DNA metabarcoding reveals vertical variation and hidden diversity of Alveolata and Rhizaria communities in the western North Pacific

Highlights

- Eukaryotic community zoned vertically, Kuroshio epi-, NPIW meso- and bathypelagic.
- Eukaryotic plankton diversity was remarkably low in the NPIW.
- Collodaria, Syndiniales and Oligohymenophorea were predominant in the NPIW.
- Acantharia, Nassellaria, Spumellaria and Phaeodaria OTUs peaked at 100 m depth.
- Taxopodia, Nassellaria and “Other Cercozoa” reads peaked in the deepest layer.

DNA metabarcoding reveals vertical variation and hidden diversity of Alveolata and Rhizaria communities in the western North Pacific

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ABSTRACT

Metabarcoding technology using high-throughput sequencing has revolutionized the current understanding of the diversity and ecology of eukaryotic microorganisms. The aim of the present study was to investigate vertical and seasonal variation in eukaryotic plankton communities and to assess the diversity of eukaryotic plankton, using 18S rRNA sequencing, over a depth gradient in subtropical waters affected by the Kuroshio Current. In particular, the present study focused on the diversity and ecology of Alveolata and Rhizaria taxa, which include a variety of plankton species with fragile skeletons or soft bodies. Three vertically distinct eukaryotic communities were identified: the Kuroshio-influenced epipelagic zone (<200 m), the North Pacific Intermediate Water (NPIW)-dominated mesopelagic zone (500–1000 m), and the bathypelagic zone (2000–3000 m). The operational taxonomic unit (OTU) richness was greatest near the surface (<200 m depth), gradually decreasing with increasing depth, and lowest in deeper layers, and OTU diversity (Pielou's evenness and Shannon-Wiener diversity indices) were lowest in the mesopelagic layer (500–1000 m depth). Hidden diversity was observed in both groups in both the surface and deeper layers of the western North Pacific, as well as in the NPIW, which was characterized by the lowest salinity and oxygen concentrations in the study area. In the NPIW, the Rhizaria yielded relatively more sequence reads than other taxa. Furthermore, specific taxa, such as Collodaria (Radiolaria), Syndiniales (dinoflagellates), and Oligohymenophorea (ciliates), were predominant, according to OTU richness and the relative abundance of sequence reads. These findings indicate that a unique ecosystem was formed over time in the NPIW-isolated water mass.

Key words:

Kuroshio

Eukaryotic plankton

Unicellular zooplankton

18S rRNA

Diversity

Abbreviations: NPIW, North Pacific Intermediate Water; OTU, operational taxonomic unit

1 Introduction

The Kuroshio Current is a warm ocean current that transfers heat and a diverse array of organisms to the high latitudes of the central North Pacific, and because many fish species spawn and migrate in the surrounding areas, the current substantially influences the ecosystem structure and fisheries in East Asia (Barkley, 1970; Qiu and Lukas, 1996; Imasaki et al., 2001; Sassa et al., 2008; Morimoto, 2010; Sugisaki et al., 2010; Harris and Lang, 2014; Yamazaki et al., 2016; Nagai et al., 2019; Kobari et al., 2020). Marine ecosystems are based on primary production by phytoplankton and bacterial plankton, and the energy and organic materials that these organisms produce are transferred to higher-trophic-level organisms, such as fishes, through intermediates, like heterotrophic plankton, that consume primary producers. Therefore, it is essential to elucidate the dynamics of lower trophic levels in the Kuroshio ecosystem, which is significant in terms of oceanography and fisheries, and to monitor its continuous changes.

Even though a large proportion of plankton species are protists (i.e., eukaryotic, predominantly single-celled organisms), most *in situ* studies of plankton communities have focused on relatively large zooplankton, such as crustaceans, or phytoplankton, such as diatoms, which can be identified using microscopy. Moreover, marine plankton are highly diverse, and advanced techniques are often required for morphological identification. Advances in molecular biological techniques, such as DNA metabarcoding using high-throughput sequencing, have enabled researchers to investigate the structures of plankton communities that contain small and amorphous plankton species (Countway et al., 2007; Schnetzer et al., 2011; Hu et al., 2016; Pernice et al., 2016; Ruppert et al., 2019; Canals et al., 2020; Brisbin et al., 2020; Ollison et al., 2021). Metabarcoding has also become a powerful and efficient tool for monitoring and detecting

hidden diversity, with community-wide taxonomic coverage (Lindeque et al., 2013; Mohrbeck et al., 2015; Valentini et al., 2016; Hirai et al., 2017; Ruppert et al., 2019; Suter et al., 2020). The taxonomic identification of Alveolata and Rhizaria, which contain many species with fragile skeletons (e.g., radiolarians) or soft bodies (e.g., ciliates), using morphological techniques has always been considered difficult. Recent DNA metabarcoding studies have revealed that Alveolata and Rhizaria are highly diverse and contribute significantly to ecological processes, such as vertical exports and trophic transfers, in marine ecosystems (Bescot et al., 2016; Gutierrez-Rodriguez et al., 2019; Preston, 2019).

The structures of Alveolata and Rhizaria communities are drastically affected by depth and season (Not et al., 2007; Nakamura et al., 2013). In the areas surrounding the Kuroshio Current, the environmental conditions of the marine environment vary both horizontally and vertically (Kuroda et al., 2018; Yasuda, 2003; Miyazawa et al., 2009). Thus, community structure may be significantly affected by environmental change. A study that used 18S rRNA sequencing to investigate the eukaryotic plankton community of coastal waters affected by the Kuroshio Current reported diverse assemblages of diatoms and dinoflagellates (Kok et al., 2012), and a more recent study, which also investigated eukaryotic plankton communities using 18S rRNA, reported finding three distinct communities (coastal, Kuroshio, and mixed water) within the surface layer of the northwestern Pacific (Wu et al., 2020). Meanwhile, Endo and Suzuki (2019), who surveyed the surface layer, reported that diatoms and haptophytes were more diverse in the Kuroshio axis. Furthermore, a water mass called the North Pacific Intermediate Water (NPIW) is formed in the mixed water region between the Oyashio and Kuroshio waters in the western North Pacific and is widely distributed in subtropical North Pacific waters between the surface and deep

layers and between 20 and 45°N (Sverdrup et al., 1942; Talley, 1993; Yasuda, 1997; Masujima et al., 2003; Shimizu et al., 2004). Most studies of plankton in the NPIW have focused on foraminiferans or large gelatinous zooplankton (Ortiz et al., 1996; Morita et al., 2017), and the comprehensive plankton community structure, especially in the southern portions of the NPIW, has yet to be reported.

The aim of the present study was to investigate the eukaryotic plankton communities in subtropical waters, in which epipelagic and mesopelagic layers are associated with different water bodies (the Kuroshio Current and NPIW, respectively). Year-round seasonal surveys of marine plankton and physicochemical environmental parameters at different depths (5–3000 m) were conducted at several sites along a transect across Kuroshio, and 18S rRNA (V7–9) DNA metabarcoding was used to assess the dynamics and diversity of eukaryotic plankton communities.

2 Material and methods

2.1 Sampling locations

Sampling was conducted from aboard the *R/V Soyo-Maru* (National Research Institute of Fisheries Science, Japan Fisheries Research and Education Agency) in the northwestern Pacific, south of Japan, adjacent to the Kuroshio Current (Fig. 1). A total of 110 samples were collected from different depths (from 5 or 10 m to 3000 m) during five cruises that were conducted from August 2015 to August 2016 (Table 1), and the locations of sampling stations were selected in order to establish a transect that crossed the Kuroshio Current axis at 138°E (Fig. 1). The sampling sites were designated as northern (north of Kuroshio), middle (Kuroshio axis, but later found to

be off-axis), or southern (south of Kuroshio), and the location of the Kuroshio Current axis was determined using hydrographic condition images based on data from vessels, satellites, and Japan Meteorological Agency products, which were published by the fisheries research institutes of Tokyo, Chiba, Kanagawa, Shizuoka, Mie and Wakayama prefectures (provided on website of Kanagawa Prefectural Fisheries Technology Center; Fig. 1).

2.2 Seawater sampling and processing

Vertical temperature and salinity profiles were measured continuously using a conductivity-temperature-depth (CTD) sensor (SBE 911plus; Seabird Co.). Seawater samples for generating discrete vertical profiles of nutrients (nitrate+nitrite, silica, and phosphate) and chlorophyll *a* concentrations were collected at several depths using Niskin bottles mounted to the rosette carrying the CTD sensor. For DNA metabarcoding analysis, seawater samples (1 L) were passed through Nucleopore membrane filters (0.2 μm), and stored at -30°C until the DNA extraction. DNA was extracted using 5% Chelex buffer, as described previously (Nagai et al., 2012; Tanabe et al., 2016). For chlorophyll *a* analysis, seawater samples (300 mL) were filtered onto Whatman GF/F filters, extracted using 6 mL of *N*,*N*-dimethylformamide (DMF), and analyzed by applying the fluorometric Welschmeyer method. Seawater samples for chlorophyll *a* and nutrient analysis were stored at -30°C until nutrient and chlorophyll concentrations were measured using a flow injection analyzer (TrAAcs 2000; Bran + Luebbe) and a fluorometer (10-AU; Turner Designs, Inc.), respectively.

2.3 DNA sequence generation and processing

Metagenomic analysis was performed using the MiSeq 300PE platform (Illumina, San Diego, CA, USA) and universal primers (SSR-F1289-sn, F: TGGAGYGATHTGTCTGGTTDATTCCG; SSR-R1772-sn, R: TCACCTACGGAWACCTTGTTACG; Sildever et al., 2019), which were designed to amplify the V7–9 hypervariable regions of the 18S-rRNA gene. A massively parallel paired-end sequencing workflow was designed by consulting the Illumina document (Illumina, 2013). Two-step PCR was used to construct paired-end libraries (Sildever et al., 2019), and the resulting PCR products were quantified, pooled in equal concentrations, and stored at -30°C until sequenced using the MiSeq Reagent Kit v3 (2 × 300 bp; Illumina).

2.4 Metabarcoding data treatment processes and operational taxonomic unit picking

Nucleotide sequences were demultiplexed using the 5-multiplex identifier tags and primer sequences. Sequences that contained palindromes of >30 bp and homopolymers of >9 bp were trimmed at both ends. The 30 tails with mean quality scores of <30 at the end of the last 25-bp window and 50 and 30 tails with mean quality scores <20 at the end of the last window were also removed. Sequences longer than 300 bp were truncated to 300 bp by trimming 30 tails, and sequences shorter than 250 bp were filtered out. Both demultiplexing and trimming were performed using Trimmomatic version 0.35 (http://www.usadellab.org/cms/?page=trimmomatic).

The trimmed and filtered sequences were merged into paired reads using Usearch version 8.0.1517 (http://www.drive5.com/usearch/). After singletons were removed, the remaining sequences were aligned using Clustal Omega version 1.2.0. (http://www.clustal.org/omega/). Multiple sequences were aligned, and only sequences that were

contained more than 75% of the read positions were extracted. Filtering and part of the multiple-alignment process were performed using the *screen.seqs* and *filter.seqs* commands in Mothur, as described in the MiSeq standard operating procedure (http://www.mothur.org./wiki/MiSeq_SOP; Schloss et al., 2011), and erroneous and chimeric sequences were detected and removed using the *pre.cluster* (*diffs=4*) and *chimera.uchime* (*minh=0.1*) commands in Mothur, respectively (http://drive5.com/usearch/manual/uchime_algo.html; Edgar et al., 2011).

The sequence data were divided into several groups and treated separately, owing to the limited memory capacity of the server. The FASTA file (*result.fasta*) of each group was merged, and identical sequences were collated into operational taxonomic units (OTUs) using the *unique.seqs* command in Mothur. Because representative sequences from different Miseq runs could contain identical sequences, the sequences were clustered to re-select representative sequences at a 0.99 level of sequence identity using CD-HIT-EST version 4.6.8 (Li and Godzik, 2006) with command-line parameters *'-c 0.99 -n 11 -d 0 -p 1'*. Representative sequences, which were designated as OTUs, were counted using the *count.seqs* command, and sequences clustered into OTUs between different runs were counted by referring to both *count.seqs* and CD-HIT data. These sequences were used for subsequent taxonomic identification analyses, and demultiplexed and filtered, but untrimmed, sequence data were deposited into the DDBJ Sequence Read Archive (access no. DRA010320).

2.5 OTU identification

To taxonomically identify the selected OTUs, a subset of nucleotide databases that satisfied the chosen conditions (described below) were prepared for BLAST analysis. One keyword was

selected from among “ribosomal,” “rrna,” and “rdna,” but “protein” protein was not included in the title. For the taxonomy search, the keywords “metagenome,” “uncultured,” and “environmental” were not included. The sequences of retrieved GenBank IDs from the nucleotide database downloaded from the NCBI FTP server on March 22, 2019, were used to construct a reference sequence database.

Each OTU was then identified by BLAST search (Cheung et al., 2010) using NCBI BLAST+ 2.2.30+ (Camacho et al., 2009), with the default parameters, and nucleotide subset described above as the database. For each query sequence, taxonomic information was obtained from BLAST hits with the highest bitscores, and OTUs with the same top hit were merged. When an OTU matched several data points with the same bitscore and top hit similarity, the OTUs were merged. Therefore, in some cases, several data were merged in a single OTU. Because the removal of error-containing sequences was imperfect and error-containing sequences remained in the dataset, error-containing sequences were detected as artificial OTUs with the same top BLAST hit name but with slight differences. To avoid the overestimation of OTU richness, artificial OTUs were merged and represented by the OTU with the highest similarity score. One OTU with abnormally high read numbers was excluded from data analysis because it was thought to contain chimeric sequences.

2.6 Data analysis

All multivariate analyses of plankton community structure and diversity were performed using PRIMER version 7 with the PERMANOVA+ add-on software (Anderson et al., 2008; Clarke and Gorley, 2015). For multivariate analyses, Bray-Curtis similarity among samples was calculated

using log-transformed sequence abundance data. Non-metric multidimensional scaling ordination was used to visualize differences between the plankton communities of seawater samples, and similarity analysis was used to identify differences between samples collected at different depths or during different months (Clarke, 1993). To investigate the effects of environmental variables on Alveolata and Rhizaria communities, multicollinearity was addressed by removing variables with correlations of >0.95, and the data were processed using distance-based linear modeling (DistLM) and redundancy analysis (dbRDA). Plankton diversity was assessed by calculating richness (number of OTUs), Shannon-Wiener diversity (Shannon and Weaver, 1949), and evenness (Pielou, 1966).

3 Results

3.1 Physical and chemical characteristics of the water masses

Environmental variation was observed among the sampling areas, northern sites, and southern sites of each cruise (Fig. 2 and Fig. S1). Vertical mixing in the surface layer was observed in March (up to ~100 m at the northern site and ~200 m at the southern site; Fig. 2). The maximum salinity was observed in the surface layer, whereas the salinity minimum layers were observed at 750–1000 m in the southern sites and 300–500 m in the northern and middle sites (Fig. 2). A potential density of 26.8 σ_θ (range: 26.6–26.9 σ_θ), which indicated NPIW, was also observed at the salinity minimum layers (Fig. 2). Oxygen decreased with increasing depth, and oxygen minimum layers were observed just below the salinity minimum layers (1250 and 750–1000 m at the southern and northern sites, respectively; Fig. 2). In contrast to oxygen levels, nutrient

(nitrate+nitrite, silica, and phosphate) concentrations increased with increasing depth (Fig. S1), and maximum nitrate+nitrite and phosphate concentrations were observed in the oxygen minimum layers (Fig. S1).

3.2 Eukaryotic plankton community structure and diversity

The present study detected a total of 1956 OTUs of oceanic plankton, including 872 OTUs attributed to supergroup Alveolata, which was represented by dinoflagellates and ciliates; 202 OTUs attributed to Rhizaria, which was represented by radiolarians and cercozoans; 275 OTUs attributed to Opisthokonta, which was represented by metazoans and fungi; 323 OTUs attributed to Stramenopiles, which was represented by diatoms; and 133 OTUs attributed to Archaeplastida (Fig. 3). Alveolata accounted for the largest proportion (nearly half) of all OTUs, and Rhizaria and Alveolata accounted for 33 and 40% of all sequence reads, respectively (Fig. 3).

Non-metric multidimensional scaling ordination and analysis of similarity revealed significant differences in oceanic plankton communities with respect to water depth (global R=0.751, p=0.001, no. permutations=999; Fig. 4). Pairwise tests revealed significant differences between shallow and deep communities (Table 2). There were significant differences (R=0.92–1, p=0.001) between communities from surface layers (5–10, 50, and 100 m) and deeper layers (500, 1000, 2000, and 3000 m) and small differences (highly overlapped) between communities collected at 5, 10, and 50 m (R=0.13, p=0.009), 500 and 1000 m, and 2000 and 3000 m (R=0.18–0.22, p=0.001). However, no significant differences were detected in the plankton communities collected during different months (global R=0.031, p>0.05; pairwise tests R<0.07, p>0.05, no. permutations=999).

More OTUs were detected in surface layers (<200 m depth) than in deeper layers (>200 m depth), however, other diversity measures failed to decrease with increasing depth that Pielou's evenness was higher at 2000 and 3000 m but lower at 500 and 1000 m, and Shannon-Wiener diversity was higher in surface layers but lower at 500 and 1000 m (Fig. 5). The mean number of OTUs for most groups, including Alveolata, Archaeplastida, Excavata, Haptophyta, Opisthokonta, and Stramenopiles, were consistently low in deep layers and were three to 19 times higher in surface layers. However, the richness of Rhizaria peaked at 100 m (41 OTUs; Fig. 6a). The proportion of sequence reads attributed to Alveolata was relatively constant (35–45%), regardless of depth, whereas the proportions attributed to Opisthokonta and Archaeplastida were higher at depths of <100 m, and the proportion attributed to Rhizaria was higher at depths of >100 m, especially at 500 and 1000 m (Fig. 6b).

3.3 Alveolata and Rhizaria communities

Among the Alveolata taxa, dinoflagellates (i.e., Dinoflagellata) represented the largest number of OTUs (659) and proportion of sequence reads (91.6%), followed by ciliates (i.e., Ciliophora; 167 OTUs and 5.4% of sequence reads; Fig. 7). Among the dinoflagellates, Gymnodiniales, Syndiniales, and Peridiniales accounted for relatively high proportions of sequence reads (32.9, 29.2, and 19.5%, respectively; Fig. 7), and Peridiniales and Gymnodiniales also accounted for relatively large numbers of OTUs (240 and 152, respectively; Fig. 7). Among the ciliates, Oligohymenophorea and Oligotrichia accounted for relatively high proportions of sequence reads (45.7 and 27.1%, respectively) and numbers of OTUs (27 and 66, respectively; Fig. 7).

Among the Rhizaria taxa, Radiolaria and Cercozoa accounted for nearly half of all OTUs (111 and 90 OTUs, respectively; Fig. 8), and no OTUs were attributed to Foraminifera, which is another major group in Rhizaria. Among Radiolaria, Spumellaria accounted for the greatest number of OTUs (n=37), followed by Acantharia (34 OTUs), Collodaria (24 OTUs), Nassellaria (12 OTUs), and Taxopodia (4 OTUs; Fig. 8). However, for Cercozoa, most OTUs were categorized as "Other Cercozoa" (86 OTUs, 95.6%), and the remaining four were attributed to Phaeodaria (4.4%; Fig. 8). Radiolaria accounted for most of the sequence attributed to Rhizaria (97.2%), whereas Cercozoa accounted for only 2.8% (Fig. 8), and among the Radiolaria sequence reads, Collodaria accounted for the highest proportion (48.7%), followed by Taxopodia (20.6%), Acantharia (15.0%), Spumellaria (12.7%), and Nassellaria (3.1%; Fig. 8). For the Cercozoa sequence reads, "Other Cercozoa" accounted for the highest proportion (93.7%), and Phaeodaria accounted for only 6.3% (Fig. 8).

Cluster analysis classified the 110 Alveolata community samples into two large clusters, the surface layer (<200 m) and deeper layer (>200 m) at 29% similarity, accordingly to the similarity profile test (SIMPROF; Clarke, 1993), and the 110 Rhizaria community samples into three large clusters, the surface layer (<200 m), mainly composed of samples from 50 m and 100 m layers (with one 500 m layer sample), and a deeper layer (>200 m), at 22.5 and 37% similarities based on the SIMPROF test (Fig. 9). Further community structure analysis was conducted each on the surface layer (<200 m) and the deeper layer (>200 m), and only surface layer communities showed significant differences between sampling months: March *vs.* other months (ANOSIM pairwise test, R=0.40–0.57, p=0.001) for the Alveolata community and March

vs. August (both 2015 and 2016; ANOSIM pairwise test, R=0.44–0.47, p=0.001) for the Rhizaria community.

DistLM analysis revealed significant associations between both the Alveolata and Rhizaria communities and the ten (<200 m) or eight (>200 m) of the tested environmental variables (Table 3). One variable (phosphate concentration) was excluded prior to analysis of surface layer communities because it was strongly correlated ($|r|>0.95$) with both nitrate+nitrite and silica, and two variables (phosphate and silica) were excluded from analysis of deeper layer communities because they were strongly correlated with nitrate+nitrite ($|r|>0.95$). In the marginal tests of DistLM analysis, depth, temperature, nitrate+nitrite, and silica individually explained 10.4–13.3% of variation in surface layer Alveolata community structure, and depth, temperature, and salinity individually explained 8.1–10.7% of variation the deeper layer Alveolata community structure (Table 3). Meanwhile, depth, temperature, nitrate+nitrite, and chllophyll *a* individually explained 8.2–16.5% of variation in the surface layer Rhizaria community structure, and depth, temperature, and salinity individually explained 12.8–16.7% of variation in the deeper layer Rhizaria community structure (Table 3).

For the Alveolata community structure, the first two dbRDA axes explained 65.5 and 24.1% of the fitted and total variation in the surface layer, respectively, and 68.2 and 15.3% of the fitted and total variation in the deeper layer. For the Rhizaria community structure, the first two dbRDA axes explained 76.1 and 31.1% of the fitted and total variation in the surface layer, respectively, and 76.7 and 22.2% of the fitted and total variation in the deeper layer. In the analysis of surface layer Alveolata and Rhizaria communities, vector overlay indicated that axis 1 was strongly correlated with depth and that axis 2 was strongly correlated with month, whereas in the

analysis of deeper layers, axis 2 was strongly correlated with temperature and nitrate+nitrite for the Alveolata community and with temperature for the Rhizaria community (Fig. 9). In addition to depth, season was also a variation factor for Alveolata and Rhizaria communities in the surface layer.

The mean OTU richness and sequence read proportions of Alveolata at different depths were compared with respect to class and order (Figs. S2 and S3). Most Alveolata taxa yielded greater OTU richness in surface layers, although Syndiniales OTUs were more evenly distributed and peaked at 500 m (Fig. S2). The proportions of sequence reads of some dinoflagellates (i.e., Gymnodinales, Peridiniales, Coccidinales, Prorocentrales, and Gonyaulacales), ciliates (i.e., Oligotrichia, Mesodiniea, and Nassophorea), and Apicomplexa in surface layers gradually decreased with increased depth, to 1000 m depth, and then increased at 2000 and 3000 m (Fig. S3), whereas proportions of sequence reads for both Syndiniales (dinoflagellates) and Oligohymenophorea (ciliates) peaked at both 500–1000 m depths (Fig. S3).

Mean numbers of Acantharia, Nassellaria, Spumellaria, and Phaeodaria OTUs peaked at 100 m depth, whereas Collodaria OTUs peaked at 500 m depth (Fig. S4). The mean number of Collodaria OTUs was relatively low in the upper layer, whereas the number of 'Other Cercozoa' OTUs decreased gradually from the surface layers to the deeper layers. The vertical dynamics of Acantharia, Nassellaria, and Spumellaria OTU number in the present study suggests that these groups are more diverse in surface layers than in deeper layers. The proportions of Acantharia, Nassellaria, Taxopodia, and "Other Cercozoa" sequence reads peaked at 2000 or 3000 m (Fig. S5). Meanwhile, the proportion of Collodaria sequence reads peaked at 500 m, and similar to Collodaria OTU numbers, the mean proportions of sequence reads were relatively small at 5–10,

50, and 100 m. In contrast, proportion of Phaeodaria OTUs and sequence reads peaked at 50 and 100 m, respectively.

4 Discussion

The present study revealed that there are clear differences in the community structure and diversity of eukaryotic plankton found at different depths and in different water masses in the western North Pacific. The eukaryotic plankton community differed distinctly between the epipelagic layer (<200 m), which was strongly influenced by the Kuroshio Current, and deeper layers (>200 m). These findings are similar to those of a study conducted in the Atlantic, which also reported distinct protistan assemblages in the euphotic zone and deep sea and a vast diversity of Alveolata and Rhizaria (Countway et al., 2007; Not et al., 2007). In addition, the present study also noted a significant difference in the eukaryotic plankton communities of the mesopelagic layer (500–1000 m) and bathypelagic layer (2000–3000 m). In general, the plankton community varied in accordance with vertical changes in environmental conditions. For example, nutrient concentrations and salinity increased with increasing depth, and temperature and chlorophyll concentration decreased with increasing depth. However, diversity did not exhibit a linear gradient with depth. The number of OTUs was greatest in the surface layer (<200 m depth), decreasing with increasing depths, and lowest in the deepest layer (3000 m), whereas the diversity indexes (Pielou's evenness and Shannon-Wiener diversity index) were remarkably low in the mesopelagic layers (500–1000 m depth), where the NPIW water mass was distributed (Reid, 1965; Sverdrup et al., 1942). The NPIW is formed in the mixed water region between the

Kuroshio Extension and the Oyashio Front in the western North Pacific and spreads southward (Talley, 1993; Watanabe et al., 1995). The plankton community structures of the Oyashio and Kuroshio water areas are distinct, and the plankton community of the nutrient-rich Oyashio area is generally characterized by fewer species (lower diversity) and higher abundance, whereas the plankton community in the nutrient-poor Kuroshio area is characterized by more species (higher diversity) and lower abundance (Sogawa et al., 2013; Morita et al., 2017; Ohtsuka and Nishida, 2017; Matsumoto and Yamaguchi, 2020). A study that used a video plankton recorder reported that Hydrozoa, Ctenophora, Copepoda, and Rhizaria were most abundant within the NPIW and emphasized that radiolarians may have physically accumulated in the water mass (Ichikawa, 2008; Ichikawa et al., 2007). The estimated residence time of NPIW, which spreads southward to Japan, is 20 years, with a subsequent increase in salinity and reduction of oxygen (Shimizu et al., 2004; Talley, 1993; Talley et al., 1995). The low mesopelagic diversity in the study area could be due to the special formation process and environmental characteristics (salinity minimum and oxygen minimum) of the NPIW and long residence time that maintains a uniform environment. These results suggest that unique isolated ecosystems form over time in NPIW. For example, several radiolarian species were detected almost exclusively in the NPIW (e.g., *Collophidium ovatum*, *Thalassicolla pellucida*, and *Thalassicolla melacapsa*, Collodaria). Furthermore, Collodaria, which include a large number of mixotrophs that harbor algal symbionts, are frequently reported in the surface layers (Biard et al., 2016; Nakamura et al. 2019; Suzuki and Not, 2015), even though they retain high species diversity in both mesopelagic and deep layers (Biard et al., 2015; Pernice et al., 2016). In the present study, the OTU and sequence read numbers attributed to Order Collodaria were highest in the NPIW, whereas Order Orodaria, which is

closely related to the Collodaria, has only been detected in the deep sea (Nakamura et al. in press). Therefore, it is quite possible that unknown Collodaria taxa dominate subsurface layers. Furthermore, some collodarian species are known to exhibit two (or possibly more) morphologies (Biard et al., 2015), and it is possible that they change their distribution depth, depending on life stage. For example, Collodaria increase their colony size *via* asexual reproduction in surface layers and by sexual reproduction in deeper layers. Further studies are needed to clarify the life cycle of radiolarians.

The vertical distribution of Gymnodinales and Syndiniales sequence reads showed the opposite trend; the relative abundances of Gymnodinales (and Peridiniales) were smallest at 1000 m depth layer, higher closer to the surface, and peaked at surface, whereas, those of Syndiniales sequence reads peaked at 1000 m, lower closer the surface, and lowest at the surface. Many *Karenia* and *Noctiluca* species that cause algal blooms belong to Gymnodiniales, which are either autotrophs that perform photosynthesis or heterotrophs that feed on phytoplankton in the euphotic zone of the surface layer (e.g., Gomez, 2007; Guiry and Guiry, 2015; Landsberg et al., 2009). Syndiniales taxa, which parasitize crustaceans, radiolarians, algae, ciliates, and other dinoflagellates, exhibit high diversity and sequence abundance in recent 18S rRNA studies (Bråte et al., 2012; Guillou et al., 2008; van den Hoek et al., 1995; López-García et al., 2001; Strassert et al., 2018). The relatively high abundance of Syndiniales (Alveolata) sequence reads, along with those of radiolarians, at the NPIW suggests that parasitic infections of radiolarians by Syndiniales might play a crucial role in determing the patterns observed in the NPIW ecosystem.

It is interesting to note that the relative abundances of Peridiniales sequence reads were highest in the bathypelagic layers (2000–3000 m). Gymnodiniales also exhibited relatively high

read numbers in the bathypelagic layers. Syndiniales exhibited relatively high read numbers in the deep layers. Most previous studies of Alveolata have focused on coastal areas or euphotic zones (e.g., Anderson et al., 1998; Gomez, 2007). However, in the present study, Alveolata exhibited consistently large proportions of OTUs and sequence reads, regardless of depth, and accounted for 41–47% of all OTUs and 35–45% of all sequence reads. These results suggest that a few groups of Alveolata (e.g., Peridiniales, Gymnodiniales, and Syndiniales) are abundant in both the surface layer and deeper layers and that such taxa may substantially affect marine ecosystems in relatively shallow coastal areas and euphotic zones, as well as in the entire open ocean ecosystem.

Foraminifera are one of the main taxa in the supergroup Rhizaria, which contains more than 4000 species (mostly benthic; Nakamura et al., 2019). However, no Foraminifera sequence reads were detected in the present study, likely because the ribosomal DNA sequences of foraminiferans differ largely from those of other rhizarians (e.g., Radiolaria and Phaeodaria; Ishitani and Takishita, 2015). Radiolaria contain ~1000 described species (excluding extinct species), whereas Cercozoa contain fewer (Nakamura et al., 2015). Radiolarians generally possess solid siliceous skeletons and are larger than cercozoans, which generally have fragile or absent skeletons. Therefore, more radiolarian species have been described, presumably because they are more recognizable microscopically. Considering that the metabarcoding results of the present study indicated similar proportions of Radiolaria and Cercozoa OTUs, previous studies might have underestimated the species diversity of Cercozoa, which are generally parasitic (except for phaeodarians; Nakamura and Suzuki, 2015a).

Even though only a single species, *Sticholonche zanclea* Hertwig 1877, is classified in the Order Taxopodia (Suzuki and Not, 2015), four distinct Taxopodia OTUs were detected in the present study. Thus, some genetically distinct lineages may exist within the Taxopodia. The total read numbers of Taxopodia were generally higher than those of other radiolarian orders and peaked at a depth of 3000 m. These results suggest that Taxopodia taxa are abundant in deep layers, despite previous reports that they are mainly distributed in the surface and mesopelagic layers (e.g., Suzuki and Not, 2015). Because Taxopodia taxa are genetically and morphologically distinct from other radiolarian orders (Suzuki and Not, 2015), the DNA copy numbers of Taxopodia taxa might differ from those of other radiolarian orders. It is difficult to ascertain the vertical profile of Phaeodaria OTU numbers since only four OTUs were detected. However, it is worth noting that the OTUs and sequence reads of Phaeodaria were mainly detected in surface layers (50 and 100 m), which corresponds to recent reports that Phaeodaria are occasionally found in shallow layers (Biard and Ohman, 2020). Since several new Phaeodaria species were recently described near the survey area (Nakamura et al., 2013, 2016, 2018), some species might be highly abundant in the surface layers.

5 Conclusions

The marine ecosystem is greatly affected by physical changes, such as oceanographic structure variation and climate change. Microplankton and primary producers with rapid life cycles are the first to be affected by physical changes and subsequently affect the productivity of meso-macrozooplankton and fishery species located at higher trophic levels. Here, the present study

demonstrates that the plankton community structure is significantly affected by vertical marine structure in the waters around the Kuroshio Current. In the North Pacific Intermediate Water (NPIW), which is formed in the northwestern Pacific Ocean and extends to the mesopelagic layer around the Kuroshio Current, the diversity of eukaryotic plankton communities is lowest vertically, and the community of the NPIW, isolated from the other vertical layers (surface and bathypelagic), form a unique ecosystem. The Alveolata and Rhizaria communities in the Kuroshio-influenced epipelagic zone exhibited seasonal variation. In oligotrophic waters, such as those around the Kuroshio Current, marine production is especially supported by microplankton. Thus, integrated research on the whole ecosystem, from microplankton to fish species, together with changes in the oceanographic structure, are needed to understand the variation of fishery resources, and to conduct effective fisheries management. In addition, for changes that involve anthropogenic effects, such as climate change and marine resources reduction, it is essential to conduct interdisciplinary research of human societies and natural ecosystems (socio-ecological systems), as well as to implement specific countermeasures based on the findings of such studies.

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Figure captions

Figure 1. Survey area and locations of sampling stations in the western North Pacific. Dotted lines indicate the Kuroshio Current axis of each cruise.

Figure 2. Vertical profiles of the physical and chemical environmental variables in the survey area. The sampling sites were categorized as northern (north of the Kuroshio Current), middle (near the Kuroshio Current axis), or southern (south of the Kuroshio Current).

Figure 3. Taxonomic compositions of operational taxonomic units (OTUs) and sequence reads detected by 18S rRNA gene metabarcoding using high-throughput sequencing.

Figure 4. Non-metric, multi-dimensional scaling ordination of oceanic plankton communities. Each plot represents samples collected at a specific depth. Bray-Curtis similarity among the samples was calculated from log-transformed sequence abundance data.

Figure 5. Diversity of oceanic plankton communities detected by metagenetic analysis with respect to depth.

Figure 6. Vertical distribution of the eukaryotic plankton community. **(a)** Mean number of operational taxonomic units (OTUs) detected in each depth layer. The upper axis and bar graph represent OTUs of all eukaryotic plankton communities, and the lower axis and line plots represent OTUs of each taxon. **(b)** Distribution of oceanic plankton communities among different depths.

Figure 7. Taxonomic compositions of Alveolata operational taxonomic units (OTUs) and related sequences detected by 18S rRNA gene metabarcoding using high-throughput sequencing.

Figure 8. Taxonomic compositions of Rhizaria operational taxonomic units (OTUs) and related sequences detected by 18S rRNA gene metabarcoding using high-throughput sequencing.

Figure 9. Cluster dendrogram of Alveolata and Rhizaria. Bray-Curtis similarity among the samples was calculated using log-transformed sequence abundance data.

Figure 10. Distance-based redundancy analysis (dbRDA) ordination of Alveolata and Rhizaria communities. Each plot represents the samples collected from a specific depth. Bray-Curtis similarity among the samples was calculated using log-transformed sequence abundance data. The dbRDA was constrained by best-fit explanatory variables from a distance-based multivariate linear model (DistLM). In the dbRDA ordination, axes indicate percentage variation, in terms of total community structure, and vector overlays indicate the strength and direction of relationships between individual variables and axes.

Figure S1. Vertical profiles of the chemical environmental variables in the survey area.

Figure S2. Numbers of operational taxonomic units (OTUs) in Alveolata communities at different depths.

Figure S3. Relative abundance of Alveolata community sequences at different depths.

Figure S4. Numbers of operational taxonomic units (OTUs) in Rhizaria communities at different depths.

Figure S5. Relative abundance of Rhizaria community sequences at different depths.

Figure1

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Figure 1

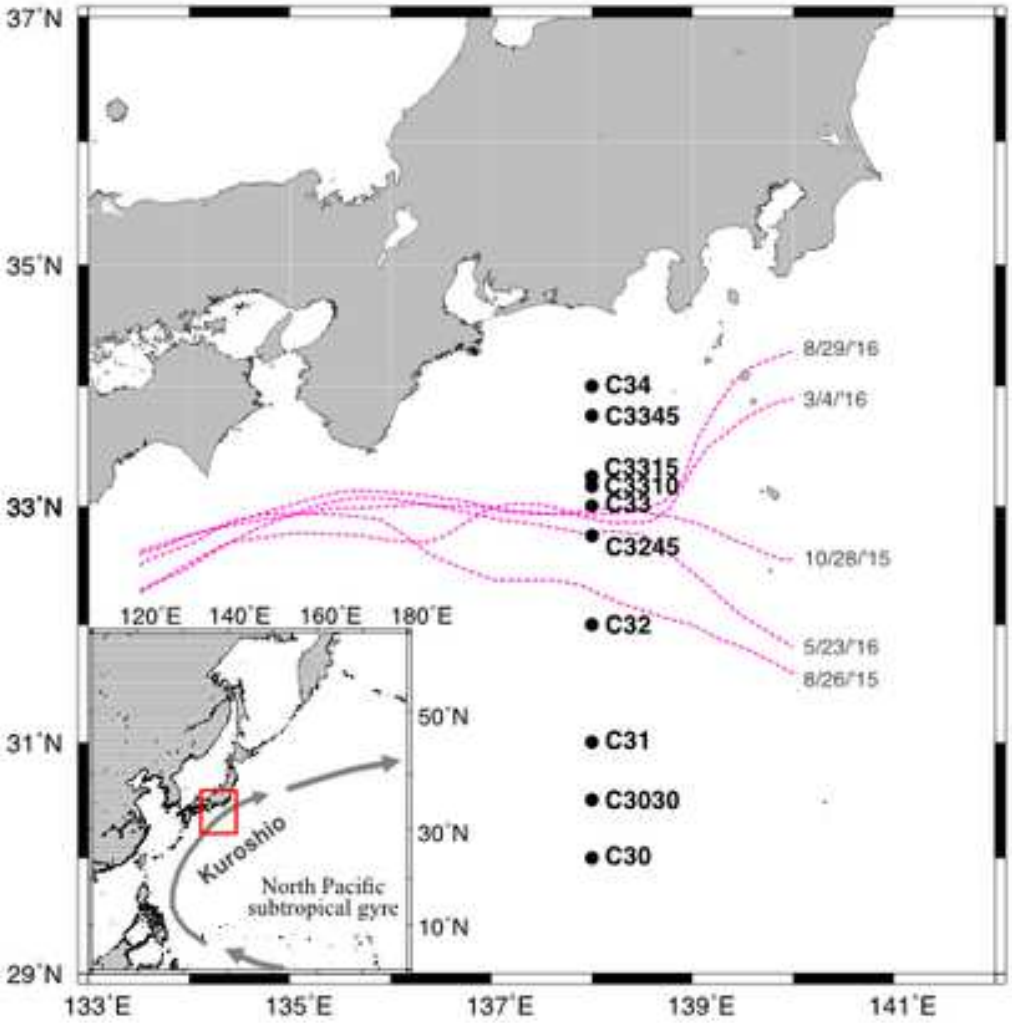

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Figure 2

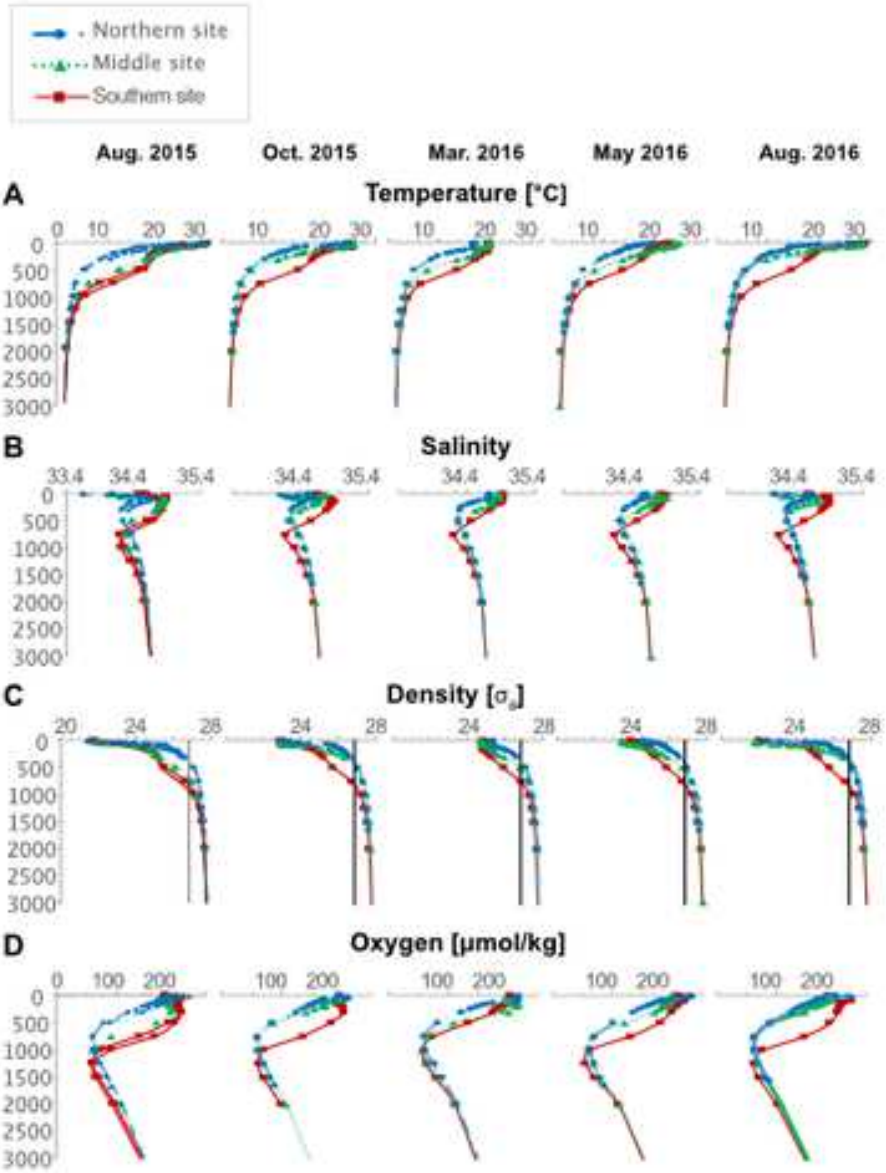

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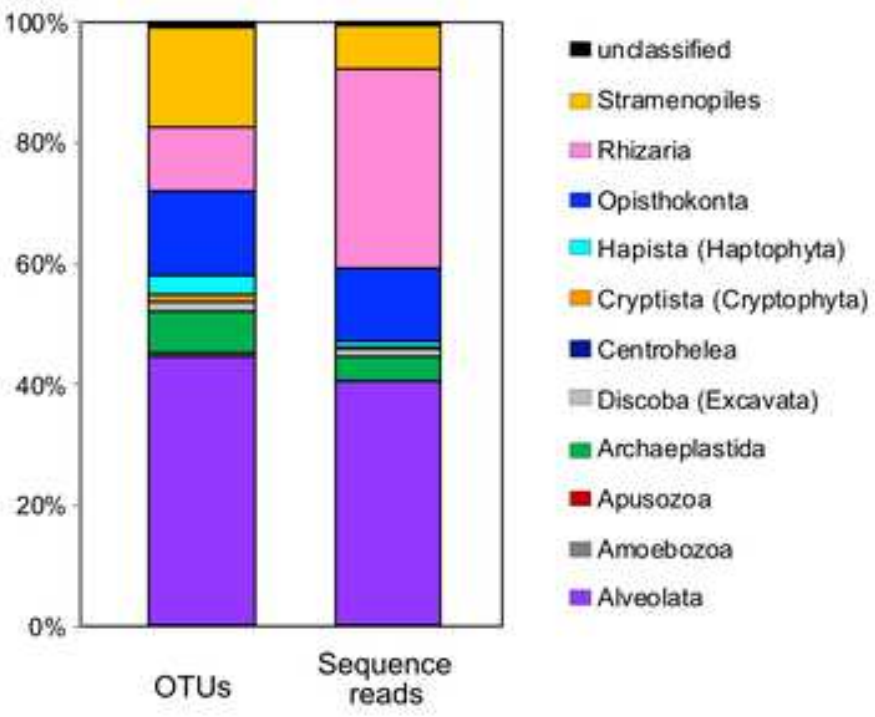
Figure 3



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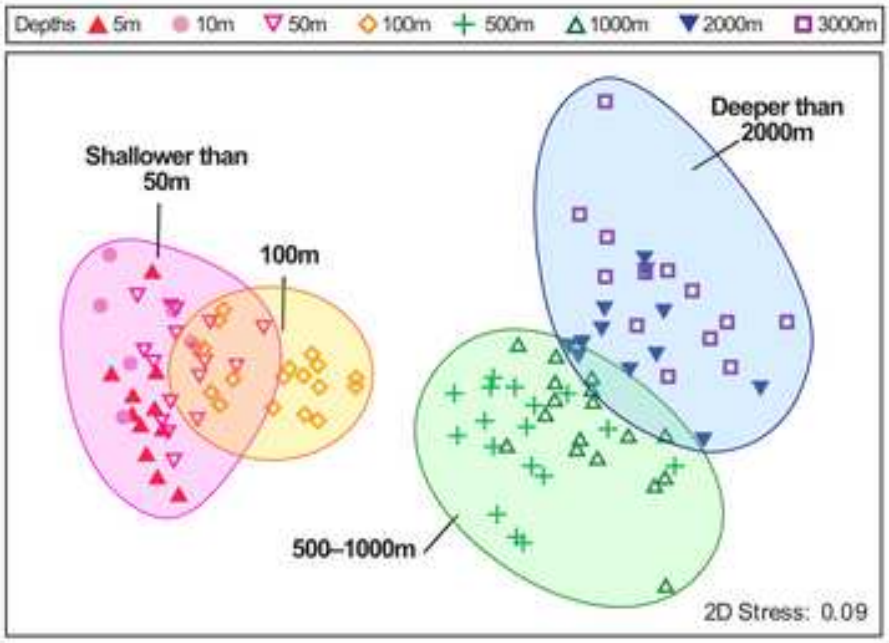

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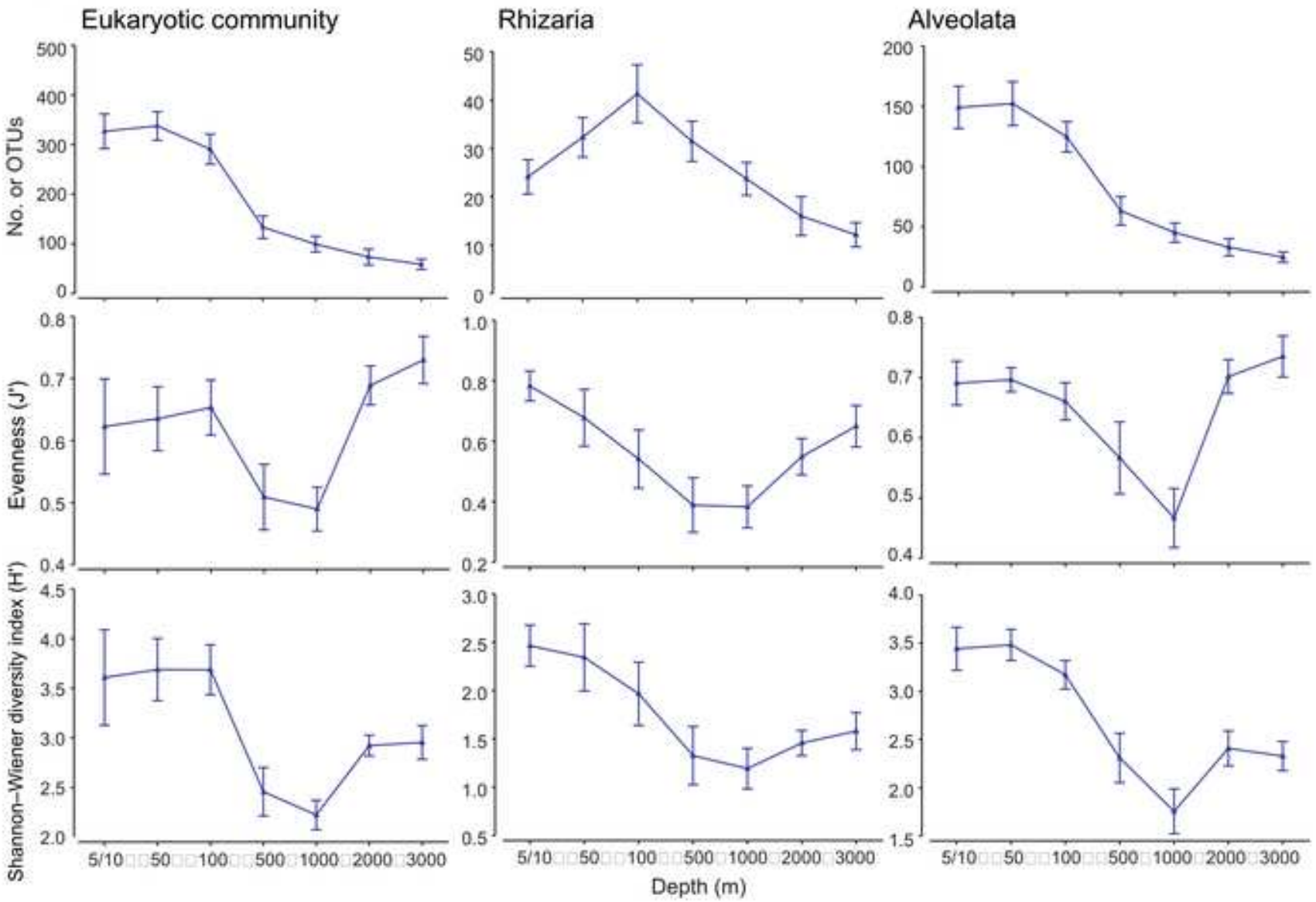

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Figure 6

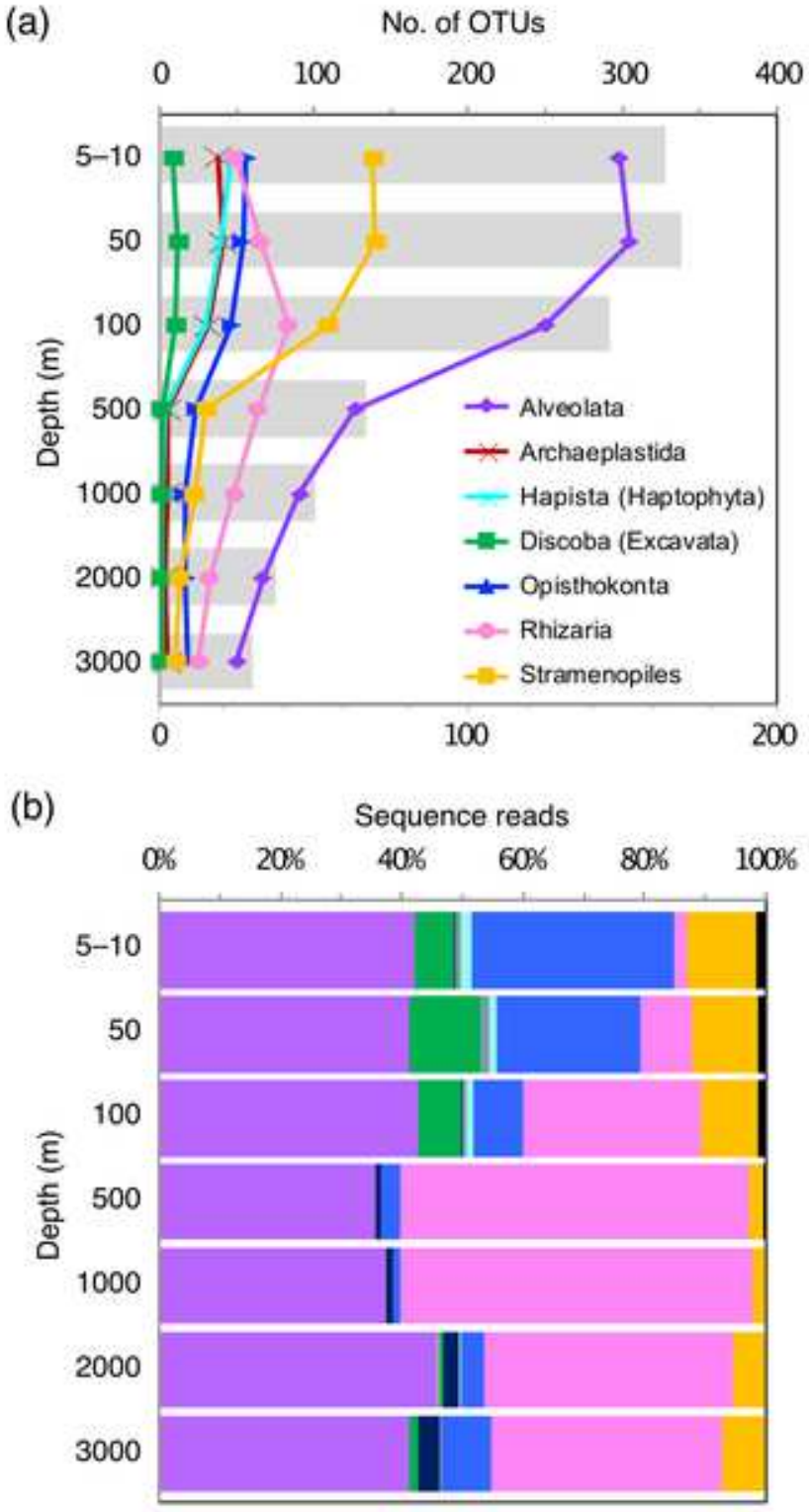

Figure7

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Figure 7

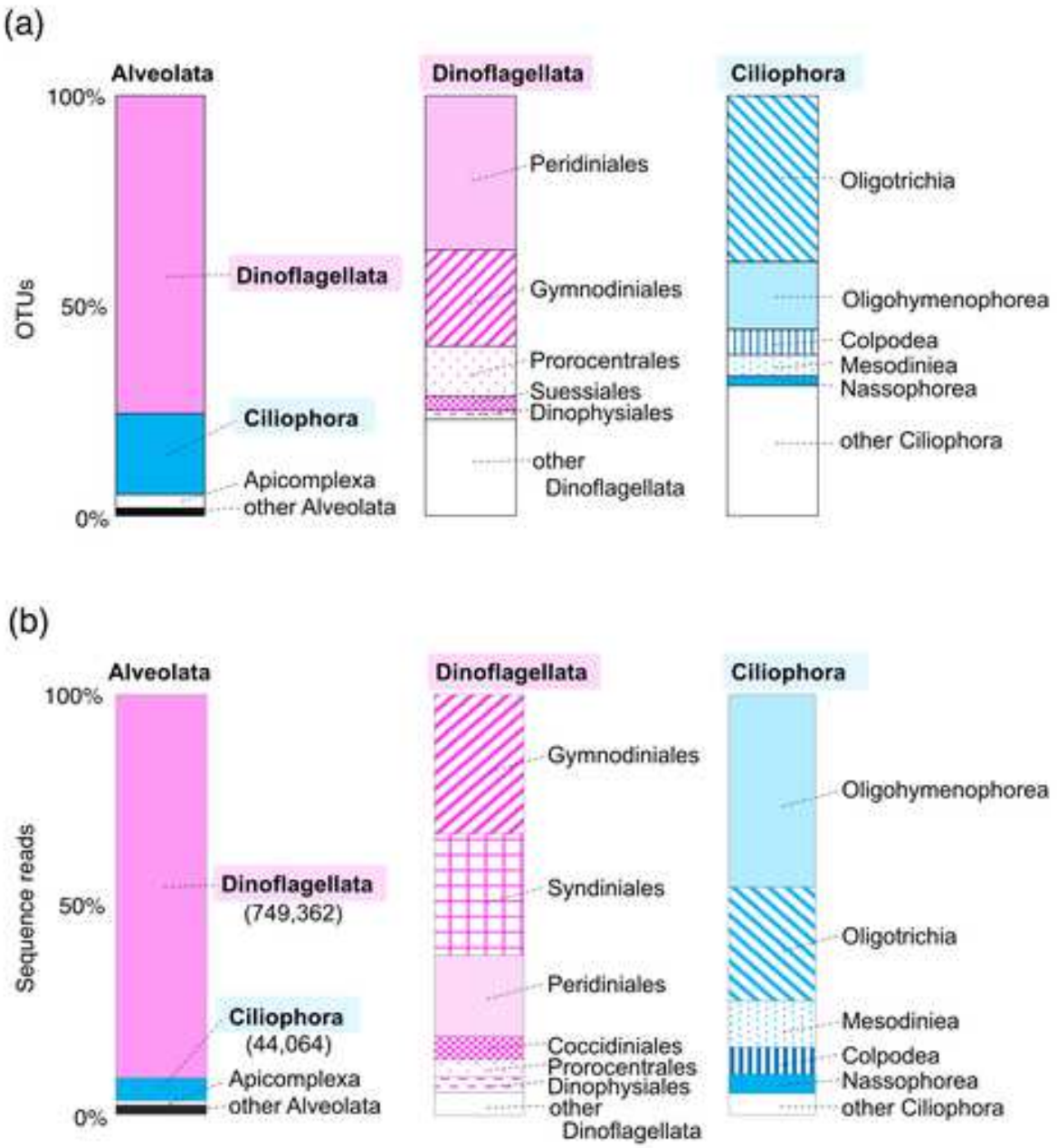

Figure8

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Figure 8

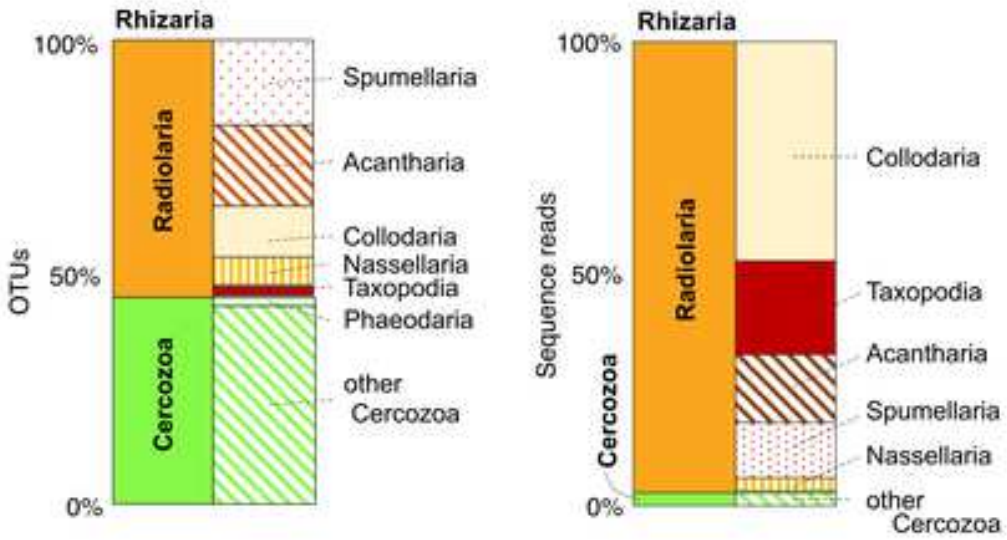

Figure9

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Figure 9

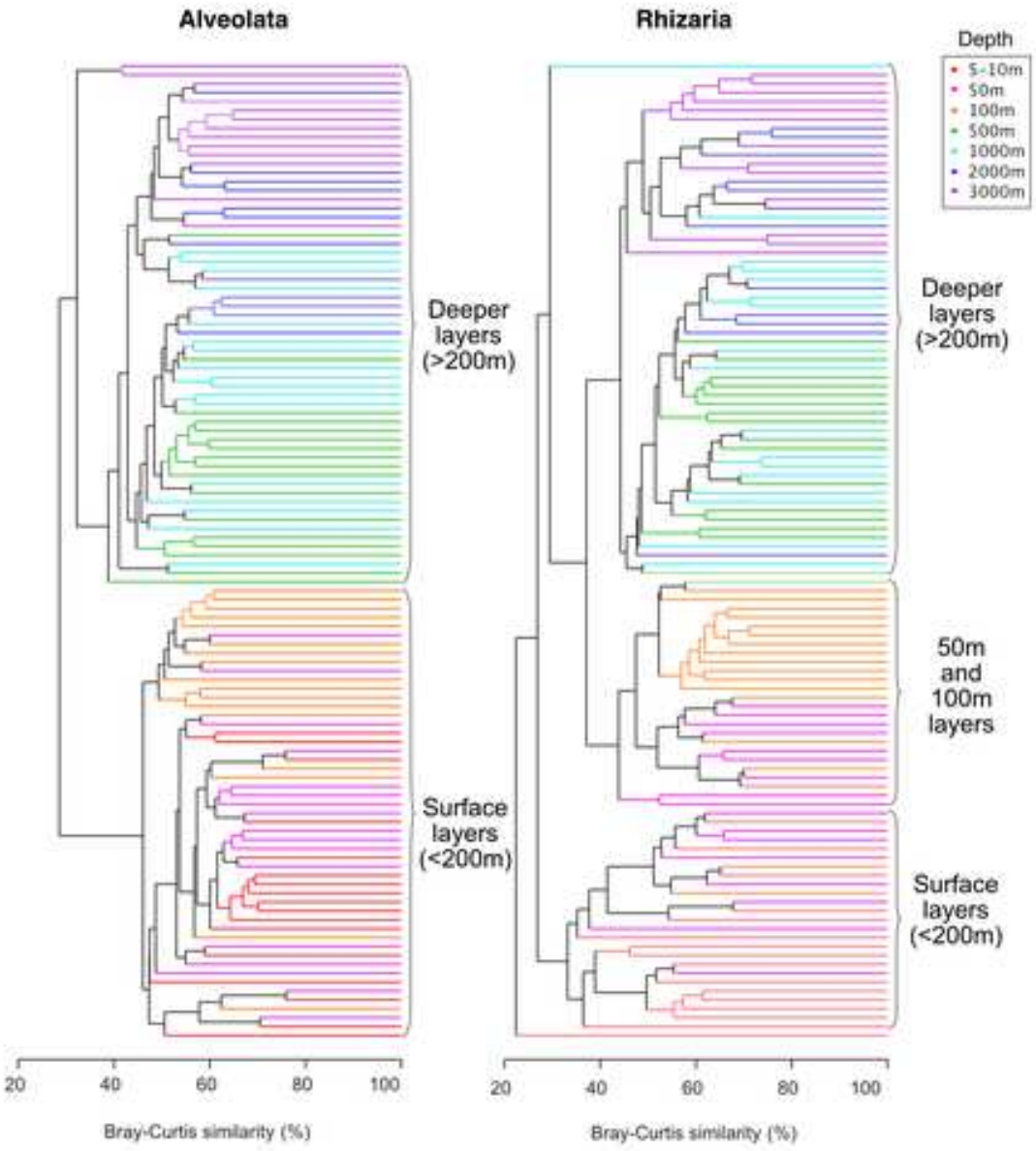

Figure10

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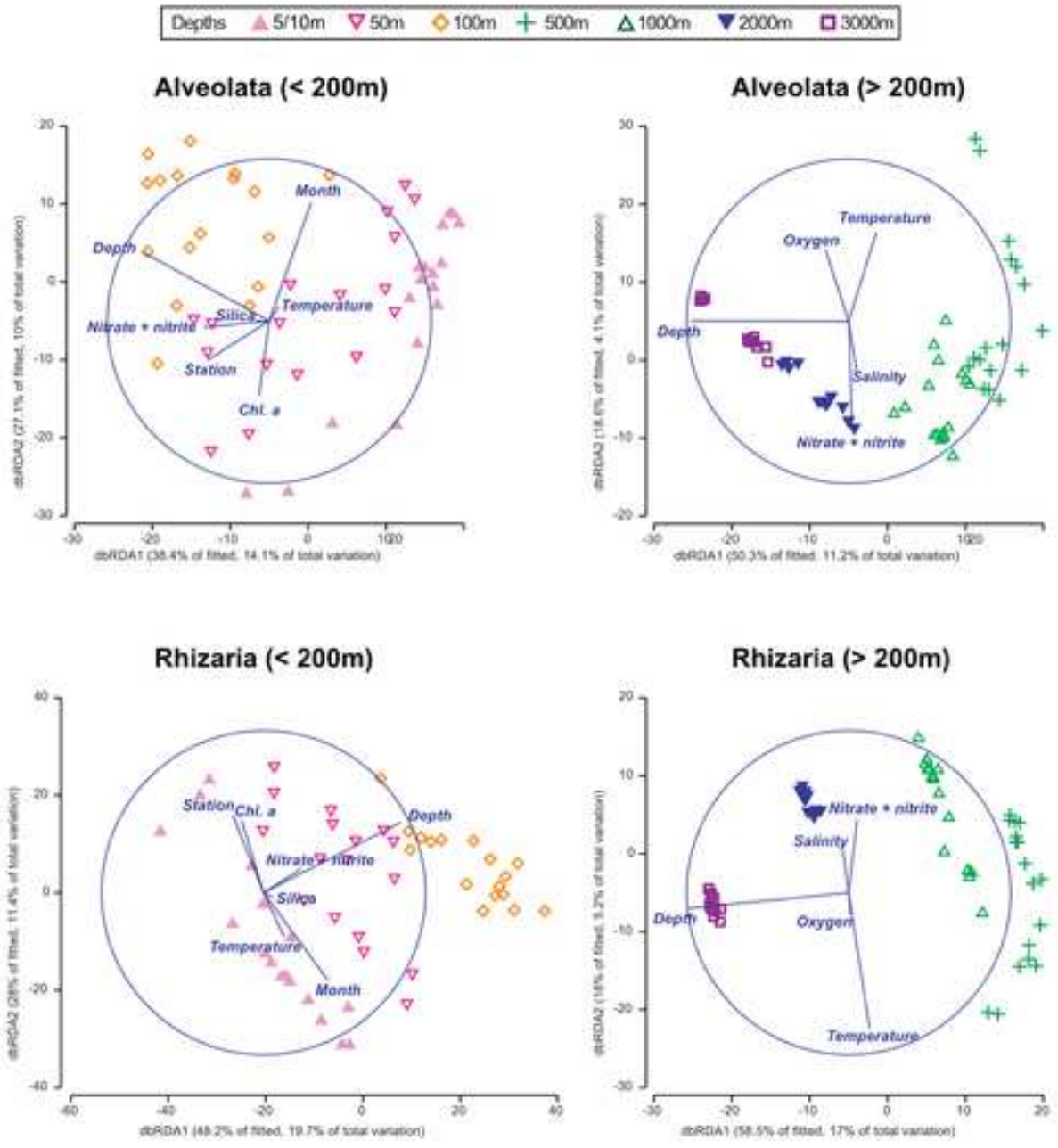
Figure 10



TABLES

Table 1. Survey cruises, sampling stations, and water depths of ~~eDNA~~ DNA maetabarcoding samples from the area adjacent to the Kuroshio Current

Cruise no.	Cruise dates	Sampling stations	Sampling water depths (m)[a]
SY1508	21 Aug to 2 Sep 2015	5 stations (C34, C33, C32, C31, C30)	5, 50, 100, 500, 1,000, 2,000, 3,000
SY1510	23 Oct to 4 Nov 2015	3 stations (C34, C3315, C3030)	5, 50, 100, 500, 1,000, 2,000, 3,000
SY1603	29 Feb to 9 Mar 2016	3 stations (C3345, C3310, C30)	10, 50, 100, 500, 1,000, 2,000, 3,000
SY1605	9 to 19 May 2016	3 stations (C34, C3245, C30)	10, 50, 100, 500, 1,000, 2,000[b], 3,000
SY1608	24 Aug to 5 Sep 2016	3 stations (C34, C33, C32)	5, 50, 100, 500, 1,000, 2,000, 3,000

[a] Sampling depths up to 1,000 m at stations C34 and C3345

[b] Not determined at C3245

Table 2. Pairwise analysis of similarities among depths

	5–10 m	50 m	100 m	500 m	1,000 m	2,000 m	3,000 m
5–10 m	-	-	-	-	-	-	-
50 m	0.128*	-	-	-	-	-	-
100 m	0.623*	0.346**	-	-	-	-	-
500 m	1**	0.999**	0.919**	-	-	-	-
1,000 m	1**	1**	0.98**	0.183**	-	-	-
2,000 m	1**	1**	0.998**	0.634**	0.394**	-	-
3,000 m	1**	1**	0.998**	0.798**	0.619**	0.221**	-

* $p < 0.001$, ** $p = 0.0001$.

Table 3. Percentage of variation explained in a distance-based multivariate linear model (DistLM) of Alveolata and Rhizaria community

Alveolata (< 200 m)			Alveolata (> 200 m)		Rhizaria (< 200 m)		Rhizaria (> 200 m)	
Variable	*p*	Variation explained (%)	*p*	Variation explained (%)	*p*	Variation explained (%)	*p*	Variation explained (%)
Depth	0.0001	11.2	0.0001	10.7	0.0001	16.5	0.0001	16.7
Temperature	0.0001	11.2	0.0001	9.6	0.0001	9.0	0.0001	14.5
Nitrate+nitrite	0.0001	13.3	0.0001	5.0	0.0001	11.3	0.0001	6.2
Salinity	0.0129	3.7	0.0001	8.1	0.0144	4.3	0.0001	12.8
Station	0.0001	5.6	0.0042	3.4	0.0086	4.8	0.09	2.6
Month	0.0001	7.5	0.4672	1.7	0.0002	7.6	0.0633	2.8
Oxygen	0.0087	3.9	0.0001	5.6	0.0003	7.2	0.0002	5.4
Position	0.0007	5.2	0.0248	2.8	0.0149	4.4	0.4255	1.7
Silica	0.0001	10.4	-	-	0.0001	7.7	-	-
Chllophyll *a*	0.0002	6.1	-	-	0.0001	8.2	-	-

Supplementary Material1

Supplementary Material2

Supplementary Material3

Supplementary Material4

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Supplementary Material
Fig_Sup4_depth_OTU_Rhiza.tiff

Supplementary Material5