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Phylogenetic Affiliations of Mesopelagic Acantharia and Acantharian-like Environmental 18S rRNA Genes off the Southern California Coast

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Incomplete knowledge of acantharian life cycles has hampered their study and limited our understanding of their role in the vertical flux of carbon and strontium. Molecular tools can help identify enigmatic life stages and offer insights into aspects of acantharian biology and evolution. We inferred the phylogenetic position of acantharian sequences from shallow water, as well as acantharian-like clone sequences from 500 and 880 m in the San Pedro Channel, California. The analyses included validated acantharian and polycystine sequences from public databases with environmental clone sequences related to acantharia and used Bayesian inference methods. Our analysis demonstrated strong support for two branches of unidentified organisms that are closely related to, but possibly distinct from the Acantharea. We also found evidence of acantharian sequences from mesopelagic environments branching within the chaunacanthid clade, although the morphology of these organisms is presently unknown. HRP-conjugated probes were developed to target Acantharea and phylotypes from Unidentified Clade 1 using Catalyzed Reporter Deposition Fluorescence In Situ Hybridization (CARD-FISH) on samples collected at 500 m. Our CARD-FISH experiments targeting phylotypes from an unidentified clade offer preliminary glimpses into the morphology of these protists, while a morphology for the aphotic acantharian lineages remains unknown at this time.

Key words: 18S rRNA phylogeny; Acantharea; CARD-FISH; deep-sea protist; novel protistan lineage; sarcodine.

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Introduction

Acantharia are amoeboid, mixotrophic protists and common constituents of the microplankton community in euphotic oceanic ecosystems. They have traditionally been distinguished from other sarcodine protists such as the polycystine and phaeodarian radiolaria and foraminifera by the composition of their exquisite skeletons, which are comprised of $SrSO_4$ in a Müllerian arrangement of 20 (in rare cases, 10) spicules (Fig. 1). Most acantharia possess tens to hundreds of photosynthetic eukaryotic symbionts per cell (Michaels 1991; Michaels et al. 1995). Total primary production within the cytoplasmic network of these and other sarcodines has been measured at more than four orders of magnitude greater than that of an equivalent volume of surrounding water (Caron et al. 1995). High rates of primary productivity in these associations reflect the fact that the microenvironment of the sarcodine is highly enriched in algal biomass relative to the surrounding oligotrophic environment, and to the observation that symbionts are typically at or close to their maximal gross growth rates within these symbiotic associations (Stabell et al. 2002). Together, these factors suggest that symbiont photosynthesis can represent a significant portion of open ocean primary production when sarcodine abundances are high.

Acantharia often numerically dominate other sarcodines in open ocean ecosystems (Caron and Swanberg 1990). During "bloom-like" conditions (Massera Bottazzi and Andreoli 1981, 1982; Zas'ko and Vedernikov 2003), acantharian abundances have been reported at integrated densities from 1.53 to $5.34 \times 10^5\ m^{-2}$. It is estimated that they comprise up to 41% of total integrated production during these events (Michaels 1988). These values represent significant amounts of carbon fixation in the oligotrophic euphotic oceans and demonstrate the potential for acantharia to exert a substantial influence on marine carbon budgets. Furthermore, acantharian blooms may contribute significantly to export flux (particularly Sr flux) from surface waters given their large cell size and dense skeletons (Michaels and Silver 1993; Michaels et al. 1995).

Acantharia are thought to exert the most significant biological influence on strontium budgets in the ocean due to their unique celestite skeletons (Bernstein et al. 1987). Acantharia are the only protists known to make their skeletons entirely from $SrSO_4$, although some radiolarian swarmer cells produce individual crystals of $SrSO_4$ (Anderson 1983). Strontium was considered a conservative element in marine environments until slight variations in the Sr/Cl ratios of the upper 400 m were detected (Brass and Turekian 1974). Studies that followed implicated the crystallization and dissolution of acantharian

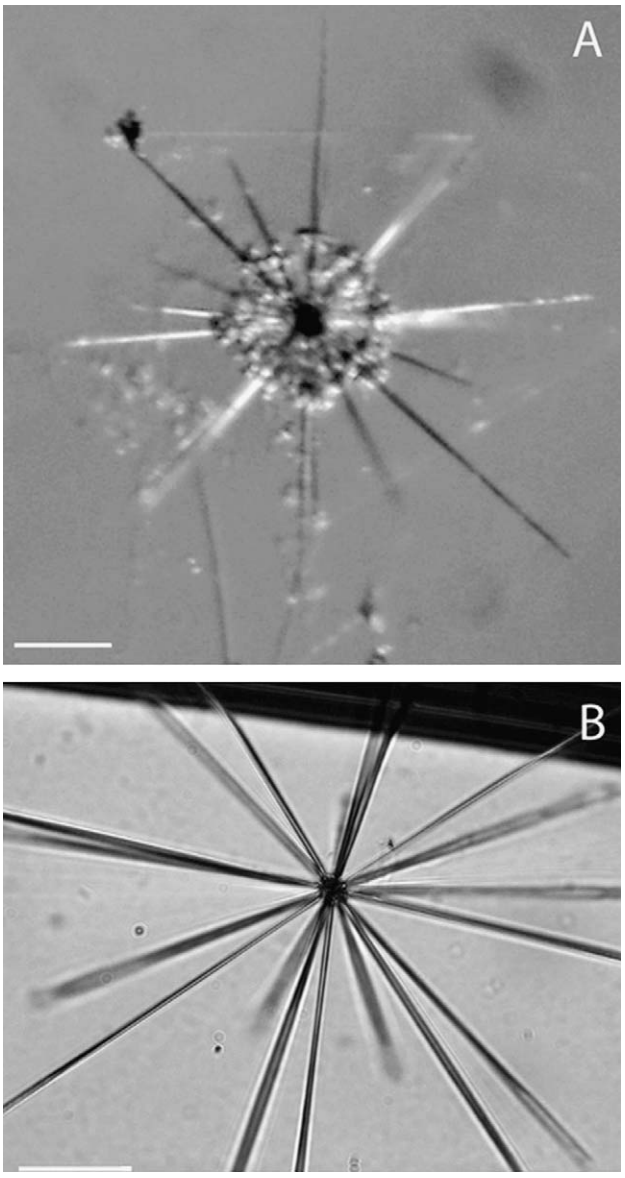

Figure 1. (**A**) An example of *Acanthometra* sp. 1 in its vegetative state. (**B**) The same organism, after releasing swarmers. The swarmers are visible as small dots around the skeleton. Note that the central skeletal junction, an important feature for taxonomic identification, is only visible after swarmers have been released. Scale bars=50 μm.

celestite as the cause of this strontium flux (Bernstein et al. 1987; Bernstein and Byrne 2004; De Deckker 2004). Seawater is highly undersaturated with respect to strontium, so acantharia must continuously expend energy to create the unique cellular structures that constitute their skeletons. Sinking acantharian celestite from both skeletons and cysts is thought to dissolve by 900 m, where stabilization of the Sr/Cl ratios has been observed (Bernstein et al. 1987). The selective removal of Sr from surface waters, where vegetative acantharia are most abundant, also accounts for the variability of Sr/Ca ratios in the euphotic zone (De Deckker 2004) and helps interpret anomalies in historical sea-surface data sets constructed from Sr/Ca ratios from corals. Acantharia have also been implicated in other elemental cycling, most notably barium, which can comprise up to 0.4% of the skeleton. These concentrations are ten times greater than that of seawater (Bernstein et al. 1999) and may be the source of mysteriously abundant strontium-rich barite particles in the deep ocean (Bernstein and Byrne 2004).

Despite their important biogeochemical influences in the ocean, relatively little is known about acantharian biology or ecology. Study of their biology has been hindered by the fact that they, like most large planktonic sarcodine protists, cannot be maintained in culture through successive generations or even maintained very long after capture. Although acantharia have not been cultured in the laboratory, formation and release of 'swarmer' cells from freshly-collected organisms has been observed. This process involves the reorganization of the multinucleated vegetative cytoplasm or cyst into tens of thousands of flagellated, mononuclear cells (Caron and Swanberg 1990). It has been hypothesized that certain acantharia sink before swarmer production occurs in their natural environment, either by forming cysts or contracting their ectoplasm through microfibrillar myonemes (Febvre and Febvre-Chevalier 1978). However, the next stage in the acantharian life cycle is entirely unknown. Knowledge of the complete life cycle could be especially useful for more accurate estimates of nutrient flux models, as current models consider only the export of carbon from sinking surface-dwelling acantharia out of the euphotic zone in the open ocean (Michaels 1988; Michaels and Silver 1993).

Environmental clone libraries have revealed the presence of acantharian-like 18S rRNA genes from depths where acantharia have rarely, if ever, been observed (Alexander et al. 2009; Edgcomb et al. 2002; Lopez-Garcia et al. 2001; Lovejoy et al. 2006; Not et al. 2007). Acantharian-like clones from deep-sea and mesopelagic environmental clone libraries have been observed in the Atlantic (Countway et al. 2007) and in the San Pedro Channel off the coast of California (Countway et al. submitted; Schnetzer et al. in preparation) respectively. Countway et al. (2007) reported significant percentages (up to 17%) of the total protistan phylotypes from 2500 m in the Sargasso Sea belonging to acantharia or acantharian-like organisms. Acantharian-like sequences have also been reported in clone libraries from the Sargasso Sea (Moon-van der Staay et al. 2001; Not et al. 2007) from 2000 m at the Antarctic polar front (Lopez-Garcia et al. 2001), sediment collected from the mid-Atlantic ridge (Lopez-Garcia et al. 2003), sediment in the southern Guaymas vent field (Edgcomb et al. 2002), aphotic Arctic waters (Lovejoy et al. 2006), the anoxic Caricao basin (Stoeck et al. 2003), and from an anoxic hypersaline basin (Alexander et al. 2009). This is striking because, to our knowledge, acantharia have not been observed at such depths based on microscopical analyses. Heavily skeletonized forms such as reproductive cysts have been observed as deep as 900 m (Bernstein et al. 1987) but dissolution of the skeleton is presumed to occur below this depth and the fate of the cells is unknown. The abundance of acantharian-like sequences at depths greater than 900 m suggests that there may be uncharacterized mesopelagic or deep-sea life cycle stages that are presently unrecognizable by morphology alone.

Molecular diversity studies that have identified possible acantharia often have not presented complete sequences of these phylotypes and some have not performed the phylogenetic analyses necessary for conclusive identification. There are also conflicting interpretations of the phylogenetic relationships between the Acantharea and the Polycystinea (Amaral Zettler et al. 1997; Danelian and Moreira 2004; Kunitomo et al. 2006; Lopez-Garcia et al. 2001; Nikolaev et al. 2004; Oka et al. 2005; Pawlowski and Burki 2009; Takahashi et al. 2004). Historically, the classes Acantharea, Phaeodarea and Polycystinea were merged into the superclass "Radiolaria" based on the presence of a central capsule (Haeckel 1887). The Acantharea were later removed from Haeckel's Radiolaria based on differences in central capsule morphology and skeletal composition (Schewiakoff 1926). Definitions of groups comprising the Radiolaria have varied in the literature and at times have included

the Polycystinea and Acantharea, Polycystinea and Phaeodarea, and more recently the Polycystinea alone. Our goal was to clarify phylogenetic relationships among acantharia and acantharian-like phylotypes and to investigate the possibility of deep-sea stages in the life cycle of acantharia. We report here phylogenetic analyses of 18S rRNA gene sequences from representatives of the Polycystinea and Acantharea along with new full-length validated acantharian and acantharian-like sequences from the San Pedro Channel, California, in order to reveal more accurate phylogenetic associations among these species, and to examine the identity of sequences obtained from deep water in the basin.

Results

Bayesian analysis (BA) of 18S rRNA genes strongly supported the monophyly of the Acantharea (Fig. 2). The analysis also supported the shared ancestry of solitary skeleton-bearing spumellarid Polycystinea and Taxopodida initially observed by Nikolaev et al. (2004). However, unlike previously reported phylogenies, the relationship of the Acantharea to other groups of Radiolaria was poorly resolved in our analysis. Our analysis supported the polyphyly of the Polycystinea with the skeleton-bearing spumellarian and taxopodid lineages branching away from the Nassellarida and colonial skeleton-bearing and non-skeletal polycystines as previously reported (Kunitomo et al. 2006; Nikolaev et al. 2004).

Three San Pedro clones from 880 m (MO010.880.00150, MO010.880.00119 and MO010.880.00133) and four from 500 m (MO010.500.00049, MO010.500.00040, MO010.500.00038 and MO010.500.000116) fell within the core acantharian clade in our analyses (Fig. 2). All but MO010.880.00119 were closely related to Chaunacanthid 6200, a *Stauracon*-like acantharian isolated from the surface waters of the San Pedro Channel. Although we were unable to make a definitive identification of this organism, it most resembled the genus *Stauracon* and possessed the grape seed-shaped base of radial spines that is the defining characteristic for all Chaunacanthida.

Bayesian inference indicated strong support for the formation of two lineages basal to identified acantharia, characterized in our analysis as Unidentified Clade 1 (UC1) and Unidentified Clade 2 (UC2) (Fig. 2, PP=0.89 and 1.00 respectively). These clades support the findings of Not et al. (2007) whose novel groups RAD-1 and RAD-II roughly correspond to UC1 and UC2 respectively. Three clones from 500 m in the present study (MO010.500.00307, MO010.500.00043 and MO010.500.00014) along with 9 Sargasso Sea clones (see Table 1, Not et al. 2007) comprised UC1. UC2 contained one 880 m clone from the present study (MO010.880.00323), a 500 m Sargasso Sea clone (SSRPB51, Not et al. 2007), an Antarctic deep-sea environmental clone (DH145-EKD17, Lopez-Garcia et al. 2001), a 75 m clone from the Equatorial Pacific Ocean (OLI11032, Moon-van der Staay et al. 2001) and a clone from a hypersaline, anoxic basin (UI13C08, Alexander et al. 2009).

Three more clones from 880 m in the present study and clone AT4-94 (Lopez-Garcia et al. 2003) branched basal to known spumellarian polycystines. One 880 m sequence branched among a clade encompassing the only known member of the Taxopodida, *Sticholonche zanclea*, and a select group of spumellarian radiolaria.

Cells from the January 2006 sample collected at 500 m successfully hybridized to the UC1 CARD-FISH probe (Fig. 3). Despite our detection of acantharian 18S rRNA gene sequences in clone libraries, the acantharian-specific probe a497 did not detect these phylotypes on filters collected at 500 m from May 2005 through January 2006.

Discussion

Phylogeny of Haeckel's Radiolaria

Hypotheses on the phylogeny of "Haeckel's Radiolaria" have changed repeatedly in recent years through the application of DNA sequence information. Amaral Zettler et al. (1997) were the first to infer a partial molecular phylogeny of representatives of these species based on 18S

Figure 2. Phylogenetic relationships among 134 eukaryotic 18S rRNA gene sequences inferred from a Bayesian analysis with 5,000,000 generations. 1115 positions were included in the analysis. Posterior probabilities >0.5 are displayed at the nodes. Sequences from this study are presented in boldface, ● indicates environmental sequences from this study collected at 500 m, and ▲ indicates sequences from the same location collected at 880 m. *Clades were collapsed into single groups for this figure when sequence similarity resulted in poor internal branch resolution. Specific sequences falling within these clades may be viewed in Table 1.

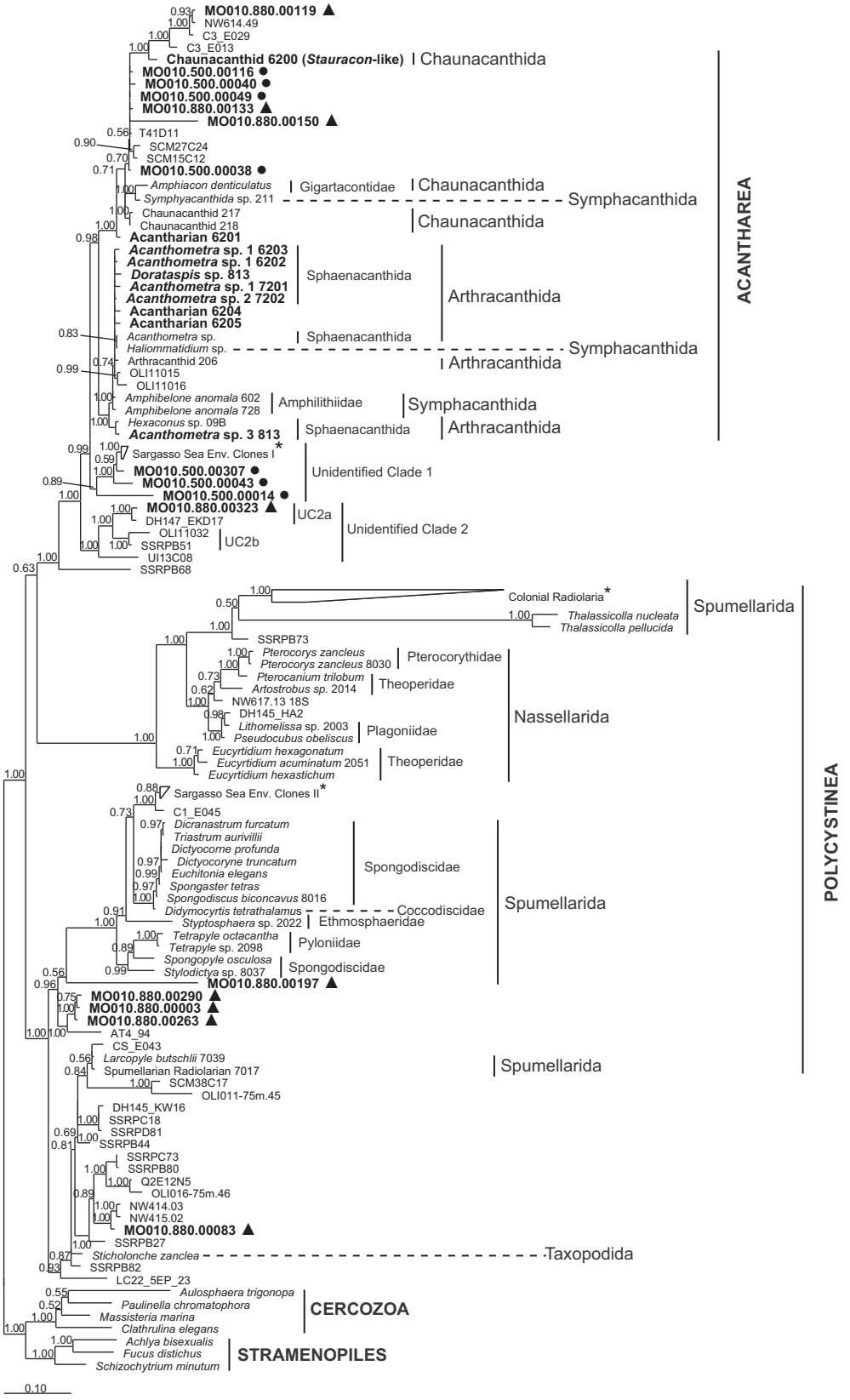
MO010.880.00119 ▲
NW614.49
C3_E029
C3_E013
Chaunacanthid 6200 (Stauracon-like)
Chaunacanthida
MO010.500.00116 ●
MO010.500.00040 ●
MO010.500.00049 ●
MO010.880.00133 ▲
MO010.880.00150 ▲
T41D11
SCM27C24
SCM15C12
MO010.500.00038 ●
Amphiacon denticulatus
Gigartacontidae
Chaunacanthida
Symphyacanthida sp. 211
Symphacanthida
Chaunacanthid 217
Chaunacanthid 218
Chaunacanthida
Acantharian 6201
Acanthometra sp. 1 6203
Acanthometra sp. 1 6202
Doratraspis sp. 813
Acanthometra sp. 1 7201
Acanthometra sp. 2 7202
Acantharian 6204
Acantharian 6205
Sphaenacanthida
Arthracanthida
ACANTHAREA
Acanthometra sp.
Haliommatidium sp.
Sphaenacanthida
Symphacanthida
Arthracanthid 206
Arthracanthida
OLI11015
OLI11016
Amphibelone anomala 602
Amphibelone anomala 728
Amphilithiidae
Symphacanthida
Hexaconus sp. 09B
Acanthometra sp. 3 813
Sphaenacanthida
Arthracanthida
Sargasso Sea Env. Clones I *
MO010.500.00307 ●
MO010.500.00043 ●
MO010.500.00014 ●
Unidentified Clade 1
MO010.880.00323 ▲
DH147_EKD17
UC2a
OLI11032
SSRPB51
UC2b
Unidentified Clade 2
UI13C08
SSRPB68
Colonial Radiolaria *
Thalassicolla nucleata
Thalassicolla pellucida
Spumellarida
SSRPB73
Pterocorys zancleus
Pterocorys zancleus 8030
Pterocorythidae
Pterocanium trilobum
Artostrobus sp. 2014
Theoperidae
NW617.13 18S
DH145_HA2
Lithomelissa sp. 2003
Pseudocubus obeliscus
Plagoniidae
Nassellarida
Eucyrtidium hexagonatum
Eucyrtidium acuminatum 2051
Eucyrtidium hexastichum
Theoperidae
Sargasso Sea Env. Clones II *
C1_E045
Dicranastrum furcatum
Triastrum aurivillii
Dictyocorne profunda
Dictyocoryne truncatum
Euchitonia elegans
Spongaster tetras
Spongodiscus biconcavus 8016
Spongodiscidae
Didymocyrtis tetrathalamus
Coccodiscidae
Styptosphaera sp. 2022
Ethmosphaeridae
Spumellarida
POLYCYSTINEA
Tetrapyle octacantha
Tetrapyle sp. 2098
Pyloniidae
Spongopyle osculosa
Stylodictya sp. 8037
Spongodiscidae
MO010.880.00197 ▲
MO010.880.00290 ▲
MO010.880.00003 ▲
MO010.880.00263 ▲
AT4_94
CS_E043
Larcopyle butschlii 7039
Spumellarian Radiolarian 7017
SCM38C17
OLI011-75m.45
Spumellarida
DH145_KW16
SSRPC18
SSRPD81
SSRPB44
SSRPC73
SSRPB80
Q2E12N5
OLI016-75m.46
NW414.03
NW415.02
MO010.880.00083 ▲
SSRPB27
Sticholonche zanclea
Taxopodida
SSRPB82
LC22_5EP_23
Aulosphaera trigonopa
Paulinella chromatophora
Massisteria marina
Clathrulina elegans
CERCOZOA
Achlya bisexualis
Fucus distichus
Schizochytrium minutum
STRAMENOPILES
0.10

Table 1. Sequences within collapsed clades (*) in Figure 2.

Condensed Clade from Figure 2	Clone Name	Accession Number
Sargasso Sea Env. Clones I	SSRPC49	EF172929
	SSRPC83	EF172911
	SSRPC07	EF172910
	SSRPC45	EF172909
	SSRPC15	EF172908
	SSRPB59	EF172842
	SSRPB70	EF172841
	SSRPB05	EF172840
	SSRPB06	EF172828
Colonial Radiolaria	*Collozoum pelagicum*	AF091146
	Collozoum inerme	AY266295
	Collozoum serpentinum	AF018162
	Collosphaera globularis-huxleyi	AF018163
	Acrosphaera sp. CR6A	AF091148
	Rhaphidozoum acuferum	AF091147
	Sphaerozoum punctatum	AF018161
	Siphonosphaera cyathina	AF091145
Sargasso Sea Env. Clones II	SSRPC01	EF172890
	SSRPC12	EF172891
	SSRPC17	EF172895
	SSRPC19	EF172905
	SSRPC22	EF172899
	SSRPC26	EF172901
	SSRPC34	EF172903
	SSRPC42	EF172902
	SSRPC52	EF172930
	SSRPC53	EF172914
	SSRPC59	EF172932
	SSRPC76	EF172900
	SSRPC78	EF172896
	SSRPC85	EF172915
	SSRPC87	EF172894
	SSRPD81	EF172984

rRNA genes. They concluded that the Acantharea and Polycystinea formed distinct lineages, and did not comprise a monophyletic assemblage. Other molecular phylogenies restored the monophyletic association of the Polycystinea with Acantharea (Danelian and Moreira 2004; Lopez-Garcia et al. 2002). With the addition of the taxopodid *Stichololonche zanclea*, the monophyly of Acantharea and spumellarian Polycystinea was again supported (Nikolaev et al. 2004). Subsequent molecular phylogenetic studies (Takahashi et al. 2004) revealed that polycystines are comprised of at least two paraphyletic lineages: 1) colonial polycystines with and without skeletons and solitary polycystines lacking skeletons and 2) shell-bearing solitary polycystine spumellarians. With the addition of spongodiscid spumellarians and nassellarian sequences (Kunitomo et al. 2006; Yuasa et al. 2005), the monophyletic relationship between the Acantharea and collodarian Polycystinea reported by Lopez-Garcia et al. (2002) has not been supported.

The addition of environmental sequences in the present study has destabilized the overall placement of the Acantharea relative to the other Radiolaria. Unlike previously reported phylogenies which analyzed rRNA gene and actin sequences (Nikolaev et al. 2004), rRNA gene and polyubiquitin sequences (Pawlowski and Burki 2009) or rRNA genes alone (Kunitomo et al. 2006; Takahashi et al. 2004; Yuasa et al. 2005), we did not observe a monophyletic relationship between the

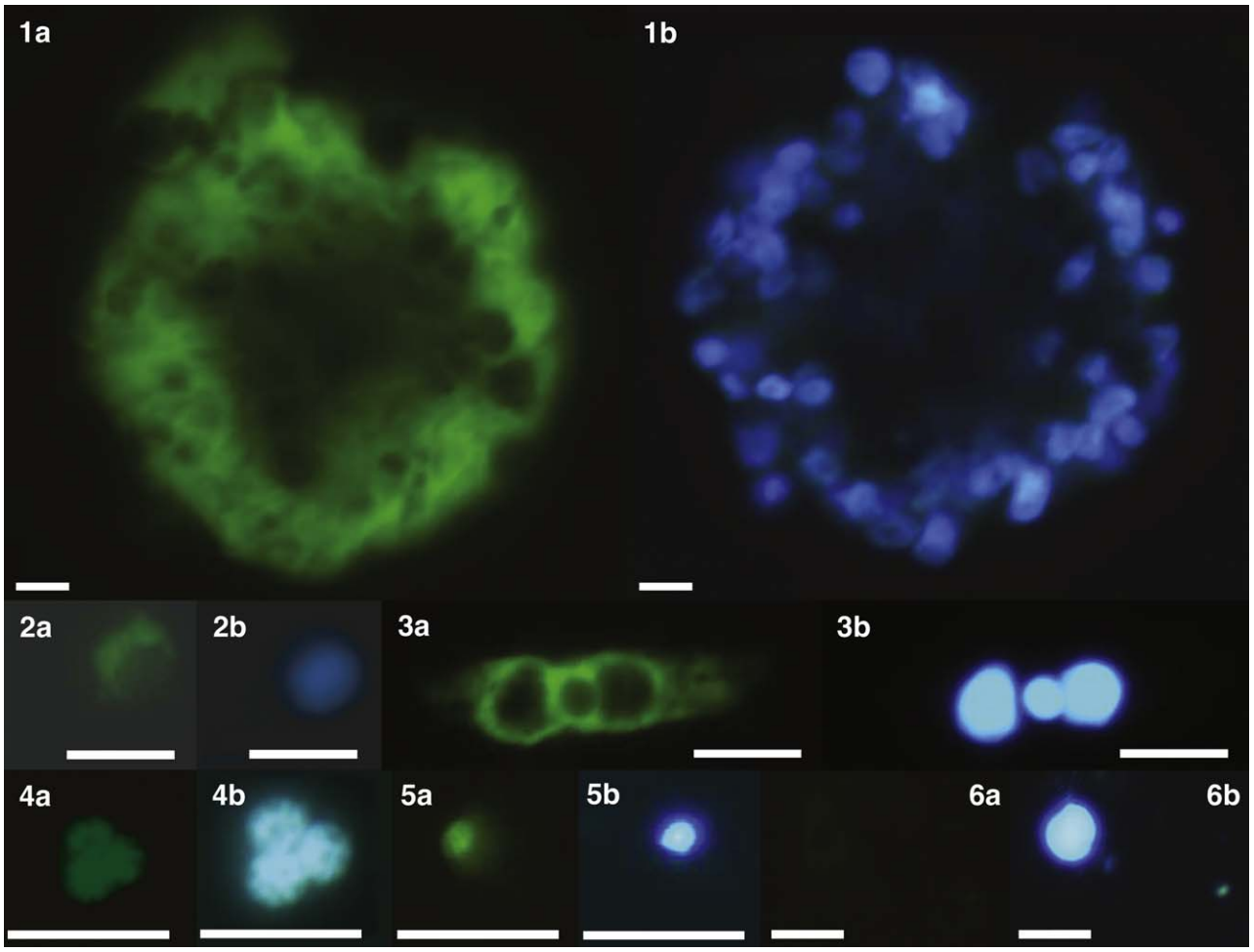

Figure 3. Photomicrographs of acantharia and relatives. CARD-FISH samples were hybridized to HRP-conjugated taxa-specific oligonucleotide probes and visualized with Alexa Fluor® 488. All scale bars=10 μm. Each "a" image shows a hybridized cell and each "b" image shows the corresponding DAPI image. (1) A vegetative acantharian (*Acanthometra* sp. 1) hybridized with acantharian-specific probe a497. All but one known species of acantharian is polynucleated in the vegetative stage. Skeletal disassociation was observed because Sr was not added to the fixation medium. (2) An acantharian swarmer, targeted with a497. Note the single nucleus at this stage. (3) An example image from a positive control, targeted with the universal eukaryotic probe 1209R. This mid-water ciliate was imaged from an environmental sample taken at 500 m in January, 2006. Positive controls revealed a mid-water community of dinoflagellates, ciliates, and nanoflagellates, as well as many other unidentifiable eukaryotes. (4-5) Cells targeted by UC1-899, a probe specific for Unidentified Clade 1. Both images were taken from an environmental sample collected in January 2006 from 500 m. (6) Negative control from January 2006 sample.

Acantharea, the spongodiscid Spumellarida and the clade bearing *Sticholonche zanclea*. With the addition of our new environmental sequences along with newly reported radiolarian-related sequences, our data may lack the phylogenetic signal necessary for resolving higher-order taxonomic relationships.

Our findings supported the polyphyletic nature of the Spumellarida (Kunitomo et al. 2006; Yuasa et al. 2005) and the paraphyly of the Spongodiscidae (Kunitomo et al. 2006), with a spongodiscid *Larcopyle butschlii* and Spumellarian Radiolarian 7017 branching among the larger clade that included *S. zanclea.* These results call into question the classification of this clade, which is often labeled "Taxopodida" in the literature (Not et al. 2007; Pawlowski and Burki 2009), especially given that the majority of members in this clade are unidentified.

Phylogeny of Schewiakoff's Acantharea

Our analysis indicated that the traditional classification of the Acantharea is not completely consistent with their molecular phylogeny, but is applicable in certain cases. For instance, our tree lends support to the traditional classification of the Chaunacanthida, which remain a discrete lineage in all analyses along with Symphacanthid 211. The association of Symphacanthid 211 with the Chaunacanthida has been reported elsewhere (Oka et al. 2005), so it is possible that Symphacanthid 211 was originally misidentified. However, the classification of Arthracanthida sensu Schewiakoff is not supported by our data, a finding which also corroborates the analyses of Oka et al. (2005). The Acantharean orders Arthracanthida and Symphacanthida appear to be polyphyletic in our analysis and probably require revision. *Amphibelone anomala, Amphibelone cultellata* and *Haliommatidium* sp., are traditionally classified as Symphacanthida, an Order characterized by the basal fusion of spines and a lack of capsular membrane (Schewiakoff 1926). In contrast, Arthracanthida is characterized by discrete but tightly joined radial spines, the base of which reassemble arrowheads (Schewiakoff 1926). Amaral Zettler and Caron (2000) reported the first molecular justification for the placement of *Haliommatidium* among the Arthracanthida. They noted that *Haliommatidium* shares morphological features with the Arthracanthida, including an occasionally observed central capsule wall (Febvre et al. 1990) which is an unusual feature for Symphacanthida *sensu* Schewiakoff, and the formation of a latticed shell by fusion of apophyses. Curiously, there are few morphological characteristics shared by *Amphibelone* and Arthracanthida. One potential synapomorphy is the lack of cyst formation. Amphilithiidae is the only family of Symphacanthida that does not form cysts (Cachon and Cachon 1982) and direct swarmer formation without encysting is one of the defining characteristics of Arthracanthida.

There is little resolution within much of the Arthracanthida clade in our analysis, although a more targeted analysis might reveal more insight. *Dorataspis*, several species of *Acanthometra* and *Haliommatidium* branch together (Fig. 2) and comprise the core of this lineage. However, there was strong support (PP=1) for a distinct clade containing *Hexaconus* and *Acanthometra* sp. 3 (Fig. 2), indicating that the genus *Acanthometra* is polyphyletic. Within the suborder Sphaenacanthina, Hexalaspitidae may need to be expanded to include some *Acanthometra* species. At present, there is no molecular justification for the families Acanthometridae and Dorataspitidae within Sphaenacanthida.

Phylogeny of Deep-Sea Acantharia and Close Relatives

We have uncovered full-length 18S rRNA gene sequences from the deep-sea environment that are presently best classified as members of the Acantharea. These clones, MO010.880.00150, MO010.880.00133, MO010.500.00040, MO010.500.00116, MO010.500.00038 and MO010.500.00049, may be chaunacanthids, based on their position in the tree (Fig. 2). Environmental clones C3_E013, C3_E029, and NW614.49 have occasionally been identified as acantharia in previous studies (Not et al. 2007), but the depth to which they branch away from Chaunacanthid 6200 in our analysis casts uncertainty onto that identification. Not et al. (2007) reported other clones (First Sargasso Sea group in Table 1) within the Acantharean clade among selected topologies. Our data strongly support (PP=0.99) the placement of these sequences in a clade basal to the known acantharia which casts doubt on their identity.

Acantharia are common in the San Pedro Channel year-round. *Acanthometra* and *Dorataspis* species were frequently found among the surface-dwelling acantharian population at our sampling site. It is therefore possible that our chaunacanthid-like sequences from aphotic depths came from sinking cellular debris, cysts less than 200 μm in diameter or swarmers, as Chaunacanthids encyst before releasing swarmers. However, if these organisms are vegetative, their occurrence at these depths would represent a newly recognized ecosystem for this taxon. The dominant species of acantharia at the surface of the channel from 2003-2005 (*Acanthometra* sp. 1, *Dorataspis* sp. 813 and Chaunacanthid 6200) were not the same as those detected at depth in June 2001. Unfortunately, we do not know the dominant species of acantharia in surface waters during June 2001, our environmental sampling period, because surface samples for microscopy were not treated with excess strontium, a necessary additive for the preservation of acantharian skeletons (Beers and Stewart 1970). It is therefore possible that our clone sequences were derived from sinking acantharian cells or cysts. However, we did not detect the putative chaunacanthid or

MO010.880.00119 phylotypes among the surface clones in our 2001 libraries.

We present further evidence of and strong support for novel, undescribed phylogenetic lineages basal to the Acantharea (Unidentified Clades 1 and 2), which corroborate the findings of Not et al. (2007). Organisms in Unidentified Clade 2 (UC2) are related to Acantharea, but given their evolutionary distance from the core Acantharea and depth of branching, it is more likely that they represent a novel group. The members of this group may even be endemic to mesopelagic marine environments. With the exception of OLI11032, all clones within this clade were obtained from mesopelagic to abyssal depths. UC2 phylotypes were not detected among surface sequences in clone libraries from our sample site over a two-year period. However, these phylotypes were detected repeatedly in our clone libraries at depth over the same sampling period. Although there are relatively few representatives for UC2, our data provide preliminary support for the formation of 2 subgroups within this clade (UC2a and UC2b) although more sequences of related taxa will be needed to validate this proposal. It appears that we are only just beginning to uncover the diversity of these organisms.

Unidentified Clade 1 (UC1) has a shorter evolutionary distance from the core Acantharea than UC2 (Fig. 2). Our San Pedro clones branch deeper than the Sargasso Sea I clones (Table 1, Not et al. 2007) and may represent more divergent members of UC1. Whether or not these organisms are acantharia remains unresolved. One possibility is that this clade represents the Holacanthida, a class of Acantharea that currently lacks representation in public gene databases. The placement of these sequences is consistent with the current taxonomic classification of Holacanthida, the most ancestral acantharian order (Schewiakoff 1926). However, we did not observe Holacanthida in our net tows during our sampling period. It is also possible that UC1 may represent another group of arthracanthids, given its position basal to known Arthracanthida. There are 30 described genera within Arthracanthida (Schewiakoff 1926), only four of which have representation from identified organisms in GenBank. While the identity of phylotypes in UC1 remains unknown, we tentatively consider these phylotypes novel until further information is available.

All environmental clones in this study were obtained from 500 and 880 m at our sample site in an environment that experiences persistent hypoxia. The dissolved oxygen concentrations at 500 m at the San Pedro Ocean Time-series site ranged from 0.14 to 0.78 ml l^{-1} (Countway et al. submitted; Schnetzer et al. in prep). Values at 880 m typically do not exceed 0.22 ml l^{-1}. Interestingly, other studies have detected acantharian and acantharian-like sequences in deep anoxic environments. T41D11 (Stoeck et al. 2006) and UI13C08 (Alexander et al. 2009) are clones from two separate anoxic basins, while C3_E013 and C3_E029 were extracted from anoxic sediments (Edgcomb et al. 2002). It is unknown if these sequences came from living organisms or simply represent sinking debris or cysts. However, if they represent active members of the microbial community in these environments, it would suggest that these species may have the ability to adapt to hypoxic or anoxic conditions.

Fluorescence In Situ Hybridization

Two acantharian-specific regions of the 18S rRNA gene at base pair 497 (probe a497) and base pair 899 (probe a899) were initially reported by Amaral Zettler et al. (1997). These probe locations remain phylogenetically informative when compared to current acantharian sequences in GenBank. A BLAST analysis at the time of this study revealed that among the validated sequences possessing the signature region of a497, all were acantharia. However, we detected occasional polymorphisms in a497 in some known acantharian sequences. One polymorphism was detected at the thirteenth base of the probe region in *Amphibelone*AB178584, and at the fourth base in *Hexaconus*AB178587 and *Amphiacon*AB178585 (data not shown). Among all validated and putative acantharian sequences from our dataset, all but one possessed the 'a899' marker. Only C3_E029 exhibited a single mutation in a899 (data not shown). All the clones comprising UC2 also possessed the region a899 without polymorphism. BLAST analysis revealed that only the core Acantharea and UC2 sequences possessed the a899 sequence. However, phylotypes from UC2 demonstrated significant polymorphisms (6 bases) of a497. This finding is echoed in the phylogenetic placement of these sequences, which lies between the core Acantharea and their relatives, the polycystines. Interestingly, members of UC1 had two unique and phylogenetically informative polymorphisms in a497 and 1-2 unique polymorphisms in a899. There were no sequences in GenBank containing these unique variations in a497 and a899 at the time of our analysis. It therefore appeared that sequences at a497 and a899 were informative not only for most

members of the Acantharea, but also for their unknown relatives in UC1. Because UC2 shared a899 with known acantharia, only a497 was used to search for known acantharia at depth. UC1-899, a derivative probe of a899 containing the UC1-specific polymorphisms, was used to search for UC1 phylotypes. We did not design a probe for UC2 phylotypes in this study.

We successfully hybridized phylotypes from UC1 (Fig. 3) with CARD-FISH (Pernthaler et al. 2002) using the unique UC1 probe UC1-899 to bind to SSU rRNA. It is unclear if the hybridized UC1 phylotype detected in frame 4 of Figure 3 represented a single cell or a cluster of cells. We also detected solitary cells (or perhaps a fragment) in the same sample (Fig. 3, frames 5a and b). Based on the size and shape of the solitary hybridized objects and the fact that they have what appears to be a single nucleus, it is possible that they were swarmers.

It is not a forgone conclusion that the source of these phylotypes are metabolically active members of the community. It is possible that template DNA for the San Pedro clones came from cysts, fecal pellets or cellular debris from the surface. However, since FISH probes target rRNA, it is unlikely that we would observe hybridization from acantharian-derived material in fecal pellets or decaying matter because of the rapid degradation of extracellular RNA. Positive CARD-FISH results were never observed with acantharian cysts in preliminary tests. Theoretically, a positive hybridization should occur only with metabolically active cells. It's therefore likely that the organisms we detected are active members of deep protistan communities.

The acantharian-specific probe (a497) did not hybridize to cells in our slide preparations from deep samples, even with increased sample volumes. It is possible that we missed these organisms because they are transient members of the protistan assemblage at depth or because they are rarely encountered. It is also possible that the putative acantharian clones from our library came from cysts, fecal pellets other aggregated particles such as marine snow containing nuclear remains of acantharia. Although it is possible for these particles to contain acantharian DNA which could be cloned, they would most likely not contain intact rRNA and would therefore not hybridize to our probe. The nature of these organisms at depth remains unresolved and awaits further investigation.

Estimates of total eukaryote abundances at 500 m, based on cell counts of CARD-FISH eukaryotic positive controls from May 2005–January 2006 ranged from 225 to 469 cells l^{-1}. The sample in which UC1 organisms were detected (January 2006) yielded an abundance estimate of 8 cells l^{-1} of the novel phylotype, or approximately 2-4% of the total eukaryote population. This was near the limit of detection by standard methods, so cells in samples from previous months may have evaded detection because abundances were below our limit of detection.

We conducted an analysis of all eukaryotic clone libraries collected at our San Pedro sampling location during 2000-2001 (Countway et al. submitted; Schnetzer et al. in prep). The UC1 phylotype was detected among sequences in 2001 at 150 m on 27 July and at 150 and 500 m on 29 October. These sequences comprised approximately 1% of the total eukaryotic phylotypes from each sample. Core acantharian sequences were also present in San Pedro eukaryotic clone libraries from mesopelagic depths (150 and 500 m). These sequences averaged approximately 4% of the total eukaryotic sequences respectively, although Acantharian phylotypes in one sample (27 July 2001 500 m) comprised over 15% of total eukaryotic sequences. This finding demonstrates that these organisms, cysts or decaying cells were at times a significant fraction of the mesopelagic eukaryotic clone libraries at our study site. It is therefore possible that aphotic acantharian populations, if they are metabolically active members of the protistan assemblage, undergo 'boom and bust' cycles, dictated by currently undetermined cues.

Overall, our study indicated the presence of two protistan clades with ancestral affinities to the Acantharea, as well as core acantharian sequences, in mesopelagic environments. The depth and physical water parameters at which these phylotypes were collected indicate that there is still much to be discovered about acantharian biology and their ecological roles.

Methods

Acantharian sample collection in surface waters: Acantharia were collected in plankton nets (200 μm mesh) from the San Pedro Channel, between Los Angeles and Santa Catalina Island, California, on multiple cruises during 2003 and 2004. Live vegetative acantharia were isolated and identified to family or genus based on the criteria of Schewiakoff (1926). Conclusive identification was difficult with live cells because taxonomically important features of the skeleton were often obscured by the cellular matrix (Fig. 1). Cells were characterized

Table 2. List of 18S rRNA gene sequences used in this study.

Clone or Organism Name	Accession Number	Clone or Organism Name	Accession Number	Clone or Organism Name	Accession Number
Acantharian 6201	**GU246567**	LC22_5EP_23	DQ504355	*Spongodiscus biconcavus*	AB246695
Acantharian 6204	**GU246568**	*Lithomelissa* sp. 8012	AB246694	*Spongodiscus resurgens*	AB246696
Acantharian 6205	**GU246569**	*Massisteria marina*	AF174370	*Spongopyle osculosa*	AB246689
Acanthometra sp.	AF063240	**MO010.500.00014**	**GU246576**	*SSRPB22*	EF172808
***Acanthometra* sp. 1 6202**	**GU246570**	**MO010.500.00038**	**GU246577**	SSRPB27	EF172806
***Acanthometra* sp. 1 6203**	**GU246571**	**MO010.500.00040**	**GU246578**	SSRPB44	EF172834
***Acanthometra* sp. 1 7201**	**GU246572**	**MO010.500.00043**	**GU246579**	SSRPB51	EF172802
***Acanthometra* sp. 1 813**	**GU246574**	**MO010.500.00049**	**GU246580**	SSRPB68	EF172847
***Acanthometra* sp. 2 7202**	**GU246573**	**MO010.500.00116**	**GU246581**	SSRPB73	EF172833
Achlya bisexualis	M32705	**MO010.500.00307**	**GU246582**	SSRPB80	EF172807
Acrosphaera sp. CR6A	AF091148	**MO010.880.00003**	**GU246583**	SSRPB82	EF172835
Amphiacon denticulatus	AB178585	**MO010.880.00083**	**GU246584**	SSRPC01	EF172890
Amphibelone anomala 728	AB178584	**MO010.880.00119**	**GU246585**	SSRPC07	EF172910
Amphibelone cultellata 602	AB178580	**MO010.880.00133**	**GU246586**	SSRPC12	EF172891
Arthracanthid 206	AF063239	**MO010.880.00150**	**GU246587**	SSRPC15	EF172908
Artostrobus sp. 2014	AB246685	**MO010.880.00197**	**GU246588**	SSRPC17	EF172895
AT4 94	AF530525	**MO010.880.00263**	**GU246589**	SSRPC18	EF172906
Aulosphaera trigonopa	AY266292	**MO010.880.00290**	**GU246590**	SSRPC19	EF172905
C1_E045	AY046642	**MO010.880.00323**	**GU246591**	SSRPC22	EF172899
C3_E013	AY046843	NW414.03	DQ314831	SSRPC26	EF172901
C3_E029	AY046858	NW414.03	DQ314831	SSRPC34	EF172903
Chaunacanthid 217	AF063241	NW415.02	DQ314830	SSRPC42	EF172902
Chaunacanthid 218	AF018158	NW415.02	DQ314830	SSRPC45	EF172909
Chaunacanthid 6200	**GU246574**	NW614.49	DQ314821	SSRPC52	EF172930
Clathrulina elegans	AY305009	NW614.49	DQ314821	SSRPC53	EF172914
Collosphaera globularis-huxleyi	AF018163	NW617.13	DQ314838	SSRPC59	EF172932
Collozoum inerme	AY266295	NW617.13	DQ314838	SSRPC73	EF172907
Collozoum pelagicum	AF091146	OLI011-75m.45	EU287807	SSRPC74	EF172920
Collozoum serpentinum	AF018162	OLI016-75m.46	EU287808	SSRPC76	EF172900
CS_E043	AY046664	OLI11015	AJ402332	SSRPC78	EF172896
DH145 HA2	AF382824	OLI11016	AJ402333	SSRPC83	EF172911
DH145 KW16	AF382825	OLI11032	AJ402342	SSRPC85	EF172915
DH147 EKD17	AF290072	*Paulinella chromatophora*	X81811	SSRPC87	EF172894
Dicranastrum furcatum	AB179733	*Pseudocubus obeliscus*	AB246692	*SSRPD81*	EF172984
Dictyocorne profunda	AB101540	*Pterocanium trilobum*	AB246682	*Sticholonche zanclea*	AY268045
Dictyocoryne truncatum	AB101541	*Pterocorys zancleus*	AB179736	*Stylodictya* sp. 8037	AB246698
***Doratasp*is sp. 813**	**GU246566**	*Pterocorys zancleus* 8030	AB246697	*Styptosphaera* sp. 2022	AB246686
Euchitonia elegans	AB179732	Q2E12N5	EF173011	Symphyacanthid 211	AF063242
Eucyrtidium acuminatum 2051	AB246687	*Rhaphidozoum acuferum*	AF091147	T41D11	AY882491
Eucyrtidium hexagonatum	AB179735	*Schizochytrium minutum*	AB022108	*Tetrapyle* sp. 2098	AB246688
Eucyrtidium hexastichum	AB246681	SCM15C12	AY665098	*Thalassicolla nucleata*	AF018160
Fucus distichus	M97959	SCM27C24	AY665095	*Thalassicolla pellucida*	AY266297
Haliommatidium sp.	AF018159	SCM38C17	AY665073	*Triastrum aurivillii*	AB179734
Hexaconus serratus 09B	AB178587	*Siphonosphaera cyathina*	AF091145	UI13C08	EU446342
Hexaconus serratus 725	AB178588	*Sphaerozoum punctatum*	AF018161	Undetermined Spumellarian 7017	AB246691
Larcopyle butschlii	AB246693	*Spongaster tetras*	AB101542		

Sequences in boldface were obtained for this study.

only as 'acantharia' when critical characteristics were not visible. One organism bore a strong resemblance to the genus *Stauracon*, although it did not quite meet all the criteria for this designation. Therefore, it was labeled 'Chaunacanthid 6200', even though we strongly suspect it was a *Stauracon*. Upon isolation, each cell was rinsed 6 times in fresh 0.2 μm filtered seawater to dilute contaminants, then placed individually into 6-well culture plates containing 0.2 μm filtered seawater. Acantharia were maintained at 16 °C in an incubator with a 12 h light/dark cycle until they formed swarmers, or for a maximum of one week to allow sufficient time for digestion of ingested prey prior to molecular analyses. Swarmer cells were preferred over vegetative cells for analysis because symbionts were consumed prior to formation. Vegetative cells were amplified without the concentration step described below if swarmers did not form after 1 week.

Acantharian DNA amplification, cloning and sequencing: Live swarmers were concentrated by centrifugation and 1 μl concentrate was added to 4 μl Lyse-N-Go® PCR reagent (Pierce Biotechnology, Rockford IL). Samples were lysed in an iCycler or MyCycler automated thermocycler (BioRad, Hercules CA). Polymerase Chain Reaction (PCR) amplification, cloning and partial-length sequencing of 18S rRNA genes were conducted according to protocols outlined in Countway (2005) and Countway et al. (2007). Briefly, full-length eukaryotic 18S rRNA genes were amplified by PCR with eukaryotic specific primers EukA (5′-AACCTGGTTGATCCTGCCAGT-3′) and EukB (5′-GATCCTTCTGCAGGTTCACCTAC-3′) (Medlin et al. 1988). Pre-mixed PCR reagents (0.5 μM of each primer, 2.5 mM $MgCl_2$, 250 μM each dNTP, 300 ng μl^{-1} BSA, 1X Promega buffer B) and 2.5 U of Taq DNA polymerase in 1X buffer B (Promega, Madison WI) were added directly to each tube in the thermocycler. Amplification took place according to the following thermal protocol: Initial denaturing at 95 °C for 2 min, followed by 35 cycles of: denaturing at 95 °C for 30 sec, 60 °C annealing for 30 sec and 72 °C extension for 2 min, with a final extension at 72 °C for 10 min. PCR amplicons were purified from gels with Zymoclean™ Gel DNA recovery kits (Zymo Research, Orange CA). Ligation reactions were set up using the TOPO-TA Cloning® kit for sequencing with the PCR® 4-TOPO® cloning vector (Invitrogen, Carlsbad CA) using 4.6 μl of PCR product. Ligation reactions were run with undiluted Invitrogen salt solution (1.2 M NaCl, 0.06 M $MgCl_2$) and purified prior to electroporation because this method resulted in substantially higher transformation efficiencies than the manufacturer's recommendation (Countway 2005). Ligations were purified with DNA Clean and Concentrator™-5 spin columns (Zymo) and eluted in 12 μl of sterile water before transformation into One Shot TOP10 Electrocompetent *E. coli* (Invitrogen) with a Gene Pulser Xcell (BioRad). Aliquots of transformed cells were diluted 1:10 with fresh SOC medium and 15 μl of diluted transformants were plated onto LB agar containing ampicillin (50 μg ml^{-1}) for overnight growth at 37 °C. Colonies were picked the following day into deep-well culture blocks containing 1.25 ml of TB medium and ampicillin (50 μg ml^{-1}), covered with AirPore™ tape (Qiagen, Valencia CA) and grown for 18-24 hours. Plasmid DNA was extracted using the Wizard SV96™ kit (Promega). Plasmid DNA for sequencing was eluted in 100 μl of sterile water. Plasmids were stored at −20 °C until ready for DNA sequencing. Sequencing was performed on a CEQ 8000 automated DNA sequencer (Beckman Coulter, Fullerton CA). All reads were manually trimmed and edited based on quality before assembly in MacVector™ v8.0. All sequences were run through Bellerophon (Huber et al. 2004) and Chimera-Check (Cole et al. 2003) servers to look for chimeric artifacts. One chimeric sequence was identified and discarded.

Environmental sample collection and DNA extraction: Environmental samples were collected on 28 June 2001 from the San Pedro Channel (33 33 N, 118 24 W) at 880 m and 500 m as part of the ongoing San Pedro Ocean Time-series (SPOT) conducted by the University of Southern California Wrigley Institute for Environmental Studies and an NSF-funded Microbial Observatory (http://www.usc.edu/dept/LAS/biosci/Caron_lab/MO/) led by Drs David Caron and Jed Fuhrman. Seawater was collected in 10 l Niskin sampling bottles on a CTD rosette (General Oceanics, Miami FL). Triplicate 2 l samples were fractionated successively through 200 μm and 80 μm Nitex mesh housed in 47 mm inline filter-holders before final filtration onto GF/F filters. The pre-filtration of samples through Nitex reduced the contribution of metazoa to subsequent DNA extracts. Filters were placed into 15 ml falcon tubes pre-loaded with 2 ml lysis buffer (40 μM EDTA, pH 8; 100 mM Tris, pH 8; 100 mM NaCl and 1% SDS) and frozen in liquid N_2. Samples were stored at −80 °C in the laboratory until ready for processing. Further details regarding sample collection, processing, and microbial diversity at this site are described elsewhere (Countway 2005; Schnetzer et al. in prep).

For community DNA extraction, tubes were thawed at 70 °C, and vortexed with 0.5 mm silica/zirconium beads for 30 seconds to mechanically disrupt cells. This heating and bead-beating cycle was repeated 3 times to maximize the release of genomic DNA. Lysates were filtered through a sterile 0.2 μm filter to remove the beads and debris into a clean tube to which a CTAB/NaCl solution was added (0.01% CTAB, 0.7 M NaCl, final concentration). Nucleic acids were extracted with three phenol:-chloroform:isoamyl alcohol (25:24:1) extractions, and a single chloroform–isoamyl alcohol (24:1) extraction. The nucleic acids in the final aqueous phase were precipitated overnight at −20 °C by adding 1X volume 95% ethanol and 0.1X volume 10.5 M ammonium acetate. The following day, nucleic acids were pelleted by centrifugation (14,000 × g) for 30 min at 4 °C, decanted and washed in 70% ethanol during an additional 15-min centrifugation, after which samples were decanted and inverted to dry. Dried DNA pellets were resuspended in 100 μl of sterile TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and stored frozen at −20 °C until further analysis.

18S rDNA PCR amplification, cloning and sequencing was conducted using the methods described for the acantharian sequences above. Partial sequences were initially obtained on a Beckman CEQ 8000 using the internal primer Euk-570F (5′-GTAATTCCAGCTCCAATAGC-3′) (Weekers et al. 1994) to read through one of the most variable regions of the gene. These reads were trimmed and subjected to a BLAST search against sequences in GenBank in May 2005. Clones with top BLAST hits matching acantharia in the database were selected for full-length sequencing. Archived clones were re-grown, the plasmids were purified and full-length sequencing was carried out using an ABI 3730XL (Applied Biosystems, Foster City, CA) at the Marine Biological Laboratory W. M. Keck Ecological and Evolutionary Genetics Facility. Individual sequence reads were assembled and edited using AlignIR (LI-COR Biotech, Lincoln, NE) software.

Phylogenetic analyses. We obtained sequences from public databases to include representative rhizaria that encompass the Polycystinea/Taxopodida/Acantharea, as well as certain environmental sequences of interest (Table 2). The core rhizarian alignment was obtained from Nikolaev et al. (2004). The Nikolaev alignment along with all acantharian and environmental samples were imported into ARB v. 05.05.26 (Ludwig et al., 2004) and the alignment was adjusted using a combination of the Fast Aligner feature in ARB along with

manual refinement. A sequence mask was applied to retain regions of unambiguous alignment and only those positions were included in subsequent phylogenetic analyses. A data set including just acantharian, polycystine sequences and select outgroups (a total of 134 taxa and 1115 positions) was subjected to Bayesian (BA) inference methods.

We conducted our Bayesian analysis using MrBayes, Version 3.0b4 (Ronquist and Huelsenbeck 2003) under the GTR model of substitution (Lanave et al. 1984; Rodriguez et al. 1990; Tavare 1986), considering invariants and a gamma-shaped distribution of the rates of substitution among sites. The chain length for our analyses was 5,000,000 generations with trees sampled every 100 generations using Markoff Chain Monte Carlo (MCMC) analysis. Chain parameters appeared to be stationary after several thousand sampled trees; the first 10,000 trees (1×10^6 generations) were discarded as burn-in for the tree topology and posterior probability. Acantharian and environmental sequences were deposited in GenBank under the Accession numbers GU246566-GU246591.

CARD-FISH for the detection of acantharian deep-sea stages: Two rRNA-targeted 5′HRP-conjugated probes were developed to specifically target organisms of interest from acantharian and unidentified clades. Probe A497, 5′-TCATTC-CAATCAACTCAC-3′ (Amaral Zettler et al. 1997) was used to target acantharia and UC1-899, 5′-TCATYATACAAAGGTCCA-3′, was designed to target Unidentified Clade 1 phylotypes. The eukaryote-specific, 5′HRP probe 1209R, 5′-GGGCATCA-CAGACCTG-3′ (Lim et al. 1993) served as the positive control. A HRP-conjugated sense probe of UC1-899 (5′-TGGACCTTTGTATRACGA-3′), served as the negative control. Before collecting samples, a series of stringency tests were conducted on environmental samples enriched with vegetative acantharia and acantharian cysts to empirically determine conditions necessary to minimize background and non-specific binding. Monthly <80 μm fractions of seawater from 500 m were collected at the SPOT sampling site from May 2005–January 2006. Cells were fixed at 4 °C for exactly 1 h in 2% formaldehyde and gently filtered onto 25 mm white GTTP filters (Millipore) and immobilized with 0.2% Metaphor agarose at 40 °C. Once dry, filters were dehydrated in 80% EtOH at room temperature for 1 minute, blotted and air-dried. Samples were then stored dry at −20 °C for several weeks. Our CARD-FISH protocol was adapted from Pernthaler et al. (2002) with some modifications. Filters were treated with proteinase K solution (2 μl proteinase K in 0.05 M EDTA and 0.1 M Tris [pH 8]) at 37 °C for 1 h. Filters were washed with dH_2O for 1 min, incubated in 0.01 M HCl for 20 min, rinsed 3 times with fresh dH_2O and air-dried. Filters were placed on clean glass slides and hybridized with 2 μl of 50 ng/μl probe stock in 20 μl 40% formamide hybridization buffer (1.5 M NaCl; 33 mM Tris [pH 8]; 1 g dextran sulfate, 40% formamide; 0.83% blocking reagent; 0.016% SDS). Negative controls received the nonsense probe, blanks received only buffer. Filters were covered with glass cover slips and incubated in humidified falcon tubes at 35 °C for 2-3 h. Filters were submerged in wash buffer (37 mM NaCl; 200 mM Tris [pH 8]; 10 mM EDTA [pH 7.5]; 0.01% SDS) at 37 °C for 10 min, then blotted on blotting paper and incubated in 0.05% Triton X-100/PBS at room temperature for 15 min with mild agitation. Filters were blotted, but not allowed to dry, before tyramide amplification using a TSA™ Kit #22 with HRP—streptavidin and Alexa Fluor® 488 tyramide (Invitrogen). We prepared amplification buffer according to the first steps of the kit protocol: 5 μl of tyramide working stock was combined with 450 μl amplification buffer and 50 μl 10% blocking solution and 100 μl of this tyramide solution was added to each filter on a fresh slide. Filters were then covered with HybriSlips™ (Sigma) and incubated in the dark for 10 min at 37 °C. Filters were washed with fresh 0.05% Triton X-100/PBS in the dark at room temperature for 15 min, then washed with dH_2O for 1 min, 80% EtOH for 1 min and dried at 37 °C in the dark. Samples were mounted and stained with SlowFade® Gold antifade reagent with DAPI (Invitrogen) and visualized on a Leica DM IBRE equipped with excitation and emission filter sets for FITC and DAPI, respectively.

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