Molecular ecology and biogeography of methanotrophic bacteria in wetland rice fields

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Methanotrophic bacteria perform a central function in our climate system representing the only biogenic sink for the greenhouse gas methane. In wetland rice fields, they function as bio-filters preventing methane produced in anoxic layers escaping into the atmosphere, attenuating the potential methane emission by up to 90%. Despite intensive studies in the past, molecular approaches have barely started to explore the full diversity of methanotrophs. Furthermore, only little is known on their ecological niche differentiation and the factors influencing their community structure. This thesis focuses on the aerobic methanotrophic communities in the wetland rice ecosystem using the *pmoA* gene as a functional and phylogenetic marker to detect these bacteria in the environment. A high diversity could be recorded dominated by *Methylocystis* and *Methylosinus* species (type II) and yet uncultivated bacteria grouping within type Ib methanotrophs. The rice paddy cluster 1 (RPC-1) forms the largest cluster consisting entirely of sequences obtained from paddy fields located around the world. It is only distantly related to cultivated species and might form a new genus of methanotrophs specifically adapted to wetland rice fields.

Methanotrophic communities showed no large scale horizontal distribution patterns within an Italian paddy field; thus, a reduced sampling effort is sufficient to extrapolate to the field scale. However, different methanotrophic communities were detected on the rice roots compared to the field soil and the communities in different fields differed significantly. The rice roots were characterized by a high abundance of type I methanotrophs and different rice cultivars were shown to have an effect on these communities. This effect could be correlated to the plant genotype and enables to select specific cultivars for in-depth studies.

Re-evaluating the *pmoA* gene as a phylogenetic marker for methanotrophs revealed a good correlation of the *pmoA* to the 16S rRNA phylogeny. Nevertheless, some exceptions suggests that methanotrophy might be evolutionary more complicated having been even exchanged between species. Furthermore, a meta-analysis of *pmoA* sequences from various environments revealed distinct correlations of genotypes and habitats.

Methanotrophe Bakterien nehmen eine zentrale Funktion innerhalb unseres Klimasystems ein, indem sie die einzige biogene Senke für das Treibhausgas Methan darstellen. In gefluteten Reisfeldern fungieren sie als Biofilter und oxidieren bis zu 90% des in den anoxischen Bereichen produzierten Methans. Trotz intensiver Studien ist die Diversität dieser Mikroorganismen in der Umwelt noch nicht annähernd erfasst. Des Weiteren ist nur wenig über ihre ökologischen Nischen sowie den Einfluss abiotischer und biotischer Faktoren auf ihre Populationsstruktur bekannt. Die vorliegende Arbeit befasst sich mit den aeroben methanotrophen Bakteriengemeinschaften in gefluteten Reisfeldern. Das *pmoA* Gen wurde als funktioneller und phylogenetischer Marker verwendet, um methanotrophe Bakterien in Umweltproben zu detektieren. Die untersuchten Habitate wiesen eine hohe Diversität auf, wobei *Methylosinus* und *Methylocystis* (Typ II) und bisher nicht-kultivierte Methanotrophe des Typ Ib dominierten. Das größte Cluster repräsentierte das Reis-Paddy-Cluster 1 (RPC-1), dessen Sequenzen in Reisfeldern weltweit detektiert wurden. Es weist nur eine geringe Sequenzähnlichkeit zu bisher kultivierten Vertretern auf und stellt vermutlich eine neue Gattung innerhalb der methanotrophen Bakterien dar, die eventuell spezifisch an geflutete Reisfelder adaptiert ist.

Methanotrophe Bakterien wiesen keine großflächige Strukturierung in ihrer räumlichen Verteilung auf. Es konnte gezeigt werden, dass eine geringe Probenanzahl ausreicht, um repräsentative Aussagen über das Gesamtsystem zu treffen. Jedoch zeigten sich deutliche Unterschiede in der Zusammensetzung der methanotrophen Bakteriengemeinschaft an der Reiswurzel und im Reisfeldboden. Das Habitat Wurzel war von einer hohen Dominanz an Typ I geprägt. Außerdem konnte ein Einfluß des Reiskultivars auf die Zusammensetzung der Methanotrophen Bakterien gezeigt werden. Dieser Effekt korrelierte mit dem Genotyp der Reispflanze.

Die Re-Evaluierung des *pmoA* Gens als phylogenetischer Marker zeigte eine gute Korrelation zwischen der *pmoA* und der 16S rRNA Phylogenie. Einige Ausnahmen lassen jedoch vermuten, dass das Gen horizontal zwischen Arten

ausgetauscht wurde und die Evolution der Methanotrophie sich komplizierter darstellt als bisher angenommen. Eine Meta-Analyse mit *pmoA* Sequenzen aus verschiedenen Habitaten ergab weiterhin eine deutliche Korrelation zwischen dem *pmoA* Genotyp und dem Habitat.

1 INTRODUCTION

1.1 Methane in the atmosphere

Methane is one of the most important greenhouse gases playing an essential role in atmospheric chemistry. Its mixing ratio changed from a pre-industrial value of 715 ppb in 1750 to a concentration of 1,774 ppb in 2005, resulting in the second largest contribution to global warming after carbon dioxide (Intergovernmental Panel on Climate Change, 2007). Ice core studies have indicated that consistently lower concentrations were present in the atmosphere over the last 650,000 years, varying between 400 ppb and 770 ppb (Spahni et al., 2005). Although the total concentration more than doubled during the industrial era, the annual growth rates decreased substantially from about 1% to nearly zero since 1999 (Blake and Rowland, 1988; Dlugokencky et al., 1998; Dlugokencky et al., 2003). Several controversial theories have been proposed to explain the decreased growth rates and the contribution of human activities to it (Bekki et al., 1994; Bousquet et al., 2006; Hansen et al., 2000; Lelieveld et al., 1998; Lowe et al., 1997; Worthy et al., 2009). Nevertheless, a very recent study from Rigby and colleagues indicates a renewed growth of methane in the atmosphere (Rigby et al., 2008).

Over 70% of atmospheric methane originates from biogenic sources including natural wetlands, rice agriculture, livestock, landfills, termites and oceans. Natural wetlands represent the largest single source accounting for about 35% of total emissions. Non-biogenic sources include burning and mining of fossil fuel, waste treatment, biomass burning and geological sources such as geothermal or volcanic methane (Intergovernmental Panel on Climate Change, 2007). About 60% of the total emission is ascribed to anthropogenic activities. Recently, an additional new source for methane was described by Keppler and co-workers (Keppler et al., 2006). They

reported methane emission from living vegetation and estimated a contribution of 10-30% to the global budget. However, this finding was not supported by other studies and its evidence remains uncertain.

The major sink for atmospheric methane, accounting for 90% of the total, is the reaction with hydroxyl radicals in the troposphere. A small part is also lost to the stratosphere. Biological methane oxidation in soils represents an additional important sink (Intergovernmental Panel on Climate Change, 2007).

Biogenic methane is produced by methanogenic archaea as the final step in anaerobic degradation of organic matter. These strictly anaerobic Euryarchaeota mainly use carbon dioxide and hydrogen or acetate as substrates for methane formation (Conrad, 1997; Thauer et al., 2008). Roughly 1% of the primary productivity is estimated to result in methane production (Reeburgh, 2003). However, only about half of the produced methane is emitted to the atmosphere, while the remainder is oxidized. The proportion of oxidized methane varies depending on the environment (Reeburgh, 2003). Biological methane oxidation is performed by a diverse group of bacteria referred to as methane oxidizing or methanotrophic bacteria.

1.2 Methanotrophic microorganisms

Methylotrophic prokaryotes are able to use reduced carbon substrates without carbon-carbon bonds as their sole carbon and energy source (Lidstrom, 1992). Methanotrophic bacteria and archaea form a subgroup of the methylotrophs and are capable of growth with methane as one-carbon compound. For a long time, only aerobic methanotrophic bacteria were known to exist, however, anaerobic methanotrophs have been characterized more recently. Ammonia oxidizers are also able to convert methane to methanol by an enzyme homologous to the methane monooxygenase of methanotrophs. It seems, however, that they cannot use this process for growth (Hyman and Wood, 1983; Jones and Morita, 1983).

AEROBIC METHANOTROPHS

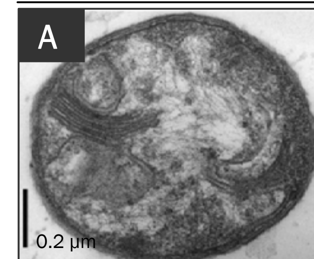
The process of aerobic methane oxidation by bacteria is known for a long time. The first isolates were described already beginning of the 20th century (Kaserer, 1905; Kaserer, 1906; Söhngen, 1906, Söhngen, 1910). However, interest in these microorganisms decreased and most cultures were apparently lost (Trotsenko and Murrell, 2008). It was not before the 1970s that an increasing number of studies focused on methanotrophic bacteria again. Their biotechnological potential for production of single cell protein or the use as biocatalysts was explored (Higgins et al., 1980; Higgins et al., 1981). Since then, many new isolates belonging to different genera were obtained and described (Bodrossy et al., 1997; Bowman et al., 1993; Bowman et al., 1997; Dedysh et al., 2002; Dedysh et al., 2007; Dunfield et al., 2003; Heyer et al., 1984; Heyer et al., 2002; Whittenbury et al., 1970). Recently, increasing interest focuses on the ecology of methanotrophs and is driven by their significant role in controlling the methane cycle and influencing global climate change.

To date, methanotrophic bacteria belonging to 16 genera within the α- and γ-proteobacteria are described (**Table 1**). In addition, three obligate methanotrophs of the phylum *Verrucomicrobia* were discovered recently (Dunfield et al., 2007b; Islam et al., 2008; Pol et al., 2007). However, the latter seem to be restricted to extreme environments growing at pH values of approximately 1 and temperatures of over 50°C. Complete genome sequencing of one isolate indicated the acquirement of genes essential for methanotrophy by horizontal gene transfer from the proteobacteria (Hou et al., 2008).

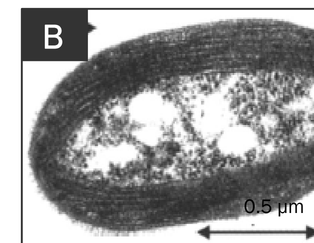
Table 1 | Phylogenetic, morphological and physiological characteristics of aerobic methanotrophs. Facultative and putative facultative methanotrophs are highlighted in bold. The pictures of intercytoplasmatic membrane arrangements were adopted from the following publications: **A**: Wartiainen *et al*., 2006; **B**: Dalton 2005; **C**: Dedysh *et al*., 2002. PLFAs - Phospholipid fatty acids; RuMP pathway - Ribulose monophosphate pathway; p.d. - poorly developed.

	γ-Proteobacteria (Type I)	α-Proteobacteria (Type II)		Verrucomicrobia
Family	*Methylococcaceae*	*Methylocystaceae*	*Beijerinckiaceae*	*Verrucomicrobiaceae*
Genera	*Methylomonas* *Methylobacter* *Methylomicrobium* *Methylosarcina* *Methylosphaera* *Methylosoma* *Methylococcus* *Methylocaldum* *Methylothermus* *Methylohalobium* ***Crenothrix*** ***Clonothrix***	*Methylosinus* ***Methylocystis***	*Methylocapsa* ***Methylocella***	*Methylacidiphilum*
Resting stages	*Azotobacter*-type cysts/none	Cysts/Exospores	Exospores/*Azotobacter*-type cysts	
Intracytoplasmic membranes	Type I (**A**)	Type II (**B**)	Type III (**C**)/p.d.	different
Major PLFAs	C16:1ω7c, C16:1ω8c, C16:0, C14:0	C18:1ω8c, C18:1ω7c, C18:2ω7c,12c	C18:1ω7c	C18:0, C16:0, aC15:0, C14:0,
Activity of				
sMMO	Yes/No	Yes/No	Yes/No	No
pMMO	Yes	Yes	Yes/No	Yes
Carbon Assimilation Pathway	RuMP pathway	Serine cycle	Serine cycle	Alternative serine cycle

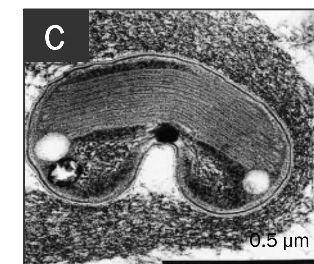
Intracytoplasmic Membrane Arrangements



Methylobacter tundripaludum



Type II methanotroph



Methylocapsa acidiphila

Proteobacterial methanotrophs were classified into two groups, type I and type II methanotrophs. This classification proposed by Whittenbury in the 70s (Whittenbury et al., 1975) was based on morphological, physiological and phylogenetical characteristics. While providing a meaningful system in the past, an increasing number of exceptions indicate that this classification might no longer be valid. The genera *Methylocapsa* and *Methylocella* share several characteristics with type II methanotrophs; however, they differ in their major phospholipid fatty acids (PLFA) and *Methylocapsa* possesses a different intracytoplasmic membrane arrangement referred to as membrane type III (**Table 1**; Dedysh et al., 2000; Dedysh et al., 2002). Another exception is the type II methanotroph *Methylocystis heyeri* sharing the PLFA profile of classical type I methanotrophs (Dedysh et al., 2007). Furthermore, nitrogen fixation was for a long time thought to be a specific trait of type II. However, a number of recent studies could demonstrate this function also in type I species. In addition, the number of environmental sequences without any cultured representative increases constantly in public databases. They cannot be assigned to type I or type II as no further information about the species is available. For these reasons, the type I and type II classification in this work refers only to phylogenetic inferences and does not allow conclusions on physiology or morphology. Type I methanotrophs are furthermore divided into type Ia and type Ib. Type Ib includes besides clusters of uncultivated methanotrophs the genera *Methylococcus* and *Methylocaldum* which were described as type X in earlier publications (Bowman, 2000; Hanson and Hanson, 1996). These genera do not only possess enzymes catalyzing the RuMP pathway reactions of formaldehyde fixation (characteristic for type I), but also enzymes of the serine pathway (characteristic for type II) (Trotsenko and Murrell, 2008).

Besides the verrucomicrobial methanotrophs, species belonging to the Proteobacteria are also known to be adapted to extreme environments. *Methylococcus*, *Methylocaldum* and *Methylothermus* form the group of thermotolerant or moderately thermophilic proteobacterial methanotrophs (Trotsenko et al., 2009). They are able to grow at temperatures up to 65°C.

Methanotrophs adapted to cold environments (growth in temperature range of 0-30°C) were also described and include *Methylobacter psychrophilus*, *Methylobacter tundripaludum*, as well as *Methylocella* and *Methylocapsa* species (Trotsenko and Khmelenina, 2005; Wartiainen et al., 2006). *Methylohalobius crimeensis* represents a halophilic methanotroph obtaining a growth optimum at salt concentrations of 0.2-2.5 M (Heyer et al., 2005). Some *Methylomicrobium* species are furthermore known to be halotolerant (Kalyuzhnaya et al., 2008; Khmelenina et al., 1997). *Methylocapsa* and *Methylocella* are acidophilic methanotrophs able to grow at pH values down to pH 4 (Dedysh et al., 2000; Dedysh et al., 2002).

Methanotrophs are generally defined as obligate methylotrophs restricted to the utilization of methane, methanol and a narrow range of C1 compounds (e.g. Bowman, 2000). Discussions about the existence of facultative methanotrophs have a long history; however, it was only recently that Dedysh and colleagues provided the first proof with the characterization of the facultative *Methylocella* species (Dedysh et al., 2005). Nevertheless, the sheathed γ-proteobacteria *Crenothrix polyspora* might present a further facultative methanotroph (Stoecker et al., 2006). Together with *Clonothrix fusca*, it was only recently found to utilize methane (Stoecker et al., 2006; Vigliotta et al., 2007) although its morphology and complex life cycle is well known since more than one century (Cohn, 1870). Beside these rather unusual methanotrophs, *Methylocystis* species seem also capable of using multi-carbon substrates such as ethanol and acetate (Dedysh, personal communication). These findings indicate that facultative methanotrophy might be more common than previously thought.

In aerobic methanotrophs, methane is oxidized to carbon dioxide via the intermediates methanol, formaldehyde and formate (**Figure 1**).

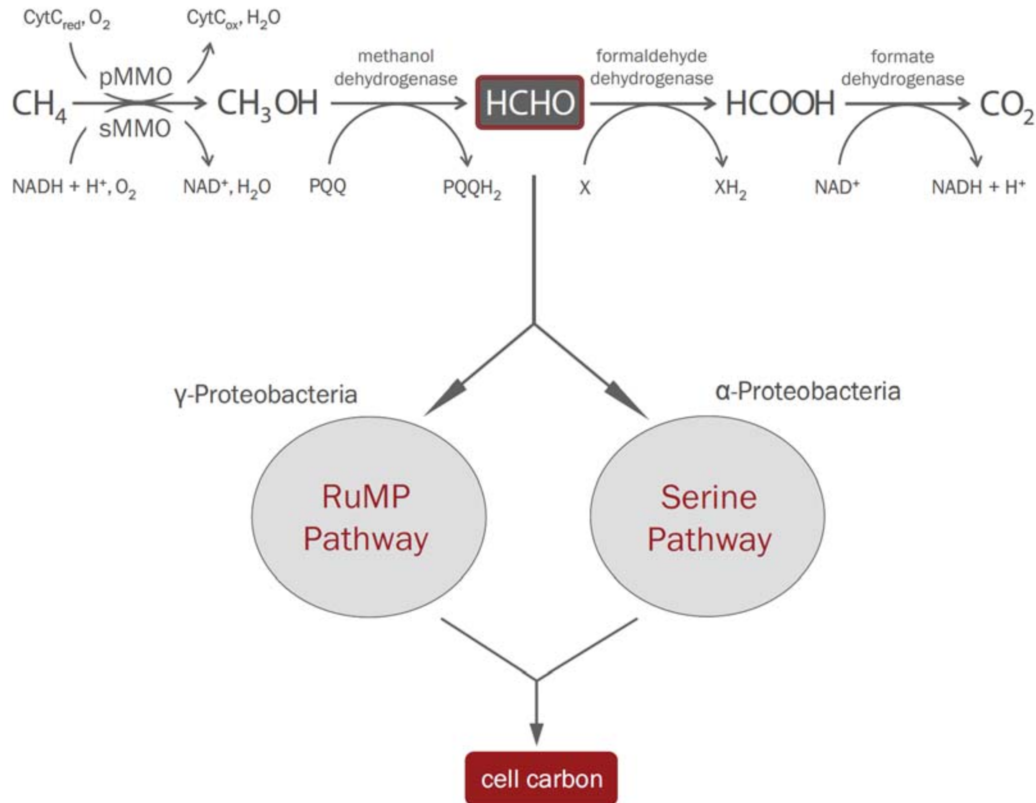


Figure 1 | Pathway for methane oxidation and assimilation of formaldehyde. Abbreviations: CytC = cytochrome *c*; PQQ = pyrroloquinoline quinine; X = $NADP^+$ or cytochrome linked; RuMP Pathway = Ribulose monophosphate Pathway. Modified from Hanson and Hanson, 1996.

The first step, the oxidation of methane to methanol, is catalyzed by the methane monooxygenase (MMO). Two forms of this enzyme are described: the membrane-bound or particulate MMO (pMMO) and the soluble enzyme (sMMO). All known methanotrophs except *Methylocella* possess the pMMO (Dedysh et al., 2000) whereas the sMMO is only present in some species. In bacteria holding both enzymes, expression is controlled by copper concentrations in the growth medium (Nielsen et al., 1996; Prior and Dalton, 1985; Stanley et al., 1983).

The two enzymes are distinct and present the result of two evolutionary independent processes. The sMMO is characterized by a wide substrate spectrum including a variety of alkanes, alkenes and aromatics, whereas the pMMO is only able to oxidize methane and short-chained alkanes and alkenes up to five carbons in length (Burrows et al., 1984; Colby et al., 1977). Although the pMMO is prevalent in nature, more is known about the biochemistry of the soluble enzyme, a fact that can be attributed to the difficulties of working with an integral membrane protein. Great progress has been made lately by obtaining the first crystal structure of the pMMO (Lieberman and Rosenzweig, 2005). Nevertheless, the active site has not been identified yet and is still actively discussed (Hakemian and Rosenzweig, 2007; Himes and Karlin, 2009). However, most data support a copper-mediated catalytic mechanism (Himes and Karlin, 2009).

In various upland soils, methane oxidation kinetics were measured holding an unexpected high apparent affinity for methane compared to pure cultures of methanotrophs or wetland soils (Bender and Conrad, 1992; Bender and Conrad, 1993; Benstead and King, 1997; Gulledge et al., 1998). These soils act as sinks for atmospheric methane and high-affinity methanotrophs were assumed to be responsible for methane oxidation at trace concentrations (Bender and Conrad, 1992). However, the mechanism and the organisms involved are still unknown (Dunfield, 2007a). Some uncultured methanotrophs were found abundant and active in these soils and are therefore proposed as the promising candidates (Holmes et al., 1999; Knief et al., 2003). Very recently, Baani and Liesack could furthermore show that a second pMMO isoenzyme found in several type II methanotrophs enabled growth at atmospheric methane concentrations for over three months (Baani and Liesack, 2008).

ANAEROBIC METHANOTROPHS

The first geochemical evidence for anaerobic oxidation of methane was found already 30 years ago, when observing the removal of methane from deeper marine sediments before any contact to oxygen (Barnes and Goldberg, 1976; Martens and Berner, 1974; Reeburgh, 1976). It is estimated to account for removing up to 90% of the methane produced in oceans thereby reducing ocean contribution to 2% of the global methane sources. However, the process involved remained unknown until Hoehler and colleagues proposed a reverse methanogenesis mediated by a consortium of methanogenic archeaea and sulfate reducing bacteria (Hoehler et al., 1994). Detecting archeal lipids highly depleted in ^{13}C carbon provided the first evidence for anaerobic methane consumption by archaea (Hinrichs et al., 1999). To date, three distinct clusters of methanotrophic archaea (ANME-1, ANME-2 and ANME-3) are described, all related to *Methanosarcinales* and *Methanomicrobiales*, two orders of methanogens with many cultivated representatives. They were mostly found in cell aggregates together with sulfate reducing bacteria belonging to the δ-proteobacteria (Knittel and Boetius, 2009). However, none of them could be cultivated so far. ANME organisms seem to be globally distributed and were not only detected in marine environments, but also in limnic water columns and sediments, landfills, soils and aquifers (Cadillo-Quiroz et al., 2008; Castro et al., 2004; Eller et al., 2005b; Grossman et al., 2002; Maclean et al., 2007). The presence of the methyl-coenzyme M reductase (MCR) catalyzing the last step in methanogenesis suggests that anaerobic methane oxidation is indeed performed by reverse methanogenesis in ANME organisms (Krüger et al., 2003). Furthermore, fosmid libraries of ANME enrichment cultures revealed the presence of nearly all genes associated with methanogenenis (Hallam et al., 2003). However, the detailed mechanism is still under discussion.

Theoretically, anaerobic methane oxidation could be also coupled to other electron acceptors such as iron (Fe^{3+}), manganese (Mn^{4+}) and nitrate (NO_3^-). All these processes are energetically more favorable than reduction of sulfate, however,

experimental evidence was only found very recently. Raghoebarsing and co-workers obtained an enrichment culture from anoxic sediments of the Twentekanaal that coupled methane oxidation to denitrification (Raghoebarsing et al., 2006). Approximately 80% of the consortium was composed of bacteria belonging to the candidate division 'NC 10' whereas approximately 10% consisted of archaea closely related to the ANME-2. Labeled methane was incorporated into bacterial and archaeal biomarkers indicating the involvement of both groups. However, the physiological process remains unknown as MCR inhibition had no influence on methane consumption and the bacteria were also able to perform the reaction in absence of the archaea (Ettwig et al., 2008). Beal and colleagues found anaerobic oxidation of methane coupled to manganese and iron in marine sediments (Beal et al., 2009). However, although energetically more favorable, both processes were shown to occur at much smaller rates. If the microorganisms involved consists of an archaeal and bacterial consortium, or if bacteria alone are responsible, remains unknown.

Ammonia oxidizing bacteria

The lithoautotrophic ammonia oxidizing bacteria (AOB) use ammonia as sole energy source and are able to fix carbon dioxide using the Calvin Benson cycle (Bock and Koops, 1999). Three main genera are described to date: the *Nitrosomonas* and *Nitrosospira* belonging to the β-proteobacteria and *Nitrosococcus* clustering phylogenetically within the γ-proteobacteria (Koops et al., 2006). The first step, the oxidation of ammonia to hydroxylamine is catalyzed by the ammonia monooxygenase. This enzyme is evolutionary related to the pMMO, the membrane bound methane monooxygenase (Holmes et al., 1995). The ammonia monooxygenase is not highly substrate specific and is able to oxidize several apolar compounds such as carbon monoxide and some hydrocarbons (Hooper et al., 1997). It is also able to oxidize methane, however, at much lower rates than the methane monooxygenase (Bedard and Knowles, 1989). It was furthermore shown that ammonia oxidizers probably play

no significant role in global methane oxidation (Bender and Conrad, 1994; Bodelier and Frenzel, 1999; Bosse et al., 1993; Jiang and Bakken, 1999).

1.3 The wetland rice ecosystem

Wetland soils are seasonally or permanently water saturated and form the largest single source of atmospheric methane (Intergovernmental Panel on Climate Change, 2007). Rice paddies represent a unique form of wetland characterized by the dominance of one plant species. The three main types of wetland rice agriculture include (i) deep water rice which is permanently flooded, (ii) rain-fed rice which is only flooded after heavy rainfall and (iii) irrigated rice which is artificially flooded during the season and lays fallow during the winter (Neue and Roger, 2003). Wetland rice was estimated to account for approximately 15% of the global methane emission (Intergovernmental Panel on Climate Change, 2007) and its influence on the methane budget will even increase in future in correlation with the food demands of the growing human world population.

The biogeochemistry in rice paddies is mainly controlled by the input of organic carbon and oxygen and by the availability of alternative electron acceptors such as Fe^{3+}, nitrate, Mn^{4+}, and sulfate (Conrad and Frenzel, 2002). Besides soil organic matter, the organic carbon originates from decay of plant material or is released from the plant through root exudation (Hartmann et al., 2009). Addition of rice straw represents a common fertilization practice resulting in a strong increase of methane production (Denier van der Gon and Neue, 1995; Sass et al., 1991). Oxygen is a limiting factor in flooded paddy fields. It only penetrates the first millimeters of the soil where it is rapidly consumed (Frenzel et al., 1992). Furthermore, rice plants act as conduit for oxygen transport through the intercellular aerenchyma system thereby providing oxygen to deeper anoxic soil compartments (Armstrong, 1979; Frenzel et al., 1992; Große and Bauch, 1991). As result, three major habitats for microorganisms

in paddy fields can be specified: (i) the anoxic bulk soil, (ii) the oxic surface soil, and (iii) the partially oxic rhizosphere with increased substrate concentration (**Figure 2**).

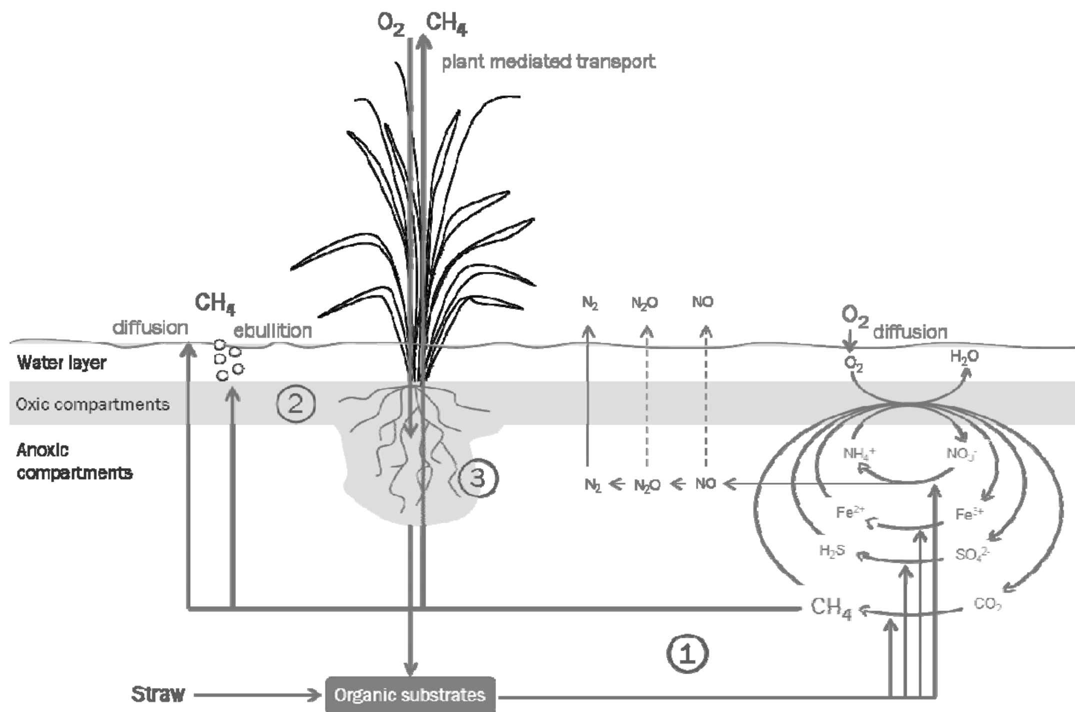


Figure 2 | Scheme of the main habitats for biogeochemical active microorganism in a flooded rice field. 1= anoxic bulk soil; 2= oxic surface soil; 3= rhizosphere. Furthermore, the simplified redox cycling taking place at the oxic-anoxic interface and the emission pathway for methane are depicted. Modified from: Conrad, 2007; Conrad and Frenzel, 2002.

In the presence of oxidants, the organic carbon is completely oxidized to carbon dioxide. If oxygen is not available, alternative electron acceptors are reduced according to their redox potential: nitrate is thermodynamically preferred over Mn^{4+}, followed by Fe^{3+} and sulfate (Patrick, Jr. and Reddy, 1978; Ponnamperuma, 1972). Fe^{3+} represents the most abundant electron acceptor in paddy fields (Yao et al., 1999). Whereas oxygen and nitrate are rapidly consumed after flooding, the following Fe^{3+}

reduction can persists for several weeks (Patrick, Jr. and Reddy, 1978; Ponnamperuma, 1972). At the oxic-anoxic interfaces prevalent at the rhizosphere and surface soil, electron acceptors can be regenerated and a redox cycling of N, Fe, and S takes place (**Figure 2**; Conrad and Frenzel, 2002).

In the absence of oxygen or alternative electron acceptors, organic carbon is disproportionated to carbon dioxide and methane. Methane as the end product of organic matter degradation serves as substrate for methanotrophic bacteria (see **1.2**). Methanotrophs can be found in habitats where methane and oxygen gradients overlap, in particular the surface of the paddy soil and the rhizosphere (Bosse and Frenzel, 1997; Eller and Frenzel, 2001; Gilbert and Frenzel, 1995; Henckel et al., 2001). However, a large amount of methanotrophs can be detected in the anoxic bulk soil (Eller et al., 2005a; Eller and Frenzel, 2001). Both, type I and type II methanotrophs were found in paddy fields, attenuating the potential CH_4 emission by up to 90% (Frenzel et al., 1992; Frenzel, 2000; Gilbert and Frenzel, 1998). Uncultured methanotrophs assumed being responsible for methane oxidation at atmospheric levels and verrucomicrobial methanotrophs were not detected in rice fields until now.

1.4 Marker genes for studying methanotroph diversity

The 16S rRNA gene is by far the most frequently used phylogenetic marker for studying microbial ecology and diversity in the environment. An additional approach includes the sequencing of functional genes that are unique to the physiology of the studied group of microorganisms. The enzyme unique to methanotrophs is the methane monooxygenase. The *pmoA* and *mmoX* gene encoding a subunit of the pMMO and the sMMO, respectively, are the most frequent targets for methanotroph diversity studies (Dumont and Murrell, 2005; McDonald et al., 2008). As the pMMO is present in nearly all methanotrophs (see **1.2**), the current *pmoA* sequence database is by far larger than the amount of public available *mmoX* sequences. The pMMO gene

cluster in type I and type II methanotrophs contains three open reading frames arranged as *pmoCAB* with a putative transcriptional start upstream of the *pmoC* gene (Gilbert et al., 2000; Semrau et al., 1995; Stolyar et al., 1999). In these organisms, two nearly identical copies of *pmoCAB* have been found. However, several type II methanotrophs were shown to harbor an additional different *pmoA* copy referred to as *pmoA-2* (Dunfield et al., 2002; Tchawa Yimga et al., 2003). In *Methylocystis* strain SC2, *pmoA-2* was shown to be part of a complete gene cluster that is responsible for oxidation of methane at atmospheric concentrations (Baani and Liesack, 2008; Ricke et al., 2004).

The active site of the pMMO enzyme has not been identified yet; however, four sites containing highly conserved amino acids are currently discussed as potential candidates (Hakemian and Rosenzweig, 2007). Two of these sites include residues located within the *pmoA* gene. Furthermore, the *pmoA* phylogeny is largely congruent to the 16S rRNA gene phylogeny (Kolb, 2003) making *pmoA* a suitable phylogenetic marker gene for methanotrophs.

1.5 Aims of this study

Methanotrophic bacteria perform a key function in the global carbon cycle by controlling the methane emissions released to the atmosphere. They have been studied intensively in the past; however, molecular approaches have barely started to explore their full diversity. Furthermore, the biotic and abiotic factors determining the niche differentiation of different species are only poorly understood. *Verrucomicrobia*, *Methylothermus* and *Methylohalobius* species seem to be only found in extreme environments whereas the uncultured 'upland soil methanotrophs' might be adapted to methane oxidation at atmospheric concentrations. However, the ecology of the mesophilic and neutrophilic type I and type II methanotrophs coexisting in many environments is largely unknown.

In this PhD work, the *pmoA* gene was used as a functional and phylogenetic marker for studying methanotrophic communities in wetland rice fields. This ecosystem represents a well studied environment and an important source of atmospheric methane. The following questions were addressed to gain further insights into the ecology of methanotrophs:

CHAPTER 2 | SPATIAL HETEROGENEITY OF METHANOTROPHS

Microorganisms are not homogeneously distributed in nature. Communities might occur in patches according to physiological properties or they might be structured along environment gradients. These patterns have strong influence on the sampling strategy and the data interpretation. *Are methanotrophic communities spatially structured within a paddy field?*

CHAPTER 3 | BIOGEOGRAPHY OF WETLAND RICE METHANOTROPHS

First molecular studies already revealed the presence of type I and type II methanotrophs in wetland rice fields. *Which species can be detected in detail? Are*

there different species on the plant roots than in the soil? And are there different communities in different paddy fields?

CHAPTER 4 | METHANOTROPHS ASSOCIATED TO RICE ROOTS

The rhizosphere represents a major habitat for methanotrophs in a paddy field. *Are there different communities on the roots of different rice cultivars? And do different molecular techniques (T-RFLP and microarray) lead to the same results?*

CHAPTER 5 | THE PMOA GENE AS PHYLOGENETIC MARKER

The *pmoA* gene is widely used as a marker in diversity studies of methanotrophs. Recently, new methanotrophic species only distantly related to canonical methanotrophs have been described. *Does the pmoA phylogeny still correspond to the 16S rRNA phylogeny? Are pmoA genotypes correlated to specific environments?*

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2 SPATIAL HETEROGENEITY OF METHANOTROPHS:

A GEOSTATISTICAL ANALYSIS OF *PMOA*-BASED T-RFLP PATTERNS IN A PADDY SOIL

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2.1 Abstract

Despite numerous studies on methanotrophs, virtually nothing is known about their spatial heterogeneity in nature. These patterns, however, have strong influences on the interpretations made from analyzing microbial processes and community structure. Here we report the first use of geostatistics to analyze the spatial heterogeneity of methanotrophs in a rice field soil (Vercelli, Italy). We used the gene encoding the particulate methane monooxygenase, *pmoA*, for terminal restriction fragment length polymorphism (T-RFLP) analysis. The profiles obtained were compared using a pseudo-variogram analysis to study autocorrelation as a function of distance. We demonstrated that there was no large-scale spatial structure at this study site, but a micro-scale spatial structure could not be excluded. A species accumulation curve with all TRFs revealed that even 75 samples were insufficient to cover the diversity of methanotrophs in a rice field. However, a species accumulation curve of methanotrophs defined as operational taxonomic units validated from a clone library with 90 % coverage demonstrated saturation after approximately 15 samples. The results of this study have consequences for studying the diversity and function of methanotrophs. In this agroecosystem no environmental gradients were found indicating that the sample size is of minor importance.

2.2 Introduction

Methanotrophs are a physiologically unique group of bacteria that utilize methane as sole carbon and energy source. They belong to the *Gammaproteobacteria* (type I methanotrophs) and *Alphaproteobacteria* (type II methanotrophs). Both groups oxidize methane via methanol and formaldehyde to carbon dioxide (Hanson and Hanson, 1996; Bowman, 2006; McDonald et al., 2008; Trotsenko and Murrell, 2008). A key enzyme in this pathway is the particulate methane monooxygenase (pMMO), which is present in all known methanotrophs except the acidophilic *Methylocella* spp. (Theisen et al., 2005). Hence, the *pmoA* gene, which encodes the α subunit, can be used as a molecular marker for the identification of methanotrophs in environmental samples (McDonald and Murrell, 1997).

In wetland rice fields, methanotrophs can reduce the potential methane emissions up to 80 % (Conrad and Rothfuss, 1991) and thus play an important role in the global methane budget. Consequently, the physiology, diversity, and ecology of methanotrophs have been studied in detail (Hanson and Hanson, 1996; Conrad, 2007; McDonald et al., 2008). However, information on their spatial heterogeneity in nature is lacking and has been rather neglected when studying methanotrophs.

The distribution of microorganisms in the environment is heterogeneous (Franklin and Mills, 2003). Bacterial communities are structured not only by the physiology and ecological properties of the members, but also by environmental parameters. These gradients have to be evaluated when designing field studies of bacterial diversity and function.

A powerful tool for gaining insight into the spatial structure is geostatistics. Geostatistics originate from soil science and are widely used for quantifying spatial patterns (Legendre and Legendre, 1998; Ettema and Wardle, 2002). It is based on the assumption that spatial variability is autocorrelated, i.e., locations close to each other are more similar than those further apart. A method to identify the spatial structures is variogram analysis. Generally, semi-variances between samples are calculated and

plotted against their spatial separation; the slope indicates whether a spatial structure is present (Ettema and Wardle, 2002).

In microbial ecology, so far, only a few studies have used geostatistics, e.g., Franklin et al. (2002), Mummey and Stahl (2003), Nicol et al. (2003), Ritz et al. (2004) and Bengtson (2007). For example, the use of geostatistics has shown that the spatial structure of microbial communities in agricultural soils can greatly differ from site to site. Grundmann and Debouzie (2000) demonstrated with a pure culture experiment of ammonia and nitrite oxidizers using an agricultural soil cultivated with maize that they aggregate at a millimeter scale. In a field study, Franklin and Mills (2003) applied amplified fragment length polymorphism (AFLP) to the total bacterial community and pointed out that the bacterial distribution can be highly structured over a distance of 30 cm to more than 6 m in a wheat field. In sharp contrast to both of these studies, Robertson et al. (1997) observed no spatial variability in the culturable bacterial population in a monospecific crop field, even though soil properties varied. Hence, the possible spatial variability has to be evaluated when microbial processes are analyzed (Robertson et al., 1997).

Our study was performed to address the general need for more information about the spatial heterogeneity of bacterial populations in agricultural systems. We used methanotrophs from a rice field as a model system. We measured *pmoA* based terminal restriction fragment length polymorphism (T-RFLP) and applied geostatistics to provide insights in the spatial structure. Moreover, this work could be applied to develop an optimal sampling strategy for diversity studies in rice fields.

2.3 Results and discussion

Spatial structure

The applied sampling scheme ensured that a representative data set was recorded (**Figure 1**). Our geostatistical analyses resulted in a pseudo-variogram depicting the spatial organization of methanotrophs (**Figure 2**). The Jaccard dissimilarities of *pmoA*-based T-RFLP patterns averaged 0.64. There were no trends towards a change in dissimilarities with increasing separation distance, which implied no spatial structure at the study site sampled.

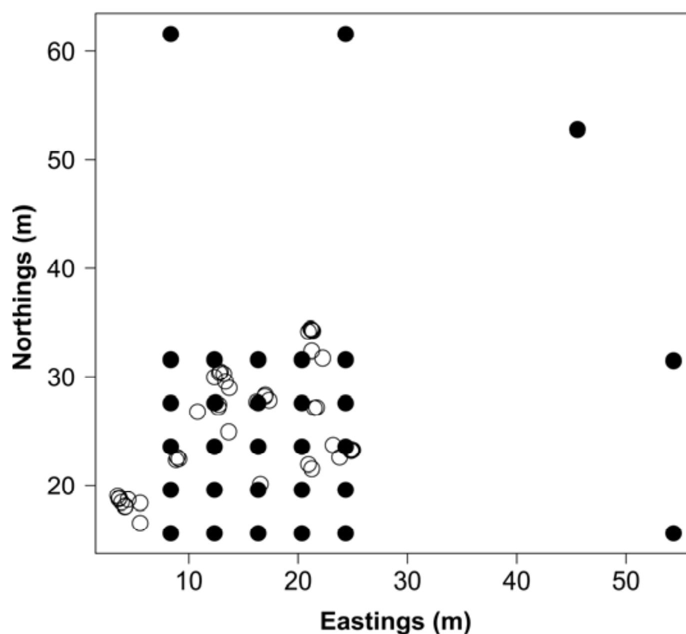

Figure 1 | **Sampling design, illustrating the location of the sampling points.** Filled circles represent grid cells and samples spaced further apart; open circles show samples of the random-walk transects. Soil samples were collected from a paddy rice field of the C.R.A. Unità di Ricerca per la Risicoltura (Vercelli, Italy) in autumn 2006 after drainage and harvest. A 60×60 m area of a rice field was sampled. In this area, 25 points were marked with 4 m between each point, forming a regular grid. Five points were chosen at random as the starting points of independent random-walk transects, as described elsewhere (Ritz et al., 2004). In addition, five samples, 30 m apart, were taken. In total, 75 samples were collected. Each sample consisted of a 64 mm core taken from the rice field surface to a depth of approximately 6 cm.

Considering the history of the study site, we can postulate a possible explanation for this result. The site has been planted with rice for more than 100 years (Lupotto, personal communication). Plowing and puddling of the flooded soil has effectively homogenized the topsoil, and when flooded this soil lacks horizontal gradients, i.e., the soil properties are fairly constant. If the plant root system and rhizosphere affects the distribution of microorganisms, as suggested by a study in a grassland (Mummey and Stahl, 2003), these effects would be even more similar throughout the site in the monospecific rice culture. Hence, the methanotrophic community would not be spatially structured. A second factor may be similarly important: by volume, the largest fraction of the flooded soil is anoxic enabling aerobic methane oxidation only at the soil surface layer and in the rhizosphere. Methanotrophs can form drought-resistant cysts and exospores (Whittenbury et al., 1970; Bowman et al., 1993) that are assumed to make up the largest fraction of the population (Eller and Frenzel, 2001). Methanotrophs have been reported to survive unfavorable conditions for up to 170 years (Rothfuss et al., 1997). Hence, this long persistence in the environment will level out actual population changes, particularly if not the active but the total population is analyzed.

In contrast, the medians of the boxes in the pseudo-variogram were significantly different at small distance classes (2.7 m to 5.4 m, **Figure 2**) and at large distance classes (18.9 m to 32.5 m, **Figure 2**). No significant differences for intermediate distance classes were observed. Differences at short distances pointed towards a slight spatial structure (**Figure 2**), which is also underlined by the results of a local regression analysis (**Figure S1**). Previous work has shown that on the micro-scale, e.g., comparing roots to the surrounding soil, the population structures can differ (Eller and Frenzel, 2001). However, an additional pseudo-variogram analysis on a small scale (< 10 m) did not reveal any spatial structure (data not shown). Hence, we assume that the scales at which spatial heterogeneity might occur are mainly smaller than those measured. We cannot exclude a spatial structure at the millimeter scale, as shown for instance by Grundmann and Debouzie (2000).

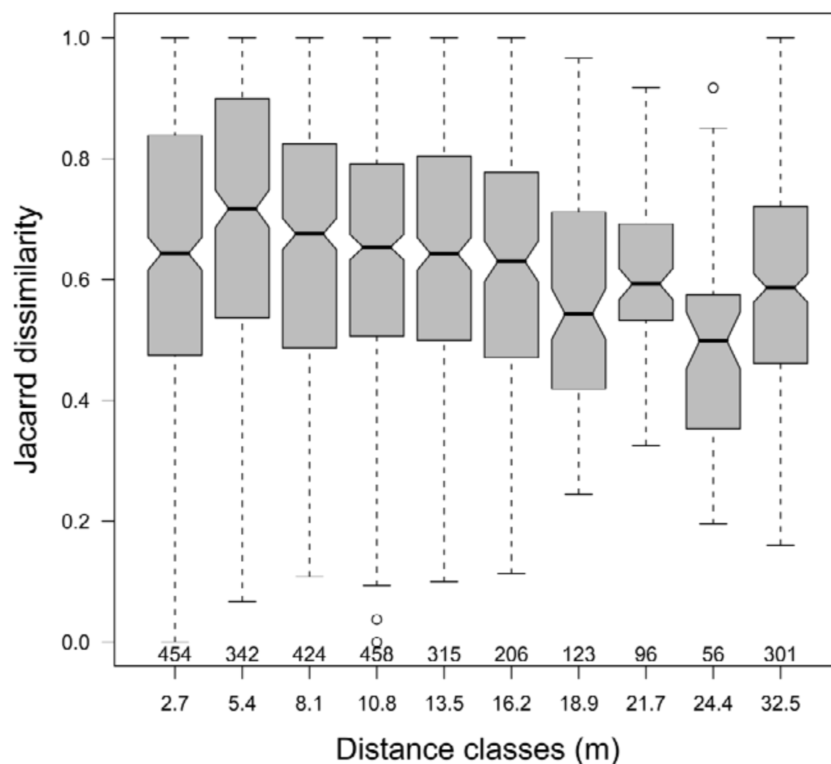

Figure 2 | Spatial heterogeneity, shown as a pseudo-variogram. Since T-RFLP analysis generates multivariate data, we followed the approach of Franklin et al. (2002) using the Jaccard coefficient as a measure of dissimilarity. Dissimilarities are shown as a series of box and whisker plots binned to distance classes. The horizontal lines in the boxes indicate the median. The bottom and top of each box indicate the 25 and 75 percentiles, respectively. Whiskers are 1.5 times the interquartile range of the data, and points outside this range are classified as outliers. Notches are shown around each median. If the notches do not overlap, the medians are roughly significantly different at about a 95 % confidence level (McGill et al., 1978). The numbers above the distance classes indicate the sample size. DNA was extracted following the protocol of Stralis-Pavese et al. (2004). Amplification of the *pmoA* gene was carried out in triplicates per sample and pooled afterwards followed by a *pmoA*-based T-RFLP analysis (Horz et al., 2001). T-RFLP data were standardized using the relative abundances of TRF peak heights (Lüdemann et al., 2000).

Species (OTU) coverage

The assignment is based on a *pmoA* database containing more than 4000 sequences. From these sequences, 500 were obtained from Vercelli rice fields and a rarefaction analysis, an estimation of the total diversity based on a sub sample, indicated a good coverage of methanotrophic diversity in this agroecosystem (data not shown). TRFs could be affiliated to the following operational taxonomic units (OTUs): *pmoA*/*amoA*-like RA21 cluster (58 bp) *Methylococcus/Methylocaldum* (80 bp), *Methylosinus trichosporium* OB3B (146 bp), *Methylosinus/Methylocystis* (245 bp), *Methylomicrobium album* (350 bp). In addition, the 47 and 113 bp TRFs were affiliated to the *amoA* gene, which is partially covered with the applied primers. This assignment is consistent with previous work (Holmes et al., 1999; Horz et al., 2001; Reay et al., 2001; Shrestha et al., 2008). With this information, an additional pseudo-variogram analysis was performed using only the assigned OTUs. The pseudo-variogram was the same as that shown in Fig. 2, with a mean Jaccard dissimilarity of 0.59. Although there was no obvious spatial structure, we identified some indications of species interactions between type I and type II methanotrophs (Table S1). Henckel (2000) found that type II methanotrophs are usually present or most active when environmental conditions in a rice field have become fairly constant, whereas type I methanotrophs are more active when environmental conditions are more variable, e.g., during drainage or flooding. They suggested that generally type I and II methanotrophs occupy different niches and coexist. Additionally, the impact of protistan grazing might have an effect on the distribution and abundance of methanotrophs, as shown by Murase et al. (2006).

Species accumulation curve

The results of our study have general implications for the study of diversity and function of methanotrophs. Demonstrating that a small sample size can representatively cover the *pmoA*-based TRFs in a rice field would simplify sampling

effort and save costs and time. In our first analysis, all fragments were included and the curve did not flatten, which indicated that 75 samples are insufficient to cover all TRFs (data not shown). Along these lines, Schloss and Handelsmann (2004) illustrated that even approximately 56,000 partial 16S rRNA gene sequences did not cover the microbial census and are far from complete, as shown by a sharp slope of the rarefaction curve, which is similar in application to the species accumulation curve. Hence, we limited the analysis to the previously assigned OTUs. This species accumulation curve flattened, i.e., indicated that the curve was saturated at a sample size of approximately 60 (Fig. S2), and a manageable sampling unit of approximately 15 was found when a limit was set at 90 % OTU coverage. We believe that it is currently not feasible to cover an entire ecosystem, even for just a single functional group like the methanotrophs. Hence, the focus should be more on different community patterns, or on species and their ecological relevance than on covering every single species in an ecosystem. There are consequences for the sampling effort.
In ecosystems with gradients the sampling strategy is of major importance, because all gradients have to be considered and a high number of samples are required. However, in systems, without gradients the samplings strategy seems to be irrelevant and a small sample size can representatively cover the study site.

In summary, our results demonstrate that the methanotrophic community in the rice field studied had no obvious spatial structure. A structure on the millimeter scale is possible, but this still needs to be investigated. We were able to limit the sample size without losing important fragments. Although no spatial structure was found at the level of T-RFLP patterns, we demonstrated that there is a noteworthy difference in the occurrence of distinct methanotrophic phylogenetic groups. The pseudo-variogram analysis in connection with T-RFLP analysis is a suitable method for the identification of spatial structures. Hence, this method can be adapted to every kind of environment. Since this study is based on DNA, i.e., on the presence of gene copies, the results do not necessarily reflect the active community. Future analyses

should concentrate on active methanotrophs since remarkable differences in the community structure based on the activity of single species are expected.

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2.5 Supplementary material

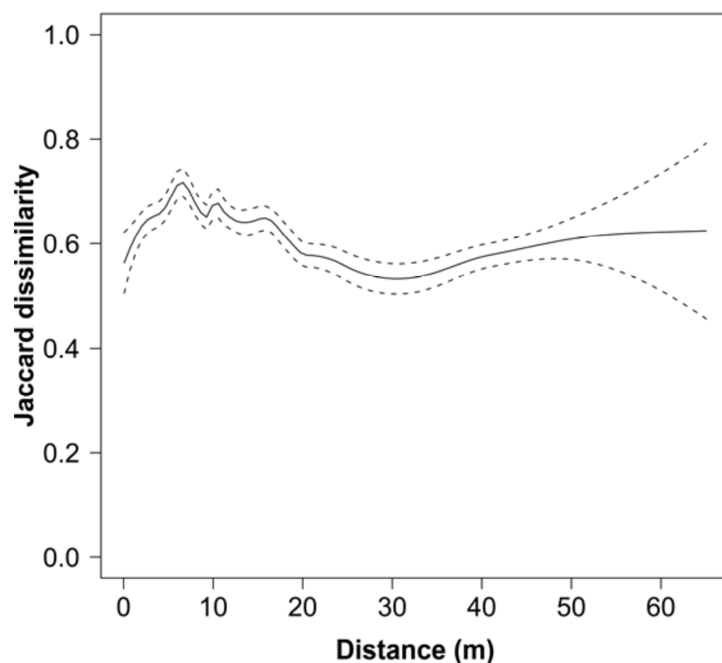

Figure S1 | Local regression fit of Jaccard dissimilarities plotted against spatial distance. The nearest neighbor bandwidth (α = 0.3; proportion of data used in each fit) was used. Dashed lines represent approximate 95 % point-wise confidence intervals for the mean. Local regression was performed using the local regression software LOCFIT as implemented in the statistical software R (R Development Core Team, 2008; Loader, 1999).

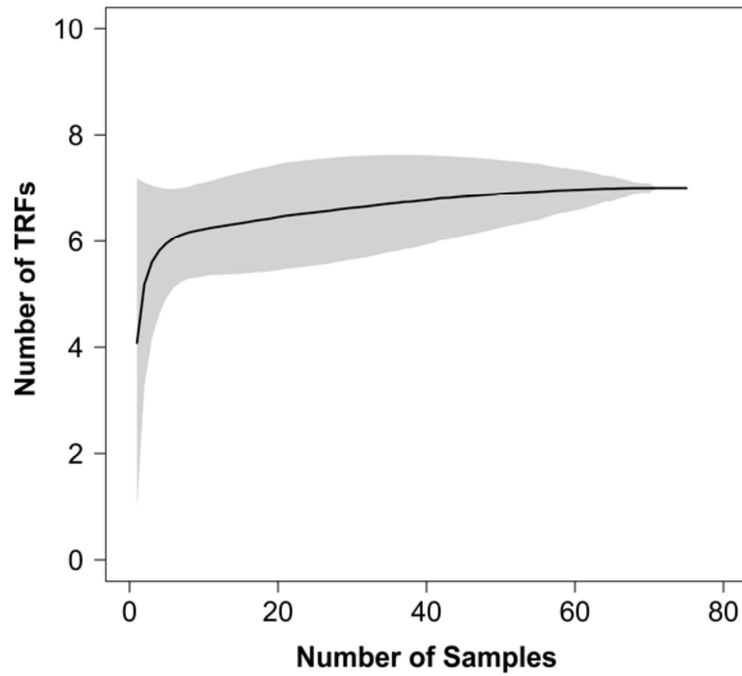

Figure S2 | Species accumulation curves of all OTUs using random accumulation of sites and a 95 % confidence interval (shaded area). A randomization approach was used where the average species richness is calculated for a series of randomly pooled sites (Kindt and Coe, 2005). Each TRF was considered as an operational taxonomic unit (OTU).

Table S1 | Spearman's rank correlation coefficients between TRFs using relative abundances; TRF affiliation: 47 bp, *Nitrosomonas / Nitrosospira*; 80 bp, *Methylococcus / Methylocaldum*; 113 bp, *Nitrosospira*; 245 bp, *Methylosinus / Methylocystis*; 350 bp, *Methlyomicrobium* album; 58 bp, *pmoA/amoA*-like RA21 cluster; and 146 bp, *Methylosinus trichosporium* OB3B.

TRFs	47	58	80	113	146	245	350
47							
58	-0.06						
80	-0.12	-0.15					
113	0.02	0.02	.-0.24				
146	*-0.34	**0.39	-0.21	***0.62			
245	0.18	*-0.32	-0.01	***-0.50	0.60		
350	0.12	.0.26	0.09	-0.18	-0.04	0.20	
531	*-0.30	-0.21	-0.04	0.21	0.21	**-0.36	-0.16

Signif. codes: '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1

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3 BIOGEOGRAPHY OF WETLAND RICE METHANOTROPHS

CLAUDIA LÜKE, SASCHA KRAUSE, STEFANO CAVIGIOLO, DIEGO GREPPI, ELISABETTA LUPOTTO, AND PETER FRENZEL.

SUBMITTED TO ENVIRONMENTAL MICROBIOLOGY. IN PRESS.

3.1 Abstract

We focused on the functional guild of methane oxidizing bacteria (MOB) as model organisms to get deeper insights into microbial biogeography. The *pmoA* gene was used as a functional and phylogenetic marker for MOB in two approaches: (i) a *pmoA* database (>4000 sequences) was evaluated to obtain insights into MOB diversity in Italian rice paddies, and paddy fields world-wide. The results show a wide geographical distribution of *pmoA* genotypes that seem to be specifically adapted to paddy fields (e.g. Rice Paddy Cluster 1 and Rice Paddy Cluster 2). (ii) On the smaller geographical scale, we designed a factorial experiment including three different locations, two rice varieties and two habitats (soil and roots) within each of three rice fields. Multivariate analysis of T-RFLP profiles revealed different community patterns at the three field sites, located 10 to 20 kilometres apart. Root samples were characterized by high abundance of type I MOB whereas the rice variety had no effect. With the agronomical practice being nearly identical, historical contingencies might be responsible for the field site differences. Considering a large reservoir of viable yet inactive MOB cells acting as a microbial seed-bank, environmental conditions might have selected and activated a different subset at a time thereby shaping the community.

3.2 Introduction

Next to CO_2, methane is the most important greenhouse gas contributing substantially to radiative forcing (Intergovernmental Panel on Climate Change, 2007). Natural wetlands and rice fields belong to the major sources of atmospheric CH_4 (Intergovernmental Panel on Climate Change, 2007). In contrast, upland soils function as a sink to atmospheric CH_4 due to the uptake by methane oxidising bacteria (MOB) (Conrad, 1996; Knief et al., 2003). On a global scale, however, MOB are even more important in wetlands, where they function as a bio-filter preventing CH_4 produced in anoxic layers escaping into the atmosphere. MOB in natural wetlands and rice paddies attenuate the potential CH_4 emission by up to 40% (Frenzel, 2000; Reeburgh et al., 1993). In particular situations, e.g. at the oxic-anoxic boundary near the very surface of sediments or water-saturated soils, an attenuation of more than 90% may be achieved (Frenzel et al., 1990; Gilbert and Frenzel, 1998). Another oxic-anoxic boundary is formed in the rhizosphere of wetland plants, where O_2 diffusing through the aerenchyma may be released from the roots supporting root-associated CH_4 oxidation (Armstrong, 1971; Conrad and Frenzel, 2002). In rice fields, root-associated CH_4 oxidation is the most important CH_4 sink. A couple of case studies in wetland rice fields have reported on CH_4 oxidation rates of 10 to 30% per season (Denier van der Gon and Neue, 1996; Eller et al., 2005; Krüger et al., 2002). However, considerable differences emerge comparing studies on different rice cultivars (Bilek et al., 1999; Bosse and Frenzel, 1998; Denier van der Gon and Neue, 1996; Eller et al., 2005; Marik et al., 2002; Tyler et al., 1997): CH_4 oxidation may occur more or less throughout the season, decline with the onset of the reproductive period, but sometimes become re-established at the very end of the season. Only part of this pattern can be attributed to agricultural practice like fertilisation with mineral nitrogen (Bodelier et al., 2000a; Bodelier et al., 2000b): this effect tends to be transient (Krüger et al., 2002) becoming unimportant late in the season (Dan et al., 2001). How

the populations of MOB growing on and in rice roots are affected by cultivars, and how in turn they may affect emissions, is largely unknown.

Basically, two types of MOB are distinguished: type I and type II corresponding to the families *Methylococcaceae* (type I, γ-proteobacteria), *Methylocystaceae* and *Bejerinckiaceae* (type II, α-proteobacteria) (Bowman, 2000). This classification was originally based on phenotypical traits, but corresponds well to the phylogeny of the *pmoA* gene encoding the α-subunit of the particulate methane monooxygenase. Type I MOB can be further divided into type Ia comprising the genera *Methylomonas*, *Methylobacter*, *Methylosoma*, *Methylosarcina* and *Methylomicrobium*, and type Ib characterized by *Methylococcus* and *Methylocaldum*. Type Ib was previously also referred to as type X. Recently, MOB belonging to the phylum *Verrucomicrobia* were isolated, however, they seem to be restricted to extreme environments (e.g. Dunfield et al., 2007). *pmoA* was found to be an excellent functional marker (McDonald and Murrell, 1997) becoming the most frequently used target in molecular ecology studies of MOB (Dumont and Murrell, 2005). Moreover, all MOB known so far except the acidophilic *Methylocella* (Dedysh et al., 2000) possess the *pmoA* gene and the phylogeny corresponds largely to the 16S rRNA gene phylogeny (Kolb et al., 2003).

Due to a large number of sequences available from various habitats world-wide, the *pmoA* gene is an excellent proxy to study the correlation between MOB, environmental factors, and geographical regions. Microbes are often perceived as opportunistic, fast-growing organisms responding quickly to environmental changes. This may be a misconception: because microbes are excellent survivors when conditions become unfavourable, the imprint of past events may be preserved in contemporary communities. A variety of MOB are known to form cysts or exospores (Whittenbury et al., 1970), making them candidates for studying microbial biogeography.

We used a database-driven approach for analysing the *pmoA* genotypes found in Italian rice paddies compared to other rice growing areas. We constructed clone libraries to expand our knowledge on *pmoA* diversity in the Italian paddy fields. We studied large-scale geographical patterns by compiling meta-information about geographical origins of paddies fields world-wide and combined it to the phylogenetic analysis of the respective *pmoA* sequences. On the smaller scale, we designed a factorial experiment to get deeper insights into the correlation of MOB with habitat and sites: within each of three Italian rice fields, 11-21 km apart, experimental plots were planted with the cultivars ROMA and KORAL. Fertilisation and water management were similar in all three fields. This design aimed to distinguish between actual effects, *e.g.* the cultivar planted, and contingencies due to the different pre-experimental histories of the different fields, if any. The choice of cultivars was motivated by previous work showing both cultivars supporting MOB and CH_4 oxidation (Bosse and Frenzel, 1998; Eller et al., 2005).

MOB populations are fully developed at late tillering/panicle initiation (Eller et al., 2005; Eller and Frenzel, 2001). Hence, we sampled at that growth stage rice roots and paddy soil. Because our focus was on population structures, we extracted DNA and used *pmoA* as a functional marker gene. Fingerprints from terminal restriction fragment analysis (T-RFLP) were used to explore the association between methanotrophic communities and cultivars, microhabitats, and/or fields by multivariate analyses.

3.3 Results

An existing *pmoA* nucleotide sequence database with approximately 600 sequences (Knief et al., 2006) was extended with publicly available and new sequences from this study (n ≈ 4000). It comprises *pmoA* sequences from various environments also covering part of the *amoA* diversity (about 6% of the total database). The *amoA* gene (encoding the ammonia monooxygenase subunit A) is homologous to the *pmoA* gene and is often co-amplified with *pmoA* primers (Holmes et al., 1995). This database was the backbone for the following analyses. Phylogenetic clusters referred to below are defined and named according to cultured MOB or, for environmental clusters without cultured representatives, in relation to the nomenclature of microarray probes targeting the respective groups (Bodrossy et al., 2003; Stralis-Pavese 2004; Vishwakarma et al., 2009).

MOB in Italian wetland rice: comparative sequence analysis

To get deeper insights into *pmoA* diversity in Vercelli rice fields, we combined 292 clone sequences obtained in this study with about 200 sequences from previous work (Henckel et al., 1999; Henckel et al., 2000a; Henckel et al., 2001; Horz et al., 2001; Shrestha et al., 2008). Phylogenetic analysis showed a wide distribution of Vercelli-sequences throughout the entire *pmoA* diversity (Figure 1). However, sequences belonging to upland soil clusters that are assumed to be responsible for the consumption of atmospheric methane, for example USC-α, USC-γ (Knief et al., 2003) and the clusters JR-1, JR-2 and JR-3 (Horz et al., 2005), were not detected. One exception might be a tropical upland soil cluster (TUSC) in which one sequence from Vercelli is so far the only representative from a high-methane environment. This cluster together with the environmental clusters RA21, M84-P22 and M84-P105 are phylogenetically positioned between *pmoA* and *amoA*. Within type II MOB, the sequences from Vercelli showed a close relationship to *Methylocystis* while

Methylosinus-related sequences were not detected. Furthermore, one sequence fell within the MO3 cluster consisting of sequences obtained from various low and high-methane environments. Most Vercelli sequences affiliated to type Ib were related to *Methylocaldum,* or fell into an environmental cluster showing no close relationship to any cultivated MOB. This cluster is composed entirely of rice field sequences from various geographical origins. We therefore refer to it as RPC-1 (Rice Paddy Cluster 1; **Figure 1**). Most of the sequences affiliated to type Ia grouped with *Methylomonas*. Further sequences showed a close relationship to *Methylobacter* species or grouped within a second environmental cluster dominated by *pmoA* sequences from rice paddies (Rice Paddy Cluster 2, RPC-2; **Figure 1**). Like RPC-1, these sequences originate from various geographical regions: Uruguay (Ferrando and Tarlera, 2009), China (Zheng et al., 2008) and Italy.

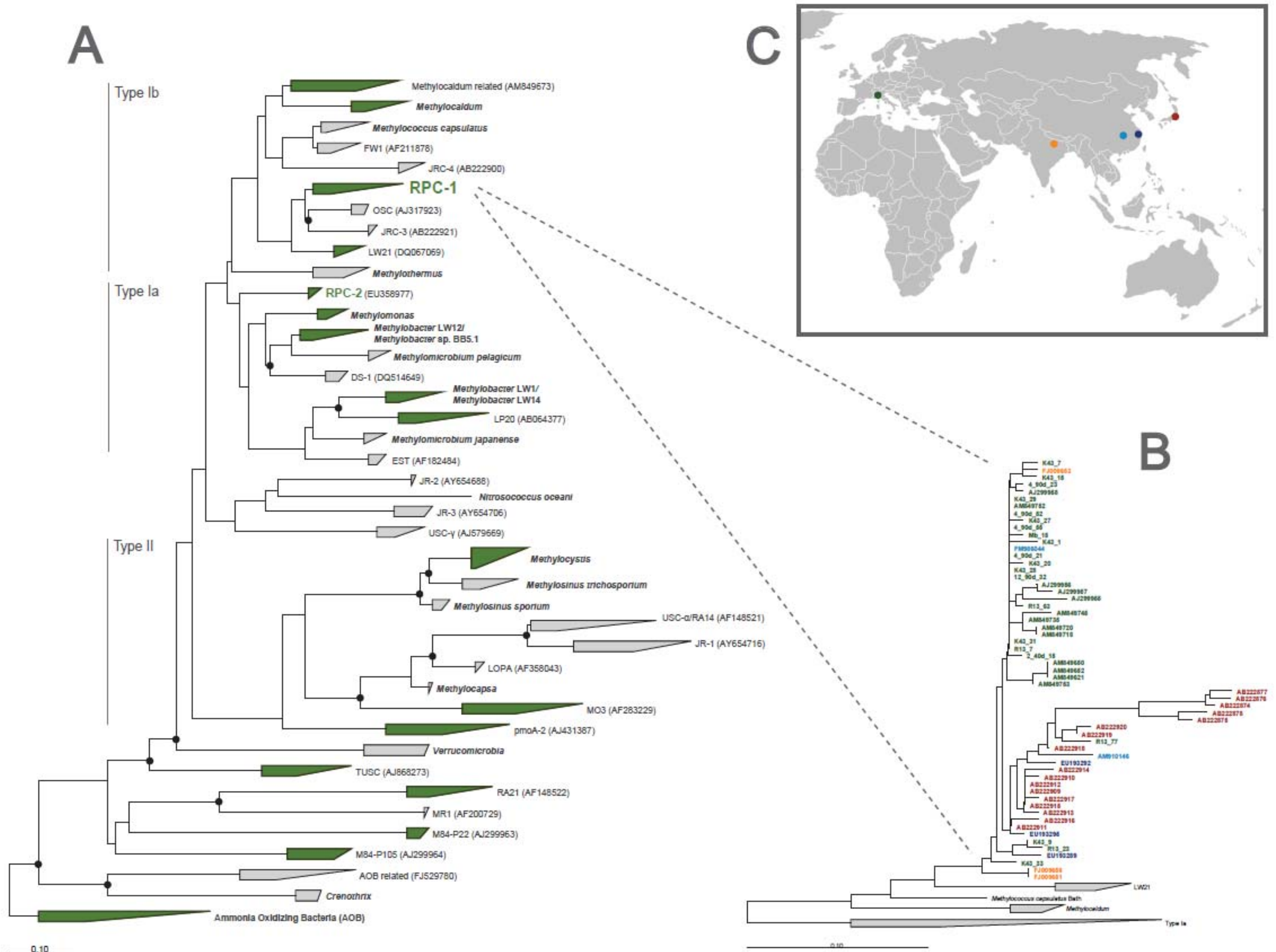

Figure 1 | **Phylogenetic relationship of partial *pmoA* sequences based on deduced amino acid residues.** Only sequences containing at least 140 amino acid positions were included in the analysis. **A.** Neighbor Joining tree showing the distribution of *pmoA* sequences retrieved from Vercelli rice paddies compared to public database sequences from various environments: Clusters containing Vercelli clone sequences are shown in green. Environmental clusters were named according to representative clones and/or in relation to the denotation of *pmoA* microarray probes. GenBank accession numbers of representative clones are given in brackets. Closed circles mark nodes that were verified by a Maximum likelihood tree. The scale bar represents 0.1 changes per amino acid position. **B.** Detailed view on the Rice Paddy Cluster 1 (RPC-1) which is composed entirely of rice paddy sequences from various geographical origins **C.** Geographical origins of the RPC-1 sequences.

MOB in Italian wetland rice: fingerprint analysis

T-RFs were binned according to phylogenetic affiliations into a total of 10 operational taxonomical units (OTUs; **Table 1**). After binning and standardization, two to eight OTUs per individual sample were retained. Constrained correspondence analysis (CCA) showed two clusters separating soil and root samples (**Figure 2**). OTUs affiliated to *Methylocaldum*, *Methylomonas* and *Methylobacter* (type I MOB) were almost exclusively detected in root samples, whereas soil samples were characterized by a higher abundance of type II MOB, the RA21 group and ammonia oxidizers. Soil samples from different locations were clearly separated, while different rice cultivars were not. This basic pattern was also preserved in NMDS analysis (supplementary material, **Figure S1**).

Table 1 | Affiliation of operational taxonomic units (OTUs) to phylogenetic groups of methanotrophs (Type I and Type II) or ammonium oxidizing bacteria (AOB). Binning was based on an *in-silico* analysis and cross-checked by T-RFLP analysis of clones. Clusters are defined in **Figure 1**.

OTU	Genus/Cluster	Subdevision of Proteobacteria	Type
46	*Nitrosospira*	beta	AOB
58	RA21 group		Others*
79	*Methylocaldum* related, RPC-1	gamma	Ib
114	*Nitrosospira,* M84-P22 group, TUSC	beta	AOB Others*
241,349, 505,531	*Methylobacter / Methylomicrobium,* LP20 group, RPC-2	gamma	Ia
244	*Methylocystis*	alpha	II
437	*Methylomonas*	gamma	Ia

* These sequences cluster between methanotroph *pmoA* sequences and *amoA* sequences from ammonium oxidizers and lack cultivated representatives.

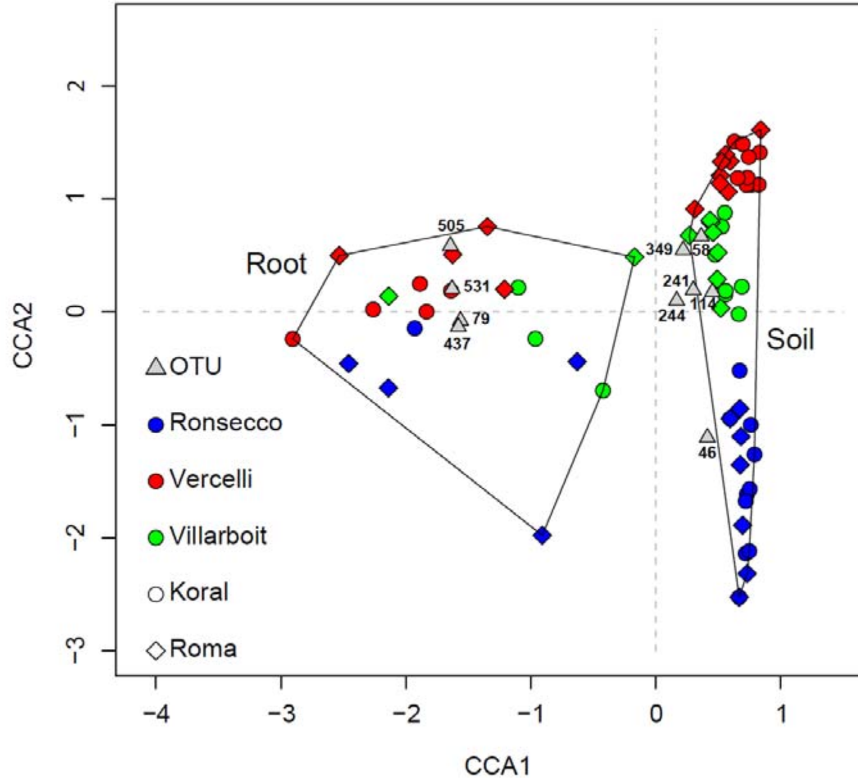

Figure 2 | Constrained correspondence analysis (CCA) calculated from standardized T-RFLP data. Constraints used: Micro-niche (Roots *vs.* Soil), Geographical location (Ronsecco *vs.* Vercelli *vs.* Villarboit), and rice variety (Roma *vs.* Koral). Explained variance: 58% of total inertia (p=0.005). OTUs are affiliated to MOB in **Table 1**.

The hierarchy of factors explaining the variation of MOB communities was explored with a multivariate regression tree (MRT) (De'Ath, 2002). An MRT explains the variation of a multivariate response (the relative abundance of OTUs) using numeric or categorial explanatory variables (e.g. different locations or cultivars). The binary splits are produced by minimizing the impurity within, and maximizing the heterogeneity between groups.

The primary split separated soil and root samples (**Figure 3**) consistent with the separation in ordination analysis (**Figure 2**). This split is mainly characterized by the presence (on roots) or absence (in soil) of type I related OTUs, and of *Nitrosospira* which was present in soil, but not on roots. Soils were further separated according to sites, while only the fourth-level split indicated a slight effect of cultivars on the

methanotrophic community (**Figure 3**). Hence, in spite of different algorithms, all analyses produced very similar results.

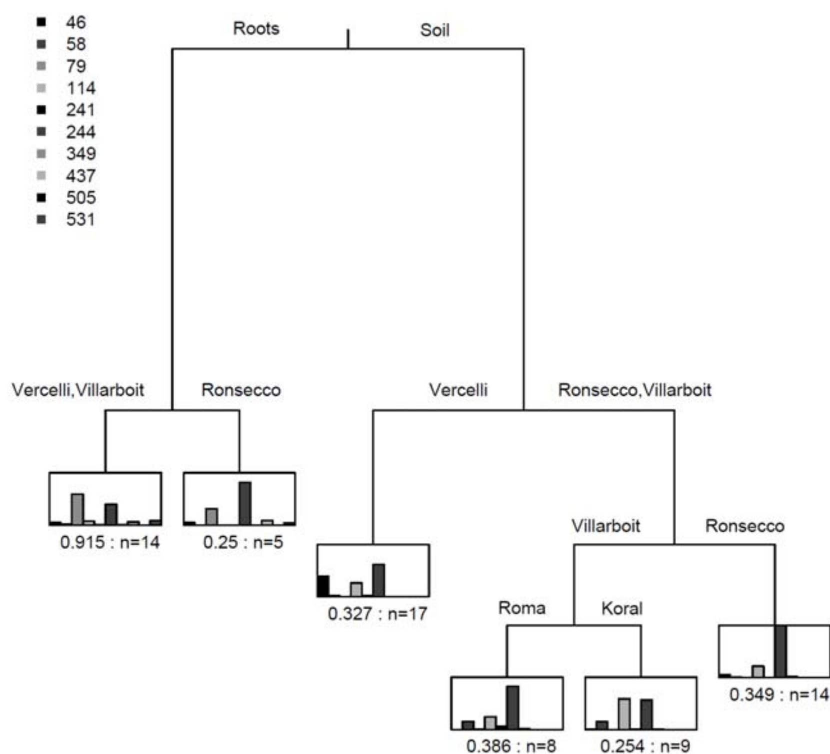

Figure 3 | Multivariate regression tree calculated from standardized T-RFLP data. The tree is based on the sums of squares in one group about the group mean. The bar plots show the multivariate species mean at each leaf and n depicts the numbers of sites at each leaf. OTUs are affiliated to MOB in **Table 1**.

3.4 Discussion

Previous studies on MOB diversity in specific environments are based on comparably small clone libraries. We combined data from several studies comprising about 500 *pmoA* sequences in total to obtain a more detailed view on MOB in Vercelli rice fields. The results indicate the presence of a highly diverse, but distinct community. They furthermore show a wide geographical distribution of *pmoA* genotypes that seem to be adapted to paddy fields. However, focusing on a smaller geographical scale, differences in the MOB communities were detected even at fields located only a few kilometers apart. Soil and root samples were characterized by different MOB communities, whereas the rice variety had no effect.

MOB in wetland rice: Biogeography at the global scale

The MOB population in Vercelli rice fields shares some components that are common to wetland rice fields around the world. Within the type Ia MOB, a large amount of *pmoA* sequences retrieved from paddy fields show high identity (91-92%) to *Methylomonas methanica*. Furthermore, several environmental *pmoA* clusters consist entirely of, or are dominated by rice field sequences. The RPC-1 represents the largest cluster comprising *pmoA* clones from rice fields distributed over all Eurasia (this study, Horz et al., 2001; Jia et al., 2007; Qiu et al., 2008; Shrestha et al., 2008; Zheng et al., 2008; Vishwakarma, unpublished) and might reflect the spread of *O. sativa japonica* from its native area in East Asia throughout the tropics and subtropics. RPC-2 is dominated by paddy field sequences from Italy, Asia and South America, whereas cluster JRC-3 consists of *pmoA* sequences retrieved so far only from Japanese and Chinese rice paddies (Jia et al., 2007; Qiu et al., 2008; Zheng et al., 2008). For the JRC-4 comprising sequences from Asia and South America, an isolate could be obtained very recently from Uruguayan rice fields (Ferrando and Tarlera, 2009). It showed congruent phylogeny of *pmoA* and 16S rRNA with *Methylococcus capsulatus* and

Methylocaldum szegediense as closest cultivated relatives (92 and 91% 16S rRNA gene identity, respectively) and might be the first representative of a new MOB genus. A lot of sequences from nearly all studied paddy field sites cluster near *Methylocaldum*. All of these rice clusters, apart from the RPC-2, could be affiliated to type Ib MOB based on *pmoA* phylogeny. RPC-2 shows an ambiguous relation to either type Ia or type Ib, depending on the method used for tree construction.

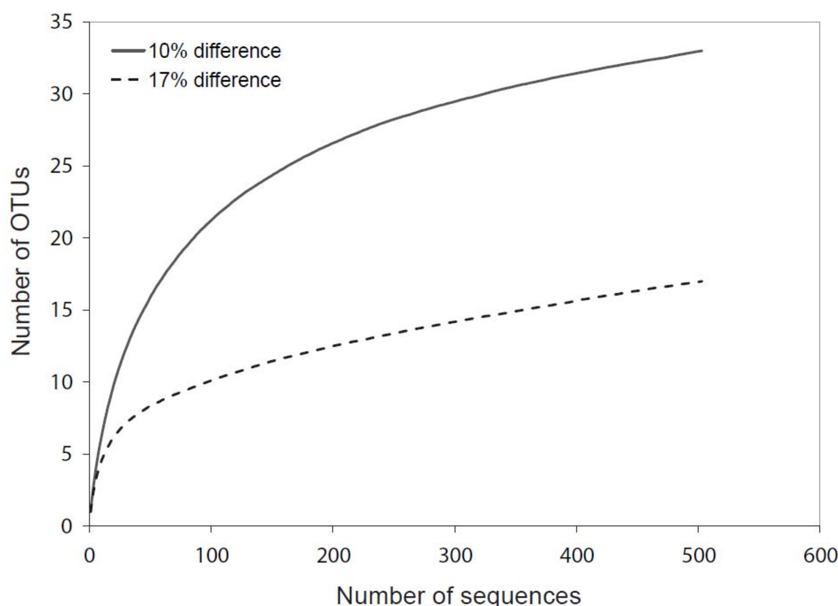

Figure 4 | Rarefaction analysis of collected *pmoA/amoA* sequences from Vercelli rice fields. The analysis is based on sequences retrieved with both primer pairs (A189f/mb661 and A189f/A682r). Nucleotide sequences were grouped as OTUs using the distance levels 10% and 17%. These *pmoA* distances correspond to the 3% and 5% 16S rRNA distance assuming a 3.5 times higher nucleotide substitution rate (Heyer *et al*. 2002).

All rice clusters combine sequences from at least three geographically separated paddy fields suggesting a certain adaptation to the rice field environment. The studies are based on clone libraries of very different sizes ranging from 30-40 clones (Zheng et al., 2008; Vishwakarma et al., 2009; Jia et al., 2007; Ferrando et al., 2009) to approximately 500 in this work. Even for the Italian clone library, rarefaction curves did not level off (**Figure 2**), indicating a still incomplete picture of total MOB diversity and a

considerable contribution of the 'rare biosphere'. Hence, an increasing sampling effort might detect some clusters in other geographical regions or in environments others then paddy fields. However, in-depth studies using the recently developed high resolution *pmoA* microarray (Bodrossy et al., 2003; Stralis-Pavese et al., 2004) did not detect these clusters in peat, landfill and gleyic soil (Chen et al., 2008; Héry et al., 2008; Cebron et al., 2007), whereas they were detected in an Indian rice paddy (Vishwakarma et al., 2009). These studies strongly support our view of a rice-specific methanotrophic community.

The primer choice has a large impact on the detectable diversity. A189f/A682r (Holmes et al., 1995) and A189f/mb661r (Costello and Lidstrom, 1999) are the predominant primers used for studying *pmoA* diversity. Whereas A682r was widely used in earlier studies (Kalyuzhnaya et al., 2002; Radajewski et al., 2002; Reay et al., 2001), more recent publications show a preference for mb661 (Chen et al., 2008; Ferrando and Tarlera, 2009; Zheng et al., 2008). One reason for this change is the discrimination of mb661r against the homologous *amoA* of ammonia oxidizers. However, mb661r also discriminates against the clusters USC-α and RA21 (Bourne et al., 2001). For the Vercelli sequence pool, several other clusters located in-between the MOB and AOB (TUSC, M84-P22, and M84-P105) have only been retrieved using A682r (Table S1, supplementary material). However, clusters within the type Ia methanotrophs, such as the RPC-2 and *Methylobacter* related genotypes, seem to be preferentially amplified by the mb661 primer. Therefore, the combined use of both primer sets will reveal the most complete picture of methanotrophic diversity (McDonald et al., 2008).

That no sequences belonging to any of the upland soil clusters were obtained might seem trivial, but crop rotation between wetland rice and other cereals is quite common, and the Italian fields lay fallow during winter. Hence, rice field MOB may at least temporarily be exposed to low methane concentrations. Indeed, re-analyzing the phylogenetic position of sequences retrieved from a Chinese paddy field (Zheng et al., 2008) revealed two sequences grouping within USC-γ.

MOB in Italian wetland rice: biogeography at the small geographical scale

Besides considering the large geographical scale, we compared the MOB communities in three very closely located paddy fields within Northern Italy. This comparison was carried out by T-RFLP analysis resulting in similar T-RFs found in all soils. However, in spite of a nearly identical agricultural treatment, the three sites showed significantly different community patterns based on the relative abundance of specific T-RFs (Figure 2&3). The reason for these differences might be found related to the history of the soils. A variety of MOB are able to form cysts or exospores (Bowman et al., 1993; Whittenbury et al., 1970) and are believed to remain viable for more than a century (Rothfuss et al., 1997). Rice soils contain a large yet inactive population of MOB acting as a seed bank (Eller et al., 2005; Eller and Frenzel, 2001). From this seed bank, past events might have activated different sub-populations from time to time. These events could be of different nature and we can only speculate about it. Different agricultural practices in the past might be of importance as well as natural variability at the three sites. A combination of all effects might have resulted in different community compositions at each site which were conserved in the seed bank. Changes in this seed bank may be slow, but once established, they might persist for quite a time providing a complex community from which the actual environment selects a subset.

MOB at the field scale: Influence of habitat and rice variety

The T-RFLP profiles did not indicate an effect of the rice varieties on MOB communities. However, both cultivars studied belong to *O. sativa* ssp*. japonica.* The Italian germplasm in particular has recently been found to be genetically quite homogeneous (Lupotto, unpublished). Analysing more distantly related cultivars may give further insights into the potential role of rice varieties shaping MOB communities.

Ordination methods as well as multivariate regression analysis all resulted in a clear separation of soil and root samples. Growth proliferation of type I MOB on rice roots has been reported previously (Bodelier et al., 2000b; Eller and Frenzel, 2001; Horz et al., 2001; Shrestha et al., 2008). The rhizosphere is a dynamic environment providing highly variable concentrations of oxygen and methane (Gilbert and Frenzel, 1998). In soil microcosms, type I methanotrophs respond most rapidly to different O_2/CH_4 ratios, whereas type II are apparently less responsive becoming active only with time (Henckel et al., 2000b). Considering the transient nature of rhizosphere oxygenation (Flessa and Fischer, 1992), type I may be pre-adapted to this ephemeral microenvironment.

Conclusions

At the large geographical scale, a number of environmental clusters could be observed that were predominantly found in paddy fields around the world. Remarkably, nearly all clusters could be assigned to type Ib MOB. This suggests the existence of MOB diversity patterns not only for low-methane environments – characterised by the predominance of upland soil clusters – but also for a high-methane environment.

At the small geographical scale, we found different MOB community compositions at three closely located sites. Considering the ability of many MOB to survive adverse conditions for decennia, we suggest that historical contingencies are of major importance shaping a particular population (Ge et al., 2008; Martiny et al., 2006). With the current agronomical practice being nearly identical, these forces may act even at the scale of some kilometres. The concept of a microbial seed-bank describes best the co-existence of an active population on rice roots with a large soil-borne reservoir of viable yet inactive cells (Eller et al., 2005; Lliros et al., 2008; Pedros-Alio, 2006). This case suggests that changing conditions may select in the future other MOB from the seed bank. Nevertheless, each paddy field stays unique holding its specific and characteristic MOB fingerprint.

3.5 Experimental Procedures

Sampling site

The samples were collected from rice fields situated on alluvial soils in the lowlands of the rivers Po and Sesia (Vercelli, Italy). Three locations, located 11-21 km apart and characterized by similar soil textures (**Table 2**), were chosen.

Table 2 | Location and soil parameters of sampling sites.

Location	Geographic coordinates	Texture [%]			Soil Analyses		
		Sand	Silt	Clay	Organic Carbon	C/N	pH
Vercelli	08°22'25.89''E 45°19'26.98''N	67	30	3	2.5	9.0	6.2
Ronsecco	08°15'16.13''E 45°16'37.48''N	61	36	3	2.4	9.6	5.9
Villarboit	08°19'19.94''E 45°27'27.07''N	61	35	4	2.6	13.1	5.7

Sampling procedure

All fields have been planted to wetland rice since the beginning of last century at least. It was sampled in July 2006. In that year, fields were flooded in April with rice being sowed at end of April/beginning of May. In each field, two plots were laid out planted to cultivars Roma and Koral, respectively. Plot width was 1.2-1.5 m, while length varied from 8 to 35 m. MOB in Italian rice fields have no spatial structure (Krause et al., 2009). Hence, a simple transect sampling was used taking core and root samples along the main axis of the plots with a sample-to-sample distance of 1-3 m.

Depending on the size of the plot, 4 to 5 samples were taken. Soil was sampled with a corer (inner diameter 6.5 cm) down to a depth of 10 cm and divided into two layers equally deep, the upper and lower representing the rooted and the bulk soil, respectively. Soil was transferred into plastic bags, kneaded to homogenize, and sub-sampled (1.5 ml). Roots from two rice plants were cut with scissors into pieces 2 cm long, mixed and packed in plastic bags. Samples were frozen on site with dry ice. Before handling the next samples, all instruments were cleaned with ethanol. Samples were kept on dry ice during transport and stored at -20°C later on till processing.

DNA extraction

DNA extraction from root samples

Total DNA from roots was isolated using the DNAeasy® Plant Maxi Kit (Qiagen). It was followed the manufacturers' instructions for manual plant tissue disruption under liquid nitrogen. DNA was purified afterwards using the Wizard® DNA Clean Up System (Promega) according to the manufacturers' instructions.

DNA extraction from soil samples

DNA isolation from soil samples was performed as previously described by Stralis-Pavese and colleagues (Stralis-Pavese et al., 2004). Briefly, 0.3 g soil was re-suspended in sodium phosphate buffer (pH 7.0) supplemented with CTAB and lysozyme. Cells were disrupted by beat beating followed by proteinase K treatment. DNA was further purified by phenol-chlorophorm-isoamyl alcohol and chlorophorm-isoamyl alcohol extraction. Potassium acetate was added for humic acids precipitation. DNA was bound to a silica matrix (FastDNA spin kit for soil, QBiogene) and washed with ethanol. Elution of DNA was performed in EB buffer (Qiagen).

Cloning, sequencing and phylogenetic analysis of *pmoA* genes

pmoA clone libraries were generated from field samples (soil and roots from different locations) and amended by clones retrieved from greenhouse experiments including rice field soil from Vercelli. In total, 292 clones were randomly selected for comparative sequence analysis. *pmoA* genes were amplified using the forward primer A189f (5'-GGN GAC TGG GAC TTC TGG) and the reverse primers A682r (5'-GAA SGC NGA GAA GAA SGC) (Holmes 1995) or mb661 (5'-CCG GMG CAA CGT CYT TAC C) (Costello 1999). Three reactions of 50 µl were carried out per sample. 100 ng template DNA was mixed with 2.5 U of Taq Polymerase (Invitrogen), 66 pmol of each primer, 0.02 mg bovine serum albumine (Roche), 5 % (v/v) DMSO, 25 µl Masteramp 2x PCR Premix F (Epicentre Biotechnologies) and filled up with molecular grade water (Sigma). The touchdown PCR was carried out with an initial denaturing step at 94°C for 5 min, followed by 11 cycles of 1 min at 94°C, 1 min at 62°C (touchdown 1°C per cycle) and 1 min at 72°C. Further 24 cycles were carried out for 1 min at 94°C, 1 min at 52°C and 1 min at 72°C. Final elongation was performed for 10 min at 72°C. PCR products were analysed by 1% agarose gel electrophoresis and visualized by staining with ethidium bromide. PCR products of parallel samples were pooled and purified using the GenElute PCR clean up Kit by Sigma. Purified PCR products were cloned into the vector pGEM-T (Promega) and transferred into competent cells of *E. coli* JM109. Transformants were selected by blue-white screening and analyzed by colony PCR using the primers T7 (5'-TAA TAC GAC TCA CTA TAG GG) and M13rev_29 (5'-CAG GAA ACA GCT ATG ACC) (MWG Biotech).

PCR products of the appropriate size were sequenced at ADIS, MPI for Plant Breeding Research (Cologne, Germany). Sequencing was carried out in both directions. Sequences were assembled and vector sequence was deleted using the SeqMan software (DNA-Star software package, Lasergene). Sequences were compared to the GenBank database using the NCBI BLAST.

Phylogenetic tree construction was based on 140 deduced amino acid residues. The analysis was performed using the Neighbor Joining method implemented in the ARB software package (Ludwig et al., 2004). The overall tree topology was compared to a tree calculated using Maximum Likelihood and nodes verified by both methods were marked.

The *pmoA* and *amoA* sequences obtained in this study were deposited at the EMBL nucleotide sequence database under the accession numbers FN599861-FN600113, and FN600122-FN600155.

T-RFLP analysis of *pmoA* genes

pmoA genes were amplified using the FAM-labeled forward primer A189f_FAM and the reverse primer A682r as described for the clone library. 100 ng PCR product was digested by mixing with 10 U of MspI enzyme (Fermentas) and 1 µl Tango buffer + BSA (Fermentas) filled up to 10 µl with molecular grade water (Sigma) and incubated at 37°C for 3 hours. The enzyme was inactivated at 65°C for 20 min. The following purification was performed using the AutoSeq G-50 columns (Amersham Biosciences). 1 µl of each purified sample was mixed with 0.2 µl of DNA fragment length standard (MapMarker 1000; Bioventures) and 11 µl Hi-Di Formamid (Applied Biosystems). The samples were denatured for 3 min at 94°C and T-RFLP analysis was carried out using the GeneScan ABIPrism 310 (Applied Biosystems). Analysis of the T-RF patterns was carried out using the appropriate analysis software (GeneScan Analysis Version 2.1, Applied Biosystems).

Statistical analysis of T-RFLP profiles

Statistical analysis was performed using the R software environment for statistical computing and graphics (version 2.8.1) (R Development Core Team, 2008). The terminal restriction fragments (T-RF) were binned to OTUs based on an *in-silico*

analysis of ca. 500 sequences from field and greenhouse experiments with soil from Vercelli. After binning, a data set was generated consisting of T-RF sizes in bp and peak heights in fluorescence units for each sample. T-RF profiles were quality-checked as described before (Krause et al., 2009) and standardized (Dunbar et al., 2000).

Non-metric multidimensional scaling (NMDS) and constrained correspondence analysis (CCA) was performed using *metaMDS* and *cca* provided by the *vegan* package (version 1.15-1) (Oksanen, 2008). In NMDS analysis, the Bray-Curtis distance was chosen for creating the dissimilarity matrix. The multivariate regression tree (MRT) was constructed using the *mvpart* package (version 1.2-6) (De'Ath, 2007).

Rarefaction curves were computed using DOTUR (version 1.3) and the furthest neighbour algorithm (Schloss and Handelsman, 2005).

3.6 Supplementary Material

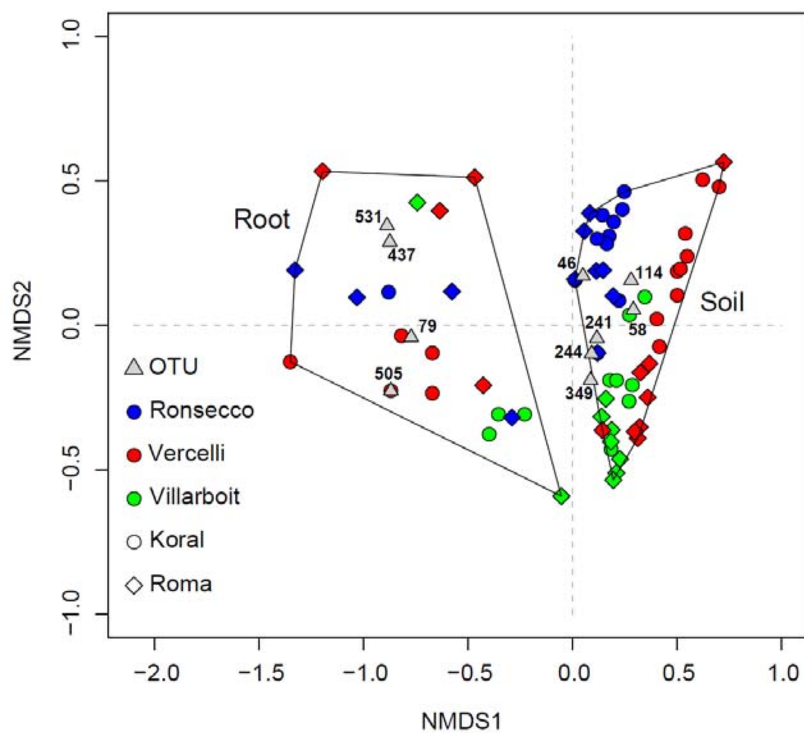

Figure S1 | Non-metric multidimensional scaling (NMDS) plot calculated from standardized T-RFLP data. The calculation is based on the Bray-Curtis distance (Plot stress=0.14).

Table S1 | Phylogenetic distribution of *pmoA/amoA* sequences from Vercelli rice fields detected by the different reverse primers A682r and mb661. Clusters are defined in **Figure 1**.

GENUS/CLUSTER	TYPE	REVERSE PRIMER	
		mb661	A682r
Methylomonas	Ia	55	13
Methylobacter LW12/BB5.1	Ia	11	0
Methylobacter LW1/LW14	Ia	22	0
LP20	Ia	0	4
RPC-2	Ia	1	0
Methylocaldum	Ib	4	1
Methylocaldum related	Ib	36	22
RPC-1	Ib	10	24
LW21	Ib	1	0
Methylocystis	II	93	151
MO3	II	0	1
pmoA-2	II	0	1
TUSC	Others*	0	1
RA21	Others*	0	6
M84-P22	Others*	0	2
M84-P105	Others*	0	2
Ammonia oxidizers	AOB	0	42
Total		233	270

* These sequences cluster between methanotroph *pmoA* sequences and *amoA* sequences from ammonium oxidizers

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4 METHANOTROPHIC BACTERIA ASSOCIATED TO RICE ROOTS:

THE RICE CULTIVAR EFFECT ASSESSED BY T-RFLP AND MICROARRAY ANALYSIS

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IN PREPARATION.

4.1 Abstract

Rice plants play a key role in regulating methane emissions from paddy fields by affecting both underlying processes: methane production and oxidation. Cultivar specific differences were reported for methane oxidation rates; however, studies on the bacterial communities involved are rare. Here, we analyzed the methanotrophic community on the roots of 18 different rice cultivars by *pmoA* based T-RFLP and microarray analysis. Both techniques showed comparable and consistent results revealing a high diversity dominated by type II and type Ib methanotrophs. The microarray has been successfully used to study MOB in various environments; still, its full phylogenetic resolution potential has not been exploited in data analysis yet. Here, we provide an example on how to include this information into multivariate statistics. The analysis revealed a rice cultivar effect on the methanotroph community composition that could be affiliated to the plant genotype. This effect became only pronounced using the high resolution analysis.

4.2 Introduction

Wetland rice agriculture accounts for a major proportion of global methane emission, one of the most important greenhouse gases contributing substantially to radiative forcing (Intergovernmental Panel on Climate Change, 2007). Estimates of annual emission from rice fields are ranging from 30-112 Tg including seasonal variations largely correlated to plant growth (Bosse and Frenzel, 1998; Holzapfel-Pschorn and Seiler, 1986; Krüger et al., 2001; Schütz et al., 1989). Emissions were furthermore shown to be influenced by agricultural practice such as fertilization and land management (Bodelier et al., 2000; Wassmann et al., 2000).

Rice plants (*Oryza* ssp.) play a key role in regulating CH_4 emission. They affect CH_4 production by releasing carbon compounds from roots being used as substrate for methanogenesis (Dannenberg and Conrad, 1999). They further act as a conduit for CH_4 transport through the intercellular aerenchyma system thereby providing the main path for CH_4 release to the atmosphere (Holzapfel-Pschorn et al., 1986; Seiler et al., 1984). Consequently, studies comparing emission from planted and unplanted rice soils revealed higher CH_4 emissions from vegetated soils (Bosse and Frenzel, 1998; Dannenberg and Conrad, 1999; Holzapfel-Pschorn et al., 1986). However, rice plants also deliver O_2 to the flooded and anoxic soil compartments by aerenchyma transport to the rhizosphere resulting in stimulation of CH_4 oxidation. This reduces the amount of CH_4 released to the atmosphere up to 90% (Frenzel et al., 1992).

The genus *Oryza* is highly diverse comprising about 80,000 cultivars that are characterized by various morphological and physiological traits (Wassmann and Aulakh, 2000). Plant traits might have a large impact on CH_4 emission, e.g. significant differences were found between cultivars characterized by varying aerenchyma and root morphologies or showing differences in root exudation (Aulakh et al., 2002; Butterbach-Bahl et al., 1997; Wang et al., 1997). Cultivar specific differences were also reported for the process of CH_4 oxidation in particular (Bilek et al., 1999; Denier van der Gon and Neue, 1996). However, studies of the bacteria involved are rare.

Methane oxidizing bacteria (MOB) can be divided into two major groups, type I and type II, corresponding to the families *Methylococcaceae* (type I, γ-proteobacteria), *Methylocystaceae* and *Bejerinckiaceae* (type II, α-proteobacteria) (Bowman, 2000). Type I MOB can be further divided into type Ia comprising the genera *Methylomonas*, *Methylobacter*, *Methylosoma*, *Methylosarcina* and *Methylomicrobium*, and type Ib (also referred to as type X) characterized by *Methylococcus* and *Methylocaldum*. Recently, MOB belonging to the phylum Verrucomicrobia were isolated, however, they seem to be restricted to extreme environments (Dunfield et al., 2007; Islam et al., 2008; Pol et al., 2007).

Early work on MOB in rice paddies based on MPN counts indicate a high number of MOB associated with rice roots that increase in correlation to plant growth (Bosse and Frenzel, 1997; Gilbert and Frenzel, 1995). First studies using culture independent methods focused on phylogeny of the 16S rRNA gene as well as marker genes such as *pmoA* showing the presence of type I and type II MOB in rice field soil (Eller and Frenzel, 2001; Henckel et al., 1999). The *pmoA* gene encoding a subunit of the particulate methane monooxygenase is present in virtually all MOB and was found to be an excellent functional marker for studying MOB in the environment (McDonald and Murrell, 1997).

After establishing the terminal restriction fragment length polymorphism (T-RFLP) analysis for the *pmoA* gene (Horz et al., 2001), this technique became one of the most widely used fingerprinting methods for microbial ecology studies of MOB. T-RFLP based studies showed amongst others the preference of type I MOB associated with the roots of rice plants (Horz et al., 2001; Wu et al., 2009a; Lüke et al., 2010). More recently, a microbial diagnostic microarray targeting the *pmoA* gene was developed and further optimized (Bodrossy et al., 2003; Stralis-Pavese et al., 2004). This method allowing for specific detection of MOB down to the species level has been successful used to study MOB in various environments (Cebron et al., 2007; Chen et al., 2008; Hery et al., 2008). Nevertheless, the full phylogenetic resolution potential of this technique has not been exploited in data analysis yet.

Here, we studied MOB communities on the roots of 18 Italian rice cultivars belonging to *Oryza sativa* japonica. The cultivars were grown in an one field experimental plot. Rice roots were harvested at late tillering/panicle initiation as MOB populations are fully developed at this time (Eller et al., 2005; Eller and Frenzel, 2001). MOB populations in rice fields were shown to have no spatial structure (Krause et al., 2009); therefore, the sampling strategy was of minor importance. The *pmoA* gene was used as a phylogenetic marker for MOB in T-RFLP and microarray analysis. The results of the two techniques were compared. Furthermore, microarray data analysis was improved attaining the full phylogenetic resolution power in multivariate statistics.

4.3 Results

MOB detected by T-RFLP and microarray

Microarray and T-RFLP analysis revealed a high diversity of MOB associated with the roots of Italian rice varieties. However, the microarray provided a clearly higher resolution (**Figure 1**). One of the main fragments detected in T-RFLP analysis is the T-RF of 79 bp (**Figure S1**, supplementary material) representing the entire type Ib MOB. The microarray enables a more specific differentiation. It showed hybridization only to a subset of type Ib probes targeting *Methylocaldum* related clusters, the Rice Paddy Cluster 1 (RPC-1; Lüke et al., 2010) and the cluster LW21 comprising sequences mainly from lake sediments (Nercessian et al., 2005; Pester et al., 2004). No *Methylococcus* was detected. Within type II MOB, highest hybridization was observed for probes targeting *Methylocystis* whereas *Methylosinus* was only detected in low amounts (**Figure S2**, supplementary material). The T-RFLP analysis does not differentiate between these genera as they possess the same MspI restriction site (244 bp). A high variety of detected T-RFs could be affiliated to type Ia. However, most of these T-RFs do not reflect the MOB phylogeny and therefore do not allow the affiliation to specific species or environmental clusters. The microarray showed hybridization mainly against one specific group of type Ia: *Methylobacter* LW1/LW14. Furthermore, the tropical upland soil cluster (TUSC) and the RA21 cluster were detected by microarray analysis. These clusters are phylogenetically located between the *pmoA* and the homologous *amoA* gene of AOB. The corresponding T-RFs of 58 bp (RA21) and 33 bp (TUSC) were also detected in T-RFLP analysis. However, the fragments are not restricted to the two groups: the 33 bp fragment in particular can also be assigned to a variety of type II environmental clusters such as the cluster MO3 upland soil clusters USC-α and JR-1.

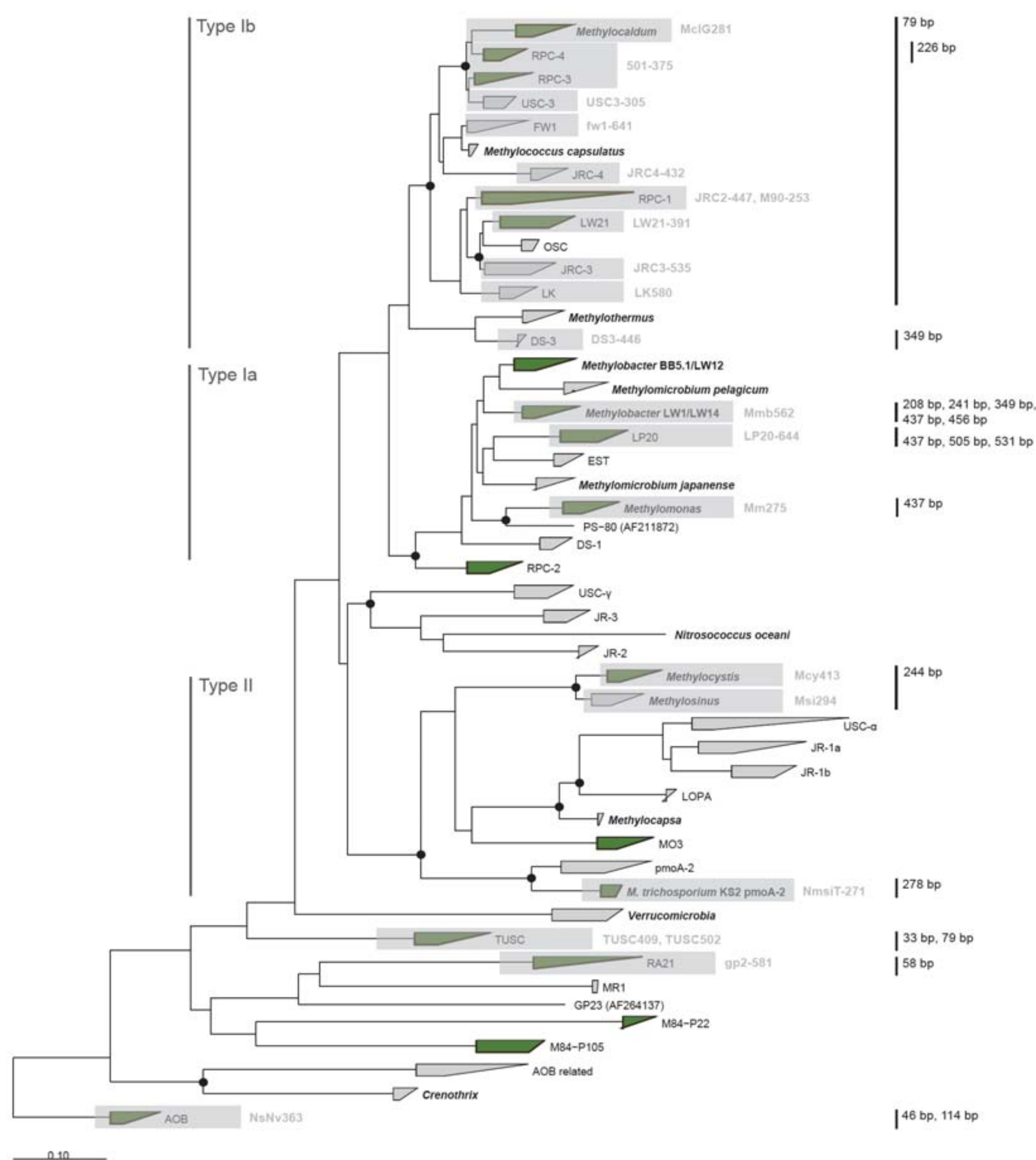

Figure 1 | Phylogenetic relationship of partial *pmoA* sequences based on deduced amino acid residues. Only sequences containing at least 140 amino acid positions were included in the analysis. Clusters targeted by microarray probes showing positive signals for the rice root samples are highlighted. Only the probes selected for statistical analysis (Table S2) are shown. Corresponding T-RFs detected in T-RFLP analysis are depicted on the right. Environmental clusters were named according to representative clones and/or in relation to the denotation of the microarray probes. GenBank accession numbers of representative clones are given in brackets. Closed circles mark nodes that were verified by a Maximum likelihood tree. The scale bar represents 0.1 changes per amino acid position.

Comparison of relative species abundances detected by the two different methods revealed similar proportions: type II were found to be most abundant, followed by type Ib MOB (**Figure 2**). Type Ia, AOB and environmental clusters located between the MOB and AOB were detected in minor amounts. For the dominant species, relative abundances detected by T-RFLP and microarray showed a positive linear correlation (**Figure 2**).

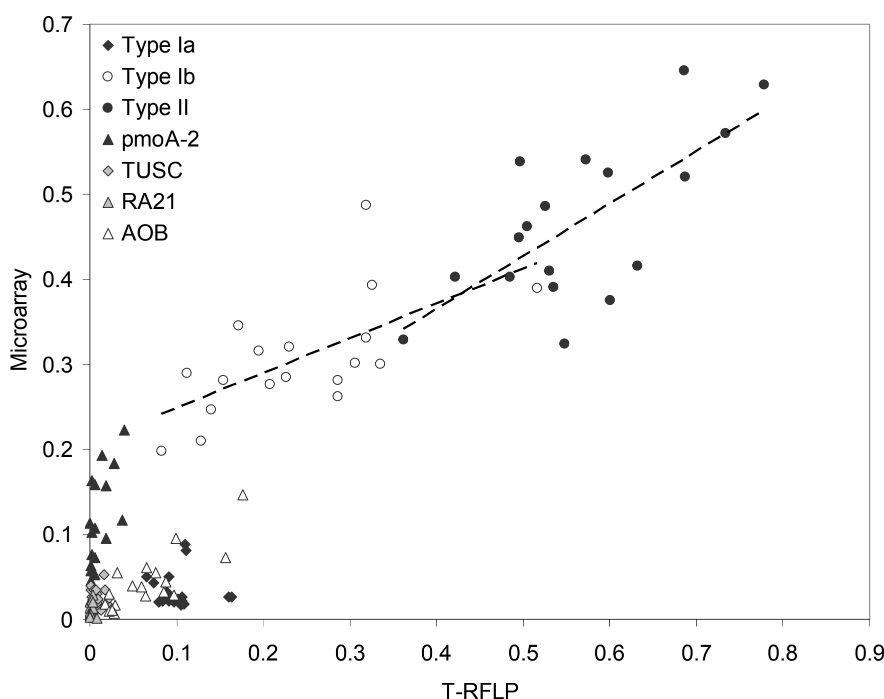

Figure 2 | Relation of relative species abundances detected in T-RFLP and microarray analysis. The following microarray probes were included in the analysis: Ia575 (type Ia), Ib453 (type Ib), II509 (type II), NMsiT.271 (pmoA-2), TUSC409/TUSC502 (TUSC), gp2.581 (RA21), NsNv363 (AOB). For T-RFLP, the relative abundance of the following T-RFs were combined: 208 bp, 241 bp, 349 bp, 437 bp, 505 bp and 531 bp (type Ia), 79 bp and 226 bp (type Ib), 244 bp (type II), 278 bp (pmoA-2), 33 bp (TUSC), 58 bp (RA21), 46 bp and 114 bp (AOB). Pearson correlation coefficients: Type Ib: 0.638; Type II: 0.70.

The effect of rice cultivars on MOB communities

MOB communities on the roots of the different rice varieties were analyzed by multivariate statistics. Constrained correspondence analysis (CCA) using the microarray data was at first calculated for probes reflecting a low species resolution comparable to the resolution provided by the T-RFLP method (**Figure 3**). Out of the four tested parameters characterizing the rice varieties (**Table S1**, supplementary material), only the rice genotype revealed a significant result in CCA (p=0.1). The ordination resulted in a cluster tendency for varieties of the same genotype alongside a gradient mainly formed by the first axis (explaining 12.6% of total inertia). CCA calculated for the T-RFLP data showed a similar result (**Figure S3**, supplementary material).

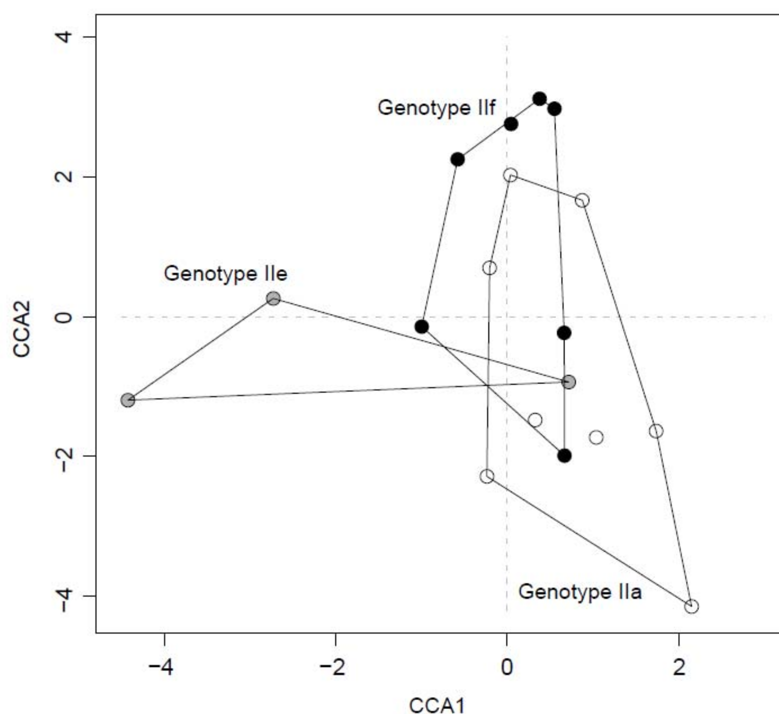

Figure 3 | Constrained correspondence analysis calculated from standardized microarray data. The probes included in the analysis correspond to the low resolution of the T-RFLP method. Probes used: Ia575 (type Ia), Ib453 (type Ib), II509 (type II), NMsiT.271 (pmoA-2), TUSC409/TUSC502 (TUSC), gp2.581 (RA21), NsNv363 (AOB). The genotype was used as a constraint explaining 18.7% of the total inertia (p=0.1).

In a second step, the high resolution of the microarray was exploited, allowing for differentiation of MOB down to the species/environmental cluster level. Probes for the analysis were selected according to the following criteria: (i) they should target a very specific species/cluster and cover nearly all sequences within that cluster. (ii) They should have a low tendency to give false positive results. It was tried to find a single probe for each cluster. However, where two probes provided the same coverage and low tendency to false hybridization, the average value of the two probes was used (**Table S2**). The probe coverage was evaluated using a *pmoA* database comprising over 4000 sequences from various environments including 500 sequences from Italian rice fields (Lüke et al., 2010). The phylogenetic resolution was adjusted to the *pmoA* diversity found in Italian rice field based on *pmoA* genotypes in the database and positive hybridization in microarray analysis.

CCA calculated from the standardized data of the selected probes showed a highly significant result for the genotype as constraint (**Figure 4**). In comparison to the ordination performed at low phylogenetic resolution (**Figure 3**), the clustering was clear and three separated groups could be observed. To identify the MOB species characteristic for each genotype, indicator species were determined. These are species that should be ideally found on plants from only one genotype and on all plants within this genotype (Dufrene and Legendre, 1997). Determination of indicator species resulted mainly in type Ib MOB being indicative for the different genotypes (**Figure 4**). The *pmoA-2* of type II MOB was found to be an indicator species for the genotype IIa.

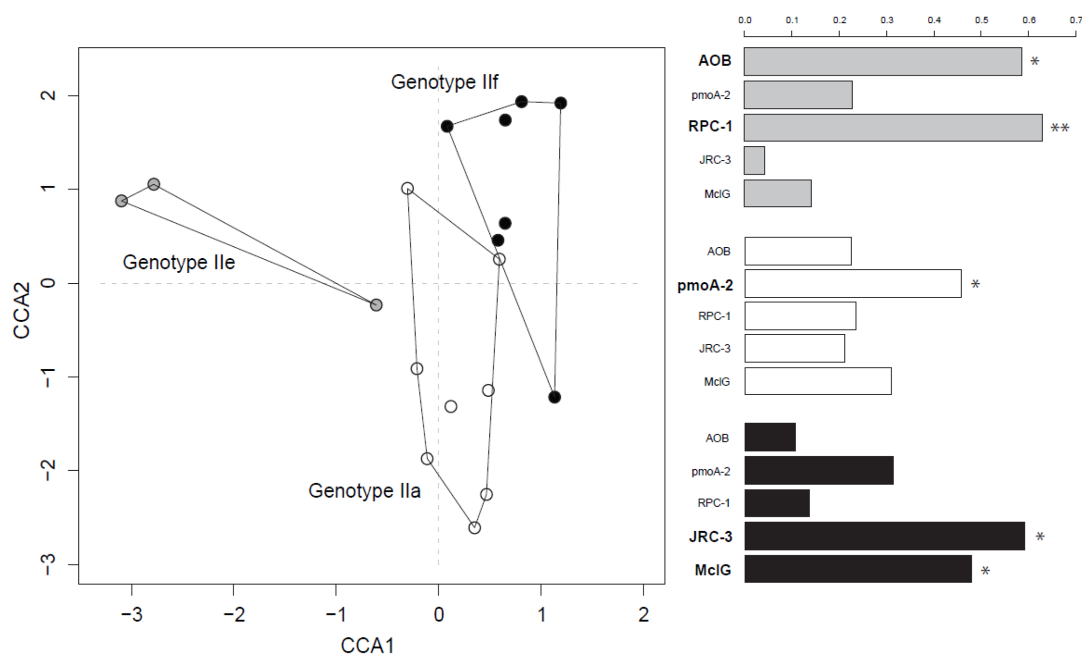

Figure 4 | Constrained correspondence analysis (CCA) calculated from standardized microarray data. The probes included in the analysis are listed in **Table S2**. The rice genotype was used as a constraint explaining 27.7% of the total inertia (p=0.005). **Left**: CCA ordination plot. **Right**: Indicator values for MOB species associated with the three rice genotypes. Grey=genotype IIe, white= genotype IIa, black= genotype IIf. Indicator species are marked in bold (* $p \leq 0.05$; ** $p \leq 0.001$; iteration=10,000).

4.4 Discussion

MOB detected by T-RFLP and microarray

T-RFLP and microarray analysis showed comparable and consistent results for MOB communities detected on the rice roots. T-RFLP analysis has been widely used in microbial ecology and was shown to be a highly reproducible and robust method for profiling microbial communities (Blackwood et al., 2003; Hartmann and Widmer, 2008; Osborn et al., 2000). Establishing the method for the *pmoA* gene enabled a reasonable differentiation and characterization of MOB communities in environmental samples (Horz et al., 2001). However, the assignment of T-RFs to phylogenetic groups of MOB can be ambiguous: the growing number of sequences in public databases revealed T-RFs that could not be clearly affiliated to one specific group of MOB, but are characteristic for a wide range of even distantly related MOB (e.g. T-RFs 33 and 79). This attaches great importance to a representative clone library for an accurate assignment. Furthermore, T-RFLP analysis represents a rather rough method providing only limited phylogenetic resolution and differentiating between species or distinct environmental clusters is mostly not possible. In contrast, the *pmoA* microarray allows a very precise distinction of MOB down to the species level. Therefore, a clone library might be not as essential as for T-RFLP analysis. Nevertheless, with growing number of environmental sequences, some microarray probes might become less specific. The *pmoA* clone library from Italian rice fields showed unspecific matching of the type II MOB probes MsT343 and MM_MsT343 to a variety of type Ib MOB including the RPC-1, RPC-3 and RPC-4. Furthermore, the two probes targeting the lake Washington sediment cluster LW21 (LW21_474 and LW21_391) also partly cover the RPC-1.

T-RFLP and microarray provide no absolute quantitative data on species abundances; however, they are often rated as semi-quantitative methods. Comparing relative abundances of the species detected by both methods showed a large consistence (**Figure 2**). This indicates robustness and reliability of the methods and

allows the comparison of results obtained by both techniques. Nevertheless, both methods are PCR-based and subject to the same biases such as the primer bias. In this study, we used the A189f/A682r primer set that is known to discriminate against a variety of type Ia MOB (Bourne et al., 2001; McDonald et al., 2008; Lüke et al., 2010). Consequently, we detected only a low type Ia diversity and abundance (**Figure S1&S2**, supplementary material).

The species detected by the fingerprinting methods are in large agreement with the clone library described previously (Lüke et al., 2010). The microarray showed hybridization to few more clusters without any representative clones from Italian rice fields (**Figure 1**). However, this is not surprising as the clone library does not completely cover the *pmoA* diversity in this environment (Lüke et al., 2010). The true diversity might be even higher as the detection limit of the microarray approach is 2-5% of the total MOB community, therefore excluding the 'rare biosphere'.

The effect of rice cultivars on MOB communities

Statistical analysis of the microarray data at low species resolution level suggests differences in MOB communities at the roots of the different rice genotypes (**Figure 3**). This result is comparable with T-RFLP analysis. However, it accounts for hardly any of the microarray resolution power. The *pmoA* microarray probes are designed as a nested set meaning not all probes provide the same resolution and many probes overlap to a high extent. This is mandatory for the detection of false positive hybridization events. However, it will strongly bias multivariate statistics based on relative abundances and will complicate the estimation of single species importance. Here, we selected a subset of probes that seem best suitable for statistical analysis. Nevertheless, we have to be aware that this procedure might also introduce a certain bias. We detected a high diversity of type Ib MOB on the rice roots that could be assigned very specifically. Type II MOB were detected in even higher amounts. However, only *Methylocystis* and *Methylosinus* contributed to the high abundance

and thus, only two probes for type II affected the final result. In comparison, type Ib were represented by ten probes (**Figure 1**). Combining all relative abundance of type Ib probes results in a slightly higher overall abundance than combining the abundance of the two type II probes. Thus, the procedure inverted the true abundances of these MOB groups. However, the all samples were subject to the same methodological bias which is therefore considered of minor importance.

Considering the full species resolution power of the microarray, constrained correspondence analysis resulted in a distinct clustering of root samples from the different rice genotypes (**Figure 4**). Previous studies of microbial communities associated with rice cultivars showed different results: A cultivar effect was observed for diazotrophic communities (Tan et al., 2003; Wu et al., 2009b), also shown on the transcriptional level based on T-RFLP analysis of *nifH* mRNA (Knauth et al., 2005). Furthermore, microbial communities of ammonia oxidizing bacteria were different on roots of modern and traditional rice varieties, shown by DGGE and FISH analysis (Briones et al., 2002). This could be linked to different O_2 concentrations on the root surface of the different cultivars. However, methanogenic archaea were not influenced by cultivated or wild rice (Conrad et al., 2009). Also for MOB, no cultivar effect could be observed in a Chinese study including an *Indica*, a *Japonica* and a hybrid rice variety as well as a wild rice (Wu et al., 2009a). The latter studies were again based on T-RFLP analysis.

In this study, differences of MOB communities could be detected even for cultivars belonging to the closely related Italian germplasm. However, they were only pronounced using the high resolution microarray technique whereas T-RFLP was inefficient for observing a significant pattern. Compared to diazotrophs, for which a variety effect could be shown at T-RFLP level, MOB might show a less pronounced effect to different cultivars. On the other hand, T-RFLP resolution for the *nifH* gene might be also more efficient in unraveling community patterns.

Of the recorded physiological or morphological plant traits, only the genotype showed a significant influence on MOB. The stem morphology that might be

correlated with aerenchyma size and O_2 transport did not show an effect. Aromatic varieties, known to produce about 100 volatile flavor compounds from which 2-acetyl-1-pyrroline (2-ACP) was identified as the main compound (Buttery et al., 1983; Lorieux et al., 1996), did also not select for specific MOB. However, these substances were found in the entire plant, but could not be detected in root exudates (Sood and Siddiq, 1978). Roots exudates largely influence the ambient soil environment and might play a crucial role in selection for different MOB communities. Large differences in exudates composition were shown for different rice cultivars (Wassmann and Aulakh, 2000). This includes a variety of compounds such as carbon compounds, amino acids, phytohormones or ectoenzymes (Hartmann et al., 2009). To which extent the exudation of the tested genotypes resemble or differ from each other, and which effect they have on MOB communities, we don't know. However, plant-microbe interactions are highly complex and a lot of yet unknown factors might play a role. Therefore, the importance of the MOB indicator species is also hard to assess. Nevertheless, it is remarkable that two of the clusters (RPC-1 and JRC-3) were exclusively found in rice paddies so far suggesting that they might play an important role in this specific environment.

Conclusions

As shown before only for diazotrophs and ammonia oxidizers, we could detect an influence of different rice cultivars on the MOB community associated to their roots. This effect could be affiliated to the plant genotype. However, it became only pronounced by using the high resolution microarray technique. About the phenotypical traits associated with the rice genotypes, we can only speculate. Nevertheless, our study enables to select for specific cultivars holding the most different MOB communities and study them in detail, such as recording and comparing the root exudation.

We used the *pmoA* microarray as an advanced high resolution technique to study MOB communities and provided an example on how to exploit its full resolution power. The physiological group of MOB is highly diverse and knowledge on their ecology is still rare. Molecular studies on the high resolution level might give more insights into the ecology of specific species and/or clusters and might result in a deeper understanding on why their diversity is so redundant and high.

4.5 Experimental procedure

Sampling

The samples were collected from a paddy rice field situated in the lowlands of the rivers Po and Sesia (Vercelli, Italy; 08°22'25.89''E45°19'26.98''N). It was sampled in July 2006. In that year, fields were flooded in April with rice being sowed at end of April/beginning of May. The 18 different rice cultivars (*Oryza sativa japonica*) were planted to one field following a randomized block design. Roots from two-three rice plants of the same cultivar were cut with scissors into pieces of 2 cm length, mixed and packed in plastic bags. Samples were frozen on site with dry ice. Before handling the next samples, all instruments were cleaned with ethanol. Samples were kept on dry ice during transport and stored at -20°C later on till processing.

DNA extraction and T-RFLP analysis

1 g of root biomass was ground to a powder under liquid nitrogen using mortar and pistil. The powder was transferred into a beat beating vial filled with Zirconium beats and it was followed the protocol described by Lueders and colleagues (Lueders et al., 2004). The replicate extractions were performed for each rice cultivar. For T-RFLP analysis, the *pmoA* gene was amplified using the primers A189f/A682r (Holmes et al., 1995) and MspI digested as described before (Lüke et al., 2010).

Microarray analysis

Microarray and target preparation was performed as described by Stralis-Pavese and co-workers (Stralis-Pavese et al., 2004) using the primers A189f/A682r (Holmes et al., 1995). The hybridization was performed over night in a hybridization oven containing a shaking platform (Thermo Scientific). The washing steps and the

scanning of the hybridized slides were again carried out as by Stralis-Pavese et al., 2004.

Phylogenetic and statistical analysis

Phylogenetic tree construction was based on 140 deduced amino acid residues. The analysis was performed using the Neighbor Joining method implemented in the ARB software package (Ludwig et al., 2004). The overall tree topology was compared to a tree calculated using Maximum Likelihood and nodes verified by both methods were marked.

The results of the T-RFLP and microarray analysis were analysed using the R software environment for statistical computing and graphics (version 2.8.1) (R Development Core Team, 2008). The terminal restriction fragments (T-RF) were binned to OTUs based on an *in-silico* analysis of ca. 500 sequences from field and greenhouse experiments with soil from Vercelli. After binning, a data set was generated consisting of T-RF sizes in bp and peak heights in fluorescence units for each sample. T-RF profiles were quality-checked as described before (Krause et al., 2009) and standardized (Dunbar et al., 2000). The values of the microarray analysis were first standardized against the mean of the overall array intensities and in a second standardization against an experimentally determined reference value of positive detection (Bodrossy et al., 2003). Constrained correspondence analysis (CCA) was performed using *cca* provided by the *vegan* package (version 1.15-1) (Oksanen, 2008) and the heatmaps were constructed using *heatmap2* provided by the *gplots* package (Venables, 2009). Indicator species were determined using *duleg* (Dufrene and Legendre, 1997) provided by the *labdsv* package.

4.6 Supplementary Material

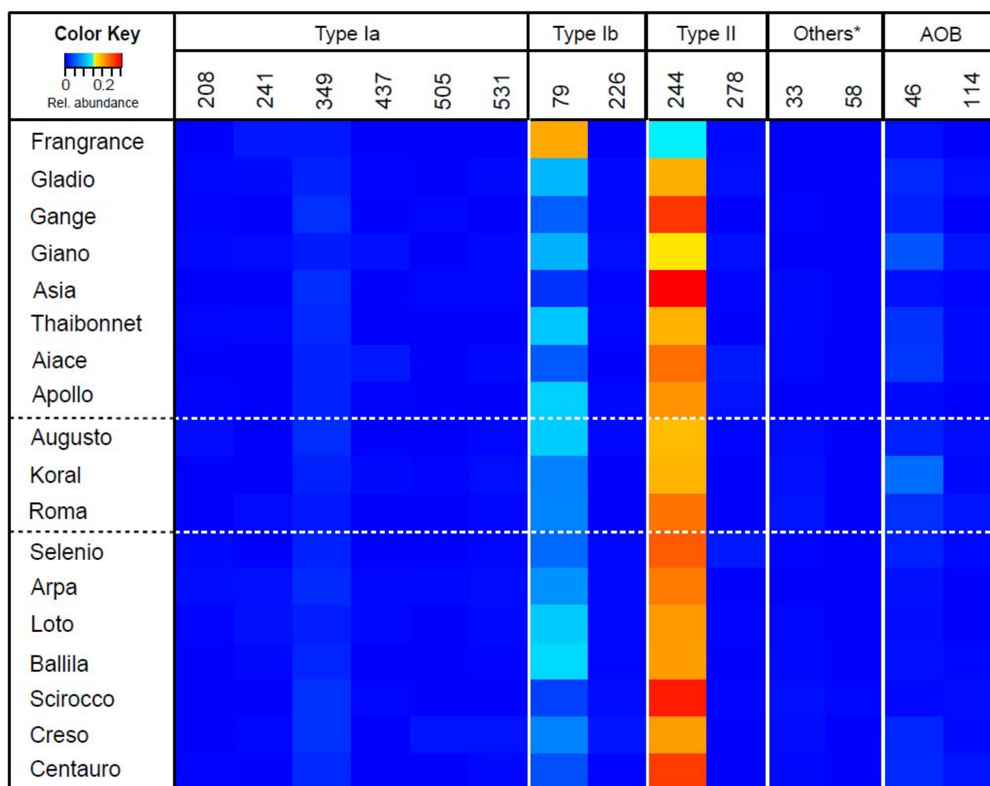

Figure S1 | T-RFLP analysis of MOB communities associated with the roots of different rice cultivars. T-RFLP results were standardized (Dunbar 2000) and relative fractions were calculated. The rice cultivars were grouped according to their genotype (IIa, IIe and IIf, respectively).

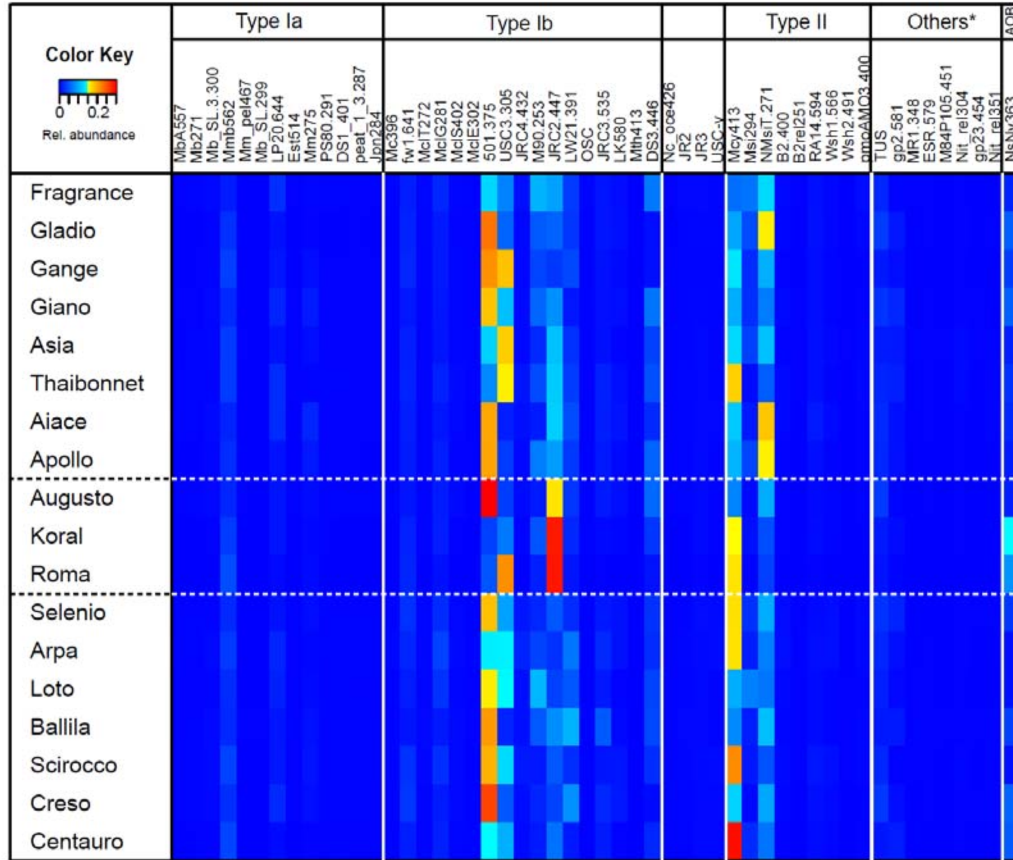

Figure S2 | Microarray analysis of MOB communities associated with the roots of different rice cultivars. Microarray results were normalized against reference values determined for each probe individually (Bodrossy 2003) and relative fractions were calculated. Only the probes selected for constraint Correspondence analysis (**Table S2**) were included in the heatmap. The rice cultivars were grouped according to their genotype (IIa, IIe and IIf, respectively).

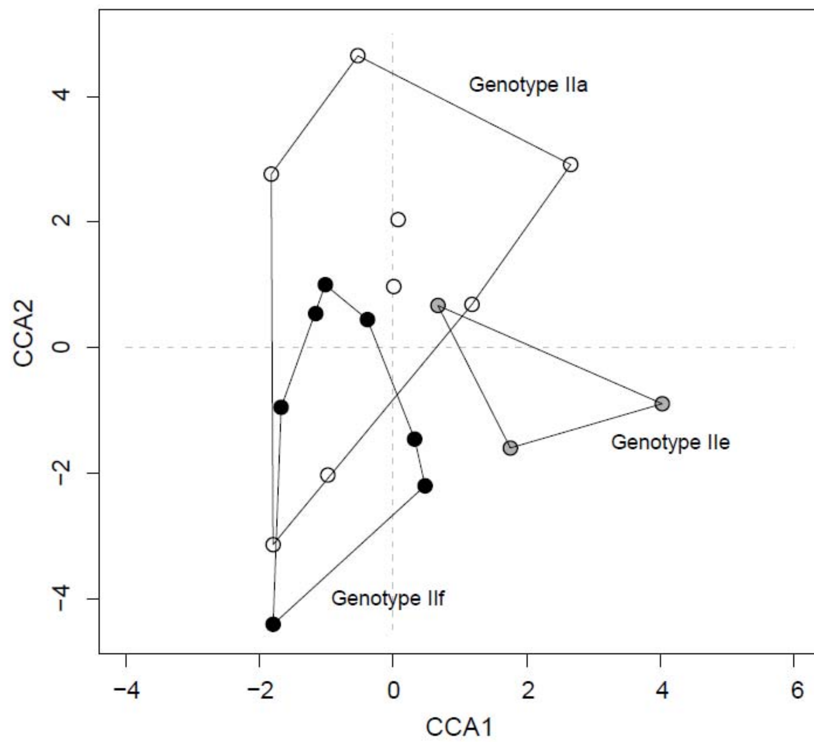

Figure S3 | Constrained correspondence analysis calculated from standardized T-RFLP data. The T-RFs were combined as described in **Figure 2**. The genotype was used as a constraint explaining 14.6% of the total inertia (*p*=0.26).

Table S1 | Characteristic traits of the rice varieties.

CULTIVAR	GENOTYPE	GRAIN	STEM	AROMATIC
Frangrance	Iia	Long B	Medium	Yes
Gladio	Iia	Long B	Thin	No
Gange	Iia	Long B	Medium	Yes
Giano	Iia	Long B	Thin	Yes
Asia	Iia	Long B	Medium	Yes
Thaibonnet	Iia	Long B	Medium	No
Aiace	Iia	Long A	Thin	No
Apollo	Iia	Long B	Medium	Yes
Augusto	Iie	Long A	Medium	No
Koral	Iie	Long A	Medium	No
Roma	Iie	Long A	Medium	No
Selenio	Iif	Round	Thin	No
Arpa	Iif	Round	Medium	No
Loto	Iif	Long A	Thick	Yes
Ballila	Iif	Round	Thick	No
Scirocco	Iif	Long A	Medium	No
Creso	Iif	Long A	Medium	No
Centauro	Iif	Round	Medium	No

Table S2 | Microarray probes selected for statistical analysis. MOB species / genera / clusters targeted by the individual probes are defined in **Figure 1.**

SPECIES/GENUS/CLUSTER	PROBES USED
Type Ia	
Methylomonas	Mm275
Methylobacter sp. BB5.1	MbA557
Methylobacter sp. LW12	Mb271
Methylobacter sp. LW1/LW14	Mmb562
Methylobacter LW12 related	Mb_SL#3-300
Methylomicrobium japanense	Mb_SL-299
Methylomicrobium pelagicum	Mm_pel467
DS-1	DS1_401
EST	Est514
LP20	LP20-644
PS-80	PS80-291
Peat	Peat_1_3-287
JPN	Jpn284
Type Ib	
Methylococcus capsulatus	Mc396
Methylocaldum gracile	MclG281
Methylocaldum tepidum	MclT272
Methylocaldum szegediense	MclS402
Methylocaldum sp. E10a	MclE302
Methylothermus	Mth413
Methylocaldum related	501-375
RPC-1a	M90-253
RPC-1b	JRC2-447
OSC	OSC220, OSC300)*
LW21	LW21-391
FW1	fw1-641
USC-3	USC3-305
JRC-3	JRC3-535
JRC-4	JRC4-432
LK	LK580
Type Ic	
Nitrosococcus oceani	Nc_oce426
JR-2	JR2-409, JR2-468)*
JR-3	JR3-505, JR3-593)*
USC-γ	USCG-225, USCG-225b)*
DS-3	DS3-446

* average of both probe values was used

Continuation Table S2 | Microarray probes selected for statistical analysis.

SPECIES/GENUS/CLUSTER	PROBES USED
Type II	
Methylocystis	Mcy413
Methylosinus	Msi294
Methylocapsa	B2-400
LOPA	B2rel251
MO3	pmoAMO3-400
pmoA-2	NmsiT-271
USC-α	RA14-594, RA14-591)*
JR-1a	Wsh1-566
JR-1b	Wsh2-491
Others	
TUSC	TUSC409, TUSC502)*
M84-P22	ESR-579
M84-P105	M84-P105
Gp23	gp23-454
RA21	gp2-581
MR1	MR1-348
AOB related	Nit_rel351
Crenothrix	Nit_rel304
AOB	NsNv363

* average of both probe values was used

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5 THE PMOA GENE AS FUNCTIONAL AND PHYLOGENETIC MARKER FOR METHANOTROPHIC BACTERIA

A SEQUENCE DATABASE ANALYSIS

CLAUDIA LÜKE AND PETER FRENZEL. IN PREPARATION.

5.1 Abstract

The *pmoA* gene encoding a subunit of the methane monooxygenase is widely used as marker for studying methane oxidizing bacteria (MOB) in the environment. We re-evaluated its value for phylogenetic inferences of MOB. We analysed the correlation of the 16S rRNA gene sequence distance and the corresponding *pmoA* sequence distance of cultivated MOB including the only recently described *pmoA* sequences of *Crenothrix polyspora* and *Verrucomicrobia* species. The distance comparison revealed overall a good correlation indicating that *pmoA* represents a suitable functional and phylogenetic marker gene. However, the *pmoA* of *Burkholderia* sp. and *C. polyspora* showed no correlation to 16S rRNA suggesting earlier lateral gene transfer events. In the second part of this study, we analysed the environmental distribution patterns of *pmoA* genotypes from uncultivated MOB. We included approximately 3400 *pmoA* sequences from public databases showing a distinct clustering of upland soil sequences and sequences retrieved from halophilic environments, respectively. Furthermore, wetland rice and freshwater sequences dominate different type Ib clusters that offer only a distant relationship to any cultivated MOB. This analysis emphasises the need for increasing cultivation efforts as the characterized isolates seem to only poorly represent the known MOB diversity.

5.2 Introduction

Methane (CH_4) is one of the most important greenhouse gases contributing substantially to global warming (Intergovernmental Panel on Climate Change, 2007). However, Shindell and co-workers (2009) reported an even higher impact when including gas-aerosol interactions into the calculations. Methane oxidizing bacteria (MOB) are able to use CH_4 as their sole source for carbon and energy metabolism (Bowman, 2000; Trotsenko and Murrell, 2008). They thereby function as a biofilter in various environments reducing CH_4 emission up to 90% (Reeburgh, 2003). They furthermore act as a sink in various upland soils by taking up CH_4 from the atmosphere (Dunfield, 2007a). MOB can be divided into two major phylogenetic groups belonging to the γ-proteobacteria (*Methylococcaceae;* type I), and α-proteobacteria (*Methylocystaceae* and *Beijerinckiaceae*; type II) (Bowman, 2000). Type I MOB can be further divided into type Ia comprising the genera *Methylomonas*, *Methylobacter*, *Methylosoma*, *Methylosarcina* and *Methylomicrobium*, and type Ib characterized by *Methylococcus* and *Methylocaldum*.

The most important tool for studying microbial diversity in the environment is the comparative analysis of 16S rRNA genes (Fox et al., 1980; Maidak et al., 1999; Pruesse et al., 2007). However, the phylogenetic relationship of microorganisms does not allow conclusion on their physiology. Targeting functional marker genes that are indicative for important biogeochemical processes enables the focus on bacteria carrying out a specific function. The *pmoA* gene is widely used as marker to detect methanotrophic bacteria in the environment (Dumont and Murrell, 2005; McDonald and Murrell, 1997). It encodes a subunit of the particulate methane monooxygenase (pMMO) catalyzing the first step in methane oxidation. This enzyme is specific for methanotrophs and can be found in all species except *Methylocella* which only possesses an alternative, soluble methane monooxygenase (Dedysh et al., 2000).

Many genes encoding metabolic key functions such as nitrogen fixation, denitrification, ammonia oxidation, methanogenesis, and sulphate reduction were

approved for diversity studies (Braker et al., 2000; Friedrich, 2005; Perez-Jimenez et al., 2001; Rotthauwe et al., 1997). They were in general shown to be congruent to the 16S rRNA gene phylogeny, thus, allowing the use not only as a functional, but also as a phylogenetic marker. Nevertheless, some of these genes such as the nitrogenase encoding *nifH* gene (Zehr et al., 2003) and the dissimilatory sulphate reductase genes *dsrAB* (Klein et al., 2001) are also mobile across species thereby preventing a reliable phylogenetic assignment. The *pmoA* gene was shown to reflect well the 16S rRNA gene phylogeny of MOB for 31 pure cultures (Kolb, 2003). However, a large number of new species were isolated recently including some unusual MOB. Three isolates of the phylum *Verrucomicrobia* represent the first non-proteobacterial MOB (Islam et al., 2008; Pol et al. 2009; Dunfield et al., 2007b). They possess *pmoA* copies that are phylogenetically distinct from the proteobacterial *pmoA* genes. Furthermore, *Crenothrix polyspora* and *Clonothrix fusca* were found to utilize methane (Stoecker et al., 2006; Vigliotta et al., 2007). These sheathed γ-proteobacteria are well known since more than one century and their morphology and complex life cycle have been studied in detail (Cohn, 1870; Volker et al., 1977). Whereas *Clonothrix* harbors a conventional *pmoA* gene, the *pmoA* of *Crenothrix* was shown to be unusual and more related to the *amoA* of ammonia oxidizers than to the *pmoA* of other methanotrophs.

In this study, we re-evaluated the value of the *pmoA* gene as phylogenetic marker for MOB by analysing the correlation of 16S rRNA phylogeny and *pmoA* phylogeny including newly described MOB species. Furthermore, the vast majority of *pmoA* sequences in public databases belong to uncultivated bacteria and knowledge about their function and ecological niche differentiation is rare. We performed a phylogenetic analysis of approximately 3400 publicly available *pmoA* sequences from various environments to gain deeper insight into the overall diversity. We then compared the phylogeny to the environmental origin of the sequences to learn more about possible environmental distribution patterns and ecological niches of uncultivated methanotrophs.

5.3 Results

This study is based on a *pmoA* database containing more than 3400 sequences that have a sequences length of more than 140 amino acids. 6% of the sequences belong to cultivated MOB species whereas the vast majority represents to-date uncultivated species found in various environments. The *pmoA* of most cultivated MOB (69%) cluster within the type II *Methylosinus* and *Methylocystis* group, 17% represent type Ia and 8% belong to type Ib. Furthermore, *Methylocapsa acidiphila* B2 (Dedysh et al., 2002), *Methylothermus* species (Bodrossy et al., 1995; Tsubota et al., 2005), *Methylohalobium* species (Heyer et al., 2005; Hirayama et al., 2007), *Crenothrix polyspora* (Stoecker et al., 2006), *Burkholderia* sp. TS2 (Islam, unpublished) and the verrucomicrobial MOB (Dunfield et al., 2007; Islam et al., 2008; Pol et al., 2007) are comprised in the database.

Cultivated methanotrophic bacteria

Out of all cultivated species in the database, 79 isolates covering the known MOB diversity were chosen for comparative sequence analysis of the 16S rRNA and the *pmoA* gene. Furthermore, two ammonia oxidizers, *Nitrosococcus oceani* (U96611; *pmoA*) and *Nitrosospira multiformis* (DQ228454; *pmoA*), were included in the analysis. *Nitrosococcus* represents a γ-proteobacterium whereas *Nitrosospira* belongs to the β-proteobacteria. For the selected species, the corresponding 16S rRNA gene sequence was downloaded from the SILVA database (Pruesse et al., 2007; release 100) and distance matrices were calculated based on the 16S rRNA and the *pmoA* sequence.

To get insights into the correlation between the 16S rRNA gene phylogeny and the corresponding *pmoA*/PmoA phylogeny, the pairwise sequence distances were plotted. The distance comparison revealed a good correlation (**Figure 1**). Nevertheless, within the type I and type II MOB, the PmoA protein is more conserved between the

species than the *pmoA* gene: the sequence distances for the protein are much smaller than the corresponding distances on the nucleotide level (marked boxes in **Figure 1**). Furthermore, the protein sequences offer a stronger linear relationship with the 16S rRNA gene phylogeny (r^2=0.93). The linear regression line shows a slope of 1.88 indicating an approximately two times higher average distance of the PmoA compared to the 16S rRNA (**Figure 1b**).

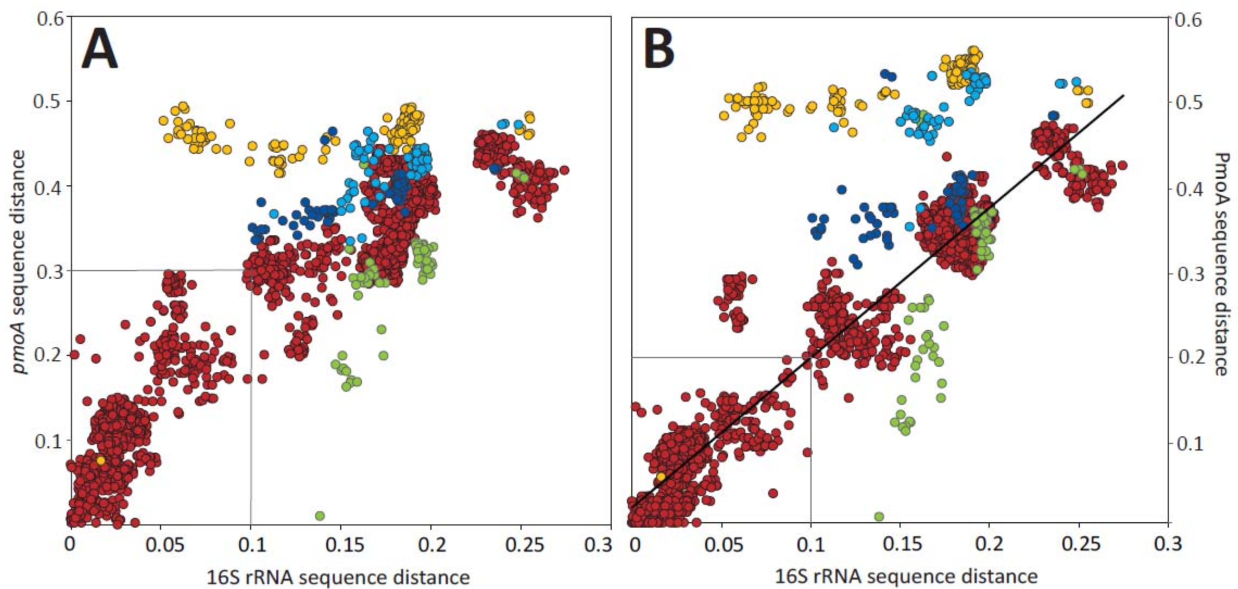

Figure 1 | Correlation between pairwise 16S rRNA gene distances and *pmoA*/PmoA distances of cultivated MOB and selected ammonia oxidizers. A. Comparison based on *pmoA* nucleotide sequences. **B.** Comparison based on PmoA protein sequences (regression line: r^2=0.93).The boxes mark the distance results within the subgroup of type Ia, type Ib and type II, respectively. ● =pairwise distance comparison within type I&2 and verrucomicrobial methanotrophs. ● =pairwise distance comparison of *Crenothrix polyspora* to all others. ● =pairwise distance comparison of *Burkholderia* sp. to all others. ● =pairwise distance comparison of *Nitrosococcus oceanus* to all others. ● =pairwise distance comparison of *Nitrosospira multiformis* to all others.

However, the pairwise sequence comparisons of three species did not fit into the regression. *Crenothrix polyspora* is closely related to type I MOB based on 16S rRNA gene identity. Yet, it possesses an unusual *pmoA* different from all known MOB, but similar to the *amoA* of *Nitrosospira multiformis* (Stoecker et al., 2006). Remarkably, the sequence distance of both species to the other MOB is higher on the amino acid

level than on the nucleotide level (**Figure 1**). This observation does not apply to the γ-proteobacterial ammonia oxidizer *Nitrosococcus oceanus*.

Furthermore, *Burkholderia* sp. TS2 represents a β-proteobacterium distantly related to the α- and γ-proteobacterial MOB on the 16S rRNA level, but possessing a *pmoA* nearly identical to *Methylococcus capsulatus* (Islam, unpublished).

Environmental distribution patterns of MOB

The vast majority of *pmoA* sequences in the database belong to uncultivated MOB obtained from various environments. The studied habitats and corresponding references included in this meta-analysis are summarized in **Table S1**. We classified available habitat information into 11 environmental categories (**Table S1**).

Phylogenetic analysis of the total dataset resulted in a high diversity of *pmoA* genotypes (**Figure 2**). Assigning the sequences to the environment they were obtained from, the most prominent pattern was the grouping of upland soil sequences within seven distinct clusters (**Figure 2**). Moreover, the sequences from arid soils are low in diversity and could be assigned to only three of the upland soil clusters: JR-3, USC-γ and TUSC (Angel and Conrad, 2009; Xhou, unpublished).

The remaining type I and type II MOB consist nearly entirely of sequences from high-methane environments. The *Methylocapsa* and the MO3 cluster (type II) comprise only a small amount of sequences originating mostly from peatland (*Methylocapsa*) and landfill soil (MO3). The *Methylosinus/ Methylocystis* group and type Ib MOB are overall dominated by wetland rice sequences. Within type Ia, sequences obtained from aquatic environments contribute the largest proportion. Nevertheless, wetland rice sequences are also represented in high amounts.

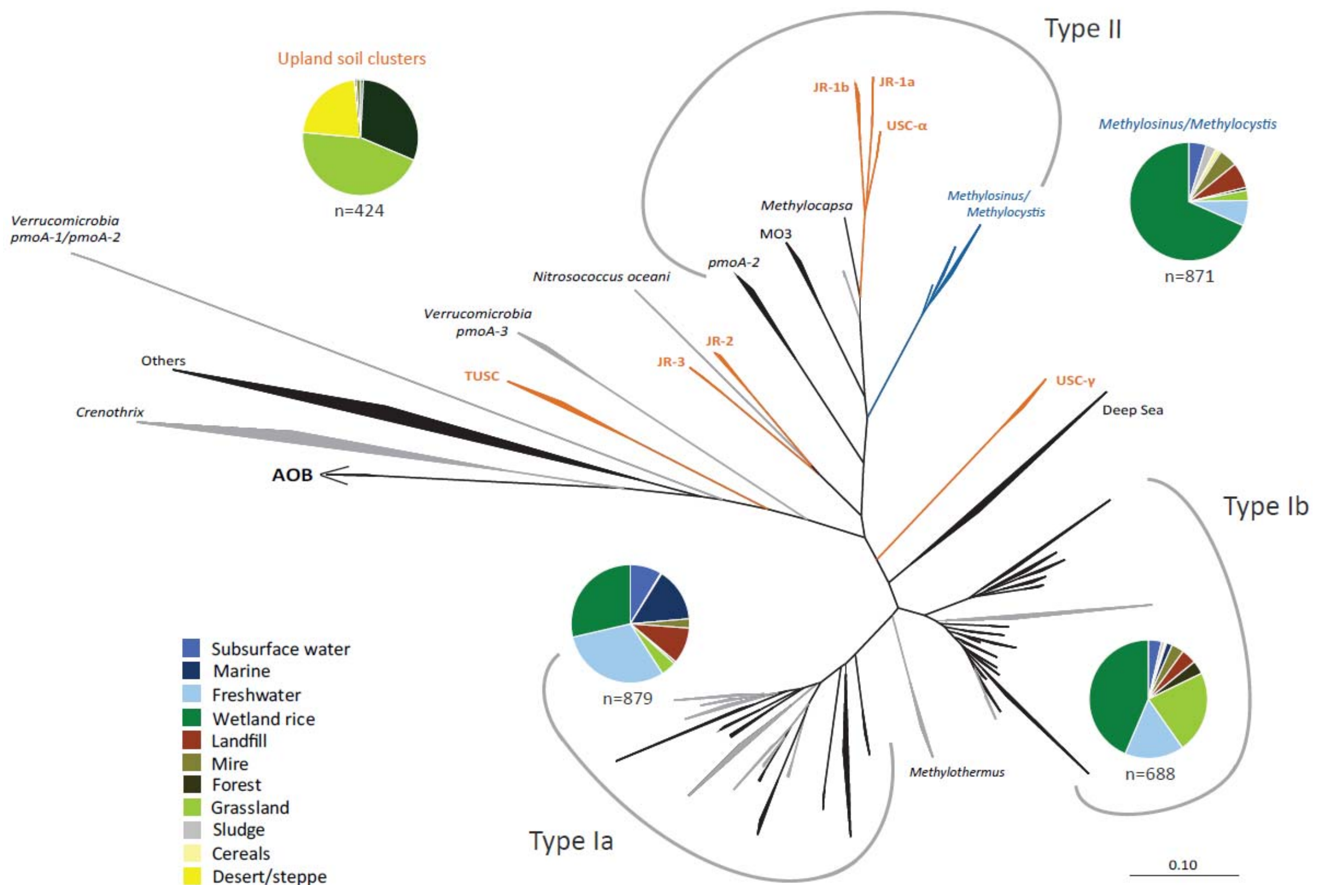

Figure 2 | Phylogenetic relationship of partial *pmoA* sequences based on deduced amino acid residues. The neighbor joining tree was calculated from a representative subset of the database containing approximately 3400 *pmoA* sequences. The ARB software package was used for tree construction (Ludwig et al., 2004). Only sequences over 140 amino acid positions in length were included in the analysis. Clusters comprising isolated species or highly enriched methanotrophs (*Crenothrix*) are depicted in grey, in case of the *Methylosinus* and *Methylocystis* group in blue. 'Upland soil clusters' are shown in orange. The pie charts illustrate the environmental distribution patterns within the type I and type II methanotrophs and the upland soil clusters.

Comparing the composition of sequence origins within type I MOB in more detail, some distinct distribution patterns can be observed (**Figure 3**). Nearly all type Ia clusters without cultivated representatives are dominated by sequences obtained from marine or limnic environments. The same applies to the clusters *Methylobacter* sp. BB5.1, *M. psychrophilus*, *M. tundripaludum* and *Methylomicrobium japanense*. Limnic environments can be furthermore divided into freshwater and soda lake habitats. Sequences retrieved from soda lakes often group with sequences from marine

origins. The *M. japanense* group exclusively consists of sequences from these saline environments. The *pmoA* sequences from wetland rice concentrate within the known MOB belonging to *Methylomonas* or *Methylosarcina*. Furthermore, landfill cover soil sequences seem to occur predominately within type Ia, also dominating one separate cluster (Landfill Cluster-1).

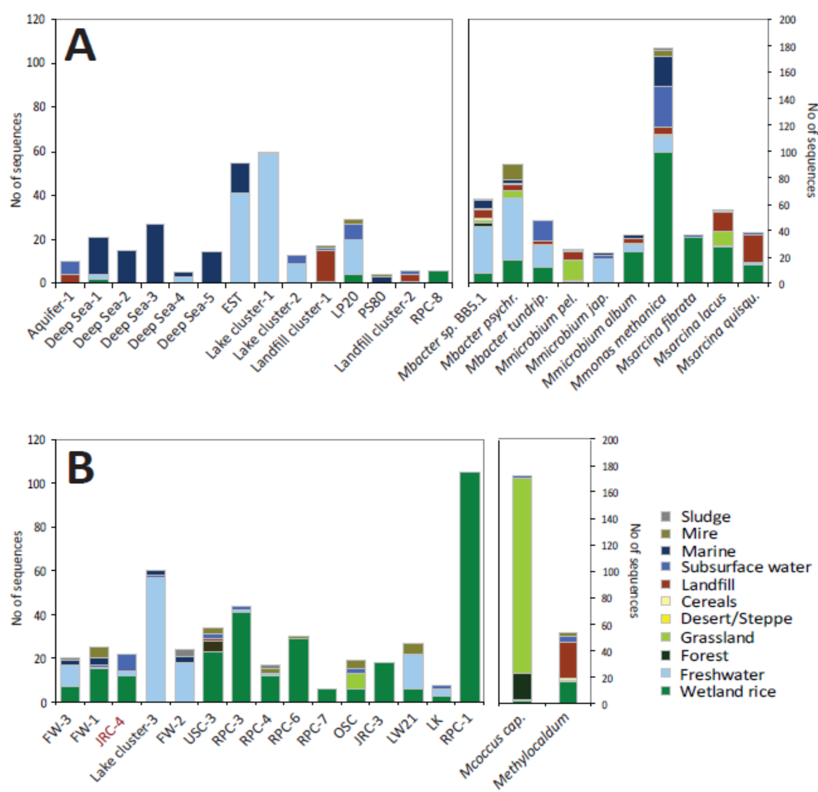

Figure 3 | Environmental distribution patterns of type Ia (A) and type Ib (B) methanotrophs. **Left:** clusters without cultivated representatives; the cluster JRC-4 contains one isolate; however, this strain is not further described to date. **Right:** clusters containing cultivated methanotrophs.

Type Ib MOB comprise only a few isolates. Whereas the *Methylococcus* group forms the only cluster dominated by sequences retrieved from low-methane environments (Bourne et al., 2001; Singh et al., 2007; Singh and Tate, 2007), the *Methylocaldum* clade is characterized primary by landfill or wetland rice origin (e.g. Bodrossy et al., 2003; Lin et al., 2009; Shrestha et al., 2008). The type Ib clusters

without isolates are strongly dominated by wetland rice sequences from various geographical sites (Lüke et al, 2010). Four clusters are furthermore characterized by sequences nearly exclusively obtained from freshwater lakes (e.g. Bussmann et al., 2006; Kim et al., 2006; Pester et al., 2004).

5.4 Discussion

Cultivated Methanotrophic bacteria

The pairwise sequence comparison of cultivated MOB revealed a good correlation between the 16S rRNA gene and the *pmoA* gene. This analysis has been done before for type II MOB resulting in a better correlation of *pmoA* nucleotide sequences than PmoA amino acid sequences to the 16S rRNA (Heyer et al., 2002). However, type II MOB are closely related and thus, the higher resolution of the nucleotide sequences leads to a more reliable phylogeny (Heyer et al., 2002). Here, we show a stronger correlation on the amino acid level including more distantly related type I and type II MOB. The result supports the use of deduced amino acid sequences for phylogenetic tree inferences spanning a wider range of MOB.

Nevertheless, C*renothrix polyspora* and *Burkholderia* sp. TS2 represent clear exceptions to the correlation (**Figure 1**). An interspecies gene transfer can be a possible explanation for such a non-correlation. The *Burkholderia* strain isolated by Islam and coworkers possesses a *pmoA* gene nearly identical to *Methylococcus capsulatus* (99% sequence identity); however, it belongs to the β-proteobacteria and is only distantly related to *Methylococcus* based on 16S rRNA phylogeny. This suggests the acquisition of the *pmoA* gene due to lateral gene transfer (LGT) between these species. The *pmoA* gene is part of the *pmoCAB* operon that is transcribed into a single polycistronic mRNA encoding the pMMO (Nielsen et al., 1997). Furthermore, genes involved in formaldehyde oxidation were shown to be organized in clusters (Boucher et al., 2003). The clustering of genes required for a function clearly facilitates LGT and might be

seen as an indirect evidence of such an event (Boucher et al., 2003; Lawrence and Roth, 1996). Hence, methanotrophy might be far more widespread among prokaryotes than assumed. Nevertheless, for *Burkholderia* sp. TS2, the presence and phylogeny of the *pmoB* and *pmoC* genes remains to be investigated as well as the synthesis of a functional methane monooxygenase protein.

Crenothrix harbors an unusual *pmoA* more related to the *amoA* of ammonia oxidizers than to the *pmoA* of type I and type II MOB. However, *Crenothrix* was shown to oxidize and incorporate methane and methanol confirming the enzyme as a methane monooxygenase (Stoecker et al., 2006). Yet, comparing the sequence distances to the other MOB on the nucleotide and amino acid level showed - similar to *Nitrosospira multiformis* - increased distances on the amino acid level. This might suggests a positive selection pressure acting on both proteins and leading apart from the other methane monooxygenases. It is interesting to speculate if the unusual *pmoA* of *Crenothrix* has been once retrieved from ammonia oxidizing bacteria by LGT.

The observation of the *Crenothrix's pmoA* grouping close to *amoA* genotypes and the *Nitrosococcus' amoA* grouping within *pmoA* genotypes suggests that sequence comparison might not allow conclusive interpretations on the function of the monooxygenase. Early studies on the pMMO showed a labeling of the *pmoA* encoded subunit using ^{14}C-acetylene as suicide substrate (DiSpirito et al., 1992). Thus, this subunit was thought to harbor the active site of the protein. However, great progress has been made lately obtaining the first crystal structure of the pMMO (Lieberman and Rosenzweig, 2005). Four metal sites are currently discussed as potential active sites with two sites that seem most promising (Hakemian and Rosenzweig, 2007). One site is located within the PmoB subunit and the other site is formed by three PmoC amino acids and only one glutamic acid from PmoA. Thus, the *pmoA* gene might be not the ideal target for function-related interpretations.

The verrucomicrobial methanotrophs were suggested to have acquired the genes essential for methane oxidation by LGT from proteobacteria (Hou et al., 2008). However, the comparison of 16S rRNA sequence distances and corresponding *pmoA*

sequence distances matches the overall regression (**Figure 1**). It suggests that a possible LGT event has occurred, if at all, very early in evolutionary history of these bacteria.

Uncultivated methanotrophic bacteria

The *pmoA* gene has been widely used as functional and phylogenetic marker for methanotrophs resulting in a large number of clusters without any cultivated representatives. Some clusters contain few isolates whereas the only two clusters dominated by cultivated strains are formed by *Methylosinus sporium* (96% are isolates) and *Methylosinus trichosporium* (50% are isolates). They were isolated from various environments including rice fields e.g. (Ferrando and Tarlera, 2009; Heyer et al., 2002), marine environments and lakes (Heyer et al., 2002); however, *pmoA* clones from these environments were only retrieved in low numbers. This suggests a method depending bias that might underestimate the true number of *Methylosinus* revealed in cultivation-independent approaches.

The clone libraries obtained from the different environments do not show the same coverage. MOB in rice fields have been studied in detail whereas rather few sequences are available for Northern wetlands and permafrost soils. These habitats represent the largest single CH_4 source and deeper knowledge about MOB diversity in these soils would be desirable. However, despite the unequal representation of the studied habitats, some distinct distribution patterns could be observed. Nearly all sequences retrieved from upland soils group within a few clusters and only a small amount cluster within the *pmoA-2* involved in atmospheric CH_4 oxidation (Baani and Liesack, 2008). This suggests that the *pmoA-2* might only play a minor role for atmospheric CH_4 oxidation in upland soils. In these habitats, uncultivated MOB grouping within the upland soil clusters seem to be responsible. It was suggested that soil pH might play a role in selection of these atmospheric MOB as USC-α seem to occur more frequently in acidic soils (Knief et al., 2003; Knief et al., 2005). Comparing the origin of the upland soil cluster sequencing in the database, it furthermore shows

the concentration of the arid or semi-arid soil sequences within a few clusters. None of these sequences group within the type II related clusters USC-α and JR-1. Thus, besides the pH, the soil moisture might also affect these atmospheric MOB.

Evaluating the high-methane environments, sequences retrieved from paddy fields concentrate within the *Methylosinus* and *Methylocystis* group (type II) and the type Ib MOB. They dominate in particular the clusters without any cultivated MOB (**Figure 5**). Only few isolates exist for type Ib MOB despite this very high number and diversity of *pmoA* genotypes retrieved from environmental samples. The *Methylococcus* group is dominated by grassland sequences whereas the *Methylocaldum* group contains a high number of landfill sequences that could not be found in any of the remaining type Ib clusters. Thus, the known isolates seem to only poorly represent the to-date uncultivated species hidden behind this large diversity.

The type Ia MOB are characterized by a high abundance of sequences retrieved from aquatic environments. In particular, marine and soda lake sequences were found in high numbers. A variety of *Methylomicrobium* species have been isolated from saline environments e.g. (Kalyuzhnaya et al., 2008) and some details on their adaptation to high salt concentrations have already been described (Trotsenko and Khmelenina, 2002). The only further known halophilic MOB is *Methylohalobius crimeensis* (Heyer et al., 2005). It forms together with the halotolerant *Methylothermus thermalis* (Tsubota et al., 2005) a monophyletic group that consists nearly entirely of environmental sequences retrieved from marine environments and soda lakes e.g. (Lin et al., 2004; Nercessian et al., 2003). The meta-analysis of the *pmoA* database confirms this low diversity of halophilic MOB. They group in only a few clusters and seem to be already well characterized by the known *Methylomicrobium* and *Methylohalobius* isolates. Only one group moderately related to the USC-γ contains no associated cultivated representative.

Furthermore, most *pmoA* clones obtained from landfill soils grouped within the type Ia MOB. In particular the *Methylosarcina* group consists of a high number of sequences retrieved from various landfill studies e.g. (Bodrossy et al., 2003; Cebron et

al., 2007a; Hery et al., 2008a). The type species *Methylosarcina fibrata* and the species *M. quisquilarium* were also isolated from landfill cover soil confirming that they indeed seem to play an important role in this environment.

Conclusions

To date, a large amount of *pmoA* genotypes are deposited at public databases from which the vast majority belongs to yet uncultivated MOB. Phylogenetic inferences in MOB diversity studies are often based on the *pmoA* sequence taking for granted that it reflects the 16S rRNA gene phylogeny. We re-evaluated the phylogenetic information derived from *pmoA* analyses showing that it still remains a suitable marker for studying MOB in the environment. Nevertheless, methanotrophy might be evolutionary more complicated than assumed and this function might have been even exchanged between species.

The comparative sequence analysis of uncultivated MOB revealed a high diversity of *pmoA* genotypes. Environmental distribution patterns indicate an adaptation of specific genotypes to low-methane concentration or high salinity, respectively. Furthermore, a high number of environmental type Ib clusters, most only distantly related to cultivated species, are nearly exclusively composed of sequences retrieved from wetland rice and freshwater lakes suggesting a certain adaptation to these high-methane environments. Our meta-analysis clearly demonstrates the need for increasing isolation efforts as to date cultivated MOB seem to only poorly represent the *pmoA* genotypes retrieved from the environment.

5.6 Supplementary material

Table S1 | Summary of environmental *pmoA* studies included in the meta-analysis.

PROPORTION OF SEUENCES IN DATABASE	COMBINED HABITAT IN META-ANALYSIS	STUDIED HABITAT	REFERENCE
5%	Subsurface water	Aquifer	Fru, 2008 Erwin et al., 2005 Newby et al., 2004 Baker et al., 2001
		Contaminated groundwater	Yan, unpublished Urmann et al., 2008)
		Movile cave	Hutchens et al., 2004
		Artesian well	Vigliotta et al., 2007
4%	Marine	Marine sediment	Nold et al., 2000 Wasmund et al., 2009 Jensen et al., 2008 Tavormina et al., 2008 Hayashi et al., 2007a
		Estuarine sediment	McDonald et al., 2005
		Hydrocarbon seeps	Inagaki et al., 2004 Yan et al., 2006
		Hydrothermal environment	Nercessian et al., 2005a Kato et al., 2009
		Sediment-water interface	Kim et al., 2008
		Oxygen minimum zone	Hayashi et al., 2007b
		Hydrothermal shrimp	Zbinden et al., 2008
		Mussel	Duperron et al., 2007 Spiridonova et al., 2006
15%	Limnic	Soda lake sediment and water column	Lin et al., 2004 Lin et al., 2005 Bodrossy et al., 2003
		Freshwater lake sediment and water column	Rahalkar and Schink, 2007 Costello and Lidstrom, 1999 Bussmann et al., 2004 Bussmann et al., 2006 Pester et al., 2004 Kim et al., 2008 Nercessian et al., 2005b Tavormina et al., 2008 Junier, unpublished
37%	Wetland rice	Rice field soil and rice roots	Henckel et al., 1999 Henckel et al., 2000b Henckel et al., 2001 Horz et al., 2001 Shrestha et al., 2008 Lüke et al., 2010 Ferrando and Tarlera, 2009

Continuation Table S1 | Summary of environmental *pmoA* studies included in the meta-analysis.

Proportion of seuences in database	Combined habitat in meta-analysis	Studied habitat	Reference
37%	Wetland rice	Rice field soil and rice roots	Zheng et al., 2008 Jia et al., 2007 Qiu et al., 2008
5%	Landfill	Landfill cover soil	Cebron et al., 2007b Nevin, unpublished Tseng, unpublished Stralis-Pavese, unpublished Lin et al., 2009 Hery et al., 2008b Bodrossy et al., 2003 Chen et al., 2007
4%	Mire	Peatland soil	Chen et al., 2008 Morris et al., 2002 Dedysh et al., 2001
		Raised mire soil	Jaatinen et al., 2005
		Permafrost soil	Pacheco-Oliver et al., 2002
7%	Forest	Forest soil	Jaatinen et al., 2004 Tsutsumi Singh and Tate, 2007 Kolb et al., 2005b Knief et al., 2005 Knief et al., 2003 Holmes et al., 1999 Henckel et al., 2000a Jensen et al., 2000 King and Nanba, 2008
16%	Grassland	Grassland soil	Knief et al., 2005 Knief et al., 2003 Knief et al., 2006 Kolb et al., 2005a Ogram et al., 2006 Horz et al., 2005 Levine, unpublished Singh et al., 2007 Bourne et al., 2001 Vasara, unpublished
0.7%	Sludge	Activated sludge	Osaka et al., 2008
0.8%	Cereals	Agricultural soil	Knief et al., 2005 Dubey, unpublished
3%	Desert/Steppe	Desert soil	Angel and Conrad, 2009
		Steppe soil	Zhou, unpublished

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6 GENERAL DISCUSSION AND OUTLOOK

Methanotrophic bacteria perform a central function in our climate system representing the only biogenic sink for the greenhouse gas methane. Even though they have been studied intensively in the past, only little is known about their ecological niche differentiation and the factors influencing their community structure. New molecular techniques based on cultivation independent approaches allow a constantly deeper analysis of microbial populations in the environment. However, we have just started to explore their full diversity and to understand the complex interaction between these bacteria and their biotic and abiotic environment.

This PhD thesis focused on the microbial ecology of methanotrophic bacteria in the wetland rice ecosystem. Rice agriculture represents a major source for atmospheric methane and will even increase in importance in order to fulfill the food demands of the growing human world population. In the first part of this thesis, the diversity and distribution patterns of methanotrophs were investigated within an Italian paddy field and compared to paddy fields worldwide (**Chapters 2&3**). Furthermore, the community fingerprints of three closely located Italian rice fields were studied. The second part concentrated on the influence of different rice cultivars on the methanotrophic community composition by using two different fingerprinting techniques: the low-resolution T-RFLP analysis and the high-resolution microarray (**Chapter 4**). Finally, the value of the *pmoA* gene as phylogenetic marker for methanotroph was re-evaluated and environmental distribution patterns of publicly available *pmoA* sequences from various habitats were investigated (**Chapter 5**).

6.1 Diversity of methanotrophs in the wetland rice ecosystem

Rice paddies are characterized by a high diversity of aerobic methanotrophs (**Chapter 3**). However, despite intensive studies over nearly two decades, extremophilic species such as the methanotrophic *Verrucomicrobia*, *Methylohalobius* or *Methylothermus* have not been detected so far. The *pmoA* gene of the *Verrucomicrobia* species is only distantly related to the common *pmoA* and cannot be amplified using the standard PCR conditions (Dunfield et al., 2007; Pol et al., 2007). Therefore, the commonly used primers might have just failed to detect these species in moderate environments. However, for the Italian paddy soil, a PCR performed with *Verrucomicrobia*-specific *pmoA* primers confirmed the absence of these methanotrophs (data not shown).

Furthermore, environmental clusters pre-dominantly found in upland soils (e.g. USC-α and USC-γ) seem to play no important role in the wetland rice ecosystem. These uncultivated methanotrophs are assumed to be responsible for oxidation of methane at atmospheric trace level concentrations. Irrigated rice fields are dry and aerated during the winter and can even act as sink for atmospheric methane during this time (Singh et al., 1998). As upland soil cluster methanotrophs seem to be virtually absent in paddy fields, other species might be responsible for atmospheric methane oxidation in these environments. Type II methanotrophs belonging to the *Methylosinus* and *Methylocystis* group were detected in high abundance. Several of these species harbor a second *pmoA* (*pmoA-2*) encoding for a subunit of a pMMO isoenzyme (Tchawa Yimga et al., 2003). For *Methylocystis* sp. SC2, this second isoenzyme has been shown to have a different apparent K_m ($K_{m(app)}$) comparable to values determined for soils consuming atmospheric methane. It furthermore enables growth on atmospheric methane for over three month (Baani and Liesack, 2008). The *pmoA-2* genotype was retrieved in culture-independent studies of Italian rice field samples (Horz et al., 2001; Tchawa Yimga et al., 2003) and was also detected in high amounts in rice root samples using the *pmoA* microarray (**Chapter 4**). Methanotrophs

harboring both isoenzymes offer an optimal adaptation to the wetland rice ecosystem which shows great changes of *in situ* methane concentrations over the seasons. They seem to be promising candidates responsible for the observed methane uptake in rice paddies.

In addition to type II, type Ib methanotrophs were found to be highly abundant in paddy fields. This group is characterized by a high diversity of environmental *pmoA* sequences for which only few isolates are available (**Chapter 5**). Nearly all type Ib clusters are dominated by sequences obtained from rice fields worldwide indicating a specific adaptation to this environment. The rice paddy cluster 1 (RPC-1) forms the largest cluster and consists entirely of sequences obtained from paddy fields so far (**Chapter 3**). It is only distantly related to the cultivated species *Methylocaldum* and *Methylococcus* (10-16% amino acid sequence identity). Assuming an approximately two times higher average distance of the PmoA protein compared to the 16S rRNA (**Chapter 5**) results in a corresponding 16S rRNA sequence difference of 5-8% of RPC-1 to the next cultivated methanotrophs. Thus, RPC-1 might represent a new genus harboring specific traits that enable an optimal adaptation to the environmental conditions found in the wetland rice ecosystem.

Within type Ia methanotrophs, *Methylomonas* and *Methylosarcina* species seem to be dominant. However, sequences clustering with *Methylobacter tundripaludum* and *M. psychrophilus*, isolated from artic soil (Omelchenko et al., 1996; Wartiainen et al., 2006), were also found in paddy fields. Thus, these species seem to be more widespread and not restricted to cold environments. Halophilic species of the genus *Methylomicrobium* were not detected in rice fields so far. Only *Methylomicrobium album*-like sequences were retrieved. However, *M. album* represents a non-halophilic species clustering close to *Methylosarcina lacus* based on 16S rRNA gene phylogeny and a reclassification of the type Ia taxonomy was already suggested (Kalyuzhnaya et al., 2008).

Furthermore, a variety of sequences were found that group phylogenetically between the *pmoA* gene of methanotrophs and the *amoA* gene of ammonia oxidizers.

These clusters include the RA21, M84-P22 and the M84-P105 group (**Chapter 2**). They share conserved amino acid residues with the *pmoA* as well as the *amoA* and could therefore not be affiliated to any of the two corresponding functions (e.g. Holmes et al., 1999). Clone libraries of the *pmoB* gene might allow more function related insights as the active site of the pMMO might be located in this subunit (Hakemian and Rosenzweig, 2007; **Chapter 5**).

6.2 Niche differentiation of wetland rice methanotrophs

Rice fields are characterized by the dominance of one plant species and regular plowing and puddling of the soil surface. Thus, they represent a comparably homogeneous environment. Consistent to this, the methanotrophic community showed no horizontal distribution patterns within an Italian paddy field (**Chapter 2**). Therefore, large scale environmental gradients can be neglected for the sampling strategy and a reduced sampling effort is sufficient to extrapolate to the field scale. However, the methanotrophic communities in defined micro-habitats differ from each other (**Chapter 3**). Type II methanotrophs of the *Methylosinus* and *Methylocystis* group were found in high abundances in all three major habitats that can be defined in paddy fields: the anoxic bulk soil, the oxic surface soil and the partially oxic rhizosphere (see **Figure 2** in **Introduction**). This finding is consistent with previous studies of methanotrophs within a paddy field (Eller et al., 2005; Eller and Frenzel, 2001). Type II methanotrophs form a phylogenetically closely related group performing the serine cycle for primary C1 assimilation. Many type II species possess the second pMMO isoenzyme enabling growth at atmospheric methane concentrations (Baani and Liesack, 2008). Furthermore, some strains are able to grow with other carbon sources than methane (Dedysh, personal communication). These traits might allow the bacteria to remain physiologically active during the winter season when the fields are aerated and methane concentrations might be close to

atmospheric levels. In contrast, type I methanotrophs seem to be restricted to higher methane concentrations and thus, the activity might be limited during the winter. A variety of methanotroph are able to form resting stages that might even stay viable for more than a century in the anoxic sediment (Rothfuss et al., 1997). *Methylosinus* species (type II) are able to form exospores surviving at least 18 month in the dried state (Whittenbury et al., 1970). On the contrary, the cysts of *Methylosarcina* and *Methylomonas* species, which seem to be the most dominant type Ia methanotrophs in rice fields, were shown to be sensitive to desiccation (Whittenbury et al., 1970; Wise et al., 2001). Hence, only a subset of the type I population might survive the winter season. Increasing methane concentrations upon flooding in spring might support a renewed growth at the oxic-anoxic interfaces such as the plant roots.

Microorganisms are phylogenetically and physiologically considerably more diverse than plants and animals and microbial interactions with their biotic and abiotic environment are more complex. Thus, understanding microbial ecology and interpreting experimental observations in the context of ecological concepts is challenging (Jessup et al., 2005; Martiny et al., 2006; Prosser et al., 2007). However, the concept of differentiating between generalists and specialists might be partly applicable to methanotrophic bacteria. With their adaptation to consume methane at elevated and atmospheric concentrations and the capability to survive on other substrates than methane, type II methanotrophs are adapted to a wider range of substrate conditions than the very specialised type I methanotrophs. Beside the substrate range, this relationship can be further observed for other abiotic factors. The halophilic *Methylomicrobium* species, the psychrophlilic (psychrotolerant) *Methylobacter tundripaludum* and the termophilic *Methylococcus* and *Methylocaldum* species belong exclusively to type I methanotrophs. The yet uncultivated species of the RPC-1, clustering within the type Ib methanotrophs, demonstrate furthermore a specific adaptation to the wetland rice environment.

Hence, within the physiological distinct group of methanotrophs, type I species might be classified as specialists whereas type II methanotrophs might represent the generalists.

Furthermore, a growing number of studies provide evidence for the existence of biogeographical patterns for microorganisms (Papke and Ward, 2004; Whitaker et al., 2003). This indicates that 'everything' might not just be found 'everywhere'; but instead, certain dispersion limitations might also exist for bacteria (Martiny et al., 2006). Studying the methanotrophic community in three different Italian rice paddies revealed a distinct fingerprint for each field (**Chapter 2**). However, these distinct patterns were the result of different species abundances, not of different species present in the fields. Nevertheless, comparing the *pmoA* genotypes within the RPC-1 (**Figure 1b** in **Chapter 3**), a large group of sequences originating from Japanese paddy fields cluster separately from the group of the Italian paddy field sequences. This might suggest a partly independent evolution of these methanotrophs at both geographical locations.

6.3 Factors influencing methanotrophs in wetland rice fields

Nitrogen fertilization plays an important role in the rice field environment. It has been shown to directly affect the methanotrophic community by selectively stimulating type I methanotrophs (Bodelier et al., 2000; Mohanty et al., 2006). In contrast, type II species seem to be inhibited. Furthermore, the effect of temperature on methanotrophs was investigated in rice field and forest soil (Mohanty et al., 2006) as well as in a gradient system using soil from a contaminated aquifer (Urmann et al., 2008). Whereas Urmann and co-workers found no significant effect, the *pmoA* T-RFLP patterns for the rice field and forest soil changed depending on the incubation temperature. However, both soils showed different responses. In addition, protists were shown to selectively graze on different methanotrophs. Whereas *Methylocystis*

species (type II) seem to be rejected, *Methylobacter* species (type I) seem to be particularly preferred (Murase and Frenzel, 2008). Furthermore, a significant effect of the rice genotype on methanotrophs could be demonstrated (**Chapter 4**). Type Ib methanotrophs showed the largest changes in response to the rice genotype. In general, changing environmental factors seem to greater influence type I populations whereas type II populations seem to remain comparably resistant.

6.4 Outlook

Comparative sequence analysis of the *pmoA* gene revealed a great diversity of methanotrophs. Adaptation to specific environmental parameters has been described for some isolated species, and distinct environmental distribution patterns could be observed for several *pmoA* genotypes. The *pmoA* microarray (Bodrossy et al., 2003) represents a high resolution technique detecting methanotrophs down to the species or cluster level. Furthermore, next generation sequencing techniques allow the acquisition of gigabases of sequence information within one single run (MacLean et al., 2009). These methods allow in-depth studies of methanotrophic populations in the environment and might give further insights into ecological niche adaptation of distinct species or clusters.

This PhD work focused on the methanotrophic community that is *present* in paddy fields. Only a subset of this community might be metabolically *active* and responsible for observed methane oxidation rates. Stable isotope probing (SIP) of DNA/RNA or phospholipid fatty acids (PLFA) represent techniques enabling to link the metabolic activity with the identity of the corresponding microorganisms (Radajewski et al., 2000). They were successfully used to investigate the active population of methanotrophs in various environments (Hery et al., 2008; Hutchens et al., 2004; Murase et al., 2007) and indicates a dominant activity of type I methanotrophs in a Chinese paddy field (Qiu et al., 2008). Furthermore, studying

pmoA transcripts retrieved from environmental samples might allow conclusions on the metabolic activity of methanotrophs (Chen et al., 2008; Kolb et al., 2005).

However, cultivation-independent studies using the 16S rRNA and functional marker genes have also limitations. These approaches enable great insights into the vast diversity of bacteria, yet fundamental conclusions on their genetic and metabolic versatility are not possible. In case of the *pmoA/amoA* gene, the sequence information seems not to be even indicative for the specific function of the corresponding monooxygenase: no sites could be identified that are clearly linked to the function of methane oxidation, atmospheric methane oxidation or ammonia oxidation, respectively. Such conclusions on the physiology of the bacteria can only be drawn from pure culture studies. The vast majority of the *pmoA* diversity is dominated by clusters without any cultured representative clearly demonstrating the need for increasing isolation efforts. The meta-analysis in **Chapter 5** has identified those habitats that are not well represented by the existing pure cultures so far. In rice fields, type Ib methanotrophs seem to play an important role, but only few type Ib isolates are described. Moreover, these isolates might only poorly represent the environmental clusters found in paddy fields. The RPC-1 in particular might be even a new genus with special adaptation to rice fields.

Summarized, we have only started to get insights into the diversity and ecology of this environmentally important group of bacteria. It is not known to what extend this great diversity affects the resilience and resistance of the methane oxidation process. This function is essential for our climate system and deeper understanding of methanotrophic diversity, activity and their reaction to perturbations is therefore needed.

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Lüke, C, and Frenzel, P. The *pmoA* gene as functional and phylogenetic marker for methanotrophic bacteria: a sequence database analysis. **In preparation.**

BEITRÄGE ZU WISSENSCHAFTLICHEN TAGUNGEN

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Ich versichere, dass ich meine Dissertation

'Molecular ecology and biogeography of methanotrophic bacteria in wetland rice fields'

selbständig und ohne unerlaubte Hilfe angefertigt habe und mich keiner als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe. Diese Dissertation wurde in der jetzigen oder ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

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