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Deciphering Community Structure of Methanotrophs Dwelling in Rice Rhizospheres of an Indian Rice Field Using Cultivation and Cultivation-Independent Approaches

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Abstract Methanotrophs play a crucial role in filtering out methane from habitats, such as flooded rice fields. India has the largest area under rice cultivation in the world; however, to the best of our knowledge, methanotrophs have not been isolated and characterized from Indian rice fields. A cultivation strategy composing of a modified medium, longer incubation time, and serial dilutions in microtiter plates was used to cultivate methanotrophs from a rice rhizosphere sample from a flooded rice field in Western India. We compared the cultured members with the uncultured community as revealed by three culture-independent methods. A novel type Ia methanotroph (Sn10-6), at the rank of a genus, and a putative novel species of a type II methanotroph (Sn-Cys) were cultivated from the terminal positive dilution (10^{-6}). From lower dilution (10^{-4}), a strain of *Methylomonas* spp. was cultivated. All the three culture-independent analyses, i.e., *pmoA* clone library, terminal restriction fragment length polymorphism (T-RFLP), and metagenomics approach, revealed the dominance of type I methanotrophs. Only metagenomic analysis showed significant presence of type II methanotrophs, albeit in lower proportion (37 %). All the three isolates showed relevance to the methanotrophic community as depicted by uncultured methods; however, the cultivated members might not be the most dominant ones. In conclusion, a combined cultivation and cultivation-independent strategy yielded us a broader picture of the methanotrophic community from rice rhizospheres of a flooded rice field in India.

Keywords Methanotrophs · Indian rice fields · Metagenome

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Introduction

Methane is the second most important anthropogenic greenhouse gas after CO_2. Rice fields are one of the most important anthropogenic sources of atmospheric methane, and they contribute to 10 % of the total methane budget [8]. Microbial methane oxidation plays a very important role in the rice rhizospheres where methanotrophs or aerobic methane-oxidizing bacteria consume methane and attenuate the net flux from rice fields to the environment [9]. Mainly, molecular methods have been used to investigate the community structure of methanotrophs dwelling in rice rhizospheres [15, 27, 28, 35, 41]. Only a few studies have used culture-dependent and culture-independent approaches in methanotroph community analysis [14, 16]. As a result of two such studies, two methanotrophic species have been formally described which had been originally isolated from rice field environments [17, 31]. India has the world's largest area under rice cultivation and is estimated to contribute considerable methane from flooded rice fields [2]. Native and relevant methanotrophic isolates from flooded rice fields can be excellent models to understand effect of ammonium fertilizers, increasing temperatures (due to global warming) on methanotrophs which would help in planning strategies to reduce methane emissions. Cultivation and isolation of methanotrophs are difficult

due to a variety of reasons, such as resistance growing on solid media, requirement of special growth environment, heterotrophic contaminants, etc. [5, 7]. To the best of our knowledge, there have been no methanotrophs isolated from Indian rice fields.

Rhizospheres and root environments are hotspots of methanotrophic activities [15], and type I methanotrophs dominate in these habitats ([9] and references therein). Pulse-labeling experiments using RNA-SIP and PLFA labeling have identified that especially within type I methanotrophs, type Ia methanotrophs (*Methylosarcina*, *Methylomicrobium*, *Methylomonas*, *Methylobacter*, *Methylosoma*, and related genera) were found to be important in the Chinese rice fields irrespective of the fertilizer used [32]. Studies from Northern India have also indicated the importance of type I methanotrophs in rice rhizospheres of fertilized as well as non-fertilized soils [40]. Nevertheless, most of the type Ia methanotrophs have resisted isolation. To the best of our knowledge, all of the cultivated type Ia methanotrophs from rice fields belong to the genus *Methylomonas* [14].

Methanotrophs from rice rhizosphere sample of a flooded rice field in India were enriched, isolated, and characterized. Further, in order to determine whether the obtained isolates were relevant members of the methanotrophic community, these were compared with the community obtained by culture-independent methods. Three culture-independent approaches were used: *pmoA* clone libraries, terminal restriction fragment length polymorphism (T-RFLP), and metagenomics. The first two involve amplification of partial *pmoA* gene. Particulate methane monooxygenase (pMMO) enzyme is present in majority of the methanotrophs except *Methylocella*, and *pmoA* gene is used for community analysis of methanotrophs [19]. Metagenomics approach was used to get a broader overview of the community.

Materials and Methods

Sampling and Soil Analysis Sampling was done in the wet season of 2014 where five rice plants were sampled with their intact roots and attached soil from a flooded rice field in Western India, 19.2° N 73.88° E. Rice variety planted here was *Oryza sativa indica* variety 'Saal,' which is a traditional variety. After transporting to the laboratory the same day, soil was shaken off from the roots by beating on a hard surface for eight to ten times followed by brief washing to remove the extra attached soil. The soil tightly attached to the roots was scraped and collected in sterile petri plates which we refer as the rhizospheric soil. Soils from all the root samples were mixed together thoroughly and stored as a pooled sample. Details of soil physicochemical analysis can be found in Supplementary Information.

Cultivation of Methanotrophs

Serial Dilution Enrichment in Microtiter Plates Within 2–3 days of collection, liquid enrichments for methanotrophs were set up in microtiter plates. Dilute nitrate mineral salt medium (dNMS) [13] was modified, and constituents per liter were as follows: 1 g $MgSO_4 \cdot 7H_2O$, 0.2 g $CaCl_2$, 0.25 g KNO_3, 600 μM KH_2PO_4/Na_2HPO_4 buffer, pH 7.2, 10 mM HEPES buffer, pH 7.2, 1 mg $FeNH_4EDTA$, and 1 ml l^{-1} trace element solution (SL10). This medium was amended from [13], and concentration of KNO_3 used was 0.25 g/l instead of 0.05 g/l, the later used in the original reference. After autoclaving, filter-sterilized solution of 1 ml of vitamin solution [43] amended with additional vitamin B12 (total 20 mg/l) was added. The sample was serially diluted in 1:10 dilution steps from 10^{-1} to 10^{-8} to make a total volume of 1.0 ml in microtiter plate in triplicates. These plates were incubated in glass desiccators at 28 °C for 10 weeks. The gas atmosphere was maintained as ~20 % methane, 25–30 % air, and rest nitrogen. Desiccators were opened after every 2 weeks and checked for growth, by visual inspection for turbidity or pellicle formation, and microscopy and observations were recorded.

Enrichments in Roll Tubes and Agarose Columns Roll tubes were prepared by mixing 0.1 ml of diluted sample and 10 ml of the same medium supplemented with 1.2 % agarose and rolled on ice [30]. Similar gas atmosphere conditions were maintained as mentioned above, and bottles were incubated inverted for 10 weeks. Similarly, agarose columns (1.2 %) with same medium composition were prepared in sealed glass tubes gassed with methane, and air and N_2 mixture were inoculated with 0.1 ml of each dilution and incubated for 10 weeks.

Isolation and Identification In case of microtiter plates, after 10 weeks of incubation, the wells which showed positive growth were again used for serial dilutions in microtiter plates and incubated for 3–4 weeks. Subsamples from positive wells were simultaneously streaked on agarose plates (same medium with 1.5 % agarose, Sigma) and incubated in desiccators with same gas atmosphere as used for the enrichments. The last positive dilutions from the grown wells (second transfer) were used for direct streaking on agarose plates with the same medium. Desiccators were opened regularly, i.e., after 1 week, and checked for growth of colonies. Similarly, colonies obtained in roll tubes and agarose columns were subcultured in the same manner (rolled or inoculated in columns) as well as streaked on plates. Purification of the methanotrophic colonies was done by repeated subculturing. In some cases, colonies were inoculated in a liquid dilution series followed by streaking on agarose plates. This process was repeated until a pure culture was obtained. Heterotrophic contamination was

checked by streaking each methanotroph colony on diluted nutrient broth (1:10) supplemented with 0.1 % glucose. Methanotroph isolates obtained were regularly checked under phase contrast microscope for purity.

Growth of Cultures All the methanotrophic isolates were regularly maintained on modified dil. NMS agarose plates and grown in liquid in flasks incubated inside desiccators with the same gas atmosphere as described above. Presence or absence of *pmoA* gene was checked in all strains which grew in the presence of methane. For testing the methane oxidation capacities of each culture, the culture was grown in serum bottles of 65-ml capacity with 10-ml medium. The proportion of the gases was maintained as 10 or 20 % methane, 25 % air, and rest nitrogen. In this case, the bottles were flushed with nitrogen gas for 10 min and calculated amount was removed and replaced by methane and air using a syringe and a sterile filter (0.2 μm). For this purpose, methane from the gas cylinder was displaced in sealed sterile serum bottles and used. Using gas chromatography (Chemito, Thermo Fisher Scientific), the decrease of methane over time was followed for confirming methane oxidation by pure cultures.

Culture-Independent Analysis

DNA Extraction and PCR Amplification DNA was extracted from pooled Sn rhizosphere sample by using FAST DNA Spin kit for soil (MP Biomedicals, USA) using the FastPrep-24 instrument (MP Biomedicals, USA). DNA was extracted from pure cultures using GenElute™ Bacterial Genomic DNA kit (Sigma). After checking on gel, partial *pmoA* gene was amplified using the universal *pmoA* primer pair A189f-mb661r [11] which amplifies partial *pmoA* gene from all alpha and gammaproteobacterial pMMO-containing methanotrophs. 16S rDNA gene was amplified using 27f and 1492r primers as described earlier [7]. All amplifications were carried out in 25-μl total volumes in an Eppendorf or ABI thermal cycler (Veriti) using recombinant Taq DNA polymerase (Sigma). After purification of the PCR products using Favorgen PCR purification kit (Progene Life Sciences), the products were sent for sequencing to Progene Life Sciences, APS Life Technologies (Eurofins, India), or GeneOmbio CRS, Pune, India. Quality of the sequences was checked using inspection of the peaks using the Bioedit program, and then, a BLAST search was performed at the NCBI site (http://www.ncbi.nlm.nih.gov/) [3]. For 16S ribosomal RNA (rRNA) genes, closely related sequences with complete 16S rRNA genes were retrieved. Sequences were also blasted in EZtaxon server (http://www.ezbiocloud.net/eztaxon) and similarity values obtained. For *pmoA* gene, sequences from known species were retrieved from the NCBI database. Alignments were done using MAFFT online aligner, and phylogenetic trees were constructed using the

maximum likelihood algorithms with Kimura-2 parameter, maximum parsimony, NJ, unweighted pair group method with arithmetic mean (UPGMA), and minimum neighborhood methods as implemented in MEGA 5.05 [38] using nucleotide data and 1000 bootstrap replications.

Clone Libraries, Restriction Fragment Length Polymorphism, and Phylogenetic Analyses

pmoA clone libraries from rhizospheric samples were prepared by cloning the partial *pmoA* gene product (508 bp) obtained after amplification with primers A189f-mb661r [10] and purification of the PCR products. PCR products were purified using the Favorgen PCR purification kit (Progene Life Sciences). All cloning steps were carried out using the StrataClone PCR cloning kit (Agilent technologies) and transformed in JM109 *E. coli* competent cells. Colony PCR was performed to identify clones that carried the insert. A total of 70 positive clones were used for RFLP analysis using *MspI* enzyme as described in [7]. Cloning and assigning clones to RFLP groups were done as described before [7]. All sequencing reactions were carried out either at Progene Life Sciences, Pune, India (first base), or APS Life Technologies (Eurofins, India) or GeneOmbio CRS, Pune, India. A BLAST search was performed at the NCBI site (http://www.ncbi.nlm.nih.gov/), and closely related sequences were retrieved. *pmoA* sequences of strains and clones were phylogenetically analyzed using MEGA 5.05 software [1, 31]. Alignments were done using MAFFT online aligner, and phylogenetic analysis was done using the maximum likelihood algorithms with Kimura-2 parameter as implemented in MEGA using nucleotide data with 1000 bootstrap replications. Additionally, tree was also constructed using neighbor-joining methods with 1000 bootstraps to confirm the branches. For assigning clones to T-RFs, clone sequences were digested in silico with *MspI* enzyme and the T-RFs were predicted for each RFLP clone group using "Editseq" program within DNASTAR (Lasergene software). A rarefaction analysis was done using PAST software (Past 3).

T-RFLP Analysis Using 5′ 6-carboxyfluorescein-labeled A189f primer (Sigma) and mb661r primer, a total of four to five PCR reactions were set and the products were checked, pooled, and purified. Around 100 ng of DNA was used for digestion with the restriction endonuclease *MspI* (3 U each, Merck Millipore). The digested products were given to a company GeneOmbio CRS, Pune, for analyzing on the DNA sequencer (Applied Biosystems, 3130 Genetic Analyzer). Peaks were analyzed by the program Gene Mapper version 3.7 (Applied Biosystems).

Metagenome Analysis Genomic DNA was used for shotgun metagenomic sequencing using the 316™ sequencing chip

and 200 bp chemistry using Ion Torrent PGM 2 platform (Life Technologies, USA) using the manufacturer's instructions. The data analysis and annotation were done using MG-RAST [29] online server. Taxonomic assignment of the sequences was performed using "organism abundance" function and "group table using family and genus" as an option, within MG-RAST. The gene abundance values were obtained using GenBank annotation with maximum e value cutoff $1e^{-5}$, minimum alignment length of 15 amino acids, and minimum 60 % similarity. Classical methanotrophs belong to the families *Methylococcaceae* and *Methylocystaceace* within gammaproteobacteria and alphaproteobacteria, respectively. Therefore, abundance data was collected for these two families. Additionally, genes from the genera *Methylocella* (family: Beijerinckiaceae) and *Methylacidiphilum* (family: Methylacidiphilaceae) were searched. These genera of methanotrophs do not belong to the families of classical methanotrophs and therefore had to be searched separately. The metagenome has been submitted to the NCBI SRA database, and the accession number is SRP062304. The metagenome has been also submitted to MG-RAST (https://metagenomics.anl.gov/) and made public with the Id: 4623913.3. Additionally, *pmoA* sequences were extracted from the metagenome using "Search for function" option, in the MG-RAST pipeline. The partial *pmoA* genes were used for construction of a phylogenetic tree using sequences from isolates and clones obtained in this study.

Microscopy Phase contrast microscopy of the cultures was performed using a Nikon microscope and a camera.

Results

Enrichment of Methanotrophs

Growth was seen in microtiter plates up to 10^{-6} dilution. Growth was in the form of thick, pink/orange pellicles in the first four dilutions, whereas in the fifth and sixth dilutions, growth was in the form of a light pink surface pellicle or a thin layer of cells and a bottom biofilm. Colonies were seen in roll tubes and agarose columns up to 10^{-4} dilutions.

Isolation and Characterization of Methanotrophs

A total of three strains of methanotrophs were isolated from the microtiter plate enrichments. No methanotrophic isolates were obtained from the roll tube and agarose column-based enrichments. In the last positive wells (10^{-6}), fat, big, dark, motile elliptical rods were observed as the dominant morphotype by phase contrast microscopy. Methanotrophs usually appear darker than non-heterotrophic bacteria due to their intracytoplasmic membranes [12], which was one of the first indications after doing phase contrast microscopy. After streaking, pale pink colonies appeared on agarose plates which showed the presence of the same organism. The culture had to be purified, as it showed presence of smaller rods and cocci. After a pure culture was established on plates, the strain was designated as Sn10-6 and was composed of big, elliptical to cucumber-shaped, motile cells of size 3.5–4 to 1.2–1.5 μm (Fig. 1). Maximum size measured for this strain was up to 5 μm. Another methanotroph strain, Sn-Cys, was isolated from the streaked liquid enrichment of 10^{-6} dilution. Strain Sn-Cys produced white to cream colonies and showed presence of 1–2 μm of coccoid to rod-shaped cells which were morphologically similar to type II methanotrophs [42]. After longer incubations, colonies of Sn-Cys turned dark red-purple. In the wells with lower dilutions, i.e., 10^{-3} and 10^{-4}, pink surface pellicles typical of *Methylomonas* spp. were seen [42]. A strain forming pink mucoid colonies was isolated from the 10^{-4} dilution. This strain was designated as Sn-4. All the three cultures grew on plates or in liquid, i.e., in flasks inside desiccators and in closed serum bottles with 10–20 % of methane, 25 % of air, and rest nitrogen and oxidized methane. More inoculum (20 %) was required for good liquid growth of Sn10-6. Further, taxonomic characterization of Sn10-6 using 16S rRNA gene and partial *pmoA* gene sequence showed a distant relationship with cultivated methanotrophs. Its 16S rRNA gene showed 93.5 % similarity to a type Ia methanotroph, *Methylosarcina lacus*, and around 90–93 % similarity with other type Ia methanotrophic genera (Table 1). The partial *pmoA* gene of Sn10-6 showed only 85 % sequence homology to *Methylovulum miyakonese* (Table 1) followed by 83 % homology to *Methylobacter tundripaludum*. Based on complete 16S rRNA gene sequences from related cultivated gammaproteobacterial methanotrophs, a phylogenetic analysis was done and Sn10-6 was identified as a member of a novel putative genus-species forming a new lineage, close to the branch of *Methylovulum-Methylosoma* genera in the phylogenetic tree (Fig. 2a). The position was confirmed by using various treeing options (neighbor joining, maximum likelihood, minimum likelihood, and UPGMA) where the branching was confirmed except the bootstrap values differed from one tree method to other. Phylogenetic tree based on partial *pmoA* sequences also placed Sn10-6 close to *Methylovulum miyakonese* branch within type Ia methanotrophs (Fig. 3). Strain Sn-Cys, the second strain isolated from the terminal dilution, showed 98.4 % similarity to *Methylocystis parvus* and 98.1 % similarity to *Methylosinus sporium* on the basis of 16S rRNA gene homology (Table 1). Nucleotide sequence of partial *pmoA* gene in Sn-Cys was similar to *Methylocystis parvus*
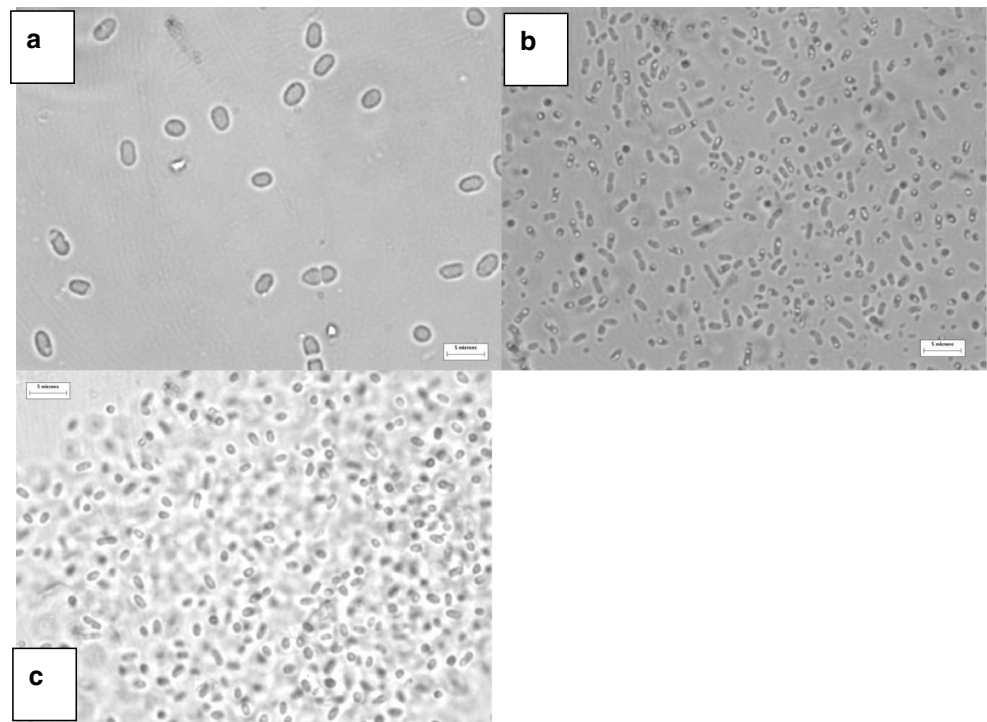
by 94 % and to other species of *Methylocystis* up to 90 %. Similarity of Sn-Cys *pmoA* to that of *Methylosinus* spp. was 89 % or less. In the 16S rRNA gene-based phylogenetic tree (Fig. 2b), Sn-Cys groups in between *Methylocystis* and *Methylosinus* spp. whereas in the *pmoA* tree, the strain grouped close to *Methylocystis*. The third isolate, *Methylomonas* Sn-4, showed 97.8 % 16S rRNA sequence homology with *Methylomonas methanica* S1. Sn-4 grouped close to *Methylomonas methanica* and *Methylomonas koyamae* in the 16S rRNA gene-based phylogenetic tree (Fig. 2a). *pmoA* sequence of Sn-4 showed 94 % similarity to *Methylomonas koyamae*. Based on 16S rRNA gene and *pmoA* gene sequences and phylogenetic analysis, all the three isolates showed taxonomic novelty. Sn10-6 clearly belonged to a

putative novel genus, a branch close to the *Methylovulum-Methylosoma* genera within type Ia methanotrophs.

Culture-Independent Analysis of Methanotrophic Communities

The uncultured methanotroph community structure was deciphered using three molecular tools: *pmoA* clone libraries, T-RFLP, and metagenomics.

***pmoA*-Based Clone Library and T-RFLP** Both of these methods involve amplification of *pmoA* gene and have been extensively used in community analysis of methanotrophs [19, 25]. In the clone library approach, a total of 70 positive

Table 1 Characteristics of isolates with phylogenetic affiliation

| Strain designation (dilution from which isolated)	Type I/type II	In silico T-RF in bp of partial *pmoA* gene with *MspI* enzyme	Next cultivated type species 16S rRNA gene sequence, similarity	Next cultivated type species by BLAST (partial *pmoA* gene), similarity
Sn10-6 (10^{-6})	Type Ia	510	*Methylosarcina lacus* AY007296.1, 93.5 %	*Methylovulum miyakonese* AB501288.1, 85 %
Sn-Cys (10^{-6})	Type II	244	*Methylocystis parvus* OBBP NRO44946. 1, 98.4 %	*Methylocystis parvus* AF533665.1, 94 %
Sn-4 (10^{-4})	Type Ia	437	*Methylomonas* sp. methanica S1 NR041815.1, 97.8 %	*Methylomonas koyamae* AB538965.1, 94 %
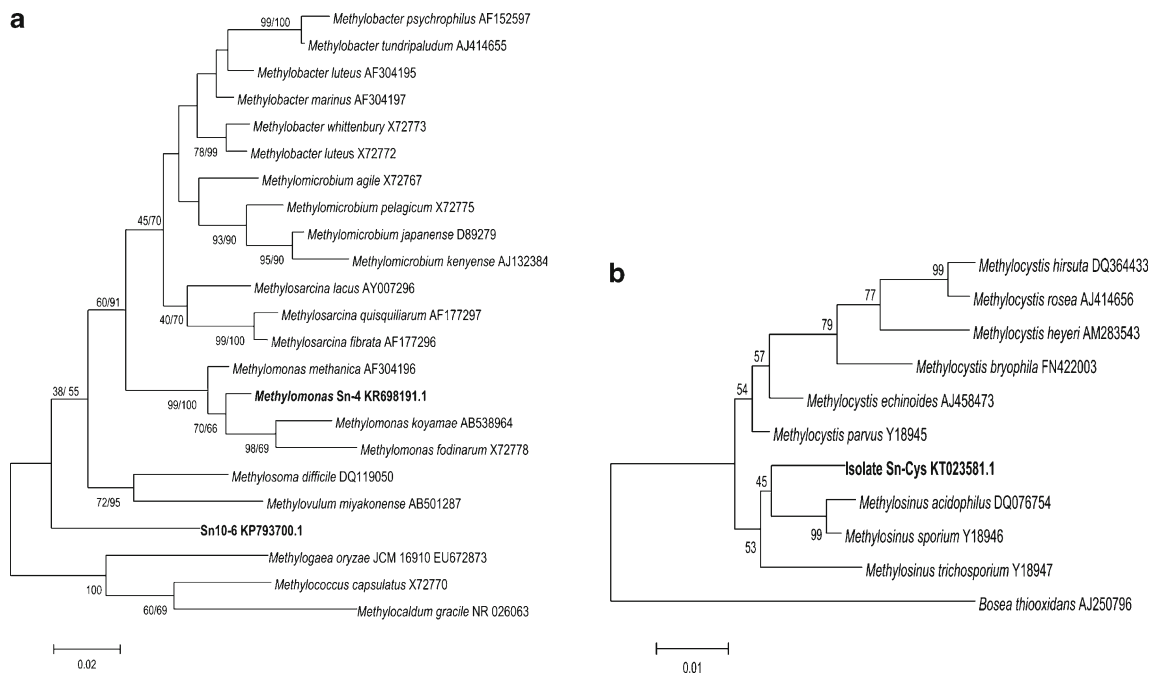
Fig. 2 **a** Maximum likelihood tree of 16S rRNA gene sequences of isolates Sn10-6 and Sn-4 in comparison with other type I methanotrophs. Alternative treeing methods gave similar branches. Bootstrap values obtained by maximum likelihood/neighbor joining are shown with a *solidus sign. Bar* represents 2 % divergence. **b** Maximum likelihood tree of 16S rRNA gene sequences of Sn-Cys in comparison with other type II methanotrophs using MEGA 5.05 and 1000 bootstraps is shown. *Bar* represents 1 % divergence

clones were screened by RFLP analysis with *MspI* enzyme. In total, six RFLP groups were present, and from each group, at least 20 % clones were sequenced. Rarefaction analysis using "Past" software showed that sufficient number of clones had been screened as plateau was reached (data not shown). The BLAST analysis and phylogenetic analysis indicated that 97 % of the clones belonged to type I methanotrophs and only 3 % constituted type II methanotrophs (Table 2). Type I methanotrophs were further separated in four clades represented by five RFLP groups: A to E (Fig. 3). RFLP group A (in silico T-RF=510 bp) showed clones which were most similar to *Methylobacter* spp. and *Methylosoma difficile*. RFLP group B (in silico T-RF=437 bp) clones were similar to *Methylomonas* spp. RFLP group C clones (in silico T-RF= 456 bp) showed similarity to *Methylosarcina* and *Methylomicrobium* spp. RFLP groups D and E (in silico T-RF=76 or 350) consisted of clones showing no close relationship to cultured representatives and only distant relationship (80–82 % on nucleotide basis) with *Methylomicrobium album* or *Methylosoma difficile*. The type II methanotrophs, group F (in silico T-RF=244 bp) clones, showed maximum similarity to *Methylocystis* spp.

T-RFLP analysis using partial *pmoA* gene fragment digested with *MspI* enzyme confirmed the results obtained by clone library approach. Major peaks were obtained at 510, 437, 350, and 76 bp, and minor peaks were obtained at 370 and 450 bp (Supplementary Fig S1). Affiliation of

majority of the peaks with clones and isolates could be done which is shown in the figure. No peak characteristics of type II methanotrophs (244 bp) were obtained.

Metagenomics A total of 58,007,061-bp data was obtained after QC, and a total 417,331 -bp with 343,455 proteins were obtained. Four genera from the Methylococcaceae family (*Methylobacter*, *Methylococcus*, *Methylosarcina*, and *Methylomonas*) and three genera belonging to Methylocystaceae (*Methylocystis*, *Methylosinus*, and *Methylocella*) were detected (Table 3). Methylococcaceae members showed higher abundance (1.7 times) compared to Methylocystaceae. Within Methylococcaceae, type Ia methanotrophs related to *Methylobacter tundripaludum* were the most abundant (abundance=307), 61 % of the total type I methanotrophs. Methanotrophs related to *Methylococcus capsulatus* occupied the rest 38 % within type I with abundance value 190. Abundance value of one was obtained for *Methylomonas* and *Methylosarcina* genera. Type II methanotrophs related to *Methylocystis* spp. and *Methylosinus* spp. showed abundance values of 167 and 128, respectively. In addition, a considerable abundance of *Methylocella silvestris* (176 abundance) was also detected. This genus shows absence of particulate methane monooxygenase. Presence of Verrucomicrobial methanotrophs was detected albeit in lower abundance, i.e., 67 [21]. Two *pmoA*-related sequences were
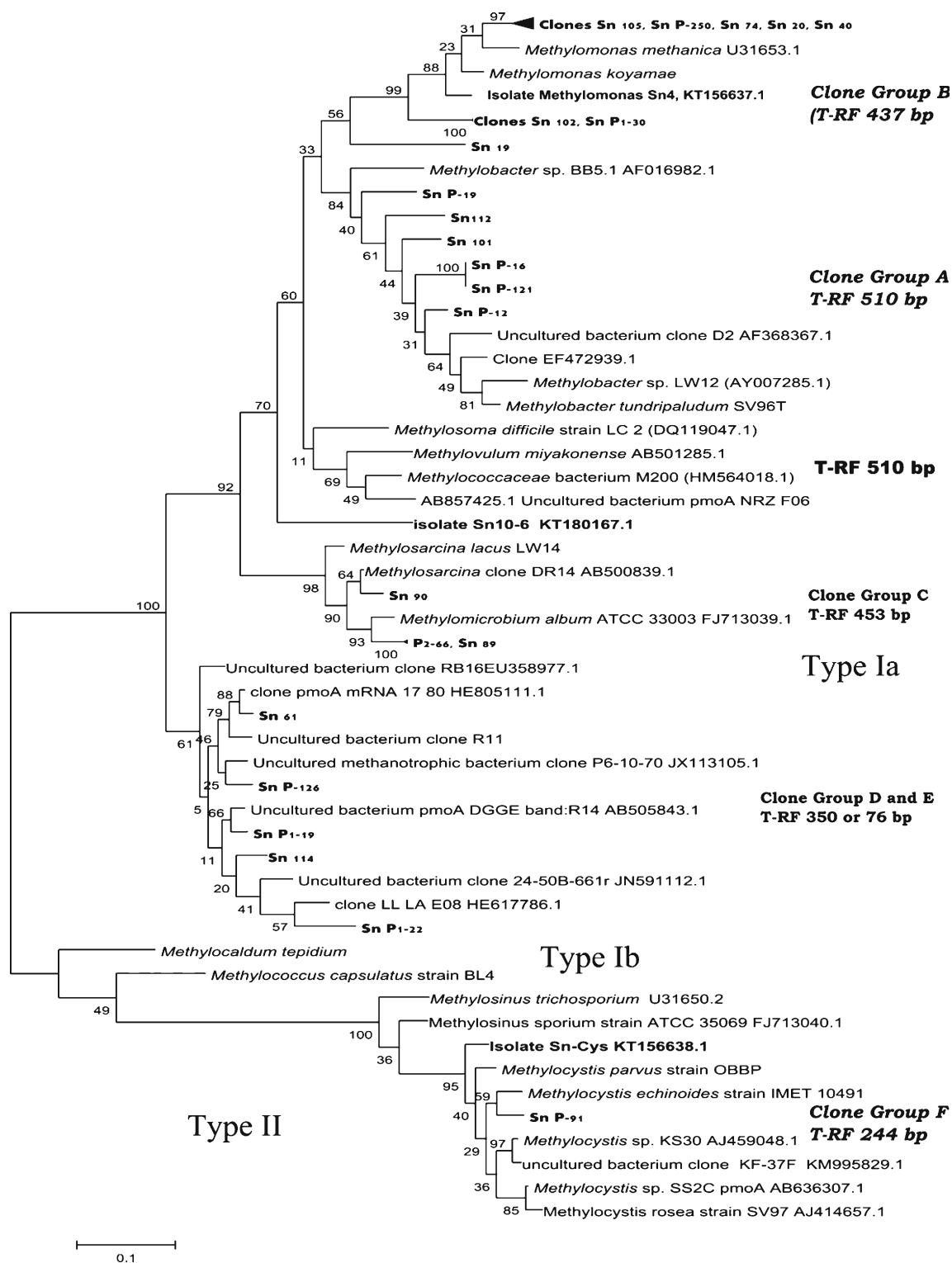
Fig. 3 Phylogenetic dendrogram based on the nucleotide sequences of cloned partial *pmoA* genes from the rice rhizosphere sample, from the three isolates in comparison with *pmoA* of known species and related clones. The tree was constructed using maximum likelihood analyses using MEGA 5.05 and 1000 bootstraps. All clones in this study are prefixed with Sn. Clone groups are indicated as clone group A to F. Type Ia, type Ib, and type II methanotrophic groups are shown. *Bar* represents 1 % divergence

extracted from the metagenome, and a phylogenetic tree was constructed using the partial *pmoA* sequences extracted from

the metagenome and using *pmoA* from isolates, known species and few clones from the clone library. In the phylogenetic

Table 2 Relative abundance of clones in the *pmoA* clone libraries and their phylogenetic affiliation

Phylogenetic group (type I /type II)	Clone group (in silico T-RF)	No. of clones (total clones=70)	Relative abundance (%)	Clone with accession number	Next cultivated type species *pmoA* gene sequence, similarity
Type I	A (510)	18	27	Sn P-19, KR698182.1	*Methylosoma difficile* strain LC 2 (DQ119047.1), 88 %
Type I	B (437)	33	49	Sn 74, KR698177.1	*Methylomonas methanica* MC09 (CP002738.1), 94 %
Type I	C (456)	3	4	Sn 90, KR698178.1	*Methylomicrobium album* strain ATCC 33003 (FJ713039.1), 94 %
Type I	D (350)	6	9	Sn 114, KT588705.1	*Methylomicrobium album* (FJ713039.1), 81%
Type I	E (76)	5	7	Sn P1-22, KR698185.1	*Methylomicrobium album* (FJ713039.1), 81%
Type II	F (244)	2	3	Sn P-91, KT588706.1	*Methylocystis echinoides* IMET10491 (AJ459000.1), 95 %

tree, the metagenome-derived *pmoA* sequences grouped closely with the *pmoA* of Sn10-6.

Discussion

At present, there are very few studies in which both cultivation and cultivation-independent approaches are used in combination for studying methanotroph community structure from rice field habitats in particular [16]. Methanotroph isolation and cultivation are challenging due to several problems encountered in isolation procedures and additionally due to the problems faced in purification of methanotrophs from heterotrophs and need up to several years [12]. The present study would be the first systematic study to the best of our knowledge, where methanotrophs from an Indian flooded rice field rhizospheric environment were enriched, isolated, and characterized. Methanotrophs from Italian rice fields (Vercelli, Italy), one of the most well-studied rice fields in the world, have been quantified by real-time PCR [23]. In this work, it was indicated that the total methanotrophs were in the range of 2.5×10^6

copies of *pmoA* per gram fresh soil which is one of the most accurate estimates of methanotrophs in rhizosphere environments, as other estimates are based on MPN counts [40]. The results match with this estimate because, statistically, 10^6 methanotrophs had to be present in the original sample to result in growth in a 10^{-6} dilution of this sample. Both type I and type II methanotrophs were isolated from the terminal dilution which indicated that both of these types were present in the original sample in fairly high numbers. The dilute NMS medium used here has been successfully used in the isolation and cultivation of novel type Ia methanotrophic genera, *Methylosoma* [34] and *Methyloglobulus* [13], from Lake Constance, an oligotrophic lake [13, 34]. Since the samples were from agricultural soil, five times more nitrate was used in comparison with the original dilute NMS medium [13]. Mimicking the natural conditions is a good approach which might result in isolation of new methanotrophs [12, 34]. Sequences of 16S rRNA gene of all the three isolates were 98 % or less similar to cultivated species, and partial *pmoA* sequences showed 94 % or less relatedness to that of cultured species, reflecting their taxonomical novelty [37]. We could cultivate a

Table 3 Abundance of genes affiliated to methanotrophs in the metagenome

Family	Methanotroph genes affiliated to species	Abundance in the metagenome[a]
Methylococcaceae	*Methylobacter tundripaludum*	307
Methylococcaceae	*Methylococcus capsulatus*	190
Methylococcaceae	*Methylomonas methanica*	1
Methylococcaceae	*Methylosarcina lacus*	1
Methylocystaceae	*Methylocystis* sp. *ATCC 49242*	167
Methylocystaceae	*Methylosinus trichosporium*	128
Beijerinckiaceae	*Methylocella silvestris*	176
Methylacidiphilaceae	*Methylacidiphilum* spp.	63

[a] Using MG-RAST pipeline and "organism abundance" function

novel type Ia methanotroph at the rank of a genus as the dominant morphotype in the 10^{-6} grown enrichment. Sn10-6 was most related to *Methylovulum miyakonese*, *Methylosoma difficile*, and *Methylobacter tundripaludum* based on the 16S rRNA gene- and *pmoA*-based phylogenetic tree forming a separate branch. Additionally, the *pmoA* sequences extracted from the metagenome (4) showed close phylogenetic affiliation to Sn10-6 *pmoA* sequence, revealing that Sn10-6 could be an important part of the community. Even though a *pmoA* sequence similar to that of Sn10-6 *pmoA* was not revealed in the clone library, Sn10-6 showed an in silico T-RF of 510 bp which is characteristic of clone group A and related type Ia genera *Methylobacter tundripaludum*, *Methylosoma difficile*, and *Methylovulum miyakonese*, indicating its phylogenetic relationship to this clade. Thus, Sn10-6 might not be one of the most dominant type I methanotrophs present, but based on the dilution from which it was isolated, its in silico T-RF, and relevance to the metagenome-derived sequence, it would be one of the relevant ones cultivated from the environment. Strains from only one type Ia methanotroph, i.e., *Methylomonas* spp., have been cultivated so far from rice fields over the world [14, 31]. Presence of many novel taxa has been detected by methods like stable isotope probing [32], although none of these has been cultivated. Thus, isolating a putative novel genus of type Ia methanotroph is an important result and could help us in understanding the physiology of this group and related species. Similarly, strain Sn-Cys cultivated in this study showed close relationship to the type II clone obtained in the clone library (93 % nucleotide similarity and 99 % amino acid similarity). Metagenomic analysis had confirmed that type II methanotrophs were present in considerable numbers in the sample. Phylogenetically, Sn-Cys was found to have a unique position in between *Methylocystis* and *Methylosinus* on the basis of both 16S rRNA- and *pmoA*-based trees (Figs. 2b and 3) and hence needs to be further explored for taxonomy and properties. *Methylocystis* is known to form desiccation-resistant cysts, and *Methylosinus* is known to produce exospores [42]. Such survival strategies become very important for the methanotrophs to survive the dry conditions in India, which persist throughout the year after the monsoon season ends. Recently, the presence and activity of *Methylocystaceae* members have been also demonstrated in rice roots by metaproteome analysis and in situ hybridization techniques (CARD FISH) [20]. Molecular approaches have also shown that *Methylocystis* spp. are one of the important members of rice field methanotrophic communities [24]. A strain of *Methylomonas* isolated in this study, strain Sn-4, appears to be a putatively novel species showing considerable relatedness to the *pmoA* clones in the library (Fig. 3). High abundance of *Methylomonas* spp. was also seen in the clone library (49 % abundance), and in T-RFLP analysis, there was a major peak at 437 bp, characteristic of *Methylomonas* spp. *Methylomonas* is amongst the most prominent members of

the methanotrophic communities in rice fields [16, 36] and one of the most common genus to be isolated from rice fields [14]. After comparing 16S rRNA gene of Sn-4 and Sn-Cys isolates with other non-type species and isolates in the NCBI database, it was seen that the 16S rRNA gene sequences matched 99 % to some of the strains isolated from Japanese rice fields [14].

Three different cultivation-independent methods were used to clearly understand the community composition of the methanotrophs in the sample. These three yielded slightly different but a much broader picture of the community. To summarize, the *pmoA* PCR-based culture-independent techniques (clone library and T-RFLP) showed that type I methanotrophs dominated and occupied methanotrophic community. Within type I, methanotrophs related to *Methylobacter tundripaludum*, *Methylomonas* spp., *Methylosoma difficile*, and *Methylomicrobium-Methylosarcina* were abundant. A considerable portion of the community was not related to any known genera of methanotrophs and formed a cluster which indicated presence of novel taxa. Type II methanotrophs were detected in very less abundance, only 3 % of the total community. Metagenomics approach gave us a rather complete picture, where dominance of type I methanotrophs was confirmed; however, it also reflected the presence of type II methanotrophs in considerable numbers, i.e., 37 %, which were missed out in the *pmoA* PCR-based approaches. *Methylocella* and *Methylacidiphilum* were detected only by metagenomics approach, as they would not have been detected by universal *pmoA* PCR-based analysis. The only drawback of using shotgun metagenome analysis with pipelines such as MG-RAST is that sequences of relatively very short length can be used for sequence prediction and similarity analysis [39]. Thus, the phylogenetic affiliation to a particular genus or species may not be perfect, as the sequence length is comparatively smaller. In contrast, the *pmoA* clone library approach yields complete sequences which can be compared using phylogenetic analysis.

Clone libraries of *pmoA* gene and T-RFLP have been extensively used in the community analysis of methanotrophs [19, 25]. The methanotrophic diversity as revealed by *pmoA* cloning and T-RFLP approach was similar to that obtained from Chinese, Italian, and Uruguayan rice fields [16, 19, 25, 32] except for the absence of peak characteristic for type II methanotrophs (244 bp). In a recent study conducted in Vercelli, Italy, irrespective of the growth stage and type of fertilizer used, Type Ia methanotrophs dominated the roots as revealed by *pmoA* transcript-based T-RFLP cloning/sequencing analysis [36]. Communities of Type Ia methanotrophs have been demonstrated to be responsible for methane consumption in variety of wetland ecosystems, including rice paddies, riparian wetlands [4], arctic wetlands [18], and lake sediments [33]. In many of the wetlands and lakes, a *Methylobacter tundripaludum*-related methanotroph was dominant. Shotgun metagenomics has not yet been used very commonly for

deciphering the methanotrophic communities from rice fields, and there are reports available on pyrosequencing of *pmoA* amplicons [22, 26]. It was seen that Type II methanotrophs form a substantial part of the community when metagenomics approach was used. This could probably be due to the PCR bias in not amplifying type II methanotrophs as seen before [6].

Thus, combining cultivation with cultivation-independent approaches proved to be a useful strategy in deciphering the methanotrophic community structure of a rice rhizosphere sample. The methanotrophic isolates obtained in this study would be further explored for their physiology and taxonomy and used as model organisms for studying methanotrophy.

The sequences have been submitted to GenBank, and the accession numbers are as follows: KR698177–KR698190 and KT588695–KT588706 (*pmoA* clones), KT156637 (*pmoA* of Sn-4), KT156638 (*pmoA* of Sn-Cys), KT180167 (*pmoA* of Sn10-6), KR698191 (Sn-4, 16S rRNA gene), KT023581 (Sn-Cys-16S rRNA), and KP793700 (Sn10-6, 16S rRNA gene). The metagenome has been submitted to the NCBI SRA database, and the accession number is SRP062304. In the MG-RAST database, the submitted metagenome has been made public, with the Id: 4623913.3. The bioproject has been registered as PRJNA286839 and biosample as SAMN03982392.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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