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ENVIRONMENTAL BIOTECHNOLOGY

Methanotrophic community abundance and composition in plateau soils with different plant species and plantation ways

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Abstract Aerobic methane-oxidizing bacteria (MOB) play an important role in mitigating the methane emission in soil ecosystems to the atmosphere. However, the impact of plant species and plantation ways on the distribution of MOB remains unclear. The present study investigated MOB abundance and structure in plateau soils with different plant species and plantation ways (natural and managed). Soils were collected from unmanaged wild grassland and naturally forested sites, and managed farmland and afforested sites. A large variation in MOB abundance and structure was found in these studied soils. In addition, both type I MOB (*Methylocaldum*) and type II MOB (*Methylocystis*) were detected in these soils, while type II MOB usually outnumbered type I MOB. The distribution of soil MOB community was found to be collectively regulated by plantation way, plant species, the altitude of sampling site, and soil properties.

Keywords Methane-oxidizing bacteria (MOB) · Soil · *Methylocaldum* · *Methylocystis*

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Introduction

Methane (CH_4), as one of the most important greenhouse gases, contributes approximately 20 % to the global warming (IPCC 2007). Natural environments are believed to contribute a significant proportion of the global emissions of CH_4 (Chowdhury and Dick 2013). Microbiologically mediated methane oxidation, which is carried out by aerobic methanotrophs (also methane-oxidizing bacteria (MOB)) that utilize CH_4 as carbon and energy sources (Hanson and Hanson 1996), plays a crucial role in mitigating CH_4 emissions produced in aquatic and terrestrial ecosystems to the atmosphere (Chowdhury and Dick 2013; Rahalkar et al. 2009). So far, the cultivated aerobic methanotrophs are known to be affiliated within phyla *Proteobacteria* and *Verrucomicrobia*, while verrucomicrobial MOB have been only found in extreme conditions in geothermal environments (Liu et al. 2015). The MOB species belonging to phylum *Proteobacteria* can be further classified into two main groups (type I and type II), based on their cell morphology and physiology (Hanson and Hanson 1996). Type I MOB are composed of microorganisms from a number of gammaproteobacterial genera adopting the ribulose monophosphate pathway for further catalyzing the intermediate formaldehyde, while type II MOB include microorganisms from several alphaproteobacterial genera assimilating the formaldehyde via the serine pathway (Deng et al. 2013; Liu et al. 2015; Zheng et al. 2008).

The initial step of aerobic methane oxidation in soil ecosystems is catalyzed by methane monooxygenase (MMO), and almost all of the known MOB species harbor the particulate MMO (pMMO) (Deng et al. 2013; Pan et al. 2014). The *pmoA* gene, encoding the β-subunit of pMMO, has been used as a preferential phylogenetic biomarker to detect the presence and distribution of MOB in a variety of soil ecosystems (Bissett et al. 2012; Dörr et al. 2010; Shrestha et al. 2012; Yang et al. 2014; Yun et al.

Table 1 Geographic and physicochemical features of soil samples

Sample	Latitude	Longitude	Altitude (m)	Vegetation type	pH	TN (g kg^{-1})	TP (g kg^{-1})	OM (g kg^{-1})	NO_3^--N (mg kg^{-1})	NH_4^+-N (mg kg^{-1})	C/N
VA	25° 02′ 08″	102° 51′ 20″	1904.6	*Brassica oleracea var. capitata*	5.23±0.13	1.69±0.03	3.15±0.13	19.90±0.43	18.30±0.09	5.56±0.03	11.78±0.04
VB	25° 02′ 17″	102° 51′ 36″	1911.9	*Eriobotrya japonica*	5.4±0.05	1.41±0.12	1.00±0.06	12.81±0.22	27.00±0.18	4.59±0.08	9.09±0.13
VC	25° 02′ 41″	102° 52′ 20″	1922.8	*Salix cavaleriei*	4.82±0.11	2.80±0.09	1.06±0.08	18.80±0.19	17.10±0.27	5.75±0.12	6.71±0.11
VD	25° 02′ 48″	102° 53′ 36″	1933	*Zea mays*	5.25±0.09	1.53±0.01	0.96±0.08	11.63±0.09	1.07±0.07	4.61±0.11	7.60±0.06
RA	25° 01′ 37″	102° 54′ 42″	2050.9	*Cryptomeria fortunei*	5.95±0.14	2.84±0.04	0.09±0.01	41.31±0.23	38.71±0.23	7.75±0.05	14.55±0.21
RB	25° 01′ 14″	102° 55′ 12″	2015.9	*Poa annua* L.	5.09±0.20	1.80±0.11	0.14±0.02	33.70±0.52	0.92±0.02	4.11±0.02	18.72±0.19
RC	25° 01′ 26″	102° 55′ 16″	1995.3	*Tripogon bromoides*	5.83±0.06	1.34±0.01	0.51±0.04	23.85±0.18	0.46±0.01	3.79±0.06	17.80±0.05

OM organic matter, *TN* total nitrogen, *C/N* ratio of OM to TN, *TP* total phosphorous

2014). The abundance and structure of soil MOB community can be influenced by a number of factors, such as land management (Abell et al. 2009), nitrogen fertilizer (Alam and Jia 2012; Dai et al. 2013), moisture (Shrestha et al. 2012), salinity (Bissett et al. 2012), and temperature (Martineau et al. 2010). It could be assumed that these multiple environmental factors might collectively regulate the MOB distribution in soil ecosystems. Hence, so far, the MOB distribution and its influential factors in natural soils ecosystems remain still unclear. In addition, information on the impact of plant species on soil MOB distribution is still very limited (Degelmann et al. 2010; Dörr et al. 2010), and the variation of MOB communities in soils with different plantation ways (natural and managed) remains elusive. Therefore, the main objective of this current study was to investigate the MOB abundance and structure in soils with different plant species and plantation ways.

Materials and methods

Sampling site description

Seven soil samples (0–5-cm depth) in triplicate were collected in Kunming City (in the Yunnan Plateau) in August 2014. Soil RA was obtained from naturally forested site while soils RB and RC from unmanaged wild grasslands (Fig. S1). The sampling sites of soil RA, RB, and RC were adjacent to a reservoir. Soils VA and VD were collected from managed farmland sites while soils VB and VC from afforested sites. The sampling sites of soil VA, VB, VC, and VD were adjacent to a village.

The sampling sites of these soils had different plant species and plantation ways (natural and managed), and the location and physicochemical characteristics of the soil samples are described in detail in Table 1.

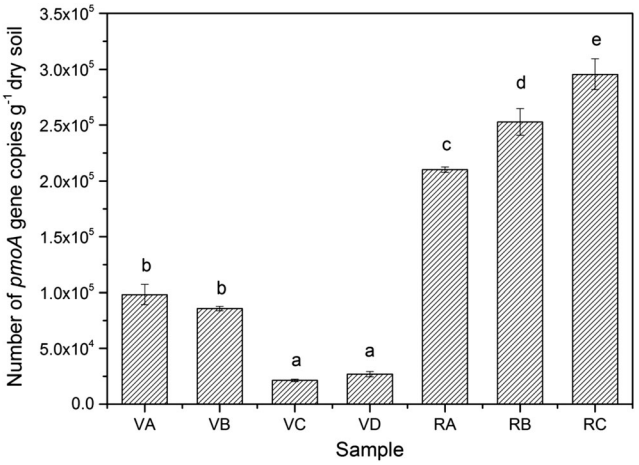

Fig. 1 Abundance of *pmoA* gene in different soils. *Different letters above the columns* indicate significant differences ($P<0.05$)

Table 2 Diversity of each soil MOB clone library

Soil	Number of clones	OTUs	Shannon index
VA	35	18	2.64
VB	30	20	2.79
VC	34	17	2.61
VD	33	23	3.04
RA	31	18	2.68
RB	32	17	2.52
RC	31	16	2.58

Molecular analyses

DNA was extracted from these seven soils, and the specific primer sets (A189F/Mb661R) targeting the *pmoA* gene of both types I and II MOB were applied for both quantitative PCR (qPCR) assay and clone library analysis, following the previously reported conditions (Liu et al. 2015; Yang et al. 2014). One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test was applied to determine the numerical difference ($P<0.05$) in the density of *pmoA* gene among different soils. Chimera-free sequences with ≥97 % similarity were grouped into operational taxonomic units (OTUs), and OTU-based Shannon diversity and rarefaction curve were generated using the MOTHUR program (Schloss et al. 2009). Phylogeny-based weighted UniFrac environmental clustering was used to discriminate the composition differences among soil MOB communities using the UniFrac program (Lozupone et al. 2006). Phylogenetic analysis of the representative *pmoA* gene sequences was conducted with the MEGA6 software using the neighbor-joining and maximum parsimony methods (Tamura et al. 2013). In addition, Pearson's correlation analysis using SPSS 20.0 software was used to discriminate the relationships between soil MOB community and the determined factors (altitude, organic matter (OM), total nitrogen (TN), ratio of OM to TN (C/N), NH_4^+-N, NO_3^--N, and total phosphorous (TP)). The links between soil MOB community composition and these factors were also identified using redundancy analysis (RDA) using CANOCO 4.5. The number of *pmoA* gene sequences in each OTU and the determined factors were used as species input and environmental input, respectively. The model of the microbe–environment relationships was selected based on the significance test of Monte Carlo permutations. The *pmoA* gene sequences obtained in the current study were deposited in the GenBank database under accession numbers KP903058–KP903283.

Results

MOB community abundance

A large variation in *pmoA* gene copy number was found in the seven studied soils, ranging from 2.12×10^4 to 2.95×10^5 copies per gram dry soil (Fig. 1). The soils from unmanaged wild grassland and naturally forested sites (soils RA, RB, and RC) showed a much higher MOB community size than those from managed farmland and afforested sites (soils VA,VB, VC, and VD) ($P<0.05$). A significant difference in the MOB community abundance was also found in soils RA, RB, and RC ($P<0.05$). In addition, soils VA and VB had a significantly higher MOB community size than soils VC and VD ($P<0.05$). However, no significant difference in MOB community abundance was found between soils VA and VB or between soils VC and VD ($P>0.05$).

MOB community diversity

In this study, a total of 226 *pmoA* gene sequences were obtained from the seven studied soils. Each soil MOB clone library was composed of 16–23 OTUs (Table 2). However, the curves for soil samples did not start to level off (Fig. S2), suggesting that further sequencing would have resulted in more OTUs. Soil VD

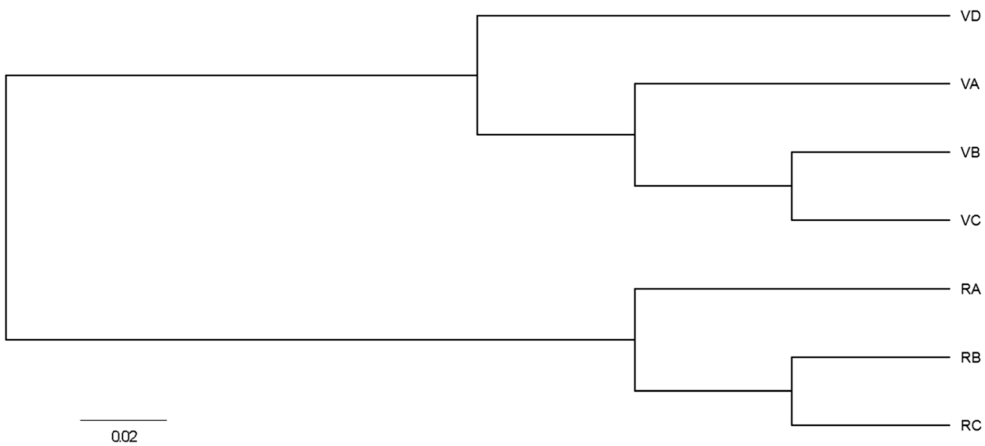

Fig. 2 Clustering of MOB clone libraries based on weighted UniFrac algorithm

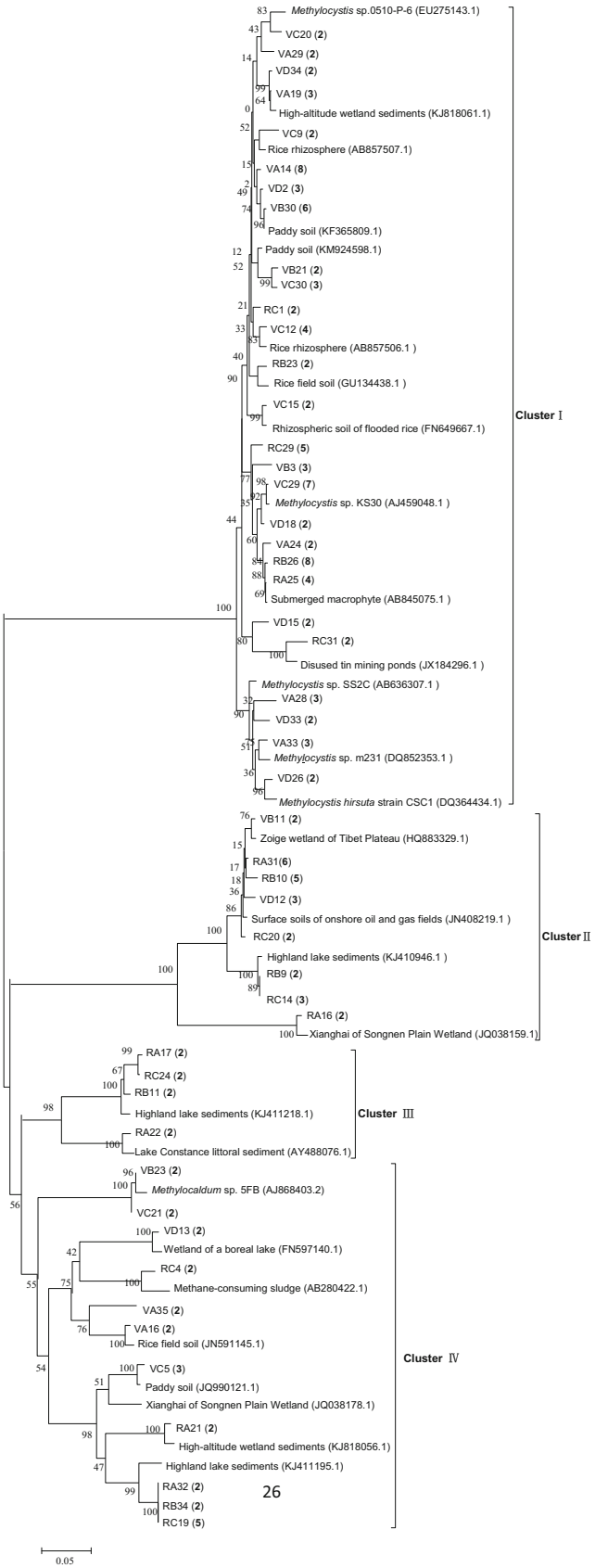

Fig. 3 Phylogenetic tree of representative *pmoA* gene sequences and reference sequences from GenBank. The obtained sequences beginning with "RA," "RB," "RC," "VA," "VB," "VC," and "VD" were referred to the sequences retrieved from soils RA, RB, RC, VA, VB, VC, and VD, respectively. The *bold number in parentheses* represents the numbers of the sequences in the same OTU in a given clone library. *Numbers at the nodes* indicate the levels of bootstrap support based on neighbor-joining analysis of 1000 resampled datasets. The *bar* represents 5 % sequence divergence

showed the highest methanotrophic community diversity (Shannon index=3.04), while other soils only showed a slight variation in community diversity (Shannon index=2.52–2.79).

Comparison of MOB community

Phylogeny-based weighted UniFrac environmental clustering analysis illustrated two distinctive MOB clades in seven soils (Fig. 2). Soils RA, RB, and RC were grouped together, while soils VA, VB, VC, and VD fell into a different clade, indicating that much different MOB community compositions could exist in these seven soils.

Phylogeny of MOB community

In this study, the representative *pmoA* gene sequences for phylogenetic analysis were selected from the OTUs containing at least two members. All the retrieved *pmoA* gene sequences from the studied seven soils could be divided into four distinctive clusters (Fig. 3). These soils showed a marked difference in the composition of MOB clusters and their relative abundance, indicating a large shift in soil MOB community structure (Fig. 4). Cluster I-like MOB predominated in soils VA, VB, VC, and VD (accounting for 72–84 %) and were also the largest group in soils RB and RC (48 or 39 %). Cluster II-like MOB were the largest group in soil RA (40 %) and also the second largest group in soil RB (33 %). Cluster III-like MOB were a minor group in soils RA, RB, and RC (9–20 %) but were not detected in soils VA, VB, VC, and VD. Moreover, cluster IV-like MOB were the second largest group in soil RC (30 %) but became less abundant in other six soils (10–20 %).

Cluster I contained a total of 88 *pmoA* gene sequences that could be grouped with those from a few cultivated *Methylocystis* MOB species (0510-P-6, KS30, SS2C, m231, and CSC1) (Chang et al. 2010; Iguchi et al. 2011; Lindner et al. 2007). Cluster II was a 25-member MOB group. The *pmoA* gene sequences in this cluster could be affiliated with the uncultured ones from various ecosystems, such as wetland soils, surface soil of onshore oil and gas fields, and highland lake sediment. Cluster III was the smallest MOB group and only contained 8 *pmoA* gene sequences that were related with the uncultured ones from lake sediments. In addition, a total of 26 *pmoA* gene sequences existed in cluster IV, and they could

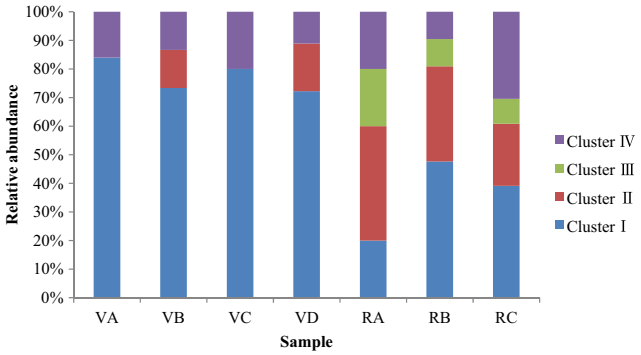

Fig. 4 Composition of MOB clusters in each soil sample

be affiliated with one cultivated *Methylocaldum* MOB species (Knief and Dunfield 2005).

Influential factors regulating MOB community

Pearson's correlation analysis indicated that MOB community abundance showed a significant positive correlation with the altitude of sampling site ($P<0.05$) and soil C/N ($P<0.01$) (Table 3). However, no significant correlation was found between MOB Shannon diversity and the determined environmental factors ($P>0.05$). The proportion of cluster I-like MOB was negatively correlated with the altitude of sampling site ($P<0.01$), and pH and OM ($P<0.05$). The proportion of cluster II-like MOB showed significant positive correlations with the altitude of sampling site ($P<0.01$) and OM ($P<0.05$), but negative with TP ($P<0.05$). Moreover, the proportion of cluster III-like MOB showed highly significant positive correlations with the altitude of sampling site and OM ($P<0.01$), while no significant correlation was found between the proportion of cluster IV-like MOB and the determined environmental factors ($P>0.05$).

The environmental factors in the first two RDA axes respectively explained 42.6 and 21.6 % of the total variance in MOB OTU composition (Fig. 5). Only TP ($F=2.806$, $P=0.010$, 499 permutations) and the altitude of sampling site ($F=2.399$, $P=0.018$, 499 permutations) were found to significantly contribute to the MOB assemblage-environment relationship.

Discussion

Soil MOB community abundance

Information on MOB community abundance in plateau soils is still very limited. Yun et al. (2010) found that *pmoA* gene of water flooded soil and surface soil in a wetland located in the Tibetan Plateau was 1.37×10^6 and 2.08×10^6 copies per gram dry soil, respectively, while Deng et al. (2013) indicated that *pmoA* gene ranged between 10^7 and 10^8 copies g^{-1} fresh soil in Riganqiao peatlands in the Qinghai-Tibetan Plateau. Yun et al. (2014) revealed 10^8 *pmoA* gene copies per gram of wet soil in soils from a lake littoral wetland in the Tibetan Plateau. Moreover, our previous study showed that the density of *pmoA* gene in agricultural soils in the Yunnan Plateau was more than 10^9 copies g^{-1} dry soil (Yang et al. 2014). However, the influential factors regulating MOB community abundance in plateau soils remain essentially unclear. In this study, *pmoA* gene in soils ranged from 2.12×10^4 to 2.95×10^5 copies per gram dry soil, much lower than those in previously studied plateau soils (Deng et al. 2013; Yang et al. 2014; Yun et al. 2010, 2014). In addition, the soils from lower altitude (soils RA, RB, and RC) showed a much higher MOB community size than those from higher altitude (soils VA, VB, VC, and VD). The results of Pearson's correlation analysis suggested that MOB community abundance was likely affected by the altitude of sampling site as well as soil C/N. To the authors' knowledge, the present study provided the first evidence for the impact of the altitude of sampling site and soil C/N on MOB community size.

Although a number of previous studies have investigated the MOB community size in various soils ecosystems (Xu et al. 2013; Zheng et al. 2008; Yun et al. 2010, 2014), only few previous studies have compared the difference of MOB community size in soils with different plant species. Degelmann et al. (2010) revealed a marked difference in MOB community abundance in European beech and Norway spruce soils. In addition, Yang et al. (2014) reported a large variation in MOB community size in rice, cabbage, and

Table 3 Pearson's correlation analysis of MOB community with the environmental factors

	Altitude	pH	TN	TP	OM	NO_3^--N	NH_4^+-N	C/N
MOB abundance	0.795*	0.616	−0.148	−0.479	0.704	−0.167	−0.161	0.974**
MOB Shannon diversity	−0.366	0.024	−0.264	0.067	−0.573	−0.016	−0.012	−0.623
Cluster I	−0.967**	−0.772*	−0.246	0.731	−0.835*	−0.168	−0.283	−0.727
Cluster II	0.926**	0.615	0.127	−0.787*	0.765*	0.085	0.173	0.675
Cluster III	0.962**	0.703	0.419	−0.629	0.932**	0.322	0.477	0.683
Cluster IV	0.245	0.533	0.107	−0.104	0.174	0.035	0.068	0.270

*Correlation is significant at the 0.05 level

**Correlation is significant at the 0.01 level

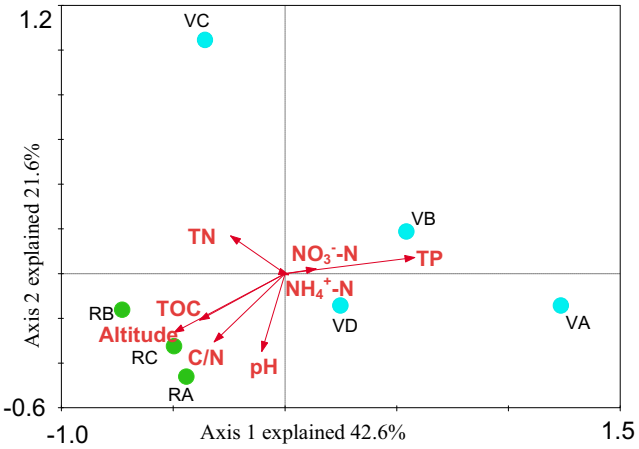

Fig. 5 RDA ordination plot for the first two principal dimensions of the relationship between MOB OTU composition and the environmental factors

garlic soils. These previous studies suggested that plant species could affect the soil MOB community abundance. However, information on the difference of MOB community abundance in soils with natural and managed plantation ways is still lacking. In this study, soils with natural plantation were found to have much higher MOB community abundance than those with managed plantation. Moreover, a significant difference in MOB community abundance could also be found in soils either with natural plantation or with managed plantation. For example, for soils with natural plantation, soil planted with *Poa annua* L. had significantly lower MOB community abundance than that with *Tripogon bromoides* but higher than that with *Cryptomeria fortune* ($P<0.05$). For soils with managed plantation, soil planted with *Brassica oleracea var. capitata* (or *Eriobotrya japonica*) significantly outnumbered that with *Salix cavaleriei* (or *Zea mays*) ($P<0.05$). These results further confirmed that soil MOB abundance could be influenced by both plant species and plantation way (natural and managed).

Soil MOB community diversity

Although numerous previous studies have investigated the MOB community diversity in various soils ecosystems (Xu et al. 2013; Zheng et al. 2008; Zhou et al. 2008), little is known about the difference of MOB community diversity in soils with different plant species. Degelmann et al. (2010) reported that MOB community diversity in spruce soils was lower than that in beech soils. Moreover, our previous study showed that rice soils had much higher methanotrophic diversity than cabbage and garlic soils (Yang et al. 2014). So far, the difference of methanotrophic diversity in soils with natural and managed plantation has not received attention. In this study, except for maize soil, soils showed similar MOB community diversity, suggesting that there was no clear link between soil MOB community diversity and plant species or plantation ways. In addition, soil MOB Shannon diversity also showed no significant correlation with the determined environmental factors ($P>0.05$).

Soil MOB community structure

Methylocystis species (type II methanotrophs) show a relatively high affinity for CH_4 and can be adapted to oligotrophic conditions (Deng et al. 2013). The high abundance or even dominance of *Methylocystis*-like MOB has been found in various agricultural soil ecosystems (Dörr et al. 2010; Vishwakarma et al. 2010). Our previous study also reported the dominance of *Methylocystis*-like MOB in two rice soils (Yang et al. 2014). In this study, *Methylocystis*-like MOB were found to dominate in soils from grassland, farmland, and naturally forested and afforested sites. *Methylocystis* species are known as acidophilic methanotrophs (Chen et al. 2008), and *Methylocystis*-like MOB showed the predominance in acidic environments (Danilova and Dedysh 2014). Hence, low-pH soil environments (4.82–5.95) might also account for the dominance of *Methylocystis*-like MOB in plateau soils in this study. The result of Pearson's correlation analysis also suggested an increase in the relative abundance of *Methylocystis*-like MOB with decreasing soil pH. Moreover, the present study provided the first evidence for the impact of the altitude of sampling site and organic matter on the proportion of *Methylocystis*-like MOB. The distribution of *Methylocystis*-like MOB was mutually shaped by pH, the altitude of sampling site, and organic matter.

Type I methanotrophs usually have a lower affinity for CH_4 than type II (Deng et al. 2013). Hence, *Methylocaldum*-like MOB (type I methanotrophs) were found to be absent in agricultural soil (Dörr et al. 2010), grassland soil (Zheng et al. 2012), and forest soil (Dörr et al. 2010). In contrast, in this study, *Methylocaldum*-like MOB organisms were found to be abundant in all of the seven plateau soils. Moreover, our previous study found that *Methylocaldum*-like MOB species were even dominant in cabbage and garlic soils in the Yunnan Plateau (Yang et al. 2014). However, the environmental factors regulating the distribution of *Methylocaldum*-like MOB in soil ecosystems remain unclear.

So far, there has been very limited information on the impacts of plantation way and plant species on soil MOB community structure. A recent study indicated a slight difference in MOB communities in soils from natural and afforested forest sites but a large difference in MOB communities in soils with different plant species (Dörr et al. 2010). Degelmann et al. (2010) also suggested the impact of plant species on soil MOB community structure. In this study, UniFrac environmental clustering analysis showed the existence of two distinctive MOB clades. The soil samples from unmanaged wild grassland and naturally forested sites were clearly separated from those from managed farmland and afforested sites,

indicating that plantation way had a profound impact on soil MOB community structure. This was not in agreement with the result reported in a previous study (Dörr et al. 2010). Moreover, the results of phylogenetic analysis further illustrated a marked difference in MOB community composition even in soils with the same plantation way. This confirmed that MOB community structure could be affected by plant species. In addition, the result of RDA suggested that the altitude of sampling site and TP might play important roles in shaping the structure of total MOB community. Therefore, plantation way and plant species as well as the altitude of sampling site and TP could collectively regulate the structure of total MOB community.

In conclusion, the soils from unmanaged wild grassland and naturally forested sites had much larger MOB community abundance than those from managed farmland and afforested sites. A distinctive difference in MOB community structure was found in soils with different plantation ways (natural and managed). A significant proportion of *pmoA* gene sequences were affiliated with *Methylocystis* (type II methanotrophs) and *Methylocaldum* (type I methanotrophs). The distribution of soil MOB community might be collectively regulated by plantation way, plant species, the altitude of sampling site, and soil properties.

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Ethical statement No conflict of interest exits in this manuscript. The work has not been published previously and not under consideration for publication elsewhere.

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