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ORIGINAL PAPER

Diversity of microbial eukaryotes in sediment at a deep-sea methane cold seep: surveys of ribosomal DNA libraries from raw sediment samples and two enrichment cultures

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Abstract Recent culture-independent surveys of eukaryotic small-subunit ribosomal DNA (SSU rDNA) from many environments have unveiled unexpectedly high diversity of microbial eukaryotes (microeukaryotes) at various taxonomic levels. However, such surveys were most probably biased by various technical difficulties, resulting in underestimation of microeukaryotic diversity. In the present study on oxygen-depleted sediment from a deep-sea methane cold seep of Sagami Bay, Japan, we surveyed the diversity of eukaryotic rDNA in raw sediment samples and in two enrichment cultures. More than half of all clones recovered from the raw sediment samples were of the basidiomycetous fungus *Cryptococcus curvatus*. Among other clones, phylotypes of eukaryotic parasites, such as Apicomplexa, Ichthyosporea, and Phytomyxea, were identified. On the other hand, we observed a marked difference in phylotype composition in the enrichment samples. Several phylotypes belonging to heterotrophic stramenopiles were frequently found in one enrichment culture, while a phylotype of Excavata previously detected at a deep-sea hydrothermal vent dominated the other. We successfully established a clonal culture of this excavate flagellate. Since these phylotypes were not identified in the raw sediment samples, the approach incorporating a cultivation step successfully found at least a fraction of the ''hidden'' microeukaryotic diversity in the environment examined.

Keywords Cultivation · Diversity · Methane seep · Microbial eukaryotes · SSU rDNA

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Introduction

Recently, sequence data on eukaryotic small-subunit ribosomal DNA (SSU rDNA) retrieved directly from various natural environments have increased rapidly. Although it remains unclear how much these types of experiments truly capture the composition of organisms in the corresponding environment, the phylotype compositions are generally considered as ''probes'' for the organismal diversity. According to the data generated from such culture-independent molecular surveys of environmental samples, the diversity of microbial eukaryotes (microeukaryotes; mostly protists) is most probably much greater than previously expected and provide detailed information on the ecology of microeukaryotes (e.g., Díez et al. 2001; López-García et al. 2001; Moon-van der Staay et al. 2001; Amaral Zettler et al. 2002). However, Stoeck et al. (2006) pointed out that the results from rDNA surveys do not necessarily reflect

the true diversity of microeukaryotes due to potential biases in the experimental techniques. For example, it is difficult to extract DNA from all the organisms present in a raw environmental sample. Similarly, all rDNA phylotypes cannot always be amplified by polymerase chain reaction (PCR) using environmental DNA as the template. In addition, the sequencing of a limited number of rDNA clones cannot exclude the possibility that some minor phylotypes escaped being surveyed (e.g., Massana et al. 2004a; Behnke et al. 2006; Zuendorf et al. 2006). Thus, conventional rDNA surveys performed to date may have partially captured the community structure of microeukaryotes in the environments examined, and the diversity and distribution were most likely underestimated.

The difficulties of culture-independent molecular surveys discussed above can be technically overcome by large-scale sequencing. In addition, cultivation or incubation of raw environmental samples was attempted to supplement the standard rDNA survey. Countway et al. (2005) reported substantial changes in the dominant microeukaryotic phylotypes through seawater incubation. Their experiments suggested that a high diversity of eukaryotic taxa existed in the original seawater sample at very low abundances, and were not observed in the community structure initially characterized by the rDNA survey. Massana et al. (2004a) investigated the eukaryotic diversity in a seawater sample from an oligotrophic coastal site using a combination of isolation/cultivation of many eukaryotic cells and rDNA survey. The molecular and culturing approaches conducted by Massana et al. (2004a) resulted in different pictures of the diversity of many eukaryotic groups. These pioneering studies strongly suggested that a combination of both culture-independent and ''culture-dependent'' rDNA surveys could reveal wider diversity of microeukaryotes in an environment than experiments utilizing either method separately.

Eukaryotic diversity in oxygen-depleted environments is as ecologically important (Fenchel and Finlay 1995) as that of oxygenated environments. However, investigations of the former environments have been poorly conducted even using traditional approaches (e.g., culturing and microscopic observation). On the other hand, extensive investigations of microeukaryotes in such environments at the molecular level have begun since culture-independent rDNA surveys have been established (Dawson and Pace 2002; Edgcomb et al. 2002; López-García et al. 2003; Stoeck and Epstein 2003; Stoeck et al. 2003, 2006). By retrieving rDNA sequences from environmental samples, we have also investigated microeukaryotic diversity in oxygen-depleted environments, such as in anoxic sediment around fumaroles on a submarine caldera floor (Takishita et al. 2005), in deep-sea methane cold-seep sediment of Kuroshima Knoll (Takishita et al. 2006), and in anoxic sediment of a saline meromictic lake (lagoon) (Takishita et al. 2007).

In the present study, we focused on another oxygen-depleted environment: the sediment at a deep-sea (about 1,170 m) ''cold seep'' (chemosynthetic ecosystem where energy-rich fluids geologically seep out of the seafloor) along the Sagami Trough subduction zone located in the northern convergence in front of the Philippine Sea plate. The seeping fluids in this area contain methane and hydrogen sulfide, and sustain large-scale megabenthos communities mainly composed of the vesicomyid clam genus *Calyptogena* with sulfur-oxidizing symbionts (Kim et al. 1995), similar to the previously investigated communities of the Kuroshima Knoll methane cold seep located in the Southern Ryukyu Arc near Ishigaki Island, Japan (Takishita et al. 2006). We extracted DNA from the cold-seep sediment, as well as from two types of anaerobic enrichment cultures. We amplified eukaryotic SSU rDNA from the three DNA samples and constructed rDNA libraries. Remarkably, surveys of the libraries from the enrichment culture samples successfully identified 29 phylotypes that were not detected in the survey of the libraries from the raw sediment samples (26% of all phylotypes recovered). As shown in Countway et al. (2005) and Massana et al. (2004a), our results presented in this study suggest that rDNA surveys of libraries from enrichment cultures can supplement the standard experimental approach (surveys of the library from raw environmental samples). Additionally, we established a clonal culture of previously undescribed protist from an enrichment culture of the raw sediment sample, with an SSU rDNA sequence that tightly clustered with a phylotype retrieved from the Guaymas Basin hydrothermal vent environment (Edgcomb et al. 2002).

Materials and methods

Sampling

The dive survey was conducted off Hatsushima in Sagami Bay, Japan, using the ROV *Hyper-Dolphin* on March 12 and 13, 2006 (cruise no. NT06-04, *Hyper-Dolphin* dives #524 and #525). Sediment samples were collected with a sterile mud sampler (Ikemoto and Kyo 1993) at two methane-seep sites: Site A (1,174 m, 35°0.09′N, 139°13.51′E) and Site B (1,178 m, 35°0.16′N, 139°13.47′E). Site A was located at a *Calyptogena* colony, while no megabenthos were observed at Site B. Although not shown here, both sediment samples were under highly reductive and oxygen-limited conditions (the detailed environmental factors will be published elsewhere). During sediment sampling we were careful not to collect sediments from the uppermost (top) surface to avoid

contamination by sinking and/or sunken cells. The samples recovered in 50 ml plastic tubes were subsequently homogenized well, and aliquots for direct DNA extraction and enrichment cultures were taken from the homogenized samples. The sediment samples subjected to the direct extraction of DNA were stored in liquid nitrogen, while the samples used for enrichment cultures were kept at 4°C.

Enrichment cultures

Two types of media, YT (100 mg yeast extract, 200 mg trypton/1 l seawater) and 5% PYNFH (ATCC medium 1034; see instructions from the American Type Culture Collection) were used for cultivation. Approximately 1 g of both sediment samples described above were inoculated into 600 ml of both media, and the enrichment cultures were maintained in BBL GasPak Anaerobic System (Becton Dickinson and Company, MD, USA). The culture media became anaerobic by this procedure (confirmed by resazurin sodium: this chemical potentially inhibits growth of microeukaryotes, and thus was not added to the cultures). These enrichment cultures were kept in dark, for 10 days at 4°C.

DNA extraction, PCR amplification, cloning, and sequencing

Total DNA was extracted from the raw sediment samples and the two types of enrichment culture of the raw sediment samples for each site using an UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). Eukaryotic SSU rDNA was amplified from the total DNA template by PCR using HotStarTaq DNA polymerase (QIAGEN, Tokyo, Japan) with the combination of a forward (18S-42F; 5′-CTCAARGAYTAAGCCATGCA, or 18S-82F; 5′-GAAACTGCGAATGGCTC) and a reverse (18S-1498R; 5′-CACCTACGGAAACCTTGTTA, or 18S-1520R; 5′-CYGCAGGTTCACCTAC) primer. Thermal cycling consisted of 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, followed by a final elongation step of 10 min at 72°C. The amplified products were visualized on 1.0% agarose gel electrophoresis. The PCR-amplified DNA fragments were cloned into the pCR2.1 vector in a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Positive transformants of the libraries were screened by PCR amplification of inserts with InsertCheck-Ready-Blue (TOYOBO, Osaka, Japan). From the libraries, amplicons of the expected size (about 1.6–1.8 kbp) were partially sequenced with an ABI PRISM 3100 Genetic Analyzer (PE Biosystems, Foster City, CA, USA) using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems) with an internal primer (EK-555F; 5′-AGTCTGGTGCCAGCAGCCGC). Clone numbers of each phylotype are presented in the supplementary data. Non-redundant clones (at a cutoff level of 99.5%) were further subjected to sequencing using M13 Forward, M13 Reverse, and a primer (EK-1269R; 5′-AAGAACGGCCATGCAC CAC) to complete both strands. Phylotypes closely related to Metazoa were discarded.

The sequences were tested using the Ribosomal Database Project CHECK_CHIMERA program (Maidak et al. 2001) and partial treeing analysis (Hugenholtzt and Huber 2003) to detect potential chimeric gene artifacts.

We designated the SSU rDNA libraries of the raw sediment samples from Sites A and B as DAL and DBL, respectively. The libraries from the enrichment cultures using YT and 5% PYNFH media from Site A were designated as CYAL and CPAL, and Site B samples as CYBL and CPBL, respectively. The phylotypes retrieved from DAL + DBL were designated as DSGM-number. The phylotypes retrieved only from CYAL + CYBL and CPAL + CPBL were designated as CYSGM-number and CPSGM-number, respectively (remarkably only two clones of CPSGM-5 were recovered from CYAL).

Phylogenetic analyses

One hundred and twelve distinct eukaryotic SSU rDNA sequences were obtained in this study (GenBank accession numbers AB275001–AB275112). Phylogenetic affiliation of each rDNA sequence was initially investigated by BLAST search. The sequences, where affinity was not clarified by BLAST search, were further assessed by preliminary phylogenetic analyses considering major eukaryotic groups (data not shown). Using CLUSTAL W version 1.8 (Thompson et al. 1994), new SSU rDNA sequences were aligned with those from phylogenetically diverged eukaryotes in the DNA Data Bank of Japan, followed by manual editing of the resultant alignments. All ambiguously aligned sites were excluded from phylogenetic analyses. Six SSU rDNA alignments were generated: (1) Alveolata (79 taxa/1,272 sites), (2) stramenopiles (74 taxa/1,358 sites), (3) Cercozoa (78 taxa/1,295 sites), (4) Opisthokonta (69 taxa/1,425 sites), (5) the representatives of most major eukaryotic groups (79 taxa/1,150 sites), and (6) Chlorophyta (37 taxa/1,539 sites). The above alignments are available on request from the corresponding author.

Maximum-likelihood (ML) analyses were performed using PhyML (Guindon and Gascuel 2003) using an input tree generated by BIONJ with general-time-reversible models (Rodríguez et al. 1990) of nucleotide substitution incorporating invariable sites and a discrete gamma distribution (eight categories) (GTR + I + Γ model). Model parameters were estimated from the dataset. ML bootstrap trees (200 replicates) were constructed as in the model and settings described above. Bayesian phylogenetic analyses

were also conducted using MrBayes version 3.0 (Ronquist and Huelsenbeck 2003) under GTR + I + Γ models. One cold and three heated Markov chain Monte Carlo (MCMC) chains with default-chain temperatures were run for 2,500,000 generations, sampling log-likelihoods (InLs) and trees at 100-generation intervals (25,000 InLs and trees were saved during MCMC). The likelihood plot for all datasets suggested that MCMC reached the stationary phase after the first 5,000 trees (i.e., the first 500,000 generations were set as ''burn-in''). Thus, clade probabilities and branch-length estimates were obtained from the remaining 20,000 trees.

Cultivation and microscopic observation of excavate-like cells

Excavate-like cells propagated in the enrichment culture of Site A sediments using 5% PYNFH medium. A single cell was isolated by micropipetting. The clonal culture was maintained in 5% PYNFH medium at 4°C under dark and anaerobic conditions as described above.

For microscopic observations, the cell suspension was mixed with the same volume of 2.5% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) at room temperature. The fixed cells were immediately observed with a Leica DMR light microscope (Leica, Wetzlar, Germany), and the images were captured using a Keyence VB6010 digital chilled CCD camera (Keyence, Osaka, Japan).

Results and discussion

Difference in phylotype composition between the raw and enrichment samples

We sequenced 307 and 337 clones from DAL and DBL, respectively [15 and 14 clones in the former and latter libraries, respectively, were derived from Metazoa (Copepoda, Nematoda, Mollusca, and Polychaeta)]. Twenty-six and 71 phylotypes of microeukaryotes were retrieved from DAL and DBL, respectively, and 14 phylotypes were shared between the two libraries. An asymptote was not observed in the phylotype accumulation curve (Fig. 1), which suggests that the true microeukaryote richness in the sediment samples was not represented by the phylotypes recovered by our sequencing. The phylotype of the basidiomycetous fungus *Cryptococcus curvatus* was the most dominant in terms of clone numbers (DAL, 83.9%; DBL, 44.6%; DAL + DBL, 63.2%; summarized in Fig. 2a). We previously observed similar results in the rDNA survey of the sediment at the Kuroshima Knoll methane seep; at the molecular level, only *C. curvatus* and foraminifers were identified (Takishita et al. 2006). Our surveys of the two

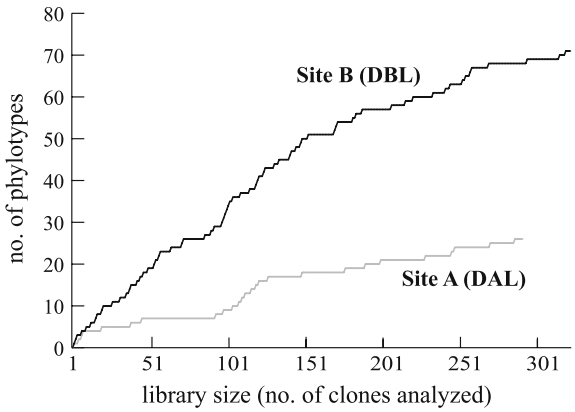

Fig. 1 Accumulation curves for rDNA libraries representing the number of phylotypes detected when increasing the number of clones analyzed. The libraries DAL and DBL were constructed from raw sediment samples of site A and site B, respectively

methane-seep sediments consistently suggest that *C. curvatus* may be a characteristic of microbial eukaryote composition in methane-seep sediments. Although *C. curvatus* is known to be an opportunistic pathogen of animals including humans (Dromer et al. 1995), the ecological role of this fungus in a methane-seep site is unclear.

Compared with the ''*C. curvatus*-rich'' libraries of the raw sediment samples, we observed a significant difference in the phylotype composition in the two enrichment samples (although it should be noted that the samples inoculated were not strictly same as the raw samples subjected to the DNA extraction without incubation) (Figs. 2b, c). Fifty-three clones were sequenced from the CYAL, CPAL, and CPBL libraries, and 49 clones from the CYBL library (no metazoan sequences were detected in these libraries). Among these 208 clone sequences, we detected 29 phylotypes that were not found in the survey of the libraries from the raw sediment samples (Fig. 2d).

The relative abundance of clone numbers does not necessarily correspond to the cell abundance in the natural environment, due to differences in rDNA copy numbers, and PCR amplification efficiency among organisms. However, as shown in Fig. 2a–d, the phylotype composition of the enrichment samples was rather different from that of the raw sediment samples. In particular, the enrichment culture using YT medium was suitable for the growth of some stramenopile species. Although the relative average abundance of the stramenopile phylotypes in DAL + DBL was 5.4% (Fig. 2a), the corresponding average value increased to 39.2% after the enrichment cultures (CYAL, 56.5%; CYBL, 20.4%; CYAL + CYBL, 39.2%; Fig. 2b). Furthermore, most of these phylotypes were unique to CYAL + CYBL, suggesting that the standard surveys using the raw sediment samples underestimated the stramenopile diversity in the sediment studied here.

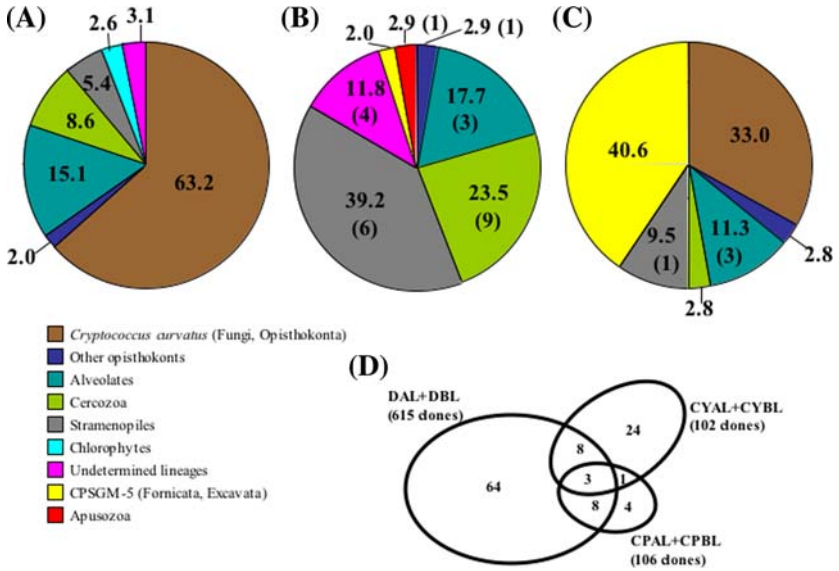

Fig. 2 Relative abundance of clone numbers belonging to each major eukaryotic group retrieved from **a** libraries from the raw samples (DAL and DBL), **b** enrichment culture using YT medium (CYAL and CYBL), and **c** enrichment culture using 5% PYNFH medium (CPAL and CPBL). *Numbers in parentheses* in **b** and **c** refer to number of original phylotypes within each major eukaryotic group. **d** Benn diagram for the clone number detected in the libraries from the raw sediment samples (DAL + DBL), those from the enrichment culture using YT medium (CYAL + CYBL), and those from the enrichment culture using 5% PYNFH medium (CPAL + CPBL)

We most frequently encountered the phylotype of a certain species of Excavata in the libraries from the enrichment cultures using 5% PYNFH medium (CPAL, 52.8%; CPBL, 28.3%; CPAL + CPBL, 40.6%; Fig. 2c). This particular phylotype was not found in the survey of DAL or DBL (Fig. 2a), suggesting that the abundance of this excavate species may have been fairly low in the raw sediment samples. On the other hand, we observed excavate-like flagellates dominating the enrichment cultures (see below for the detailed characteristics of this flagellate).

In the following sections, we describe the phylotypes recovered from both the samples of raw sediments and enrichment cultures based on some eukaryotic groups (Alveolata, stramenopiles, Cercozoa, Opisthokonta, and other lineages) in detail.

Alveolata

We detected 36 phylotypes (DSGM-1–30, CYSGM-1–3, and CPSGM-1–3) of Alveolata (Fig. 3).

Phylotypes related to Apicomplexa

DSGM-1–6 grouped robustly with *Lecudina tuzetae*, *Pterospora schizosoma*, and *Gregarina polymorpha*, which belong to Gregarinea, a group of Apicomplexa [100% ML bootstrap support (BP)]. Additionally, two gregarine-related phylotypes were detected: DSGM-8 branched with a clade including the gregarine species *Lecudina polymorpha* with 100% BP, and DSGM-14 clustered with a clade including the gregarines *Ophriocystis* and *Monocystis* with 100% BP. All gregarine species described to date are obligate unicellular parasites, having monoxenous life cycles in invertebrate hosts and extracellular trophozoite stages (Perkins et al. 2002; Leander et al. 2003, 2006). However, no multicellular organisms were identified in the sediment by our microscopic observations. Although a few invertebrate sequences were detected, these were most probably derived from dead tissues or gametes. Thus, the ecology of these organisms represented by the gregarine-related phylotypes obtained remains unclear. Furthermore, some gregarine species may be anaerobic or anoxic-tolerant, since many gregarine-related phylotypes have been recovered from the sediment samples used in this study and other oxygen-depleted environments (Dawson and Pace 2002; Edgcomb et al. 2002; López-García et al. 2003; Stoeck and Epstein 2003; Takishita et al. 2007).

DSGM9-11 and CYSGM-1 formed a robust monophyletic group with a sequence of an endobiont of *Ammonia beccarii* (a foraminifer) and environmental sequences from other oxygen-depleted sites. Curiously, we did not obtain any PCR-amplified products with a set of primers specific to foraminiferan SSU rDNA (Pawlowski et al. 1996), and did not find any foraminiferan cells under microscopic observations. Thus, the phylotype DSGM9-11 and CYSGM-6 may be derived from parasites/symbionts of non-foraminiferan organisms in the sediments.

DSGM-7 (branched with C2_E016 from deep-sea hydrothermal sediments at the Guaymas Basin), DSGM-

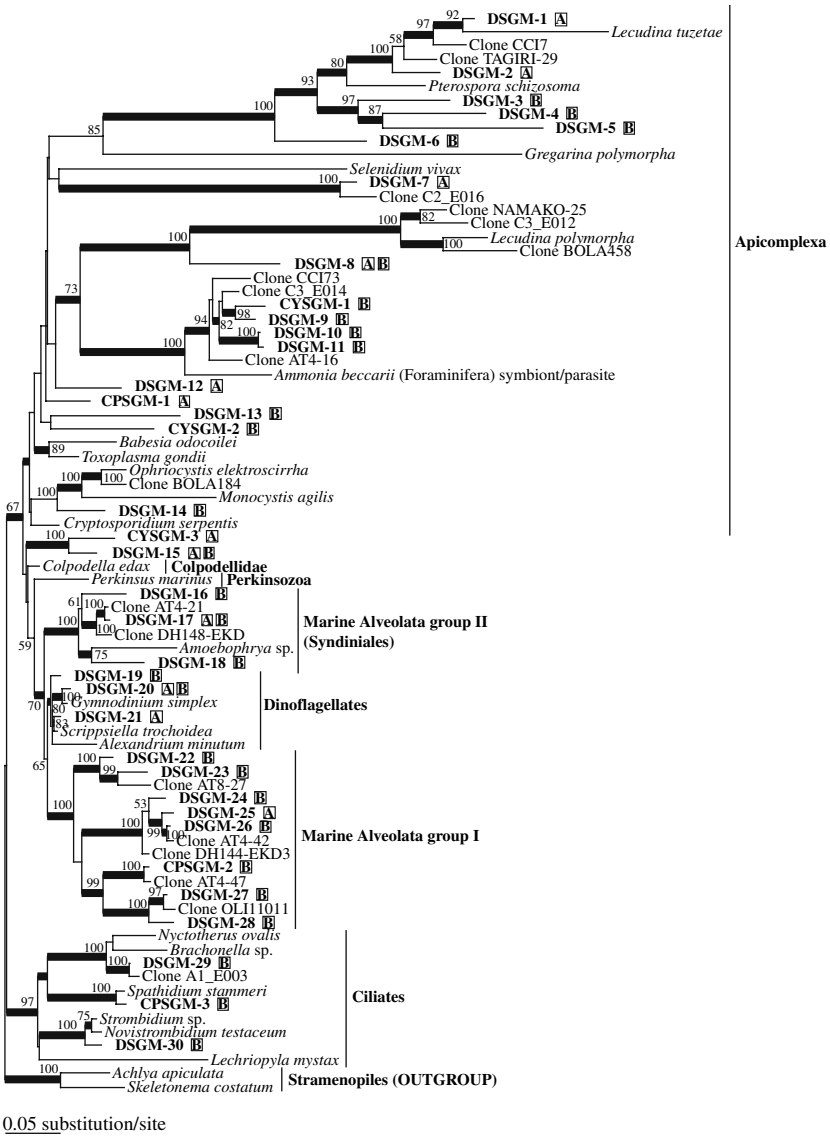

Fig. 3 Maximum likelihood phylogeny of SSU rDNA from Alveolata. Bootstrap probabilities are shown for nodes with support over 50%. The *thick branches* represent branches with over 0.90 Bayesian posterior probabilities. The phylotypes (DSGM-No. from libraries DAL and DBL, CYSGM-No. from libraries CYAL and CYBL, and CPSGM-No. from libraries from CPAL and CPBL) are represented with the sediment sites (A and/or B) from which they were retrieved

12, DSGM-13, CPSGM-1, CYSGM-2, and a cluster of DSGM-15 and CYSGM-3 showed no evolutionary affinity to any apicomplexan species known to date. The organisms represented by those ''orphan'' phylotypes are potentially novel apicomplexan lineages. Although the DSGM-15 + CYSGM-3 clade, as well as *Colpodella*, fell outside of the apicomplexan clade in Fig. 2, these sequences were positioned within the radiation of Apicomplexa in the analyses excluding the divergent gregarine sequences (data not shown). In light of the results of recent rDNA surveys, the diversity of Apicomplexa in the environment appears to be much higher than previously recognized. Thus, the novel apicomplexan species related to the orphan phylotypes need to be identified to elucidate the ecological role of this protist group.

Phylotypes related to dinoflagellates and marine Alveolata groups I and II

DSGM-19–21 appeared to be phylotypes of dinoflagellates. DSGM-20 and DSGM-21 grouped robustly with *Gymnodinium* and *Scrippsiella*, respectively. Based on their strong phylogenetic affinities to planktonic species, the two phylotypes were possibly derived from cysts or sinking cells in the sediment. DSGM-19 was not related to any dinoflagellate species sequenced to date.

DSGM-22–28 and CPSGM-2 were positioned within the cluster of marine Alveolata group I with 100% BP, while DSGM-16–18 were within the cluster of marine Alveolata group II (Syndiniales) with 100% BP. Phylotypes belonging to marine Alveolata groups I and II have been isolated

from ecologically and geographically diverse marine habitats. Our present results further confirm the ubiquity of these two Alveolata groups.

Phylotypes related to ciliates

DSGM-29, DSGM-30, and CPSGM-3 were from ciliates. DSGM-29 was closely related to Armophorida composed of anaerobic species (e.g., *Nyctotherus*). The affinity between anerobic ciliates and DSGM-29 that was identified from oxygen-depleted sediment is reasonable. DSGM-30 and CPSGM-3 were not related to known anaerobic species. These phylotypes were possibly derived from cysts or sinking cells of anoxic-tolerant ciliates.

Stramenopiles

Eighteen phylotypes (DSGM-31–41, CYSGM-4–9, and CPSGM-4) were derived from stramenopiles (Fig. 4).

Phylotypes related to determined lineages

DSGM-31–34 were positioned within the radiation of the bacillariophycean algae with 64% BP, while DSGM-35 branched with a bolidophycean species with 56% BP. It is difficult to rationalize how these phylotypes (related to planktonic and photosynthetic species) were detected in the deep-sea sediment. These sequences may be derived from sinking and dying (or dead) cells. Alternatively, some

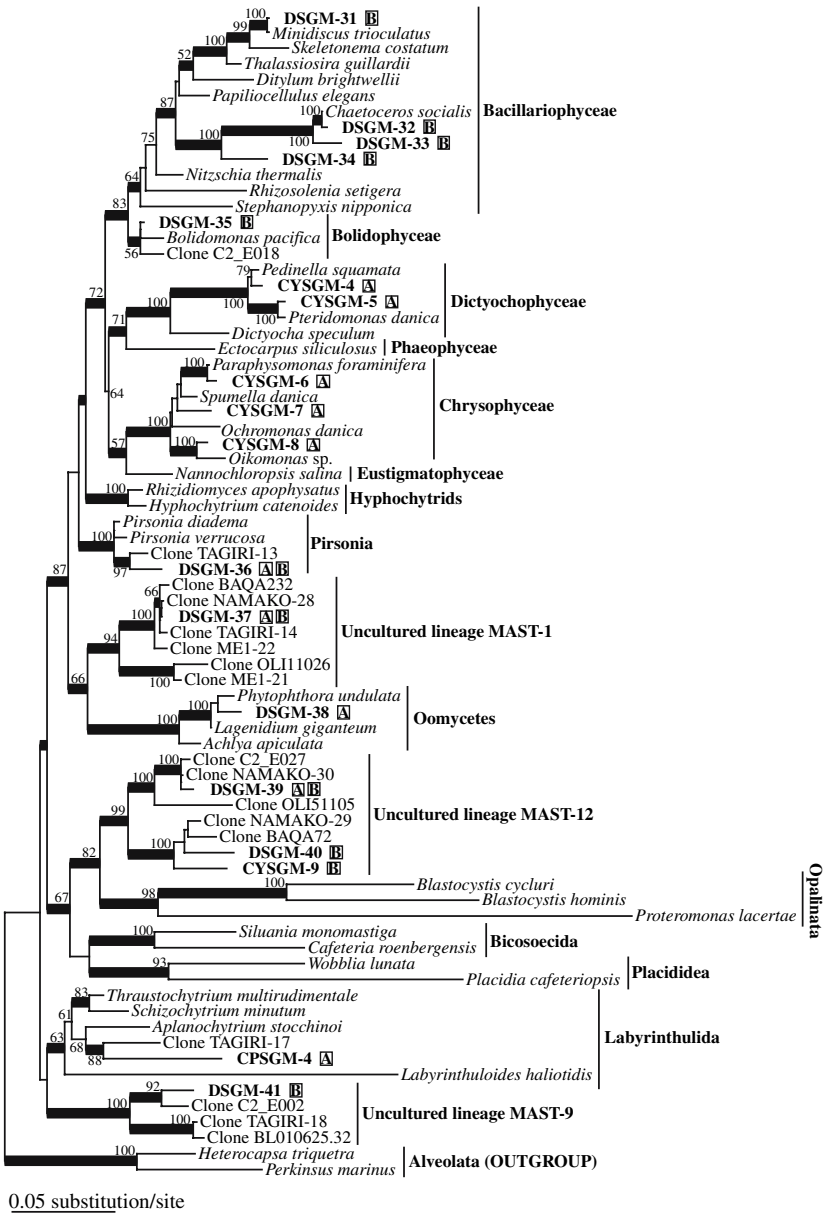

Fig. 4 Maximum likelihood phylogeny of SSU rDNA from stramenopiles. The details are the same as described in the legend of Fig. 3

bacillariophycean and/or bolidophycean cells were likely alive in the sediment, since phylotypes related to both heterokont groups have frequently been found in deep-sea and oxygen-depleted environments (Dawson and Pace 2002; Edgcomb et al. 2002; Stoeck and Epstein 2003; Stoeck et al. 2003, 2006; Takishita et al. 2005).

DSGM-36 and DSGM-38 were positioned within the radiation of *Pirsonia* and oomycetes with 100% BP, respectively. Since *Pirsonia* species are parasitoid nanoflagellates that infect members of the Bacillariophyceae (Schnepf et al. 1990; Kühn et al. 1996; Schweikert and Schnepf 1997), the organisms of DSGM-36 and the Bacillariophyceae-related phylotypes (DSGM-31–34) might be tightly associated in the environment. Oomycetes are also known to be composed of many parasites, but it remains unclear whether the organism represented by DSGM-38 is parasitic or free-living. If this phylotype is derived from a parasite, it remains unclear what the host might be.

CYSGM-4–9 were not detected in the surveys of the libraries from the raw sediment samples (DAL + DBL), and they were identified only after cultivation in YT medium. Thus, the abundance of the organisms related to these phylotypes may be low in the sediment studied. CYSGM-4 and CYSGM-5 belonged to the Dictyochophyceae with 100% BP, and CYSGM-6–8 belonged to the Chrysophyceae with 100% BP. CYSGM-5, CYSGM-6, and CYSGM-8 appeared to be closely related to the heterotrophic (probably bacterivorous) flagellates *Pteridomonas*, *Paraphysomonas*, and *Oikomonas*, respectively. Markedly, it has been reported that the heterotrophic chrysophycean flagellates grew and did not encyst at pressures up to 200 atm (Atkins et al. 1998). Thus, at least the organisms related to the phylotypes of Chrysophyceae might be grazers of small-sized cells in the ecosystem studied.

CPSGM-4 retrieved from the CPAL library nested in the labyrinthulid clade, with specific affinity to TAGIRI-17 from anoxic sediment (Takishita et al. 2005) with 88% BP. The cells of this group have usually been found in oxygen-depleted deep-sea environments (Raghukumar 2002), and some species are known to be anaerobic (Naqvi 1994). Thus, the organism related to CPSGM-4 may represent an anaerobic species of labyrinthulids.

Undetermined lineages MAST-1, MAST-9 and MAST-12

DSGM-37 branched strongly with the sequences of uncultured (undetermined) lineages of MAST-1. Because Massana et al. (2004b) hypothesized that MAST-1 is a planktonic cluster, DSGM-37 may be derived from an organism descended from the water column.

DSGM-41 was nested robustly in the MAST-9 clade. The MAST-9 lineage is composed mostly of sequences from hydrothermal vents, and therefore, MAST-9-related organisms are considered to be adapted to anoxic or suboxic habitats (Massana et al. 2004b). In addition to the MAST-9 sequences detected in the survey of anoxic sediment on a submarine caldera floor (Takishita et al. 2005), DSGM-41 in the sediment of a deep-sea methane cold seep further supports the hypothesis proposed by Massana et al. (2004b).

DSGM-39, DSGM-40, and CYSGM-9 were positioned within the radiation of MAST-12 with 99% BP. The MAST-12 phylotypes (including DSGM-39, DSGM-40, and CYSGM-9) have predominantly been identified in oxygen-depleted habitats, and the organisms related to these sequences have been suggested to be adapted to anoxic or suboxic environments similar to MAST-9 (Massana et al. 2004b).

Cercozoa

DSGM-42–61 and CYSGM-10–18 were phylotypes of Cercozoa (Fig. 5).

Phylotypes related to previously described species

2DSGM-42–50 and CYSGM-10–11 formed a monophyletic lineage with the genera *Cryothecomonas* and *Protaspis* with 99% BP. *Cryothecomonas* is an aerobic host-specific parasitoid nanoflagellate, and can cause high mortality among phytoplankton populations (Drebes et al. 1996; Tillmann et al. 1999). On the other hand, both *Cryothecomonas* and *Protaspis* have been reported to be phagotrophic and free-living (Thomsen et al. 1991; Ekebom et al. 1995; Ekelund and Patterson 1997; Tong et al. 1998). It is not clear whether the retrieved sequences represent parasites associated with phytoplankton or free-living predators.

DSGM-52–54 and CYSGM-13 branched with *Ebria* and environmental sequences (TAGIRI-1, TAGIRI-2, NAMAKO-9, and NAMAKO-10) from other oxygen-depleted sediment sites (Takishita et al. 2005, 2007) with 100% BP. Hoppenrath and Leander (2006) argued that *Ebria* species are members of planktonic communities and may produce resting cysts. Thus, the environmental sequences of this lineage including DSGM-52–54 and CYSGM-13 were possibly derived from resting cysts.

DSGM-57 was nested with *Pseudopirsonia* with 100% BP. Because *Pseudopirsonia* is an obligate parasitoid nanoflagellate that preys on marine diatoms (Kühn et al. 2004), the organism of phylotype DSGM-57 might infect either of the bacillariophycean algae represented by DSGM-31–34, like the *Pirsonia*-related phylotype DSGM-36.

No occurrence of Euglyphid species in a deep-sea and oxygen-depleted environment has been reported prior to the present study. CYSGM-17 and DSGM-59 were posi-

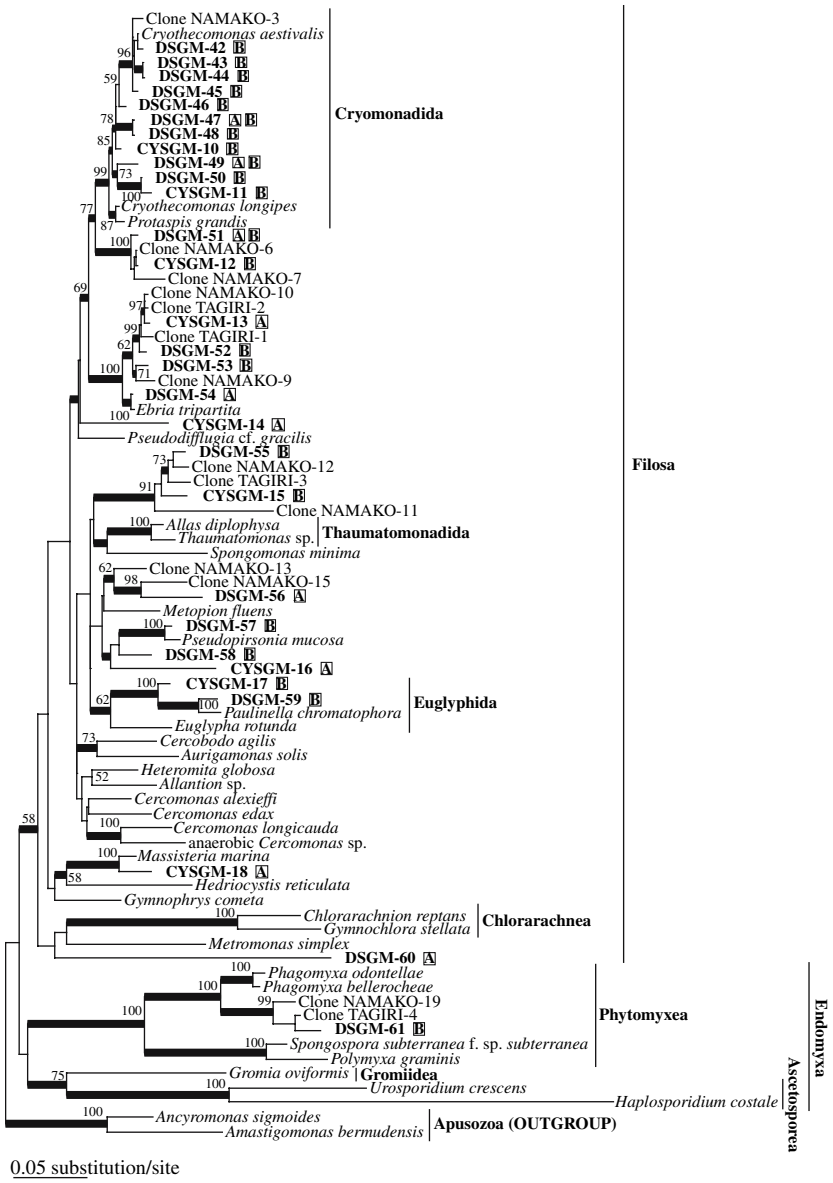

Fig. 5 Maximum likelihood phylogeny of SSU rDNA from Cercozoa. The details are the same as described in the legend of Fig. 3

tioned within the radiation of Euglyphida with 100% BP. DSGM-18 has a strong phylogenetic affinity with *Massisteria*, which was isolated from deep-sea hydrothermal vent sites (Atkins et al. 2000).

DSGM-61 was placed within the radiation of Phytomyxea, a group of Endomyxa, with 100% BP. Furthermore, a robust clade comprised of DSGM-61 and two phylotypes from oxygen-depleted sediments previously surveyed (TAGIRI-4 and NAMAKO-19; Takishita et al. 2005, 2007) was connected to the marine algal parasite genus *Phagomyxa* (Schnepf et al. 2000) with 100% BP. However, it is not clear whether the organisms represented by DSGM-61, TAGIRI-4, and NAMAKO-19 are parasites.

Phylotypes with no clear affinities to previously described species

DSGM-56, the clade of DSGM-51 and CYSGM-12, and that of DSGM-55 and CYSGM-15, were both exclusively composed of environmental sequences from oxygen-depleted habitats with ≥90% BP, suggesting that the organisms related to these phylotypes are anaerobic taxa. DSGM-58, DSGM-60, CYSGM-14, and CYSGM-16 showed no affinity to any organisms known to date. These phylotypes clearly indicate extremely high organism diversity of filosa, which may include anaerobic or anoxic-tolerant taxa.

Opisthokonta

Except for the phylotype of *C. curvatus* mentioned above (DSGM-62), five other phylotypes (DSGM-63–66 and CYSGM-19) were from Opisthokonta (Fig. 6).

Phylotypes related to fungi

CYSGM-19 was placed within the radiation of the genus *Candida* (Ascomycota) with 100% BP. It is widely held that many species of the order Saccharomycetales have a fermentative metabolism and anaerobic growth abilities, and *Candida* has been shown to grow under strictly anaerobic conditions (Dumitru et al. 2004). Thus, these previous findings agree with the recovery of the phylotype of *Candida* through anaerobic cultivation in the present study, and the organism related to this phylotype is probably involved in the fermentation of organic matter in the sediment.

DSGM-63 and DSGM-64 appeared to be from Chytridiomycota or Zygomycota, but had no clear affiliation with known fungal species sequenced to date. DSGM-64 robustly formed an independent group with environmental sequences TAGIRI-23 and NAMAKO-37 from oxygen-depleted sediments (Takishita et al. 2005, 2007) (to the exclusion of aerobic taxa), suggesting that the organisms related to these phylotypes are anaerobic.

Phylotypes related to non-fungus opisthokonts

DSGM-65 constituted a robust monophyletic clade with four environmental sequences (TAGIRI-26, TAGIRI-27,

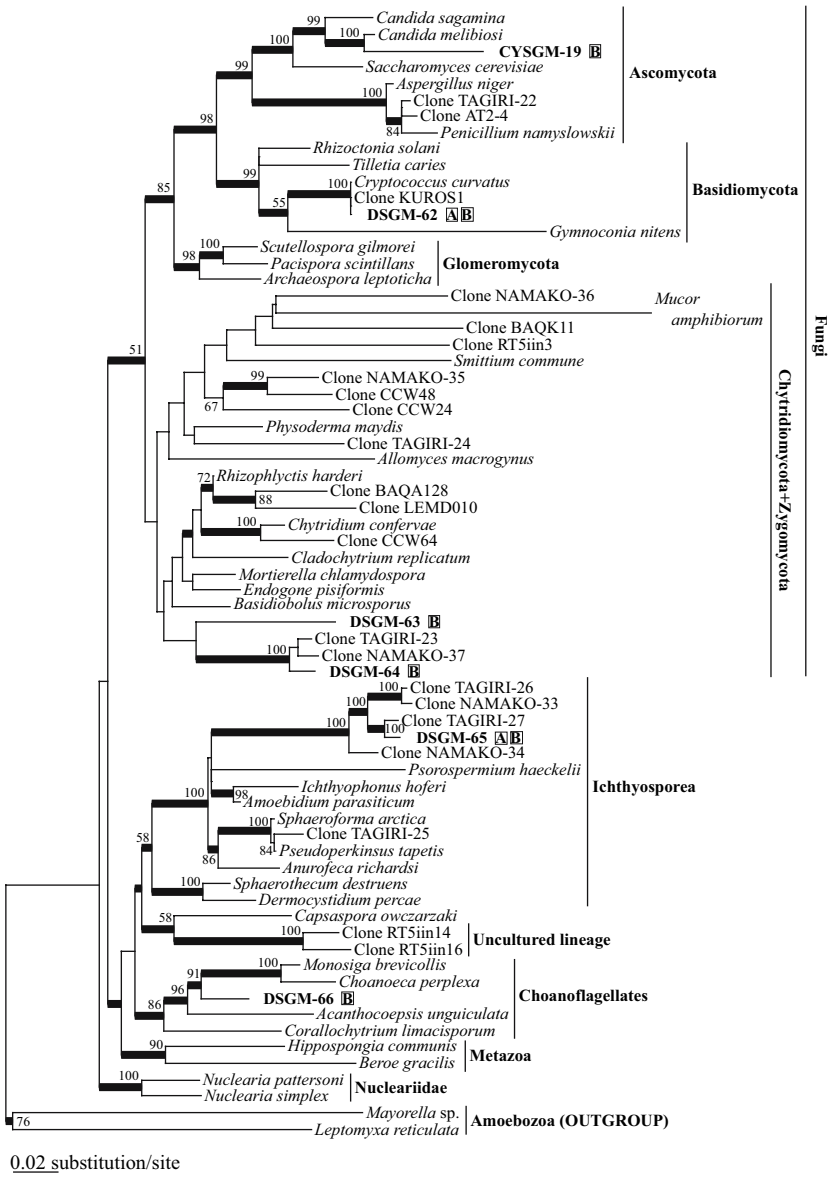

Fig. 6 Maximum likelihood phylogeny of SSU rDNA from Opisthokonta. The details are the same as described in the legend of Fig. 3

NAMAKO-33, and NAMAKO-34) from two oxygen-depleted sediment sites with different depths (20 and 200 m) (Takishita et al. 2005, 2007). The whole clade further grouped with the ichthyosporean sequences with 100% BP. Although all known species of Ichthyosporea are parasites infecting animals (Mendoza et al. 2002), we are uncertain whether the organisms related to these phylotypes [exclusively composed of sequences from oxygen-depleted sediments over a wide range of depths (20–1,170 m)] occur as parasitic or free-living forms (see Takishita et al. 2007).

DSGM-66 branched within the radiation of choanoflagellates with 86% BP. Because Atkins et al. (1998) reported that a choanoflagellate isolated from a deep-sea hydrothermal vent encysted at pressures greater than 50 atm, DSGM-66 may be derived from chanoflagellate cysts.

CPSGM-5 represents an excavate lineage

It is significant to link the phylotypes detected in an rDNA survey to the corresponding cells of organisms. Here, we successfully identified an organism that had been known only by an environmental sequence. CPSGM-5 was frequently encountered in surveys of libraries from the enrichment culture using 5% PYNFH medium (Fig. 2c). Interestingly, this phylotype appeared to be closely related to the environmental sequence C1_E027 from the Guaymas Basin hydrothermal vent, and this clade was then grouped with excavate protists *Carpediemonas* and *Retortamonas* (95% BP; Fig. 7). Based on the phylogenetic affinity described above, CPSGM-5 and C1_E027 were likely derived from excavate protists, particularly the cells belonging to the Fornicata (Simpson 2003).

Independent of the molecular surveys, we observed that one type of flagellate was dominant in the enrichment cultures using 5% PYNFH medium (Fig. 8a). Our microscopic observations revealed that the cells possessed a ventral feeding groove, and two flagella emerged from the anterior part of the cell. Since the ventral groove has been recognized as one of the morphological characteristics of typical excavates, the flagellates likely belong to Excavata (Simpson and Patterson 1999; Simpson 2003). We also isolated the excavate-like cells from the enrichment cultures and subsequently established a clonal culture (Fig. 8b). The SSU rDNA sequence of the clonal strain was almost identical to that of CPSGM-5 (data not shown). Consequently, we conclude that the excavate-like cells isolated here are very closely related to the organisms from which the SSU rDNA sequence was previously assigned to an early branching lineage (Edgcomb et al. 2002) or to a novel lineage of Excavata (Berney et al. 2004). This cultured organism will be formally described elsewhere in the future.

Other lineages

CYSGM-24 has phylogenetic affinity with *Amastigomonas*, a species of Apusozoa, with 97% BP (Fig. 7). Similarly, the members of Apusozoa have been found in deep-sea and anoxic habitats (Bernard et al. 2000; Atkins et al. 2000). DSGM-76–83 were phylotypes of trebouxiophycean or prasinophycean algae (Chlorophyta; see Supplementary data) and may be derived from sinking cells or cysts.

We recovered phylotypes that could not be assigned to major eukaryotic groups. DSGM-67–73 and CYSGM-20 formed an independent lineage with environmental sequences from both oxic and anoxic habitats previously reported in the global eukaryotic phylogeny with 100% BP (Fig. 7). Thus, these phylotypes possibly represent anoxic-tolerant taxa rather than anaerobic taxa. DSGM-75 appeared to be closely related to NAMAKO-2 (Takishita et al. 2007). The two phylotypes were both isolated from oxygen-depleted sediments, and thus organisms related to these sequences may be anaerobic. We have no theories regarding the organisms represented by DSGM-74 and CYSGM-21–23. Until organisms matching these taxonomically uncertain phylotypes are described, it is difficult to ascertain confidently whether these phylotypes are truly novel at a high taxonomic level. At least, our survey demonstrates the high levels of eukaryotic diversity in some oxygen-depleted environments.

Conclusions

In the present study, we investigated microeukaryotic diversity in deep-sea methane cold-seep sediment using the standard culture-independent procedure (survey of the library from the raw sediment samples), coupled with supplementary surveys of libraries from enrichment cultures. Significantly, the molecular surveys of the enrichment cultures detected phylotypes that were not identified in the surveys of the raw sediment samples.

In general, it is widely accepted that a survey of the rDNA library from a raw environmental sample undersamples the eukaryotic diversity (e.g., Massana et al. 2004a; Behnke et al. 2006; Zuendorf et al. 2006). Enormous amounts of rDNA clones from the library can be sequenced to counter this problem. However, surveys of both libraries from raw environmental samples and enrichment cultures may be an alternative strategy to reveal microeukaryotic diversity in an environment. In addition, in microscopic observations of enrichment cultures, there are opportunities to link phylotypes found in molecular surveys to organisms in an enrichment culture, as demonstrated in the present study. However, it may be difficult to retrieve phylogenetically divergent phylotypes from the library of a single

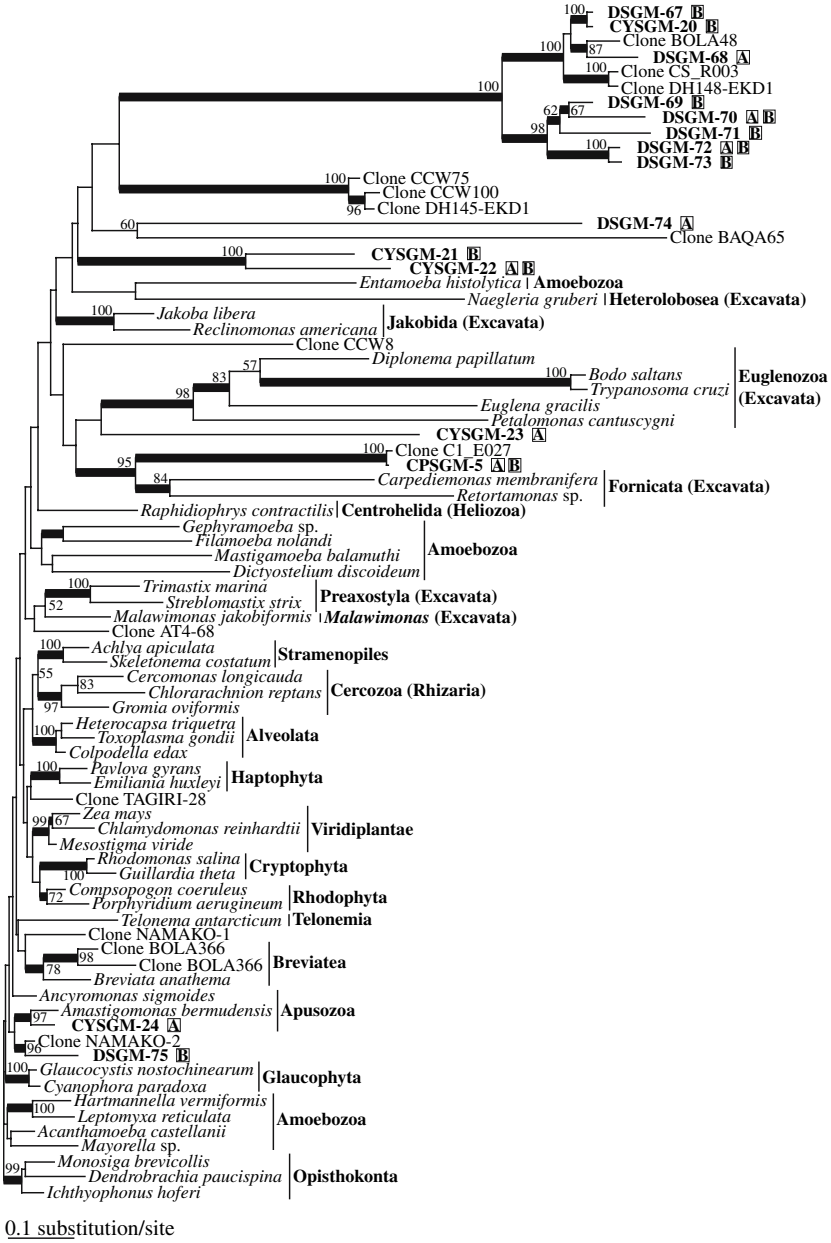

Fig. 7 Maximum likelihood phylogeny of SSU rDNA from major eukaryotic groups. The details are the same as described in the legend of Fig. 3

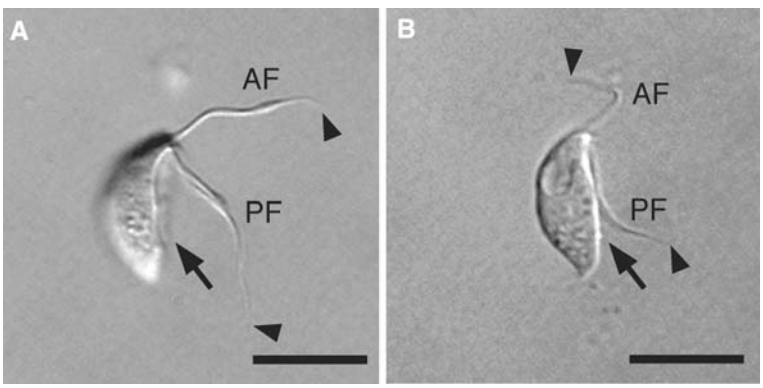

Fig. 8 Differential interference contrast (*DIC*) micrograph of a cell with a ventral feeding groove (*arrow*), anterior flagellum (*AF*), and posterior flagellum (*PF*). *Arrowheads* indicate the tips of the flagella; *scale bar* 10 μm. **a** A cell of the type found dominating the enrichment culture of 5% PYNFH medium, **b** a cell of the culture strain

enrichment culture, since certain culture conditions favor the growth of a limited number of taxa. Although we examined only two types of enrichment culture here, more varied culture conditions should be applied to future surveys of the diversity of microeukaryotes in environments.

Since anaerobic eukaryotes are generally difficult to maintain and/or grow in laboratories, the environmental molecular survey may be convenient to detect organisms adapted to oxygen-depleted environments (e.g., MAST-9 and MAST-12 of stramenopiles). Furthermore, when the molecular data accumulated from multiple oxygen-depleted environments, it is intriguing to compare ''overall'' phylotype compositions across the environments. At face value, the environments investigated possess unique overall community composition, and these differences may be attributable to differences in environmental factors (other than dissolved oxygen concentration). However, the community compositions in these environments were most probably underestimated. Indeed, the number of the rDNA clones sequenced in the present study was insufficient to cover the microeukaryotic diversity in the raw sediment sample (Fig. 1). Another potential problem is the presence of phylotypes originating from sinking and/or sunken cells that are not indigenous to the environment. We cannot definitely identify such ''contaminating'' phylotypes, and these sequences may significantly bias the comparison of microeukaryotic diversity across environments. Therefore, it is not prudent to discuss unique ecological characteristics across different oxygen-depleted environments based on incomplete composition data of microeukaryotes.

The diversity of the phylotypes related to eukaryotic parasites (such as Apicomplexa, Phytomyxea, and Ichthyosporea) identified is intriguing. The results presented here are similar to previous findings around deep-sea hydrothermal vents, where many sequences closely related to eukaryotic parasites (such as Kinetoplastida and Apicomplexa) have been detected (López-García et al. 2003). Moreira and López-García (2003) suggested that parasitic protists inhabiting these areas are possibly hosted by dense animal populations and may be responsible for the sudden massive mortality of those animals. However, the above-mentioned hypothesis cannot be directly applied to the environment studied here: no obvious animal cells were observed in the sediment at the microscopic level. In order to understand the organisms (and their life histories) corresponding to the parasite-related phylotypes detected in this study, in-depth knowledge of the entire ecosystem of deep-sea methane cold-seep sediments is essential.

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