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Comparative Analyses of Methanogenic and Methanotrophic Communities Between Two Different Water Regimes in Controlled Wetlands on the Qinghai-Tibetan Plateau, China

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Abstract

Wetlands are an important methane (CH_4) emission source. CH_4 is mainly produced during the biogeochemical process, in which methanogens and methanotrophs both play important roles. However, little is known how these two microbial communities change under different water regimes. In this study, the diversity and abundance of methanogens and methanotrophs in wetlands on Qinghai-Tibetan Plateau with different water contents (a high water content site DZ2-14-3 and a low water content site DZ2-14-4) were studied by using phylogenetic analysis and quantitative PCR based on *mcr*A gene and *pmo*A gene. A total of 16 methanogenic operational taxonomic units (OTUs) and 9 methanotrophic OTUs are obtained. For methanogens, Fen cluster (58.0%) and *Methanosaetaceae* (20.3%) are the dominant groups in high moisture samples, whereas *Methanosaetaceae* (32.4%), *Methanosarcinaceae* (29.4%), and *Methanobacteriaceae* (22.1%) are prevalent in low moisture samples. *Methylobacter* (90.0%) of type I methanotrophs are overwhelmingly dominant in high moisture samples, while *Methylocystis* (53.3%) and *Methylomonas* (42.2%) belonging to types II and I methanotrophs are the predominant groups in low moisture samples. Furthermore, qPCR analysis revealed that the abundance of methanogens and methanotrophs were higher in high moisture samples than that in low moisture samples. Overall, this comparative study between wetlands controlled by two different water regimes on the Qinghai-Tibetan Plateau provides fundamental data for further research on microbial functions within extreme ecosystems.

Introduction

Wetlands are a large source of atmospheric CH_4 and contribute 32% (~217 Tg) of global annual CH_4 emissions during the decade of the 2000s [19]. Permafrost wetlands, the most fragile ecosystem threatened by climate change [55], play a vital role in terrestrial carbon storage on the Qinghai-Tibetan Plateau [20], which lies at an altitude of 4000 m above sea level (ASL) and is the largest and highest plateau on Earth [8]. A previous study has shown that the average annual CH_4 emission from cold wetlands on the Qinghai-Tibetan Plateau is approximately 0.7–0.9 Tg [22], which is likely related to the abundant vegetation and the active microbial decomposition and transformation within wetlands. Therefore, microbial communities in wetland systems play an influential role in biogeochemical cycles and are crucial to the function of wetland systems [3].

Methanogens and methanotrophs are the major communities involved in CH_4 cycling. The diversity and community of methanogens and methanotrophs are affected by many environmental factors, which play a significant role in governing soil microorganisms and their activities [41], such as temperature [26], soil pH [23], plant species [25], soil depth [6], land use [58], and the water regime [47]. The moisture of the soil determines the wetland type and the plant communities thriving thereon, and fluctuations in the moisture can cause changes from a methanogenic environment to CH_4 oxidation environment, or vice versa [16]. Therefore, moisture is considered to have a considerable effect on CH_4

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emissions. Despite some investigations of the microbial communities in the wetlands have been conducted on the Qinghai-Tibetan Plateau [2, 8, 9, 17, 42, 51], differences between the methanogenic and methanotrophic microbial communities in the wetlands controlled by different water regimes have been mostly unexplored to date. Therefore, in this study, we elucidate the abundance and diversity of methanogens and methanotrophs between wetlands controlled by two different water regimes on the Qinghai-Tibetan Plateau using qPCR and phylogenetic analysis of *mcr*A and *pmo*A genes. CH_4 in permafrost soils is exclusively produced by anaerobic methanogens. *mcr*A gene is the functional gene encoding the α subunit of methyl-coenzyme M reductase, which catalyzes the final and key enzyme in methanogenesis [46], and *mcr*A has been widely used for phylogenetic analysis of methanogens [35, 54]. The particulate methane monooxygenase (pMMO) gene, which encodes a key enzyme involved in CH_4 oxidation [48] and Dumont and Murrell [10] found the *pmo*A gene to be a useful functional and phylogenetic marker for detecting methanotrophs.

Materials and Methods

Site Description and Sampling

In June 2014, two sites (DZ2-14-3 and DZ2-14-4) controlled by different water regimes were selected to collect samples on the Qinghai-Tibetan Plateau (N38°04′54.04″ and E99°09′15.96″; N38°05′01.97″ and E99°09′17.12″, respectively) at an elevation of 4060 m ASL (Fig. 1). Three replicates (~5 g each) were obtained aseptically and placed into sterilized centrifuge tubes. All the samples were transported on dry ice to laboratory and stored in a −80 °C freezer until further analysis.

Soil Characteristics

The CH_4 concentration was determined using gas chromatography (Agilent 7890A, US) and a granulometric analysis of sediments was conducted using a Mastersizer 2000 laser particle size analyzer. pH and moisture were measured as described by Wei et al. [51]. Total organic carbon (TOC) content of samples was determined according to Jiao et al. [21].

DNA Extraction and PCR Amplification

DNA was extracted using the Soil DNA Isolation Kit (MP) in accordance with the manufacturer's instructions, and the extracted DNA was quantified by Nanodrop (ND-1000). The DNA from each replicate was mixed in equal amounts to compose the DNA of each site. *mcr*A and *pmo*A genes sequences were amplified using the primer sets *mcr*AF/*mcr*AR [34] and 189f/661r [2], respectively. PCR amplification was performed in 50 μL of mixture (including 5 μL of Taq buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.25 μM of each primer 5 U Taq DNA polymerase (Invitrogen, USA), and 20 ng template DNA). PCR conditions were as follows: 95 °C for 4 min followed by 30 cycles for 1 min at 94 °C, 1 min at 55 °C for *mcr*A gene or 52 °C (for *pmo*A) and 2 min at 72 °C, and a final extension step of 10 min at 72 °C. PCR products were purified using a Gel Spin DNA purification kit (AxyPrep DNA Gel Extraction Kit, USA), then ligated into the pGEM-T Easy Vector and transformed into *Escherichia coli* JM109 competent cells. Clones were randomly selected and sequenced.

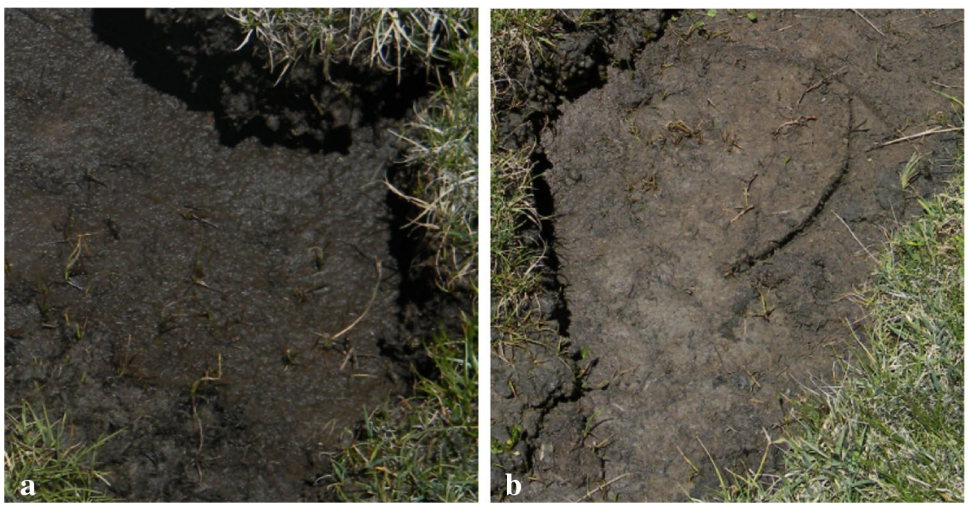

Fig. 1 Two sampling sites DZ2-14-3 (**a**) and DZ2-14-4 (**b**) on the Qinghai-Tibetan Plateau

Quantitative Polymerase Chain Reaction (qPCR)

The abundance of targeted genes were obtained using an ABI 7500 real-time PCR system (Applied Biosystems, USA). The qPCR primer pairs, ME2r/ME3mf [39] and 189f/661r [2], were used for amplifying *mcr*A and *pmo*A genes, respectively. The PCR reagent mixture contained 10 µL of SYBR Premix Ex Taq (TaKaRa, Japan), 1 µL of DNA, 10 pM of each primer, 10 pM ROX Reference Dye II, and the appropriate volume of ddH_2O (with a total volume of 20 µL for qPCR). The standards for *mcr*A and *pmo*A genes were prepared from *mcr*A and *pmo*A clones that were amplified using the primer sets of *mcr*AF/*mcr*AR and 189f/661r. Plasmid was extracted and serial diluted at 1:10 to generate a standard template and they were amplified to measure the threshold cycle (CT) for a known concentration with R^2 values greater than 0.99.

Phylogenetic Analyses

The sequences were determined using an ABI 3730 automated sequencer and the *mcr*A and *pmo*A genes clone libraries were constructed. Nucleotide sequences were assembled and edited using Sequencher v.4.1. OTUs were defined using a 14.3% cutoff value for *mcr*A and a 7% cutoff value for *pmo*A [2]. The coverage (C) was derived from the equation $C = [1 - (n/N)] \times 100$, where n is the number of clones that occurred only once and N is the total number of clones examined [37]. OTUs were determined by using the computer program DOTUR [45]. Phylogenetic trees were constructed by the neighbor-joining method using the maximum-parsimony algorithm in MEGA4 software with 1000 bootstrap replicates. The functional genes *mcr*A and *pmo*A sequences were screened and translated into correct amino acid sequences for further phylogenetic analyses using DNAman software. Nucleotide sequences were then deposited in the GenBank database, and the accession numbers were as follows: *mcr*A clones, KX609006-KX609021 and *pmo*A clones, KX609022-KX609030.

Results

Characterization of Permafrost Soil

Selected soil physiochemical properties including granulometric, pH, TOC, moisture, and CH_4 content of soil are shown in Table 1. Granulometric analysis reveals that throughout their stratigraphic profiles, the two samples DZ2-14-3 and DZ2-14-4 comprise sand ($32.5 \pm 0.2\%$, $9.6 \pm 0.3\%$), silt ($63.0 \pm 0.3\%$, $77.5 \pm 0.4\%$), and clay ($4.4 \pm 0.2\%$, $12.8 \pm 0.2\%$). pH is slightly acidic and ranges from 6.0 ± 0.1 to 5.6 ± 0.2 between DZ2-14-3 and DZ2-14-4; The soil TOC content is $12.0 \pm 0.1\%$ and $10.2 \pm 0.1\%$ at sites DZ2-14-3 and DZ2-14-4, respectively. The moisture is also higher in site DZ2-14-3 ($59.5 \pm 0.3\%$) than that in site DZ2-14-4 ($32.6 \pm 0.2\%$); the CH_4 content is 1286.7 ± 20.6 and 16.3 ± 1.2 ppm at sites DZ2-14-3 and DZ2-14-4, respectively.

Abundance of *mcr*A and *pmo*A Genes

qPCR results reveal that the *mcr*A and *pmo*A genes copies are more abundant at the high moisture site (DZ2-14-3) with $9.8 \pm 0.4 \times 10^8$ copies g^{-1} soil and $7.5 \pm 0.4 \times 10^7$ copies g^{-1} soil, respectively. In addition, the *mcr*A gene copies are 10 times higher at both sites than the *pmo*A genes.

Diversity of Methanogens Based on *mcr*A Gene in Both Sites

The structure of the methanogenic community revealed that 137 clones were selected and sequenced, and then assigned to 16 OTUs with coverages of 99.3% (Fig. 2). 69 sequences were obtained at the high moisture site (DZ2-14-3), Fen Cluster was the most abundant group and constituted approximately 58.0% of the clone sequences (Fig. 3). Other methanogen groups were also detected in the community phylogenetic analysis, including members of *Methanosaetaceae*, Rice cluster I, and *Methanobacteriaceae*, which comprised 20.3, 13.0, and 8.7% of the communities, respectively. 68 sequences were classified at the low moisture site (DZ2-14-4), which were clustered with the groups of *Methanosaetaceae* (32.4%), *Methanosarcinaceae* (29.4%),

Table 1 Physiochemical properties and gene abundance of soil samples at sites DZ2-14-3 and DZ2-14-4 (average ± SD)

Site	Sand (%)	Silt (%)	Clay (%)	pH	TOC (%)	Moisture (%)	CH_4 (ppm)	*mcr*A (10^8 copies g^{-1} soil)	*pmo*A (10^7 copies g^{-1} soil)
DZ2-14-3	32.5 ± 0.2	63.0 ± 0.3	4.4 ± 0.2	6.0 ± 0.1	12.0 ± 0.1	59.5 ± 0.3	1286.7 ± 20.6	9.8 ± 0.4	7.5 ± 0.4
DZ2-14-4	9.6 ± 0.3	77.5 ± 0.4	12.8 ± 0.2	5.6 ± 0.2	10.2 ± 0.1	32.6 ± 0.2	16.3 ± 1.2	2.3 ± 0.2	4.9 ± 0.3

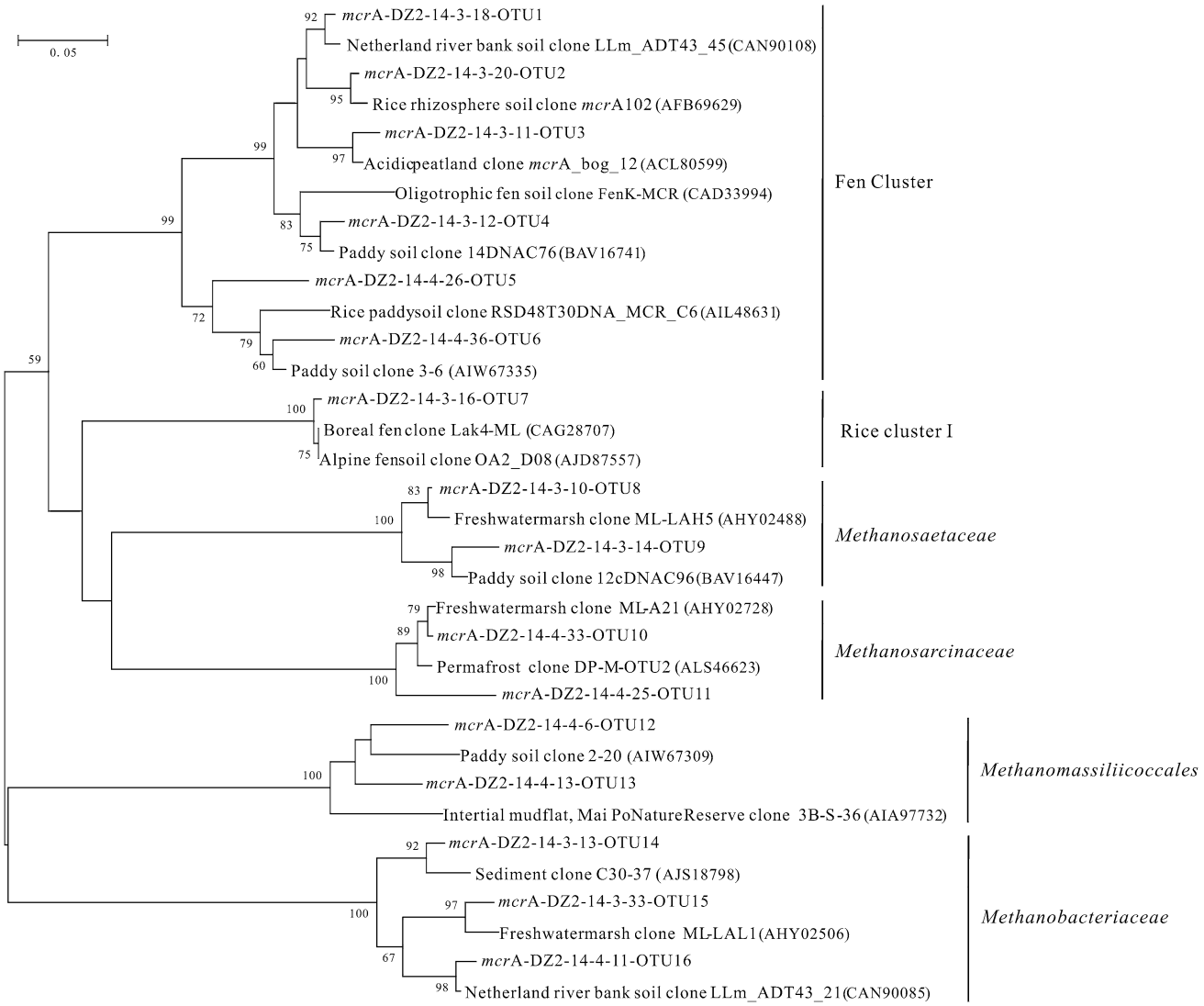

Fig. 2 Phylogenetic tree showing relationships of derived amino acid sequences encoded by *mcr*A genes to reference sequences from the GenBank

Methanobacteriaceae (22.1%), Fen Cluster (8.8%), and *Methanomassiliicoccales* (7.3%) (Fig. 3).

Diversity of Methanotrophs Based on *pmo*A Gene in Both Sites

A total of 85 sequences were obtained from both sites and affiliated to 9 OTUs with coverages of 96.5% (Fig. 4). 40 sequences in the clone library were identified at the high moisture site (DZ2-14-3), and the majority of the sequences (90.0%) belonged to *Methylobacter* of type Ia methanotrophs, and 7.5 and 2.5% of the sequences were related to *Methylomonas* and *Methylocystis*, respectively (Fig. 3). 45 sequences were analyzed at the low moisture site (DZ2-14-4). *Methylocystis* (53.3%) of type II methanotrophs and *Methylomonas* (42.2%) of type Ia were the dominant groups, and 4.5% sequences were identified as belonging to the genus *Methylococcus* (Fig. 3).

Discussion

Results showed evident differences between the two sites in terms of the properties of the soil and its biological characteristics. DZ2-14-3 site contained higher water content (59.5 ± 0.3 vs. 32.6 ± 0.2%), more sand (32.5 ± 0.2 vs. 9.6 ± 0.3%), and less clay (4.4 ± 0.2 vs. 12.8 ± 0.2%) relative to DZ2-14-4 site, indicating that soil at the DZ2-14-3 site is loose, multi-porous, and thus has a high capacity for water retention and a high moisture. Xiang et al. [53] indicated that a higher moisture can improve the activity of soil microorganisms. qPCR data showed that the abundance of *mcr*A

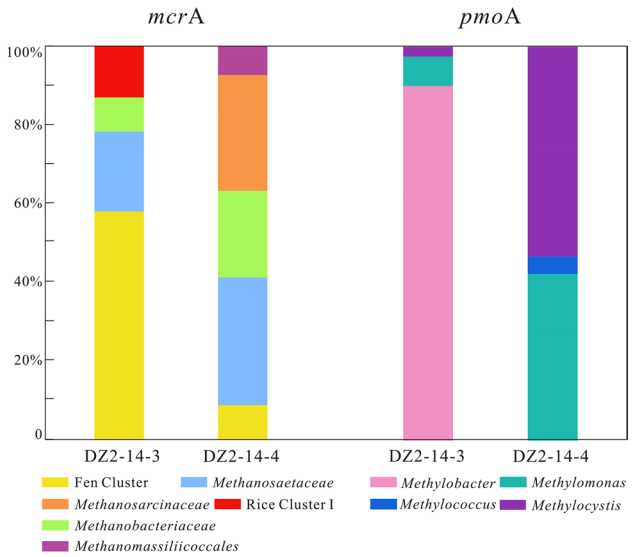

Fig. 3 Relative *mcr*A and *pmo*A genes abundance of methanogens and methanotrophs at the DZ2-14-3 and DZ2-14-4 sites based on gene clone libraries

and *pmo*A genes at the high moisture site (DZ2-14-3) were higher than that at the low moisture site (DZ2-14-4), which might indicate more active microbial community at the high moisture site (DZ2-14-3). The pH (6.0 ± 0.1 to 5.6 ± 0.2) of the soil was slightly acidic and similar to that measured at the other point in this area [30, 49].

However, there was a difference between the CH_4 content of the soil at both sites; the high moisture site (DZ2-14-3) had a higher CH_4 content than the low moisture site (DZ2-14-4). Previous research has shown that CH_4 emissions increase progressively along with an increase in moisture [11, 44]. The source and sink dynamics of CH_4 in soil are determined by the balance between CH_4 generated by methanogens and CH_4 loss caused by methanotrophic bacteria in the aerobic environment [4]. The principal influencing factor of CH_4 generation is the soil's anaerobic condition; in general, the deeper the soil layer the lower the redox potential, which provides favorable survival conditions for methanogens, leading to greater CH_4 production [13]. Additionally, the DZ2-14-3 site contains more moisture compared to the DZ2-14-4 site, and has poor breathability. Accordingly, the soil environment is anaerobic, which enables extensive breeding of methanogens within the soil. In addition, as methanotroph activity is limited because of a reduction in oxygen content, the flux of CH_4 emissions increases. The qPCR result shows that the gene abundance of *mcr*A is 10 times higher than *pmo*A gene. Meanwhile, Whalen et al. [52] found that CH_4 diffused more easily in soil with a reduced moisture content and provided a higher CH_4 oxidation rate. Despite the fact that the gene abundance of *mcr*A gene was higher than *pmo*A gene at the low moisture site, the difference was really smaller relative to that in the high moisture site (i.e., 10 times difference), which could explain that the soil moisture content might fail to meet the appropriate condition for CH_4 generation.

The *mcr*A clone library revealed that Fen Cluster (58.0%) were the dominant groups at the high moisture site (DZ2-14-3). Fen Cluster were a family level clade within the order

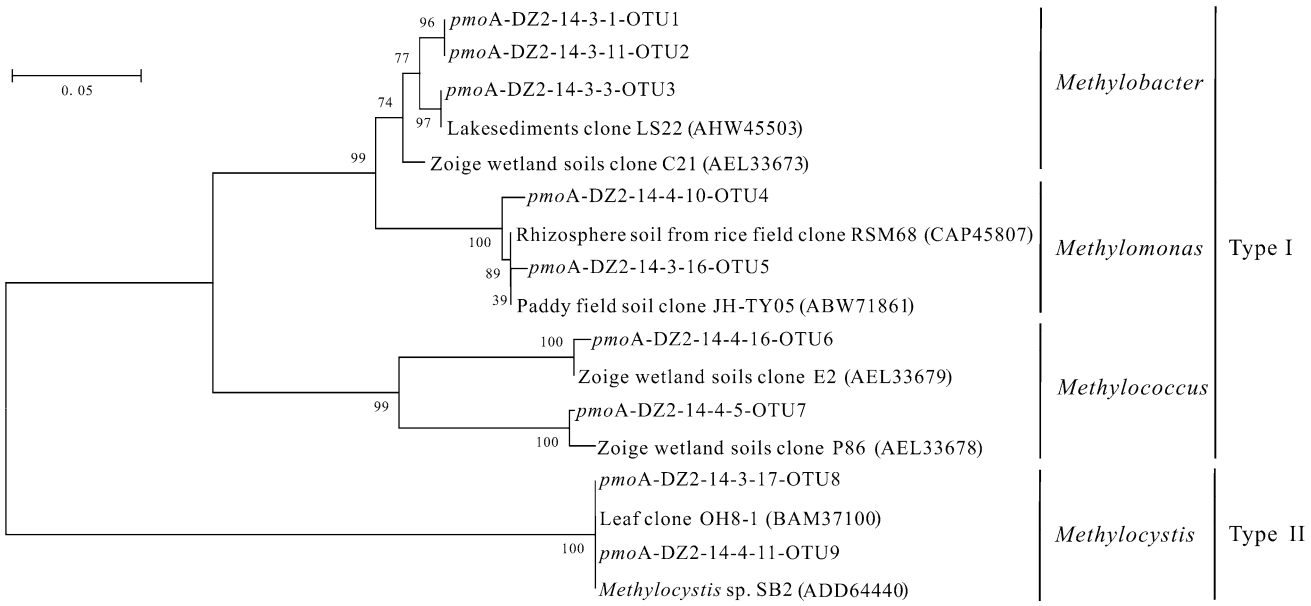

Fig. 4 Phylogenetic tree showing relationships of derived amino acid sequences encoded by *pmo*A genes to reference sequences from the GenBank

of *Methanomicrobiales* that had been widely detected where there were drastic fluctuations in the water table [54], or in acidic boreal peatlands [24] and in wetlands [31, 55]. Such results suggested that Fen Cluster methanogens were relatively tolerant to a changing water table and low pH, which agreed with previous findings determined from research on a boreal fen ecosystem [54], founding that Fen cluster characterized the dry end. *Methanosaetaceae* were found at the highest band percentages where acetate was the substrate [12], and Boone et al. [5] detected *Methanosaetaceae* utilizing only acetate. Furthermore, Peng et al. [40] suggested that *Methanosaetaceae* were usually predominant when the acetate concentration had already decreased to a lower steady state level of <0.2 mM. Our data showed that *Methanosaetaceae* occupied a higher percentage composition of methanogens (32.4%) at the low moisture site (DZ2-14-4), in accordance with the lower pH value measured at this site. Similarly, *Methanobacteriaceae* were distributed at both sites and frequently recovered on the Qinghai-Tibetan Plateau [8, 30, 51]; it proliferated preferentially when H_2 was not limited [7]. However, *Methanosarcinaceae* were only detected at the low moisture site (DZ2-14-4); it existed in extensive wetlands such as peatlands, freshwater marshes [32], and paddy soils [38] due to the existence of all three known pathways for methanogenesis [59]. In freshwater wetlands, *Methanosarcinaceae* were able to primarily utilize acetate, various methyl compounds, and hydrogen as methanogenic substrates [5, 15]. *Methanomassiliicoccus* were methyl-type methanogenic archaea; they presented unique metabolic characteristics, were different from general special methyl-type methanogenic archaea, and lacked the full pathway for reducing CO_2 into methyl-coenzyme M [28]. From a nutritional perspective, *Methanomassiliicoccus* had a mixed nutrition type [57]. Whether they played an important role in CH_4 production and emission was still unclear; however, their function was attracting increasing scientific attention. Rice cluster I were originally retrieved from rice roots [14] and had recently been isolated and shown to be CO_2 reducers that had a role in the inter species transfer of H_2 in rice paddies [43]. Watanabe et al. [50] showed that Rice cluster I members were predominantly retrieved from soil under drained conditions. In the present study, Rice cluster I comprised 13.0% of the communities at the high moisture site (DZ2-14-3).

Analysis of the methanotrophic community revealed that *Methylobacter* belonging to type Ia methanotrophs were the predominant groups at the high moisture site (DZ2-14-3) (90.0%). The group had frequently been detected on the Qinghai-Tibetan Plateau [56], and Lüke et al. [33] demonstrated that the genus *Methylobacter* affiliated with paddy soils was indicative of high CH_4 source strengths. These results were consistent with our study; the CH_4 content was higher at the high moisture site (DZ2-14-3). *Methylomonas*, type I methanotrophs, preferred an environment with low in CH_4 and high in O_2 [1], and Deng et al. [8] reported *Methylomonas* to be detected in soils with a low moisture content. In this study, *Methylomonas* had the high percentage composition (42.2%) of the methanogens at the low moisture site (DZ2-14-4) but were of low relative abundance at the high moisture site (DZ2-14-3) (7.5%). In addition, *Methylocystis* (53.3%) were the most dominant methanotrophic groups at the low moisture site (DZ2-14-4), but only a few sequences (2.5%) were detected at the high moisture site (DZ2-14-3). *Methylocystis* were ubiquitous methanotrophic inhabitants of many ecosystems [27] and had often been observed on the Qinghai-Tibetan Plateau, and Leng et al. [29] reported that CH_4 was the preferred substrate for *Methylocystis* in paddy soil. *Methylocystis* were more widely found in conditions with high O_2 and low CH_4, such as in upland agricultural soil and forestry soil [36]. Furthermore, Leng et al. [29] revealed that uncultivated *Methylocystis* species were facultative methanotrophs utilizing acetate as a secondary carbon source in the absence of CH_4 in paddy soil. This result was in accordance with the lower CH_4 content and pH value at the low moisture site (DZ2-14-4). In our study, 25 clones (29.4%) obtained from both sites had great similarities to the *Methylocystis* strain SB2 (ADD64440), Im et al. [18] demonstrated that strain SB2 was able to utilize not only CH_4 for growth, but also ethanol and acetate.

Conclusions

In this study, we found some fundamental differences in methanogenic and methanotrophic abundance and community composition between two wetland sites under different water regimes on the Qinghai-Tibetan Plateau. qPCR analysis revealed that the abundance of methanogens and methanotrophs was higher at the high moisture site (DZ2-14-3) than that at the low moisture site (DZ2-14-4). Similarly, the CH_4 content was higher at the high moisture site (DZ2-14-3). Cloning analyses of *mcr*A and *pmo*A genes revealed that Fen Cluster, *Methanosaetaceae, Methylobacter* were predominant at the high moisture site (DZ2-14-3), while *Methanosaetaceae, Methanosarcinaceae, Methanobacteriaceae, Methylocystis, Methylomonas* were the dominant groups at the low moisture site (DZ2-14-4). This comparative analysis study makes a contribution to improving scientific understanding of the microbial community structure in permafrost wetlands.

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