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Full Paper

Comparison of the microbial diversity in cold-seep sediments from different depths in the Nankai Trough

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We have investigated the molecular phylogeny of cold-seep sediments obtained from the Nankai Trough, at depths of about 600, 2,000, and 3,300 m, and compared the microbial diversity profiles of those sediments samples. The *γ-Proteobacteria* that might function as sulfide oxidizers and the symbiotically related *δ-Proteobacteria* which might function as sulfate reducers were identified amongst the bacteria from all depths of the sediments. However, anoxic methane oxidizing archaea (ANME) and methanogens were only found in the 600 m deep sediments. These results indicated that the cold-seep microbial sulfur circulation system could be functioning in the shallow seep sediment at a depth of 600 m and the microbial activities at these sites might be more dynamic than at other deeper cold-seep sites.

Key Words——accretionary prism; *Calyptogena* communities; cold-seep; deep-sea; microbial diversity; Nankai Trough; sulfur circulation

Introduction

The Nankai Trough is the subduction margin between the Shikoku Basin (Philippine sea plate) and the Southwest Honshu Arc (Eurasian plate) at 4 cm/year. The current accretionary prism is building from the trench axis and increasing in thickness landward. Several seismic profiles provide excellent images of this prism building. The Cretaceous to Tertiary Shimanto Belt is exposed from the Ryukyu Arc to the middle of the Honshu Arc. The subducting oceanic plate beneath the Shikoku Basin has spread by 15 Ma. Many geological, geophysical and geochemical data have been accumulated and are currently available for use (Kodaira et al., 2004; Kuramoto et al., 2001; Park et al., 2002). The Nankai Trough is also one of the areas where cold-seepage has been thoroughly investigated. Since 1984, the French-Japanese KAIKO Project has found several cold-seep sites in the accretionary prism by means of submersibles (Le Pichon et al., 1987). During dive surveys, chemosynthesis-based biological communities served as useful markers for mapping seep sites because anomalies of temperature and geochemistry in cold-seeps are more rarely detected than those in hydrothermal areas (Le Pichon et al., 1987). Gamo et al. (1994) reported that there might be a drastic change in pore water chemistry between the interior and exterior of *Calyptogena* communities, because the sedimentary pore water recovered only 0.3 m from the margin of the community showed little indication of *in situ* sulfate reduction. They also confirmed that surface sediment temperature is higher inside the *Calyptogena* community than on the outside.

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Calyptogena is a bivalve associated with sulfur oxidizing endosymbiont bacteria living in such environments and researchers have used its communities as markers to investigate the cold-seep environments in the Nankai Trough (Ashi, 1997; Ohta and Laubier, 1987). The description of *Calyptogena* species and their biogeographical properties were reviewed by Kojima et al. (2004).

We have carried out several studies concerning the relation between the cold-seep ecosystems and their microbial diversity in the Japan Trench (Arakawa et al., 2005; Inagaki et al., 2002; Li et al., 1999b), the Nankai Trough (Li et al., 1999a) and the Sagami Bay (Fang et al., 2005). These results suggested a sulfate circulation model utilizing cold-seep activity involving microbial communities containing methanogenic archaea, a sulfate reducing consortium (anoxic methane oxidizing archaea+sulfate reducing bacteria; ANME-SRB), and sulfur oxidizing bacteria (microbial mat forming and/or chemosynthetic symbiotic) (Boetius et al., 2000; Kato et al., 2005; Li and Kato, 1999; Orphan et al., 2001). Particularly, more abundant microbial communities capable of establishing sulfate circulation systems were identified at deeper depths in the Japan Trench land slope. In these regions, no accretionary prism structures were identified and this suggested that the deepest depths of the trench could be more dynamic in seep activity than the shallower land slope (Arakawa et al., 2005). Our former study of the Nankai Trough involved only a bacterial diversity study (Li et al., 1999a) at a depth of 3,843 m, and lacked a study concerning archaeal diversity in the cold-seep communities at different depths. To study the different cold-seep microbial systems present between the Japan Trench land slope and the Nankai Trough, we organized a cruise, called "NaBiSC" (Nankai Trough Bio-Symbiosis Cruise, YK05-08 Leg. 1, PI; C. Kato), in June 2005. In this paper, the microbial diversity of Nankai Trough cold-seep sites at different depths is described and the correlation between the microbial communities and the geological setting is discussed.

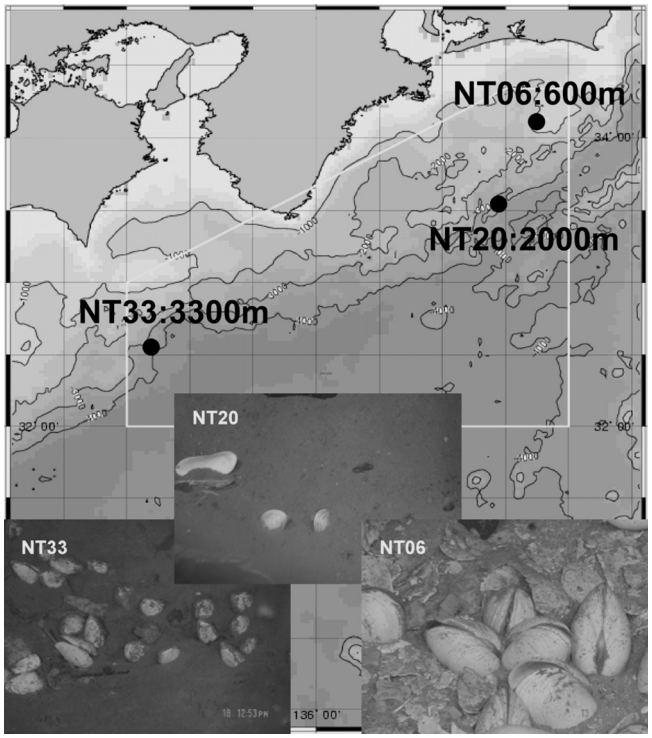

Fig. 1. Map of the sampling sites and their visualization in the cold-seep area containing *Calyptogena* communities at different depths in the Nankai Trough.

NT06: *Calyptogena* colony at a depth of 615 m, NT20: *Calyptogena* colony at a depth of 2,048 m, NT33: *Calyptogena* colony at a depth of 3,310 m. These sites correspond to the sampling sites shown in the photographs. The area in the white pentagon indicates the investigation area of the NaBiSC cruise.

Materials and Methods

Collection of the cold-seep sediment samples from the Nankai Trough. The cold-seep sediments were collected by the manned submersible *SHINKAI 6500* (6 K) at 2nd Tenryu Knoll at 615 m (34°04.30′N, 137°47.34′E, NT06 site, dive No. 881), off Shiono Cape at 2,048 m (33°15.78′N, 136°42.99′E, NT20 site, dive No. 883), and off Muroto Cape at 3,310 m (32°34.97′N, 134°41.72′E, NT33 site, dive No. 884), in the Nankai Trough, using a core sediment sampler (MBARI-type) during the cruise NaBiSC. The sampling sites are shown in Fig. 1. The core sediment samples (lengths about 15–20 cm) were each cut into 2 cm sections and treated on board for the isolation of the environmental DNA.

DNA manipulation. Total DNA was extracted directly from the sediment samples using an Ultra Clean soil DNA kit (MO Bio Laboratories, Solana Beach, CA, USA). Bacterial or archaeal 16S rRNA gene was amplified by polymerase chain reaction (PCR) from the sediment DNA using bacterial- and archaeal-specific primers (Kato et al., 1997). The PCR products were cloned into the TA Cloning vector (Invitrogen, Carlsbad, CA, USA). *Escherichia coli* transformants containing 16S rRNA gene inserts were identified by agarose

gel electrophoresis. To screen the 16S rRNA gene clones for grouping into identical clone types for DNA sequencing analysis, restriction fragment-length polymorphism (RFLP) analysis using restriction enzymes that recognize a 4-bp restriction site was performed with *Rsa*I (GT′AC) and *Msp*I (C′CGG). The selected cloned 16S rRNA gene fragments were then amplified and subsequently sequenced using the dideoxynucleotide chain-termination method with a model 3100 DNA Sequencer (Perkin-Elmer/Applied Biosystems Co., Foster City, CA, USA).

Analysis of DNA sequences and phylogenetic relationships. The sequences of 16S rRNA gene determined were checked for similarities to DNA sequences in the DNA Data Base of Japan (DDBJ) using FASTA within the GENETYX-MAC program (ver. 13.0.3, Software Co., Tokyo, Japan). Sequences were aligned and phylogenetic trees were constructed from a matrix of pairwise genetic distances by the maximum-parsimony algorithm and the neighbor-joining method using the CLUSTAL W program (Saitou and Nei, 1987). The amplified rRNA gene sequences reported in this paper have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases. The accession numbers of the 16S rRNA gene sequences of the isolated clones are AB240685 to 240731 in BNT bacterial clones and AB240732 to 240750 in ANT archaeal clones.

Terminal restriction fragment-length polymorphism (t-RFLP) analysis. The 16S rRNA gene for terminal restriction fragment-length polymorphism (t-RFLP) analysis were amplified with PCR using Bac27F and FAM-labeled Bac927R for bacterial 16S rRNA gene, and Arch21F and FAM-labeled Arch958R for archaeal 16S rRNA gene according to the procedure reported previously (Inagaki et al., 2002). Amplified rRNA gene was subjected to agarose gel electrophoresis and the labeled PCR products were purified using a Gel Spin DNA purification kit (MO Bio Laboratories). DNA was precipitated with ethanol and centrifuged, and the pellets were resuspended in double-distilled water. The purified rRNA gene was digested with *Hha*I at 37°C for 8 h. The terminal restriction fragments (t-RFs) were analyzed using a model 310 automated sequencer (Perkin-Elmer/Applied Biosystems) equipped with GENESCAN software ver. 3.1 (Perkin-Elmer/Applied Biosystems). The precise lengths of the t-RFs were determined by comparison with an internal size standard (GENESCAN-2500 ROX, Perkin-Elmer/Applied Biosystems) added to each digested sample. The electrophoresis conditions and the procedures followed were those suggested by the manufacturer.

Results

Phylogenetic analysis of 16S rRNA gene sequences

Microbial phylogenetic analyses were performed on the surface cold-seep sediment samples at depths of 0–2 cm, obtained from the sites, NT06, NT20 and NT33. The bacterial phylogenetic tree constructed demonstrated that the 58 different bacterial sequences fall into four phylogenetic group affiliations: *Proteobacteria*, *Spirochaetaceae-Cytophaga*, gram-positive bacteria, and an unknown group. There were 31 different 16S rRNA gene sequences belonging to the Proteobacteria group. Of the 42 clones, 26 were in the γ*-Proteobacteria* group, 6 in the α*-Proteobacteria*, 8 in the δ*-Proteobacteria*, and one each in the β- and ε*-Proteobacteria* groups, respectively (Fig. 2). In the γ group, most of the clones (24 clones) were closely related to the symbiotic sulfur-oxidizing bacterial branch present in chemosynthetic-based bivalves (Distel et al., 1994) and were labeled “SYM” in Fig. 2 since these might function in sulfide oxidation. The cold-seep sediment samples contained δ*-Proteobacterial* 16S rRNA gene (7 different sequences in 8 clones), which could be sulfate-reducing bacteria (SRB) that might play important roles in sulfur circulation systems in the cold-seep ecosystem (Kato et al., 2005; Li and Kato, 1999). SYM and SRB groups could be identified from any of the cold-seep sediment samples.

Nineteen different sequences were selected from among all of the 47 archaeal clones by RFLP analysis and sequenced. As shown in Fig. 3, the archaeal phylogenetic tree demonstrated 13 sequences in 35 clones clustered in the crenarchaeota marine group 1 indicated as “MG1” (DeLong, 1992), which were relatively abundant in the ocean sediment. Sequences ANT06-01, 02, 05, and 06 are related to methanogen and anoxic methane oxidizing archaea (ANME) and were found only in the NT06 sediment samples. These may function as autotrophic methane-producing organisms and could also cooperate with SRB in sulfate reduction (Boetius et al., 2000; Orphan et al., 2001).

t-RFLP profiles for the microbial communities at different sediment depths

For analysis of the microbial communities at differ-

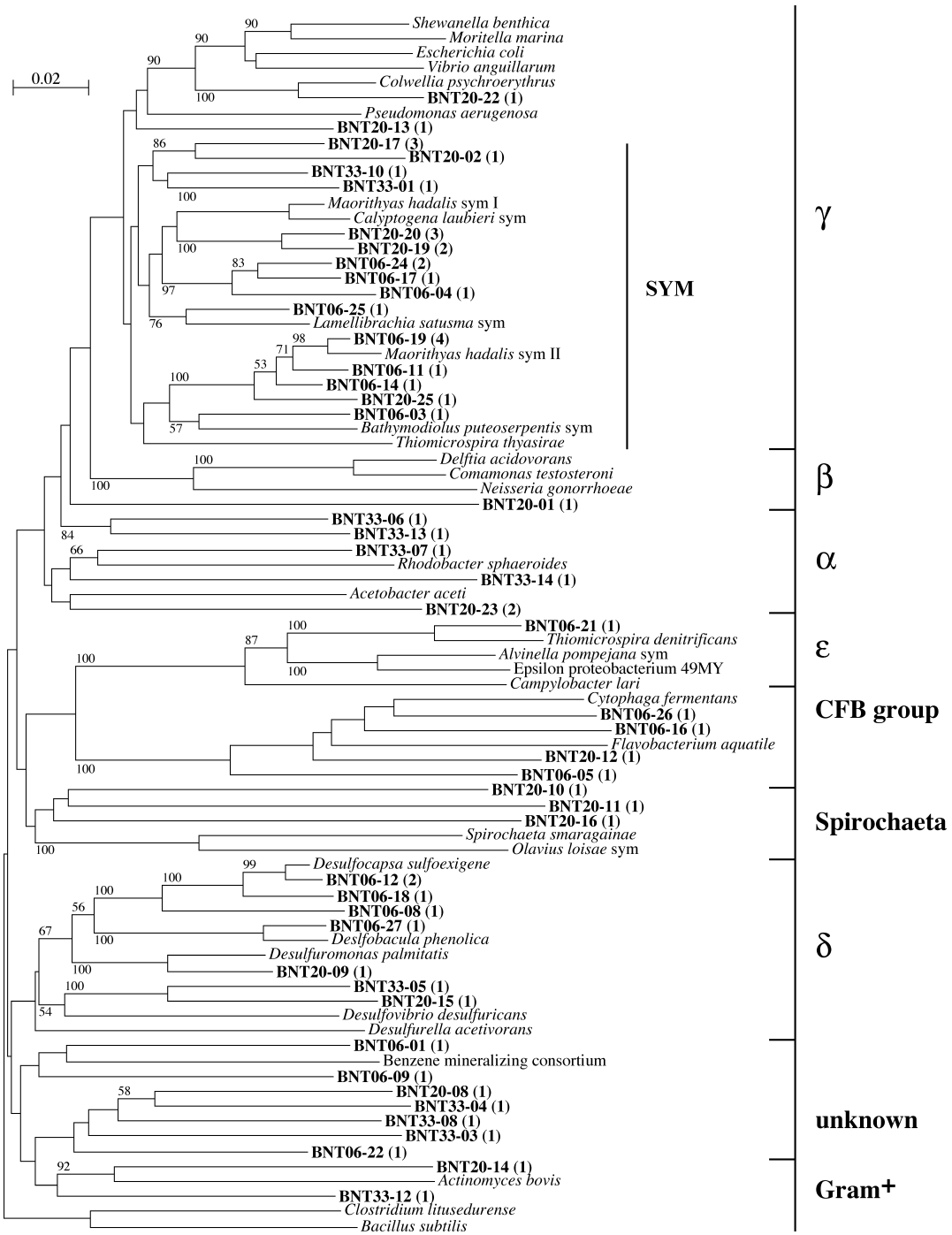

Fig. 2. Phylogenetic analysis of the cold-seep bacterial diversity based on the 16S rRNA gene sequences.

The bacterial sequences contained in the 615, 2,048, and 3,310 m sediments are indicated as BNT06, BNT20, and BNT33, respectively, shown in bold characters (numbers in parentheses show the identical clone numbers). The values of 1,000 bootstrap trial replications are given for nodes in the trees (%). The scale bar represents 0.02 nucleotide substitutions per sequence position. Greek letters indicate the group of *Proteobacteria*. SYM indicates the chemoautotrophic symbiotic related bacterial group.

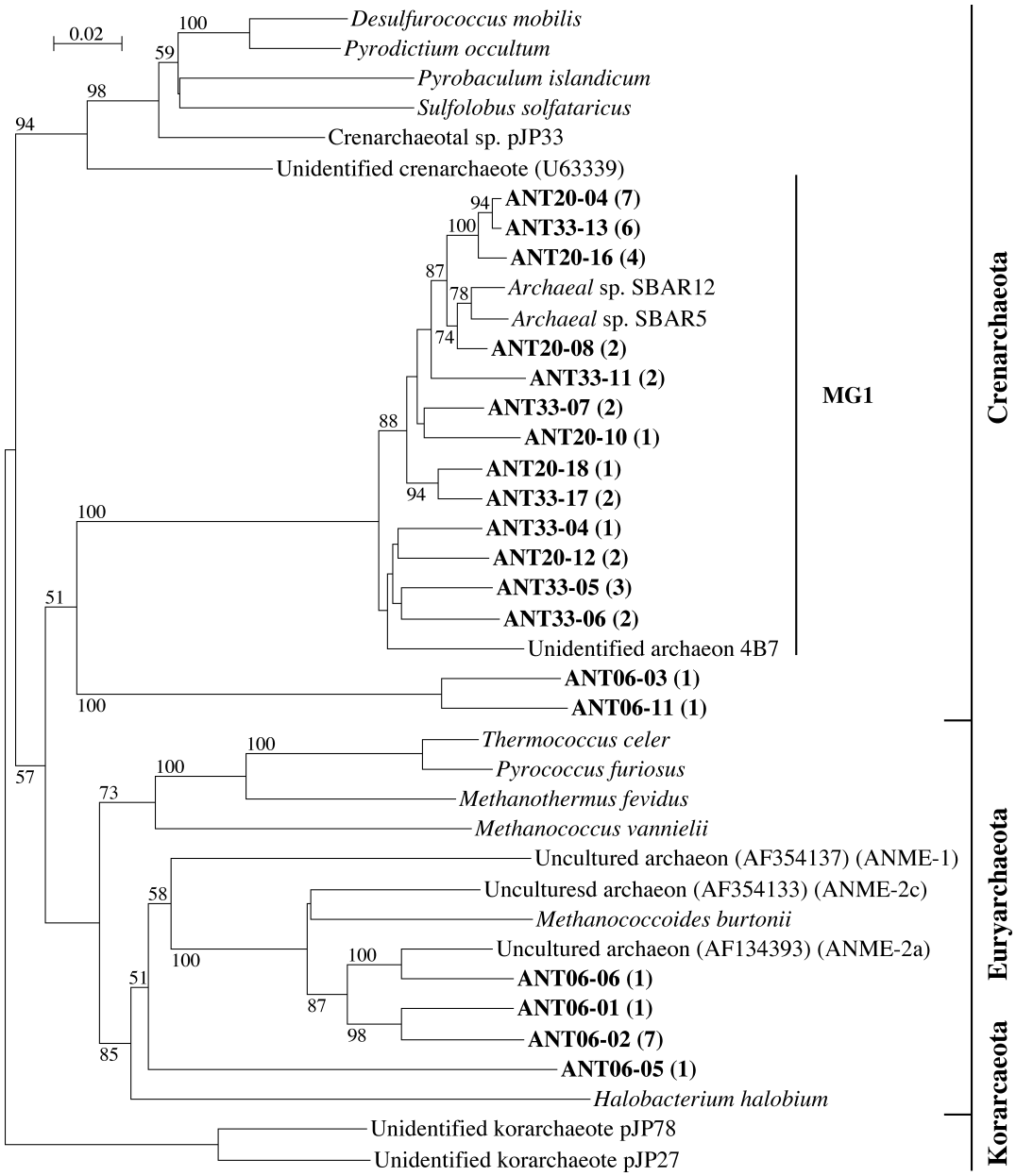

Fig. 3. Phylogenetic analysis of the cold-seep archaeal diversity based on the 16S rRNA gene sequences. The archaeal sequences contained in the 615, 2,048, and 3,310 m sediments are indicated as ANT06, ANT20, and ANT33, respectively, and shown in bold characters (numbers in parentheses depict the identical clone numbers). MG1 indicates the crenarchaeota marine group 1 reported by DeLong (1992). Other abbreviations are the same as in Fig. 2.

ent depths of the sediment, t-RFLP was performed using the three different cold-seep core samples, at depths of 0–2 cm , 4–6 cm, and 8–10 cm from the surface of the sediments, as shown in Figs. 4 and 5. As shown in Fig. 4, the bacterial t-RFLP profiles indicated similar profiles of the community structure in the surface sediments (0–2 cm depth) among the NT06, NT20 and NT33 sites and were constituted mainly of the SYM and SRB groups which are composed of marine sulfur-oxidizing bacteria and sulfate-reducing bacteria, respectively (Fig. 2). However, the community structures were more stable in the NT06 site deeper sediments, compared with the NT20 and NT33 sites, where the communities were less obvious. In the NT33 site, almost no bacterial communities were observed in the deeper subsurface sediment (Fig. 4). The archaeal t-RFLP profiles of these vertical sediment samples were more distinct in the surface sediments (0–2 cm)

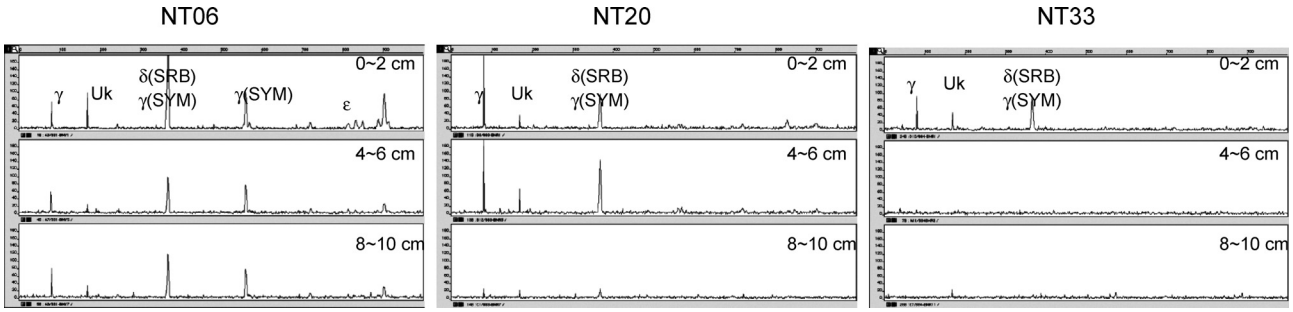

Fig. 4. t-RFLP profiles of the bacterial community structures of the NT06, NT20, and NT33 sites at different sediment depths.

Depths from the surface sediment are shown at the right side of each site profile. γ, δ, and ε indicate the corresponding Proteobacterial groups, and, SRB and SYM indicate sulfate reducing bacteria and symbiotic related bacteria, respectively. The length of fragments (*x*-axis) and relative fluorescence intensity of peaks (*y*-axis) are also displayed.

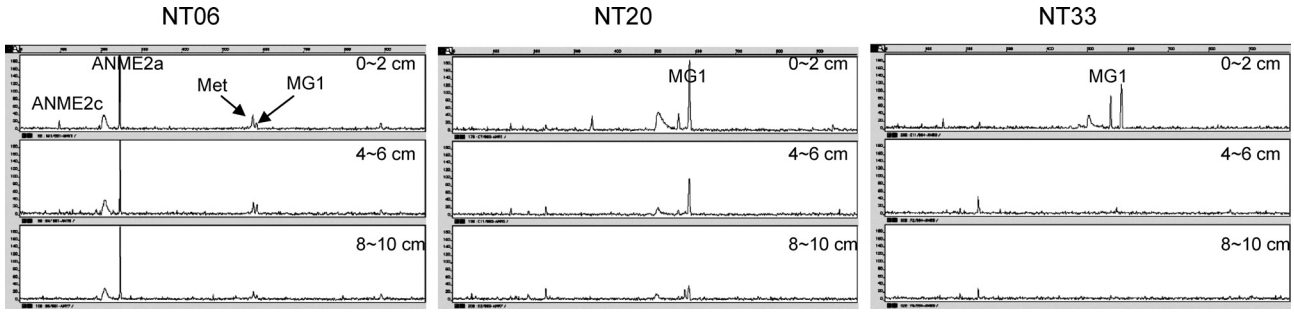

Fig. 5. t-RFLP profiles of the archaeal community structures of the NT06, NT20, and NT33 sites at different sediment depths.

Met and MG1 indicate methanogenic archaea and crenarchaeota marine group 1, respectively. Other abbreviations are the same as in Fig. 4.

compared with the case of bacterial profiles (Fig. 5). Archaeal diversity was greatest in the NT06 site compared to the other two sites and only a crenarchaeota marine group 1 peak (indicated as MG1) was identified in the NT20 and NT33 sites. However, several anoxic methane-oxidizing (ANME2a and 2c groups) and methane-producing archaea (Met) were identified in the NT06 site, even in the deeper sediments.

Discussion

Bacteria belonging to the *δ-Proteobacteria* as well as archaea belonging to the methanogens and ANME groups were previously concluded to be particularly abundant in cold-seep communities (Arakawa et al., 2005; Kato et al., 2005; Li and Kato, 1999). In this study, we identified complete cold-seep microbial communities only in the shallowest cold-seep sediments from the NT06 site at a depth of 615 m. However, we found widespread *Calyptogena* communities at the NT06 site but only a few *Calyptogena* colonies at the other two sites, NT20 and NT33. These observations including the current results indicated that the cold-seep activity of the NT06 site could be higher than at the NT20 and NT33 sites. Basically, cold-seep activity might correspond to the existence of active faults and related geological settings (Kuramoto et al., 2001; Okamura et al, 2002). Thus, our results suggested that the NT06 site could contain more active geological settings than the other two deeper sites.

In the case of the Japan Trench, we have reported that more abundant microbial communities commonly identified in cold-seep sediments are found at the deepest depths of the trench (Arakawa et al., 2005), which could be points for fast plate subduction (about 12 cm/year) by the Pacific ocean plate into the North american plate. The Nankai Trough is a slower plate of subduction (4 cm/year) for the Philippine sea plate into the Eurasian plate (Kuramoto et al., 2001) and the resulting accretionary prism is built from the trench axis

and increases in thickness landward. There is a difference in the geological setting of the Japan Trench compared to the Nankai Trough since no accretionary prism structure was identified in the Japan Trench. Several active faults have been identified in the prism structures by seismic imaging profiles (Kodaira et al., 2004; Park et al., 2002). Thus, it is possible that strong cold-seep activity might occur at even the shallower water depths on the prism structure. The NT06 site could be one such area because of the numerous *Calyptogena* colonies observed there (Kuramoto and Joshima, 1998; this study). It is interesting that complete cold-seep microbial structures are identified at the shallower depths on the accretionary prism structure in the Nankai Trough while these were identified in deeper sediments in the Japan Trench lacking prism structures.

In conclusion, the current study concerning the microbial diversity of the Nankai Trough cold-seep sediments at different depths suggests a relationship between seep microbial diversity and accretionary prism structures. This is the first observation which suggests a correspondence between cold-seep microbial communities and accretionary prism structures.

Acknowledgments

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