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ORIGINAL ARTICLE

Distribution and Diversity of Microbial Eukaryotes in Bathypelagic Waters of the South China Sea

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ABSTRACT

Little is known about the biodiversity of microbial eukaryotes in the South China Sea, especially in waters at bathyal depths. Here, we employed SSU rDNA gene sequencing to reveal the diversity and community structure across depth and distance gradients in the South China Sea. Vertically, the highest alpha diversity was found at 75-m depth. The communities of microbial eukaryotes were clustered into shallow-, middle-, and deep-water groups according to the depth from which they were collected, indicating a depth-related diversity and distribution pattern. Rhizaria sequences dominated the microeukaryote community and occurred in all samples except those from less than 50-m deep, being most abundant near the sea floor where they contributed ca. 64–97% and 40–74% of the total sequences and OTUs recovered, respectively. A large portion of rhizarian OTUs has neither a nearest named neighbor nor a nearest neighbor in the GenBank database which indicated the presence of new phylotypes in the South China Sea. Given their overwhelming abundance and richness, further phylogenetic analysis of rhizarians were performed and three new genetic clusters were revealed containing sequences retrieved from the deep waters of the South China Sea. Our results shed light on the diversity and community structure of microbial eukaryotes in this not yet fully explored area.

MICROBIAL eukaryotes are composed of morphologically, genetically, and functionally diverse group of single-celled eukaryotes that play fundamental ecological roles as primary producers, consumers, decomposers, and trophic links in aquatic food webs (Azam et al. 1983; Caron et al. 2012). Starting from the landmark works of López-García et al. (2001) and Moon-van der Staay et al. (2001), there has been a surge of studies applying rDNA analyses to reveal the biodiversity of microbial eukaryotes from a variety of oceanic environments (Countway et al. 2007; Edgcomb et al. 2002; Not et al. 2007a; Scheckenbach et al. 2010; Stoeck et al. 2003). These studies have unveiled a previously unexpected diversity such as the Marine Alveolate Group I & II (López-García et al. 2001), Marine Stramenopiles (Massana et al. 2004), and picobiliphytes (Not et al. 2007b). However, compared with surface/epipelagic waters (Bachy et al. 2011; Diez et al. 2001; Genitsaris et al. 2016; Hu et al. 2016; Li et al. 2010; Lie et al. 2013; Moon-van der Staay et al. 2001; de Vargas et al. 2015; Vigil et al. 2009; Wu et al. 2014a,b) or extreme deep-ocean environments, such as hydrothermal vents (Edgcomb et al. 2002; López-García et al. 2003), cold methane seeps (Takishita et al. 2007), or anoxic basin (Edgcomb et al. 2011a; Orsi et al. 2011, 2012; Stoeck et al. 2003), few studies have focused on microbial eukaryotes in oxygenated meso- and bathypelagic oceanic habitats (Countway et al. 2007; Lie et al. 2014; López-García et al. 2001; Lovejoy et al. 2006; Not et al. 2007a,b; Scheckenbach et al. 2010). Previous studies showed the microbial eukaryote communities in meso- and bathypelagic waters are significantly different from those in epipelagic waters with several groups, for example, Rhizaria, Excavata, dominating the deep waters, whereas the shallow waters were normally dominated by Alveolata and Stramenopiles (Countway et al. 2007; Not et al. 2007a,b; Scheckenbach et al. 2010).

Microbes are major players in the cycling of energy and matter, and mediate all biogeochemical cycles in the oceans (DeLong et al. 2006). Understanding the roles of different assemblages of microbes in a variety of different habitats is required in order to understand their functions at global scales. Massive sequencing of marker genes including ribosomal genes from diverse marine environments with simultaneous monitoring of environmental variables can provide large amounts of data that can be applied to network theory and modeling to identify ecological links and generate testable hypotheses (Worden et al. 2015). The South China Sea (SCS) is one of the largest marginal seas in the tropical Pacific Ocean. The maximum depth of SCS basin can exceed 5,000 m (Chen et al. 2001), which is permanently stratified and oligotrophic, similar to the interior of the major ocean basins (Gong et al. 1992). Despite their pivotal roles in the ocean matter and energy cycling (Arístegui et al. 2009), the diversity and community structure of microbial eukaryotes in the SCS has been rarely studied, for example, surveys of planktonic protists in the northern SCS (Li et al. 2010; Yuan et al. 2004) and of photosynthetic picoeukaryotes along transcontinental sections of the SCS (Wu et al. 2014a,b). The above studies found high diversities of microbial eukaryotes, high estimates of undiscovered diversity and the presence of previously unknown groups (Li et al. 2010; Wu et al. 2014a,b; Yuan et al. 2004). However, none of these studies has characterized diversity and community structure of microbial eukaryote assemblages dwelling in the water deeper than 100 m. Furthermore, previous studies in the SCS focused more on the pico-sized protists (Wu et al. 2014a,b), leaving diversity and distribution patterns of the whole microbial eukaryote community largely unknown, especially in the meso- and bathyal waters.

In this study, we studied the diversity and community structure of nonsize-fractionated microbial eukaryote communities in waters of the SCS at various depths, from the surface to near the sea floor (> 4,000 m). This was done by sequencing the small subunit ribosomal DNA (SSU rDNA) gene via clone library construction. With the data obtained, we aimed to address the following questions: (i) are there novel microbial eukaryotes in the deep waters of the SCS; (ii) are there distinct vertical and horizontal patterns of diversity and community composition of microbial eukaryotes in the SCS.

MATERIALS AND METHODS

Study site and sample collection

Samples were retrieved from the South China Sea (SCS) aboard the R/V Dongfanghong II in April 2012 at six sites (Fig. S1). Niskin bottles mounted on a CTD rosette system were used to collect seawater. At site O10, seawaters from eight discrete depths (5, 50, 75, 500, 800, 1,000, 2,000, and 4,276 m) were collected. At sites G3 (3,844 m), H4 (4,053 m), H3 (4,075 m), O1 (4,022 m), and O7 (4,240 m), seawater was collected from near the sea floor. Two liters of sea water were prefiltered using 200-μm Nitex (Sefar) screening to reduce the contribution of metazoa to subsequent DNA extracts and filtered onto a 0.22-μm pore size polycarbonate filter (Millipore) in a filtration unit and then stored frozen in Cryo-vials in liquid nitrogen until further treatment. Temperature and salinity were determined using an SBE-911 instrument (Sea-bird electronics).

Samples (2 ml) of 20-μm-mesh prefiltered seawater were fixed with 1% ice-cold glutaraldehyde and then deep-frozen in liquid nitrogen. Picoeukaryotes and cyanobacteria were directly counted with a Flow Cytometer (Epics Altra II, Beckman Coulter, Brea, CA). Heterotrophic bacteria were stained with SybrGreen I at 1/10,000 dilution and counted on the same flow cytometer (Li et al. 2013).

DNA extraction, cloning, and sequencing of PCR-amplified SSU rDNA

Genomic DNA was extracted from the filters using PowerWater DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) following the manufacturer's instructions but with the following modifications: (i) the filters were cut into small pieces using sterile scissors before bead-beating; (ii) instead of bead-beating for 5 min, 15-min (5 min each) beating were employed with 2 min of cooling at room temperature between each beating; (iii) in order to obtain a higher concentration of genomic DNA, at the final step, 40 μl of elution buffer was used rather than 100 μl. Extracted DNA was stored at −20 °C for later processing. The SSU rDNA gene was amplified using eukaryotic-specific forward primer E360F (5′-CGG AGA RGG MGC MGA GA-3′; Medlin et al. 1988) and universal reverse primer U1517R (5′-ACG GCT ACC TTG TTA CGA ACT T-3′; Shopsin et al. 1999). The PCR protocol employed TaKaRa Ex Taq DNA polymerase (TaKaRa, Dalian, China) in all cases. For each sample, the PCR products from three separate reactions were pooled for clone library construction in order to minimize reaction-level PCR bias (Bates et al. 2013). The clone libraries were constructed using TA cloning kit (TaKaRa) according to the manufacturer's instructions and 120 positive clones at each depth were commercially sequenced using M13F and M13R primers from both ends using an ABI 3730xl DNA Analyzer.

Sequence data processing, taxonomic assignment, and statistical analysis

The obtained sequences were automatically assembled and then manually checked using Sequencher 5.0 (GeneCodes Corp., Ann Arbor, MI). Potential chimeric sequences were detected using KeyDNATools (Guillou et al. 2008) and ChimeraSlayer (Haas et al. 2011) and then removed from the analysis. Sequence data from this study were deposited in GenBank (accession numbers KX532240–KX533445).

The clone libraries from all sites were then combined and pairwise sequence alignments performed using

MUSCLE (Edgar 2004). The aligned sequences were used as input for QIIME (Caporaso et al. 2010) in order to group sequences in operational taxonomic units (OTUs) at ≥ 95% different identity level to obtain approximate species-level OTUs for most taxa (Caron et al. 2009) and to generate an OTU table for downstream analysis. Representative sequences for each OTU were used in a BLASTn search against Protist Ribosomal Database 2 (PR^2) (Guillou et al. 2013) to provide taxonomic information on the OTUs.

To normalize the sampling effort, all datasets were rarefied to 59 sequences for diversity and community composition analyses, this being the lowest number of sequences recovered from the 13 samples. Rank abundance lists of OTUs served as input files for diversity estimation in SPADE (Chao and Shen 2003). Shannon's (H′) and Simpson's (Ds^{-1}) diversity indexes were calculated from OTU abundance data along with the nonparametric richness estimators, Chao1. Beta diversity was calculated on Bray–Curtis distances matrix and visualized with nonmetric multidimensional scaling ordination (nMDS). Simple and partial Mantel tests were performed in R with Vegan package to explore correlations between bacterial abundance and community variability (Legendre and Legendre 1998). Pairwise distance (*p*-distance) between representative sequences of each rhizarian OTUs with their first BLAST hit, as well as the first named BLAST hit, were calculated using MEGA 6 (Tamura et al. 2013).

Phylogenetic analyses

One representative sequence for each OTU was selected for phylogenetic construction. Other sequences included in phylogenetic analyses were obtained from the GenBank database as shown in fig. 4. The sequences of the SSU rDNA gene were aligned using Clustal W, as implemented in BioEdit v.7.0.0 (Hall 1999; Thompson et al. 1997). Modeltest and MrModeltest v. 2 were used to select the best models for the Maximum likelihood (ML) and Bayesian inference (BI) analyses (Nylander 2004; Posada and Crandall 1998). The ML tree was constructed with the PhyML 3.0 program (http://www.atgc-montpellier.fr/phyml/) using a GTR + I + G model, which performed ML analysis with a heuristic search and 1,000 bootstrap replicates (Guindon et al. 2005). The BI tree was constructed with MrBayes v. 3.1.2 (Ronquist and Huelsenbeck 2003) under a GTR + I + G model. Four simultaneous Markov chain Monte Carlo (MCMC) chains were run for 3,500,000 generations, sampling every 100 generations. The first 25% trees were discarded as burn-in. The 50% majority rule consensus tree was determined to calculate the posterior probabilities for each node.

RESULTS

Sampling sites and environmental factors

Six sites were chosen to represent the central part of the deep basin in the SCS, with geographical distances between the paired near-sea-floor sampling sites ranging from 67 km to 506 km (Fig. S1). Samples collected from site O10 were at a specific depth from surface to bottom waters (ca. 4,200 m). The temperature at O10 ranged from 2.48 to 29.82 °C and salinity ranged from 33.31 to 34.61 psu. The bacterial abundance decreased with increasing water depth, from ca. 1.7–6.0 × 10^5 cells/ml to ca. 1.5 × 10^4 cells/ml. The abundances of *Prochlorococcus*, *Synechcoccus*, and picoeukaryotes were ca. 3.9–4.4 × 10^4 cells/ml, 65–289 cells/ml, and 109–508 cells/ml, respectively, at depths above 75 m. None of these three groups was detected at depths below 500 m. At sites G3, H4, H3, O1, and O7, samples were collected near the sea floor with the depth ranging from 3,844 to 4,240 m. Water temperature (ca. 2.403–2.487 °C) and salinity (ca. 34.621–34.623 psu) were relatively constant between near-sea-floor sites. The lowest abundance of bacteria was found at H3 (ca. 1.3 × 10^4 cells/ml) and the highest at O1 and O7 (ca. 5.0 × 10^4 cells/ml) (Table 1).

Taxonomic distribution of sequences

A total of 1,206 partial-length SSU rDNA eukaryotic sequences with an average length of 1,300 bp from 13 clone libraries were obtained for downstream analysis after removing potential chimeric sequences and the sequences that were too short (< 800 bp). Although 200-μm-mesh prefiltering was used to minimize the contribution of metazoa from subsequent DNA extraction, a total of 74 clones were found belonging to metazoan phyla including Arthropoda (mainly crustaceans), Cnidaria (mainly hydrozoans), and Urochordata (mainly appendicularians). At site O10, metazoan sequences were detected in all libraries except samples below 1,000 m and its contribution to each of the clone libraries were 23.7% at 5 m, 3.7% at 50 m, 35.9% at 75 m, 7.1% at 500 m, and 4.0% at 800 m. No metazoan sequences were retrieved from the near-sea-floor samples.

After removing metazoan sequences, 1,132 microbial eukaryote sequences were left ranging from 59 to 100 per library (Table 2). Vertically, members of the Alveolata, which was composed primarily of dinoflagellates, ciliates, and syndiniales (including Syndiniales Group I, II, III, IV, and V) were represented at all depths and dominated the microbial eukaryote community in the euphotic zone (Fig. 1). Within Alveolata, syndiniales outnumbered the rest and contributed most significantly (ca. 50.8%) at the depth of 75 m. Syndiniales Group I and II were the major contributors of syndiniales, with the former having the highest sequence abundance at the depth of 75 m, whereas the latter contributed ca. 1.3% of library constructed from this depth (Fig. 1). Stramenopiles, together with the chlorophytes, haptophytes, cryptophytes, holozoans, and centrohelids, were restricted to the euphotic zone with a small number of apicomplexan sequences detected at depths of 50 and 1,000 m (Fig. 1). No radiolarian sequences were found at 5 m and only a few were found at 50 and 75 m. The relative number of radiolarian sequences increased with depth, replacing the alveolates

Table 1. Coordinate of sampling locations, physical parameters of the water column in the sampling location

Sampling sites	Date (2012)	Latitude (N)	Longitude (E)	Depth (m)	Water temperature (°C)	Salinity (psu)	Bacterial abundance ($\times 10^3$ ml^{-1})	*Prochlorococcus* abundance ($\times 10^3$ ml^{-1})	*Synechcoccus* abundance (ml^{-1})	Pico-eukaryotic cells (ml^{-1})
O10	23 April	14°34′48″	116°59′24″	5	29.820	33.305	169	43.5	65	109
				50	28.395	33.485	665	41.0	289	508
				75	27.968	33.637	626	39.3	196	400
				500	8.895	34.427	60	/	/	/
				800	5.873	34.472	39	/	/	/
				1,000	4.445	34.524	32	/	/	/
				2,000	2.497	34.609	30	/	/	/
				4,276	2.483	34.621	15	/	/	/
								Phosphate (μmol/l)	Nitrate (μmol/l)	
G3	12 April	18°00′00″	117°00′00″	3,844	2.403	34.623	22	2.672	39.39	
H4	17 April	17°00′00″	116°00′00″	4,053	2.424	34.622	16	2.843	41.20	
H3	18 April	17°00′00″	117°00′00″	4,075	2.423	34.622	13	2.498	38.55	
O1	18 April	16°24′00″	117°00′00″	4,022	2.419	34.622	50	2.749	40.03	
O7	22 April	13°34′48″	116°58′48″	4,240	2.487	34.622	49	2.789	40.80	

as the dominant group at depths of 500, 1,000, 2,000 m, and near the sea floor. Their contributions to the microbial eukaryote community reached ca. 94% at 2,000 m (Fig. 1). Radiolarian sequences overwhelmed all the near-sea-floor samples, ranging from ca. 77.5% (O1) to 92.7% (G3) of all clones detected in each library. The number of sequences affiliated with other taxa was significantly lower than radiolarian sequences (Fig. 1).

Taxonomic distribution of OTUs

Using a threshold of 95% identity, all microbial eukaryote sequences were grouped into 262 OTUs ranging from 11 to 46 OTUs per library (Table 2). Overall, Dinophyta made the highest contribution to the total microbial eukaryote community forming 106 out of 262 OTUs followed by rhizarians (75 OTUs), comprising radiolarians (69 OTUs) and cercozoans (6 OTUs), and then Stramenopiles (15 OTUs). Within Dinophyta, Syndiniales Group II (44 OTUs), Syndiniales Group I (35 OTUs), and Dinophyceae (16 OTUs) contributed strongly followed by Syndiniales Group III, Syndiniales Group IV, and Syndiniales Group V. Within the radiolarians, Polycystinea had the highest number of OTUs (41 OTUs), 34 of which were affiliated with Spumellarida, 4 were affiliated with Collodaria, and 3 were affiliated with Nassellaria. Acantharea, RAD A, RAD B, RAD C had much fewer OTUs (7, 6, 12, and 2 OTUs, respectively). Members in Excavata (7 OTUs), Ciliophora (6 OTUs), Chlorophyta (4 OTUs), Hacrobia (4 OTUs), Opisthokonta (4 OTUs), and Streptophyta (1 OTU) made only minor contributions to the diversity of the microbial eukaryote communities.

Microbial eukaryotic communities from the upper water column (5 m, 50 m, and 75 m) were characterized by high

Table 2. Microbial eukaryotes diversity estimates for the 13 clone libraries

Sample	*N*	OTUs (95%)	OTUs (95%)[a]	H′[a]	Ds^{-1}[a]	Chao1 (95% CI)[a]	ACE (95% CI)[a]
O10–5	71	43	39	4.19	37.20	109.1 (64.0–235.5)	137.3 (77.5–289.6)
O10–50	79	31	25	3.10	8.91	106.0 (43.9–371.9)	77.0 (42.0–184.1)
O10–75	59	42	42	4.42	57.03	127.3 (73.1–276.0)	134.4 (78.8–274.0)
O10–500	92	46	30	3.82	12.28	168.5 (89.5–391.3)	212.4 (113.5–456.5)
O10–800	95	45	35	3.84	12.77	245.3 (87.9–871.4)	160.6 (78.5–397.7)
O10–1k	100	24	17	2.37	4.29	33.7 (20.7–91.5)	42.5 (24.3–105.5)
O10–2k	97	19	13	1.77	2.26	– (–)	– (–)
O10–B	89	23	18	2.22	2.54	67.0 (28.9–239.1)	71.6 (31.8–225.3)
G3	96	11	9	1.07	1.34	– (–)	– (–)
H3	96	17	12	1.49	1.65	– (–)	84.8 (23.6–469.5)
H4	90	13	10	1.42	1.91	– (–)	70.9 (19.1–416.7)
O1	80	32	26	3.11	6.76	226.0 (61.0–1168.8)	90.1 (46.5–227.0)
O7	88	24	15	1.85	1.98	87.0 (26.8–455.2)	64.9 (25.8–245.1)
Total	1,132	262	210	3.86	5.12	614.2 (490.5–804.8)	692.2 (558.7–885.5)

N is the number of sequences. H′ is the Shannon diversity index, and Ds^{-1} is the inverse Simpson's diversity index. Chao1 and ACE are nonparametric species (OTU) richness estimators calculated from the OTU rank abundance data for each set of sequences.

[a]Normalized numbers based on subsampling of 59 sequences.

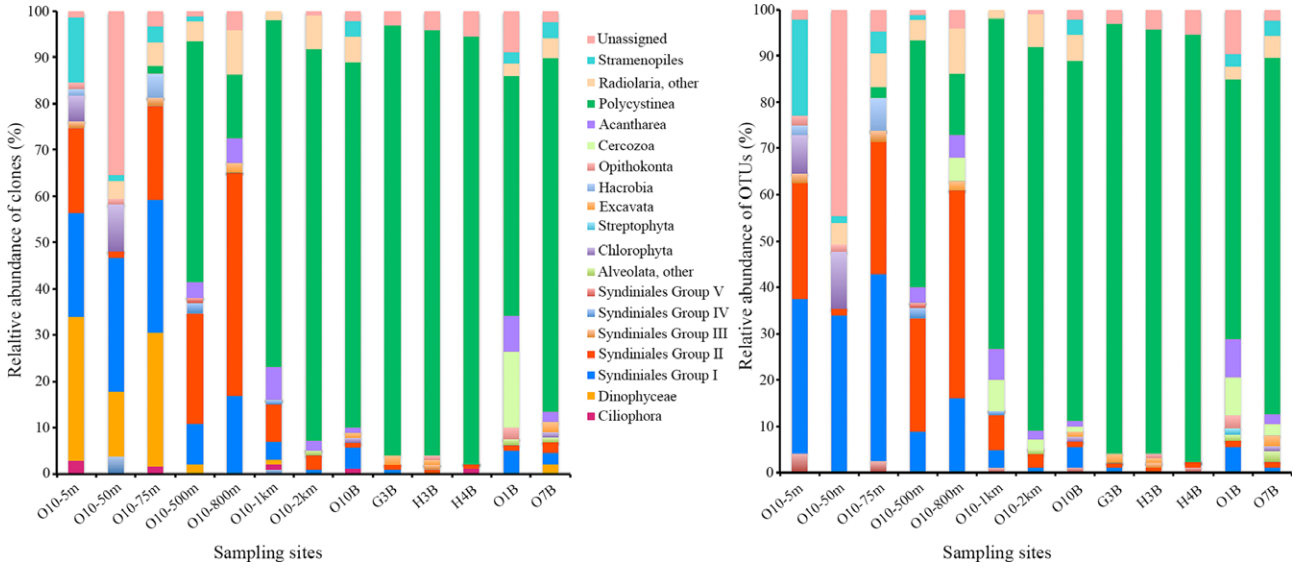

Figure 1 Relative abundance of major microbial eukaryote assemblages (sequences, left; OTUs, right) in each of the 13 clone libraries constructed from environmental SSU rDNA.

relative numbers of dinophyta and stramenopile OTUs (Fig. 1), while chlorophyte, haptophyte, cryptophyte, and ciliate OTUs, which are common members in upper layer of coastal waters, were rarely encountered in our study. The highest contributions of dinophyta OTUs to the total microbial eukaryotic OTUs were at depths of 50 m and 75 m (ca. 20% of total OTUs), with Syndiniales Group II contributing the most at 800-m depth (ca. 48% of total OTUs). In waters below 1,000 m, the proportion of radiolarian-affiliated OTUs to total microbial eukaryote OTUs ranged from 61% to 94% (Fig. 1).

A total of 91 OTUs were found in the six near-sea-floor samples. The two most dominant OTUs were affiliated with Radiolaria, which were present in all near-sea-floor sampling sites. Few OTUs were found in more than two sites (only 1 OTU was found in 4 sites and 18 OTUs were encountered in 2 or 3 sites, respectively), thus the majority of OTUs were detected in one site only (Fig. S2). Notably, 6 OTUs affiliated with Cercozoa were detected at site O1 only.

Alpha- and beta-diversity of microbial eukaryotes

To normalize sampling effort, all datasets were rarefied to 59 sequences per sample to examine and compare the alpha- and beta-diversity of microbial eukaryotes. Generally, alpha diversity (Richness, Shannon, Simpson, and Chao1 diversity estimators) of microbial eukaryotes took the form of a single-peak curve with the highest diversity found at a depth of 75 m and the lowest found at the near-sea-floor depths (Table 2). The only exception was O1, the diversity indexes of which were higher than those of O10–1 km and O10–2 km, and even comparable to those of O10–50 m. These trends were apparent in rarefaction curves (Fig. S3). Generally, rarefaction curves for the upper water column samples (e.g. 5–800 m at site O10 and bottom sample at site O1) rose more steeply than those for the deeper water column samples indicating higher evenness within the deep-sea communities.

Nonmetric multidimensional scaling ordination (nMDS) analysis revealed a depth-oriented trend that all samples clustered into three groups, that is, shallow (5, 50, 75 m), middle (500, 800 m), and deep (1,000, 2,000 m, and near-sea-floor samples) (Fig. 2). The Bray–Curtis clustering showed the same pattern (Fig. S4). The Venn diagram showed that 8 OTUs were shared between shallow and mid-depth waters, 22 OTUs were shared between mid-depth and bottom waters, and 7 OTUs were shared between shallow and bottom waters. Only 3 OTUs, OTU_30, OTU_31, and OTU_180, were encountered in all three groups (Fig. S5). OTU_30 was affiliated with Syndiniales Group I, close to one environmental sequence from Cariaco Basin (98.9% similarity) but had low similarity with its closest cultured organism *Eudubosquella*

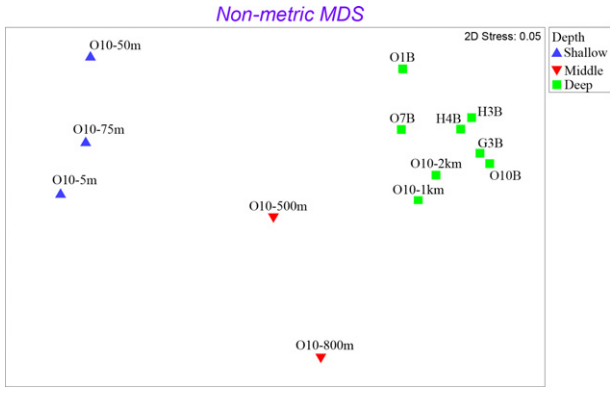

Figure 2 Plots of nonmetric multidimensional scaling (nMDS) ordination based on community distance matrices.

Table 3. List of the 3 OTUs that were shared between shallow, middle, and deep waters with the number of clones, taxonomic identification, and similarity (%-S) with the nearest neighbor (NN) and the nearest named neighbor (NNN)

OTU ID	Clones	Group	NN	%-S	NNN	%-S
OTU_30	9	Syndiniales; Syndiniales Group I;	GU819951	98.9	*Eudubosquella costata* (KP749831)	92.8
OTU_31	7	Syndiniales; Syndiniales Group II;	KF130546	99.5	*Amoebophrya* sp. (KF791347)	92.8
OTU_180	4	Syndiniales; Syndiniales Group II;	KJ761177	99.1	*Amoebophrya* sp. (KF791347)	92.0

costata (92.8% similarity). OTU_31 and OTU_180 were both affiliated with Syndiniales Group II and had high similarities with their closest environmental sequences (99.5% and 99.1%, respectively). The closest cultured organism of OTU_31 and OTU_180 I was *Amoebophrya* sp. (KF791347) but the similarities were rather low (92.8% and 92.0%, respectively) (Table 3).

To further explore correlations between bacterial abundance and community variability, Mantel and partial Mantel tests were conducted. The results showed that bacterial abundance always showed strong correlations with community variability both before ($r = 0.908$, $p < 0.001$) and after ($r = 0.5387$, $p < 0.001$) controlling for water depth.

Novel rhizarian phylotypes

Due to the prominent contributions of rhizarian (including Radiolaria and Cercozoa) sequences, a BLAST search against GenBank was performed to find their closest matches of named and environmental sequences. We defined the nearest neighbor as the BLAST hit with the highest score and no more than 5.21% *p*-distance to the respective clone. The nearest named neighbor was accordingly defined as the highest scoring BLAST hit with no more than 5.21% *p*-distance to the respective clone and representing a sequence with full species name annotated (Scheckenbach et al. 2010). The mean genetic *p*-distances between representative sequences of each rhizarian OTU and its first BLAST hit, as well as its first named BLAST hit, were 3.3% and 7.2%, respectively. The rank abundance curve for all the rhizarian-affiliated OTUs, and the pairwise genetic *p*-distances with their first BLAST hit and first named BLAST hit, are shown in Fig. 3. The four most abundant OTUs affiliated with rhizaria had both a nearest neighbor and a nearest named neighbor (Fig. 3). Comparisons of all rhizarian OTUs with published sequences showed that 28.9% of OTUs have both a nearest neighbor and a nearest named neighbor. Approximately 51.3% of OTUs have a nearest neighbor only. Fifteen OTUs (accounting for 19.7% of all OTUs) were found to have neither a nearest named neighbor nor any nearest neighbor in GenBank (Fig. 3). The locations of the environmental nearest neighbor sequences for each OTU are shown in Fig. 4, most of which are from East Pacific Rise and Cariaco Basin. The rests are from the Gulf Stream, Scotia Shelf, Coastal North Pacific, Sargasso Sea, South China Sea, Arctic Ocean, central Pacific Ocean, Lost City hydrothermal field, and deep hypersaline anoxic basins (Fig. 4).

Phylogenetic diversity of rhizarian-affiliated sequences

To explore phylogenetic diversity, representative sequences of 76 rhizaria-affiliated OTUs from this study plus 113 nearly full-length sequences from GenBank were used to reconstruct phylogenetic trees of Rhizaria. Phylogenetic analyses showed that the rhizarian sequences from this study were well represented in the major clades of Rhizaria (Fig. 4). Cercozoa-affiliated sequences were basally placed within Rhizaria, which were exclusively retrieved from deep (> 1,000 m depth) waters. Within radiolaria, Polycystinea were polyphyletic and separated into two branches. The placement of Polycystinea, including Spumellarida (colonial and naked) and Nassellarida, being next to Cercozoa were highly supported in both ML and BI trees. OTUs from our study that were affiliated within this group were all retrieved from middle waters (500- and 800-m depth) except one which was from deep water (Fig. 4). Three environment clades, RAD A, B, and C were represented by OTUs coming from different depths. Within Acantharea, OTUs from this study were placed in Acanth I and Clade C, respectively.

Approximately 46.7% of all rhizarian OTUs representing 87.9% of all clones fell into Polycystinea (solitary and shell bearing). Within this group, some OTUs (e.g. OTU_120, 188, 152, 147, 174, 182, and 115) fell within the core Polycystinea clade and grouped with their known congeners (Fig. 4). Fourteen OTUs, which were all from deep waters, formed an unidentified clade (Unidentified Clade-I) that was sister to the one containing sequences of Astrosphaeridae and environmental sequences from the Sargasso Sea and Cariaco Basin. Notably, the most abundant and most widely distributed OTU in our study, OTU_215 which represented 494 clones, was placed outside of the Unidentified Clade-I with low support. Both ML and BI trees showed generally strong support for two clades being basal to Polycystinea, and characterized these as Unidentified Clade II & III, all members of which were from deep waters (Fig. 4).

DISCUSSION

Depth-related microbial eukaryote community in the South China Sea

In this study, SSU rDNA clone libraries were employed to study the diversity and community structure of microbial eukaryote assemblages in samples from surface to near-sea-floor waters (3,844–4,240 m) in the SCS. Overall, both the diversity and community structure varied with

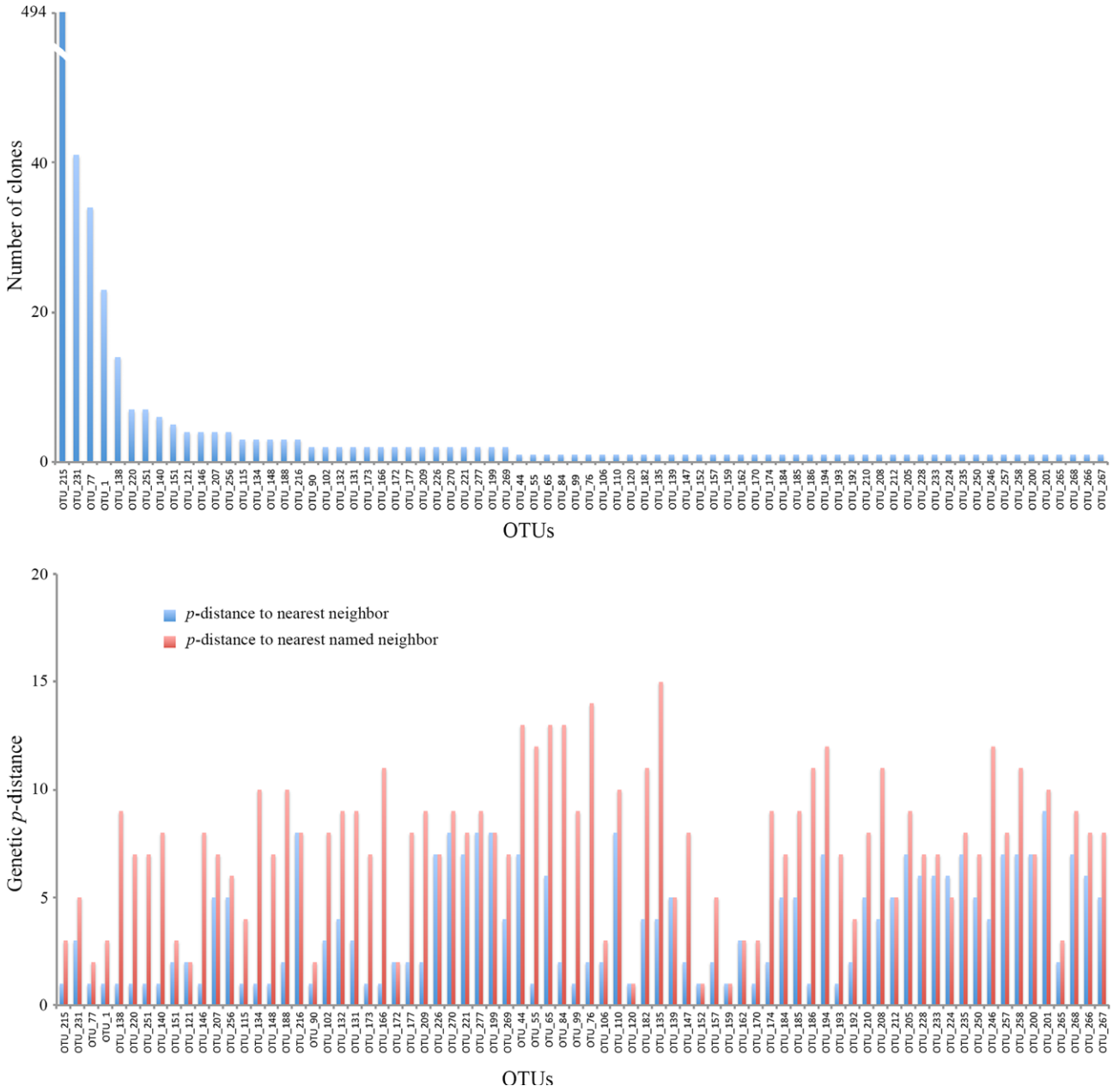

Figure 3 Rank abundance curve for the Rhizaria-affiliated sequences at a threshold for OTU delineation of 5% *p*-distance (upper). Pairwise genetic *p*-distance between representative clones of each Rhizaria-affiliated OTU and its first BLAST hit (blue) and first named BLAST hit (red) (lower). Note the OTU numbers in both figures correspond to each other.

increasing water depth. Alpha diversity took the form of a single-peak curve and the highest diversity was found at depth of 75 m. All samples were clustered into three groups, the shallow, middle, and deep water group according to the depth of the waters from which they were collected. Shallow-water samples (5, 50, and 75 m) were characterized by dominance of alveolate groups, especially Dinophyta, which resembles the community structure of other oceans (Bachy et al. 2011; Countway et al. 2007; Diez et al. 2001; Not et al. 2007a,b). Middle water samples (500, 800 m) were dominated by mixture of rhizaria and alveolate groups, with community compositions more similar to deep samples as indicated by clustering analysis (Fig. S4). Rhizaria sequences overwhelmed deep water samples making it the most dominant taxon of the microbial eukaryote sequence pool in bathypelagic depths. Similar results were also found in the Sargasso Sea (Countway et al. 2007; Not et al. 2007a,b) but neither of these studies reported such a high proportion of radiolarian sequences as that in SCS, that ca. 94% of all

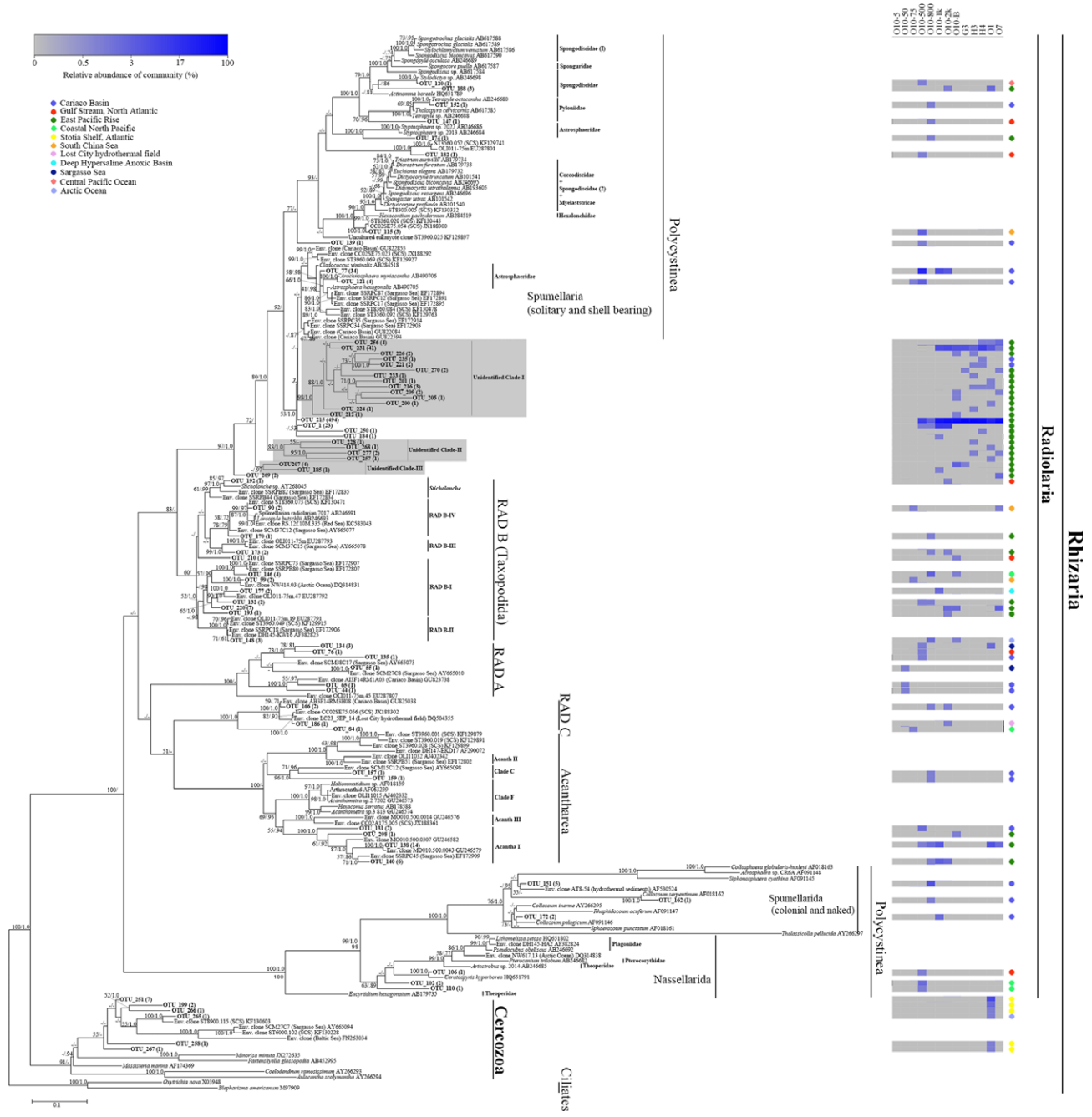

Figure 4 Heatmap-combined phylogenetic tree among 187 rhizarian SSU rDNA gene sequences inferred from a Maximum Likelihood (ML) analysis including 1,226 positions. Sequences from this study are presented in boldface. ML bootstrap values > 50% are shown at the nodes, followed by Bayesian posterior probabilities. Numbers in the brackets show the sequence number belonging to each OTU and the water depth from which it was collected. The colored scale bar from 0 to 100 indicates the relative number of clones for each OTU in the different clone libraries. Colored dots indicate the locations of the closest environmental sequences for each OTU.

sequences detected at 2,000-m depth. Our results provide further evidence that community composition of microbial eukaryotes in deep waters differs significantly from shallow and middle waters as reported previously (Countway et al. 2007, 2010; Not et al. 2007a,b; Schnetzer et al. 2011).

Patterns of diversity and community structure in the deep SCS

The perceived homogeneity of deep-sea environments, with little environmental variation, has led to the assumption that species have broad distribution ranges. To study

the patterns of microbial eukaryote communities in the deep waters of SCS, samples were collected from six near-sea-floor sites located in the SCS basin that are 50–500-km apart. The most abundant phylogenetic groups dwelling in deep SCS were Radiolaria (including Polycystinea, Acantharea, and RAD A, B, C) followed by Cercozoa, Alveolata, and Stramenopiles, which resembles the phylogenetic composition of previous deep-sea studies (Buck et al. 2000; Countway et al. 2007; Not et al. 2007a, b; Schnetzer et al. 2011). Euglenozoa is another taxon that was frequently reported to contribute strongly in deep-sea microbial eukaryote community (Buck et al. 2000; Orsi et al. 2011). Recently, a large amount of novel euglenozoan phylotypes that accounted for 36% of all the clones retrieved using general eukaryotic primers, was reported from the abyssal plains of the southeastern Atlantic Ocean (Scheckenbach et al. 2010). Later, a putative novel euglenozoan class and three Symbiontida sister clades were reported from the deep Cariaco Basin (Orsi et al. 2011). Furthermore, novel active kinetoplastids specialized in hypersaline anoxic basins in the Eastern Mediterranean were reported (Edgcomb et al. 2011b). Although euglenozoans have been implicated in protistan-prokaryotic symbiosis in the deep sea (Buck et al. 2000; Edgcomb et al. 2011b; Yubuki et al. 2009), the ecology of most of these deep-sea forms is currently unknown. In our study, eight Euglenozoa clones representing seven OTUs were found only in the deep samples. The nearest named neighbor of five out of seven OTUs is *Diplonema ambulator* (AY425009) and their nearest neighbors are sequences from deep Atlantic and Pacific Oceans with high genetic similarities (ranging 95–99%) (Lie et al. 2014) indicating their prevalence in deep oceans globally. The nearest neighbor of one of the two remaining OTUs is a sequence from deep sea of Bannock hypersaline anoxic basin (FJ000261) (Edgcomb et al. 2011b). Considering their relatively low genetic similarity (94%), it is unlikely that these two OTUs represent the same species. The last OTU showed high genetic similarity (96%) with a known flagellated protist *Neobodo designis* (AY753622). None of the OTUs retrieved in the deep SCS showed close relationships with symbiotic euglenozoan indicating differences in the euglenozoan assemblages between deep SCS and Cariaco Basin. This also suggests habitat specialization of euglenozoans in the Cariaco Basin and in hypersaline anoxic basins (Edgcomb et al. 2011b; Orsi et al. 2011).

Among the near-sea-floor samples, the numerically dominant OTUs were generally present at more than one sampling site, suggesting similarities in the community structure of microbial eukaryote studied. This finding is consistent with a study carried out in the abyssopelagial zone of the northwestern Atlantic Ocean (Countway et al. 2007). Interestingly, among all bottom samples, 10 of the 11 OTUs affiliated with Cercozoa were only found at O1, leading to the higher estimated alpha diversity of site O1 compared to other bottom sites. There were no significant differences in the environmental parameters between O1 and the other sites apart from higher bacterial abundances. However, no Cercozoa sequences were found at the nearby site O7 which had comparable bacterial abundances. Thus, the microbial eukaryote community in deep waters of SCS showed a mosaic distribution pattern. It is likely that other environmental factors shaped and maintained the distribution of microbial eukaryote communities on regional scales (Scheckenbach et al. 2010).

The dominance of Radiolaria-affiliated sequences in deep waters of the South China Sea

In our study, radiolarian sequences and OTUs were the most abundant in all deep samples, with the representative sequences of OTUs widespread across the major lineages of Radiolaria. Previous studies showed that some surface-dwelling radiolarian species can produce reproductive cysts that sink rapidly through water columns. The cysts then release flagellated cells (called swarmers) that are several micrometers in diameter in the deep waters (Decelle et al. 2013), potentially contributing to the heterotrophic nanoflagellates (HNF) community. Although still controversial, in the bathypelagic zone, HNF are argued to consume almost half of the prokaryote production and are the major cause of prokaryote mortality (Nagata et al. 2010). Furthermore, Acantharia have been shown to contribute to mesopelagic and bathypelagic particulate organic carbon (POC) flux via cyst formation (Bernstein et al. 1987; Decelle et al. 2013; Martin et al. 2010), thus playing an important role in the biological pump of carbon. However, in a recent study, radiolarian sequences were found to overwhelmingly dominate rDNA libraries in the < 0.8-μm faction, but were absent in the 0.8–3-μm faction in shallow waters (< 140 m depth) (Not et al. 2009). Also, far fewer radiolarian sequences were found in the 18s rRNA library than in the 18s rDNA library, which leads to the assumption that the radiolarian sequences detected in the 18s rDNA library come from extracellular material from larger cells (Not et al. 2009). In the present study, a large number of radiolarian sequences were found in water deeper than 1,000 m (ca. 85–97% of total microbial eukaryote sequences). More important, a large portion of radiolarian-affiliated sequences have neither a nearest named neighbor nor a nearest neighbor in the public database, suggesting the presence of new phylotypes in SCS. Because the morphology of these new phylotypes is currently unknown, and given the large genetic distance they have with other radiolarian sequences, we cannot rule out the possibility that some small, but active, radiolarians do dwell in the deep SCS and play a potentially important role in biogeochemical processes in this area.

New radiolarian OTUs and clades

The most abundant OTU, OTU_215, was affiliated with Radiolaria and was encountered in all except shallow water samples. The closest BLAST matches of this OTU were sequences from the anoxic basin in Cariaco Basin, Caribbean Sea (Orsi et al. 2011), and the Sargasso Sea, northern Atlantic (Lie et al. 2014; Not et al. 2007a,b). Their sequence dissimilarities were < 1 which indicate the wide

distribution of this taxon in the global oceans. The similarity of OTU_215 with its nearest named neighbor in GenBank is 97%, which indicates that this sequence likely belongs to the well-studied species *Cladococcus viminalis* (accession number: HQ651782) or represents one of its close congeners.

In our study, approximately 80% of OTUs affiliated with Rhizaria had a nearest neighbor, but about 51% of those had no nearest named neighbor. This suggests that despite their prevalence in environmental samples there is a lack of detailed morphological studies. Notably, about 20% of rhizarian OTUs have neither a nearest named neighbor nor a nearest neighbor in the GenBank database which suggests that there are new phylotypes that have yet to be reported from other ocean environments.

Hypotheses on the phylogeny of "Haeckel's Radiolaria" have changed repeatedly in recent years owing to the application of rDNA gene sequencing (Amaral-Zettler et al. 1997; Danelian and Moreira 2004; Krabberød et al. 2011; Nikolaev et al. 2004; Takahashi et al. 2004; Yuasa et al. 2005). The inclusion of environmental sequences in the Radiolaria phylogeny led to the findings of several new clades/lineages (Gilg et al. 2010; Not et al. 2007a,b; Orsi et al. 2011). Five environmental radiolarian clades, RAD 1–5, were reported from the Sargasso Sea (Not et al. 2007a, b). Later, 14 more radiolarian lineages, RAD 6–19, were reported from Cariaco Basin, several of which were possibly restricted to anoxic habitats (Orsi et al. 2011).

Radiolarian sequences detected in this study were widespread across the major lineages of Radiolaria. Generally, our findings supported the basal placement of Cercozoa in Rhizaria, the polyphyletic nature of Spumellarida, and the well supported environmental clades, RAD A, B, and C. In this study, we discovered three moderately supported lineages, Unidentified Clade I–III (Fig. 4, in gray). Unidentified Clade-I was sister to a clade consisting of solitary and shell-bearing Spumellaria species and environmental sequences from other ocean environments. Unidentified Clades-II & -III were basal to Polycystinea. Notably, each of these three clades included novel sequences, indicating the presence of potential new clades restricted to the deep waters of SCS. The new phylotypes, and even new clades, indicate the possible presence of endemic species in SCS, which supports the moderate endemicity theory of protists by previous studies (Foissner 2006; Orsi et al. 2011). The most abundant and widely distributed OTU, OTU_215, was placed outside Unidentified Clade-I with low support, therefore its phylogenetic position remained unresolved. One possibility could be that the close relatives of this OTU are still missing from the tree. Further sampling efforts need to be applied to add more sequences to the phylogenetic tree to help solve this problem.

The new phylotypes, perhaps even new clades found in our study as well as new clades discovered in previous studies, indicate that the unknown radiolarian diversity is likely enormous (Burki and Keeling 2014). Further efforts encompassing morphological studies as well as environmental surveys combined with molecular methods (e.g. high-throughput sequencing which possesses the nature of deep coverage) in various environments will surely reveal more "hidden" diversity and community structure of this poorly understood group.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Geographic locations of the sampling sites.
Figure S2. Rank abundance curves for a threshold for OTU delineation of 5% *p*-distance and for each near seafloor sampling site.
Figure S3. Rarefaction curves calculated for each of the 13 clone libraries for values for OTU delineation of 5%.
Figure S4. Cluster diagram of Bray–Curtis similarities calculated from square-root-transformed relative OTU abundances for each clone library.
Figure S5. Venn diagram showing the OTUs shared among Upper, Mid, and Deep microbial eukaryote communities as indicated in Fig. 2 and S4.