Seasonal Change in Methanotrophic Diversity and Populations in a Rice Field Soil Assessed by DNA-Stable Isotope Probing and Quantitative Real-Time PCR

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The community structure of methane-oxidizing bacteria (methanotrophs) is affected by concentrations of methane and oxygen. In rice fields, concentrations of both gases differ significantly between the flooded and drained seasons. We investigated the active methanotrophic community structures in flooded and drained soils by DNA-based stable isotope probing. Active methanotrophic diversity was assessed with clone library-based analyses of the 16S rRNA gene and the particulate methane monooxygenase gene (*pmoA*). The active methanotrophic populations were also estimated by group-specific quantitative real-time PCR assays targeting the 16S rRNA gene and the *pmoA* gene in ¹³C-labeled DNA. These molecular biological analyses showed that the flooded rice field soil was dominated by Type II methanotrophs closely related to the genera *Methylocystis* and *Methylosinus*, whereas the drained rice field soil was dominated by Type I methanotrophs closely related to the genera *Methylobacter*, *Methylosarcina*, and *Methylobacterium*. The alternating conditions in a rice field select for methanotrophs adapted to each environment, resulting in a dramatic change in methanotrophic community structure from one season to another.

Key words: methanotrophs, DNA-SIP, clone library, quantitative real-time PCR, rice field soil

Methane is an important greenhouse gas with a global warming potential about 25 times higher per molecule than that of CO₂ (33). Rice fields are major sources of methanogen-derived methane, representing 7% to 17% (about 40–100 Tg year^{−1}) of worldwide methane emissions (26). However, much of the methane derived from rice fields is oxidized and consumed by methane-oxidizing bacteria called methanotrophs (39). Methanotrophs also contribute to reductions in global methane emissions from sources other than rice fields (15).

Methanotrophs are a physiologically distinct group of Gram-negative bacteria that can utilize methane as their sole carbon and energy source. Methanotrophs are subdivided into Type I (*Gammaproteobacteria*) and Type II (*Alphaproteobacteria*), on the basis of their phylogeny, chemotaxonomy, intracellular membrane arrangement, carbon assimilation pathway, and phospholipid fatty acid (PLFA) composition (15). In rice fields, both Type I and Type II methanotrophs have been detected using culture-dependent techniques (12, 23) and culture-independent techniques (21).

Although both types of methanotrophs exist together in the same rice fields, they occupy different niches with different CH₄ and O₂ concentrations. For example, during the flooded period, Type I methanotrophs are more active and abundant on the rice roots, where a high O₂ concentration is provided by rice plants. In contrast, Type II methanotrophs are more active and abundant in the rhizospheric soil some distance from the rice roots, where the soil air tends to be micro-aerobic but has a high CH₄ concentration (34). Seasonally the

methanotrophic community structure in rice fields has been well studied in flooded soils (3, 11, 17). However, there are only a few reports describing the methanotrophic populations in drained soils. Macalady *et al.* (28) investigated the transition of methanotrophic community structure in a rice field throughout the year, including in the drained soil, by PLFA analysis, a powerful tool for the quantification of methanotrophic populations because of its high sensitivity (2). However, since the PLFA database for methanotrophs is much less extensive than the 16S rRNA gene and functional gene databases (6, 10), PLFA profiles provide no detailed phylogenetic information, especially on uncultured methanotrophs for which no information exists in the PLFA database.

Comparing the methanotrophic community structure in rice fields during drained and flooded periods should improve our understanding of microbial methane oxidation in rice ecosystems. To date, there has been little phylogenetic information about methanotrophs in drained rice fields, and thus, molecular biological analysis is currently the most appropriate method for obtaining information on community structure (29). In this study, we investigated the change in active methanotrophic community structure between flooded and drained rice fields by DNA-based stable isotope probing (SIP), a powerful tool for investigating active micro-organisms (32). In addition, we assessed active methanotrophic populations in rice field soils by combining DNA-SIP with quantitative real-time PCR using the 16S rRNA gene and *pmoA* gene as molecular biomarkers.

Materials and Methods

Soil samples and SIP incubation

Rice field soil samples were taken in August 2007 (flooded rice field soil) and December 2007 (drained rice field soil) in Tsukuba,

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Japan. During the past 20 years, the field sampled has been used as a paddy field and left untreated during the winter after the year's harvest. The flooded soils were collected in the fourteenth week after planting and the drained soils were collected in the sixteenth week after drainage. Soils were collected at a distance of 10 to 20 cm from rice plants and at a depth of 5 to 15 cm.

Immediately after sampling, soil slurries for DNA-SIP were prepared as follows. The soil slurries from the flooded rice field consisted of 30 g of bulk soil plus water derived from the flooded rice field; the soil slurries from the drained rice field consisted of 10 g of bulk soil plus 20 mL of sterile distilled water. Soil slurries were placed in 150-mL glass bottles, and sealed with butyl rubber stoppers. In the headspace, 15 mL of air was replaced with 15 mL of $^{13}\text{CH}_4$ (>99% pure, Tokyo Gas, Tokyo, Japan). In total, 15 bottles of each rice field soil were incubated at 150 rpm and 25°C. The incubated soils were collected by centrifugation everyday during the first week and every 3 days thereafter.

Extraction of DNA and separation of ^{13}C -labeled DNA

Environmental DNA was extracted from 3 g of the incubated soil using Isoil for Beads Beating (Nippon Gene, Tokyo, Japan) and eluted in 400 μL of sterile distilled water. The ^{13}C -labeled DNA was separated by CsCl density gradient centrifugation as described by Radajewski *et al.* (32). Briefly, the environmental DNA was centrifuged in small-scale (1 mL) CsCl/ethidium bromide gradients at $541,000\times g$ for 24 h. After the centrifugation, light DNA (unlabeled) and heavy DNA (^{13}C -labeled) were collected with a syringe and needle on a transilluminator. To minimize contamination from unlabeled DNA, the separated ^{13}C -labeled DNA was purified a second time by CsCl density gradient centrifugation. Ethidium bromide was removed from the collected DNA fractions by butanol extraction. After ethanol precipitation, the ^{13}C -labeled DNA was dissolved in 50 μL of sterile distilled water.

16S rRNA gene amplification and denaturing gradient-gel electrophoresis (DGGE)

The nearly full-length 16S rRNA genes were amplified from each 1 ng of unlabeled and ^{13}C -labeled DNA retrieved from the flooded and drained soils using the bacterial universal primer set 8f (38) and 1494r (4). Ex Taq DNA polymerase (Takara Bio, Otsu, Japan) was used for PCR amplification. PCR conditions were as follows: 1 cycle of 2 min at 94°C, then 30 cycles of 20 s at 94°C, 10 s at 55°C, and 1.5 min at 72°C. A nested PCR strategy was used to ensure amplification of the 16S rRNA gene. The second PCR was performed with 1 μL of the first PCR product as a template and with 357f-GC and 518r (30). The PCR products containing a GC clamp were separated on an 8% (w/v) polyacrylamide gel with a urea-formamide denaturing gradient (30% to 60%, w/v). The gel was run at 36V for 18 h at 60°C in $1\times\text{TAE}$ buffer. The gel was then stained for 1 h with 1:10,000 (v/v) SYBR Gold, rinsed with $1\times\text{TAE}$, and scanned on a transilluminator. Bands of interest were excised from the gel and DNA was dissolved in 10 μL of sterile distilled water. 1 μL of the dissolved DNA was used for PCR amplification with the 357f and 518r primer set, and PCR products were then directly sequenced with the 357f primer.

Construction of the 16S rRNA gene and *pmoA* clone libraries

For the construction of clone libraries, the nearly full-length 16S rRNA gene was amplified from 1 ng of ^{13}C -labeled DNA using the 8f and 1494r primer set. PCR conditions were as described above. The *pmoA* gene was amplified with the primer set A189f (20) and mb661r (8). Ex Taq DNA polymerase was used for PCR amplification. PCR conditions were as follows: 1 cycle of 2 min at 94°C, then 30 cycles of 20 s at 94°C, 10 s at 60°C, and 1 min at 72°C, and then 5 min at 72°C for final extension. PCR products from the 16S rRNA gene and *pmoA* gene amplifications were purified and ligated into the pGEM-T Easy cloning vector (Promega, Madison, WI, USA) according to the manufacture's instructions. Forty-two clones from the 16S rRNA gene clone library and thirty-two clones from

the *pmoA* gene clone library were randomly selected, and single strands of inserted DNA fragments were sequenced with pUC/M13 Forward primer and pUC/M13 Reverse primer (Promega).

DNA sequencing and phylogenetic analysis

DNA sequencing was performed by cycle sequencing using the BigDye Terminator Cycle Sequencing ver. 3 Kit (Applied Biosystems, Foster City, CA, USA) and analyzed with an ABI Prism 310 DNA sequencer (Applied Biosystems). Resulting sequences were compared with similar sequences in public databases by BLAST searches and then aligned using the ClustalX program, version 2.0.10 with similar sequences downloaded from GenBank. The aligned sequence length was 420 bp for the 16S rRNA gene sequences, and 163 residues for the *PmoA* amino acid sequences. The phylogenetic trees were constructed using the NJplot program, version 2.2 (35).

Design of 16S rRNA gene primer sets for quantitative real-time PCR

Two group-specific primer sets were designed to selectively amplify the 16S rRNA genes from Type I and Type II methanotrophs. Reference sequences were selected on the basis of a phylogenetic analysis, from the 16S rRNA gene clone library used in this study (Fig. 2). Twenty-five reference sequences from Type I methanotrophs (nine strains), Type II methanotrophs (nine strains), and non-methanotrophs (seven strains) were downloaded from GenBank, aligned with clone sequences from this study using ClustalX 2.0.10, and manually verified for alignment accuracy. Potential primers were manually identified and further tested using the probe match program in Ribosomal Database Project (RDP) II (7) to ensure their specificity for each methanotroph. Type I56f (AAC GGT ARC RGG CCT TCG GG) and Type I239r (GCT AAT CYR ACR YAG GCT CAT CT) were designed as Type I methanotroph-specific primers; Type II432f (RCC WGG GAM GAT ART GAC GG) and Type II659r (ACC TCT CCC GRA CTC KAG AYC) were designed as Type II methanotroph-specific primers. The group specificity of each primer set was confirmed by sequencing the PCR products amplified with the set and 1 ng of ^{13}C -labeled DNA from the flooded and drained soils. Ex Taq DNA polymerase was used for PCR amplification. PCR conditions were as follows: 1 cycle of 2 min at 94°C, then 30 cycles of 20 s at 94°C, 10 s at 55°C, and 30 s at 72°C, and then 5 min at 72°C for final extension. PCR products were purified and ligated into the pGEM-T Easy cloning vector according to the manufacture's instructions. Four clone libraries were constructed with amplified PCR products, and twenty clones were randomly selected from each library and sequenced. The derived 16S rRNA gene sequences were compared with similar sequences in public databases using BLAST searches and classified on the basis of the highest percent identity to a cultivated representative.

Real-time PCR assays

Quantitative real-time PCR assays for the 16S rRNA gene and *pmoA* gene were performed for total bacteria (the 16S rRNA gene only), Type I methanotrophs, and Type II methanotrophs using a LightCycler 1.5 (Roche, Mannheim, Germany). All PCR mixtures were prepared using a LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit: 4 μL of $5\times$ reaction solution, 1 μL of each primer (final concentrations of 500 nM), 5 μL (1 ng) of ^{13}C -labeled DNA, and 9 μL of PCR-grade water. The 16S rRNA gene assays were performed with a four-step protocol as follows: initial denaturation for 10 min at 94°C followed by 40 cycles of 20 s at 94°C, annealing of primers for 10 s at 55°C, elongation for 10 s at 72°C, and fluorescence data acquisition for 1 s at 82°C. Copy numbers of 16S rRNA genes from all bacteria, Type I methanotrophs, and Type II methanotrophs were determined by amplification using primer sets 357f and 518r, Type I56f and Type I239r, and Type II432f and Type II659r, respectively. Assays for the *pmoA* gene were performed with the primer concentrations and PCR conditions described by Kolb *et al.* (25). In this study, the copy numbers of

Type I methanotrophic *pmoA* genes were calculated from the sum of results from the MBAC and MCOC assays, and the copy numbers of Type II methanotrophic *pmoA* genes were determined by TYPEII assay (25). Each measurement was performed in triplicate. Data analysis was carried out with LightCycler software version 3.5.3.

To construct calibration standard curves, dilution series of positive control DNA were used. For positive control DNA, the 16S rRNA gene and *pmoA* gene fragments amplified from *Methylosarcina lacus* (JCM 13284) were used for total bacteria and Type I methanotrophs, and those amplified from *Methylocystis* sp. strain M (37) were used for Type II methanotrophs. PCR products were then separated by gel electrophoresis and extracted and purified with a QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany). The DNA concentrations were measured with a PicoGreen dsDNA Quantification Kit (Invitrogen, Carlsbad, CA, USA).

Nucleotide sequence accession numbers

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *pmoA* sequences determined in this study are AB500710–AB500793 and AB500794–AB500857, respectively.

Results

DNA-SIP

To investigate the active methanotrophic community structure in flooded and drained rice fields, each bulk soil was incubated with $^{13}\text{CH}_4$ at 25°C. The incubated soils were periodically collected and subjected to DNA extraction followed by CsCl density gradient centrifugation in order to separate ^{13}C -labeled DNA from total DNA. The incubation was continued until a band of ^{13}C -labeled DNA could be visualized below the unlabeled DNA in the centrifuge tube after the ultracentrifugation. The shortest incubation period necessary for visualization of ^{13}C -labeled DNA was 27 d for the flooded soil and 5 d for the drained soil. PCR-DGGE showed different band patterns between unlabeled DNA and ^{13}C -labeled DNA within each soil type, and between ^{13}C -labeled DNA in the flooded and drained soils (Fig. 1). Seven major bands of ^{13}C -labeled DNA recovered from flooded and drained soils were closely related to known methanotrophic microorganisms.

Phylogenetic analysis of the 16S rRNA gene in ^{13}C -labeled DNA

To enable a more detailed phylogenetic analysis of active methanotrophs in the flooded and drained soils, 16S rRNA gene clone libraries were constructed from ^{13}C -labeled DNA. The phylogenetic analysis revealed a substantially different methanotrophic community structure between the flooded and drained soils (Fig. 2). In the flooded soil, almost all of the 16S rRNA gene sequences were affiliated with Type II methanotrophs closely related to the genera *Methylocystis* (52% of all the 16S rRNA gene sequences) and *Methylosinus* (43%). No sequences affiliated with Type I methanotrophs were detected within 42 randomly selected clones derived from the flooded soil. In contrast, the 16S rRNA gene sequences in the drained soil were affiliated with Type I methanotrophs closely related to the genera *Methylomonas* (17%), *Methylosarcina* (17%), and *Methylomicrobium* (2%) and with Type II methanotrophs closely related to the genus *Methylocystis* (9%). A large proportion of sequences in the drained soil were affiliated with non-methanotrophs (50%), which were also detected in the flooded soil but at a

Fig. 1. Denaturing gradient gel electrophoresis of unlabeled and ^{13}C -labeled DNA recovered from incubated soils of flooded and drained rice field. The predominant DGGE bands from the ^{13}C -labeled DNA were sequenced and the genus name was determined on the basis of the highest homology to a cultivated representative. Lane 1, marker; Lanes 2 and 4, unlabeled DNA; Lanes 3 and 5, ^{13}C -labeled DNA. Band 1, Y18946 *Methylosinus sporium* (98% identity); Band 2, AM285681 *Methylocystis heyerii* (97% identity); Band 3, AF150792 *Methylomonas* sp. LW13 (98% identity); Band 4, AF150807 *Methylomonas rubra* (96% identity); Band 5, AF150807 *Methylomonas rubra* (96% identity); Band 6, AY007296 *Methylosarcina lacus* (95% identity); Band 7, X72777 *Methylomicrobium album* (89% identity).

much lower frequency (5%). Some non-methanotrophs were closely related to the genera *Methylophilus* and *Hyphomicrobium*, which are able to grow on methanol (16, 24). The other non-methanotrophs were related to the genera *Nitrosolobus* and *Novosphingobium*. The genus *Nitrosolobus* has been reported as ammonia-oxidizing bacteria, which can oxidize methane and incorporate carbon dioxide into cell material (15), but the genus *Novosphingobium* has not been reported to grow on either methane or methanol.

Phylogenetic analysis of the *pmoA* gene in ^{13}C -labeled DNA

pmoA gene clone libraries were also constructed from ^{13}C -labeled DNA in order to investigate the active methanotrophic community structure in the flooded and drained soils (Fig. 3). The *pmoA* gene sequences in the flooded soil were affiliated with Type II methanotrophs closely related to the genus *Methylocystis* (31% of all the *pmoA* gene sequences) and with Type I methanotrophs closely related to the genera *Methylosarcina* (22%), *Methylomonas* (13%), and *Methylococcus* (3%). Phylogenetic analysis of the methanotrophic community in flooded soil showed a higher diversity than that in drained soil. This diversity resulted primarily from the presence of various Type I methanotrophs, represented by uncultured environmental *pmoA* clones (a total of 31%) that were classified into four groups (OTU1–OTU4 in Fig. 3). The *pmoA* gene sequences in the drained soil were affiliated with Type I methanotrophs closely related to the genera *Methylosarcina* (47%) and *Methylomonas* (38%) and with Type II methanotrophs closely related to the genus *Methylocystis* (3%). The remainder of the *pmoA* gene

Fig. 2. Phylogenetic neighbor-joining tree of the partial 16S rRNA gene sequences from two clone libraries representing the diversity of active bacteria (^{13}C -labeled DNA fractions) present in flooded and drained rice fields. Sequences derived from clone libraries are indicated by boldface type and begin with “FL” and “DR” to indicate the soil type (FL, flooded soil; DR, drained soil). The columns of numbers on the right indicate the proportion of each type of clone represented within each of the libraries. Reference sequences were downloaded from GenBank and are designated by their accession numbers. These reference sequences were also used to design methanotrophic primer sets for quantitative real-time PCR. The numbers at the branch points are bootstrap values derived from 1,000 replicates. The bar represents 2% sequence divergence.

sequences in the drained soil were closely related to uncultured environmental *pmoA* clones classified into the OTU3 group (12%). No sequences affiliated with the genus *Methylosinus* were found in either *pmoA* gene clone library, although they were abundant in the 16S rRNA gene clone library in the flooded soil.

Development of quantitative real-time PCR assays targeting the methanotrophic 16S rRNA genes identified in this study

To quantify the methanotrophic 16S rRNA genes in the

^{13}C -labeled DNA, we designed group-specific primer sets for Type I and Type II methanotrophs (See Materials and Methods). Primer specificity was tested by the sequencing and identification of PCR products amplified from ^{13}C -labeled DNA from the flooded and drained soils. Forty randomly selected sequences amplified using the Type I primer set (Type I56F and Type I239R) were affiliated with the family *Methylococcaceae*, classified into the Type I methanotrophs (Table 1). Similarly, 40 randomly selected sequences amplified using the Type II primer set (Type

Fig. 3. Phylogenetic neighbor-joining tree of the partial PmoA amino acid sequences from two clone libraries representing the diversity of active methanotrophs (¹³C-labeled DNA fractions) present in flooded and drained rice fields. Sequences derived from clone libraries are indicated by bold-face type and begin with “FL” and “DR” to indicate the soil type (FL, flooded soil, DR, drained soil). The columns of numbers on the right indicate the proportion of each type of clone represented within each of the libraries. Reference sequences were downloaded from GenBank and are named by their accession numbers. The numbers at the branch points are bootstrap values derived from 1,000 replicates. The bar represents 2% sequence divergence.

II432F and Type II659R) were affiliated with the family *Methylocystaceae*, classified into the Type II methanotrophs. These results indicated that each primer set was specific for either Type I or Type II methanotrophs and was able to detect a diversity of methanotrophs within that family. In contrast, no non-methanotrophic sequences were detected in

either of the 16S rRNA gene clone libraries constructed with the group-specific primer sets. The 16S rRNA gene-targeted real-time PCR assays were experimentally optimized for primer concentrations and PCR conditions using the 16S rRNA gene fragment of *Methylosarcina lacus* and *Methylocystis* sp. strain M as

Table 1. Composition of clone libraries constructed from PCR products amplified using primer sets specific to Type I and Type II methanotrophs.

Affiliation ^a	No. of detected sequences			
	Type I primer set		Type II primer set	
	Flooded	Drained	Flooded	Drained
<i>Methylococcus</i>	2	2	0	0
<i>Methylobacterium</i>	11	1	0	0
<i>Methylobacter</i>	4	10	0	0
<i>Methylomonas</i>	3	6	0	0
<i>Methylosarcina</i>	0	1	0	0
Betaproteobacterial methyloproteobacteria	0	0	0	0
<i>Methylosinus</i>	0	0	8	5
<i>Methylocystis</i>	0	0	10	15
<i>Methylocella</i>	0	0	2	0
Alphaproteobacterial methyloproteobacteria	0	0	0	0
Others	0	0	0	0

^a Determined on the basis of the highest homology to a cultivated representative.

positive control DNA of Type I and Type II methanotrophs, respectively. The total copy numbers of bacterial 16S rRNA genes were estimated using 357f and 518r, a bacterial primer set (30). The standard curves showed good linearity ($R=0.99$) for up to 10^3 copies per reaction in the assays for all bacteria and for up to 10^2 copies per reaction in the assays for Type I and Type II methanotrophs.

Quantification of active methanotrophs in the flooded and drained rice fields

Active Type I and Type II methanotrophic populations in the flooded and drained soils were measured by the 16S rRNA gene-targeted real-time PCR assays using ^{13}C -labeled DNA as templates. The real-time PCR assays of flooded soil showed that the relative abundance of copy numbers of Type II methanotrophic 16S rRNA genes accounted for 69% ($[6.9\pm0.1]\times10^4$ copies per ng of ^{13}C -labeled DNA) of total bacterial 16S rRNA gene copy numbers ($[1.0\pm0.1]\times10^5$ copies per ng of ^{13}C -labeled DNA) (Fig. 4A), whereas the relative abundance of copy numbers of Type I methanotrophic 16S rRNA genes accounted for only 9% ($[9.3\pm0.0]\times10^3$ copies per ng of ^{13}C -labeled DNA) of the total. In contrast, the real-time PCR assays of drained soil showed that the relative abundance of copy numbers of Type I methanotrophic 16S rRNA genes accounted for 56% ($[3.8\pm0.0]\times10^5$ copies per ng of ^{13}C -labeled DNA) of the total ($[6.8\pm0.1]\times10^5$ copies per ng of ^{13}C -labeled DNA), whereas the relative abundance of copy numbers of Type II methanotrophic 16S rRNA genes made up 13% ($[8.5\pm0.4]\times10^4$ copies per ng of ^{13}C -labeled DNA) of the total. The remaining copy numbers, representing non-methanotrophic bacteria, accounted for 24% and 30% of total bacterial 16S rRNA gene copy numbers in the flooded and drained soils, respectively.

The *pmoA* gene copy numbers in ^{13}C -labeled DNA were measured by real-time PCR assays developed by Kolb *et al.* (25). The relative abundance of copy numbers of Type II

Fig. 4. Relative abundance of Type I and Type II methanotrophs in flooded and drained soils, estimated by the 16S rRNA gene-targeted real-time PCR assay (A) and *pmoA*-targeted real-time PCR assay (B). In the 16S rRNA gene assay, the abundance of non-methanotrophs was calculated by subtracting Type I and Type II methanotrophic copy numbers from the total bacterial copy number.

methanotrophic *pmoA* genes accounted for 99% ($[8.7\pm0.7]\times10^4$ copies per ng of ^{13}C -labeled DNA) of the total copy numbers of *pmoA* genes detected in the flooded soil, but only about half ($[6.1\pm0.4]\times10^4$ copies per ng of ^{13}C -labeled DNA) of the total copy numbers in the drained soil (Fig. 4B). The copy numbers of Type I methanotrophic *pmoA* genes were $(1.2\pm0.0)\times10^3$ and $(5.9\pm0.0)\times10^4$ copies per ng of ^{13}C -labeled DNA in flooded and drained soils, respectively.

Discussion

Previous studies have shown that methanotrophic community structure depends on CH_4 and O_2 concentrations (1, 18, 19). Methanotrophic communities in rice fields have often been studied during flooded seasons, so the ecotypes under those conditions are well understood. However, the environmental conditions in flooded soil differ markedly from those in drained soil. Although these differences would be expected to affect the methanotrophic community structure, few studies have addressed this issue. Therefore, we compared the active methanotrophic populations in flooded and drained soils by DNA-SIP. Although DNA-SIP is a powerful tool for assessing active microorganisms, a possible limitation to this approach is that microbial diversity and populations might change during long SIP incubation periods (31). To address that concern, we incubated soil samples with the stable isotope under water-supplemented conditions to reflect the *in situ* methanotrophic community structure as closely as possible (5). In addition, periodic sampling was conducted to minimize the incubation period. The flooded soil required a longer incubation period than the drained soil, implying that the methanotrophs in the two soils had different assimilation capacities for $^{13}\text{CH}_4$. Type I methanotrophs have a more efficient carbon assimilation pathway than Type II methanotrophs, resulting in higher cell yields when grown

on methane (9, 13). Indeed, the PCR-DGGE and band sequencing showed that ^{13}C -labeled DNA from flooded and drained soils contained different methanotrophic 16S rRNA genes (Fig. 1). A detailed analysis of phylogenetic diversity using the 16S rRNA gene clone library showed that the active methanotrophs in the flooded soil were mainly of Type II, whereas the drained soil contained mainly Type I methanotrophs (Fig. 2). The same degree of diversity was also observed in the *pmoA* gene clone libraries from each soil type. In addition, some uncultured Type I methanotrophic *pmoA* sequences were detected in both flooded and drained soils. This may mean that these uncultured methanotrophs are important parts of active methanotrophic populations throughout the year.

It should be noted that the genus *Methylosinus* was not found in either *pmoA* gene clone library, despite its abundance in the 16S rRNA gene clone library from flooded soil. Previous studies have also reported differing results for the 16S rRNA gene compared with the *pmoA* gene. These differences are considered to be caused by bias in PCR performed with different primer sets (5). Thus, a phylogenetic analysis using either the 16S rRNA gene or the *pmoA* gene alone may overlook important information about methanotrophic community structure.

The analysis of phylogenetic diversity with the 16S rRNA gene clone libraries revealed the presence of non-methanotrophs in both soil types, which would have resulted from cross-feeding via methanol and other metabolites produced by primary consumers of methane (27). For the quantitative analysis of methanotrophs, we developed group-specific primer sets for Type I and Type II methanotrophs instead of using the known methylotrophic primer set 10 γ and 9 α (14, 36). Our group-specific primer sets enabled us to assess active methanotrophic populations without the overestimation caused by the amplification of non-methanotrophs, even in the 16S rRNA gene-targeting assay (Table 1). Note that these primer sets are specific for the methanotrophic community at our study site because they were designed on the basis of the 16S rRNA gene clone libraries from this site. Indeed, the probe match program in RDP showed that some methanotrophs were not targeted by these primer sets (data not shown). The quantitative real-time PCR assays using these 16S rRNA gene-targeting primer sets showed that the flooded soil was dominated by Type II methanotrophs, whereas the drained soil was dominated by Type I methanotrophs (Fig. 4A). This observation is consistent with the result of a previous study of methanotrophs in rice soils (28). The quantitative analysis of the *pmoA* gene appeared to underestimate the copy numbers of the Type I methanotrophic *pmoA* gene compared with the clone frequencies in *pmoA* gene clone libraries. This seems to be due to the presence of uncultured environmental *pmoA* genes that are not targeted by MBAC and MCOC assays. However, the quantitative analysis also revealed the transition of the active methanotrophic population between the flooded and drained soils. These results indicate quantitatively that the methanotrophic community structure in rice fields is altered by agricultural seasons.

The preference of methanotrophs for CH_4 and O_2 has been studied extensively in laboratory cultures (13), rice fields

(19), and landfills (5). Generally, Type I methanotrophs outcompete Type II methanotrophs in high- O_2 and nutrient-rich environments, whereas Type II methanotrophs tend to dominate when CH_4 availability is high (28). Flooded soils contain high levels of CH_4 because vigorous methanogenesis occurs in anaerobic areas of the soil (22). On the other hand, drained soil is supplied with O_2 from the atmosphere and will be also supplied with nitrogen from postharvest straw. Methane-oxidizing activities and total methanotrophic populations of the flooded and drained soils were not estimated in this study. However, previous studies of rice soils indicated that the total methanotrophic population increased during a flooded period and decreased during a drained period (28). Thus, it is likely that methane produced during a flooded season is actively oxidized mainly by Type II methanotrophs. Meanwhile, a drained season provides Type I methanotrophs with optimum conditions for methane oxidation, and Type I methanotrophs seem to dominate through a reduction in the size of the Type II methanotrophic population. The drained soil appears to serve as a reservoir for Type II methanotrophs that survive as resting cells until the next flooding (11). Interestingly, some uncultured Type I methanotrophs (OTU1, -2 and -4 in Fig. 3) were detected only in the *pmoA* gene clone libraries from flooded soil. This may suggest that these uncultured Type I methanotrophs exceptionally favor high- CH_4 and low- O_2 conditions. It seems that these methanotrophs are also important for the oxidation of methane in rice fields, and they will be investigated in further studies.

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