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Molecular phylogenetic position of *Hexacontium pachydermum* Jørgensen (Radiolaria)

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ABSTRACT

The taxonomic affiliation of *Hexacontium pachydermum* Jørgensen, specifically whether it belongs to the order Spumellarida or the order Entactinarida, is a subject of ongoing debate. In this study, we sequenced the 18S rRNA gene of *H. pachydermum* and of three spherical spumellarians of *Cladococcus viminalis* Haeckel, *Arachnosphaera myriacantha* Haeckel, and *Astrosphaera hexagonalis* Haeckel. Our molecular phylogenetic analysis revealed that the spumellarian species of *C. viminalis*, *A. myriacantha*, and *A. hexagonalis* form a monophyletic group. Moreover, this clade occupies a sister position to the clade comprising the spongodiscid spumellarians, coccodiscid spumellarians, and *H. pachydermum*. This finding is contrary to the results of morphological studies based on internal spicular morphology, placing *H. pachydermum* in the order Entactinarida, which had been considered to have a common ancestor shared with the nassellarians.

1. Introduction

Hexacontium pachydermum was originally described from the west coast of Norway by Jørgensen (1900). The species has three spherical shells, consisting of two medullary shells and one cortical shell, with six radial three-bladed spines oriented perpendicular to each other (Cortese and Bjørklund, 1998). The outer cortical shell has somewhat regular rounded pores and is furnished with numerous needle-like byspines in all pore corners (Cortese and Bjørklund, 1998).

Since the work of Haeckel (1881, 1887), the taxonomy of the genus *Hexacontium* has generally been based on features of the morphology of the skeleton, such as the outer cortical shell, medullary shells, radial spines, and by-spines (e.g., Boltovskoy, 1999; Anderson et al., 2002). Based on these morphologic features, some researchers have placed the genus *Hexacontium* in the order Spumellarida (Boltovskoy, 1999; Anderson et al., 2002).

In contrast, De Wever et al. (2001) classified the genus *Hexacontium* as belonging to the family Hexalonchidae, order Entactinarida, which was established by Kozur and Mostler (1982) as a new suborder of Radiolaria based on morphologic features of the initial skeleton. According to Kozur and Mostler (1982) and De Wever et al. (2001), members of the order Entactinarida are characterized by an initial skeleton with a variable number of spines arising from the two ends of "a median bar" or from a center. Accordingly, the authors concluded that the order Entactinarida has an inner spicular system homologenous with that of the order Nassellarida.

The question of whether *Hexacontium pachydermum* Jørgensen belongs to the order Spumellarida or to the order Entactinarida remains a topic of debate.

The method of molecular phylogeny has been used to define the relationships among radiolarians (e.g., Amaral Zettler et al., 1997; Polet et al., 2004; Yuasa et al., 2005). Such sequence data have revealed four distinct genetic groups of radiolarians: the Nassellarida, Spumellarida, Acantharea, and Taxopodida. Moreover, some researchers have reported a large number of radiolarian-affiliated sequences recovered by analyzing 18S rDNA clone libraries of marine picoeukaryotes (e.g., Díez et al., 2001; López-García et al., 2001; Moon-van der Staay et al., 2001; Massana et al., 2002; Not et al., 2007). The libraries that included samples from sediments and deep-sea environments were rich in radiolarians and the sequences were branched among the clade of the radiolarians as the Polycystinea and the Acantharea.

In the present study, we sequenced the 18S rRNA gene of four spherical radiolarians: *Cladococcus viminalis* Haeckel, *Hexacontium pachydermum* Jørgensen, *Arachnosphaera myriacantha* Haeckel, and *Astrosphaera hexagonalis* Haeckel (Fig. 1). Given their disputed taxonomic and phylogenetic placement (which until now has been based on the presence or absence of cytoplasmic and skeletal features), we sought to investigate whether all these species are spumellarian species or whether some (e.g., *Hexacontium pachydermum*) are more closely related to the nassellarians, as suggested by De Wever et al. (2001).

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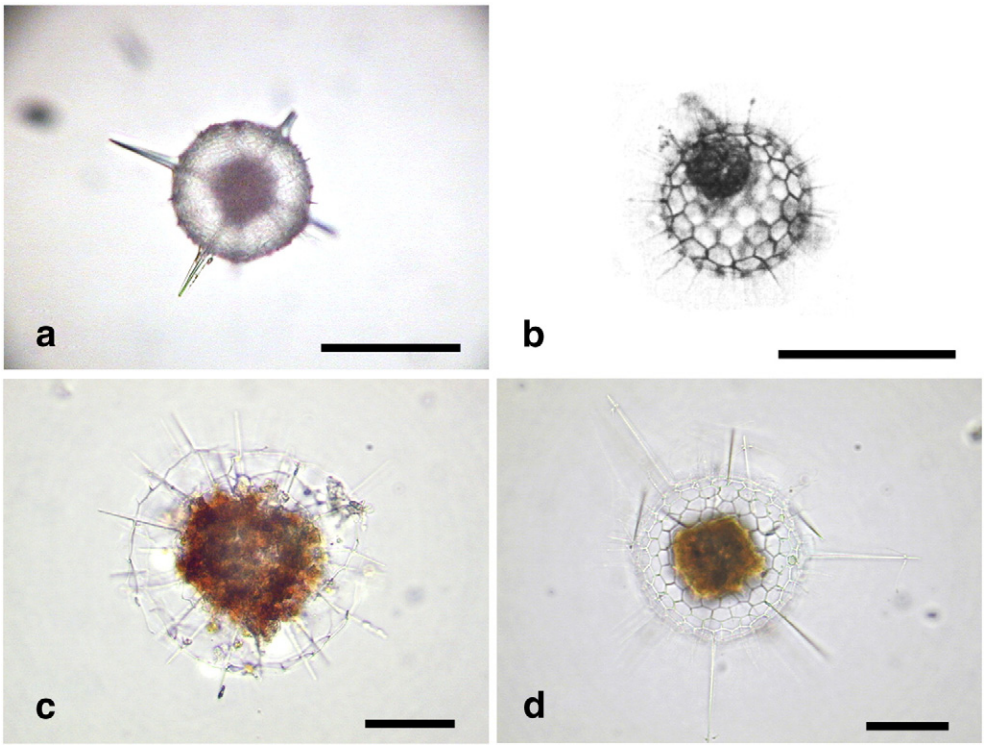


Fig. 1. Light micrographs of radiolarians used in this study. Scale bars indicate 100 µm. (a) *Hexacontium pachydermum* Jørgensen from the Sogndalsfjord, Norway. (b) *Cladococcus viminalis* Haeckel from the Sogndalsfjord, Norway. (c) *Arachnosphaera myriacantha* Haeckel from the East China Sea. (d) *Astrosphaera hexagonalis* Haeckel from the East China Sea.

2. Materials and methods

Cladococcus viminalis Haeckel and *Hexacontium pachydermum* Jørgensen were collected using a 45-µm-mesh plankton net at Sogndalsfjord, a tributary fjord to the Sognefjord, southwestern Norway, on 18 June and 27 October 2003, respectively. The water depth at the sampling site (61°12′32″N, 7°05′55″E) was about 270 m. The water column was sampled from 250 to 25 m, with the net being closed at 25 m to avoid the diatomaceous surface layer. *Arachnosphaera myriacantha* Haeckel and *Astrosphaera hexagonalis* Haeckel were collected using a 37-µm-mesh plankton net at a location site (26°37′N, 127°50′E) approximately 5 km northwest of Okinawa Island, Japan, on 6 October 2008.

The collected samples were stored at about 5 °C and immediately brought to the laboratory, where individual species were transferred to culturing dishes containing filtered seawater and stored in a refrigerator until further analysis.

2.1. DNA extraction and amplification

A single cell of each species was rinsed twice in filtered seawater, and the central capsule was physically separated (using a sterile razor blade) from the ectocytoplasm, which contained endosymbiotic algae. The central capsule was rinsed twice more in distilled water, and then incubated for 30 min at 37 °C in 0.2 µg/µl proteinase K solution. This sample was used as a template for the amplification of 18S rRNA gene regions.

The first PCR amplification was performed using a MiniCycler (MJ Research, Massachusetts, USA) with an initial denaturation step of 95 °C for 3 min, followed by 35 amplification cycles each consisting of 95 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min with the eukaryotic-specific forward primer 90F (Hendriks et al., 1989): 5′-GAAACTGCGAATGGCTCATT-3′ and the reverse primer B (Medlin et al., 1988): 5′-CCTTCTGCAGGTTCACCTAC-3′. In the second PCR, 1.0 µl of the first PCR products was added to a new reaction mixture. PCR conditions were an initial denaturation step of 95 °C for 3 min, followed by 35 amplification cycles each consisting of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min using the primers 90F & 1130R: 5′-CCGTCAATTCCTTTAAGTTT-3′, and 570F (Hendriks et al., 1989): 5′-CGCGGTAATTCCAGCTCC-3′ & B for *H. pachydermum*, *A. myriacantha*, and *A. hexagonalis,* and 191F (Yuasa et al., 2004): 5′-GCGACTYACGAAGCCCTGTA-3′ and 1731R (Yuasa et al., 2004): 5′-ACTTCGRGCCTCCCGTT-3′ for *C. viminalis*. The PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA), and then cloned in the pGEM-T Easy Vector System (Promega) using *E. coli* JM109 Competent Cells (Promega).

2.2. Sequencing

Sequencing was performed using the DYEnamicET terminator or BigDye terminator v3.1 cycle sequencing kit (Perkin-Elmer, Forster City, CA, USA) and analyzed using the ABI PRISM 377 or 3130 Genetic Analyzer (Perkin-Elmer), according to the manufacturer's instructions. Sequences of the *C. viminalis*, *H. pachydermum*, *A. myriacantha*, and *A. hexagonalis* have been submitted to GenBank under accession numbers AB284518, AB284519, AB490705, and AB490706, respectively.

2.3. Alignment

The obtained sequences were aligned with other sequences of polycystines, acantharians, phaeodarians, and cercozoans retrieved from GenBank using Clustal W ver. 1.81 (Thompson et al., 1994). The accession numbers of the 18S rRNA gene sequences used in this study are listed in Table 1.

2.4. Phylogenetic analysis

Molecular phylogenetic relationships were inferred from Bayesian analysis using MrBayes ver. 3.0b4 (Huelsenbeck and Ronquist, 2001), and from neighbor-joining (NJ) (Saitou and Nei, 1987) and maximum-likelihood (ML) (Felsenstein, 1981) methods using PAUP* version 4.0b10 (Swofford, 2002). Bayesian analysis employed a data set of 91

Table 1
Accession numbers of 18S rRNA gene sequences of Acantharea, Polycystinea, Phaeodarea, *Sticholonche*, Cercozoa, and environmental samples used in this study.

	Taxon	Accession number
Acantharea	*Acanthometra sp.*	AF063240
	Arthracanthid	206 AF063239
	Amphiacon denticulatus	AB178588
	Amphibelone anomala	AB178582
	Chaunacanthid 217	AF063241
	Chaunacanthid sp. 218	AF018158
	Haliommatidium sp.	AF018159
	Hexaconus serratus	AB178587
	Symphyacanthid 211	AF063242
Cercozoa	*Haplosporidium nelsoni*	X74131
	Phagomyxa odontellae	AF310904
	Polymyxa betae	AF310902
	Urosporidium crescens	U47852
Polycystinea	*Acrosphaera* sp. CR6A	AF091148
	Arachnosphaera myriacantha	AB490705
	Astrosphaera hexagonalis	AB490706
	Artostrobus sp. 2014	AB246685
	Cladococcus viminalis	AB284518
	Collosphaera globularis-huxleyi	AF018163
	Collozoum pelagicum	AF091146
	Collozoum serpentinum	AF018162
	Dicranastrum furcatum	AB179733
	Dictyocoryne profunda	AB101540
	Dictyocoryne truncatum	AB101541
	Didymocyrtis tetrathalamus	AB193605
	Euchitonia elegans	AB179732
	Eucyrtidium acuminatum	AB246690
	Eucyrtidium hexagonatum	AB179735
	Eucyrtidium hexastichum	AB246681
	Hexacontium pachydermum	AB284519
	Lithomelissa sp. 8012	AB246694
	Pterocanium trilobum	AB246682
	Pterocorys zancleus	AB179736
	Pseudocubus obeliscus	AB246692
	Rhaphidozoum acuferum	AF091147
	Siphonosphaera cyathina	AF091145
	Sphaerozoum punctatum	AF018161
	Spongaster tetras	AB101542
	Spongodiscus biconcavus	AB246695
	Spongodiscus resurgens	AB246696
	Spongopyle osculosa	AB246689
	Spumellarian radiolarian 7017	AB246691
	Stylodictya sp. 8037	AB246698
	Styptosphaera sp. 2022	AB246686
	Tetrapyle octacantha	AB246680
	Thalassicolla nucleate	AY266297
	Thalassicolla pellucida	AY266297
	Thalassophysa pelagica	AY266296
	Triastrum aurivillii	AB179734
Taxopodida	*Sticholonche* sp. JJP-2003	AY268045
Phaeodarea	*Aulosphaera trigonopa*	AY266292
	Coelodendrum ramosissimum	AY266293
	Conchellium capsula	AB218766
	Protocystis xiphodon	AB218767
Environmental samples	AT4_94	AF530525
	AT8_54	AF530524
	DH145-HA2	AF382824
	DH145-KW16	AF382825
	DH147-EKD17	AF290072
	OLI11015	AJ402332
	OLI11016	AJ402333
	OLI11032	AJ402342
	Q2E12N5	EF173011
	SSRPB22	EF172808
	SSRPB27	EF172806
	SSRPB44	EF172834
	SSRPB51	EF172802
	SSRPB73	EF172833
	SSRPB82	EF172835
	SSRPC12	EF172891
	SSRPC34	EF172903
	SSRPC45	EF172909
	SSRPC53	EF172914
	SSRPC81	EF172892
	SSRPC85	EF172903
	SSRPC87	EF172894
	SSRPC73	EF172833
	SSRPD81	EF172984
	OLI011-75m.A	EU287797
	OLI011-75m.2(5)	EU287801
	OLI011-75m.12(5)	EU287794
	OLI011-75m.19	EU287791
	OLI011-75m.26(5)	EU287799
	OLI011-75m.32	EU287800
	OLI011-75m.45	EU287807
	OLI016-75m.46	EU287808
	OLI011-75m.47	EU287792
	OLI011-75m.50	EU287795
	OLI011-75m.58	EU287793
	OLI011-75m.62	EU287798
	OLI011-75m.82	EU287796

taxa (1209 bp) (Table 1). NJ and ML analyses were run while omitting the partial sequences for environmental samples to determine the effect of their absence on the tree topology. A data set of 47 taxa (1260 bp) (Table 1) was used for NJ and ML analyses.

Bayesian analysis was performed using the general time reversible (GTR) model (Lanave et al., 1984) with gamma-distributed site-to-site rate variation (G) and allowance for invariant sites (I) of substitution among sites. Five million generations were run and 50000 trees were sampled, 10000 of which were discarded as burn-in.

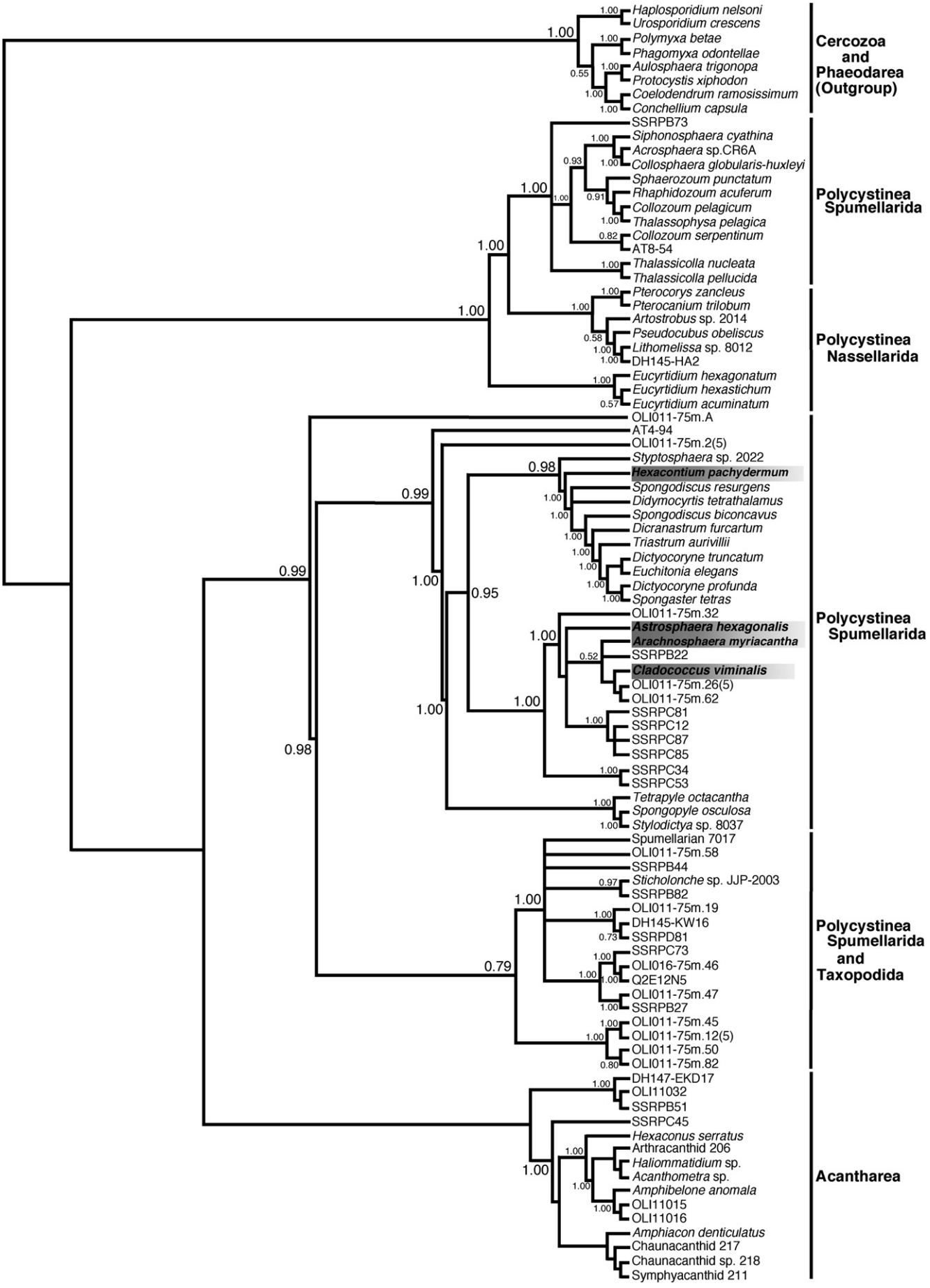
The evolutionary distance decided from the GTR + G + I distance matrices models was used for NJ and ML analyses. The ML analysis was initially obtained from the transition/transversion (ts/tv) ratio of the GTR + G + I distance matrices model, which was estimated by maximizing the likelihood value for the NJ topology. The ML tree was then analyzed using a heuristic search method with a TBR branch-swapping option and random taxon addition.

The reliability of internal branches was assessed using the posterior probabilities (PP) calculated with MrBayes ver. 3.0b4 (Fig. 2), and the bootstrap values of the NJ and ML trees were obtained from 1000 and 100 replicates, respectively (Fig. 3). Only the ML tree with bootstrap values for both ML and NJ is shown in Fig. 3.

3. Results

Our analyses (Figs. 2 and 3) reveal that all four analyzed radiolarian species (*Hexacontium pachydermum*, *Cladococcus viminalis*, *Arachnosphaera myriacantha*, and *Astrosphaera hexagonalis*) clearly belong to the Spumellarida, a clade supported by high PP and bootstrap values (PP: 0.99, ML: 100%, NJ: 100%); however, the four species do not form a monophyletic group: *H. pachydermum* branches at the base of the family Spongodiscidae (*Dictyocoryne truncatum*, *D. profunda*, *Spongaster tetras*, *Euchitonia elegans*, *Dicranastrum furcartum*, and *Triastrum aurivillii*) and the family Coccodiscidae (*Didimocyrtis tetrathalamus*) lineage. Therefore, *H. pachydermum* groups together with the "spongodiscid and coccodiscid spumellarian" clade with high PP and bootstrap values (PP: 0.98, ML: 100%, NJ: 100%).

On the other hand, *C. viminalis*, *A. myriacantha*, and *A. hexagonalis* form a sister group with the clade comprising the spongodiscid spumellarians, the coccodiscid spumellarians, and *H. pachydermum*. The clade was supported with PP of 0.95 for Fig. 2, but it was not well supported with BV of 54% and 63% for Fig. 3. The position of the acantharian clade changes depending on the employed phylogenetic method. Concerning the four spherical spumellarians, however, the topologies of the Bayesian, NJ, and ML trees are all similar.


Haplosporidium nelsoni
Urosporidium crescens
Polymyxa betae
Phagomyxa odontellae
Aulosphaera trigonopa
Protocystis xiphodon
Coelodendrum ramosissimum
Conchellium capsula
Cercozoa and Phaeodarea (Outgroup)
SSRPB73
Siphonosphaera cyathina
Acrosphaera sp.CR6A
Collosphaera globularis-huxleyi
Sphaerozoum punctatum
Rhaphidozoum acuferum
Collozoum pelagicum
Thalassophysa pelagica
Collozoum serpentinum
AT8-54
Thalassicolla nucleata
Thalassicolla pellucida
Polycystinea Spumellarida
Pterocorys zancleus
Pterocanium trilobum
Artostrobus sp. 2014
Pseudocubus obeliscus
Lithomelissa sp. 8012
DH145-HA2
Eucyrtidium hexagonatum
Eucyrtidium hexastichum
Eucyrtidium acuminatum
Polycystinea Nassellarida
OLI011-75m.A
AT4-94
OLI011-75m.2(5)
Styptosphaera sp. 2022
Hexacontium pachydermum
Spongodiscus resurgens
Didymocyrtis tetrathalamus
Spongodiscus biconcavus
Dicranastrum furcartum
Triastrum aurivillii
Dictyocoryne truncatum
Euchitonia elegans
Dictyocoryne profunda
Spongaster tetras
OLI011-75m.32
Astrosphaera hexagonalis
Arachnosphaera myriacantha
SSRPB22
Cladococcus viminalis
OLI011-75m.26(5)
OLI011-75m.62
SSRPC81
SSRPC12
SSRPC87
SSRPC85
SSRPC34
SSRPC53
Tetrapyle octacantha
Spongopyle osculosa
Stylodictya sp. 8037
Polycystinea Spumellarida
Spumellarian 7017
OLI011-75m.58
SSRPB44
Sticholonche sp. JJP-2003
SSRPB82
OLI011-75m.19
DH145-KW16
SSRPD81
SSRPC73
OLI016-75m.46
Q2E12N5
OLI011-75m.47
SSRPB27
OLI011-75m.45
OLI011-75m.12(5)
OLI011-75m.50
OLI011-75m.82
Polycystinea Spumellarida and Taxopodida
DH147-EKD17
OLI11032
SSRPB51
SSRPC45
Hexaconus serratus
Arthracanthid 206
Haliommatidium sp.
Acanthometra sp.
Amphibelone anomala
OLI11015
OLI11016
Amphiacon denticulatus
Chaunacanthid 217
Chaunacanthid sp. 218
Symphyacanthid 211
Acantharea

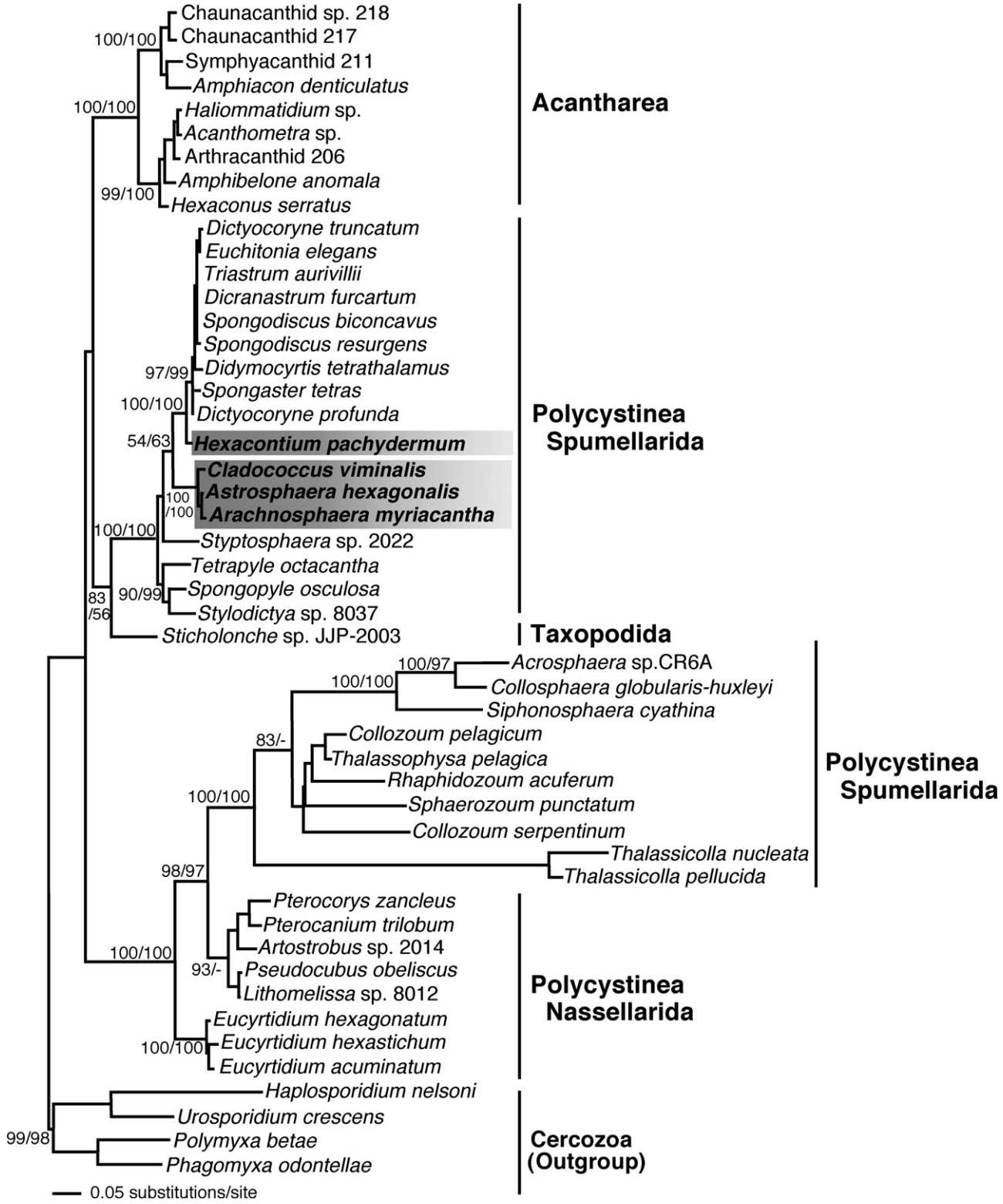


Fig. 3. Phylogenetic tree of 18S rRNA genes obtained from 49 taxa (1260 nucleotide sites). The tree was constructed using the maximum likelihood (ML) method. Bootstrap values exceeding 50% are given at the respective nodes obtained from both ML and neighbor-joining methods (ML/NJ %).

4. Discussion

Our molecular phylogenetic analysis revealed that the spumellarian species of *Cladococcus viminalis*, *Arachnosphaera myriacantha*, and *Astrosphaera hexagonalis* form a monophyletic group. Moreover, this clade occupies a sister position to the clade comprising the spongodiscid spumellarians, coccodiscid spumellarians, and *Hexacontium pachydermum*.

Fig. 2. Phylogenetic tree of 18S rRNA genes obtained from 91 taxa (1209 nucleotide sites). The tree was constructed based on Bayesian analysis. See the 'Material and methods' section for additional information. Posterior Probabilities (PP) exceeding 0.50 are given at the respective nodes.

This finding is contrary to the results of morphological studies based on internal spicular morphology, placing *H. pachydermum* within the order Entactinarida (Kozur and Mostler, 1982; De Wever et al., 2001), which has been suggested to share a common ancestor with the nassellarians.

The internal spicule is considered an important taxonomic feature, especially for the nassellarians (e.g., Riedel, 1971), and it has been used to group all radiolarians possessing this feature in higher-rank categories (e.g., De Wever et al., 2001). According to Dumitrica (2001), the Entactinaria have the following diagnostic features: 1) species having a simple structure in the center, consisting of an initial spicule and some connecting arches, never a true latticed shell; and 2) initial spicule commonly consisting of a median bar with six spines (two equal apical spines and four basal spines, with the latter connected to one another by a system of arches bearing 1–10 antapical spines). However, an accurate analysis of the features of the internal skeleton is generally demanding, requiring dedicated efforts to understand the complex spatial relationships (e.g., Anderson et al., 2002).

Data arising from cytoplasmic studies and molecular phylogenetic analyses point to the same conclusion: *H. pachydermum* is closely related to the species from the order Spumellarida. That is, although most of the entactinarians (e.g., *Stigmosphaera*) have a nucleo-axopodial system of peri-axoplastidian type (Hollande and Enjumet, 1960; De Wever et al., 2001), some (e.g., *Rhizosphaera* and *Hexacontium*) have a centro-axoplastidian type nucleo-axopodial system (Hollande and Enjumet, 1960), with the latter also being the typical cytoplasmic feature of the spumellarians. This observation suggests that it would be impossible to reach the conclusion that entactinarians are closely related to the nassellarians based solely on a comparison of the intra-spicule-like form within the initial position of the skeleton.

The origin of *Hexacontium pachydermum* can be explained according to the following hypothesis. Many of the Paleozoic spherical radiolarians, previously considered to be spumellarians, are now placed in the order Entactinarida and do contain the remains of an inner spicule (De Wever et al., 2001). During the Paleozoic, the primitive nassellarians (with their characteristic internal spicules) also appeared but with skeletons having some entactinarian characteristics (De Wever et al., 2003). It is therefore possible that both the nassellarians and the spumellarians originated from a common ancestor during the Paleozoic.

The present radiolarian taxonomy requires an extensive revision in light of newly acquired data from fine structural and molecular taxonomic analyses of living radiolarian species (e.g., Sugiyama and Anderson, 1998; Yuasa et al., 2005). Thus, radiolarians provide a great opportunity for the use of both molecular and paleontological data in a single protist group. Classical morphological approaches, combined with modern, fine structural and molecular genetic analyses, show great promise in clarifying unresolved issues in radiolarian systematics.

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