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ORIGINAL ARTICLE/SHORT PAPER

Methanogenic archaeal communities developed in paddy fields in the Kojima Bay polder, estimated by denaturing gradient gel electrophoresis, real-time PCR and sequencing analyses

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Abstract

Methanogenic archaeal communities inhabiting the paddy field soils in the Kojima Bay polder were investigated using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), real-time PCR and sequencing analyses. Soil samples of the plow and subsoil layers were collected in 2006 from four paddy fields that were reclaimed between 1692 and 1954. The DGGE band patterns of the targeted 16S rRNA genes amplified from the extracted DNA from the samples were different from the patterns from the paddy field soils in diluvial and alluvial areas. The numbers of targeted 16S rRNA genes, which were involved with methanogenic archaeal and other archaeal sequences, were approximately 10^7–10^8 and 10^6 g^{-1} dry soil in the plow and subsoil layers, respectively. Sequences of methanogenic archaeal 16S rRNA genes belonging to Methanocellales (Rice cluster I), Methanosarcinales and Methanobacteriales were obtained from the major DGGE bands. Whereas sequences in Methanomicrobiales, which were predominant methanogens in the diluvial and alluvial paddy fields, were not recovered. Known halophilic and methylotrophic methanogens, which are characteristic of saline and marine environments, were not detected. These results indicate that distinctive methanogenic archaeal communities have developed in the paddy field soils in the Kojima Bay polder.

Key words: methanogenic archaea, paddy field soil, PCR-DGGE, polder, real-time PCR.

INTRODUCTION

Methanogenesis is the final degradation pathway of organic matter in flooded paddy field soils (Conrad and Frenzel 2002; Kimura 2000). Methane emitted from flooded paddy fields is a major greenhouse gas and contributes to global warming (Denman *et al.* 2007). Therefore, it is important to elucidate the mechanisms of methanogenesis and the ecology of the methanogenic archaea that monopolize biological methane production in paddy fields. In previous studies, we have investigated the community structures of methanogenic archaea in two Japanese paddy fields located in central (Anjo; diluvial area) and southwestern (Chikugo; alluvial area) Japan during an annual cycle of cropping with rice and wheat using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), real-time PCR and sequencing analyses (Watanabe *et al.* 2006, 2007).

However, rice cultivation in Japan has occurred not only in diluvial and alluvial areas, but also in polders that were originally tidal flats (e.g. the Kojima Bay and the Ariake Sea) or brackish lagoons (e.g. the Hachirogata). In general, because minerals flowed into the tidal flat/lagoons from upstream and algae and plankton accumulated in these areas, polders are potentially rich in nutrients and have been used for agriculture, particularly as paddy fields, in Japan. The coastal region of the Kojima Bay located in Okayama prefecture in western Japan is one of the largest polders. Reclamation projects started in the 16th century and approximately

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20,000 hectares have been hitherto reclaimed in several big projects (Yoneda 1964). As these areas were in coastal salty environments, the polder soils were formed through unique processes under the influence of alkali salts (Yoneda 1964). In addition, the soils in the Kojima Bay polder originally exhibited the same base composition and physicochemically similar characteristics, irrespective of the site. Therefore, the processes of the soil formations are chronologically comparable among the sites and have been studied from the aspect of pedogenesis (Kawaguchi and Matsuo 1955a;b; Kawaguchi *et al.* 1957; Yoneda 1964). Although distinctive bacterial communities might develop under this unique pedogenesis, microbial communities inhabiting soils in the Kojima Bay polder have not been clarified. A number of studies have investigated microorganisms in soils that were in sea sediments. However, specific bacteria, for example, iron-oxidizing and sulfur-oxidizing bacteria, have been only focused since acidification of soil caused by oxidization of pyrite (FeS_2) was of a major problem for agriculture in such areas (Ogawa *et al.* 1990; Ohba and Owa 2005; Takai *et al.* 1989; Wainwright 1984). As distinct bacterial and archaeal communities inhabit estuaries and tidal flats (Freltag *et al.* 2006; Munson *et al.* 1997), comprehensive investigations of microbial communities in polders are important to promote a greater understanding of microbial ecology in this environment.

In the present study, we focused on methanogenic archaea in paddy fields. In saline environments, such as tidal flats, estuaries and brackish lakes, halophilic and methylotrophic methanogens (e.g. *Methanococcides* spp. and *Methanolobus* spp.) are common because these methanogens do not compete with sulfate-reducing bacteria over substrate (Munson *et al.* 1997; Purdy *et al.* 2002; Wilms *et al.* 2006; Zinder 1993). These halophilic and mehylotrophic methanogenic archaea might have survived even after the reclamations and the methanogenic archaeal community might be different from that in diluvial and alluvial paddy fields. Therefore, we investigated the community structure of methanogenic archaea in four paddy fields in the Kojima Bay polder, which were reclaimed between 1692 and 1954, using PCR-DGGE, real-time PCR and sequencing analyses and compared these with the communities in diluvial (Anjo) and alluvial (Chikugo) paddy fields in Japan.

MATERIALS AND METHODS

Sampling sites

Soil samples were collected from four paddy fields located in the Kojima Bay polder in Okayama prefecture; site 1, Fujita Omagari (34°35′N, 133°51′E); site 2, Fujita Nishiki-rokku (34°35′N, 133°54′E), site 3, Kuwano (34°37′N, 133°58′E); site 4, Kawauchi (34°38′N, 133°59′E). Sites 1 and 2 were reclaimed in 1912 and 1954, respectively, whereas both sites 3 and 4 were reclaimed in 1692. Soils from the plow layer (0–10 cm) and subsoil layer (below 20 cm) were taken on 16 March 2006. Soil samples from sites 1 and 2 were collected again on 14 November 2006. Manganese (Mn) nodules from sites 1 and 2 (Cahyani *et al.* 2007) and ferric-oxide mottle from site 4 were collected from the illuvial horizons in the subsoil with a micro spatula and used because 16S rRNA gene sequences closely related to sulfate-reducing bacteria were retrieved from the Mn nodules (Cahyani *et al.* 2007), indicating that the nodules were a possible habitat for obligate anaerobes. The chemical properties of the investigated sites are shown in Table 1.

DNA extraction, PCR-DGGE, real-time PCR and sequencing analyses

The DNA was extracted from the soil samples using the FastDNA SPIN Kit for Soil (BIO 101; Qbiogene, Carlsbad, CA, USA) with three replicates (Cahyani *et al.* 2007). The PCR-DGGE analysis was carried out using the primer 1106F-GC/1378R, which targeted methanogenic archaeal 16S rRNA genes, as described by Watanabe *et al.* (2006). The PCR program had an initial denaturation at 95°C for 90 s, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 90 s, and a final extension at 72°C for

Table 1 Chemical qualities of the paddy soils in the Kojima Bay polder

Site no.	Site name	Year	Soil depth (cm)	pH (H_2O)	pH (KCl)	EC (dS m^{-1})	Total C (%)	Total N (%)	CEC ($cmol_c$ kg^{-1})
1	Fujita Omagari	1994	0–16	6.56	5.54	0.129	1.60	0.14	20.1
			>16	6.83	5.48	0.097	0.56	0.05	17.4
2	Fujita Nishiki-rokku	2002	0–13	6.35	4.99	0.115	1.71	0.14	18.8
			13–23	6.58	5.04	0.074	1.28	0.12	18.5
			>23	6.87	5.11	0.157	0.66	0.08	15.4
3	Kuwano	1994	Plow layer	5.42	4.57	nd	1.63	0.14	15.5

Data from site 4 (Kawauchi) is unavailable. CEC, cation exchange capacity; EC, electrical conductivity; nd, not determined.

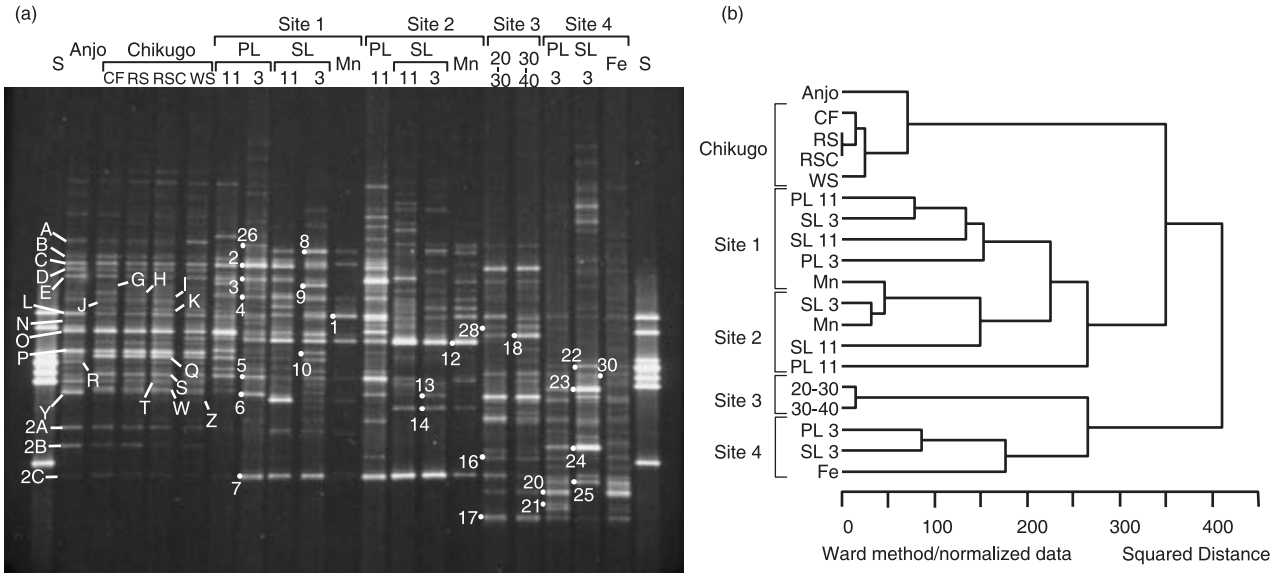

Figure 1 (a) Denaturing gradient gel electrophoresis (DGGE) band patterns of methanogenic archaeal 16S rRNA genes obtained from paddy field soils in the Kojima Bay polder. (b) Cluster analysis of the DGGE band patterns. S, a mixture of equal amounts of polymerase chain reaction (PCR) products from the genomic DNAs of 13 methanogenic archaeal strains (Watanabe *et al.* 2004); Anjo, paddy field plot at Anjo; CF, chemical fertilizer plot; RS, rice straw plus chemical fertilizer plot; RSC, rice straw compost plus chemical fertilizer plot; WS, wheat straw plus chemical fertilizer paddy field plot at Chikugo (Watanabe *et al.* 2006); Site 1, Fujita Omagari; Site 2, Fujita Nishiki-rokku; Site 3, Kuwano; Site 4, Kawauchi in the Kojima Bay polder in Okayama prefecture; PL, plow layer soil; SL, subsoil layer; Mn, Mn nodule; Fe, ferric-oxide mottle; 20–30 and 30–40, soil from a depth of 20–30 cm and 30–40 cm, respectively. 3 and 11, samples collected on 16 March and 14 November 2006, respectively. The denaturant gradient ranged from 32 to 62%. The letters and numbers indicated by lines and symbols show the bands that correspond to the bands on the phylogenetic trees (Fig. 2).

6 min. The DGGE was carried out with a Dcode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) and the denaturant gradient range of the gel was from 32 to 62%, in which 100% denaturant contained 7 mol L^{-1} urea and 40% (v/v) formamide. The methanogenic archaeal 16S rRNA genes amplified from the extracted DNAs in the Anjo and Chikugo paddy field soils (Watanabe *et al.* 2006) were used for comparison. The DGGE bands observed after running were classified into four categories for cluster analysis depending on their intensities (0 = no band; 1 = weak; 2 = moderate; 3 = strong). A cluster analysis with normalized data was carried out using the Black Box program and the Ward method (Aoki 1996).

Real-time PCR was carried out to quantify the number of methanogenic archaeal 16S rRNA genes in the samples using LightCycler ST300, LightCycler Software Ver. 3.5 (Roche Diagnostics, Basel, Switzerland) and SYBR *Premix Ex Taq* (TaKaRa, Siga, Japan), as described by Watanabe *et al.* (2007).

The nucleotide sequences of the 16S rRNA gene fragments recovered from the DGGE bands and their phylogenetic affiliations were determined using the direct sequencing method, as described by Watanabe *et al.* (2004). A phylogenetic tree was constructed by 1,000-fold bootstrap analysis using the neighbor-joining method in the ClustalW program on the website of the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/Welcome-j.html) and NJ plot software (Perrière and Gouy 1996). The distance matrix was computed using the Jukes–Cantor correction. All sequences determined in the present study have been submitted to the DDBJ/EMBL/GenBank database under accession numbers AB442058–AB442082.

RESULTS AND DISCUSSION

Partial 16S rRNA genes were amplified from all extracted DNAs, including the samples of Mn nodules and ferric-oxide mottle. Figure 1a shows the DGGE band patterns of the amplified 16S rRNA gene fragments. In total, 66 bands were observed from the paddy field samples in the Kojima Bay polder at different positions and the patterns were different from the patterns of the Anjo (diluvial) and Chikugo (alluvial) paddy fields (Watanabe *et al.* 2006), in particular, the patterns from sites 3 and 4 were distinctly dissimilar to them. At the different sites, the band patterns derived from the paddy

field soils in the Kojima Bay polder were different from each other. Several bands (8–25) were obtained even from the Mn nodules and ferric-oxide mottle. Only bands 1 and 7 were commonly observed and some of the other bands were specific to the respective sites (e.g. band 12 for sites 1 and 2 and bands 22, 24 and 25 for site 4). In the patterns from site 3, both bands that were observed from sites 1 and 2 (i.e. bands 2 and 6) and those from site 4 (i.e. bands 16 and 17) were observed.

The cluster analysis based on the DGGE band patterns showed that a cluster formed consisting of samples from sites 3 and 4, and that the samples from sites 1 and 2 and the Anjo and Chikugo soils were divided into two clusters (Fig. 1b). The samples from each site of the Kojima Bay polder tended to form distinct clusters and the squared distances among the sites in the polder were larger than those between the Anjo and Chikugo samples. Sites 3 and 4 were reclaimed 220–262 years earlier than sites 1 and 2. The elapsed years after the reclamation might have influenced the community structures of methanogenic archaea in the paddy soils.

The number of targeted 16S rRNA genes in the soils, which were collected from sites 1 and 2 in November 2006, was estimated using real-time PCR. The numbers of genes in the plow layer soils were 2.8×10^8 (site 1) and 2.9×10^7 (site 2) g^{-1} dry soil, whereas the numbers in the subsoils were 1.1×10^6 (site 1) and 1.4×10^6 (site 2) g^{-1} dry soil. Although several unknown archaeal 16S rRNA genes other than methanogenic archaea were recovered from the DGGE band patterns, the numbers of methanogenic archaeal 16S rRNA genes in the plow layer at sites 1 and 2 were comparable to those in the Anjo and Chikugo paddy field soils (5.9×10^7–1.3×10^9 g^{-1} dry soil; Watanabe *et al.* 2007) because the sequence of major DGGE bands obtained from the plow layer samples was mostly affiliated with methanogenic archaeal lineages. Numbers in the subsoils were 10–100-fold smaller than those in the plow layer soils and some of the major DGGE bands were derived from crenarchaeotal 16S rRNA genes (e.g. bands 7, 8 and 12). These findings suggest that the populations/total biomass of methanogenic archaeal community in the subsoil layer were originally small and did not proliferate and predominate after reclamation. Substrates for methanogenesis derived from rice roots and plant residues might be limited in the subsoil layer as total C and N are low in the subsoil (Table 1).

Phylogenetic affiliations of the nucleotide sequences from the major DGGE bands are shown in Fig. 2 (closed circles). The sequences obtained from the Anjo and Chikugo paddy field soils (Watanabe *et al.* 2006) are also shown on the tree (closed triangles). Sequences belonging to Methanocellales (Rice cluster I), *Methanosarcina* spp., *Methanosaeta* spp., Methanosarcinales and Methanobacteriales were recovered from the paddy soils in the Kojima Bay polder. Crenarchaeotal and unknown euryarchaeotal 16S rRNA genes were also obtained. However, sequences of Methanomicrobiales, which were the dominant methanogenic archaea in the Anjo and Chikugo paddy soils, were not retrieved from the paddy soils of the polder. In the paddy soils at sites 1 and 2, where the DGGE band patterns were relatively similar to those from the Anjo and Chikugo paddy fields, methanogenic archaeal 16S rRNA genes that were closely related to Methanocellales (Kojima-2, 3 and 4), *Methanosarcina* sp. (Kojima-5) and Methanobacteriales (Kojima-10) were retrieved. In the subsoil of site 3, sequences closely related to *Methanosaeta* spp. were recovered (Kojima-18 and 28). In contrast, the sequences in unidentified Methanosarcinales (Kojima-22–25 and 30) and Crenarchaeota (Kojima-20 and 21) were mainly obtained from the soils at site 4. That is, different members of methanogenic archaea inhabit each site, indicating that distinct methanogenic archaeal communities have developed under the different environmental conditions. Methanogenic archaeal 16S rRNA genes were not recovered from the Mn nodules and ferric-oxide mottle, indicating that methanogenic archaea do not survive under such oxic conditions, although some sulfate-reducing bacteria inhabited the Mn nodules by using Mn oxide as an electron acceptor as discussed by Cahyani *et al.* (2007).

Halophilic and methylotrophic methanogens (e.g. *Methanococcides* spp. and *Methanolobus* spp.) inhabit tidal flats, estuaries and brackish lakes because they do not compete with sulfate-reducing bacteria over substrates, that is, H_2 and acetate (Munson *et al.* 1997; Purdy *et al.* 2002; Wilms *et al.* 2006; Zinder 1993). In the paddy field soils, where at least 50 years have passed since reclamation, sequences of known halophilic or methylotrophic methanogenic archaea were not obtained from the DGGE bands, although sequences (Kojima-5) that were closely related to acetoclastic and methylotrophic *Methanosarcina acetivorans* (99.3% identity [277/279 bp]) were retrieved. In the meantime, several methanogenic archaeal 16S rRNA fragments that were closely related to members of novel methanogenic archaea (ZC-I cluster) were recovered, mainly from the subsoil at site 4 (96.8% maximum identity [270/279 bp]). In addition, in the subsoil of site 3, a band corresponding to Kojima-6 at site 1 (ZC-1 cluster) was observed. Members of the ZC-I cluster are enriched from the Zoige wetland of the Tibetan plateau (Zhang *et al.* 2008) and are assumed to use acetate, H_2/CO_2, methanol and trimethylamine as substrates for methanogenesis. These findings suggest that novel methanogenic archaea that can use versatile substrates, including

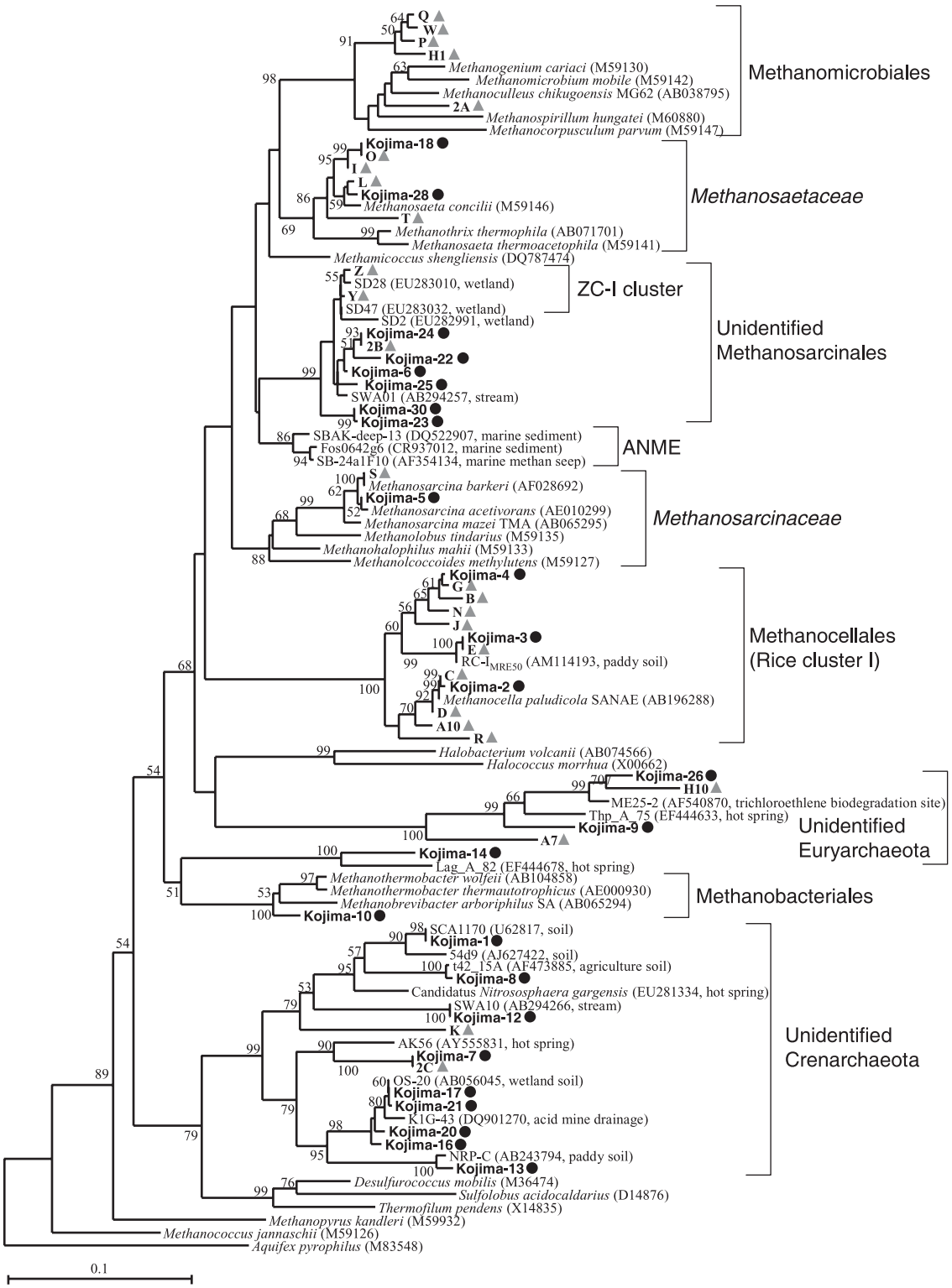

Figure 2 Phylogenetic relationships of the methanogenic archaeal 16S rRNA genes retrieved from the denaturing gradient gel electrophoresis (DGGE) with 1106F-GC/1378R (*Escherichia coli* positions 1107–1377) from the paddy field soils. The sequences obtained from the paddy field soils in the Kojima Bay polder are shown in bold letters with closed circles. The sequences from Anjo and Chikugo are shown in bold letters with closed triangles (Watanabe *et al*. 2006). Bootstrap values (%) are shown at the branch points (when the values are greater than 50%). *Aquifex pyrophilus* was used as an outgroup. The scale bar represents 0.1 substitutions per nucleotide. The accession numbers of the reference sequences are indicated in parentheses.

methyl compounds, have survived in the subsoil of paddy fields in the Kojima polder for a long time under the assumption that these methanogenic archaea originally inhabited the sea floor of Kojima Bay.

In the present study, several crenarchaeotal 16S rRNA genes, which were closely related to 16S rRNA genes recovered from terrestrial and marine environments, were obtained, although the physiological characteristics of these members have not been clarified. As certain members of the Crenarchaeota are recognized as ammonium oxidizers (Hayatsu *et al*. 2008; Treusch *et al*. 2005) and the identity between the sequence of Kojima-1 and that of the estimated ammonium-oxidizing archaea (fosmid library 54d9) was 95% (267/281 bp), some crenarchaeaotal members in the paddy fields in the Kojima Bay polder might be involved in ammonium oxidation in the soils.

In conclusion, 16S rRNA genes that were closely related to known halophilic and methylotrophic methanogenic archaea were not obtained from the main DGGE bands derived from paddy fields in the Kojima Bay polder, irrespective of site or soil layer. In contrast, several novel uncultured methanogenic archaeal 16S rRNA genes, which were assumed to be methylotrophic methanogenic archaea, were recovered from some sites. The community structures of methanogenic archaea were different from those in the diluvial and alluvial paddy fields and tended to be distinct at each site depending on the elapsed years since reclamation. The numbers of methanogenic archaeal and other archaeal 16S rRNA genes were higher in the plow layer soils than in the subsoil layer soils. These findings indicated that distinct methanogenic archaeal communities developed under the unique pedogenesis processes in the paddy field soils in the Kojima Bay polder. Further investigations are required to reveal the relationships between methanogenic archaeal communities and the processes of paddy soil formation in the polder.

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