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Genetic diversity of microbial eukaryotes in anoxic sediment around fumaroles on a submarine caldera floor based on the small-subunit rDNA phylogeny

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Abstract Recent culture-independent molecular analyses have shown the diversity and ecological importance of microbial eukaryotes (protists) in various marine environments. In the present study we directly extracted DNA from anoxic sediment near active fumaroles on a submarine caldera floor at a depth of 200 m and constructed genetic libraries of PCR-amplified eukaryotic small-subunit (SSU) rDNA. By sequencing cloned SSU rDNA of the libraries and their phylogenetic analyses, it was shown that most sequences have affiliations with known major lineages of eukaryotes (Cercozoa, Alveolata, stramenopiles and Opisthokonta). In particular, some sequences were closely related to those of representatives of eukaryotic parasites, such as *Phagomyxa* and *Cryothecomonas* of Cercozoa, *Pirsonia* of stramenopiles and Ichthyosporea of Opisthokonta, although it is not clear whether the organisms occur in free-living or parasitic forms. In addition, other sequences did not seem to be related to any described eukaryotic lineages suggesting the existence of novel eukaryotes at a high-taxonomic level in the sediment. The community composition of microbial eukaryotes in the sediment we surveyed was different overall from those of other anoxic marine environments previously investigated.

Keywords Anoxic · Diversity · Eukaryotes · Parasites · Phylogeny · Protists · SSU rDNA

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Introduction

Over the past several years there has been an increasing interest in the diversity of microbial eukaryotes, especially protists from various marine environments based on culture-independent molecular surveys. An unexpectedly high amount of diversity of eukaryotic small-subunit (SSU) rDNA sequence recovered from a 'pico-sized' fraction of the deep-sea (López-García et al. 2001) and the ocean surface (Díez et al. 2001; Moon-van der Staay at al. 2001) has been reported, and some of the SSU rDNA sequences represented novel lineages within known eukaryotic groups, such as alveolates (including dinoflagellates, ciliates and apicomplexans) and stramenopiles (including diatoms, brown algae, labyrinthurids and oomycetes etc.). This approach was applied to surveys of eukaryotic diversity in some extreme environments, such as: acidic and iron-rich rivers (Amaral Zettler et al. 2002); anoxic shallow sediments of marine water and freshwater (Dawson and Pace 2002); suboxic waters and anoxic sediments in a salt marsh (Stoeck and Epstein 2003); permanently anoxic deep-sea waters (Stoeck et al. 2003); and deep-sea hydrothermal vents (Edgcomb et al. 2002; López-García et al. 2003); as well as 'benign' environments of open sea (Massana et al. 2002); coasts (Massana et al. 2004); and a river (Berney et al. 2004). All these studies changed our view of the biosphere, revealing the extraordinary diversity of previously undetected eukaryotic lineages. In particular, the analyses for anoxic environments detected many novel phylotypes at a high-taxonomic (kingdom) level (Dawson and Pace 2002; Edgcomb et al. 2002; López-García et al. 2003; Stoeck et al. 2003; Stoeck and Epstein 2003), although some of them have been suggested to be artifacts caused by the failure to detect chimeric sequences, phylogenetic misplacement of fast-evolving sequences and incomplete sampling of described but yet as unsequenced eukaryotes (Berney et al. 2004). Eukaryotes may have evolved before the planet's biosphere became oxy-

genated (Knoll 1999), but previous evolutionary studies of anaerobic or anoxic-tolerant eukaryotes have been dominated by parasites of human and commercial animals, such as diplomonads, parabasalids, microsporidia and *Entamoeba*. Thus, such surveys of unknown eukaryotic microbes inhabiting natural anoxic environments have enhanced, and will continue to enhance, our understanding of the diversity and evolution of eukaryotes. A recent increase in molecular data from various environments also provides us with ecological information regarding uncultured eukaryotes. For example, several novel lineages of stramenopiles have been found in anoxic environments and probably represent truely anaerobic or anoxic-tolerant organisms rather than invaders originating from other environments, while other novel lineages of stramenopiles have been reported from geographically distant marine surface waters and are indeed cosmopolitan plankters (Massana et al. 2004).

In this study we focused on an anoxic marine environment in Kagoshima Bay in Japan. Kagoshima Bay is characterized by two gigantic calderas, which form the southern and the northern areas of the bay, respectively (Matsumoto 1943). The northern bay-head area is separated from the southern area by an active volcano, Mt. Sakurajima, connected by a shallow, narrow strait. The last big eruption of the northern caldera is thought to have occurred about 22,000 years ago (Aramaki and Ui 1966). In the eastern part of the northern bay-head area, two submarine fumaroles at depths of 80 m and 200 m are recognized by the appearance of released gas bubbles containing carbon dioxide, methane and hydrogen sulfide (Ossaka et al. 1992) and are called 'Tagiri' by local fishermen (Oki and Hayasaka 1978). The word 'Tagiri' means 'bubble and boil' in Japanese. Although the 'Tagiri' sites are quite shallow when compared with conventional deep-sea hydrothermal vents, the geochemical characteristics of these sites are believed to be the same or similar to those of the deep-sea vents. In fact, around the fumaroles on the caldera floor, petroleum of hydrothermal origin 'hydrothermal petroleum', as seen near deep-sea hydrothermal vents such as the Guaymas Basin (Simoneit and Lonsdale 1982), has been discovered (Yamanaka et al. 1999, 2000). Furthermore, a representative chemosynthetic animal typically associated with the deep-sea vents, a vestimentiferan tubeworm, was found around the 80 m fumarole site (Hashimoto et al. 1993). In addition, solemyid bivalves, polychaetes, palaemonid shrimps and galatheids were also identified at the same site (Hashimoto et al. 1993). On the contrary, it is curious that no megabenthos were found at the other fumarole's site on a flat bottom at 200 m depth, although filamentous bacteria swaying in a current of fumarolic gas and fluid were observed at this site (Naganuma 1991). The 200 m fumarole site is much more anoxic than the 80 m fumarole site where vestimentiferan tubeworms and other animals were found. This is attributed to the fact that seawater in the northern bay-head area deeper than 100 m is quite stagnant, being in a semi-closed basin (Takahashi 1981). This unusual anoxic environment is still enigmatic in terms of an ecosystem, prompting us to investigate which microbial eukaryotes naturally occur there.

In the present study, we directly isolated genomic DNA from the anoxic sediment of the 'Tagiri' site at 200 m, cloned and sequenced PCR-amplified products of eukaryotic SSU rDNA. Based on the retrieved sequences, phylogenetic analyses were performed to examine the diversity of eukaryotes in the sediment. The phylogenetic information obtained in our analyses was compared with those of other environments, especially anoxic ones.

Materials and methods

Sampling and measurement of environmental factors

The dive survey was conducted off Fukuyama in Kagoshima Bay using the ROV *Hyper-Dolphin* on November 22, 2003 (Cruise No. NT03-11, Hyper-Dolphin Dive#252). A sediment sample was collected with a sterile mud sampler (Ikemoto and Kyo 1993) at the 'Tagiri' site (204 m, 31° 39.747′N, 130° 46.285′E: WGS-84 Datum). Vertical profiles of environmental factors (depth, temperature, salinity and dissolved oxygen concentration) were measured and calculated using a CTD-DO meter (Seabird: SBE19 + SBE23) attached to the Hyper-Dolphin.

DNA extraction, PCR-amplification, cloning and sequencing

Total DNA from the sediment was extracted with UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). PCR-amplification using the total DNA as a template was performed with HotStarTaq DNA polymerase (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. Nuclear SSU rDNA were amplified by using different combinations of the primers 18S-42F (5′-CTCAARGAYTAAGCCATGCA-3′), 18S-82F (5′-GAAACTGCGAATGGCTC-3′), 18S-1498R (5′-CACCTACGGAAACCTTGTTA-3′), and 18S-1520R (5′-CYGCAGGTTCACCTAC-3′), under the following thermal cycle conditions: 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 confirmed on 1.0% agarose gel electrophoresis. The PCR-amplified DNA fragments were cloned into the pCR2.1 vector of the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Positive transformants of the libraries were screened by PCR amplification of inserts with Insert-Check-Ready-Blue (TOYOBO, Osaka, Japan). A total of 252 expected-size amplicons from the libraries was partially sequenced with an ABI PRISM 3700 DNA Analyzer (PE Biosystems, Foster City, CA, USA) using a BigDye Terminator Cycle Sequencing Ready Reaction

Kit (PE Biosystems) with either primer 18S-42F or 18S-82F. Non-redundant clones were chosen for complete sequencing. M13 Forward (−20), M13 Reverse and the internal primers EK-555F (5′-AGTCTGGTGCCAGCAGCCGC-3′) and EK-1269R (5′-AAGAACGGCCATGCACCAC-3′) were used to complete and overlap insert sequences. The sequences were analyzed using GENETYX-MAC version 8.0 (Software Development, Tokyo, Japan). The sequences were tested by 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.

Phylogenetic analyses

Low-quality and chimeric sequences were excluded from further phylogenetic analyses. The phylogenetic analyses included 29 distinct eukaryotic SSU rDNA sequences obtained in this study (GenBank under accession numbers AB191409-AB191437). By using CLUSTAL W version 1.8 (Thompson et al. 1994), new SSU rDNA sequences were aligned with those from various eukaryotes that were retrieved from the DNA Data Bank of Japan (DDBJ). The generated alignments were inspected by eye and manually edited; all ambiguous sites of the alignments were removed. Consequently, five different alignment datasets of SSU rDNA were generated for phylogenetic analyses: (1) Cercozoa (41 taxa/1556 sites); (2) Alveolata (40 taxa/1629 sites); (3) stramenopiles (46 taxa/1527 sites); (4) Opisthokonta (48 taxa/1575 sites); (5) representatives of most major eukaryotic groups (37 taxa/1401 sites). The alignment datasets are available on request from the corresponding author. Each dataset was tested for optimal fit of various models of nucleotide evolution using MODELTEST version 3.06 (Posada and Crandall 1998). The proportion of invariable sites, a discrete gamma distribution (four categories) and base frequencies were estimated from the dataset. Maximum-likelihood (ML) distance was calculated under an optimal model. A distance tree was constructed using the neighbor joining (NJ) (Saitou and Nei 1987) method. The ML distance bootstrap analysis (Felsenstein 1985) (1,000 replicates) was also performed. Furthermore, for the alignment including representatives of most major eukararyotic groups, ML analysis with 20 random additions and the tree bisection-reconnection branch-swapping option was also performed under the model selected by MODELTEST. For all phylogenetic analyses in this study, PAUP* version 4.0 was used.

Results and discussion

Temperature, salinity and dissolved oxygen concentration

The vertical profiles of environmental factors are shown in Fig. 1. Temperature and salinity were 21.7°C and 33.2–33.3 PSU on the surface, respectively. A thermocline and a halocline existed between 60 m and 90 m depths, with the temperature and salinity just under the layer approximately 17.2°C and 34.0 PSU, respectively. Below the layer, the temperature and salinity stabilized at 17.0–16.4°C and 34.0–34.1 PSU, respectively. Dissolved oxygen was 4.0–4.1 ml/l on the surface and decreased rapidly at 25 m and 170 m depths. Dissolved oxygen was 0.05 ml/l at the bottom (200 m depth). The temperature, salinity, and dissolved oxygen concentration in water just above (approximately 1.5 m) the sampling site (at 204 m depth) were 16.4°C, 34.1 PSU, and 0.04 ml/l, respectively.

Model selection for phylogenetic analyses

Five alignment datasets were independently analyzed using MODELTEST. Through calculation of log-likelihood scores, this program found that a TrN model of nucleotide evolution (Tamura and Nei 1993) incorporating invariable sites and a discrete gamma distribution (four categories) (TrN + I + Γ) was better than other models examined for all alignment datasets. Each dataset was therefore analyzed using an ML distance method under this model. ML analysis for the fifth dataset including major eukaryotic groups was also performed under this model.

Cercozoa

The relative abundance of clones of Cercozoa (five phylotypes, TAGIRI-1∼5) was 12.5%. The NJ tree (Fig. 2) was composed of three major distinctly separate subgroups of Cercozoa: the parasitic Haplosporidia; the parasitic Phytomyxea and the Filose (Cavalier-Smith and Chao 2003) including the chlorarachniophyte algae, testate amoebae and biciliate zooflagellates. In the tree two species of Apusozoa (*Ancyromonas sigmoides* and *Amastigomonas bermudensis*) were used as outgroups. Three phylotypes TAGIRI-1, TAGIRI-2 and TAGIRI-3 belonged to the Filosan clade. Two of them, TAGIRI-1 and TAGIRI-2 were monophyleic with 100% bootstrap value, and this monophyletic branch clustered with the genus *Cryothecomonas* with 86% bootstrap value. *Cryothecomonas* is a host specific parasitoid nano-flagellate and can cause great mortality among phytoplankton populations (Drebes et al. 1996; Tillmann et al. 1999). It is uncertain whether the phylotypes TAGIRI-1 and TAGIRI-2 originated from the *Cryothecomonas*-like parasites or not. The other filosan phylotype TAGIRI-3, did not show supportable phylogenetic affinity with any described species or with the other uncultured environmental clones of Filosa. Two phylotypes, TAGIRI-4 and TAGIRI-5, were within the radiation of Phytomyxea with 100% bootstrap support. The phytomyxean linages is divided into two clades, Plasmodiophorida (land plant parasites) and Phagomyxida (marine algal parasites) (Bulman et al. 2001;

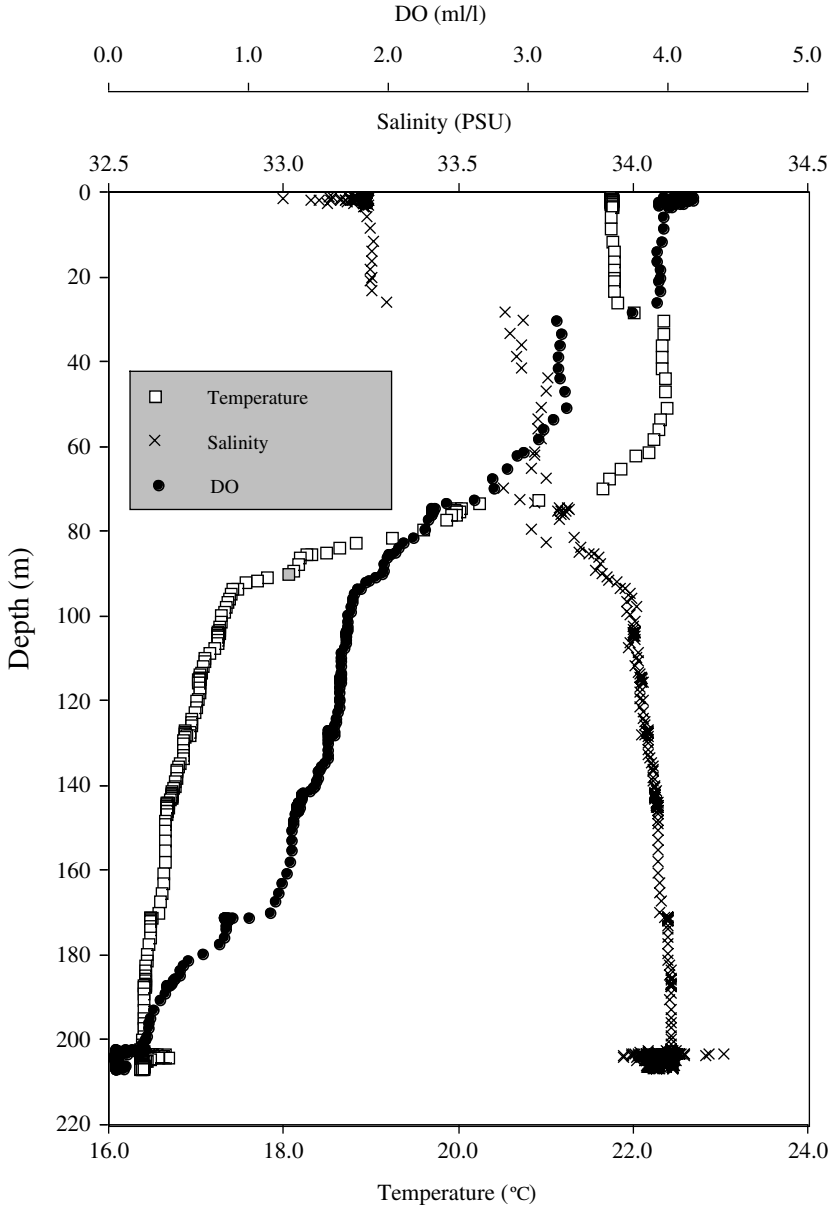

Fig. 1 Vertical profiles of environmental factors (temperature, salinity and dissolved oxygen concentration)

Cavalier-Smith and Chao 2003). TAGIRI-4 branched with members of the Phagomyxida, the genus *Phagomyxa*, with 100% bootstrap support, and TAGIRI-5 was a sister to the lineage comprising *Phagomyxa* and TAGIRI-4 with 70% bootstrap support. This is the first report of uncultured environmental clones from Phytomyxea and it is unclear whether the organisms of these two phylotypes are free-living or parasitic in the sediment.

Alveolata

The relative abundance of clones of Alveolata (five phylotypes, TAGIRI-6∼10) was 8.4%. This value was rather low compared to environmental molecular surveys previously reported (López-García et al. 2001, 2003; Dawson and Pace 2002; Stoeck and Epstein 2003; Berney et al. 2004). The NJ tree (Fig. 3) was composed of six major independent subgroups of Alveolata: dinoflagellate; ciliates; apicomplexans; Perkinsozoa; marine alveolate group I and marine alveolate group II. In the tree two species of stramenopiles (*Skeletonema costatum* and *Achlya apiculata*) were used as outgroups. Three phylotypes TAGIRI-6, TAGIRI-7, and TAGIRI-8 were placed within the dinoflagellate radiation with 81% bootstrap support. Two of them, TAGIRI-6 and TAGIRI-7 did not specifically nest with described species of dinoflagellates, and could not be attributed to any already known dioflagellate genera. The other phylotype TAGIRI-8 was very closely related to *Gymnodinium beii* (99.8% identity). Although *G. beii* is known to be an intracellular symbiont of foraminifera (Spero 1987), we could not identify any foraminifera in the sediment

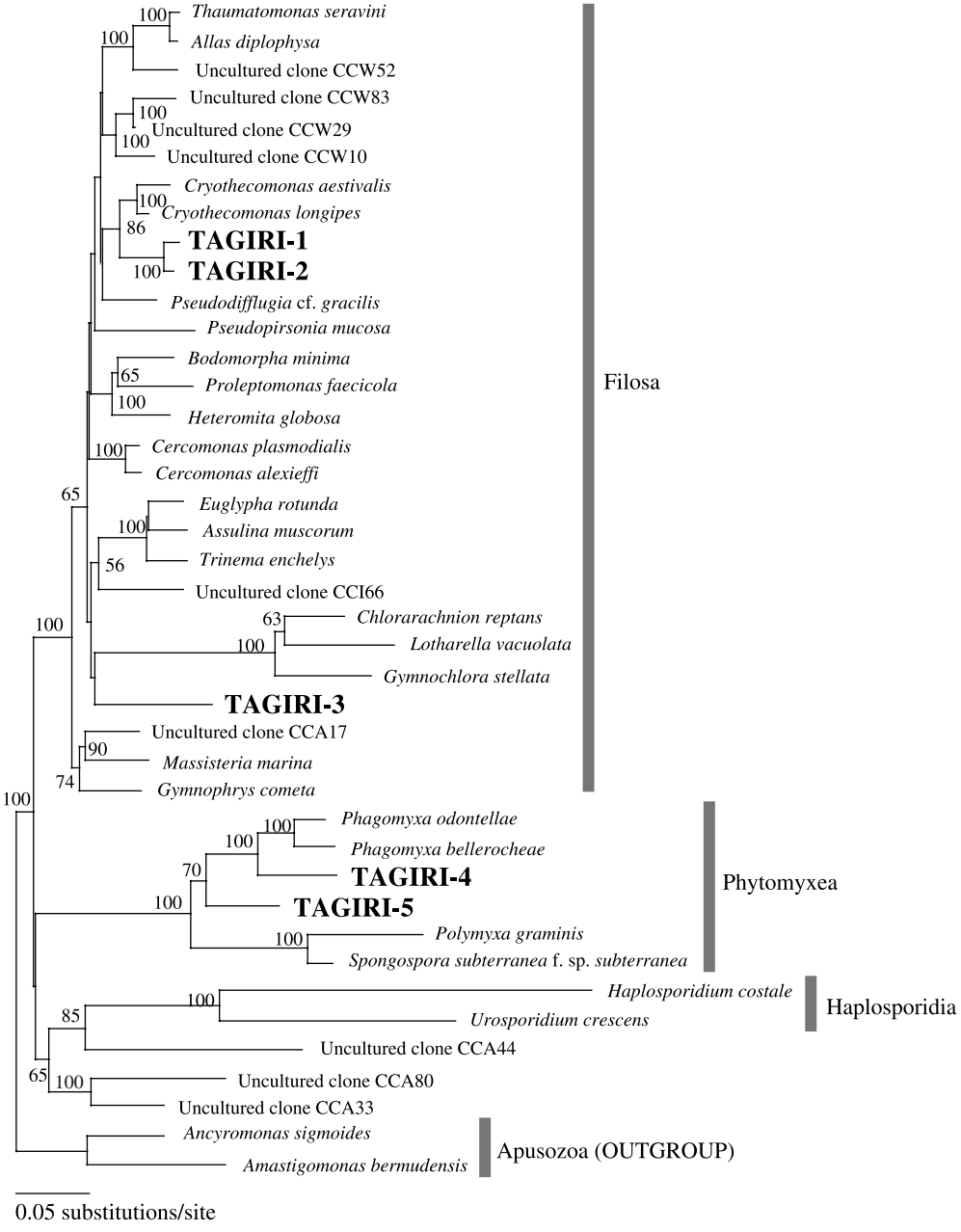

Fig. 2 The maximum-likelihood distance tree of Cercozoa (41 taxa/1556 sites) reconstructed under a TrN nucleotide substitution model incorporating the proportion of invariable sites (I = 0.2793) and a discrete gamma distribution (four categories) (parameter $\alpha = 0.5184$). Two species of Apusozoa, *Ancyromonas sigmoides* and *Amastigomonas bermudensis*, were used to root the tree. Numbers at the nodes refer to the percentage (> 50%) of bootstrap support of maximum-likelihood distance analysis

sample with light microscopy, and could not obtain any PCR-amplified products with the set of primers specific to foraminiferan SSU rDNA (Pawlowski et al. 1997). Thus, it is possible that the dinoflagellate of phylotype TAGIRI-8 occurs as a free-living cell or a symbiont of organisms other than foraminifera in the sediment. Although many dinoflagellates are known to be benthic, we cannot exclude the possibility that these three dinoflagellate phylotypes have planktonic origins: They may occur as a cyst or a detrital precipitation in the sediment. Two phylotypes TAGIRI-9 and TAGIRI-10, belonged to the lineage of marine alveolate group I with a bootstrap value of 100%. The SSU rDNA sequences of marine alveolate group I have been retrieved from diverse marine environments, including anoxic ones. Especially, TAGIRI-9 was very closely related to the phylotypes AT4-42 from deep-sea hydrothermal sediment at the Mid-Atlantic Ridge (López-García et al. 2003), C1_E008, C1_E020, C2_E006, C2_E017 and C3_E003 from deep-sea hydrothermal sediment at the Guaymas Basin (Edgcomb et al. 2002) and D12, D51, D188, D205, D254, E27, E214, E228 and H61 from anoxic water in the Cariaco Basin in the Caribbean Sea (Stoeck et al. 2003). Of the previously reported sequences above, only AT4-42 was included in our phylogenetic analysis, as the other sequences are short. Therefore, this phylotype may be ubiquitous in anoxic habitats. Sequences from ciliates, apicomplexans, Perkinsozoa and marine alveolate group II were not obtained in the present study.

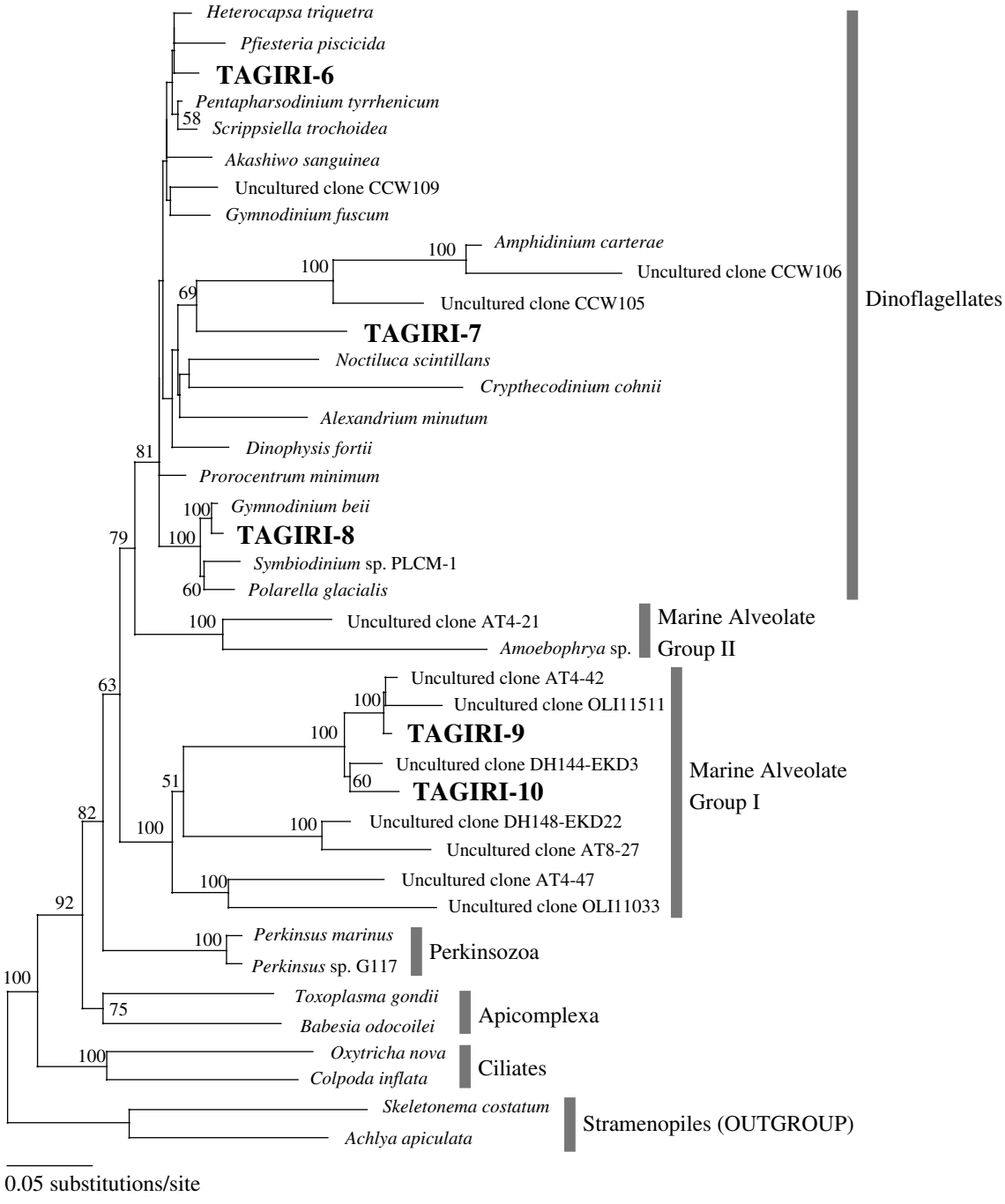

Fig. 3 The maximum-likelihood distance tree of Alveolata (40 taxa/1629 sites) reconstructed under a TrN nucleotide substitution model incorporating the proportion of invariable sites (I = 0.2950) and a discrete gamma distribution (four categories) (parameter $\alpha = 0.6592$). Two species of stramenopiles, *Skeletonema costatum* and *Achlya apiculata*, were used to root the tree. Numbers at the nodes refer to the percentage (> 50%) of bootstrap support of maximum-likelihood distance analysis

Stramenopiles

The relative abundance of clones of stramenopiles (eight phylotypes, TAGIRI-11∼18) was 15.4%. The NJ tree (Fig. 4) encompassed several photosynthetic, heterotrophic and two uncultured (novel) lineages of stramenopiles. In the tree, two species of Alveolata (*Heterocapsa triquetra* and *Perkinsus marinus*) were used as outgroups. The phylotype TAGIRI-13 affiliated with the genus *Pirsonia* with 100% bootstrap support. *Pirsonia* species are parasitoid nanoflagellates that infect planktonic diatoms (Schnepf et al. 1990; Kühn et al. 1996; Schweikert and Schnepf 1997), like *Cryothecomonas* of Cercozoa. This phylotype may be attributed to the genus *Pirsonia*, but its ecological role in the sediment remains unknown. Two phylotypes TAGIRI-11 and TAGIRI-12 were placed within the diatom radiation with a bootstrap value of 100%. Specifically, TAGIRI-11 and

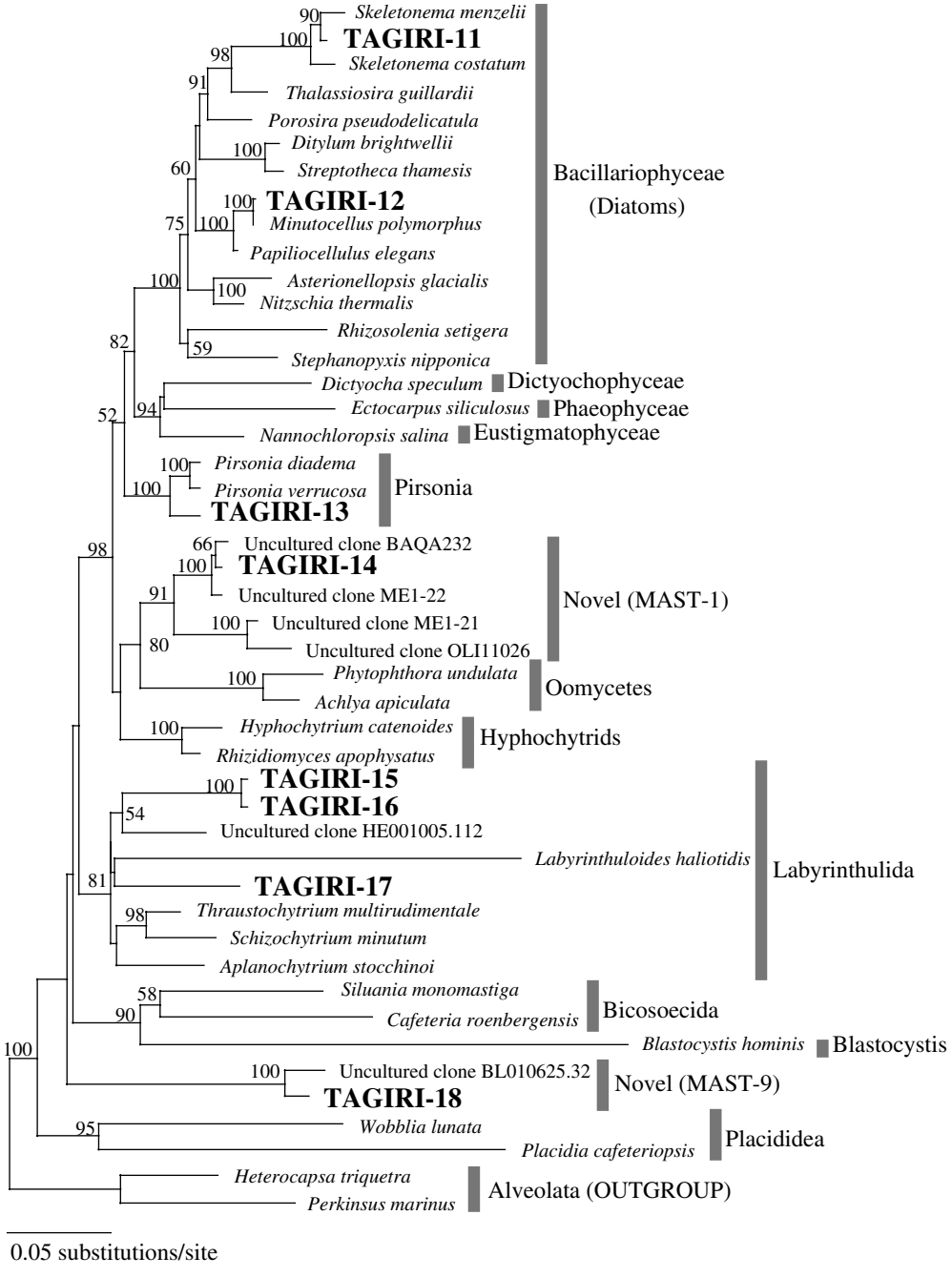

Fig. 4 The maximum-likelihood distance tree of stramenopiles (46 taxa/1527 sites) reconstructed under a TrN nucleotide substitution model incorporating the proportion of invariable sites (I = 0.3376) and a discrete gamma distribution (four categories) (parameter $\alpha = 0.5646$). Two species of Alveolata, *Heterocapsa triquetra* and *Perkinsus marinus*, were used to root the tree. Numbers at the nodes refer to the percentage (> 50%) of bootstrap support of maximum-likelihood distance analysis

TAGIRI-12 were closely related the genera *Skeletonema* and *Minutocellus*, respectively. Because these genera are representatives of planktonic diatoms, TAGIRI-11 and TAGIRI-12 may originate from sinking (dead) cells. Alternatively, it is possible that they occurred as cysts in the sediment. As several phylotypes of possible diatom parasites (*Cryothecomonas*-, *Phagomyxa*- and *Pirsonia*-like organisms) were found in this study, the cells of TAGIRI-11 and TAGIRI-12 may be infected by these parasites. TAGIRI-15, TAGIRI-16 and TAGIRI-17 belonged to the labyrinthulid lineage with 81% bootstrap support, and TAGIRI-15 and TAGIRI-16 were closely related to each other. These three phylotypes were basally positioned within this lineage, although bootstrap support was rather low and the group had little structure. Neither phylotype showed phylogenetic affinity with any described species or previously reported uncultured environmental clones of Labyrinthulida. (Most previously reported environmental clones were not included in the phylogenetic tree, because their sequences are short.) TAGIRI-14 and TAGIRI-18 are robustly clustered with members of the novel stramenopile lineages, MAST-1 and MAST-9 (Massana et al. 2004), respectively. Massana et al. (2004) have argued that MAST-1 contains sequences retrieved from many pelagic oceans and a single coastal site (Roscoff),

and appears to be a planktonic cluster. However, because the phylotypes of MAST-1, TAGIRI-14 and BAQA232 (Dawson and Pace 2002) are obtained from anoxic sediment, it is possible that MAST-1 includes both planktonic and benthic organisms. Massana et al. (2004) have also argued that MAST-9 exclusively includes the sequences from hydrothermal vents, and probably represents anaerobic or anoxic-tolerant organisms. (Our phylogenetic analysis did not include the MAST-9 members, C1_E021, C1_E024, C2_E002, C2_E014, C2_E028, C2_E043, C3_E002, C3_E004, C3_E007, C3_E008, C3_E017, C3_E019, C3_E026 and C3_E044 from deep-sea hydrothermal sediment at the Guaymas Basin [Edgcomb et al. 2002], because they are partial sequences.) The fact that the phylotype of MAST-9 was retrieved from the 'Tagiri' site supports their hypothesis.

Opisthokonta

The relative abundance of clones of Opisthokonta (nine phylotypes, TAGIRI-19~27) was 60.0%. The NJ tree (Fig. 5) was composed of four major clade of Opisthokonta: Ichthyosporea; choanoflagellates; fungi and Metazoa. In the tree, two species of Apusozoa (*Ancyromonas sigmoides* and *Amastigomonas bermudensis*) were used as outgroups. Three phylotypes TAGIRI-25, TAGIRI-26 and TAGIRI-27, were placed within the radiation of Ichthyosporea with 80% bootstrap support, and TAGIRI-26 and TAGIRI-27 were closely related to each other. Specifically, TAGIRI-25 clustered with the clam parasite *Pseudoperkinsus tapetis* and the amphipod parasite *Sphaeroforma arctica* with 100% bootstrap support. This is the first report of uncultured environmental clones from Ichthyosporea. The Ichthyosporea

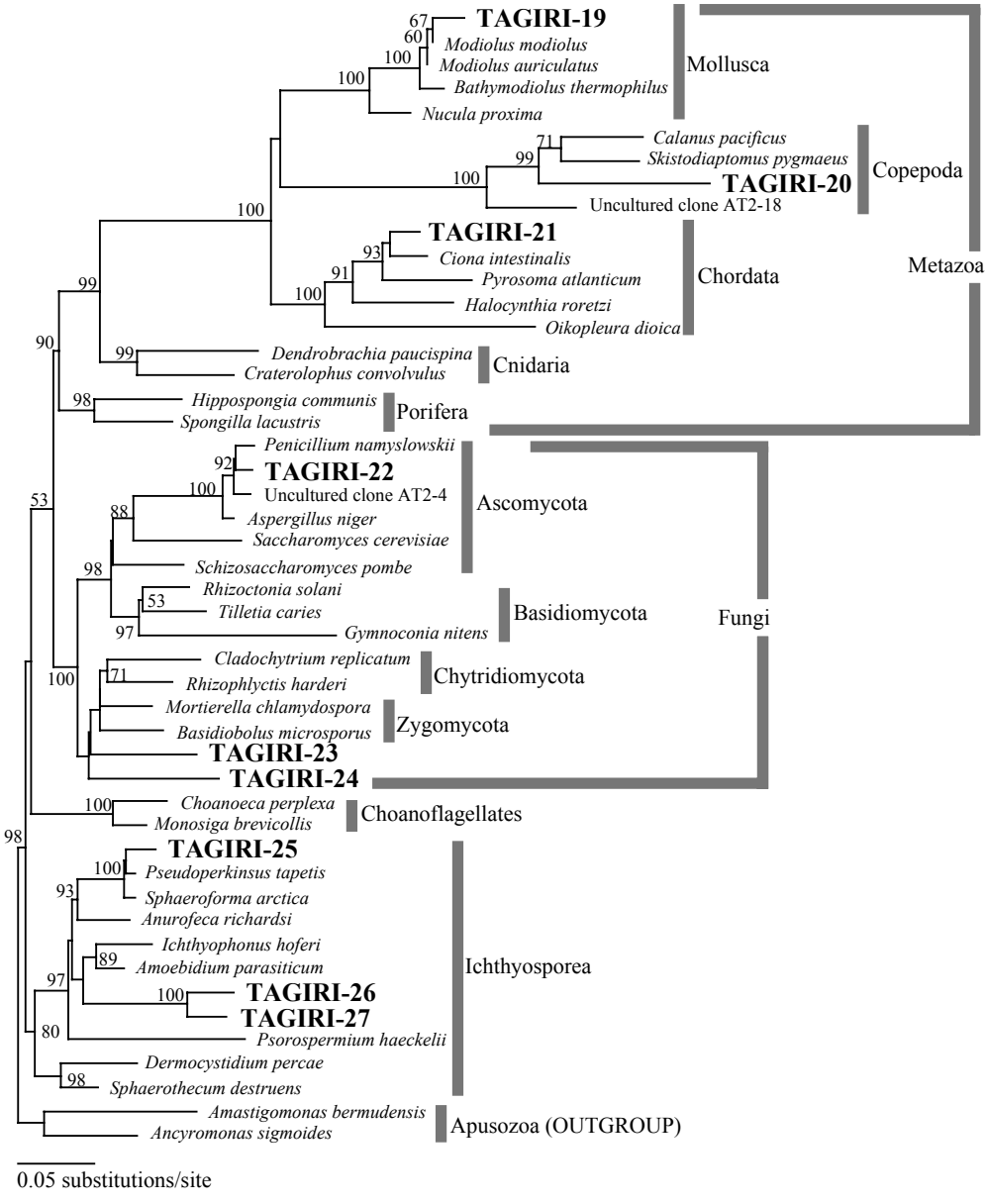

Fig. 5 The maximum-likelihood distance tree of Opisthokonta (48 taxa/1575 sites) reconstructed under a TrN nucleotide substitution model incorporating the proportion of invariable sites (I = 0.3188) and a discrete gamma distribution (four categories) (parameter $\alpha = 0.5759$). Two species of Apusozoa, *Ancyromonas sigmoides* and *Amastigomonas bermudensis*, were used to root the tree. Numbers at the nodes refer to the percentage (> 50%) of bootstrap support of maximum-likelihood distance analysis

are known to infect a variety of animals, including humans. If the organisms of Ichthyosporean phylotypes found in the present study are indeed parasites, their life cycle (especially in the sediment) should be elucidated. Three phylotypes TAGIRI-22, TAGIRI-23 and TAGIRI-24 were placed within the fungal lineage with 100% bootstrap support. TAGIRI-22 was most dominant in terms of numbers of clones (15.8%) and affiliated with Ascomycotan species, such as *Penicillium* and *Aspergillus*. Because this phylotype was also closely related to AT2-4 retrieved from hydrothermal sediment at the Mid-Atlantic Ridge (López-García et al. 2003), TAGIRI-22 and AT2-4 may be Ascomycotan fungi and be ubiquitous in anoxic hydrothermal sediment. On the other hand, two other phylotypes of fungi TAGIRI-23 and TAGIRI-24 could not be attributed to well-defined phylogenetic groups. It is likely that they belong to either Chytridiomycota or Zygomycota. (The phylogenetic positions of species belonging to Chytridiomycota and Zygomycota were unstable within the fungal lineage.) Three phylotypes of Metazoa TAGIRI-19, TAGIRI-20 and TAGIRI-21, were positioned within the lineages of Mollusca (Mytilidae), Copepoda and Chordata, respectively (all 100% bootstrap support). TAGIRI-20 was relatively abundant in the numbers of clones (13.3%). As we could not identify any multicellular animals in the sediment sample under light microscopic observation, TAGIRI-19 and TAGIRI-21 possibly originated from larvae, gametes or dead tissue of the respective animals. No megabenthos was found at 'Tagiri' site at 200 m depth, and therefore it is highly likely that the organisms (larvae or gametes or dead tissue) of TAGIRI-19 and TAGIRI-21 come from other environments.

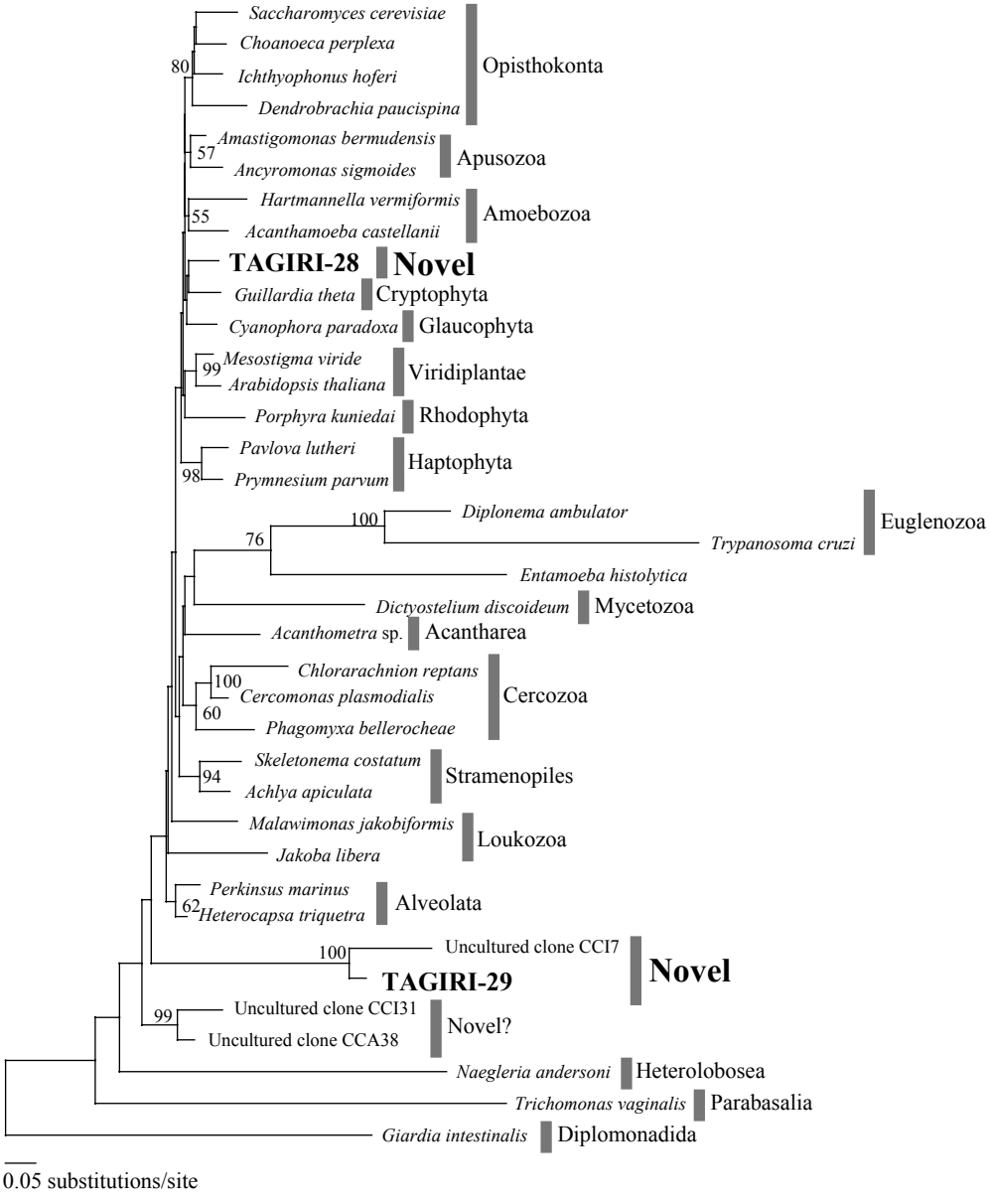

Fig. 6 The maximum-likelihood distance tree of major eukaryotic groups (37 taxa/1401 sites) reconstructed under a TrN nucleotide substitution model incorporating the proportion of invariable sites (I = 0.0889) and a discrete gamma distribution (four categories) (parameter $\alpha = 0.5467$). The diplomonad *Giardia intestinalis* was used to root the tree. Numbers at the nodes refer to the percentage (> 50%) of bootstrap support of maximum-likelihood distance analysis

Possibly novel lineages at a high-taxonomic level

Simulation studies indicate that ML method is significantly robust to 'long branch attraction (LBA)', whereby highly diverged sequences are artifactually attracted to one another (Swofford et al. 2001). Thus, not only an NJ tree (Fig. 6) but also an ML tree (Fig. 7) was constructed for the global eukaryotic phylogeny, because several sequences included in the analysis were extremely divergent. The diplomonad *Giardia intestinalis* was used to root the trees. The phylotype TAGIRI-28 deeply branched in the trees. Based on the chimera check and partial treeing analysis, it is unlikely that the sequence of TAGIRI-28 is of chimeric origin. Furthermore, because the sequence is not divergent, its phylogenetic status is most likely not caused by a phylogenetic artifact such as LBA. Although we can not exclude the possibility that TAGIRI-28 is derived from a described, but as yet unsequenced eukaryote, it is reasonable to assume that this phylotype truly represents a novel eukaryotic lineage at the high-taxonomic level. The phylotype TAGIRI-29 branched with the already published phylotype CCI7 from the anoxic sediment–water interface of the great Sippewisset salt marsh (Stoeck and Epstein 2003) with 100% bootstrap value, and this monophyletic lineage was not specifically related to any known eukaryotes at the high-taxonomic level. Stoeck and Epstein (2003) have suggested that CCI7 is closely related to CCI31 and CCA38 retrieved from the same environment, but our phylogenetic analysis did not support this affiliation. Instead, the independent lineage comprising of CCI31 and CCA38 deeply branched in the NJ tree (Fig. 6) and clustered with Alveolata in the ML tree (Fig. 7) (with no bootstrap supports). Like TAGIRI-28, TAGIRI-29 is unlikely to be a chimeric product. Although the sequence of TAGIRI-29 is relatively divergent, the monophyletic lineage of TAGIRI-29 and CCI7 is still deeply branched in the ML tree. Thus, this lineage might represent a novel eukaryotic group, although more taxon sampling of known eukaryotes, especially protists, would be required. Furthermore, because both TAGIRI-29 and

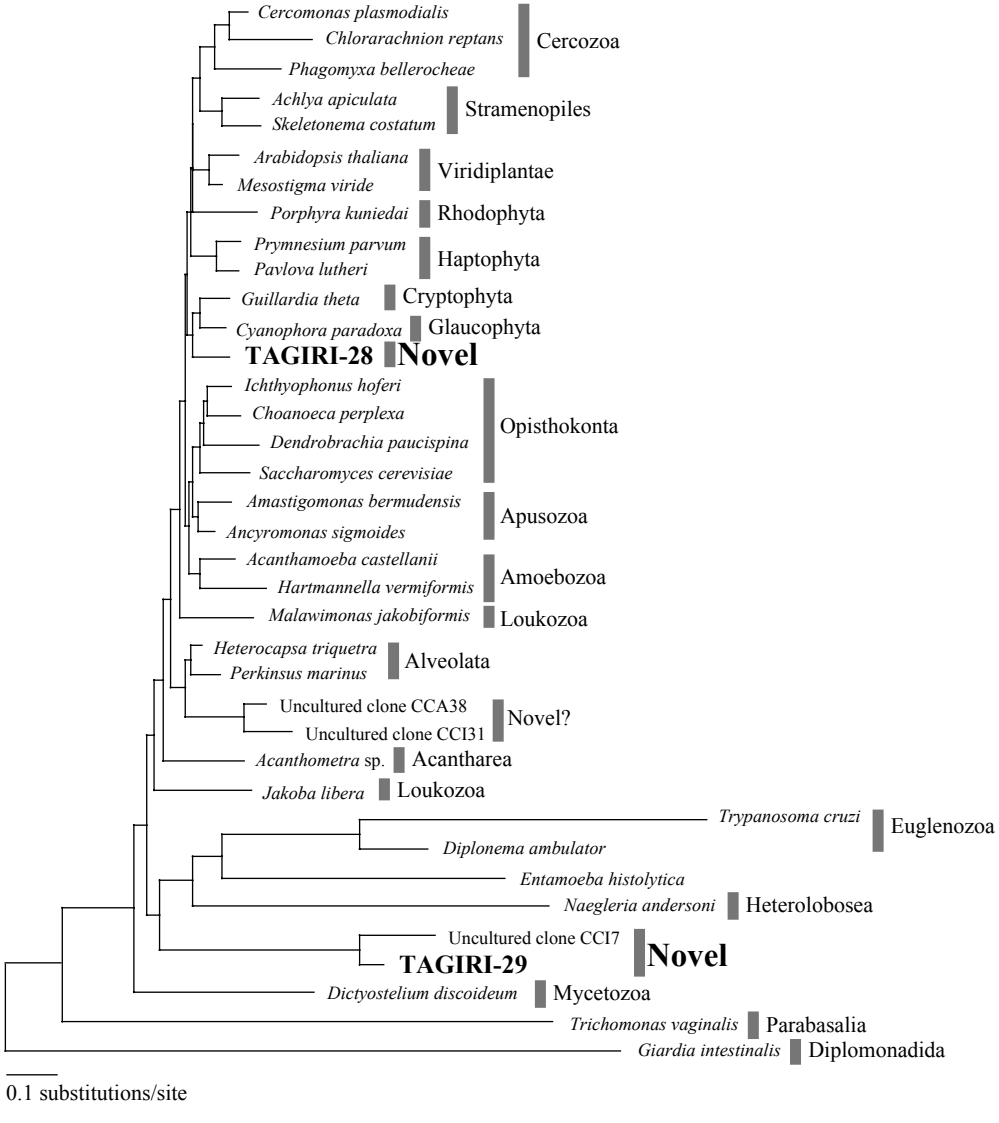

Fig. 7 The best maximum-likelihood tree inferred from the dataset including major eukaryotic groups (37 taxa/1401 sites) under a TrN nucleotide substitution model incorporating the proportion of invariable sites (I = 0.0889) and a discrete gamma distribution (four categories) (parameter $\alpha = 0.5467$). The diplomonad *Giardia intestinalis* was used to root the tree

CCI7 were retrieved from anoxic environments, these possibly novel eukaryotic organisms may be anaerobic or anoxic-tolerant. TAGIRI-28 and TAGIRI-29 were low in abundance (1.6% and 2.1%, respectively).

Comparison of eukaryotic diversity with those of other environments

The sampling of clone libraries from the sediment investigated in this study appeared to almost reach saturation in spite of sequencing of only 252 clones (data not shown), suggesting that the diversity of eukaryotes at the research site is not so high, when compared with other environmental surveys previously reported. (Of course, it is probable that some phylotypes present could not be retrieved due to problems associated with primer selection and DNA extraction.) Remarkably, no phylotype of ciliates was retrieved in the present analysis, although many phylotypes of this group have been identified from most other environments, including anoxic ones. Furthermore, we found no phylotypes belonging to apicomplexans, Perkinsozoa, kinetoplastids, bodonids, Acantharea and Polycystinea recovered from deep-sea hydrothermal vents (Edgcomb et al. 2002; López-García et al. 2003) in our clone libraries. These results, taken together with the occurrence of Phytomyxean and Ichthyosporean phylotypes lead us to consider that the community composition of eukaryotes in the sediment of the 'Tagiri' site appears to be unique despite a small fraction of phylotypes obtained in the present study being closely related to those from other anoxic environments (as mentioned above). Moreira and López-García (2003), have argued that parasitic protists inhabiting the area around deep-sea hydrothermal vents, such as Apicomplexa and kinetoplastids, are possibly hosted by dense animal populations, and may cause sudden massive mortality of these animals. However, the ecological significance of the putative eukaryotic parasites found in the present study remains unclear. Efforts toward their isolation, cultivation and infection testing are necessary.

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References

Amaral Zettler LA, Gómez F, Zettler E, Keenan BG, Amils R, Sogin ML (2002) Eukaryotic diversity in Spain's River of Fire. Nature 417:137

Aramaki S, Ui T (1966) The Aira and Ata pyroclastic flow and related caldera and depressions in southern Kyushu, Japan. Bull Volcanol 29:29–48

Berney C, Fahrni J, Pawlowski J (2004) How many novel eukaryotic 'kingdoms'? Pitfalls and limitations of environmental DNA surveys. BMC Biol 2:13

Bulman SR, Kühn SF, Marshall JW, Schnepf E (2001) A phylogenetic analysis of the SSU rRNA from members of the Plasmodiophorida and Phagomyxida. Protist 152:43–51

Cavalier-Smith T, Chao EE (2003) Phylogeny and classification of phylum Cercozoa (Protozoa). Protist 154:341–358

Dawson SC, Pace NR (2002) Novel kingdom-level eukaryotic diversity in anoxic environments. Proc Natl Acad Sci USA 99:8324–8329

Díez B, Pedrós-Alió C, Massana (2001) Study of genetic diversity of eukaryotic picoplankton in different oceanic regions by small-subunit rRNA gene cloning and sequencing. Appl Environ Microbiol 67:2932–2941

Drebes G, Kühn SF, Gmelch A, Schnepf E (1996) *Cryothecomonas aestivalis* sp. nov., a colourless nanoflagellate feeding on the marine centric diatom *Guinardia delicatula* (Cleve) Hasle. Helgol Wiss Meeresunters 50:497–515

Edgcomb VP, Kysela DT, Teske A, de Vera Gomez A, Sogin ML (2002) Benthic eukaryotic diversity in the Guaymas Basin hydrothermal vent environment. Proc Natl Acad Sci USA 99:7658–7662

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

Hashimoto J, Miura T, Fujikura K, Ossaka J (1993) Discovery of Vestimentiferan tube-worms in the euphotic zone. Zoolog Sci 10:1063–1067

Hugenholtzt P, Huber T (2003) Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. Int J Syst Evol Microbiol 53:289–293

Ikemoto E, Kyo M (1993) Development of microbiological compact mud sampler. JAMSTEC Res (In Japanese with English abstract) 30:1–16

Knoll AH (1999) A new molecular window on early life. Science 285:1025–1026

Kühn S, Drebes G, Schnepf E (1996) Five species of the nanoflagellate *Pirsonia* in the German Bight, North Sea, feeding on planktic diatoms. Helgoländer Meeresunters 50:205–222

López-García P, Rodriguez-Valera F, Pedros-Alio C, Moreira D (2001) Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. Nature 409:603–607

López-García P, Philippe H, Gail F, Moreira D (2003) Autochthonous eukaryotic diversity in hydrothermal sediment and experimental microcolonizers at the Mid-Atlantic Ridge. Proc Natl Acad Sci USA 100:697–702

Maidak BL, Cole JR, Lilburn TG, Parker CT Jr, Saxman PR, Farris RJ, Garrity GM, Olsen GJ, Schmidt TM, Tiedje JM (2001) The RDP-II (Ribosomal Database Project). Nucleic Acids Res 29:173–174

Massana R, Guillou L, Díez B, Pedrós-Alió C (2002) Unveiling the organisms behind novel eukaryotic ribosomal DNA sequences from the ocean. Appl Environ Microbiol 68:4554–4558

Massana R, Castresana J, Balague V, Guillou L, Romari K, Groisillier A, Valentin K, Pedrós-Alió C (2004) Phylogenetic and ecological analysis of novel marine stramenopiles. Appl Environ Microbiol 70:3528–3534

Matsumoto T (1943) The four gigantic caldera volcanoes of Kyushu. Jpn J Geol Geogr (Special No) 19:1–57

Moon-van der Staay SY, De Wachter R, Vaulot D (2001) Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. Nature 409:607–610

Moreira D, López-García P (2003) Are hydrothermal vents oases for parasitic protists? Trends Parasitol 19:556–558

Naganuma T (1991) Collection of chemosynthetic sulfur bacteria from a hydrothermal vent and submarine volcanic vents. JAMSTEC Deep Sea Res 7:201–219

Oki K, Hayasaka S (1978) Geological study on Kagoshima Bay, South Kyushu, Japan. Part IV - A note on the peculiar mode of occurrence of foraminifers in the bottom sediments of the bay-head area. Rep Fac Sci Kagoshima Univ Earth Sci Biol 11:1–11

Ossaka J, Hirabayashi J, Nogami K, Kurosaki M, Hashimoto J (1992) Variation of chemical composition of volcanic gases from the northern part of Kagoshima bay. JAMSTEC Deep Sea Res (In Japanese with English abstract) 8:75–80

Pawlowski J, Bolivar I, Fahrni JF, de Vargas C, Gouy M, Zaninetti L (1997) Extreme differences in rates of molecular evolution of foraminifera revealed by comparison of ribosomal DNA sequences and the fossil record. Mol Biol Evol 14:498–505

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817–818

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Schnepf E, Drebes G, Elbrächter M (1990) *Pirsonia guinardiae*, gen. et spec. nov.: a parasitic flagellate on the marine diatom *Guinardia flaccida* with an unusual mode of food uptake. Helgoländer Meeresunters 44:275–293

Schweikert M, Schnepf E (1997) Light and electron microspical observations on *Pirsonia punctigerae* spec. nov., a nanoflagellate feeding on the marine centric diatom *Thalassiosira punctigera*. Europ J Protistol 33:168–177

Simoneit BRT, Lonsdale PF (1982) Hydrothermal petroleum in mineralized mounds at the seabed of Guayman Basin. Nature 295:198–202

Spero HJ (1987) Symbiosis in the planktonic foraminifer, *Orbulina universa*, and the isolation of its symbiotic dinoflagellate, *Gymnodinium beii* sp nov. J Phycol 23:307–317

Stoeck T, Epstein S (2003) Novel eukaryotic lineages inferred from small-subunit rRNA analyses of oxygen-depleted marine environments. Appl Environ Microbiol 69:2657–2663

Stoeck T, Taylor GT, Epstein SS (2003) Novel eukaryotes from the permanently anoxic Cariaco Basin (Caribbean Sea). Appl Environ Microbiol 69:5656–5663

Swofford DL, Waddell PJ, Huelsenbeck JP, Foster PG, Lewis PO, Rogers J (2001) Bias in phylogenetic estimation and its relevance to the choice between parsimony and likelihood methods. Syst Biol 50:525–539

Takahashi T (1981) Seasonal differences of the circulation processes in a coastal basin nearly closed by land. Ocean Manag 6:189–200

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10:512–526

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nuc Acids Res 22:4673–4680

Tillmann U, Hesse KJ, Tillmann A (1999) Large-scale parasitic infection of diatoms in the Northfrisian Wad-den Sea. J Sea Res 42:255–261

Yamanaka T, Mizota C, Murae T, Hashimoto J (1999) A current forming petroleum associated with hydrothermal mineralization in a submarine caldera, Kagoshima Bay, Japan. Geochem J 33:355–367

Yamanaka T, Ishibashi J, Hashimoto J (2000) Organic geochemistry of hydrothermal petroleum generated in the submarine Wakamiko caldera, southern Kyushu, Japan. Org Geochem 31:1117–1132