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BRIEF REPORT

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DNA metabarcoding focused on difficult-to-culture protists: An effective approach to clarify biological interactions

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Abstract

DNA metabarcoding on a single organism is a promising approach to clarify the biological interactions (e.g., predator–prey relationships and symbiosis, including parasitism) of difficult-to-culture protists. To evaluate the effectiveness of this method, Radiolaria and Phaeodaria, which are ecologically important protistan groups, were chosen as target taxa. DNA metabarcoding on a single organism focused on the V9 region of the 18S rRNA gene revealed potential symbionts, parasites and food sources of Radiolaria and Phaeodaria. Previously reported hosts and symbionts (parasites) were detected, and newly recognized combinations were also identified. The contained organisms largely differed between Radiolaria and Phaeodaria. In Radiolaria, members of the same order tended to contain similar organisms, and the taxonomic composition of possible symbionts, parasites, and food sources was fixed at the species level. Members of the same phaeodarian family, however, did not contain similar organisms, and body part (i.e., the central capsule or the phaeodium) was the most important factor that divided the taxonomic composition of detected organisms, implying that the selection of appropriate body part is important when trying to ascertain contained organisms, even for unicellular zooplankton. Our results show that DNA metabarcoding on a single organism is effective in revealing the biological interactions of difficult-to-culture protists.

INTRODUCTION

The biological interactions (e.g., competition, predator–prey relationships and symbiosis, including parasitism) of protists have been widely studied, mainly focusing on 'culturable' species in the domain of microbiology or protistology. However, many protists in natural environments cannot be successfully cultured under artificial conditions, and these 'difficult-to-culture' protists are reported to play important roles in natural environments (Biard et al., 2016; Ikenoue et al., 2019; Sogawa et al., 2022).

DNA metabarcoding is an effective approach to clarify biological interactions of aquatic organisms, and the taxonomic composition (species diversity) of environmental samples can be thoroughly clarified by using this technique. For example, DNA metabarcoding has been used to clarify the food sources of crustaceans (Cleary et al., 2012, 2015). However, because multicellular organisms contain numerous cells, a blocking polymerase chain reaction (PCR) with Peptide Nucleic Acid (PNA) must also be performed to reduce the detection of host's DNA (Nakamura, Tuji, et al., 2020), which creates a bottleneck when trying to analyse numerous species at the same time. Symbionts, parasites and food sources, however, are more easily detected by DNA metabarcoding focused on unicellular eukaryotes (i.e., protists) because they have a relatively small amount of DNA. The DNA sequence of difficult-to-culture protists has generally been difficult to

clarify because of their small amount of DNA and the high risk of contamination. However, a single-cell DNA analysis method for protists was established, and the DNA sequences of numerous protistan groups have been revealed during the last decade (Decelle, Suzuki, et al., 2012; Nakamura et al., 2021; Nakamura, Sandin, et al., 2020; Pawlowski et al., 2013; Sandin et al., 2019, 2021). For these reasons, the combination of single-cell DNA analysis and DNA metabarcoding should be an effective means to clarify the biological interactions of difficult-to-culture protists and other organisms.

Radiolaria and Phaeodaria are difficult-to-culture but ecologically important protists. Radiolaria contain 6 orders and more than 1100 species (Nakamura et al., 2021; Suzuki & Aita, 2011), while Phaeodaria currently includes 18 families and about 300 species (Nakamura et al., 2015; Nakamura & Suzuki, 2015). These two groups are heterotrophic or mixotrophic unicellular zooplankton, most of which have siliceous skeletons. They are thought to be key groups in ecosystems and material cycles in the world ocean because their high abundance and large contribution to material cycles have often been reported in the past decade (Biard & Ohman, 2020; Nakamura et al., 2013; Sogawa et al., 2022). The symbiosis between these two groups and other eukaryotic organisms has also attracted attention recently. Radiolaria and Phaeodaria are reported to have a symbiotic relationship with crustaceans, which is called the 'Rhizarian rider' phenomenon (Nakamura, Minemizu, & Saito, 2019; Saito et al., 2022). Radiolaria are also known for their symbiosis with algae, and their symbiotic algae have been analysed with different approaches, such as microscopic observation (Anderson, 1983), DNA barcoding (Decelle, Siano, et al., 2012) and fluorescence pattern (Zhang et al., 2018). Their symbiosis is thought to be complicated because some Radiolaria can have more than two symbiotic algae (Decelle, Siano, et al., 2012). Closely related species have also been reported to have symbiotic algae of totally different origins. For example, *Dictyocoryne profunda* (Radiolaria) has a cyanobacterium (symbiotic alga) (Yuasa et al., 2012), whereas *D. truncata* (Radiolaria) possesses a haptophyte (symbiotic alga) (Yuasa et al., 2019). Although a great deal of knowledge has been accumulated during the past 150 years (Table S1), the taxonomic composition of radiolarian symbiotic algae has never been thoroughly clarified. Compared with the case of Radiolaria, knowledge about the symbiosis of Phaeodaria is limited, with less than 10 reports currently available (Table S1).

Radiolaria and Phaeodaria have similar cell size, body structure and ecological niches. This study therefore focused on these two groups as the target organisms and to show the first big picture, attempted to explore the interactions between Radiolaria/Phaeodaria and other eukaryotic organisms. DNA metabarcoding on a single organism was applied to detect potential symbionts, parasites and food sources, to show a comprehensive big picture of the biological interactions of these difficult-to-culture protists.

EXPERIMENTAL PROCEDURES

Field sampling, microscopy and treatment

Plankton sampling was conducted in 2012–2019 at 22 stations located in seven marine areas of the Northern Hemisphere (Figure 1). Radiolaria and Phaeodaria were manually isolated from the bulk plankton samples under a stereomicroscope or inverted microscope (e.g., TMS, Nikon, Japan). The isolated individuals were then photographed with a digital camera (e.g., Nikon 1 V3, Nikon, Japan) attached to the microscopes, and individuals were identified based on their morphological characteristics. The identified specimens were then carefully observed to confirm that no other organisms were attached to their surface. After the observation, the specimens were individually preserved in tubes filled with approximately 2.0 mL of 99.9% ethanol and stored at 4°C. Among these ethanol-preserved specimens, Orodaria and solitary Collodaria were dissected with a sterilized scalpel under a stereomicroscope, and the central area containing nuclei was isolated. Large Phaeodaria (larger than ca. 400 μm in diameter) were also dissected, and their 'central capsule' (the protoplasmic body, including the nuclei) and 'phaeodium' (mass of aggregated brown or yellowish particles) were isolated to separately perform further analyses.

After the DNA extraction (described later), some of the specimens, which have solid siliceous skeletons, were observed with a scanning electron microscope (SEM, JSM-6390LV with LaB6 gun, JEOL, Japan). The conditions and parameters were the same as those described in Nakamura et al. (2016).

DNA metabarcoding and cluster analysis

Each isolated specimen (whole cell, central capsule, or phaeodium) was individually put into 100 μL of guanidine-containing extraction buffer (GITC buffer) (Decelle, Suzuki, et al., 2012), and the DNA was extracted according to the method described in Nakamura et al. (2015). Three tubes filled with ethanol were also analysed as negative controls in the subsequent experiment. The DNA extraction was conducted in a specialized and sterilized laboratory.

Hitherto reported symbionts, parasites and prey organisms of Radiolaria and Phaeodaria were mainly eukaryotes (Table S1), and to compare with these previous studies, the eukaryote-specific primers were

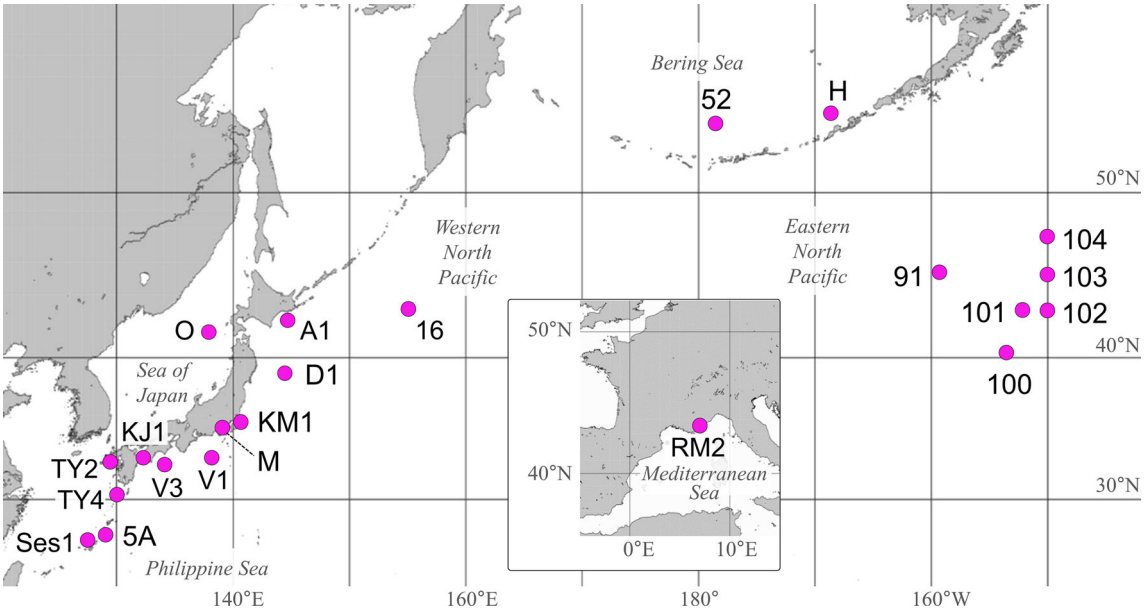

FIGURE 1 Location of the plankton sampling stations in 2012–2019. Pink dots indicate the sampling stations. The detailed information on each station is shown in Table S2.

chosen in this study. The V9 hypervariable region of approximately 315 base pairs in the 18S rRNA gene was amplified by PCR following the procedure in Toju (2016). The first fusion primers were designed by combining P5 or P7 adapters, a series of 'N' and V9-specific sequences for eukaryotes: 1389F (5′-TTGTACACACCGCCC-3′) and 1510R (5′-CCTTCYGCAGGTTCACCTAC-3′) (Amaral-Zettler et al., 2009). The structure of primers (for the first and second PCR), the contents of the reaction mixture and the thermal cycling conditions were the same as in Nakamura, Tuji, et al. (2020). Three negative controls were also contained in the PCR to check that there was no contamination of eukaryotes. After the second PCR, all of the PCR products were mixed and purified with AMPure XP (Beckman Coulter, USA). The purified mixture was adjusted to 4 pM before amplicon sequencing using MiSeq (Illumina, USA). One run of sequencing was performed with MiSeq Reagent kit v3 (600 cycles) (Illumina, USA), following the recommended protocol and default settings.

The obtained data were analysed with Claident ver. 0.2.2019.05.10 software (Tanabe & Toju, 2013) according to the Claident manual (Tanabe, 2018). Low-quality sequences, with average quality scores less than 30 were removed, and chimera sequences were also excluded. The sequences were then clustered into OTUs using a minimum identification score of 0.97. The OTU compositions of each specimen are summarized in a matrix, which lists sequences longer than 200 mer with at least 200 reads. After the treatment mentioned above, 0.01%–10.31% of the original sequence reads were removed in each sample. The OTUs were taxonomically identified until the genus or species level by the Basic Local Alignment Search Tool (BLASTN) from the U.S. National Center of Biotechnology Information (https://www.ncbi.nlm.nih.gov/) using the nr database, excluding environmental sample sequences. The taxonomic name of the registered sequence with at least 98% match was assigned to each OTU in most cases. However, some sequences difficult to identify by BLASTN were (1) further identified by SILVA (Quast et al., 2013) and/or (2) assigned taxonomic names by creating phylogenetic trees containing sequences of related organisms. The classification of phylum- or class-level taxa is referred to by Adl et al. (2019) and Nakamura, Matsuoka, et al. (2019). The relative abundance (%) was derived from the ratio of the total sequence read and the sequence read of each higher taxon.

Cluster analyses were based on the taxonomic composition of the detected organisms in each specimen. The read numbers of detected OTUs were collapsed into binary data (0 or 1), and the Euclidean distances within the resulting dataset were calculated by the statistical software College Analysis ver. 6.6 (Fukui & Hosokawa, 2004). We constructed dendrograms based on the higher taxon and habitat by Ward's method (Ward, 1963) to visualize the differences among the layers.

RESULTS

A total of 22 plankton samples were collected over 8 years (Figure 1). From these samples, 28 Radiolaria

and 56 Phaeodaria, belonging to almost all orders, were analysed by the DNA metabarcoding (Figures 2 and S1, Table S2). In the DNA metabarcoding analyses, the sequences of the hosts (Radiolaria and Phaeodaria) were often detected in most of the specimens (Figure 3, Table S3). Multiple eukaryotic organisms were detected in most of the radiolarian specimens, except for specimens Tax4, Kn10b, St2, oth5b, GS14 and Or9, in which only radiolarian sequences were detected. The same taxa tended to be detected in the same Radiolaria, such as Kinetoplastea, *Pelagomonas* and *Scrippsiella* in *Acanthoplegma krohni* (specimens Ae6 and Ae7), and *Prymnesium* in *Acanthometron pellucidum* (specimens Ae9 and Ae10). Photosynthetic organisms (e.g., Haptophyta, Pelagophyceae and Dinoflagellata) were frequently detected in the radiolarian orders Acantharia, Taxopodia, Spumellaria and Collodaria, whereas they were never found in the order Orodaria, in which non-photosynthetic Dinoflagellata and animals (Cnidaria and Chaetognatha) were detected.

Host sequences were also mainly detected in Phaeodaria, followed by other eukaryotic organisms (Figure 4). However, no or very few hosts of Phaeodaria were detected in the family Astracantha and the specimens from the phaeodium (specimens with 'phd' in their names). Similar to Radiolaria, the same taxa tended to be found in the same Phaeodaria, for example, *Cephaloidophora*/*Thiriotia* in the family Castanellidae and *Dermocystidium* in the family Astracantha. Other eukaryotic organisms were more frequently detected in specimens from the phaeodium than in specimens from the central capsules.

The cluster analysis based on the detected organisms revealed that all specimens could be categorized into two large groups: cluster A including only Phaeodaria and cluster B containing Radiolaria and Phaeodaria (Figure S2). In cluster B, Phaeodaria appeared in several limited subclusters.

Further analysis of Radiolaria clarified that they could be clustered into three large groups, and this categorization corresponded to radiolarian order-level

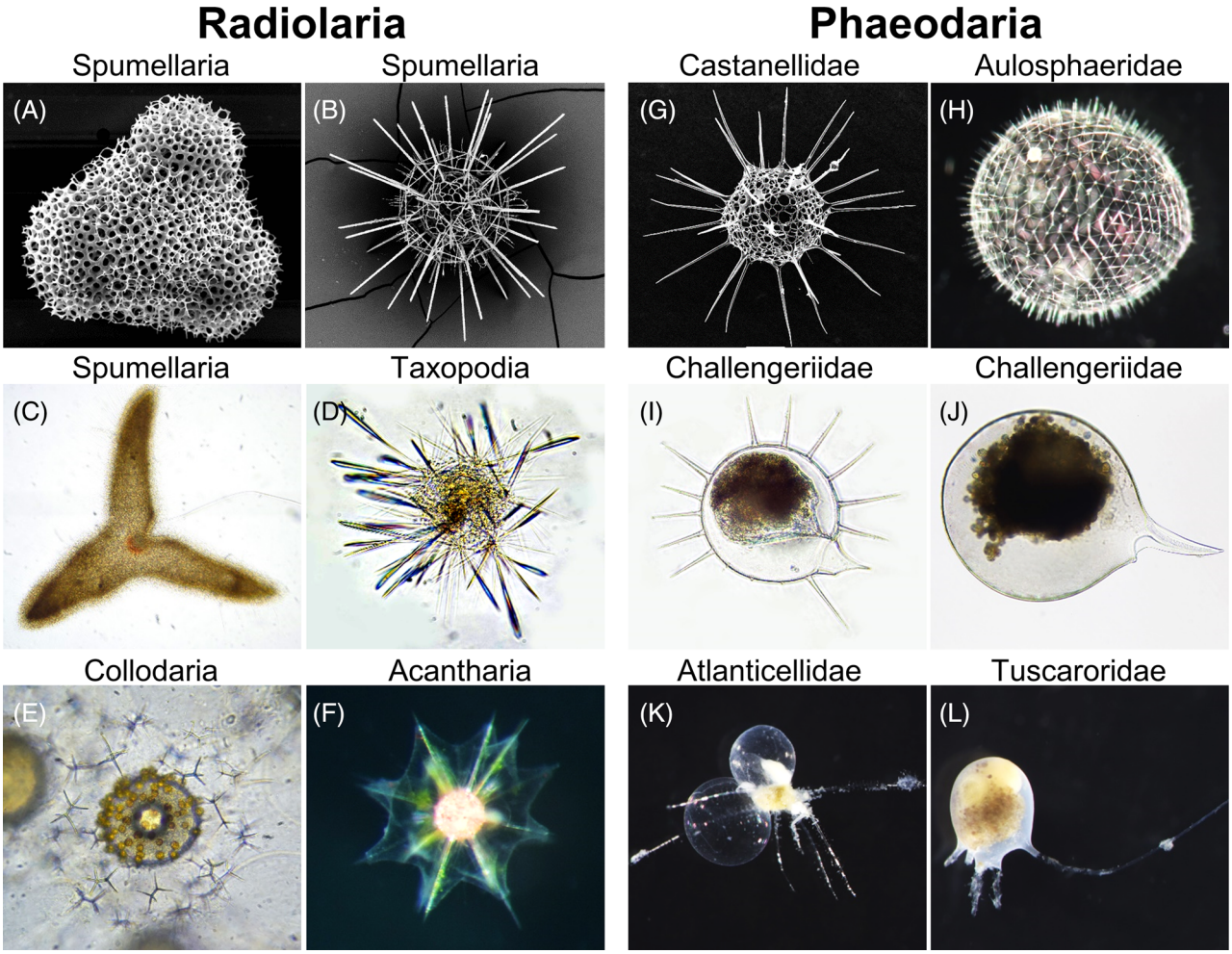

FIGURE 2 Some specimens of Radiolaria and Phaeodaria collected in this study. (A) *Dictyocoryne truncata*, (B) *Diplosphaera hexagonalis*, (C) *Myelastrum trinibrachium*, (D) *Sticholonche zanclea*, (E) *Sphaerozoum punctatum*, (F) *Acanthoplegma* sp., (G) *Castanidium longispinum*, (H) *Aulosphaera* sp., (I) *Challengeron channeri*, (J) *Challengeria naresii*, (K) *Atlanticella* sp., (L) *Tuscarora tubulosa*.

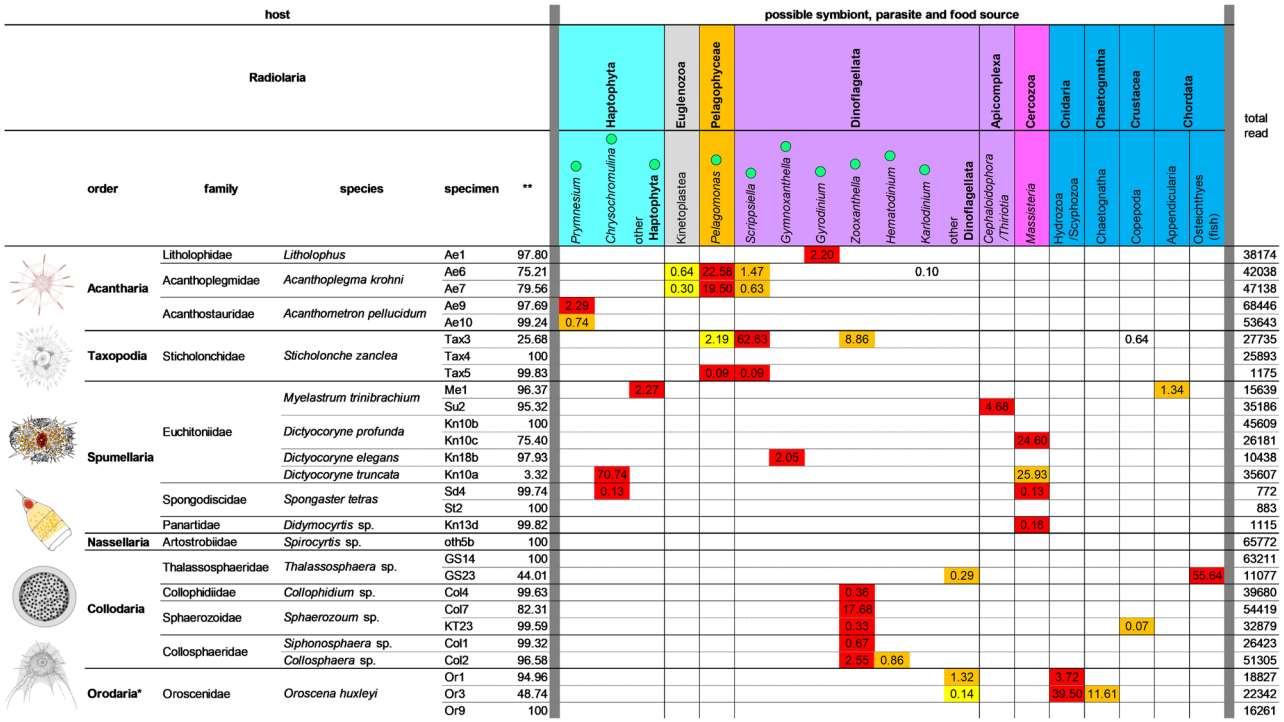

FIGURE 3 Proportion in total sequence reads (%) of Radiolaria (host) and other detected organisms (possible symbionts, parasites and food sources). The first, second and third highest values for each specimen are shown in red, orange and yellow, respectively. Taxa with green circles are photosynthetic autotrophs, which have the potential to be symbiotic algae. *18S rRNA sequences are not registered in the NCBI database. **The proportion of the host.

taxonomy (Figure S3): cluster C, which contained the orders Acantharia and Taxopodia; cluster D, which included only the order Spumellaria; and cluster E, which is mainly composed of the order Collodaria, although three specimens belonging to other orders were also present.

Unlike Radiolaria, phaeodarian clusters did not correspond to the order- or family-level taxonomy (Figure S4). Rather, the difference between body parts (central capsule vs. phaeodium) was highlighted. As a result, Phaeodaria were categorized into two large clusters: cluster F, which chiefly contained the specimens from the phaeodium; and cluster G, which mainly included specimens isolated from the central capsule.

DISCUSSION

Radiolaria

The cluster analysis based on the taxonomic composition of organisms detected in the Radiolaria and Phaeodaria specimens suggests that the organisms contained in them largely differ between these two groups (Figure S2). The high detection of algae (phytoplankton) presumably reflects their symbiosis judging from previous reports concerning the symbiosis of protists (Bjorbækmo et al., 2020; Nowack & Melkonian, 2010). The taxonomic composition of potential symbionts, parasites and food sources seems to be fixed at the species level, considering that the same species of Radiolaria contained similar organisms (Figure 3). The cluster analysis focused on Radiolaria also shows that members of the same radiolarian order tend to contain similar other organisms (Figure S3), suggesting that their biological interactions largely differ among the orders.

The following algae detected in this study have some kind of biological interaction with Radiolaria: Haptophyta, Pelagophyceae and Dinoflagellata (Figure 3). The following combinations were recognized for the first time by this study: *Gyrodinium* in *Litholophus* sp. (Acantharia); *Pelagomonas*, *Scrippsiella* and *Karlodinium* in *Acanthoplegma krohni* (Acantharia); *Pelagomonas*, *Scrippsiella* and *Zooxanthella* in *Sticholonche zanclea* (Taxopodia); and Haptophyta in *Myelastrum trinibrachium* (Spumellaria). The detected organisms may be symbiotic algae judging from the data of previous studies (Table S1), but other analyses, such as observations of substance transportation, are necessary to further clarify details on their symbiosis. The following combinations may be symbiosis with more than two algae, as suggested by (Decelle, Siano, et al., 2012): *Pelagomonas* and *Scrippsiella* in *Acanthoplegma krohni* (Acantharia) and *Sticholonche zanclea* (Taxopodia) (Figure 3). Future studies applying DNA

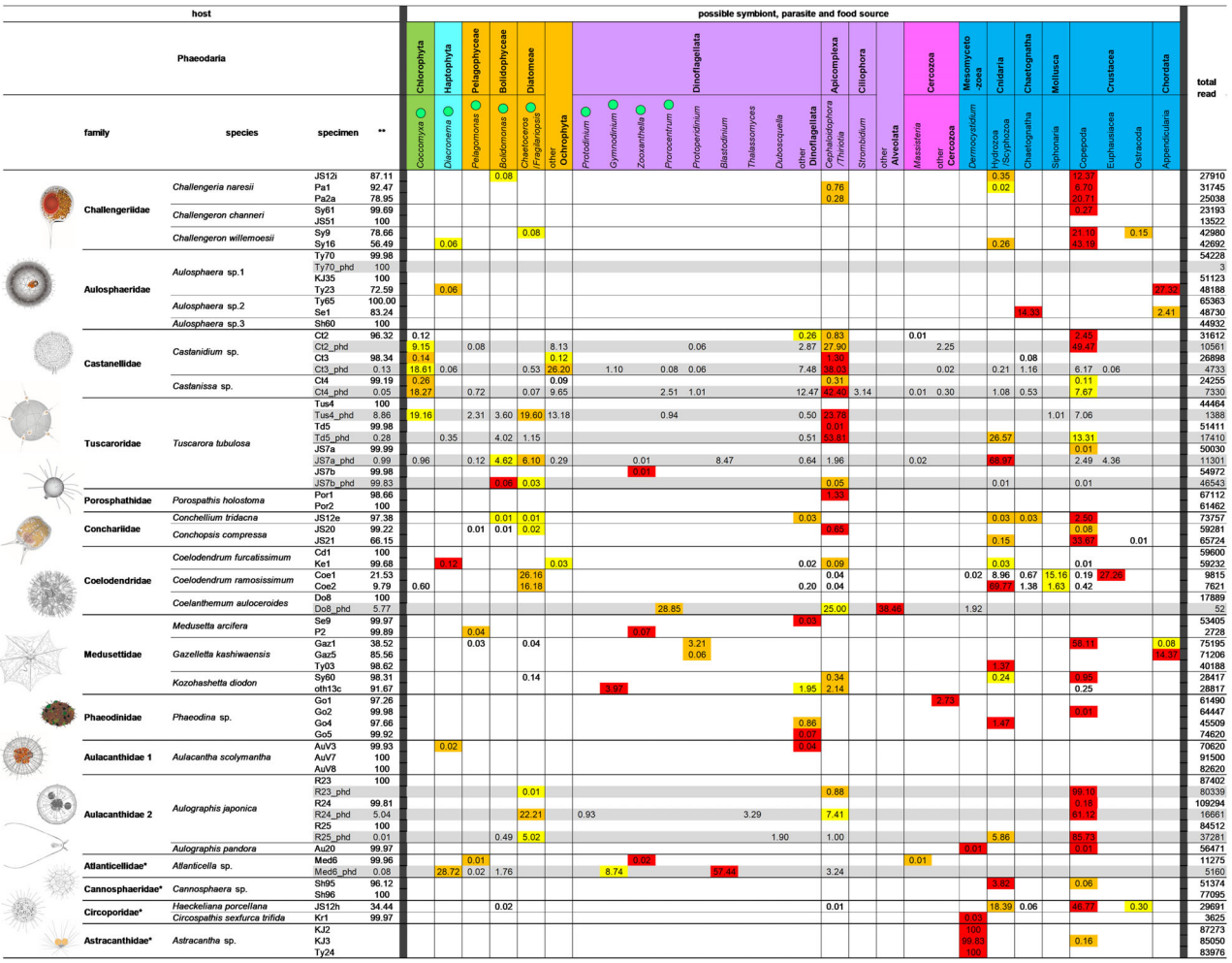

FIGURE 4 Proportion in total sequence reads (%) of Phaeodaria (host) and other detected organisms (possible symbionts, parasites and food sources). The first, second and third highest values for each specimen are shown in red, orange and yellow, respectively. Taxa with green circles are photosynthetic autotrophs, which have the potential to be symbiotic algae. *18S rRNA sequences are not registered in the NCBI database. **The proportion of the host.

metabarcoding on single organism would further reveal the symbiosis with multiple algae.

Kinetoplastea (Euglenozoa), Apicomplexa and *Massisteria* (Cercozoa), which were detected in the Radiolaria specimens (Figure 3), are known to be parasitic to some marine organisms (Gull, 2001; Mylnikov et al., 2015; Seeber & Steinfelder, 2016), and these taxa could be parasites of Radiolaria. This is the first report of parasitism of these three taxa to Radiolaria.

The detection of multicellular organisms (Cnidaria, Chaetognatha, Crustacea and Chordata, including fishes) should be interpreted carefully because these animals have a large number of cells, and they can be detected more easily than unicellular hosts. It is possible that is that some Radiolaria feed on the carcasses of multicellular animals contained in detritus or marine snow (Ikenoue et al., 2019; Nakamura et al., 2017). Another possibility is that some parts of the body of these multicellular animals were contained inside the specimens. Certain large Radiolaria have been reported to be eaten by gelatinous zooplankton, such as Cnidaria and salps (Nakamura et al., 2021), but their fragile bodies are easily damaged during the process of field sampling. They thereby become unrecognizable, but a small amount of their bodies remain inside the radiolarian specimens. This is especially the case in the order Orodaria (Or1 and Or3), which are often fed on by gelatinous zooplankton.

Phaeodaria

The cluster analysis focused on Phaeodaria suggested that, unlike the case with Radiolaria, members of the same phaeodarian family do not tend to contain similar organisms (Figure S4). The body part (i.e., the central capsule or the phaeodium) could be the most important factor dividing the taxonomic composition of detected organisms (Figure S4), implying that the selection of an appropriate body part is important when determining

contained organisms, even for unicellular zooplankton. Previous researchers have suggested that the phaeodium contains undigested prey (Gowing, 1986, 1989), and this idea is partly supported by the results of this study, which revealed that the phaeodium contains numerous small organisms (i.e., possible food sources).

There was a paucity of information about the biological interactions of Phaeodaria (Table S1). Some previous studies thoroughly reviewed the symbiosis of protists and the biological interactions were well documented for the other culturable cercozoans (Bjorbækmo et al., 2020; Nowack & Melkonian, 2010). Very little information was, however, available for Phaeodaria, which also belong to Cercozoa. This study succeeded in adding to and updating knowledge on these biological interactions. Previous studies reported that Dinoflagellata are parasitic on Phaeodaria (Cachon-Enjumet, 1961), and this was confirmed by our results. In addition, we found that Apicomplexa, *Massisteria* (Cercozoa) and *Dermocystidium* (Mesomycetozoea) may also be parasites of some Phaeodaria since these taxa are known as parasites of diverse marine organisms (Gull, 2001; Mylnikov et al., 2015; Seeber & Steinfelder, 2016).

Symbiotic algae have not previously been reported in Phaeodaria, and therefore, the detection of photosymbiotic organisms should be interpreted carefully. Most of these algae may be food sources, but it is also possible that some of them function as symbiotic algae because some host Phaeodaria were collected in euphotic zones (e.g., *Aulosphaera* sp.1, *Coelanthemum auloceroides* and *Aulacantha scolymantha*). In addition, the algae detected in these Phaeodaria (e.g., Haptophyta and some autotrophic species of Dinoflagellata) are symbionts of other marine organisms (Bjorbækmo et al., 2020; Lee et al., 2022; Takagi et al., 2019). Considering the Radiolarian results (Figure 3), Pelagophyceae may also be symbiotic algae of Phaeodaria.

Similar to the case of Radiolaria, multicellular organisms (Chaetognatha, Mollusca, Crustacea and Chordata, including fishes) were detected in Phaeodaria. These taxa are food sources or possibly contaminants in the plankton sampling process. It is noteworthy that Copepoda were more frequently detected in Phaeodaria than in Radiolaria. This crustacean taxon is one of the most abundant zooplankton in the world ocean, and consequently, contamination with their body parts during the sampling process is possible. However, some specimens of Phaeodaria and Radiolaria were collected in the same stations (Stas. 101, 102, 103, 104, KJ1 and Ses1) (Table S2), and Copepoda were rarely detected in Radiolaria (Figure 3). The high detection of Copepoda, therefore, presumably reflects an ecological characteristic of Phaeodaria. It has been suggested that Phaeodaria feed on detritus or marine snow (Gowing, 1989), and the carcasses of Copepoda and other multicellular organisms are often contained in these substances. Copepoda may thus be eaten indirectly by Phaeodaria and presumably be an important food source.

DNA metabarcoding of difficult-to-culture protists

The presence of multiple symbionts and parasites is generally difficult to detect, and simultaneous analysis of numerous specimens requires a great deal of time and effort with ordinary methods. However, by using a combination of single-cell DNA analysis and DNA metabarcoding, we were able to overcome these obstacles. This study succeeded in shedding light on the biological interactions of two groups of difficult-to-culture protists, Radiolaria and Phaeodaria. Moreover, the approach was shown to be effective enough to reveal the ecological relationships of these difficult-to-culture protists.

Future studies should focus on other difficult-to-culture but ecologically important protists such as Ciliophora, Choanoflagellata and especially Foraminifera. The last group is known as an environmental proxy because of their wide distribution, importance as microfossils and function as primary producers of symbiotic algae (Takagi et al., 2019). The symbionts of Foraminifera could be clarified more easily than those of Radiolaria and Phaeodaria because the 18S ribosomal RNA sequence of this group is largely different from other eukaryotes, and therefore, the host would not be detected. Indeed, Foraminifera are rarely detected by DNA metabarcoding using eukaryote-specific primers (Sogawa et al., 2022). In addition, more specimens of Radiolaria and Phaeodaria should be examined to further confirm the pattern and specificity of their symbionts, parasites and food sources.

AUTHOR CONTRIBUTIONS

Yasuhide Nakamura: Conceptualization (lead); data curation (lead); formal analysis (lead); funding acquisition (lead); investigation (lead); methodology (lead); project administration (lead); resources (lead); visualization (lead); writing – original draft (lead); writing – review and editing (lead). **Hiryori Itagaki:** Investigation (supporting); resources (supporting). **Akihiro Tuji:** Methodology (supporting). **Shinji Shimode:** Investigation (supporting); resources (supporting). **Atsushi Yamaguchi:** Investigation (supporting); resources (supporting). **Kiyotaka Hidaka:** Investigation (supporting); resources (supporting). **Eri Ogiso-Tanaka:** Formal analysis (supporting); methodology (supporting); software (supporting).

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

All data used are present in the paper and the Supplementary Material. Amplicon sequences generated in this study are available via the NCBI and DDBJ databases with the accession number DRA017142 (BioProject PRJDB16648): https://ddbj.nig.ac.jp/resource/bioproject/PRJDB16648.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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