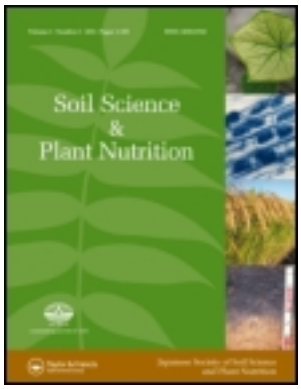

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Analysis of communities of ammonia oxidizers, methanotrophs, and methanogens associated with microcrustaceans in the floodwater of a rice field microcosm

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Analysis of Communities of Ammonia Oxidizers, Methanotrophs, and Methanogens Associated with Microcrustaceans in the Floodwater of a Rice Field Microcosm

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Ammonia oxidizers (AOB), methanotrophs (MOB), and methanogens that were associated with *Moina* sp., *Mesocyclops* sp., and *Heterocypris* sp. in the floodwater of a rice field microcosm were studied by using DGGE and sequence analysis with *amo*A / *pmo*A and methanogen-specific primer sets. Only one AOB and two MOB DGGE bands with the same mobility were detected, irrespective of the microcrustaceans. Effect of the incubation period was observed only in the AOB community associated with *Moina* sp., and an additional AOB clone appeared in the late period of incubation. Sequence analysis of the bands revealed that both of the closest relatives of the AOB clones belonged to uncultured ammonium monooxygenase genes of β-*Proteobacteria*, while those of the MOB clones to *Methylomicrobium buryatense*, an uncultured methanotroph bacterium, and *Methylococcus capsulatus* within type I methanotrophs belonging to γ-*Proteobacteria*. In contrast, no amplification products of 16S rDNA of the methanogenic archaea were obtained from any of the microcrustacean samples, indicating the absence or very small populations of methanogens.

Key Words: **ammonia oxidizer, methanogen, methanotroph, microcrustacean, *Moina*, rice field.**

Ammonia-oxidizing bacteria (AOB), methane-oxidizing bacteria (MOB), and methanogens are important groups for sound soil activities due to their significant contribution to global nitrogen and carbon cycling. Most of the molecular ecology studies on AOB, MOB, and methanogens have focused on high NH_3 or high CH_4 environments such as peat soils (McDonald et al. 1996; Utsumi et al. 2003), rice fields (Conrad and Rothfuss 1991; Henckel et al. 1999; Henckel et al. 2001), lake sediments (Costello and Lidstrom 1999), and landfill cover soil (Wise et al. 1999). And the community structure of these bacteria was studied in rice field ecosystems, e.g. methanogens in anoxic soils (Großkopf et al. 1998; Fey et al. 2001), MOB in rice roots (Horz et al. 2001), MOB in rice field soil after drainage (Henckel et al. 2001), and AOB in the rhizoplane and rhizosphere of rice (Tomiyama et al. 2001; Briones et al. 2002). However, reports on these bacteria from microcrustaceans occurring in the floodwater of rice fields are not available, although several authors reported the presence of methanotrophic symbionts with marine invertebrates (Fisher 1990; Kochevar et al. 1992; Cavanaugh et al. 1992; Fisher et al. 1993; Lee and Childress 1994) and the activity of AOB in the fecal pellets of copepods (Bianchi et al. 1992). Distel and Cavanaugh (1994) observed that Type I MOB occurred as symbionts of marine bivalves. AOB and MOB occupy similar habitats in soil and aquatic environments in counter gradients of O_2 and NH_3 or CH_4, respectively (Hanson and Hanson 1996).

Niswati et al. (submitted for publication) applied universal 16S rDNA primer sets to DNA extracted from microcrustaceans in the floodwater of a rice field microcosm to identify a wide range of associated microorganisms belonging to the Eubacteria domain. However, these primer sets were not suitable for detecting specific functional groups such as AOB, MOB, and methanogens, which were expected to be associated with the microcrustaceans. Microcrustaceans are known to excrete ammonia (Poulet et al. 1991; Carman 1994) and the activity of methanogens is considered to be expressed in the anaerobic intestinal tract (Traganza et al. 1979; Bianchi et al. 1992; Cynar and Yayanos 1991; de Angelis and Lee 1994). Therefore, the specific primer

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sets targeting the *amo*A / *pmo*A genes of AOB and MOB and 16S rDNA of methanogenic archaea were applied to the DNA extracted from microcrustaceans in the present study to analyze the phylogenetic positions of associated AOB and MOB. Holmes et al. (1995) reported that the particulate CH_4 monooxygenase (pMMO), which is present in almost all the MOB isolated, was homologous to the ammonium monooxygenase (AMO), the key enzyme of AOB. AMO catalyzes not only NH_3 but also CH_4 oxidation (Bedard and Knowles 1989). The putative active subunit of pMMO is encoded by *pmo*A, and the homologous subunit of AMO is encoded by *amo*A (Murrel et al. 2000).

The objectives of the present study were to elucidate the phylogenetic positions of AOB, MOB, and methanogenic archaea associated with *Moina* sp., *Mesocyclops* sp., and *Heterocypris* sp. in the floodwater of a rice field microcosm. In this environment, bacteria associated with microcrustaceans accounted for about 1–10% of the total bacteria in the overlying water (Taniguchi et al. 1997a, b). Since they occur as planktonic (*Moina* sp. and *Mesocyclops* sp.) and benthic (*Heterocypris* sp.) organisms (Dodson and Frey, 1991; Delorme 1991; Williamson 1991) in freshwater environments, they play several roles in nutrient cycling by grazing nitrogen-fixing blue green algae (Wilson et al. 1980; Grant et al. 1983) and by releasing nitrogen along with their grazing (Von Elert and Franck 1991; Hessen and Van Donk 1993).

MATERIALS AND METHODS

Preparation of microcrustaceans, and establishment of and incubation in a rice field microcosm

The soil samples used in this microcosm experiment were taken from a paddy field in Aichi-ken Anjo Research and Extension Center, central Japan. Three microcrustaceans (*Moina* sp., *Mesocyclops* sp., and *Heterocypris* sp.) that were collected from a rice field were cultured separately in an aqueous medium with periodical supply of *Chlorella* sp. One kilogram of each soil sample that was passed through a 4 mm sieve was mixed with chemical fertilizers first. After rice straw segments 2 cm long were mixed thoroughly into the fertilized soil, the soil was heated at 80°C for 2 h to avoid the development of indigenous microcrustaceans. Then, the soil samples were put into a container (20 cm long, 13 cm wide, and 14 cm high) with triplicates, flooded with 3 L of distilled water, and about 150 individuals of each adult microcrustacean were inoculated to the flooded rice field microcosm (Niswati et al. 2003). The incubation was conducted at 30°C in the daytime (from 4 a.m. to 8 p.m.) and at 20°C in the nighttime (from 8 p.m. to 4 a.m.). During the incubation period, the depth of water was maintained at 10 cm with distilled water. Microcrustaceans were collected from the floodwater microcosm weekly and stored at −20°C until DNA extraction.

DNA extraction and PCR amplification

DNA was extracted from 100 individuals of the respective microcrustacean samples using a high temperature and a salt and sodium dodecyl sulfate (SDS)-based lysis method (Zhou et al. 1996) with slight modifications (Niswati et al. 2002).

The primer sets for the amplification of the genes encoding AMO and pMMO were applied with slight modifications from the methods of Holmes et al. (1995) and Nold et al. (2000). The use of a GC clamp attached to the forward primer was described in the method of Henckel et al. (1999). The complete primer sequences are shown in Table 1.

The PCR reactions for the *amo*A / *pmo*A genes were carried out by using nested PCR techniques. The *amo*A / *pmo*A primer set without GC clamp was used for the first PCR with extracted DNA. The second PCR was conducted with 1 / 10 or 1 / 100 of the PCR products from the first PCR as a template using the primers with GC clamp (Table 1) and the products were applied for DGGE analysis. The first PCR was carried out in 25-μL (total volume) mixtures containing approximately 50–100 ng of DNA extracted from the microcrustaceans, 10 pmol of each primer, 1.5 mM of the dNTP mixture,

Table 1. Primers and sequences of *amo*A and *pmo*A genes used for this study.

References	Target	Primer sequences	
		Forward[a]	Reverse[b]
Holmes et al. (1995)	*amo*A (β, γ), *pmo*A (γ)	5′-GGNGACTGGGACTTCTGG-3′	5′-GAASGCNGAGAAGAASGC-3′
Nold et al. (2000)	*amo*A (β, γ), *pmo*A (γ)	5′-GGNGACTGGGACTTCTGG-3′	5′-AAVGCVGAGAAGAAWGC-3′
Modified primer pair	*amo*A (β, γ), *pmo*A (γ)	5′-GGNGACTGGGAYTTCTGG-3′	5′-AAVGCVGAGAAGAAWGC-3′

[a] Forward primer corresponding to *Nitrosomonas europaea amo*A positions 172 to 189.

[b] Reverse primer corresponding to *N. europaea amo*A positions 665 to 681.

N, A, T, C, or G; S, C or G; V, A, C, or G; W, A or T; Y, C or T.

Forward primer had a GC clamp for DGGE (CCCCCCCCCCCCCCGCCCCCCGCCCCCCGCCCCCGCCGCCC) (Henckel et al. 1999)

1 mM of TaKaRa Ex Taq™ (Takara Shuzo, Otsu, Japan) buffer, 1.25 U of Ex Taq™ polymerase, and ultra-pure water. The reaction was performed in TaKaRa PCR Thermal Cycler Dice Model TP600 (Takara Shuzo, Otsu, Japan) by using 40 cycles for the first PCR and 28 cycles for the second PCR consisting of 94°C for 5 min (initial denaturation), followed by 40 or 28 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min, with final extension at 72°C for 7 min. The presence of the PCR products was confirmed by the analysis of 2 μL by agarose gel electrophoresis. Ethanol precipitation of the PCR amplification products was carried out before the application to DGGE.

The PCR primer sets used for amplifying 16S rDNA of the methanogens were 0357F (5′-CCCTACGGGGC-GCAGCAG-3′) and 0691R (5′-GGATTACARGATTTC-AC-3′), and 0357F and 0915aR (5′-GTGCTCCCC-CGCCAATTCCT-3′) (Watanabe et al. 2004). The PCR mixture (50 μL) contained DNA extracted from the microcrustaceans (50–100 ng), 0.5–1.5 pM of each primer, 0.25 mM of the dNTP mixture, 0.2 mM of TaKaRa *Ex Taq*™ buffer, and 1.25–2.5 U of *Ex Taq*™ polymerase. Amplification conditions for the 0357F-GC and 0691R primer pairs were 94°C for 1 min, followed by 25 to 30 cycles at 94°C for 1 min, 55 to 58°C for 1 min or 1.5 min, 72°C for 2 min, with final extension at 72°C for 7 min. Amplification conditions for 0357F-GC and 0915aR were 94°C for 1 min, followed by 35 cycles at 94°C for 1 min, 66 to 70°C for 1 min, 72°C for 2 min, with final extension at 72°C for 7 min. The PCR mixture containing the 16S rDNA from three pure cultures of *Methanobrevibacter arboriphilus* SA, *Methanosarcina mazei* TMA, and *Methanoculleus chikugoensis* MG62 was used as positive control. The presence of the PCR products was confirmed on 2% agarose gel containing ethidium bromide (10 g L^{-1}).

DGGE analysis for *amo*A / *pmo*A products

DGGE was performed using the Dcode™ System (BioRad, Hercules, California), as described in the manufacturer's instructions and by Muyzer et al. (1993). PCR products (about 300 ng) were loaded onto 8% (w / v) polyacrylamide (acrylamide–bisacrylamide, 37.5 : 1) with a linear gradient of the denaturants (100% denaturant contained 7 M urea and 40% (v / v) formamide), increasing from 25% at the top of the gel to 65% at the bottom for the separation of the AOB and MOB fragments. Electrophoresis was performed at 60°C in a 7 L of 1 × TAE (40 mM Tris-acetate, 1 mM EDTA) buffer, and an electric current of 100 V was applied to the submerged gel for 14 h. After completion of electrophoresis, the gels were stained for 30 min with a 1 : 10,000 (v / v) SYBR Green I nucleic acid solution (BMA, Rockland, ME, USA) and visualized with a UV transilluminator.

Cloning and sequences of DGGE fragments

The DGGE bands were carefully excised under UV illumination and then placed in 75 μL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA was extracted from the gel piece by overnight incubation at 4°C, and the supernatant was used as template DNA in the re-amplification by PCR using the same primers as those described above. The amplicons were cloned using the pT7Blue T-vector (Novagen, Darmstadt, Germany). Randomly selected clones were screened to identify a clone aligned with the intended band by amplification with a primer set targeting the *amo*A / *pmo*A genes. Only 10% of the positive clones contained inserts of the expected size (525 bp) and the PCR products were reanalyzed by DGGE to confirm the mobility. Plasmid was isolated from the clones using a QIAprep spin miniprep kit (Qiagen, Crawley, UK) and digested with *Sal*I and *Eco*RI restriction endonucleases. Sequencing reactions were performed using the DYEnamic™ ET Terminator Cycle Sequencing kit (Amersham Biosciences, Piscataway, NJ) as specified by the manufacturer. Products from cycle sequencing were analyzed with an ABI 373S DNA sequencer (Applied Biosystems Calif., USA). The se-quences obtained were submitted to the BLAST search option of the DNA Data Bank of Japan (http://www. ddbj.nig.ac.jp). Phylogenetic trees were constructed with the *amo*A / *pmo*A nucleic acid-based gene sequences and related sequences from γ and β-*Proteobacteria* which were deposited at the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov) by using Clustal-x and Nj plot software package.

RESULTS AND DISCUSSION

Methanogenic community. Amplification products of the 16S rDNA of methanogenic archaea could not be obtained from any of the microcrustacean samples, in spite of several attempts. The PCR program and PCR mixture were modified for both primer sets, including decrease and increase of the annealing temperature, PCR cycles, primer concentration and DNA template concentration. The amplification products were obtained only from the positive control with methanogens in pure culture. These findings, therefore, indicated that the methanogenic community was absent or too small to be amplified in the microcrustaceans studied. Therefore, the absence or paucity of the associated methanogenic communities with microcrustaceans could not be confirmed in the floodwater of rice fields based on the present microcosm experiment.

DGGE patterns of AOB and MOB associated

with microcrustaceans. Bands found in the DGGE image were divided into 4 groups (A, B, C, and D) based on the mobility and sequence analysis. The DGGE analysis of the PCR products amplified with the *amo*A / *pmo*A primer set showed very few bands without any significant changes in the band patterns, irrespective of the microcrustaceans and the incubation period, except for *Moina* sp. after 8 and 12 weeks of incubation (Fig. 1, Band C). Similar findings of the effect of the incubation period were also reported by Niswati et al. (submitted for publication) for the same microcrustacean samples, in which only eubacterial communities associated with *Moina* sp. were affected by the incubation period of flooding. *Moina* sp. was reported to be a sensitive indicator for environmental change in aquatic ecosystems (Hatakeyama and Sugaya 1989; Wong 1997; Kikuchi and Wakabayashi 1997). Physicochemical conditions of floodwater and the aging of the *Moina* sp. seemed to change the band patterns after 8 weeks of incubation. Pérez-Martínez and Gulati (1999) reported that the amount of nitrogen released to the surrounding water by adult cladoceran *Daphnia* was larger than that by juvenile individuals. Since the ammonia concentration in the lake affected the density and diversity of the cladoceran (Mangas-Ramirez et al. 2002) and algal growth (Wiltshire and Lampert 1999), it might have affected the bacteria associated with *Moina* sp. In contrast, no studies on the effect of environmental changes and the aging of *Mesocyclops* sp. and *Heterocypris* sp. on their associated bacterial communities are available, except for our previous study showing that the period after flooding affected the changes in the RFLP patterns of the eubacterial communities associated with these microcrustaceans (Niswati et al. 2003). In addition, less than ten DGGE bands were identified in this study, indicating that the AOB and MOB communities were very simple and specific (Fig. 1). The populations of *Moina* sp. and *Heterocypris* sp. reached a peak at two weeks after flooding while the population of *Mesocyclops* sp. reached a peak at three weeks after flooding, respectively. Thereafter, the populations remained almost stable until 12 weeks of incubation because there was no competition with the other microcrustaceans, and they survived under the controlled conditions in the microcosm. On the other hand, the populations of microcrustaceans in the floodwater of the paddy field fluctuated depending on the rice growth period and environmental conditions (Simpson et al. 1994; Kuwabara 1999; Yamazaki et al. 2001)

Generally, the DGGE profiles of the PCR products amplified in the present study contained three major bands in the middle part and several faint bands in the upper and lower parts of the gel (Fig. 1, Bands A, B and D). Uniformly present, three dominant bands in the middle part indicated that every microcrustacean examined was inhabited by the same dominant AOB / MOB in the floodwater of paddy soil. As the DGGE patterns of the respective microcrustaceans were not studied at the time of the inoculation of the microcrustaceans to the microcosm, the possibility that their bacteria, which may have originated from the floodwater in the paddy field, existed throughout the pre-culture periods, could not be ruled out. Appearance of Band C after 8 weeks of incubation may have been affected by some changes of the floodwater conditions and the aging of *Moina* sp. as described above.

Phylogenetic analysis of DGGE bands. As shown in Fig. 1 and Table 2, Band 'C' was associated

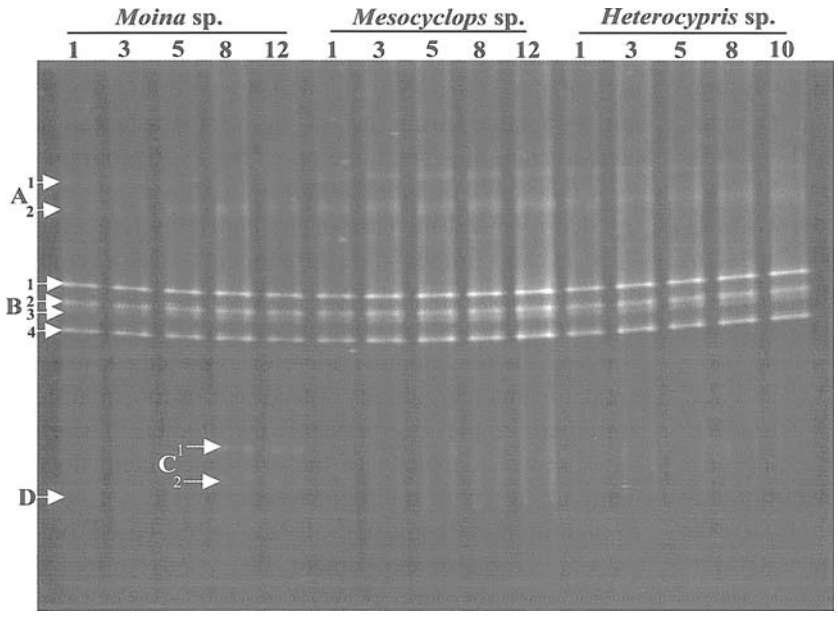

Fig. 1. DGGE patterns of methanotroph and ammonia oxidizers associated with microcrustaceans. Lane numbers correspond to the incubation period (weeks).

Table 2. Closest relatives of ammonium and methane-oxidizing bacteria associated with microcrustaceans revealed by DGGE analysis using *amo*A / *pmo*A primer sets.

Band name[a]	Amino acid Seq.	Alignment	Closest relative	Taxonomic description	Similarity (%)	Accession number	Sources	References
Band A	165	137 / 163	*Methylomicrobium buryatense*	γ-*Proteobacteria*	84	AAL09399	Alkaline Soda lake	Kaluzhnaya et al. 2001
		137 / 163	Uncultured putative methanotroph		84	AAP43772	Alkaline Soda lake	Bodrossy et al. 2003
Band B(1)	165	144 / 165	Uncultured bacterium PS-49	γ-*Proteobacteria*	87	AF64098	Marine sediment	Nold et al. 2000
		141 / 165	*Methylocaldum gracile*		85	AAC04380	Soil	Bodrossy et al. 1997
Band B(2)	167	148 / 167	*Methylococcus capsulatus*	γ-*Proteobacteria*	88	AAB51065	Culture	Stoylar et al. 1999
		146 / 167	*Methylocaldum gracile*		87	AAC04380	Soil	Bodrossy et al. 1997
Band C[b]	168	138 / 148	Uncultured bacterium ammonia monooxygenase-like protein	β-*Proteobacteria*	93	AAL87427	Forest soil	Radajewski et al. 2002
		136 / 147	Uncultured bacterium gp17 ammonia monooxygenase-like protein		91	AAK51664	Forest soil	Reay et al. 2001
		135 / 147	Uncultured bacterium gp22 ammonia monooxygenase-like protein		91	AAK51668	Forest soil	Reay et al. 2001
Band D	162	150 / 162	Uncultured ammonia-oxidizing bacterium	β-*Proteobacteria*	92	AAO60354	Agricultural soil	Avrahami et al. 2003
		150 / 162	Uncultured bacterium ammonia monooxygenase subunit A protein		92	CAE22488	Upland soil	Knief et al. 2003
		150 / 162	*Nitrosospira* sp.		92	AAA87222	Culture	Holmes et al. 1995

[a] See Fig. 1.
[b] Bands which were found in *Moina* sp. samples only.

only with *Moina* sp. The excision and re-amplification of Band D gave a single band in its rerun on the gel. However, the re-amplification of Bands A1 and A2 that were obtained after cloning or extracted directly from the DGGE gel always gave two bands with a similar mobility to the original position. The clones isolated from Band B1 gave two bands, those for Bands B2 and B3 gave three bands, and those for Band B4 also gave two bands with a similar mobility to that of the original B group bands. Re-amplification of the clones isolated from Bands C1 and C2 also gave two bands with a similar mobility to that of the original C group bands. Although no single bands were obtained after purification by cloning, five out of nine bands that were excised and cloned were successfully sequenced (one from bands 'A', two from bands 'B' [Band B(1) from bands B1, B2 and B3 and Band B(2) from band B2, B3 and B4], and one from Band 'C'). The closest relatives corresponding to these bands are shown in Table 2 and their phylogenetic relationships are depicted in Fig. 2. Sequence analysis of Bands 'A' revealed that the closest relatives belonged to *Methylomicrobium buryatense* and the closest relatives corresponding to bands 'B' belonged to an uncultured bacterium or *Methylococcus capsulatus*. The nucleotide sequences of two 'B' clones differed only in three nucleotide bases whose positions were not at the priming site (data not shown). All of the MOB clones that were identified belonged to the type I methanotrophs in γ-Proteobacteria (Fig. 2). As the closest relatives corresponding to Bands A and B were detected in aquatic environments, the clones were considered to have been adapted to the floodwater environment.

Since methane was reported to be produced in the intestine of microcrustaceans, the methanogens and methanotrophs were analyzed in the present study. Although the amplification of the methanogens from the same DNA samples was not successful, which indicated the insufficient amount of template DNA, if any, for amplification, MOB clones were detected in this study.

In the present study, only type I MOB was associated with microcrustaceans, presumably due to the very low mixing ratio of CH_4 to O_2 in the microcrustacean habitats. Methane in the microcrustaceans was emitted due to the anaerobic conditions in parts of their small intes-

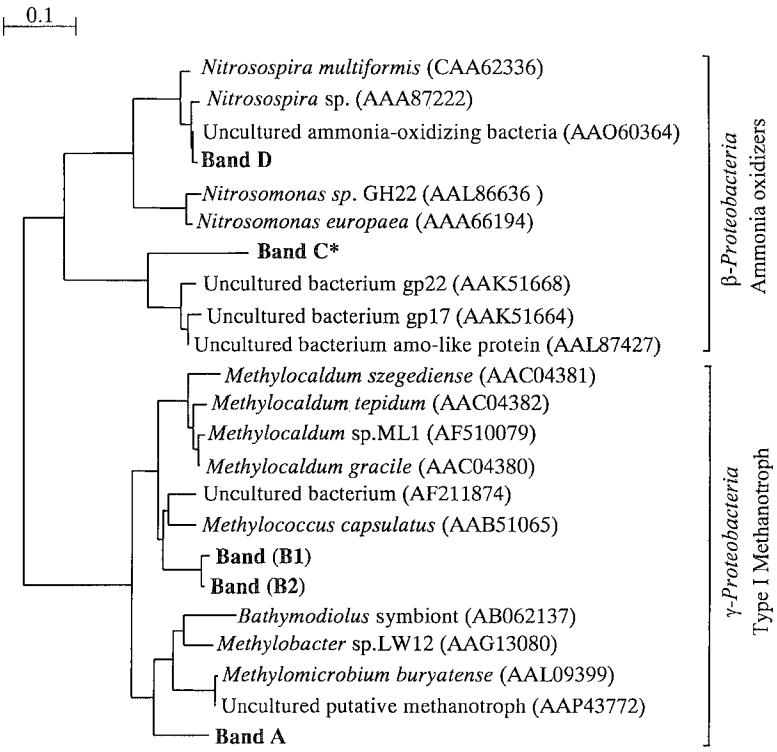

Fig. 2. Phylogenetic tree of the *amo*A / *pmo*A gene sequences from ammonium and methane-oxidizing bacteria associated with microcrustaceans in the floodwater of a paddy field microcosm. *, found in *Moina* sp. only. The scale bar denotes the estimated number of amino acid changes per *amo*A / *pmo*A gene sequence position.

tine and the concentration of CH_4 was considered to be very low. The limiting factor for methanotrophs is the availability of CH_4 and O_2. Henckel et al. (2000) reported that the aerated surface part of a rice field was dominated by type I MOB, when air with a low mixing ratio of CH_4 to O_2 was supplied ($1 \times 10^{-3}\,m^3\,m^{-3}$). Amaral and Knowles (1995) and Amaral et al. (1995) reported that in agar diffusion columns with counter gradients of O_2 and CH_4, type I MOB predominated in layers with low mixing ratios of CH_4 to O_2, whereas type II MOB predominated at high CH_4 and low O_2 mixing ratios. In the rice field soil where the concentration of CH_4 fluctuated, both types I and II MOB were detected (Henckel et al. 1999; Eller and Frenzel 2001).

The closest relatives corresponding to Bands 'C' and D belonged to an uncultured ammonium monooxygenase-like protein and uncultured ammonia-oxidizing bacterium, respectively. Band D corresponded to the same group as *Nitrosospira* sp. in β-*Proteobacteria* in the phylogenetic tree (Fig. 2), but Band 'C' did not correspond to the same cluster as *Nitrosospira* or *Nitrosomonas*. The present study indicated that microcrustaceans in the floodwater of a rice field microcosm harbored AOB. As these closest relatives were detected in soils (Table 2), they were considered to be derived from the rice field soil. Carman (1994) reported that marine copepods released NH_4^+, and this nutrient was metabolized by the bacterioplankton and phytoplankton in the surrounding water (Von Elert and Franck 1991; Wiltshire and Lampert 1999). Only *Nitrosospira* spp. were detected in the rice roots and on the surface of the flooded rice soil by using CTO primers (Ikenaga et al. 2003; Murase et al. 2003), where the soil collected from the same rice field as that in the present study was subjected to the experiments. These findings indicated that *Nitrosospira* spp. grew preferentially in this rice field environment.

Several DGGE bands appeared in the rerun of single excised clones from the DGGE gel. Sequence analysis of the two clones obtained from Bands 'B' revealed a difference in three nucleotide bases which displayed a different mobility in DGGE and their affiliation to the same phylogenetic branch, indicating that DGGE analysis was sensitive enough to detect a single base mutation on a DGGE gel. Several investigators reported that MOB contained multiple *pmo*A gene copies (Stolyar et al. 1999) while AOB contained multiple *amo*A gene copies (Norton et al. 1996; Klotz and Norton 1998; Norton et al. 2002; Nicolaisen and Ramsing 2002). The present study revealed that the number of DGGE bands obtained from the amplification of environmental DNA with the *pmo*A / *amo*A primer sets did not always reflect the population diversity.

In conclusion, DGGE and sequence analysis demonstrated that very specific AOB and MOB were associated with *Moina* sp., *Mesocyclops* sp., and *Heterocypris* sp. and that the incubation period affected only AOB and MOB of *Moina* sp. Type I MOB in γ-*Proteobacteria* and AOB in β-*Proteobacteria* were associated with the microcrustaceans studied. The predominance of type I MOB was attributed to the absence of associated meth-

anogens with these microcrustaceans, because type I MOB could grow well at low mixing ratios of CH_4 to O_2.

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