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Micro-eukaryotic diversity in the surface layer of sediments from the East China Sea

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ABSTRACT

Question: What is the micro-eukaryotic diversity in the surface layer of sediments from the East China Sea?

Methods: We sequenced 332 clones from surface sediments at three different sites, and then selected 129 representative phylotypes for phylogenetic analysis. We used culture-independent molecular approaches based on 18S rRNA genes to investigate the diversity. We also used redundancy analysis to determine the relationship between sediment microbial community diversity and environmental parameters.

Results: Protists were highly diverse. Most clone sequences were affiliated to protists (72.6%), dominated by Alveolata (57.2%) and Rhizaria (12.0%). The other clone sequences belonged to Metazoa (17.6%), Stramenopiles (3.4%), fungi (2.6%), Apusozoa (2.1%) and 'unclassified' (1.7%). A number of the clones were related to anoxic environment clones or polar region clones. Redundancy analysis showed that some environmental parameters had a marked effect on the sediment microbial community diversity.

Keywords: marine sediment, micro-eukaryotic, RDA, 18S rRNA.

INTRODUCTION

The oceans harbour most of the Earth's microbial biomass (Whitman *et al.*, 1998). Microorganisms play important ecological roles in marine ecosystems as primary producers and consumers. They are also indispensable catalysers and significant transformers in oceanic biogeochemical cycles. Micro-eukaryotes, a key group of marine microbes, have been investigated in many marine environments, including oligotrophic open oceans (Worden, 2006; Schnetzer *et al.*, 2011), the polar regions (Arctic and Antarctic oceans) (Lawley *et al.*, 2004; Lovejoy *et al.*, 2006), and marine sediments (Jiang *et al.*, 2006; Takishita *et al.*, 2007a; Park *et al.*, 2008). Micro-

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eukaryotic diversity can be analysed using either culture-dependent or culture-independent methods, and a combination of these two approaches could provide deeper insight into their diversity and ecological importance (Jebaraj *et al.*, 2010). A molecular approach (culture-independent methods) to studying micro-eukaryotic diversity through sequencing eukaryotic 18S rRNA from various marine environments has been shown to be the method of choice. It was used frequently in the above studies, and an unexpectedly high diversity of micro-eukaryotes was revealed. However, Behnke *et al.* (2006) pointed out that a large-scale multi-primer rRNA approach still misses a substantial part of a sample's rRNA diversity, so that multiple PCR primer combinations may be needed in surveys of micro-eukaryotic diversity.

The East China Sea (ECS), the largest continental marginal sea in the Western Pacific, is influenced by several water systems, including nutrient-enriched freshwater input from the Yangtze River, and warm and oligotrophic seawater from the Kuroshio Current and Taiwan Strait Current (Jiao *et al.*, 2007). The ECS has a variety of hydrological conditions, significant physical and chemical gradients, obvious seasonal variations, and high primary productivity, making it a favourable place to study microbial community diversity. Being the interface between land, freshwater, and marine environments, the ECS is extremely complicated and dynamic. But only a few molecular surveys of micro-eukaryotic diversity in ECS benthic environments have been reported (Park *et al.*, 2008). Hence, our understanding of the composition, diversity, and evolution of micro-eukaryotes in the ECS is very limited.

In this study, we used 18S rRNA analysis to examine micro-eukaryote diversity in the surface layer of sediments at three different sites in the ECS. We investigated the molecular phylogenetic relationships between environmental disturbances and patterns in microbial diversity, as well as their ultimate effects on ecosystem function.

MATERIALS AND METHODS

Sampling sites and sampling

We chose three sampling sites – DH-9 (30°45.650′N, 122°06.617′E), DH-13 (31°30.103′N, 123°29.844′E), and DH-18 (30°00.049′N, 122°52.467′E) – in Hangzhou Bay and the adjacent coastal area of the ECS (Fig. 1). We collected sediment samples using a 0.1 m^2 stainless steel Gray O'Hara box corer in April 2007. On retrieval, the surface layer (0–10 cm) of each sample was divided into four separate layers (0–3, 3–5, 5–8, and 8–10 cm). In total, we obtained 12 marine sediment samples. Each sample was then packaged into a sterile sample bag and stored in airtight sterile plastic bags in a freezer at −20°C during the cruise and at −80°C after return to the laboratory.

Environmental parameters

Subsamples from the different sediment depths were thawed and mixed. For each sample, 5 g of wet sediment was transferred into a 500 mL sterile quartz conical flask with 200 mL diluted water and rotated for 30 min. Then we left samples to stand for 10 min, before removing the supernatant vacuum over 0.45 μm membrane filters. The filtrate was collected for the determination of pore water total organic carbon (TOC), and the surplus filtrate was stored at −20°C. Pore water TOC, and total carbon (TC) and total nitrogen (TN) in the

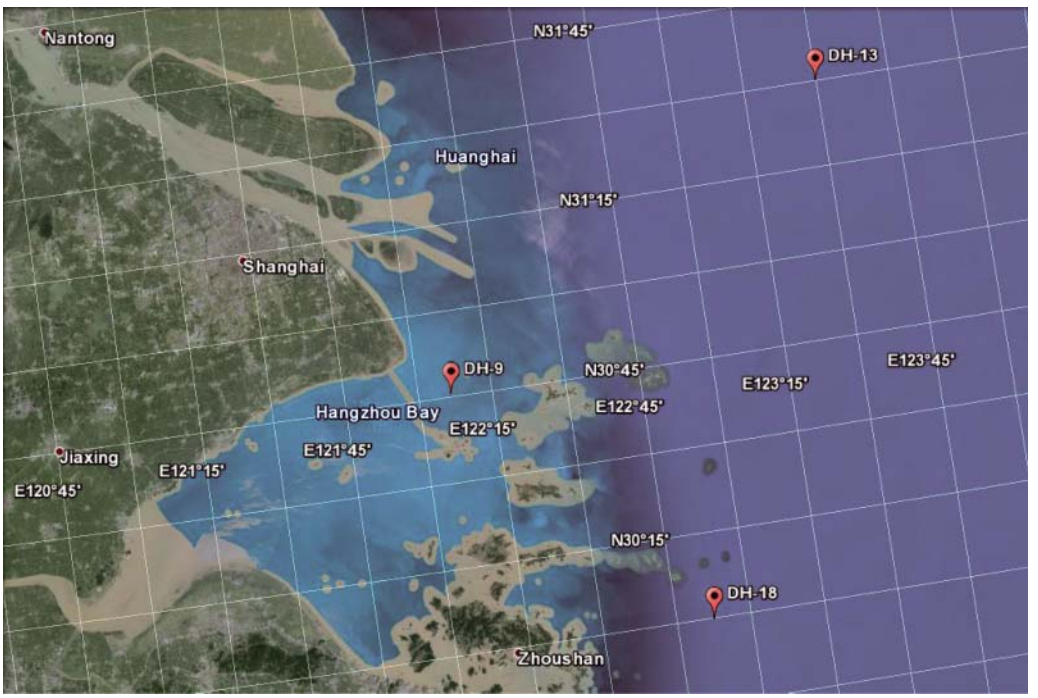


Fig. 1. Map showing the sampling sites in the East China Sea.

dried sediment were measured using a TOC analyser (Shimadzu Corporation, Kyoto, Japan). Aliquots (50 mL) of the filtrate were treated with 10 μL ultrapure 1 M HCl and stored at 4°C for the determination of total phosphorous (TP). The TP concentration was determined colorimetrically using an Automatic Discrete Analyzer SmartChem 200 (AMS, Roma, Italy).

DNA isolation and amplification of 18S rRNA gene regions, cloning and sequencing

For each sample, about 0.5 g (wet weight) of sediment was thawed on ice and transferred to a Lysing Matrix E tube for DNA extraction with a FastPrepfi-24 rapid nucleic acid extraction instrument (MP Biomedicals) and a FastDNA Spin Kit for Soil. We measured the concentration of purified DNA using a protein nucleic acid detector (Bio-Rad). DNA samples were amplified using the universal primers NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and NS4 (5′-CTTCCGTCAATTCCTTTAAG-3′) (White *et al.*, 1990) for micro-eukaryotic 18S rRNA genes, and adopting the following PCR program: 94°C for 5 min, 35 cycles of 94°C for 1 min, 58.5°C for 30 s, 72°C for 1 min, and a final elongation step of 72°C for 10 min. The PCR products were then stored at 4°C. The PCR mixtures (25 μL) contained 1 μL template DNA, 2.5 μL $MgCl_2$, 2.5 μL $MgCl_2$ buffer, 2 μL of each dNTP, 1 μL of each primer and 0.5 U of Taq DNA polymerase, and 14.5 μL ddH_2O.

The PCR products were purified using a TIANgel Midi Purification Kit following the manufacturer's instructions and quantified using NanoDrop ND-3000. Subsequently,

the PCR products were cloned into the pMD18-T vector system (TaKaRa, Japan) using *Escherichia coli* DH5α as the host. We selected positive clones using the blue-white selection method. We screened libraries for the 1.5 kb 18S rDNA inserted by PCR with M13 primers. We determined 18S rRNA gene sequences using an ABI3730XL Automated Sequencer.

Sequence and community analysis

We submitted sequences to the CHECK-CHIMERA program of the Ribosomal Database Project (rdp.cme.msu.edu) to detect chimeric artifacts, which we deleted from the dataset (Wang *et al.*, 2007). We searched for similar sequences using the National Center for Biotechnology Information (NCBI) BLASTN program (www.ncbi.nlm.nih.gov/BLAST) for phylogenetic analysis. Sequences that showed no relatedness with a known eukaryotic phylogenetic group in the NCBI Taxonomy database were listed as unclassified. Representative sequences of the dominant genera were selected and aligned using the CLUSTAL X program (Thompson *et al.*, 1994). Phylotypes closely related to Metazoa were discarded.

Simpson's diversity index and the estimated number of species [Abundance-base Coverage Estimator, ACE (Chao and Lee, 1992)] were calculated for all samples on the basis of phylotype distribution (97% similarity), then clustered as operational taxonomic units (OTUs) at an overlap identity cut-off level of 97% using the DOTUR program (Schloss and Handelsman, 2005). Sequences with greater than 97% similarity were considered to belong to the same phylotype. Accordingly, we define phylotype as a group that encompasses all sequences of at least 97% similarity to each other. Evolutionary distances of full-length sequences were calculated according to the Kimura two-parameter correction method (Kimura, 1980), after which neighbour-joining trees were constructed with 1000 bootstrap samplings using MEGA4.

The Hellinger transformation (Legendre and Gallagher, 2001) was applied to the data. Then, using the vegan package in the integrated suite of software facilities, R (Oksanen *et al.*, 2010), we used redundancy analysis (RDA) to examine the correlation between micro-eukaryotic diversity and environmental parameters. In the RDA plots, the length of the line indicated how much variance was explained by that factor, and the direction of the arrows for individual environmental factors indicated an increasing concentration of that factor.

Nucleotide sequence accession numbers

We deposited all 18S rRNA gene sequences obtained in this study in the GenBank database under accession numbers KP685238 to KP685366.

RESULTS AND DISCUSSION

Micro-eukaryotic diversity and their rarefaction curves

To compare the micro-eukaryotic diversity of the three sites, we calculated ACE and Simpson's diversity index (Table 1) using the software DOTUR. The results of this analysis were corroborated by the statistical data above. These diversity measures illustrated clear differences in sediment microbial diversity between sites and indices, and a decreasing

Table 1. Measures of micro-eukaryotic diversity in the surface layer of sediments from the East China Sea

Site	No. of clones	OTUs	ACE	Simpson index (D, $\times 10^{-2}$)
DH-9	100	31	61.45	9.17
DH-13	129	81	320.71	1.53
DH-18	59	59	108.27	1.21

Note: OTUs = operational taxonomic units; ACE = Abundance-base Coverage Estimator.

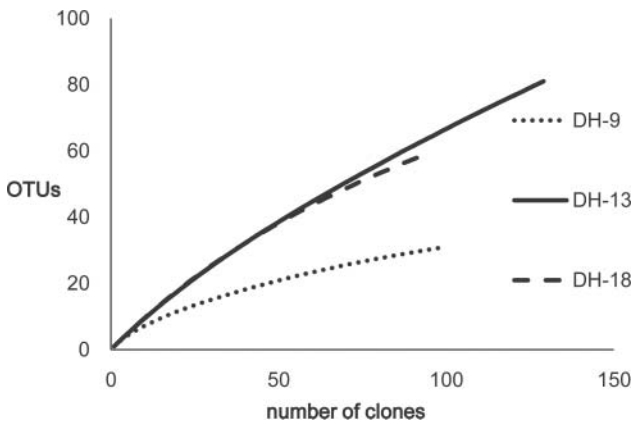


Fig. 2. Rarefaction curves for the number of clones at each of the three sites in the East China Sea (OTUs = operational taxonomic units).

diversity from DH-13 to DH-18 to DH-9. The results also showed that there were more clones at site DH-13 than at the other two sites, but the number of clones (Fig. 2) at each site was still limited. Overall, our data indicate that the sampling sites probably have higher micro-eukaryotic genotype diversity than we collected in our study. Future studies should identify more clones from different samples to help provide a more complete picture of micro-eukaryotic diversity in the ECS. The rarefaction curves (Fig. 2) also supported the conclusion that site DH-13 exhibited higher diversity than sites DH-18 and DH-9.

From the clone libraries of micro-eukaryotic 18S rRNA genes, we identified a total of 322 18S rRNA gene sequences (site DH-9: 100 clones; site DH-13: 129 clones; site DH-18: 93 clones). After BLAST sequence comparison with sequences in databanks, we obtained our first insight into the diversity of micro-eukaryotic groups represented in the sediment (Fig. 3). The majority of clone sequences belonged to protists (72.6%). The protists Alveolata (57.2%) and Rhizaria (12.0%, most of which were Cercozoa) were dominant at these three sample sites. The other clone sequences belonged to Apusozoa (2.1%) and Stramenopiles (3.4%). Metazoa constituted about 17.6% of the total clones and fungi about 2.6%, with Chytridiomycota the dominant group. Clones of Cryptophyta, Viridiplantae, and an unclassified group constituted about 5.1% of the total. Site DH-13 and site DH-18 shared similar microbial communities, whereas site DH-9 was significantly different from these two sites with only three groups present: Alveolata (92.0%), Metazoa (6.0%), and an

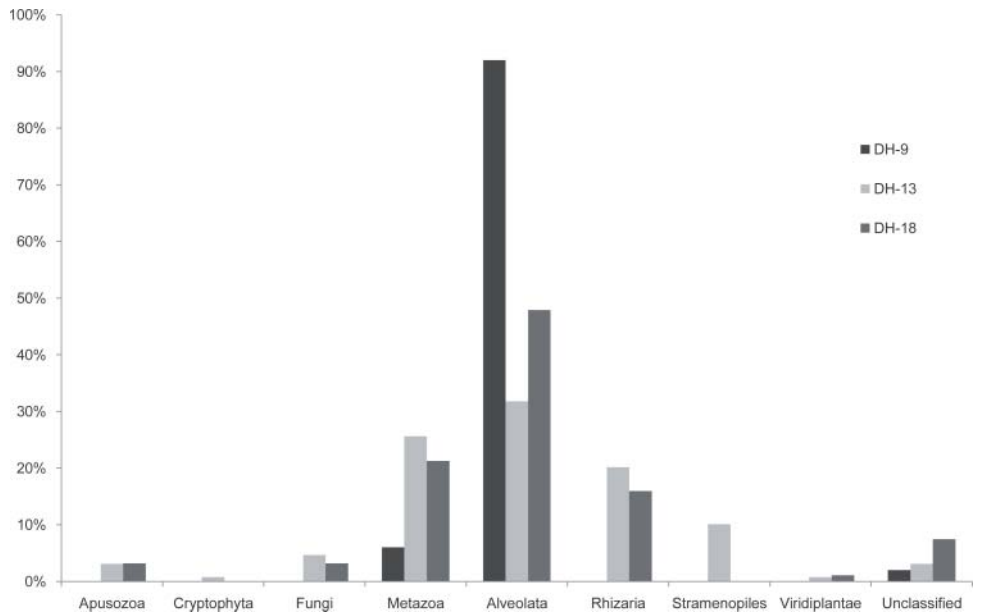


Fig. 3. Proportions of each phylotype at each of the three sites in the East China Sea.

unclassified group (2.0%). Our results indicate that the near-shore site DH-9 has the least biological diversity, probably because of poor water quality due to Changjiang Diluted Water from the mainland and near-shore environmental pollution from human activity.

After discarding the phylotypes closely related to the Metazoa of Opisthokonta, we selected representative sequences randomly from the OTUs at each site for phylogenetic analysis as three groups: (A) Alveolata, (B) Rhizaria, and (C) other phylotypes.

Alveolata

Alveolata sequences were the most abundant and diverse in our samples. We detected 67 phylotypes of Alveolata (Fig. 4).

Phylotypes related to Dinophyceae made up the largest part of the Alveolata phylogenetic tree. A total of 11 sequences from site DH-9 were closely related to *Scrippsiella* sp. SCKS 0701 (96–99%), a species of dinoflagellate found in coastal waters off western Korea. Phylotypes F.18 1132 and 1119 branched with *Pentapharsodinium tyrrhenicum*, F.13 3105, 5116, and 2064 branched with *Heterocapsa triquetra*, and F.18 492 and 1122, and F.13 519 branched with *Gonyaulax spinifera*, all of which been reported in a study that tested classical evolutionary hypotheses with molecular systematic methods (Saunders *et al.*, 1997). Phylotype F.13 643 grouped strongly with *Gyrodinium fusiforme*, a heterotrophic dinoflagellate found in Otaru Bay in Hokkaido, Japan. Phylotypes F.13 3111 and 2081, F.18 123, a cluster of F.18 478, 493, and 313 together with F.13 3123, 2133, 3132, and 3115 branched with *Woloszynskia cincta*; *W. cincta* is also found in western Korean waters. Phylotypes F.13 515 and 511 were closely related to the newly reported *Alexandrium satoanum* (Kim *et al.*, 2005). Some of our clones branched with uncultured reference sequences: F.13 3413 (clone S49), F.13 3115 and F.18 317 (clone B57), F.18 124 (clone NA2 4B10),

F.18 130 (clone AT4-98), and F.13 3142 and 3138 (clone CCW105). None of the latter clones showed close affiliation with any currently known species, though the reference sequences were all isolated from marine environments. Thus these clones may represent a new lineage of Dinophyceae.

Phylotypes related to Ciliophora were the next largest group of Alveolata. Phylotypes F.18 481, 475, and 489 were similar to *Strombidium* sp. SBB99-1, which was isolated from Buzzards Bay, USA. Phylotype F.13 2032 was closely related to *Paradiscocephalus elongates* (98%), a discocephalid ciliate found in the Yellow Sea, China. Phylotype F.9 350 grouped with *Strombidium basimorphum* (96%), which belongs to Spirotrichea, a group of Ciliophora. Phylotypes F.13 4109 and F.18 14 were closely related to the uncultured marine eukaryote clone UI13F02 (both 99%). Notably, clone UI13F02 was found in a hypersaline anoxic environment (Alexander *et al.*, 2009). Phylotype F.18 32 branched with *Eutintinnus pectinis* clone Epec99ssu_3 (93%). Phylotypes F.18 39 and 319, and F.13 1140 were closely related to *Strombidinopsis acuminata*.

A small group of phylotypes related to Apicomplexa also contributed to the Alveolata phylogenetic tree. There were four representative sequences that branched with one reference clone only. Phylotypes F.18 134 and 122, and F.13 3125 branched with *Gregarinidae* sp. with >95% similarity, but phylotype F.18 318 had relatively low similarity with *Gregarinidae* sp. (91%).

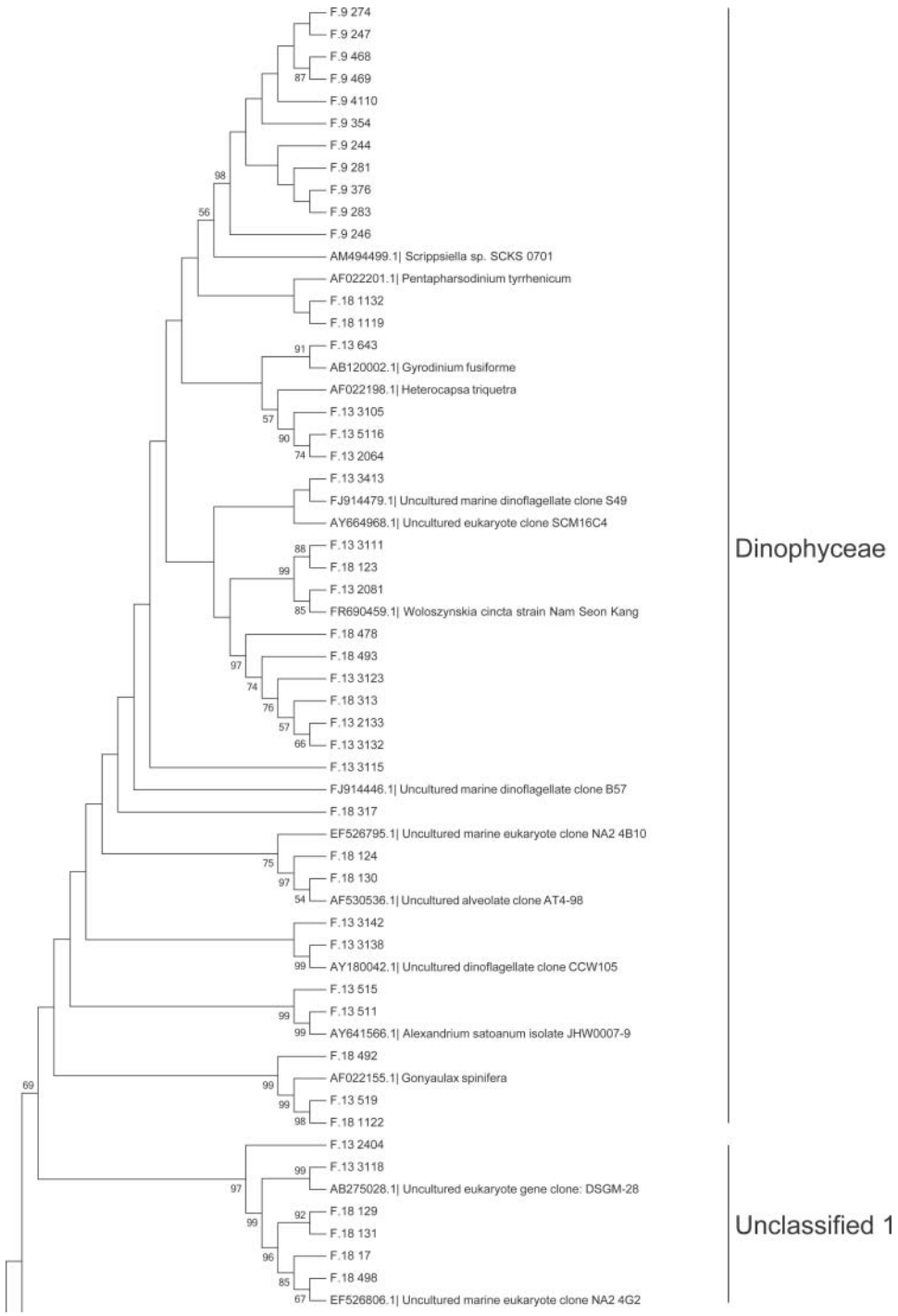
In the Alveolata phylogenetic tree, we also found several sequences grouped into independent clades that did not show a close affiliation with any currently known species. These were termed Unclassified 1 (F.13 2404 and 3118, and F.18 129, 131, 17, and 498) and Unclassified 2 (F.13 544 and 541, and F.18 480, 373, and 34). The reference clone DSGM-28 (in Unclassified 1) was found in deep-sea sediment and grouped in Marine Alveolata group I by Takishita *et al.* (2007b). Clone NA2_4G2 was found in the anoxic Framvaren Fjord (Norway). Clone 31 (in Unclassified 2) had no details, but was submitted from a study on the leaf-epiphytic community of seagrass *Posidonia oceanica* (Medina-Pons *et al.*, 2009). Unclassified 1 and Unclassified 2 may represent novel species previously unrecognized due to a lack of sequence characters.

Rhizaria

We detected 34 phylotypes of Rhizaria (Fig. 5).

Phylotypes related to Cercozoa made up the vast majority of the sequences. Phylotype F.13 4120 was closely related to *Cryothecomonas* sp. APCC MC5-1Cryo, and phylotypes F.13 2092 and 4408 formed a lineage with *Thaumatomastix* sp. CC002; these have all been used to infer the evolutionary relationships among marine cercozoans (Chantangsi *et al.*, 2010). Phylotype F.18 19 branched with *Ebria tripartita*. Hoppenrath and Leander (2006) proposed that *E. tripartita* is a phagotrophic flagellate present in marine coastal plankton communities worldwide.

In addition to the sequences mentioned above, other sequences had no clear affinities to previously described species. Phylotypes F.13 3141, 646, 1103, 2101, 5112, 642, 4132, 4115, and 2103, and F.18 33, 117, and 316 all showed affinity to uncultured cercozoan clones (clone C9, clone PP2-2C, clone 12-4.4, clone 12-3.6, clone C1, clone 12-4.1 in Fig. 5), revealing the remarkably high global biodiversity of Cercozoa (Bass *et al.*, 2004). Phylotype F.13 2125 branched with clone RM1-SGM41, and F.13 4123 and F.18 375 branched with clone DSGM-49. These two clones were found in sediments from a deep-sea methane cold seep


F.9 274
F.9 247
F.9 468
F.9 469
F.9 4110
F.9 354
F.9 244
F.9 281
F.9 376
F.9 283
F.9 246
AM494499.1| Scrippsiella sp. SCKS 0701
AF022201.1| Pentapharsodinium tyrrhenicum
F.18 1132
F.18 1119
F.13 643
AB120002.1| Gyrodinium fusiforme
AF022198.1| Heterocapsa triquetra
F.13 3105
F.13 5116
F.13 2064
F.13 3413
FJ914479.1| Uncultured marine dinoflagellate clone S49
AY664968.1| Uncultured eukaryote clone SCM16C4
F.13 3111
F.18 123
F.13 2081
FR690459.1| Woloszynskia cincta strain Nam Seon Kang
F.18 478
F.18 493
F.13 3123
F.18 313
F.13 2133
F.13 3132
F.13 3115
FJ914446.1| Uncultured marine dinoflagellate clone B57
F.18 317
EF526795.1| Uncultured marine eukaryote clone NA2 4B10
F.18 124
F.18 130
AF530536.1| Uncultured alveolate clone AT4-98
F.13 3142
F.13 3138
AY180042.1| Uncultured dinoflagellate clone CCW105
F.13 515
F.13 511
AY641566.1| Alexandrium satoanum isolate JHW0007-9
F.18 492
AF022155.1| Gonyaulax spinifera
F.13 519
F.18 1122
F.13 2404
F.13 3118
AB275028.1| Uncultured eukaryote gene clone: DSGM-28
F.18 129
F.18 131
F.18 17
F.18 498
EF526806.1| Uncultured marine eukaryote clone NA2 4G2
Dinophyceae
Unclassified 1

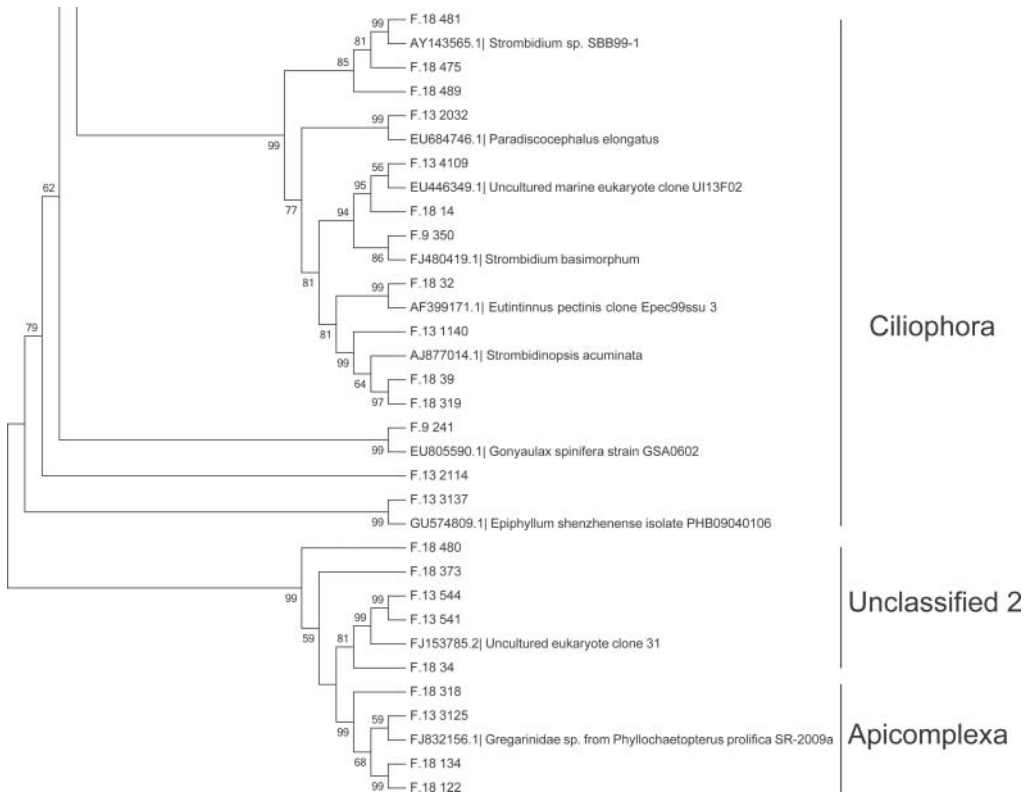


Fig. 4. Phylogenetic tree of Alveolata based on 18S rRNA gene sequences.

in Sagami Bay, Japan, during an investigation of the diversity and ecology of microbial eukaryotes in this ecosystem (Takishita *et al.*, 2007b, 2010).

Phylotypes related to Haplosporidia formed the smallest part of the Rhizaria phylogenetic tree. Phylotype F.18 293 branched with *Bonamia ostreae*, an oyster pathogen included in Haplosporidia (Carnegie *et al.*, 2000).

We also found several phylotypes (F.13 2119, 3135, 1139, 4422, 2126, and 2073 and F.18 320, 37, 473, and 395) that showed no close affiliation with any currently known species. These were termed Unclassified 3.

Other phylotypes

We also detected a further 28 phylotypes (Fig. 6).

Among these, we found phylotypes related to fungi. Phylotypes F.13 3143 and 6416 formed a monophyletic lineage with *Chytridium polysiphoniae*, a pathogen of marine microalgae (Küpper *et al.*, 2006). Phylotypes F.18 135 and 121 were related to uncultured Chytridiomycota clones, which have been shown to be a dominant fungal community in high-elevation soils (Freeman *et al.*, 2009). Phylotypes F.13 2132 and 1141, and F.18 384 branched with uncultured eukaryote clones, and had no clear affiliation with known fungal species sequenced to date; however, the environmental sequences NAMAKO-35 and DSGM-64

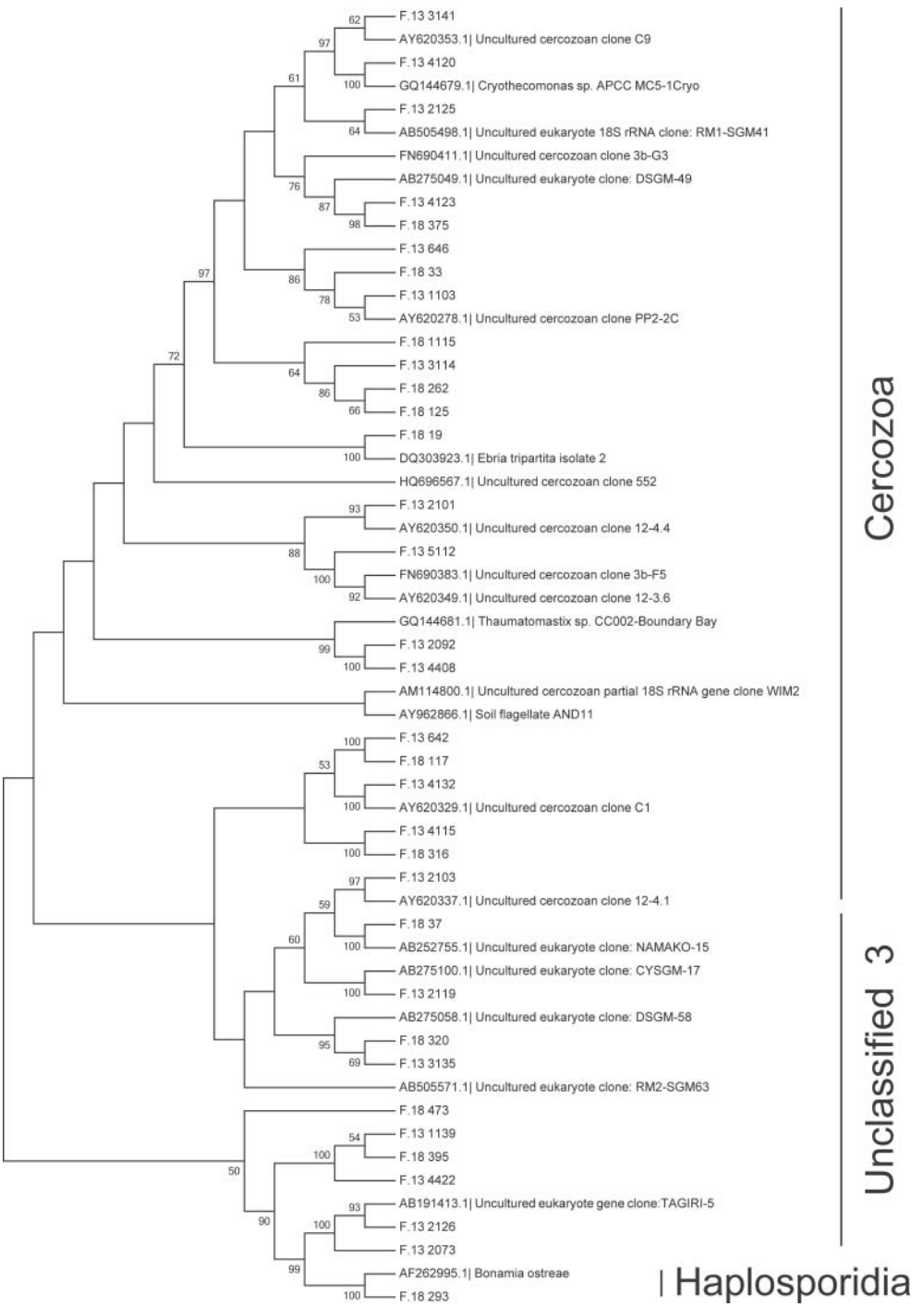


Fig. 5. Phylogenetic tree of Rhizaria based on 18S rRNA gene sequences.

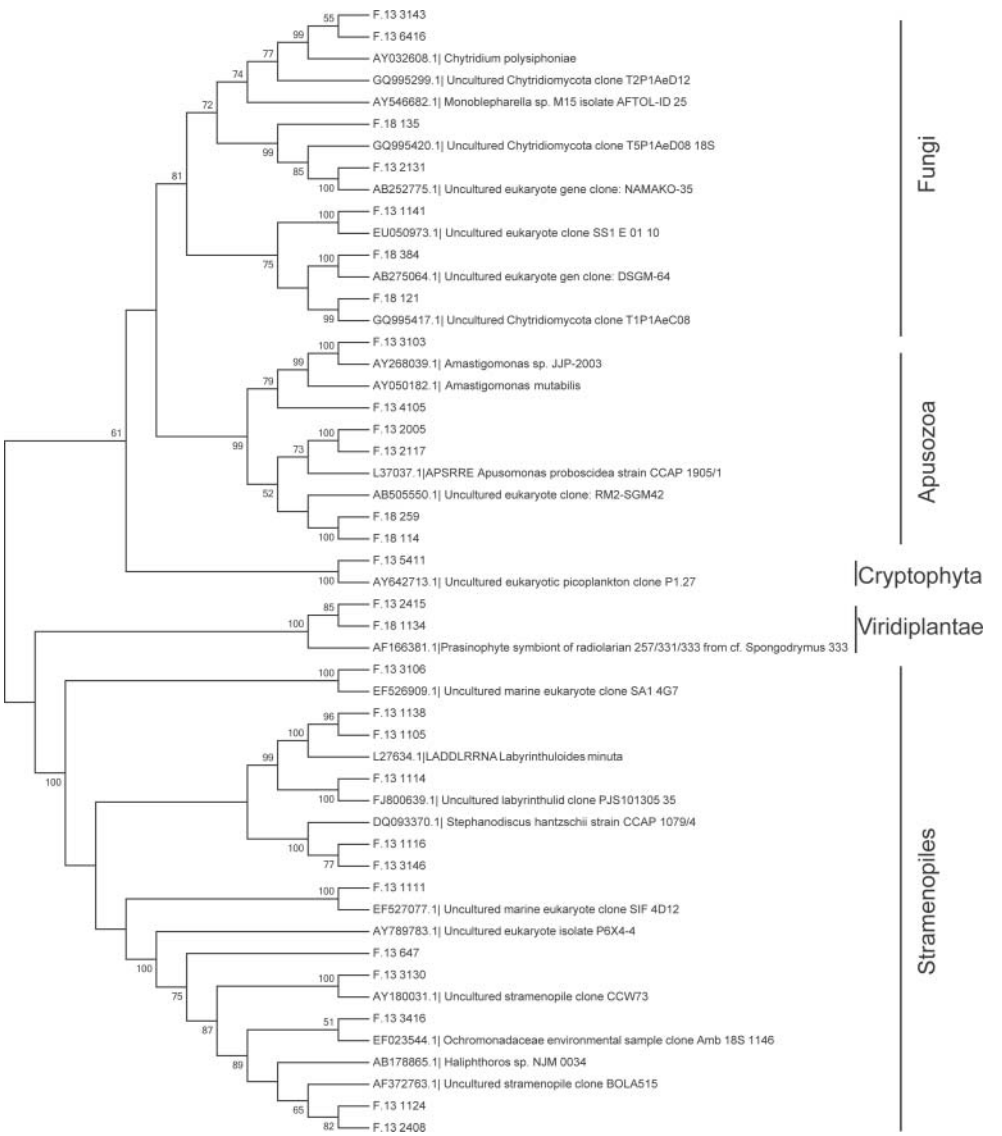


Fig. 6. Phylogenetic tree of other phylotypes based on 18S rRNA gene sequences.

were from oxygen-depleted sediments (Takishita *et al.*, 2007a), while sequence SS1_E_01_10 was from Arctic sediments (Tian *et al.*, 2009). Judging from these environmental sequences, we suggest that the organisms related to these phylotypes are anaerobic.

We also found phylotypes related to Apusozoa, Cryptophyta, and Viridiplantae. Two of the sequences belonged to the genera *Amastigomonas*: F.13 3103 was related to *Amastigomonas* sp. JJP-2003 (96%), and F.13 4105 was similar to *Amastigomonas mutabilis* (94%). Phylotypes F.13 2005 and 2117 formed a monophyletic lineage with the genera *Apusomonas* (*A. proboscidea* strain CCAP 1905/1). Phylotypes F.18 259 and 114 were related to the environmental sequence RM2-SGM42, which was found in deep-sea cold seep

sediments (Takishita *et al.*, 2010). Phylotype F.13 5411 was robustly related to the environmental sequence P1.27 (98%), which was reported to branch with Cryptophyta (Lefranc *et al.*, 2005). Phylotypes F.13 2415 and F.18 1134 belonged to Prasinophyte of Chlorophyta, found as algal symbionts of a planktonic foraminifer and a solitary radiolarian (Gast *et al.*, 2000).

Finally, we found phylotypes related to Stramenopiles. Phylotypes F.13 1138 and 1105 branched with a *Labyrinthuloides* species, while F.13 1116 and 3146 formed a monophyletic lineage with *Stephanodiscus hantzschii*. Phylotype F.13 3416 was similar to Ochromonadaceae clone Amb_18S_1146 (98%). Phylotypes F.13 3106, 1114, 1111, 647, 3130, 1124, and 2408 branched with environmental sequences that had no clear affiliation with known stramenopile species sequenced to date. However, the environmental sequences SA1_4G7, SIF_4D12, CCW73, and BOLA515 were all found in anoxic environments, suggesting that these phylotypes are anaerobic organisms.

Distribution in relation to environmental parameters

The environmental parameters of the three sites are listed in Table 2. Although the total inorganic carbon (TIC) and total phosphorous (TP) concentrations were approximately the same across the three sites, there were slight differences. Unusually, the farthest site from the mainland, DH-13, had the highest concentrations of total carbon (TC) and total organic carbon (TOC). This could be because site DH-13 is located at 123°29.844′E, where there is a confluence of Yellow Sea Cold Water from the north, Changjiang Diluted Water from the east, and the Taiwan Warm Current from the south. We observed a high density of large organic aggregates, known as marine snow, indicating a faster sinking rate of organic matter in this region. The concentration of total nitrogen (TN) was significantly different at each site. Because of the significant differences in TN, the TC/TN ratio also differed greatly among the three sites.

An RDA examined the effect of environmental parameters (TIC, TC, TOC, TN, TP, and TC/TN) on community structure (Fig. 7). The RDA results showed that Haplosporidia, Nematoda, Alveolata of Apicomplexa, Ciliophora, and uncultured Alveolata were positively correlated with TOC, TC, TN, TIC, and TP. In the RDA plot, the angle between species and environmental parameters indicated the correlation: acute angle for a positive correlation, obtuse angle for a negative correlation. Also, the longer the line the stronger the relationship. Our results suggest that these species could potentially be indicators of environmental parameters. It is noteworthy that the TC/TN ratio showed a negative correlation with the species mentioned above, but was slightly correlated with Dinophyceae. This phenomenon could be due to the significant difference in TN as the denominator of TC/TN.

Table 2. Pore water environmental parameters at each site in the East China Sea

Site	TIC ($mg \cdot L^{-1}$)	TC ($mg \cdot L^{-1}$)	TOC ($mg \cdot L^{-1}$)	TN ($mg \cdot L^{-1}$)	TP ($mg \cdot L^{-1}$)	TC/TN
DH-9	7.808	19.698	11.890	0.063	0.139	312.67
DH-13	6.576	24.476	17.909	0.120	0.107	203.97
DH-18	6.070	19.701	13.631	0.230	0.141	85.66

Note: TIC = total inorganic carbon, TC = total carbon, TOC = total organic carbon, TN = total nitrogen, TP = total phosphorus, TC/TN = ratio of total nitrogen to total carbon.

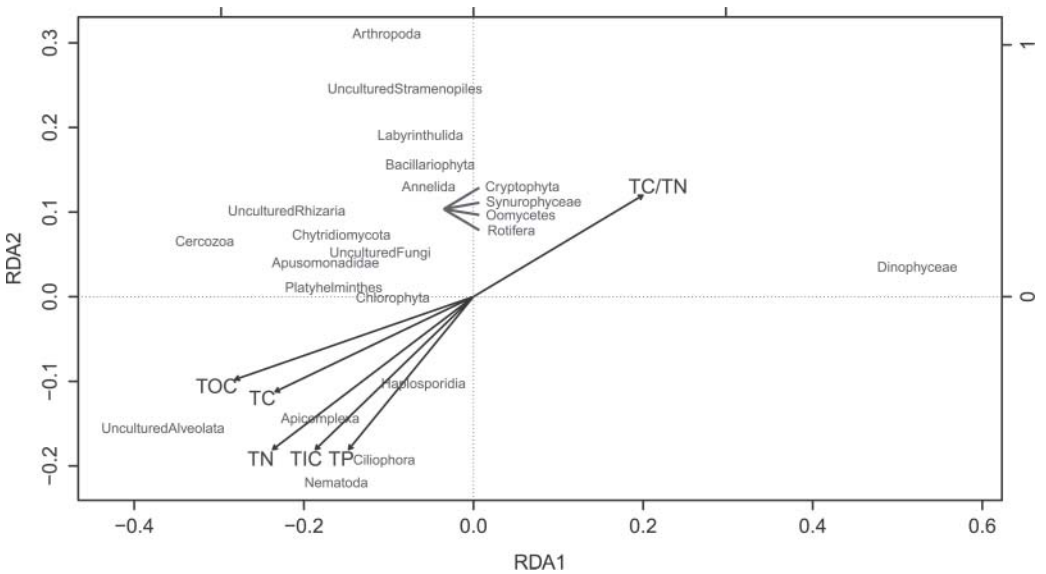


Fig. 7. Redundancy analysis (RDA) of the relationship between environmental factors and micro-eukaryotic diversity.

CONCLUSIONS

We investigated micro-eukaryotic diversity in the surface layer of sediments from the East China Sea using a molecular approach. A sister paper (Wang *et al.*, in press) examines the bacteria from these same samples.

We clustered 171 OTUs from 332 clones of the 18S rRNA gene at three sediment sites in the ECS, and then selected 129 representative phylotypes for phylogenetic analysis. Compared with other previous environmental surveys, the community composition (Alveolata, Rhizaria, Stramenopiles, Apusozoa, Metazoa, fungi, Cryptophyta, and Viridiplantae) was roughly the same at each site. Protists were also significantly dominant in the community composition of the sediments.

Some phylotypes were detected that are similar to oxygen-depleted environment clones (such as series NAMAKO, DSGM or CCW), suggesting that these phylotypes are anaerobic. We also detected some phylotypes that are related to the polar region (Arctic or Antarctic environment) clones, which are significantly different from clones related to temperate-zone environments, such as hydrothermal vents, deep-sea cold seeps, and so on.

Site DH-9 differed markedly in community composition from sites DH-13 and DH-18, possibly due to the special geographical position of site DH-9 (at the junction between the Changjiang River and Hangzhou Bay). Most clones at site DH-9 were Dinophyceae of Alveolata and they showed the lowest microbial diversity of the three sites.

In addition to the phylotypes with explicit reference sequences, we also detected 21 novel unclassified phylotypes in Alveolata and Rhizaria that showed no relatedness with a known eukaryotic phylogenetic group in the NCBI Taxonomy database, implying that there are many unknown novel micro-eukaryotes remaining to be discovered, and that more in-depth knowledge of the entire ecosystem of surface layer sediments in the ECS is essential.

Our redundancy analysis (RDA) showed TOC, TC, TN, TIC, and TP were significantly positively correlated with community diversity, whereas the TC/TN ratio was significantly negatively correlated, as can be seen in Fig. 7. This study provides more evidence of the influence of environmental parameters on community composition, diversity, and abundance of micro-eukaryotes in the surface layer of sediments in the ECS.

A single 18S rRNA survey to describe micro-eukaryotic diversity is inadequate (Anderson *et al.*, 2003, Behnke *et al.*, 2006). Huge amounts of 18S rRNA sequencing using high-throughput sequencing technology, for example, might solve this problem. Combining diverse primers to reduce bias in the amplification process is an alternative method. Overall, culture-independent molecular surveys still need to be improved, and more accurate databases and methods are necessary for future studies.

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