Progress in Earth and Planetary Science

RESEARCH ARTICLE Open Access

Phylogenetic positions of "pico-sized" radiolarians from middle layer waters of the tropical Pacific

Luyan Li[1] and Kazuyoshi Endo[2*]

Abstract

In order to determine whether truly pico-sized adult radiolarians exist, we compared spumellarian sequences from individual adult samples collected in the central Pacific, with filtered sea water samples of juvenile (5–42 μm) and gamete (0.2–5 μm) sized fractions to see whether the gene sequences are similar or different. Environmental spumellarian-affiliated sequences we sampled were mostly concentrated in samples from 250 to 400 m depth and only appeared in the RAD-III clade, which corresponds to the family Astrosphaeridae (including *Arachnosphaera*, *Astrosphaera*, and *Cladococcus*). None of the same ITS (internal transcribed spacer) sequences were found in both filter membranes of the same sea water samples. Pairwise distances among these environmental spumellarian-affiliated sequences are within or slightly above the range of intra-morphospecific variations. We propose a model to explain our observations based on the hypothesis that the "pico-sized radiolarians" represent gametes of radiolarians of normal size, assuming different sinking speeds of parents and offspring.

Keywords: 18S rDNA, Phylogenetic tree, Genetic distance, Gametes, Pico-sized spumellarians

1 Introduction

1.1 What are radiolarians?

Radiolarians are marine protist zooplankton, belonging to the classes Acantharea, Taxopodia, and Polycystinea (Adl et al. 2019). Extant Polycystinea are further subdivided into the orders Collodaria, Nassellaria, and Spumellaria (Cavalier-Smith et al. 2018; Adl et al. 2019). Extant Entactinaria of De Wever et al. (2001) are not regarded as an established taxon in the currently accepted taxonomy, due to the lack of a connection with true Devonian Entactinaria (Nakamura et al. 2020). Their sizes range from tens to hundreds of micrometers (Suzuki and Not 2015). In general, adult specimens are around 100–300 μm. Life-cycles of radiolarians are largely unknown. Small, flagellated reproductive cells (swarmers) are the most commonly observed stages (Kimoto et al. 2011; Yuasa and Takahashi 2014, 2016).

1.2 Why we focus on the pico-sized range?

DNA sequences identified with radiolarians have been found in the pico-sized fraction of marine environmental libraries sampled at the surface to thousands of meters below (< 2 μm) (Not et al. 2007) and from various geographic locales: Sargasso Sea (< 2 μm) (Not et al. 2007), Arctic Ocean (3–0.22 μm) (Lovejoy et al. 2006), and the Antarctic Ocean (5–0.2 μm) (Lopez-Garcia et al. 2001). Do living pico-sized radiolarian cells really exist, as implied by some published reports? The smallest polycystines confirmed by molecular phylogenetic analysis are reproductive swarmers 1.6–3 μm × 2.5–10 μm long (Kimoto et al. 2011; Yuasa and Takahashi 2014, 2016), small enough that they can just pass through 2–5 μm membrane pores. These sequences may also have come from unknown "new" species that are genuinely "pico-sized" as adults. Focusing on "pico-sized plankton,"

* Correspondence: endo@eps.s.u-tokyo.ac.jp
[2]Department of Earth and Planetary Science, University of Tokyo, 7-3-1 Hongo, Tokyo 113-0033, Japan
Full list of author information is available at the end of the article

considerable percentages of environmental radiolarian sequences have been found in the South China Sea (SCS) and the Atlantic and Pacific Oceans in filtered seawater samples: 15 to 20% at 75 m depth (locality A1) near the basin of SCS, 5 to 10% at 75 m depth (South East Asia Time-series Study station) (Wu et al. 2014b), 12 to 25% in clone libraries from depths of 60 m at five sites along a south–north transect at 155° E (Wu et al. 2014a), and 14 to 15% in full-length 18S rDNA sequence data from the North Atlantic and Pacific Oceans (Lie et al. 2014). In those studies, large plankton were removed by filtration, but plankton filtered at different mesh diameters were not compared.

1.3 What are the environmental radiolarian groups?

By phylogenetic analyses of 18S rRNA gene clone libraries, five environmental radiolarian clades, RAD-I to RAD-V, have been identified in the Sargasso Sea (Not et al. 2007). However, none of them completely matches sequences of individually isolated specimens. We do not know whether these environmental sequences represent "unknown" species. RAD-I and RAD-II belong to the Acantharea. It was suggested by Decelle et al. (2012) that the clade, Acanth II, corresponding to RAD II, is represented by the family Acanthoplegmidae. The taxonomic status of the environmental clade, Acanth I, corresponding to RAD I, remains undetermined. RAD-IV and RAD-V are composed of Taxopodida-like sequences. The Taxopodida are very fragile, with easily dissolved skeletons (Suzuki and Not 2015). A third environmental clade, RAD III, is neighbor to the spumellarian family Spongodiscidae and comprises deep Sargasso Sea sequences and sequences from the South China Sea (Yuan et al. 2004; Li et al. 2011). Among these five clades, RAD III most likely consists of spumellarians with siliceous skeletons, which can be preserved as fossils. In a reclassification study of Spumellaria, 1165 environmental sequences affiliated with the Spumellaria were included in a phylogenetic analysis (Sandin et al. 2020). Most environmental sequences analyzed by Sandin et al. (2020) are related to the superfamily Liosphaeroidea (Matsuzaki et al. 2015), a clade named EnV5 by Sandin et al. (2020). Considering its phylogenetic position relative to others, Env5 in Lineage II corresponds to RAD III, named by Not et al. (2007).

1.4 Why we are interested in spumellarians

Spumellaria and Nassellaria have morphologically complex siliceous architectures, and the fossil record of the former first appeared in the Cambrian (Ma et al. 2019; Zhang and Feng 2019), while the record of the latter dates to the late Devonian (Suzuki and Not 2015; Sandin et al. 2019). These fossil records provide an opportunity to understand how polycystines evolved, corresponding to the secular changes of the paleoenvironment (De Wever et al. 2001).

1.5 Previous efforts to find tiny adult radiolarians and the purpose of our study

Fossil radiolarians have been conventionally studied using 63 μm sieves to concentrate larger specimens. Smaller sieves (45 μm or even 38 μm) are regularly used to collect small species and small specimens (Matul et al. 2002; Itaki et al. 2003). Considerable effort to find tiny adult radiolarians (~ 5–45 μm in diameter) has been expended since the nineteenth century (Haeckel 1887; Frenguelli 1940, 1941; Swanberg and Bjørklund 1987; Takahashi and Honjo 1991); however, they only illustrated juveniles of Spumellaria and Nassellaria. No papers reported pico-sized radiolarians (< 5 μm). Therefore, we set out to determine whether pico-sized radiolarians exist, using a molecular biological approach, by analyzing environmental DNA collected by filtration of seawater.

Radiolarians are indicators of water masses having specific temperatures and salinities (Kamikuri et al. 2008; Suzuki and Not 2015). RAD-III sequences have been reported from warm, oligotrophic blue waters (Not et al. 2007; Li et al. 2011). Thus, we collected samples in the Central Equatorial Pacific in this study. Primers specific only for spumellarians were newly designed in this study. In the same water column, single-cell samples were obtained to compare their sequences with those of environmental DNA samples. We separated samples into three different size classes: adult (> 42 μm), juvenile (5–42 μm), and gamete or pico-sized (0.2–5 μm) samples.

2 Materials and methods

Seawater samples were collected during Cruise KH10-4 of the *R/V Hakuho-maru* operated from September to October 2010. Five stations (A, C, D, E, F) were located in the tropical Pacific (Fig. 1). Seawater was collected using a 10 L Niskin bottle, at 4 or 5 depth strata for each station.

In order to avoid contamination with free DNA from disintegrating organisms, a 42 μm mesh screen was used to remove adults, and subsequent filtrations were performed under low pressure (< 1 kPa). Two different filters were used: 5 μm MF-Millipore SMWP and 0.2 μm Whatman nuclepore PC, allowing us to fractionate the samples into juvenile and pico-sized subsamples, respectively. Total environmental DNA was extracted using a Power Water DNA Kit (MO BIO). Primers specific for spumellarians were newly designed (SITSF 5′CAGCGACGTGTCATTCAAATTTC3′ and SITS R 5′GCAGTCCCAAGCAACACGACTC3′). The 2.5 kb rDNA cistron (18S-ITS1-5.8S-ITS2-28S) regions of environmental DNA were amplified. PCR products were cloned using a Mighty TA cloning kit (TaKaRa). All clones were sequenced with the primers M13 RV and M13 M4.

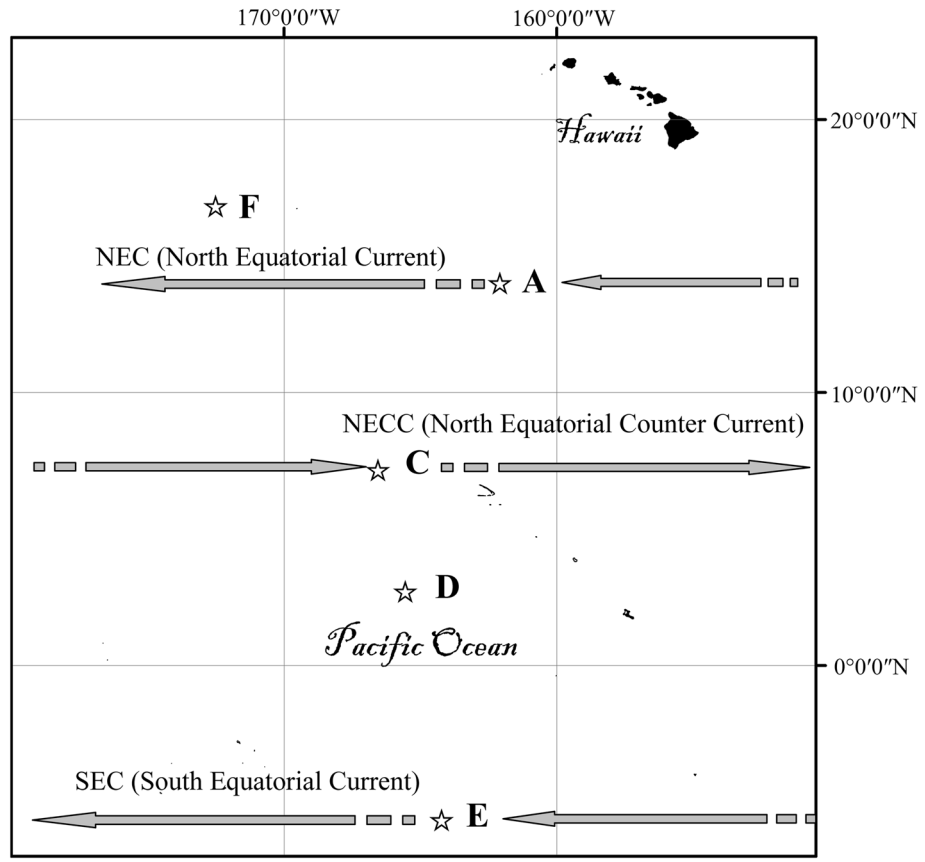

Fig. 1 Location of the 5 sampling sites in the Central Pacific Ocean

Preliminary taxonomic affiliations of the sequences were assessed using BLASTN against GenBank database. Phylogenetic relationships of the 18S rDNA part of sequences were inferred by ML analysis with IQ-Tree 2 (Minh et al. 2020) and Bayesian analysis with MrBayes Version 3.2.7 (Huelsenbeck and Ronquist 2001; Zhang et al. 2020). Genetic distances between 18S-ITS1-5.8S-ITS2-28S sequences were calculated with the distance module of Mega X (Kumar et al. 2018). Sequences provided in this paper have been submitted to the NCBI nucleotide sequence database under accession numbers: KP175029-KP175040.

3 Results and discussion

Results of our search for "pico-sized" spumellarians are summarized in Table 1. The tree in Fig. 2 shows phylogenetic positions of DNA sequences isolated from single-cell samples (Fig. 3) and membrane-filtered seawater samples. Figure 4 illustrates more detailed relationships between the DNA sequences from the membrane-filtered seawater samples and those available in public databases.

We found environmental spumellarian-affiliated sequences in the filter membranes at only two sites: a subsample in the size range between 42 and 5 μm (juvenile size) from 400 m at site C, and subsamples of both juvenile and gamete sizes from 250 m depth at site D (Table 1).

The 18S rDNA phylogenetic tree is shown in Fig. 2. Environmental spumellarian-affiliated sequences from Pacific seawater samples only appeared in the RAD-III clade named by Not et al. (2007) for environmental sequences from size-fractionated samples (< 2 μm) from the Sargasso Sea. Our spumellarian-specific primers yielded positive results from spumellarians collected at site D, but those species do not belong to RAD III: *Heliodiscus asteriscus* D397, *Spongotrochus?* aff. *Glacialis* D188, Pylonioidea gen. et sp. indet. D401 and D382 (Figs. 2 and 3), in contrast to PCR products amplified from total environmental DNA, which yielded sequences that belong only to RAD III.

Using more RAD III sequences stored in public databases to cover a broader range of different localities, a phylogenetic tree was constructed by focusing on RAD III (Fig. 4). RAD III sequences can be clearly divided into several subclades, three of which are represented by a named taxon (*Ar. myriacantha*-like clade, *As. hexagonalis*-like clade, and *C. viminalis*-like clade). The fourth

Table 1 Results of molecular cloning for the environmental DNA from five sites

Site	Depth (m)	CTD information	Volume of filtered seawater (L)	DNA (ng/μl)		PCR	Clone number	Spumellarian clones
A	1000		5	A	2.6	−		
			5	B	183.4	−		
A	400		5	A	47.0	−		
			5	B	56.5	−		
A	120	Chlorophyll max	5	A	77.9	+	0	
			5	B	48.3	−		
C	750		5	A	94.0	−		
			5	B	197.0	−		
C	400		5	A	113.8	+	5	3
			5	B	96.1	−		
C	150		5	A	79.7	+	0	
			5	B	57.0	−		
C	100	Chlorophyll max	5	A	64.3	+	0	
			5	B	100.8	−		
D	400		5	A	50.8	−		
			5	B	22	−		
D	250		5	A	58.6	+	11	2
			5	B	15.8	+	18	1
D	120		5	A	78.4	+	0	
			5	B	23.7	−		
D	90	Chlorophyll max	5	A	39.9	+	7	0
			5	B	33	+	8	0
E	400		5	A	74.0	−		
			5	B	31.5	−		
E	150		5	A	62.8	−		
			5	B	18.0	−		
E	105		5	A	47.0	+	0	
			5	B	30.4	−		
E	70	Chlorophyll max	5	A	53.1	−		
			5	B	39.5	−		
F	400		5	A	5.6	−		
			5	B	15.6	−		
F	200		5	A	89.1	−		
			5	B	60.6	−		
F	130	Chlorophyll max	5	A	93.9	−		
			5	B	23.4	−		

Site A: Sep 20, 2010, 10:30 PM, N 14′ 00″, W 162′ 05″, depth 5662 m; site C: Sep 22, 2010, 10:30 PM, N 07′ 09″, W 166′ 33″, depth 4933 m; site D: Sep 23, 2010, 9:00 PM, N 2′ 42″, W 165′ 33″, Depth 5346 m; site E: Sep 29, 2010, 8:14 PM, N 05′ 40″, W 164′ 13″, Depth 4874 m; site F: Oct 9, 2010, 9:00 PM, N 16′ 492″, W 172′ 30″; Depth 5299 m

CTD is an oceanography instrument used to determine the conductivity, temperature, and depth of the ocean; A and B represent the following fractions: 42 μm > A > 5 μm, 5 μm > B > 0.2 μm. The value "+" in PCR column means a positive result with spumellarian-specific primers. Clone column means the number of M13-primer, PCR-positive white colonies. The value in spumellarian clones means the number of spumellarian clones that were confirmed by sequencing

major clade includes no identified species (denoted here as clade A). The phylogenetic position of *Cladococcus viminalis* within the *C. viminalis*-like clade has not been well resolved, showing a polychotomous relationship together with a cluster of sequences from 100 m depths of SCS (Fig. 4). The deep-sea sequences seem to be clustered mainly in clade A and in the *C. viminalis*-like clade. Five of our environmental sequences (C400A3,

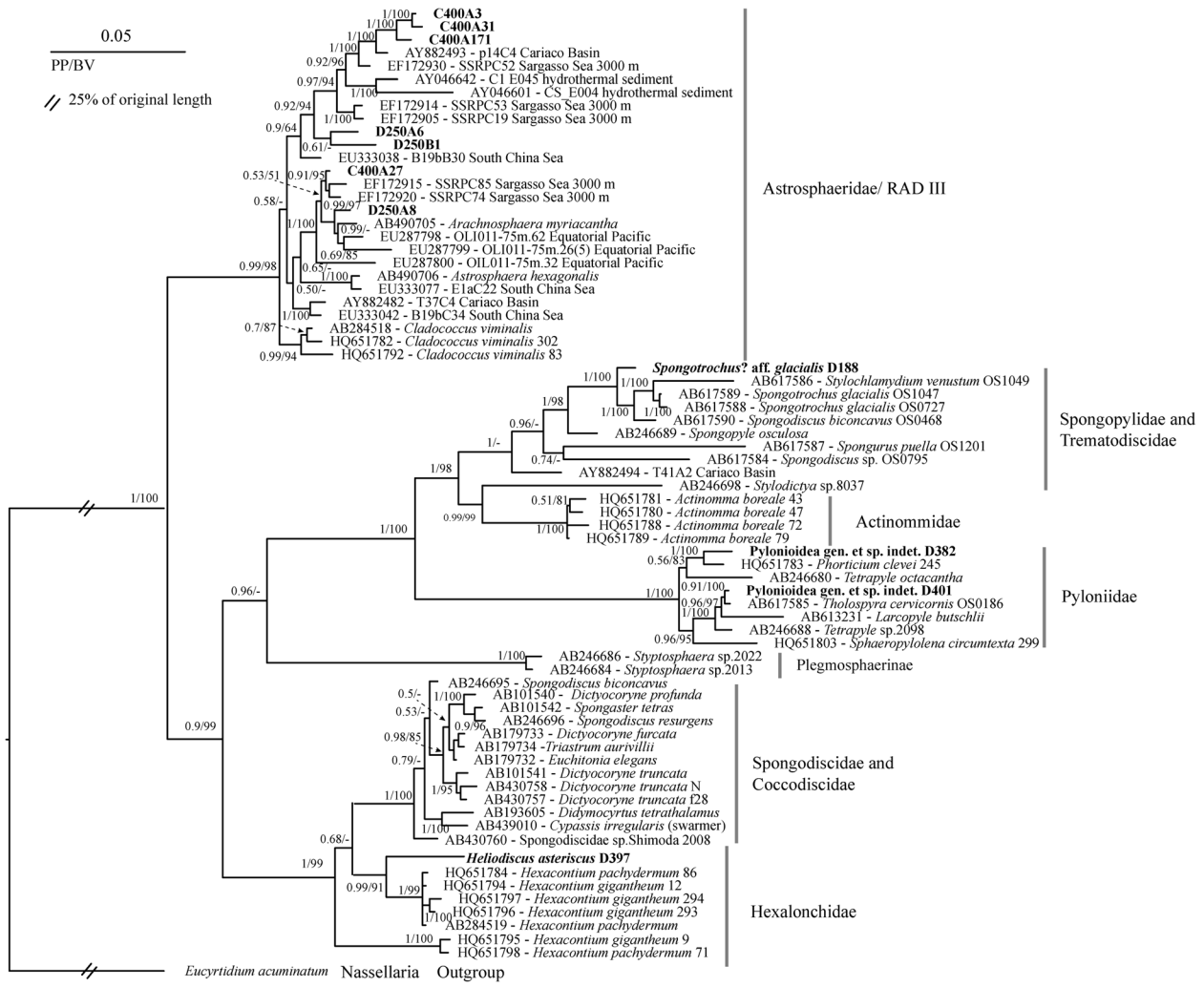

Fig. 2 Phylogenetic tree of spumellarians based on 18S rDNA sequences. Sequences obtained from the Central Pacific Ocean in this study are in bold. Numbers at nodes represent posterior probabilities (PP) of Bayesian inferences, bootstrap values (BV) of the ML analysis. The scale bar in the upper left corner is the substitution rate per site for the Bayesian analyses. Sequences obtained in this study are in bold

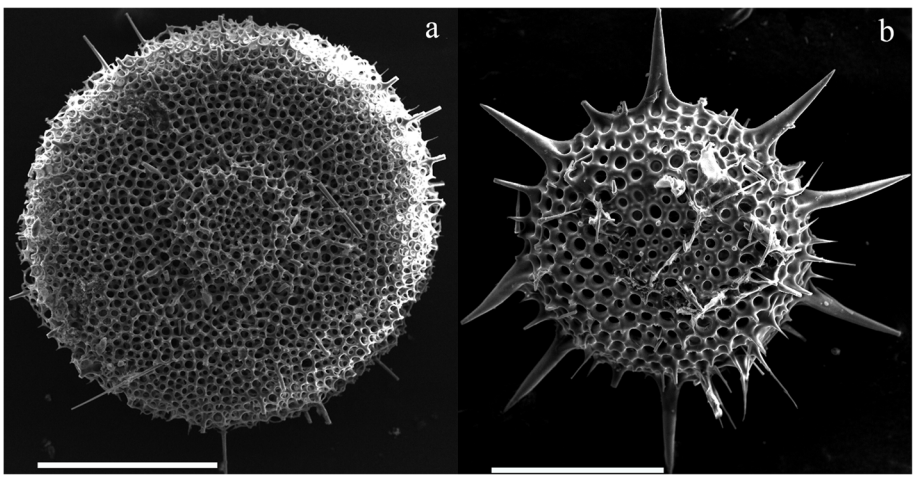

Fig. 3 Scanning electron microscope photos of **a** D188 and **b** D397. The scale bar in the bottom of photo represents 100 μm

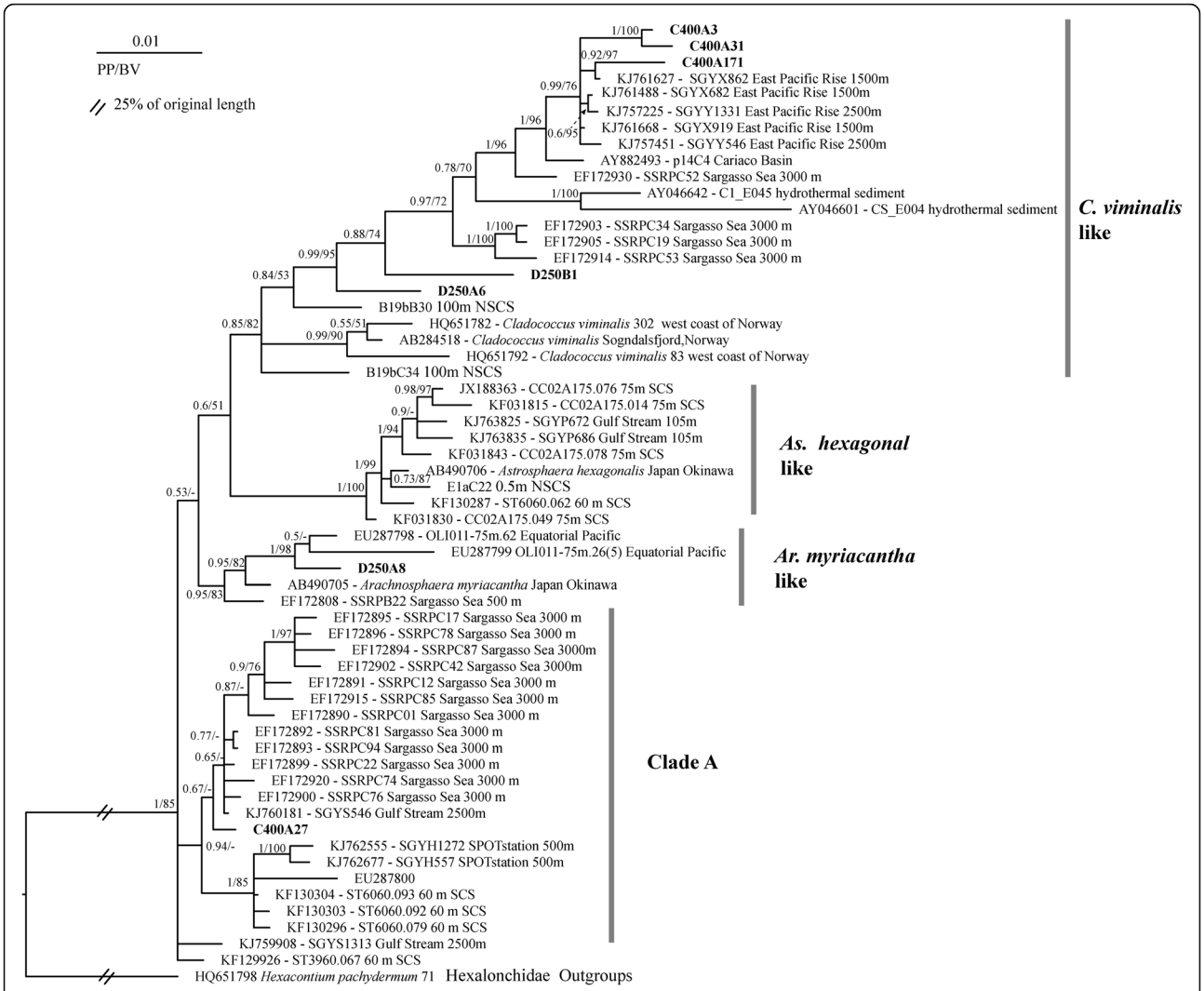

Fig. 4 A phylogenetic tree of RAD III based on 18S rDNA sequences. Sequences obtained from the Central Pacific Ocean in this study are in bold. Numbers at the nodes represent posterior probabilities (PP) of the Bayesian inferences, bootstrap values (BV) of the ML analysis. The scale bar in the upper left corner is the substitution rate per site for the Bayesian analyses. Sequences obtained in this study are shown in bold

C400A31, C400A171, D250A6, and D250B1) clustered in the *C. viminalis*-like clade and two (C400A27 and D250A8) somewhat separately in clade A and the *Ar. myriacantha*-like clade (Fig. 4).

We did not find the same ITS (internal transcribed spacer) sequences in juveniles and pico-sized subsamples. The largest of the 18S-ITS1-5.8S-ITS2-28S distances in those environmental spumellarian-affiliated sequences is in the range of 0.070 (between D250A6 and C400A31), no different than the interspecific distance observed within a family (Ando et al. 2009; Krabberød et al. 2011). The lowest is 0.008, between C400A3 and C400A31, which might have come from two different "juvenile"-filtered subsamples smaller than 42 μm. The distance between D250B1 and D250A6 is 0.062, being close to the distance between D250B1 and D250A8 (0.064). This value is close to interspecific distances among spumellarians isolated in site D in this study: 0.083, 0.084, and 0.010 in Hexalonchidae; 0.096 in Pyloniidae.

No identical rDNA sequences were found in both filter membranes used for the same sea water samples. Radiolarian skeletons are quite fragile; however, for three reasons, we think it is unlikely that the pico-sized sequences could have come from broken cells. First, a couple of previous studies (Diez et al. 2001; Not et al. 2007) indicated that the sequences of known multicellular or larger single-celled organisms constitute very small percentages of the pico-sized environmental samples (2.5% and 1.7%, respectively). Second, astrosphaerid cells are not as fragile as their skeletons. Radiolarian DNA sequences we obtained from the membranes are mainly

concentrated in the RAD-III clade of Not et al. (2007) or Lineage II of Sandin et al. (2020). Thus, those sequences could have originated from broken cells of astrosphaerids. However, the central capsule of astrosphaerids is so large (80–250 μm in diameter), robust, and rigid that it cannot be broken easily (Hollande and Enjumet 1960; Suzuki and Sugiyama 2001). Besides, astrosphaerids are generally tangled with organic matter to form aggregates or "meat balls" up to 1 cm in diameter when collected (Zhang et al. 2018), so the material inside the capsular membrane basically cannot trickle out. Therefore, we consider it unlikely that the sequences we obtained are contaminants from broken adult astrosphaerid cells. Third, we carefully treated the samples to avoid damaging the cells, eliminating the possibility, as far as possible, that DNA could be released from radiolarian cells broken during filtering. As a consequence of doing filtration at low pressure to prevent crushing the radiolarians, the amount of sample we collected was very limited. That might be the reason why we obtained only several clones from the filtered samples, making it difficult to fully assess the genetic diversity of sequences in the RAD-III clade, which now embraces a considerable number of environmental sequences stored in public databases (Fig. 4).

Pairwise distances among these environmental spumellarian-affiliated sequences (0.008–0.060) are within or slightly above the range of intra-morphospecific variation. For example, *Dictyocoryne truncata* (Ando et al. 2009; Krabberød et al. 2011) and *Actinomma boreale* (Krabberød et al. 2011) have been sequenced to estimate intraspecific distances (0.017 and 0.005, respectively). These juvenile sequences from site C may have originated from different individuals of an unidentified small (5–42 μm) spumellarian species.

Another possible origin of pico-sized radiolarian sequences could be the pico-sized swarmers or gametes of radiolarians. But the ITS region sequences of rDNA sequences we found in juveniles and pico-sized subsamples are different. One possibility for this difference between juvenile and pico-sized subsample sequences is that spumellarians have intragenomic ITS sequence variation. Intraspecific variations in the ITS region of radiolarians were analyzed and quantified by Ando et al. (2009), Krabberød et al. (2011), and Ishitani and Takishita (2015), but no intra-individual differences have been studied in radiolarians. ITS sequences evolve faster than 18S or 28S rRNA gene sequences and are most commonly used as markers to discriminate species. Multigenic family members of ITS regions are subject to concerted evolution, which homogenizes their sequences (Dover 1982). However, exceptions have been observed, and intragenomic polymorphism of ITS has been detected in many species (Wang et al. 2017; Matthias et al. 2018; Itskovich 2020), including even the Foraminifera (Pillet et al. 2012), which, together with the Radiolaria, belongs to the Retaria. If spumellarians produce gametes showing intragenomic polymorphism, the ITS sequences from each reproductive cell and juvenile could differ. This would even apply to ITS sequences of the same 18S rDNA sequences, but in radiolarians, such intracellular variability is very limited (Decelle et al. 2014). In contrast, even the 18S rDNA sequences in our samples differ between juvenile and pico-sized subsamples. Thus, it appears likely that the different ITS sequences represent different species. Since no molecular data are available for any of the 23 known species belonging to the Astrosphaeridae, these different 18S rDNA sequences from membrane samples could conceivably be pico-sized swarmers or gametes of different astrosphaerid species.

The observation that no identical sequences were found among adult, juvenile, and pico-sized subsamples can be explained by the "gametes hypothesis," assuming different sinking speeds of parents and offspring (Fig. 4). According to the results of our phylogenetic analysis (Figs. 2 and 3), only three identified species fell into the RAD III clade. They are *Astrosphaera hexagonalis*, *Arachnosphaera myriacantha*, and *Cladococcus viminalis*, in the size range 80–150 μm. They were recorded by Haeckel in the nineteenth century from warm surface waters (Boltovskoy et al. 2010). Living astrosphaerids have been collected even at depths of 500–1000 m from February to May in the Mediterranean (Hollande and Enjumet 1960). Obviously, they are not pico-sized radiolarians, but they could be the mother cells of "pico-sized radiolarians." Due to differences in size and structure, the forces that mother cells and swarmer cells experience in seawater would be different. Radiolarians float passively in convection currents of the mixed layer by hydrodynamic drag, due to their elongated pseudopodia (Ichinohe et al. 2019). Swarmers with celestine crystals inside quickly sink (Anderson 1983; Yuasa and Takahashi 2014).

In general, plankton exhibit three kinds of movement in the ocean that need to be considered to infer the whereabouts of radiolarians: (1) movement driven by the daily migration of nektons between 0 and 500 m, which swim to deeper waters in the daytime, presumably to seek refuge from large predators, and return to the surface at night to feed (Bianchi et al. 2013). Mother cells would be affected by this kind of process, but skeletons of dead cells would not. Swarmers that do not have enough buoyancy would not be able to return to the sea surface. We collected all our samples at night. Thus, mother cells should have been near the surface. (2) Vertical movement of water masses with cold or warm eddies that occur everywhere at bi-weekly to seasonal scales. This is a passive, but very powerful movement.

Eddy diffusivities for the east Pacific sector range from −0.2 to 0.1 m s^{-1} (Klocker and Abernathey 2014). Mother cells, skeletons, and swarmers of radiolarians would be equally subject to this kind of movement. This might be the reason why astrosphaerids can be collected at water depths from 500 to 1000 m. (3) Pacific circulation, which forms vertical profiles of equatorial zonal velocity, with the peak appearing at 100 to 200 m above 0.50 m s^{-1} at a longitude of 170° W (Johnson et al. 2001). Mother cells in the surface water (0–500 m) would be greatly affected by this circulation, while it would have no effect on swarmers and skeletons that have sunk to more than 500 m. Some observations in an experimental study (Yang et al. 2020) concluded that cyclonic eddies enhance the looping path, but anticyclonic eddies decrease it. If cyclonic eddies occur in a sampling site, the separation of mother cells and swarmers would increase. Combining the effects of those forces, Fig. 5 shows situations that would occur under our "gametes hypothesis." When the research vessel stopped at site A2 and sampled swarmers at B2, the skeletons of the dead mother cells would have arrived at point B1. At this time, immature cells in this population would have floated along with the current and have arrived at site A3. Therefore, in the sample at point B2 of site A2, it would be difficult to find gametes together with adults or juveniles of the same species. This model would also explain high percentages (ca. 20%) of radiolarian DNA recorded in some deep water (500–3000 m) environmental samples (Not et al. 2007) (B3 in Fig. 5).

Still another possibility, especially for those specimens that did not cluster with known shallow water species (such as C400A27), is that the sequences we found in the pico-sized samples indeed represent pico-sized adult radiolarians.

A peculiarity of RAD III is that the three identified species in it belong to the family Astrosphaeridae, the only family among extant spumellarians with no structure inside the cortical shell (De Wever et al. 2001; Sandin et al. 2020). Other groups of Spumellaria have various internal structures. Further studies on unidentified astrosphaerid species may help to reveal evolutionary relationships among radiolarians with and without internal structures and to understand how the initial spicular system developed and evolved. The existence of so many potential astrosphaerid environmental sequences indicates that a number of undescribed astrosphaerid species await morphological studies. It would be ideal to observe directly the morphogenesis of siliceous skeletons in those radiolarians by capturing the mother cells of RAD III in surface waters of deep-sea sites with rich environmental sequences.

Our results (Table 1) indicate that juvenile (5–42 μm)-, and gamete (0.2–5 μm)-sized spumellarians are

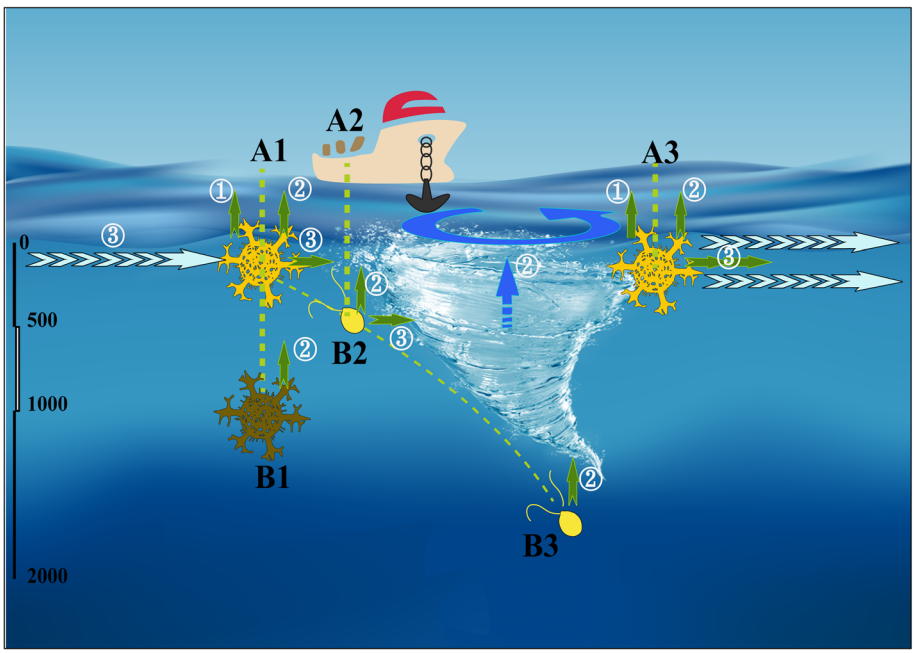

Fig. 5 Diagram showing a model based on the hypothesis that "pico-sized radiolarians" represent radiolarian gametes. Three kinds of movement in the ocean should be considered: ① the daily migration of nektons between 0 and 500 m; ② vertical movement of water masses with cyclonic eddies; ③ pacific circulation. We show a case in which cyclonic eddies occurred at a sampling site (night in the Northern Hemisphere), with cyclonic eddies enhancing the looping path, and increasing the distance between mother cells and swarmers

not restricted to particular water depths. Still, some discernible patterns exist. They are often found 100 to 300 m below the highest chlorophyll zone. Although the PCR results for samples with the highest chlorophyll peak were positive, cloning and sequencing showed that they were not spumellarian sequences but false positives caused by mismatches to other plankton sequences. The greatest depth of chlorophyll at site D was shallower than that at site C, and the spumellarian sequences also appeared at a shallower depth. But the two phenomena are not parallel. Swarmers continually sink in the sea, so they do not concentrate at a certain depth. For the three reasons discussed above, juvenile cells can also migrate up or down. The deep sea below 1000 m is a blank that we have not explored. The presence of RAD III in the deep sea can be seen from clade A (Fig. 4), or from records in the Sargasso Sea (Not et al. 2007). Their distributions and morphologies are also worthy of further study.

One possible way to clarify the origin of "pico-sized" sequences would be to do hybridization in situ. Studies have been done (Gilg et al. 2010) by hybridization to see whether they can catch unidentified acantharian cells. Samples collected at 500 m successfully hybridized to the UC1 (corresponding to RAD I) CARD-FISH probe. But the difficulty of studying living radiolarians is that until now, no lab has been able to culture radiolarians from swarmers to next generation adults. Thus, whether or how their genomes change during reproduction, when their skeletons first form, is very hard to know. Combinations of whole-cell in situ hybridization and flow cytometry may be the solution. For in situ hybridization, fluorescent oligonucleotides derived from the 18S rRNA sequences may be used as probes. For bacteria, a combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry has been employed by Fuchs et al. (1998) and Fernández-Lago et al. (2000). Therefore, we think it is theoretically practical to carry out similar assays with plankton. Our sequences offer some help to design the probes. The combination of these technologies will not only allow us to solve the problem of "pico-sized" radiolarians, but also to recover all "the fish that miss the net" (Biard et al. 2016). Even though use of fine sieves will help collect more living and fossil radiolarians, true radiolarian diversity would still be underestimated.

4 Conclusions

Considering the phylogenetic positions of the environmental sequences, as well as the genetic distances to known species, the most plausible explanation for "pico-sized radiolarians" is that they represent gametes of radiolarians of a normal size. The existence of a large number of environmental sequences in warm deep-sea water indicates that our understanding of life cycles and morphological diversity of Spumellaria is inadequate. Most of these unknown sequences can be attributed to the family Astrosphaeridae, which is of great significance for understanding evolution of radiolarian skeletons.

Abbreviations

18S rDNA: 18 subunit ribosomal DNA; DNA: Deoxyribonucleic acid; ITS: Internal transcribed spacer; RAD: Radiolarian clade

Acknowledgements

We are grateful to the captain, crew, and scientists of the cruise KH10-4 of *R/V Hakuno-Maru* for their assistance in plankton and seawater sampling. We thank Drs. Yoshiyuki Ishitani and Yurika Ujiie for their comments on the manuscript and their support in collecting samples. In addition, we also thank Dr. Ishitani and the Kochi Core Center (Kochi University/JAMSTEC) for taking SEM photos.

Authors' contributions

LL initiated this study and was primarily responsible for the sequence analysis. KE hosted LL, and they designed the experiments together. Both authors contributed to writing the paper. The authors read and approved the final manuscript.

Funding

This study was funded by the Japanese Society for the Promotion of Science (JSPS) 11P11741 and was carried out at the University of Tokyo.

Availability of data and materials

Datasets supporting this article are included within it. Sequences discussed in this paper can be found in Genbank (accession numbers: KP175029-KP175040).

Competing interests

The authors declare that they have no competing interest.

Author details

[1]State Key Laboratory of Palaeobiology and Stratigraphy (SKLPS), Nanjing Institute of Geology and Palaeontology, Chinese Academy of Sciences (CAS), Nanjing 210008, China. [2]Department of Earth and Planetary Science, University of Tokyo, 7-3-1 Hongo, Tokyo 113-0033, Japan.

Received: 12 August 2020 Accepted: 28 October 2020
Published online: 16 November 2020

References

Adl S, Bass D, Lane C, Lukeš J, Schoch C, Alexey S, Agatha S, Berney C, Brown M, Burki F, Cárdenas P, Cepicka I, Chistyakova L, Del Campo J, Dunthorn M, Edvardsen B, Eglit Y, Guillou L, Hampl V, Zhang Q (2019) Revisions to the classification, nomenclature, and diversity of eukaryotes. J Eukaryot Microbiol 66:4–119

Anderson OR (1983) Radiolaria. Springer, New York

Ando H, Kunitomo Y, Sarashina I, Iijima M, Endo K, Sashida K (2009) Intraspecific variations in the ITS region of recent radiolarians. Earth Evol Sci 3:37–44

Bianchi D, Galbraith ED, Carozza DA, Mislan KS, Stock CA (2013) Intensification of open-ocean oxygen depletion by vertically migrating animals. Nat Geosci 6(7):545–548

Biard T, Stemmann L, Picheral M, Mayot N, Vandromme P, Hauss H, Gorsky G, Guidi L, Kiko R, Not F (2016) In situ imaging reveals the biomass of giant protists in the global ocean. Nature 532(7600):504–507

Boltovskoy D, Kling SA, Takahashi K, Bjørklund K (2010) World atlas of distribution of recent polycystina (Radiolaria). Palaeontol Electron 13:18A:1–230.

Cavalier-Smith T, Chao EE, Lewis R (2018) Multigene phylogeny and cell evolution of chromist infrakingdom Rhizaria: contrasting cell organisation of sister phyla Cercozoa and Retaria. Protoplasma 255(5):1517–1574

De Wever P, Dumitrica P, Caulet JP, Nigrini C, Caridroit M (2001) Radiolarians in the sedimentary record. Gordon and Breach Science Publisher, Singapore

Decelle J, Romac S, Sasaki E, Not F, Mahé F (2014) Intracellular diversity of the V4 and V9 regions of the 18S rRNA in marine protists (radiolarians) assessed by high-throughput sequencing. PLoS One 9(8):e104297

Decelle J, Suzuki N, Mahe F, De Vargas C, Not F (2012) Molecular phylogeny and morphological evolution of the Acantharia (Radiolaria). Protist 163(3):435–450
Diez B, Pedros-Alio C, Massana R (2001) Study of genetic diversity of eukaryotic picoplankton in different oceanic regions by small-subunit rRNA gene cloning and sequencing. Appl Environ Microbiol 67(7):2932–2941
Dover G (1982) Molecular drive: a cohesive mode of species evolution. Nature 299(5879):111–117
Fernández-Lago L, Vallejo FJ, Trujillano I, Vizcaíno N (2000) Fluorescent whole-cell hybridization with 16S rRNA-targeted oligonucleotide probes to identify *Brucella* spp. by flow cytometry. J Clin Microbiol 38(7):2768–2771
Frenguelli J (1940) Consideraciones sobre los Silicoflagelados fósiles. Revista del Museo de La Plata, Nueva Serie, Sección Paleontología 27:37–112
Frenguelli J (1941) Silicoflagelados y radiolarios dl trípoli del Valle de Til-Til (Chile). Instituto del Museo de la Universidad nacional de la Plata, Notas del Museo de La Plata. Palaeontologica 62:93–100
Fuchs BM, Wallner G, Beisker W, Schwippl I, Ludwig W, Amann R (1998) Flow cytometric analysis of the in situ accessibility of *Escherichia coli* 16S rRNA for fluorescently labeled oligonucleotide probes. Appl Environ Microbiol 64(12): 4973–4982
Gilg IC, Amaral-Zettler LA, Countway PD, Moorthi S, Schnetzer A, Caron DA (2010) Phylogenetic affiliations of mesopelagic acantharia and acantharian-like environmental 18S rRNA genes off the southern California coast. Protist 161(2):197–211
Haeckel E (1887) Report on the Radiolaria collected by HMS Challenger during the years 1873-1876. Rep Voyage HMS Challenger Zool 18:1–1803
Hollande A, Enjumet M (1960) Cytologie, évolution et systématique des Sphaeroïdés (Radiolaires)Archives du Muséum National d'Histoire Naturelle, vol 7, pp 1–134 http://bibliotheques.mnhn.fr/EXPLOITATION/infodoc/ged/viewportalpublished.ashx?eid=IFD_FICJOINT_ARCHI_S007_1960_T007_N000_1
Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17:754–755
Ichinohe R, Shiino Y, Kurihara T, Kishimoto N (2019) Active floating with buoyancy of pseudopodia versus passive floating by hydrodynamic drag force: a case study of the flat-shaped spumellarian radiolarian dictyocoryne. Paleontol Res 23:236–244
Ishitani Y, Takishita K (2015) Molecular evidence for wide vertical distribution of the marine planktonic protist Larcopyle buetschlii (Radiolaria) in a semi-enclosed marginal sea. J Plankton Res 37(5):851–856
Itaki T, Ito M, Narita H, Ahagon N, Sakai H (2003) Depth distribution of radiolarians from the Chukchi and Beaufort Seas, western Arctic. Deep Sea Res Part I Oceanogr Res Pap 50(12):1507–1522
Itskovich V (2020) Intragenomic variation of rDNA internal transcribed spacers in the endemic Baikal sponge Lubomirskia baikalensis (Pallas, 1776) (Spongillida, Lubomirskiidae): implications for Porifera barcoding. J Great Lakes Res 46(1): 62–66
Johnson GC, Mcphaden MJ, Firing E (2001) Equatorial Pacific Ocean horizontal velocity, divergence, and upwelling*. J Phys Oceanogr 31(3):839–849
Kamikuri S-I, Motoyama I, Nishimura A (2008) Radiolarian assemblages in surface sediments along longitude 175°E in the Pacific Ocean. Mar Micropaleontol 69(2):151–172
Kimoto K, Yuasa T, Takahashi O (2011) Molecular identification of reproductive cells released from *Cypassis irregularis* Nigrini (Radiolaria). Environ Microbiol Rep 3(1):86–90
Klocker A, Abernathey R (2014) Global patterns of mesoscale eddy properties and diffusivities. J Phys Oceanogr 44(3):1030–1046
Krabberød AK, Bråte J, Dolven JK, Ose RF, Klaveness D, Kristensen T, Bjørklund KR, Shalchian-Tabrizi K (2011) Radiolaria divided into Polycystina and Spasmaria in combined 18S and 28S rDNA phylogeny. PLoS One 6(8):e23526
Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35(6):1547–1549
Li L-Y, Lin D, Chen J-H, Wu S-H, Huang Q-J, Zhou H, Qu L-H, Chen Y-Q (2011) Diversity and distribution of planktonic protists in the northern South China Sea. J Plankton Res 33(3):445–456
Lie A, Liu Z, Hu S, Jones A, Kim D, Countway P, Amaral-Zettler L, Cary S, Sherr E, Sherr B, Gast R, Caron D (2014) Investigating microbial eukaryotic diversity from a global census: insights from a comparison of pyrotag and full-length sequences of 18S rRNA genes. Appl Environ Microbiol 80:4363–4373
Lopez-Garcia P, Rodriguez-Valera F, Pedros-Alio C, Moreira D (2001) Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. Nature 409(6820):603–607
Lovejoy C, Massana R, Pedros-Alio C (2006) Diversity and distribution of marine microbial eukaryotes in the Arctic Ocean and adjacent seas. Appl Environ Microbiol 72(5):3085–3095
Ma Q, Feng Q, Cao W, Zhang L, Ye Y, Gu S (2019) Radiolarian fauna from the Chiungchussuan Shuijingtuo Formation (Cambrian Series 2) in Western Hubei Province, South China. Sci China Earth Sci 62(10):1645–1658
Matsuzaki KM, Suzuki N, Nishi H (2015) Middle to upper Pleistocene polycystine radiolarians from hole 902-C9001C, Northwestern Pacific. Paleontol Res 19(s1):1–77
Matthias S, Eniko H, Pfliegler WP (2018) Birth-and-death evolution and reticulation of ITS segments of Metschnikowia andauensis and Metschnikowia fructicola rDNA repeats. Front Microbiol 9:1193
Matul A, Abelmann A, Tiedemann R, Kaiser A, Nürnberg D (2002) Late quaternary polycystine radiolarian datum events in the Sea of Okhotsk. Geo-Mar Lett 22(1):25–32
Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, Von Haeseler A, Lanfear R (2020) IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. Mol Biol Evol 37(5):1530–1534
Nakamura Y, Sandin MM, Suzuki N, Tuji A, Not F (2020) Phylogenetic revision of the order entactinaria—Paleozoic relict radiolaria (Rhizaria, SAR). Protist 171(1):125712
Not F, Gausling R, Azam F, Heidelberg JF, Worden AZ (2007) Vertical distribution of picoeukaryotic diversity in the Sargasso Sea. Environ Microbiol 9(5):1233–1252
Pillet L, Fontaine D, Pawlowski J (2012) Intra-genomic ribosomal RNA polymorphism and morphological variation in *Elphidium macellum* suggests inter-specific hybridization in foraminifera. PLoS One 7(2):e32373
Sandin MM, Biard T, Romac S, O'dogherty L, Suzuki N, Not F (2020) Morpho-molecular diversity and evolutionary analyses suggest hidden life styles in Spumellaria (Radiolaria). bioRxiv. https://doi.org/10.1101/2020.06.29.176917
Sandin MM, Pillet L, Biard T, Poirier C, Bigeard E, Romac S, Suzuki N, Not F (2019) Time calibrated morpho-molecular classification of Nassellaria (Radiolaria). Protist 170(2):187–208
Suzuki N, Not F (2015) Biology and ecology of Radiolaria. In: Ohtsuka S, Suzaki T, Horiguchi T, Suzuki N, Not F (eds) Marine protists: diversity and dynamics. Springer, Tokyo, pp 179–222
Suzuki N, Sugiyama K (2001) Regular axopodial activity of Diplosphaera hexagonalis Haeckel (spheroidal spumellarian, Radiolaria). Paleontol Res 5(2):131–140
Swanberg NR, Bjørklund KR (1987) The pre-cephalic development of the skeleton of Amphimelissa setosa (Actinopoda: Nassellarida). Mar Micropaleontol 11(4):333–341
Takahashi K and Honjo S (1991) Radiolaria: flux, ecology, and taxonomy in the Pacific and Atlantic. Ocean Biocoenosis Series 3
Wang X, Chen X, Yang P, Wang L, Han J (2017) Barcoding the *Dendrobium* (Orchidaceae) species and analysis of the intragenomic variation based on the internal transcribed spacer 2. BioMed Res Int 1:1–10
Wu W, Huang B, Liao Y, Sun P (2014a) Picoeukaryotic diversity and distribution in the subtropical-tropical South China Sea. FEMS Microbiol Ecol 89:563–579
Wu W, Huang B, Zhong C (2014b) Photosynthetic picoeukaryote assemblages in the South China Sea from the Pearl River Estuary to the SEATS station. Aquat Microb Ecol 71:271–284
Yang Q, Liu H, Lin P (2020) The effect of oceanic mesoscale eddies on the looping path of the Kuroshio intrusion in the Luzon Strait. Sci Rep 10(1):636
Yuan J, Chen MY, Shao P, Zhou H, Chen YQ, Qu LH (2004) Genetic diversity of small eukaryotes from the coastal waters of Nansha Islands in China. FEMS Microbiol Lett 240(2):163–170
Yuasa T, Takahashi O (2014) Ultrastructural morphology of the reproductive swarmers of Sphaerozoum punctatum (Huxley) from the East China Sea. Eur J Protistol 50:194–204
Yuasa T, Takahashi O (2016) Light and electron microscopic observations of the reproductive swarmer cells of nassellarian and spumellarian polycystines (Radiolaria). Eur J Protistol 54:19–32
Zhang C, Huelsenbeck J, Ronquist F (2020) Using parsimony-guided tree proposals to accelerate convergence in Bayesian phylogenetic inference. Syst Biol. https://doi.org/10.1093/sysbio/syaa002
Zhang K, Feng Q-L (2019) Early Cambrian radiolarians and sponge spicules from the Niujiaohe Formation in South China. Palaeoworld 28(3):234–242
Zhang L, Suzuki N, Nakamura Y, Tuji A (2018) Modern shallow water radiolarians with photosynthetic microbiota in the western North Pacific. Mar Micropaleontol 139:1–27

Publisher's Note