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Microeukaryotic Diversity in Marine Environments, an Analysis of Surface Layer Sediments from the East Sea

Soo-Je Park[1], Byoung-Joon Park[1], Vinh Hoa Pham[1], Dae-No Yoon[1], Si-Kwan Kim[2], and Sung-Keun Rhee[1]*

[1]Department of Microbiology, Chungbuk National University, Chungbuk 361-763, Republic of Korea
[2]Department of Life Science, College of Biomedical and Health Science, Konkuk University, Chungbuk 380-701, Republic of Korea

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Molecular techniques, based on clone library of 18S rRNA gene, were employed to ascertain the diversity of microeukaryotic organisms in sediments from the East Sea. A total of 261 clones were recovered from surface sediments. Most of the clone sequences (90%) were affiliated with protists, dominated by Ciliates (18%) and Dinoflagellates (19%) of Alveolates, phototrophic Stramenopiles (11%), and Cercozoa (20%). Many of the clones were related to uncultivated eukaryotes clones retrieved from anoxic environments with several highly divergent 18S rRNA gene sequences. However, no clones were related to cultivated obligate anaerobic protists. Protistan communities between subsurface layers of 1 and 9 cm shared 23% of total phylotypes which comprised 64% of total clones retrieved. Analysis of diversity indices and rarefaction curve showed that the protistan community within the 1 cm layer exhibited higher diversity than the 9 cm layer. Our results imply that diverse protists remain to be uncovered within marine benthic environments.

Keywords: 18S rRNA, marine sediment, microeukaryotic

It has been estimated that marine seafloor habitats harbor the majority of global microbial biomass (Whitman *et al.*, 1998). As a result, microorganisms would presumably have significant involvements in oceanic biogeochemical cycles. Compared to estuary and tidal flats, ocean seafloor is characterized by low temperature, low input of organic nutrients and primary production rates (Dittman, 1999; Poremba *et al.*, 1999). This inhospitable benthic environment provides a unique habitat for psychrophilic microorganisms. Ocean seafloors exhibit low sedimentation rates and moderate chemical gradients with oxygen generally being depleted within a few centimeters below the surface (Cha *et al.*, 2005).

Molecular techniques based on 16S rRNA analysis have shown that these environments harbor tremendous diversity of prokaryotes (Whitman *et al.*, 1998). Molecular approach is also being used for phylogenetic analysis of eukaryotic 18S rRNA gene sequences that have been amplified and cloned from environmental samples (Caron *et al.*, 2004). Studies of 18S rRNA gene sequences from cultured and uncultured species imply that large evolutionary distances separate major groups of protists. There are only a few comparable molecular surveys of eukaryotic microbial diversity in ocean benthic environments, limited to specific sediment environments near hydrothermal and cold seep vents and tidal flats (Edgcomb *et al.*, 2002; Stoeck and Epstein, 2003; Wilms *et al.*, 2006; Takishita *et al.*, 2007). As such, limited information has been used to provide clues about the diversity, physiology, and evolution of microeukaryotes in plain deep ocean benthic environments. In this study, we employed 18S rRNA analysis to examine the diversity of microeukaryotes from two layers (1 cm and 9 cm) of marine sediments from the East Sea. Analysis has revealed diverse microeukaryotes including phylotypes distantly related to cultivated protists.

* To whom correspondence should be addressed.
(Tel) 82-43-261-2300; (Fax) 82-43-264-9600
(E-mail) rhees@chungbuk.ac.kr

Materials and Methods

Site descriptions and sample collection

Marine sediments were collected from a site within the East Sea, using a core sampler, in July 2005. Sampling locations (coordinates & water depth) were recorded as follows: ST3 (E 128°35', N 38°20', 650 m). The cores were obtained from the top 9 cm of sediment depth. Top (1 cm) and bottom (9 cm) layers of the core were used in this study. The bottom water characteristics of sampling sites were as follows: temperature, 0.5~0.6°C; dissolved oxygen, 6.7~6.8 mg/L; salinity, 34‰, chlorophyll, 0.2~0.02 mg/L; nitrate, 1.5~1.6 mg/L; ammonia, 0.052~0.067 mg/L; phosphate, 0.02 mg/L. The core samples were placed into sterile plastic tubes using alcohol-sterilized spatulas and stored at -80°C.

Extraction of genomic DNA and cloning of microeukaryotic 18S rRNA gene

To extract genomic DNA from frozen sediment samples, a Power Soil™ DNA Kit (Mo Bio Laboratories, USA) was used. The genomic DNA concentrations were determined in triplicate using a spectrophotometer (Nanodrop Technologies, USA). Approximately 20 ng of genomic DNA from the 1 cm and 9 cm layers was used for each PCR. For cloning, the 18S rRNA genes were amplified by PCR employing *EF*-Taq

polymerase (Solgent, Korea) and the 18S-82F and 18S-1520R primers (Lopez-Garcia *et al.*, 2001) to generate a product for the preparation of clone library. The following PCR cycles were used: 5 min at 94°C; 30 cycles of 30 sec at 94°C, 30 sec at 55°C, 90 sec at 72°C; 7 min at 72°C; and hold at 10°C. Duplicate PCR products were pooled and purified using the PCR Purification Kit (Solgent), ligated into the T&A Cloning Vector Kit (Real Biotech Corporation, Taiwan), and transformed into *E. coli* DH5α cells according to the manufacturer's instructions. The whole round of the library construction process from the genomic DNA extraction step was repeated. Putative positive clones from each layer were transferred to two 96 well plates containing Luria Broth with ampicillin (100 ng/μl), grown overnight at 37°C and store at -70°C before screening.

Sequencing and phylogeny analysis

Library clones were PCR-screened directly for the presence of inserts using the M13 universal primer set, M13F; 5'-GT TTCCCAGTCACGAC-3' and M13R; 5'-TCACACAGGAAA CAGCTATGAC-3'. PCR was performed under the following conditions: 5 min at 94°C; 30 cycles of 30 sec at 94°C, 30 sec at 55°C, 90 sec at 72°C; 7 min at 72°C; hold at 10°C. The positive clones from each library were randomly selected and purified using a PCR Purification Kit (Solgent). PCR products were sequenced directly using Bigdye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems, USA) and 18S-82F PCR primer and an ABI PRISM 3730×l DNA Analyzer (PE Applied Biosystems). The sequences were checked for possible chimeras using the CHIMERA_CHECK program at the Ribosomal Database Project Web site (http://rdp8.cme.msu.edu). Finally, we obtained a total of 261 microeukaryote clone sequences. The sequences were submitted to BLAST (http://www.ncbi.nlm.nih.gov) to identify related 18S rRNA gene sequences. Related taxa were obtained from the GenBank database and multiple alignments were performed by the CLUSTAL X program (Thompson *et al.*, 1997). The evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). Unrooted phylogenetic trees were constructed for the 261 eukaryotic clones using minimum evolution method provided in Molecular Evolutionary Genetics Analysis (MEGA, version 3) (Kumar *et al.*, 2004) with bootstrap values based on 1,000 replications (Felsenstein, 1985). All clones are affiliated to those of eukaryotes and covered all major protistan lineages.

Rarefaction analysis and estimation of microbial diversity

Microeukaryotic 18S rRNA gene diversity, richness estimation, diversity indices, and rarefaction curves for each library were determined with the ESTIMATE S software program (http://viceroy.eeb.uconn.edu/EstimateS). Phylotype of 18S rRNA gene was defined as sequence groups in which sequences differed by 2% (Stoeck and Epstein, 2003; Jeon *et al.*, 2006). The species rarefaction curve of the entire dataset of each group were plotted using the individual-based Coleman method with the PAST software program (http://folk.uio.no/ohammer/past). The percentage of coverage was calculated by Good's method with the following formula: [1-(n/N)]×100, where n is the number of phylotypes in a sample represented by one clone (singletons) and N is the total number of sequences in the sample (Good, 1953). Curves were plotted using Sigamplot (V7.101, SPSS).

Nucleotide sequence accession numbers

All partial 18S rRNA gene sequences determined in this study were deposited in the GenBank database under the accession numbers EU087181-EU087292 and EU545651-EU545799.

Results and Discussion

We compared two eukaryotic clone libraries between two layers (1 and 9 cm) of marine sediments. Physicochemical analysis of water above the sediment indicated that the East Seawater is oligotrophic and close to that of open ocean seawater. From the clone libraries of microeukaryotic 18S rRNA genes, we obtained 261 microeukaryotic sequences (136 and 125 sequences from 1 and 9 cm, respectively). The protistan sequences were dominated by Ciliates (18%) and Dinoflagellates (19%) of Alveolates, phototrophic Stramenopiles (11%), and Cercozoa (20%) (Fig. 1). Alveolates were dominant and made up more than 30% of organisms in both depth layers. Similarly, analysis of a microeukaryotic community along an O_2/H_2S gradient in a superfluidic anoxi Fjord revealed that Alveolates were the major protistan organisms (Behnke *et al.*, 2006). It is interesting that the gross composition of protists at the kingdom-level between the two layers did not appear to be significantly different (Spearman $R=0.84$, $P<0.05$). In addition to protistan clones, clones of Fungi, green algae, and Metazoa constituted about 8% of total clones, with most of them being detected in the surface layers. Retrieval of green algae-related clones was unexpected since the sediment were obtained from aphotic zone depth (650 m). Remnants of green algae ma-

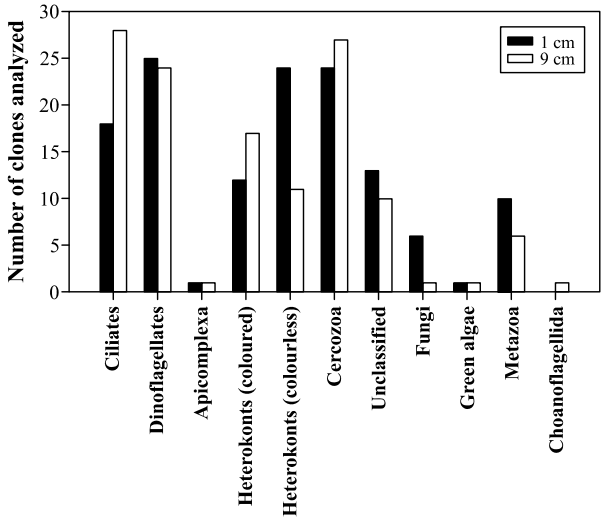
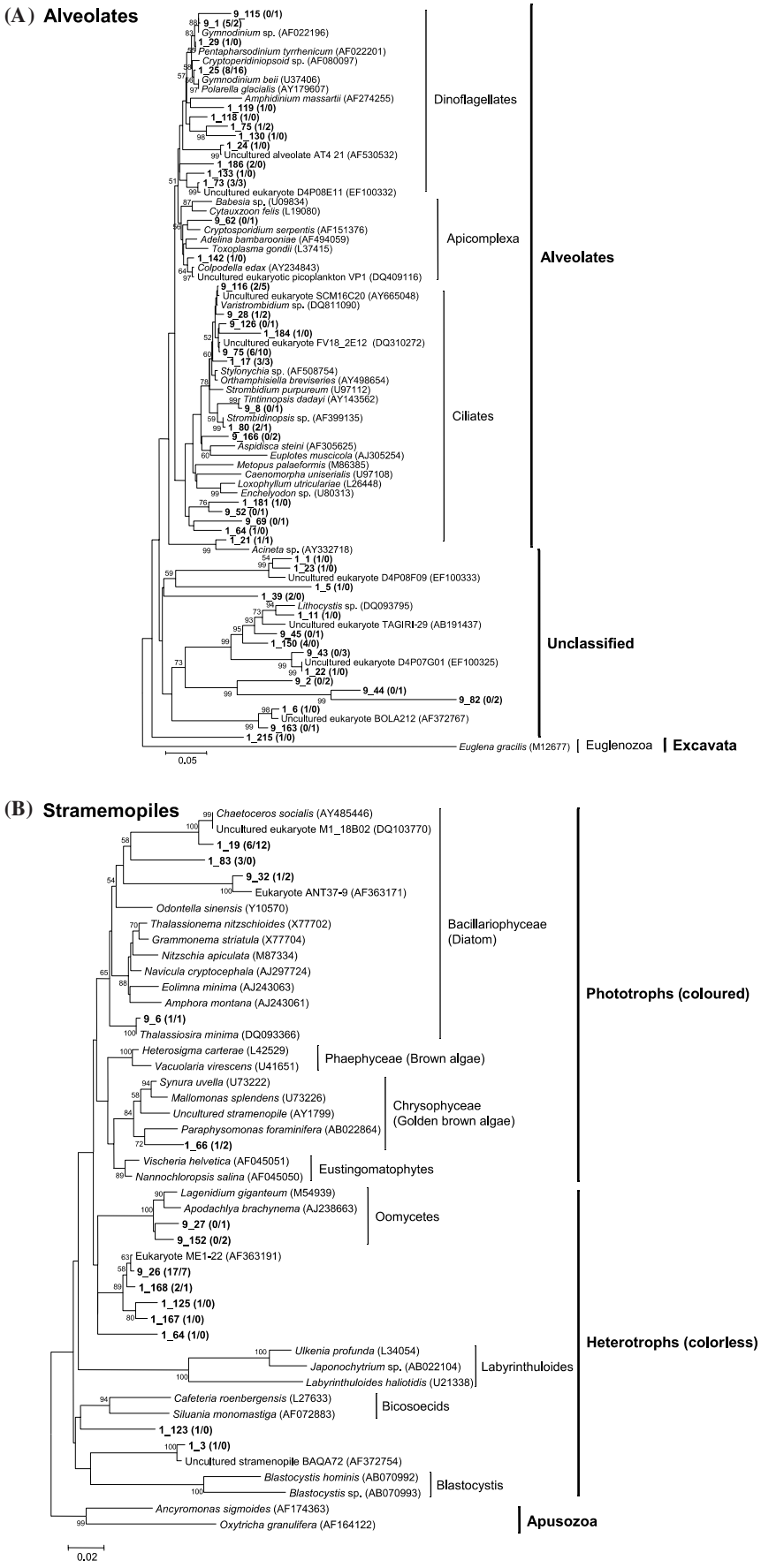


Fig. 1. Taxonomic distribution of 18S rRNA phylotypes retrieved from two protistan communities at different sediment depths (1 cm and 9 cm). Phylotypes were defined to encompass clones that exhibited at least 98.0% sequence similarity based on a pairwise comparison of the 18S rRNA gene sequences.


(A) Alveolates
9_115 (0/1)
9_1 (5/2)
Gymnodinium sp. (AF022196)
1_29 (1/0)
Pentapharsodinium tyrrhenicum (AF022201)
Cryptoperidiniopsoid sp. (AF080097)
1_25 (8/16)
Gymnodinium beii (U37406)
Polarella glacialis (AY179607)
Amphidinium massartii (AF274255)
1_119 (1/0)
1_118 (1/0)
1_75 (1/2)
1_130 (1/0)
1_24 (1/0)
Uncultured alveolate AT4 21 (AF530532)
1_186 (2/0)
1_133 (1/0)
1_73 (3/3)
Uncultured eukaryote D4P08E11 (EF100332)
Dinoflagellates
Babesia sp. (U09834)
Cytauxzoon felis (L19080)
9_62 (0/1)
Cryptosporidium serpentis (AF151376)
Adelina bambarooniae (AF494059)
Toxoplasma gondii (L37415)
1_142 (1/0)
Colpodella edax (AY234483)
Uncultured eukaryotic picoplankton VP1 (DQ409116)
Apicomplexa
Alveolates
9_116 (2/5)
Uncultured eukaryote SCM16C20 (AY665048)
Varistrombidium sp. (DQ811090)
9_28 (1/2)
9_126 (0/1)
1_184 (1/0)
Uncultured eukaryote FV18_2E12 (DQ310272)
9_75 (6/10)
1_17 (3/3)
Stylonychia sp. (AF508754)
Orthamphisiella breviseries (AY498654)
Strombidium purpureum (U97112)
Tintinnopsis dadayi (AY143562)
9_8 (0/1)
Strombidinopsis sp. (AF399135)
1_80 (2/1)
9_166 (0/2)
Aspidisca steini (AF305625)
Euplotes muscicola (AJ305254)
Metopus palaeformis (M63385)
Caenomorpha uniserialis (U97108)
Loxophyllum utriculariae (L26448)
Enchelyodon sp. (U80313)
1_181 (1/0)
9_52 (0/1)
9_69 (0/1)
1_64 (1/0)
1_21 (1/1)
Acineta sp. (AY332718)
Ciliates
1_1 (1/0)
1_23 (1/0)
Uncultured eukaryote D4P08F09 (EF100333)
1_5 (1/0)
1_39 (2/0)
Lithocystis sp. (DQ093795)
1_11 (1/0)
Uncultured eukaryote TAGIRI-29 (AB191437)
9_45 (0/1)
1_150 (4/0)
9_43 (0/3)
Uncultured eukaryote D4P07G01 (EF100325)
1_22 (1/0)
9_2 (0/2)
9_44 (0/1)
9_82 (0/2)
1_6 (1/0)
Uncultured eukaryote BOLA212 (AF372767)
9_163 (0/1)
1_215 (1/0)
Unclassified
Euglena gracilis (M12677)
Euglenozoa
Excavata
0.05
(B) Stramenopiles
Chaetoceros socialis (AY485446)
Uncultured eukaryote M1_18B02 (DQ103770)
1_19 (6/12)
1_83 (3/0)
9_32 (1/2)
Eukaryote ANT37-9 (AF363171)
Odontella sinensis (Y10570)
Thalassionema nitzschioides (X77702)
Grammonema striatula (X77704)
Nitzschia apiculata (M87334)
Navicula cryptocephala (AJ297724)
Eolimna minima (AJ243063)
Amphora montana (AJ243061)
9_6 (1/1)
Thalassiosira minima (DQ093366)
Bacillariophyceae (Diatom)
Heterosigma carterae (L42529)
Vacuolaria virescens (U41651)
Phaephyceae (Brown algae)
Synura uvella (U73222)
Mallomonas splendens (U73226)
Uncultured stramenopile (AY1799)
Paraphysomonas foraminifera (AB022864)
1_66 (1/2)
Chrysophyceae (Golden brown algae)
Vischeria helvetica (AF045051)
Nannochloropsis salina (AF045050)
Eustingomatophytes
Phototrophs (coloured)
Lagenidium giganteum (M54939)
Apodachlya brachynema (AJ238663)
9_27 (0/1)
9_152 (0/2)
Oomycetes
Eukaryote ME1-22 (AF363191)
9_26 (17/7)
1_168 (2/1)
1_125 (1/0)
1_167 (1/0)
1_64 (1/0)
Ulkenia profunda (L34054)
Japonochytrium sp. (AB022104)
Labyrinthuloides haliotidis (U21338)
Labyrinthuloides
Cafeteria roenbergensis (L27633)
Siluania monomastiga (AF072883)
Bicosoecids
1_123 (1/0)
1_3 (1/0)
Uncultured stramenopile BAQA72 (AF372754)
Blastocystis hominis (AB070992)
Blastocystis sp. (AB070993)
Blastocystis
Heterotrophs (colorless)
Ancyromonas sigmoides (AF174363)
Oxytricha granulifera (AF164122)
Apusozoa
0.02

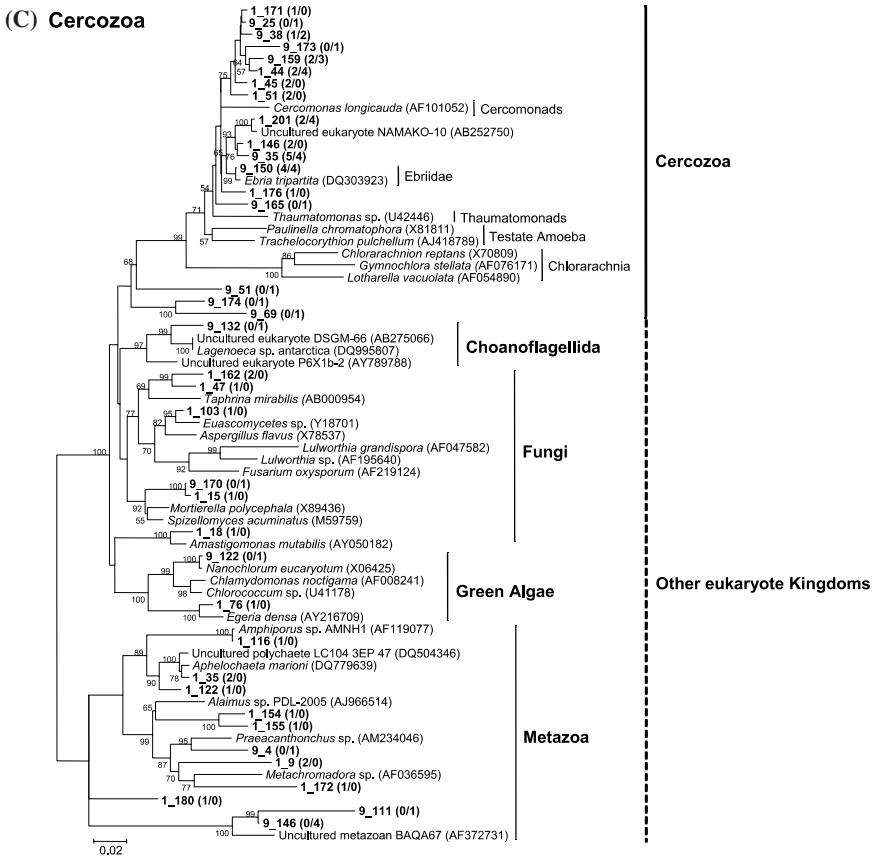


Fig. 2. Phylogenetic trees 18S rRNA gene sequences of environmental clones, closely related cultured organisms and environmental 18S rRNA signatures. (A) Alveolates, (B) Stramenopiles, (C) Cercozoa. Our environmental clones are indicated by boldface type. Each clone is designated by the library designation (1 for 1 cm layer; 9 for 9 cm layer) followed by a number after underscore. Numbers in parentheses following clone names indicate the number of times the sequences were found in the clone libraries from the two sediment layers. The tree topologies were obtained by a Tamura-Nei minimum evolutionary model, with gapped and ambiguously aligned positions excluded. Number within the dendrogram indicates the occurrence (%) of branching order in 1,000 bootstrapped trees, while only values in excess of 50% are shown. The scale bar represents nucleotide substitutions per 100 nucleotide positions. Reference sequences are followed by GenBank accession numbers.

terials might be transported from shallow coastal area to deep water by currents. However, it has been reported that extracellular DNA recovered from sediments did not contain amplifiable rRNA genes (Corinaldesi *et al.*, 2005). More than 8% of deeply branched unclassified eukaryotes, which are not in the well-established taxonomic groups, were observed (Fig. 2A). In this group, we were able to identify several subgroups. A clone of subgroup 1_6, is affiliated with the clone BOLA212 retrieved from anoxic environments, which was proposed as a novel kingdom level eukaryote (Dawson and Pace, 2002). In the subgroup, only one cultivated protist (*Lithocystis* sp. of Apicomplexa) is closely related to identified clone in this study. However, phylogenetic analysis of 18S rRNA gene of the *Lithocystis* sp. showed that the strain is distantly related to other Apicomplexa relatives (88% with a closest relative, *Pterospora floridiensis*). It is obvious that we need more detailed phylogenetic analysis of clones from these subgroups. Clones of 9_82, 9_44, 9_2, and 1_215 are not related to any other protists.

Similarity of 18S rRNA gene analysis showed that most environmental clones (74%) are regarded as novel phylotypes not related to cultivated eukaryotic relatives based on 98% 18S rRNA gene similarity as a phylotype cutoff (Webster *et al.*, 2004). Some clones (26%) were not exactly assignable, but within established protistan clades were observed in several groups as shown in the Fig. 2: 9_115 and 9_1 of Alveolates; 1_66 of phototrophic Stramenopiles; 9_26, 1_168, 1_125, 1_167, 1_64, 1_123, and 1_3 of heterotrophic Stramenopiles; 1_171, 9_25, 9_38, 9_173, 9_159, 1_44, 1_45, 1_51, 9_51, 9_174, and 9_69 of Cercozoa. This indicates that large fractions of protistan groups in the environment still remain to be isolated (Whitman *et al.*, 1998). Some clones were closely related to cultivated eukaryotes of Ciliates (*Varistrombidium*, *Strombidium*, and *Strombidinopsis*) Dinoflagellates (*Gymodinium*, *Polarella*, *Pentaparsodinium*, and *Cryptoperidiniopsoid*), Diatom (*Chaetoceros* and *Thalassiosira*), and Ebriidae (*Ebria*).

We found 94 phylotypes from two library clones (68 and 48 phylotypes from 1 and 9 cm layers, respectively) with 22 shared phylotypes. This indicates that microeukaryote community composition is different between the 1 and 9 cm layers, although kingdom-level community composition is

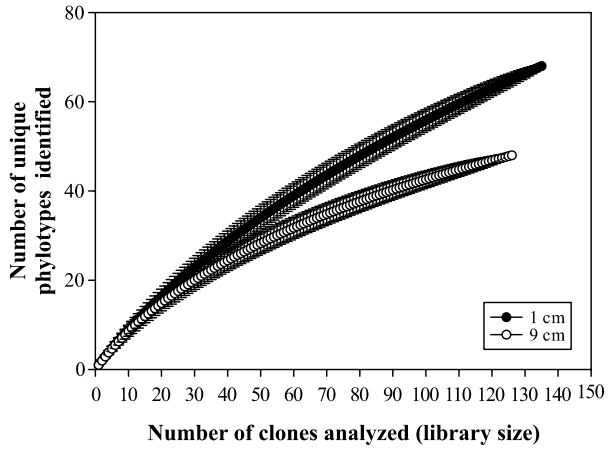


Fig. 3. Rarefaction curves indicating microeukaryotic 18S rRNA gene richness within clone libraries extracted from the East Sea. Phylotypes were defined as groups of sequences differing by 2%. The error bar represents 95% confidence intervals.

similar. Good's estimator coverage values were 68.2% and 80.9% for 1 cm and 9 cm layers, respectively. Singletons are 31.9% and 19.0% of clone libraries for 1 cm and 9 cm sediment layers, respectively. Analysis of Shannon diversity index and rarefaction curves (Fig. 3) also support the conclusion that the 1 cm layer (Shannon index: 3.85) exhibited higher protistan diversity than the 9 cm layer (Shannon index: 3.46). Rarefaction analysis showed that these are likely to be minimal estimates as the curve did not plateau with the current sequencing effort.

Comparative protistan diversity revealed that 57% and 72% of total clones from 1 cm and 9 cm layers are shared phylotypes. It is interesting that many of the protistan clones were detected both in surface and subsurface layers. In the 9 cm layer, there are no clones related to genus *Metopus*, *Caenomorpha*, or *Plagiopyla*, which are known to harbor obligate anaerobes. This result is contrasted by earlier observations which indicated that depth-dependent archaeal community composition profile changes dramatically at this depth. The majority of archaeal clones (94%) at 9 cm are anaerobic Crenarchaeotic Group I (CG I), while all of the archaeal clones at 1 cm are related aerobic CG I (submitted for publication).

Simulation study of redox gradient based on the ratio of redox Mn/Fe determination also showed that sediments of the East Sea below 3~4 cm layers are under anoxic conditions (Cha *et al.*, 2005). Protistan community of marine sediments might be less sensitive to a redox gradient in marine sediments compared with archaeal community. Regardless, small benthic animals may make the marine benthic environments mixed and more heterogenous in redox potential, thus preventing benthic sediments from being completely anoxic. In fact, it is suggested that many anaerobic environments contains very low amount of oxygen or are oxygen-fluctuating (Lloyd, 2004).

In conclusion, we detected diverse microeukaryotes in marine sediments of the East Sea using a molecular approach. Most of the clone sequences were distantly related to cultivated relatives. Our results imply that diverse protists remained to be uncovered in marine benthic environments.

Acknowledgements

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