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ORIGINAL PAPER

Stable isotope probing of active methane oxidizers in rice field soils from cold regions

Nasrin Sultana[1,2] · Jun Zhao[1] · Yan Zheng[3] · Yuanfeng Cai[1] · Muhammad Faheem[1,2] · Xianlong Peng[4] · Weidong Wang[5] · Zhongjun Jia[1]

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Abstract

DNA-based stable isotope probing (DNA-SIP) was employed to establish direct link between methane oxidation activity and the taxonomic identity of active methanotrophs in three rice field soils from Jian-San-Jiang (one baijiang origin soil, JB and one meadow origin soil, JM) and Qing-An (meadow origin soil, QA) districts in Northeastern China. Following microcosm incubation under 1% *v*/*v* $^{13}CH_4$ condition, soil organic ^{13}C atom percent significantly increased from background 1.08 to 1.21% in average, indicating the biomass synthesis supported by methanotrophy. Real-time PCR analysis of methanotroph-specific biomarker *pmoA* genes of the buoyant density for DNA gradient, following the ultracentrifugation of the total DNA extracted from SIP microcosms, indicated an enrichment of methanotroph genomes in ^{13}C-labeled DNA. It suggested propagation of microbial methane oxidizers in soils. High-throughput sequencing of 16S rRNA and *pmoA* genes from ^{13}C-labeled DNA further revealed a diverse guild of both type I and II methanotrophs in all three soils. Specifically, *Methylobacter*-affiliated type I methanotrophs dominated the methanotrophic activity in JB and JM soils, whereas *Methylocystis*-affiliated type II methanotrophs dominated QA soil. This implied the physiological diversification of soil methanotrophs that might be due to constant environmental fluctuations in paddies.

Keywords Methanotrophs · Cold-temperate paddy soil · DNA-based stable isotope probing · High-throughput sequencing

Introduction

The submerged wetlands are considered as a major source for methane emission, which contribute approximately 40% of the global methane emission (IPCC 2013). Remarkably, estimates have indicated that more than 80% of the methane gas produced from wetlands could have already been consumed by active methanotrophy at the aerobic-anaerobic interfaces into the same environment (Conrad and Rothfuss 1991; Frenzel et al. 1992). Therefore, the wetlands can also be seen as a dynamic system for vast methane consumption and support microbial guilds with high methanotrophic activities. Rice fields represent one of the most important anthropogenic wetland ecosystems which usually experience more frequent fluctuations in environmental conditions due to agricultural management, including periodic flooding, crop plantations,

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✉ Jun Zhao
zhaojun@issas.ac.cn; jun.zhao1215@hotmail.com

✉ Zhongjun Jia
jia@issas.ac.cn

[1] State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing, 210008 Jiangsu Province, People's Republic of China

[2] University of Chinese Academy of Sciences, Beijing 100049, China

[3] School of Food and Bioengineering, Zhengzhou University of Light Industry, Zhengzhou 450002, Henan Province, China

[4] College of Resources and Environment, Northeast Agricultural University, Harbin 150030, China

[5] Heilongjiang Provincial Key Laboratory of Environmental Microbiology and Recycling of Argo-Waste in Cold Region, College of Life Science and Biotechnology, Heilongjiang Bayi Agricultural University, Daqing 163319, China

and intensive fertilization. In view of these, it provides a unique model system differing from natural wetlands for studying many important biogeochemical processes, including methanotrophy, and the responsible microorganisms. China is the largest producer of rice worldwide, and the rice yield accounts for 43.7% of the total national grain production (Zhang et al. 2014). The increasing demand for rice production has accelerated the expansion of rice paddy fields in the cold northeastern area. As methane production is a temperature-sensitive process, the relatively long period of off-rice winter would potentially lead to higher CH_4 production in warm rice-growing seasons in this area than subtropical and tropical areas (Huang et al. 2015; Tang et al. 2016). Yet, the methane fluxes and responsible organisms in these cold-temperate regions have long escaped the scientific attention.

Methanotrophs (also known as methane-oxidizing bacteria (MOB)) are a unique group of bacteria growing on methane as the sole source of carbon and energy through the enzyme methane monooxygenase (MMO). They can be broadly divided into type I (*Gamma-Proteobacteria*) and type II (*Alpha-Proteobacteria*) groups, which display a distant phylogenetic relationship and distinct physiologies (Hanson and Hanson 1996). In natural wetland and lake systems, the methane oxidation can be predominated by either type I or type II methanotrophs, or co-dominated by both types (Chen et al. 2008a, 2008b; Dedysh 2009; Esson et al. 2016; Gupta et al. 2012). In paddy soils, the methane in the oxic-anoxic interface of the soil can reach as high as 50,000 ppmv (Eller and Frenzel 2001; Nouchi et al. 1990, 1994), which can support strong methanotrophic activity and growth. However, the compositions of active methanotrophs appeared less diverse, as accumulating evidence has inclined that the major contributors were type I rather than type II methanotrophs to oxidize methane at high concentrations in most paddy soils tested (Ma et al. 2013; Qiu et al. 2008; Reim et al. 2012; Shrestha et al. 2008). Nevertheless, this was only generated recently from a few numbers of studies on subtropical areas, and an extended investigation into microbes in less studied geographic regions is requisite to better understand the niche differentiation of active methanotrophs in the rice field wetland habitats. The rice fields in Northeast China face a relatively long period of frozen state and were derived from various origins, which potentially favor community compositions and population dynamics differing from those in the subtropical soils.

In this study, DNA-based stable isotope probing (DNA-SIP) technique was applied to establish a direct link between methane oxidation and the taxonomic identity of active methanotrophs in response to a simulated high methane content (10,000 ppmv) from rice field soil samples. By feeding soils with ^{13}C-labeled methane, we aimed to compare the methane oxidization and C assimilation potentials in three differently originated rice fields in Northeast China, and to further reveal the community assembly of active methanotrophs.

Materials and methods

Site description and soil sampling

Three soil samples were collected from three rice fields in Northeastern China. Two were from Jian-San-Jiang city, one classified as "baijiang" soil (JB, 133° 1′ 3″ E, 47° 2′ 48″ N) with white subsurface layer and the other one derived from meadow soil (JM, 127° 1′ 39″ E, 47° 2′ 29″ N). The third sample came from Qing-An (QA, 127° 40′ 45″ E, 46° 57′ 28″ N) which was also derived from meadow soil. These field sites have a cold-temperate continental monsoon climate, with average annual temperature of 1–2 °C. These rice fields usually experience a relatively long period of frozen state from early October till early April. During growing season, the fields usually received urea fertilization at approximately 150 kg N ha^{-1}. The soil samples were collected in harvest season from 0 to 20-cm depth by mixing three random cores. Soil samples were homogenized by passing through a 2-mm meshed sieve and stored at 4 °C before construction of microcosms. Soil properties were determined as previously described (Wang et al. 2015), including soil pH, organic matter, organic C, total N, and exchangeable NH_4^+-N and NO_2^- + NO_3^--N. Soil methane production rates were measured as described in supplementary materials.

DNA-SIP microcosm

Microcosms were performed with three treatments in triplicate including "control" (under atmosphere condition), "high $^{12}CH_4$" (incubated with 1% *v*/*v* $^{12}CH_4$), and "high $^{13}CH_4$" (1% *v*/*v* $^{13}CH_4$). For each microcosm, 6.0 g dry weight soil was incubated at approximately 60% maximum water-holding capacity and 28 °C in the dark in a 120-ml serum bottle sealed with a butyl stopper (Zhao et al. 2015). For high $^{12}CH_4$ and high $^{13}CH_4$ treatments, 1.2 ml of the headspace air in the bottles was replaced by the same volume of $^{12}CH_4$ and $^{13}CH_4$ gas, respectively, to make an initial methane mixing ratio at approximately 1% (equivalent to 10,000 ppmv) in the headspace (Cai et al. 2016; Esson et al. 2016). The $^{13}CH_4$ was > 99% ^{13}C-atom pure (Cambridge Isotope Laboratories, USA). The headspace CH_4 concentrations were measured on a daily basis by gas chromatography (Shimadzu GC12-A, Japan). Soil incubation ended when more than 90% of CH_4 was consumed (when the concentration dropped below 1000 ppmv), and soil samples were immediately collected and frozen at − 20 °C. The relative ^{13}C-atom abundance and total organic C of soil samples after microcosm incubation were then assessed by a Flash 2000 elemental analyzer coupled to a Delta V Advantage isotope ratio mass spectrometer (Thermo Scientific, USA) using approximately 1 g of vacuum freeze-dried soil.

DNA extraction and SIP gradient fractionation

DNA was extracted from 0.5 g soil of each microcosm using the FastDNA spin kit for soil (MP Biomedicals, USA), according to the manufacturer's instructions. Quality and quantity of soil DNA were assayed using a NanoDrop ND-1000 UV-visible light spectrophotometer (NanoDrop Technologies, USA). For high $^{12}CH_4$ and high $^{13}CH_4$ treatments, density gradient centrifugation of total DNA was performed to separate the ^{13}C-labeled DNA from ^{12}C DNA as previously described (Jia and Conrad 2009; Xia et al. 2011). The fractionated DNA in the CsCl medium was precipitated by polyethylene glycol 6000 (PEG 6000) and dissolved in 30 μl sterile water for downstream analyses.

Real-time quantitative PCR of *pmoA* genes

Real-time quantitative PCR (qPCR) analysis of the methanotrophic biomarker *pmoA* genes was performed with total DNA to determine the abundances of MOB stimulated by the methane amendment on a CFX96 Optical Real-Time Detection System (Bio-Rad, USA). In addition, qPCR was also conducted across fractionated DNA gradients (fraction no. 2-14) to assess the efficiency of ^{13}C incorporation into the genomic DNA of MOB communities. The primer pair and thermal condition are detailed in supplementary Table S1. PCR reaction mixtures and the standards were used as previously described (Zheng et al. 2014). Amplification efficiencies ranged from 92 to 105%, with R^2 values of 0.996 to 0.999.

MiSeq sequencing of 16S rRNA genes

MiSeq sequencing of 16S rRNA genes were performed for the total DNA extracts, as well as DNA of the "heavy" CsCl fractions (buoyant density around 1.735 g ml^{-1}) retrieved from $^{13}CH_4$-amended microcosms as previously described (Cai et al. 2016). And the fractions with the same buoyant density from $^{12}CH_4$ microcosms were used to provide background information. The generated reads were then applied to key quality control steps to remove low-quality sequences (Vestergaard et al. 2017) using QIIME pipeline (Caporaso et al. 2010). A total of 1,123,367 sequences with quality score > 20 without mismatched primers and ambiguous bases were obtained (supplementary Tables S2 and S3) and subjected to taxonomic assignment by RDP MultiClassifier (Wang et al. 2007). The relative abundance of type Ia methanotrophs was calculated as the sum of sequences affiliated to *Methylobacter* and *Methylosarcina*. And type Ib and type II were represented by *Methylocaldum* and *Methylocystis* sequences, respectively. All MOB-affiliated 16S rRNA gene sequences were further recollected and clustered into OTU at 97% sequence similarity cutoff (Schloss et al. 2009). For ^{13}C-labeled 16S rRNA genes, representative sequences of dominant OTUs (containing ≥ 2% of MOB-like 16S rRNA gene sequences in at least one of the samples) were applied to phylogenetic analysis using the neighbor-joining method in MEGA 4.0 with bootstrapping of 1000 replicates (Tamura et al. 2007).

MiSeq sequencing of *pmoA* genes

The *pmoA* genes in the ^{13}C-DNA fractions were applied to MiSeq sequencing (Cai et al. 2016). The bioinformatic processing followed the key steps described previously (Schöler et al. 2017). The raw sequences were clustered and classified following quality control by multiple software programs as detailed previously (Cai et al. 2016). A total of 19,626 high-quality sequences were included for molecular analyses (supplementary Table S3). For the major *pmoA* OTUs (containing ≥ 4% of *pmoA* gene sequences in at least one of the samples), a representative sequence was selected for phylogenetic analysis by comparing with known sequences from GenBank. All the high-throughput sequencing reads of the *pmoA* and 16S rRNA genes were deposited in NCBI Sequence Read Archive (SRA) under the project accession number PRJNA482071.

Results

Physicochemical properties

The basic physicochemical properties tested were listed in supplementary Table S4. In brief, QA soil had the highest pH, organic matter, organic C, total N, and exchangeable NH_4^+ concentration compared to JB and JM soils. In addition, QA soil had the lowest methane production rate among all three soils under flooded condition (supplementary Fig. S1).

Methane oxidation and assimilation

All the paddy soils displayed strong activities of methane oxidation and consumed more than 90% of amended methane in 10, 15, and 18 days of incubations for JB, JM, and QA soils, respectively. Assuming linear kinetics, the methane oxidation rate was the highest in JB soil, followed by JM and QA soils (Fig. 1a). No significant difference was observed between $^{12}CH_4$ and $^{13}CH_4$ treatments (supplementary Fig. S2). The methanotrophy-supported assimilation of methane-derived C by microorganisms in all soils, as the soil ^{13}C-atom abundances were significantly increased from background 1.08% to an average value of 1.21% after $^{13}CH_4$-amended microcosm incubation (Fig. 1b). The average C conversion efficiency from methane to soil organic matter was estimated to be 36.9% during microcosm incubations, with a range between 14.3 and 58.3% (calculated in supplementary Table S5).

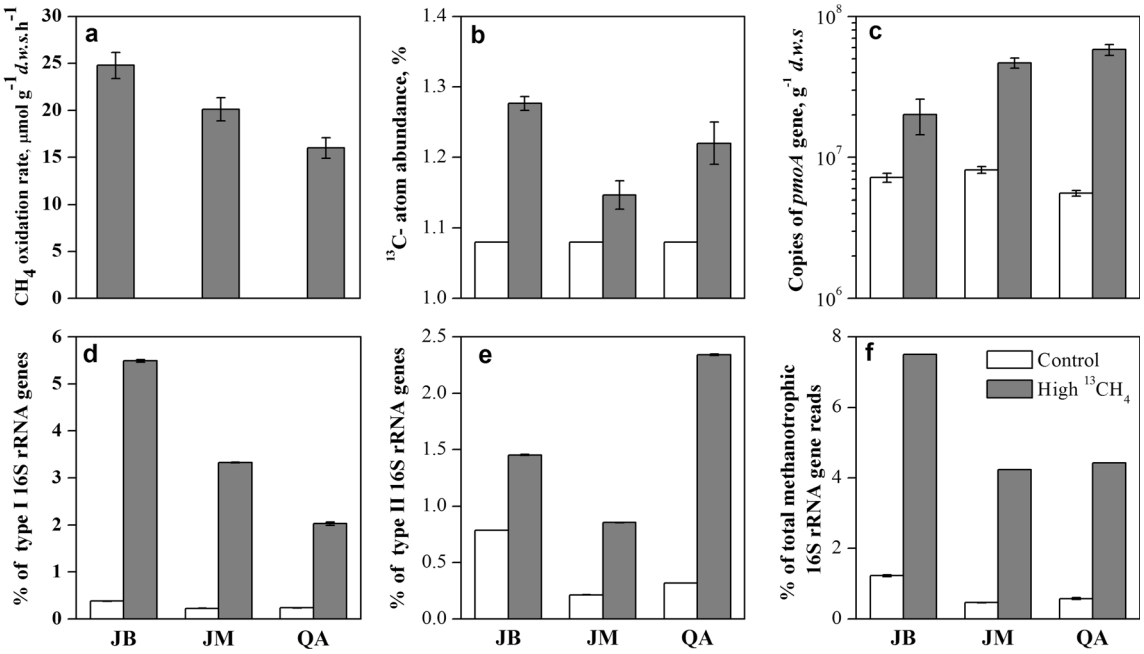

Fig. 1 Methane oxidation potentials, soil ^{13}C-atom abundances, community sizes, and relative abundances of methanotrophs following soil microcosms. **a** Methane oxidation potential was estimated as the linear rate of methane consumption by soils. **b** Soil ^{13}C-atom abundance was measured to assess methane assimilation in soil microcosms treated with 1% $^{13}CH_4$. **c** The *pmoA* gene copy numbers were estimated using real-time quantitative PCR. Illumina sequencing was performed at the whole microbial community level in microcosms targeting 16S rRNA genes, and the relative abundances of type I (**d**), type II (**e**), and total (**f**) methanotrophs are expressed as the ratio of affiliated gene reads to the total 16S rRNA gene reads in each microcosm. "Control" indicates a soil under natural atmosphere condition. "High $^{13}CH_4$" refers to soil microcosms incubated with 1% *v*/*v* $^{13}CH_4$. The error bars represent the standard errors of the triplicate microcosms

Methanotrophic abundances

Soil absolute methanotrophic abundances were assessed by real-time quantitative PCR of *pmoA* genes, which presented 2.81-, 5.72-, and 10.5-fold increases following the CH_4-amended microcosms in JB, JM, and QA soils, respectively (Fig. 1c). According to high-throughput sequencing of 16S rRNA genes, the percents of type I and type II MOB in the whole microbial community were calculated to estimate their relative abundances. The relative abundances of type I MOB increased by 14.4-, 14.6-, and 8.55-fold following the CH_4 amendment in JB, JM, and QA soils, respectively (Fig. 1d). And type II in JB, JM, and QA soils increased by 1.84-, 3.97-, and 7.41-fold, respectively (Fig. 1e). This led to increase in the relative abundance of total MOB-like genes (Fig. 1f).

SIP of methanotrophs

Following the isopycnic centrifugation of the DNA extracts, qPCR of *pmoA* genes indicated active cell propagation stimulated by ^{13}C assimilation in all three soils during methane oxidation. A peak shift of relative *pmoA* gene abundances toward heavy fractions (buoyant density around 1.735 g ml^{-1}) was clearly observed in all soil microcosms with $^{13}CH_4$ amendment, compared to corresponding $^{12}CH_4$ treatment in which the gene abundance peaked only in the "light" fractions (1.715 g ml^{-1}) (Fig. 2). The enrichment of ^{13}C-labeled MOB was further demonstrated by MiSeq sequencing of 16S rRNA genes, as the relative abundances of total MOB-like genes in heavy DNA fractions (1.735 g ml^{-1}) were greatly increased compared to the background values from the same fractions in $^{12}CH_4$ treatment (Fig. 2).

Composition of methanotrophs

The methane oxidation led to changes in compositions of methanotrophs by selection of distinct phylotypes in different soils (supplementary Fig. S3). Taxonomic analysis of ^{13}C-labeled methanotrophic 16S rRNA genes further revealed distinct proportions of active type I and II methane oxidizers (Fig. 3a). Specifically, in JB and JM soils, *Methylobacter*-like type Ia MOB were the most abundant methanotrophic phylotypes accounting for approximately 87.1% and 70.2% of the MOB-affiliated 16S rRNA genes, respectively (Fig. 3b). On the contrary, type II dominated the active methanotrophs in QA soil accounting for 99.3% of the MOB sequences (Fig. 3b). These were further confirmed by the ^{13}C-labeled *pmoA* genes. About 74.4% and 76.7% of ^{13}C-*pmoA* genes were related to *Methylobacter tundripaludum* in JB and JM soils, respectively, while in QA soil, *Methylocystis parvus*-like MOB comprised 51.8% of *pmoA* genes (Fig. 3b).

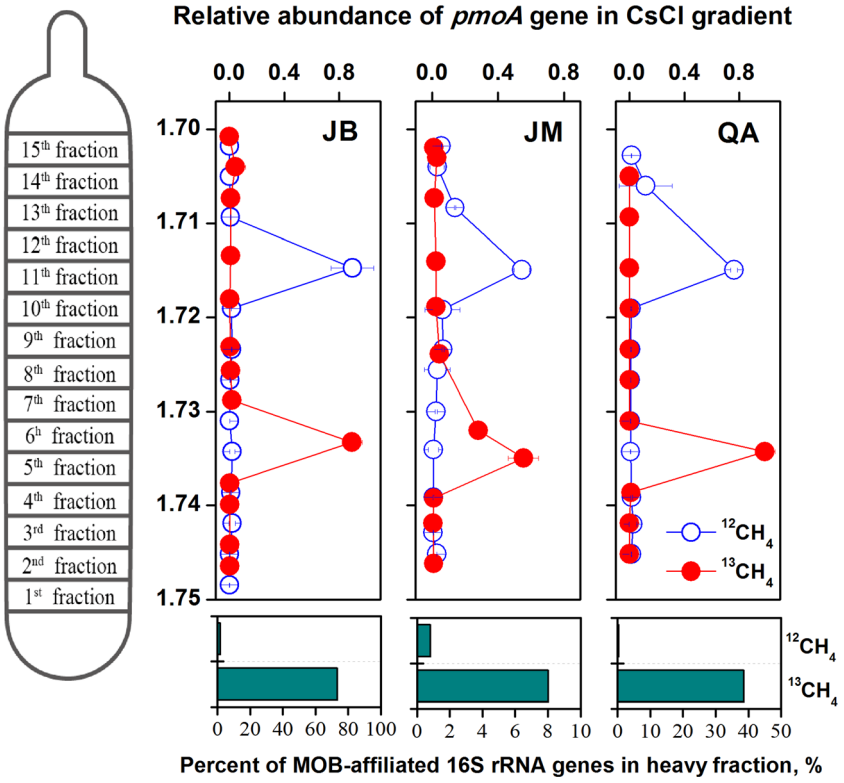

Fig. 2 The enrichment of ^{13}C-labeled methanotrophs based on qPCR of *pmoA* and sequencing of 16S rRNA genes following DNA-SIP microcosms. The relative gene abundance is the proportion of *pmoA* gene copy number in each fraction to the total abundance across the gradient. The columns beneath display the percent of methanotroph-affiliated reads in all 16S rRNA genes in the "heavy" fractions (buoyant density 1.735 g ml^{-1}) from $^{12}CH_4$- and $^{13}CH_4$-amended soil microcosms, respectively

Discussion

It is a well-known technical challenge to detect in situ activity of microbes. The constantly fluctuating environment in rice paddy fields such as temperature (Huang et al. 2015), moisture (oxygen) (Walkiewicz et al. 2018), and N level (Bodelier and Laanbroek 2004; Hahn et al. 2018) are all important factors that might influence the metabolic activities of methanotrophs. In this context, our lab microcosm cannot always reflect in situ field environments. However, our relatively favorable incubation condition indeed led to distinct compositions of active methanotrophs across different soils (two soils dominated by type I and one dominated by type II), indicating that soil intrinsic biotic and abiotic factors could have been a primary force in shaping active methanotrophic communities. In addition, the community composition of total bacteria was not disturbed by the microcosm condition (supplementary Fig. S4). Therefore, SIP microcosm might to some extent reveal the different potential activities by distinct methanotrophic groups, and our result might reflect what is largely occurring under in situ condition, particularly in regard to the relative activities of type I versus type II methanotrophs that should have been controlled by soil intrinsic factors.

The temperature variation from our soil collecting sites of Jian-San-Jiang and Qing-An can exceed more than 60 °C within 1 year, with lowest temperature below − 30 °C in the winter and highest temperature above 30 °C in the summer. Different thermal conditions might influence the relative activity of different phylotypes. Especially, we revealed the active contribution of *Methylobacter tundripaludum*-related type I lineages to the methane oxidation in all three soils. This strain was first isolated in permanently cold wetland environment (Wartiainen et al. 2006), and since then, the related lineages were found in many cold ecosystems (Graef et al. 2011; He et al. 2012). We speculate that this lineage could have played a more important role in methane oxidation under lower temperatures, although a further study should be conducted to monitor the community dynamics of this lineage in soils under varied thermal conditions.

The revelation of active type II methanotrophs from all three of our soils indicated that the actual methane oxidation activity in paddy fields was not restricted to type I methanotrophic groups as previous studies inclined. *Methylocystis*-like type II phylotypes even dominated the active methanotrophs in one of our soils. The dominance of methanotrophic activity by type II MOB at high methane concentration has been rarely reported in paddy soils (Shiau et al. 2018), although this group was often found to be numerically abundant under in situ conditions (Luke et al. 2014; Macalady et al. 2002; Mayumi et al. 2010; Zheng et al. 2014; Zheng et al. 2008). *Methylocystis* members of type II methanotrophs are capable of forming resting cells, surviving on multicarbon compounds, and using CH_4 at both high and low concentrations, which should make them especially important under frequent disturbances or unfavorable conditions (Ho et al. 2013).

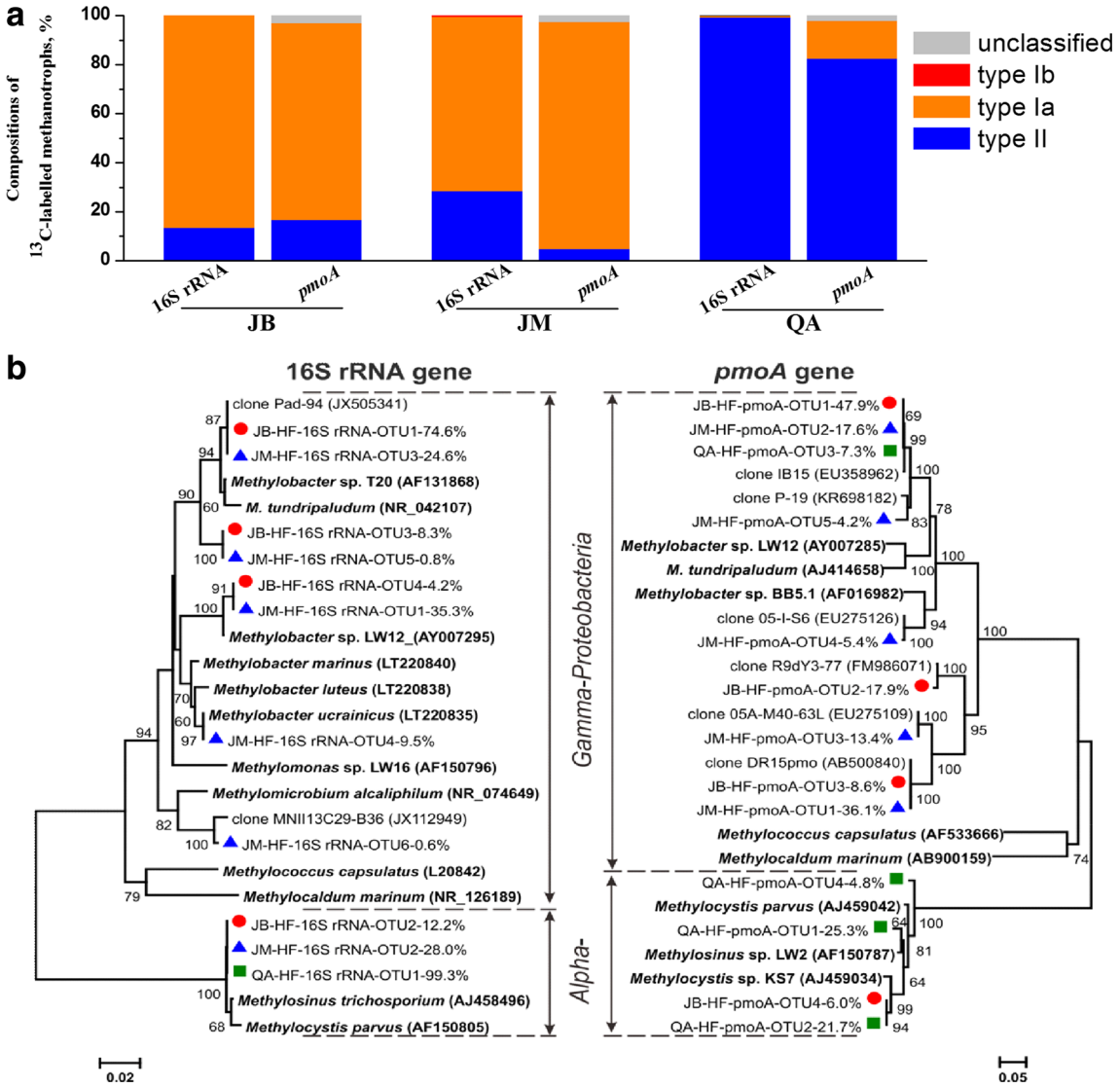

Fig. 3 The community compositions of ^{13}C-labeled methanotrophs based on phylogenetic analyses of 16S rRNA and *pmoA* genes from high-throughput sequencing. **a** The percentages of type Ia, Ib, and II methanotrophs were calculated from the ^{13}C-labeled genes following $^{13}CH_4$-amended microcosm incubation. **b** The neighbor-joining phylogenetic tree illustrates the phylogenetic relations of dominant OTU sequences with the known methanotrophic sequences from GenBank. The percentage number indicates the relative sequence abundance of each OTU to the total methanotroph-affiliated 16S rRNA or *pmoA* genes. Bootstrap values higher than 60% are indicated at the branch nodes

Type II methanotrophs dominated the methane oxidation only in QA soil. It is noteworthy that compared to the JM and JB soils, QA has the lowest methane production rate under our lab-simulated flooding condition (supplementary Fig. S1) despite its highest content of organic matter. This indicated that the methane production in this soil should be limited by the methanogenic potentials. The greater stimulation of type II than type I methanotrophs following our microcosm incubation could be a consequence of long-term adaptation to the relatively low methane availability in QA soil, which conforms to the previous finding that type II strains can grow under oligotrophic (low methane level) conditions (Knief and Dunfield 2005). Additionally, it is interesting that QA soil also had the highest N availability and pH value compared to the other two soils, which was inconsistent with previous observations that type II methanotrophs were commonly more competitive in N-limited conditions (Bodelier et al. 2000; Mohanty et al. 2006; Noll et al. 2008) and low pH environments (Chen et al. 2008a, 2008b; Dedysh 2009; Gupta et al. 2012; Kip et al. 2012). Therefore, these results from our study displayed physiological versatilities of methanotrophic phylotypes, implying that the life strategy of soil methane oxidizing communities might be more complex than previously appreciated.

In conclusion, the stable isotope probing suggested that the genomes of phylogenetically distinct methanotrophs were

labeled to different extents. Domination by type I or type II methanotrophic activities was revealed in the rice field soils from cold regions, which might be largely determined by soil intrinsic biotic and abiotic characteristics. Diverse phylotypes were demonstrated active in our soils including type I lineages closely related to the strain *Methylobacter tundripaludum* isolated from cold environment and *Methylocystis parvus*-related type II methanotrophs. This implied diversification of soil methanotrophs that might be important in coping with constantly changing environments in paddies.

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