collozoum pelagicum Haeckel, 1887
Collosphaera globularis–huxleyi Haeckel, 1887
Acrosphaera (*circumtexta*?) Haeckel, 1887
Siphonosphaera cyathina Haeckel, 1887
Collozoum serpentinum Haeckel, 1887

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