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DNA microarray analysis targeting *pmoA* gene reveals diverse community of methanotrophs in the rhizosphere of tropical rice soils

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The diversity of the methanotrophs community of two different rice fields of a typical tropical rice agroecosystem was assessed using microarray targeting *pmoA* gene-based approach. The presence of types I and II methanotrophs was observed with the dominance of *Methylocystis* in both the fields. The study revealed that the Barkachcha rice field harbours more diverse groups of methanotrophs than the Ghazipur rice field. It was also observed that in some members of types I and II methanotrophs, even the peat-associated group was present in the enriched culture of the soils. The Ghazipur soil and its enriched mixed methanotrophic culture showed higher methane oxidation potential than the Barkachcha soil. These results suggest that the methanotrophs community and its potential for methane oxidation vary with change in soil type within the same ecosystem.

Keywords. Methane oxidation, methanotrophs, microarray, *pmoA* gene, rice soil.

DUE to their significant role in global CH_4 cycling, methane-oxidizing bacteria (MOB; methanotrophs) have been the focus of several scientific researchers. Methanotrophs, abundantly found in the aerobic layer of the soil, the rhizosphere[1,2], oxidize significant amount of CH_4 generated by methanogens. It is expected that methanotrophic bacteria utilizing CH_4 as substrate in the rhizosphere will vary in the population composition and density within the rice rhizosphere[3]. The methanotrophic community is complex and diverse, containing 10 genera which belong to type I (*Gammaproteobacteria*), and four genera to group

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type II (*Alphaproteobacteria*) methanotrophs, and two recently described genera, *Crenotrichidae* and *Verrucomicrobia*[4]. For the study of methanotrophs from various habitats, phylogenetic (16S rRNA) and functional gene (*mmoX* and *pmoA*) techniques are used. The diversity of methanotrophs in enriched cultures and from environmental samples without cultivation has been assessed by several researchers using cloning, DGGE, T-RFLP, DNA microarray, RNA dot-blotting hybridization and PLFA–SIP[5–8]. The DNA microarray technology is a highly sensitive and cost-effective tool compared to traditional hybridization methods for detecting microorganisms in natural environment[9].

There are numerous studies on methanotrophic diversity in wetland rice fields of temperate soils. Nevertheless, reports on the diversity of the methanotrophic community from tropical rice agroecosystem are still inadequate. India ranks second only after China in rice production, covering a cultivated area of 42.3 m ha and accounting for 28.6% of the world's rice-cultivated area of 14.3×10^7 ha (ref. 10). Out of total 18.63 Tg yr^{-1} CH_4 emission from India, the rice fields alone contribute about 4 Tg yr^{-1} CH_4 emission[11].

Rice is invariably a major food crop of the South East Asian countries and in the decades to come the demand for rice will increase to fulfil the needs of the growing population, which in turn will result in the enhancement of methane emission. Under the above circumstances it is expected that the methanotrophs will exhibit variation in diversity and density, depending upon the physico-chemical conditions of the soil and other related factors, based especially on rice varieties under cultivation. Methanotrophs are an important component of the terrestrial ecosystem. However, difficulties in growing methanotrophs in the laboratory have hampered ecological studies. Molecular approaches such as microarray are essential tools for studying these important organisms.

India is an agriculture-based country with a variety of soil types and cultivation practices. Therefore, a better understanding of the methane oxidation activity and diversity of the methanotrophs for different rice fields is important. Our previous studies have indicated that the impact of cultivation practices and soil types on the variation of the MOB population size and activity is significant[12]. In the present study, we study the diversity and activities of the methanotrophic community in tropical rice fields.

Soil samples from two different paddy fields in a part of the Indo-Gangetic plains – Barkaccha Agriculture Farm, Banaras Hindu University, Mirzapur, Uttar Pradesh (UP), India (S1 and S2) and a farmer's field in Ghazipur District, UP, India (S3 and S4) were used. The Barkachcha soil (alfisol) was silt loam (sand 12%, silt 75% and clay 1%), and the Ghazipur soil (vertisol) was silty clay loam (sand 2%, silt 85% and clay 6%). The Barkachcha soil below 10 cm had pH 5.23, WHC 35%, organic C 0.57 mg g^{-1} and total N 0.12 mg g^{-1}, whereas the soil of Ghazipur site had pH 7.19, WHC 45.45%, organic C 0.85 mg $g^{-1}$and total N 0.16 mg g^{-1}. The Barkachcha rice field was rainfed, whereas the Ghazipur rice field was irrigated[12].

In order to get a better picture of the methanotrophic diversity in paddy fields, the rhizospheric soil sampling was done twice – at the vegetative (S1 and S3) and reproductive (S2 and S4) stages of the rice plant in 2005. The soil samples were collected randomly in triplicate and were mixed together to avoid spatial heterogeneity. Rhizospheric soil samples were dug out below 10 cm with the root on plastic sheets. Field-moist soil samples were stored at 4°C for enrichment of the methanotrophs and at –20°C for DNA analysis. The samples were kept in triplicate.

Enrichment of methanotrophs from the soil samples was done in 5 ml enrichment medium, i.e. nitrate mineral salt (NMS) medium of Whittenbury *et al.*[13] (pH 6.8), established in a 20 ml serum bottle. Bottles were supplied with 20% methane and 80% air (20% O_2). After 4 days, the headspace was replenished again with fresh methane and air. All cultures were incubated at 37°C for 4–6 weeks with shaking at 200 rpm. The entire procedure followed was essentially the same as that used by Dubey *et al.*[14]. The C1, C2, C3 and C4 cultures were enriched from soil samples S1, S2, S3 and S4 respectively.

Methane oxidation capacity of the soil in triplicate was studied in laboratory incubation assays, as detailed by Dubey[2]. In brief, 10 g of field-moist samples, after an equilibration period of 24 h at 25°C, were transferred in triplicate into gas-tight, 135 ml Erlenmeyer flasks, and incubated at 30°C for 10 days in the dark. Soil samples with or without autoclaving, were used to confirm that the methane oxidation was microbial-mediated. The flasks were sealed with rubber stoppers fitted with a silicon septum. The initial concentration in the headspace was maintained at about 50 μmol l^{-1} (equivalent to 1150 ppmv) by injecting the appropriate quantity of pure (99.999%) CH_4.

In order to test the methane oxidation capacity of methanotrophic mixed culture, 1 ml of well-grown mixed methanotrophic culture (O.D. at 600 nm = 1.2) was inoculated into 100 ml serum bottles containing 25 ml NMS medium in triplicate. After the bottles had been capped with butyl rubber stopper and aluminium caps, 20% air was withdrawn from the bottles and equal percentage of methane was injected into the serum bottles. Gas chromatograph (Model 3800 GC, Varian, BV, The Netherlands) equipped with flame ionization detector (FID) and Porapak-N column was used to analyse the residual methane concentration by taking 0.1 ml of headspace sample with a 1 ml gas-tight syringe on 0, 2, 4, 6, 8 and 10th day of incubation. The oven, injector and detector temperatures were set at 40°C, 110°C and 180°C respectively. Nitrogen (flow rate – 15 ml min^{-1}) served as the carrier gas. On the 10th day, all cultures were centrifuged

and pellets were preserved at –20°C for genomic DNA isolation.

DNA from rhizospheric soil samples in triplicate was extracted using the fast DNA spin kit (BIO 101 system, Q-Biogene Inc, Irvine, USA) using the manufacturer's protocol. Further, to separate humic acid, purification of DNA was performed with the QIAquick gel extraction kit (QIAGEN). Genomic DNA of the bacterial culture was extracted from pelleted cells of the enriched cultures[15]. The enrichment culture (final O.D. at 600 nm = 0.1–0.2) was centrifuged (10 min at 15,000 *g*, 4°C) and the cells were lysed by adding 500 µl lysozyme buffer (pH 8) containing 5 mg ml^{-1} lysozyme and 250 µl sodium dodecyle sulphate (2%) to the pellet. Thereafter, incubation was done at 37°C for 1 h. Purification was followed using sodium acetate and isopropanol precipitation. The purified triplicate DNA of the soil samples and enrichment cultures was pooled together and stored at –20°C until further use.

Amplification of *pmoA* gene was performed by the semi-nested touchdown PCR method, as described by Horz *et al.*[16] and Bodrossy *et al.*[17]. In the first-round PCR *pmoA*/*amoA* genes were amplified using primers $pmoA_{189}$ (5′-GGN GACTGGGACTTCTGG-3′) and $pmoA_{682}$ (5′-GAASGCNGAGAAGAASGC-3′). Next, 0.5 µl PCR product of the first round was used as template in the second-round PCR for amplification of the *pmoA* gene using $pmoA_{189}$ as forward primer and T7-mb_{661} (5′-TAATACGACTCACTATAGCCGGMGCAACGTCYTTACC-3′) as reverse primer. T7-mb_{661} is a modified reverse primer containing the T7 promoter site (5′-TAATACGACTCACTATAG-3′) at its 5′-end. The reactions were performed in triplicate. Each sample showed PCR amplification of 500 bp as predicted for the *pmoA* gene (Figure 1).

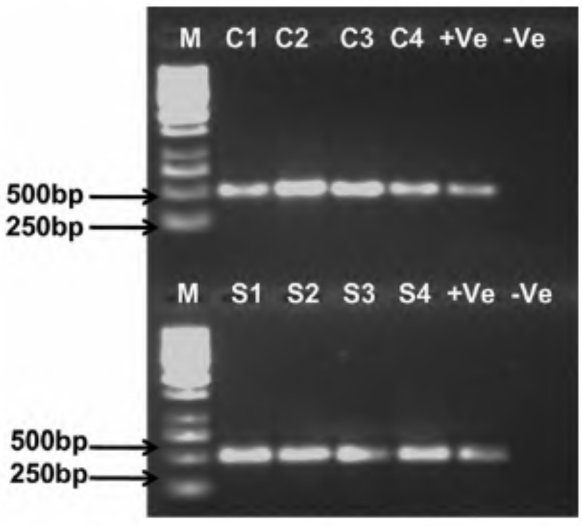

Figure 1. PCR products represented are targeted for microarray analysis. PCR amplification was done with pooled PCR product of step-I semi-nested touchdown PCR. Lane M, 1 kb DNA ladder; lanes C1–C4. From culture; lanes S1–S4. From soil; lane +ve. Positive control with genomic DNA isolated from *Methylosinus trichosporium OB3b* and lane –ve; Negative control without template in the reaction mixture.

Residual DNA, primers and unincorporated nucleotides left in the PCR products cause complication in diagnostic PCR and microarray. Therefore, PCR products were purified using QIAquick PCR purification kit prior to microarray analysis. The purified triplicate PCR products were pooled together before microarray analysis.

Microarray and target preparations, hybridization, scanning and data analysis were carried out as described by Bodrossy *et al.*[17] and Stralis-Pavese *et al.*[18]. The T7 promoter site tag on the 5′-end of the mb661 primer was used to enable a T7 RNA polymerase-based reverse transcription on the PCR products. Cy3-labelled UTP was incorporated during the *in vitro* transcription step. Purified labelled RNA was chemically fragmented to an average fragment size of around 50 nt and hybridized overnight on microarrays, and spotted in-house onto a commercial aldehyde surface. Hybridization was carried out in a custom-tailored aluminum block used as an insert for a temperature-controlled Belly Dancer. Hybridized slides were washed, dried and scanned within 2 h. Hybridized slides were scanned at three lines to average 10 µm resolution with a GenePix 4000a laser scanner (Axon, Foster City, CA) at 532 nm wavelength for Cy3. Fluorescent images were captured as multilayered tiff images and analysed with the GENEPIX PRO 6.0 software (Axon). Microsoft Excel was used for statistical analyses. The methanotrophic community structure was visualized by a colour-coded presentation of abundance as yielded by the hybridization results using GeneSpring software. The probe-set, in general, targets the species level with some probes aiming at the subspecies and some at the genus level.

Rate of CH_4 oxidation (µmol l^{-1} h^{-1} g^{-1}dry wt soil) for soil samples and enriched mixed methanotrophic culture are shown in Table 1. The effect of soil type on CH_4 oxidation rate was significant ($F_{3,11} = 99.11$; $P = 0.000$). It has been reported that the methane oxidation rate changes with change in soil type and location[19]. The CH_4 oxidation rate was significantly ($F_{3,11} = 112.9$; $P = 0.000$) higher in mixed methanotrophic culture enriched from the black soil in comparison to those from the red soil. Methane oxidation was found to be higher at the reproductive stage. It has been reported earlier that MOB population is lower in the red soil compared to the black soil[12]. This could have led to higher methane oxidation potential in methanotrophic cultures enriched from the black soil.

Table 1. Variation in methane oxidation rate (µmol l^{-1} h^{-1} g^{-1} dry wt soil) in soil and mixed methanotrophic cultures enriched from soils

Soil/study site	Sampling stage	Soil	Culture
Barkaccha	Vegetative	0.05 ± 0.01	0.03 ± 0.01
	Reproductive	0.08 ± 0.02	0.06 ± 0.02
Ghazipur	Vegetative	0.14 ± 0.1	0.09 ± 0.01
	Reproductive	0.17 ± 0.3	0.12 ± 0.08

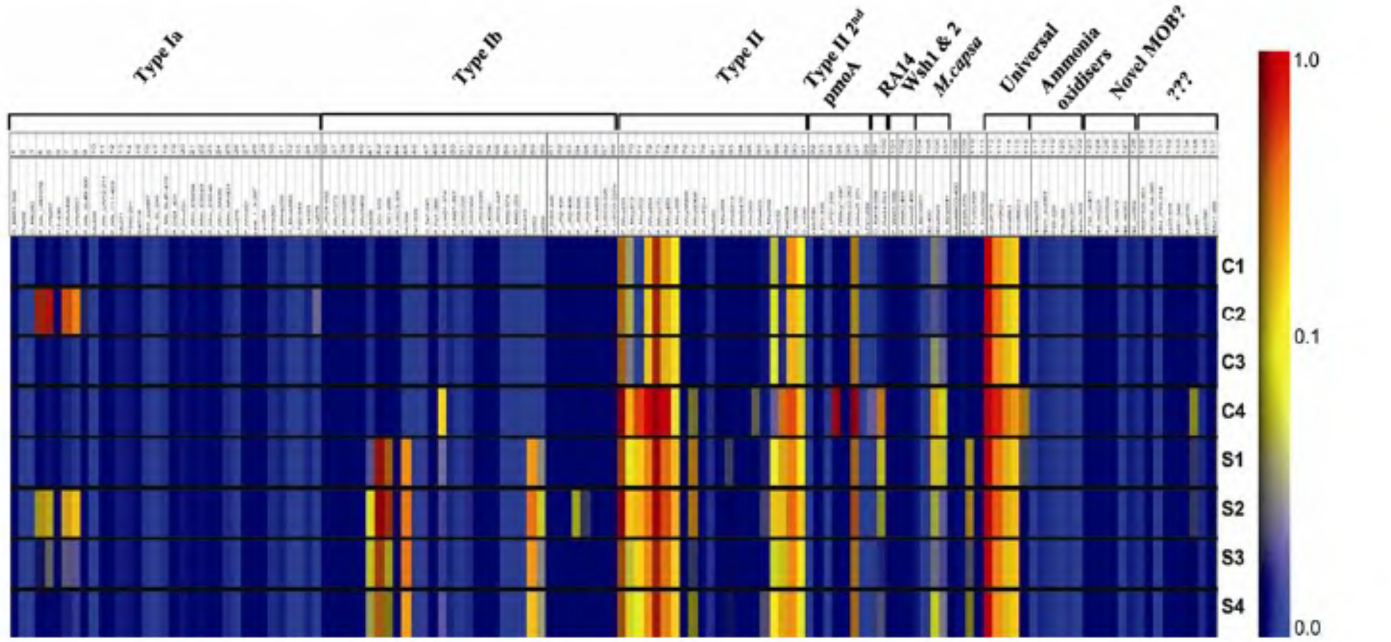

Figure 2. Methanotrophic community in Barkaccha and Ghazipur soils through *pmoA*-based microarray. Probes are arranged as target groups in columns numbered from 1 to 137 (cf. Table 2). 'Novel MOB' are target related to environmental *pmoA* sequences that are likely to belong to the methanotrophs; probes '???' again target the *pmoA* sequence but differ in phylogenetic affiliation with the methanotrophs. Colour-coding bar on the right side represents achievable signal for an individual probe (1 = maximum signal obtained, 0.1 = 10% signal meaning only 10% hybridization to that probe and 0 = no signal). C1 and C2 – methanotrophs enriched from the Barkaccha soil at vegetative and reproductive stages respectively; C3 and C4 – methanotrophs enriched from Ghazipur soil at vegetative and reproductive stages respectively; S1 and S2 – Barkaccha soil at vegetative and reproductive stages respectively; S3 and S4 – Ghazipur soil at vegetative and reproductive stages respectively.

The soil samples (S1–S4) examined in this study for the diversity of methanotrophs exhibited the presence of types I and II methanotrophs (Figure 2 and Table 2). DGGE and T-RFLP-based studies have shown the presence of types I and II methanotrophs in 10 rice field soils of China and the Philippines[19]. Jia *et al.*[20] have also reported the methanotrophic community in Japanese rice fields using cultivation-independent techniques. Type I methanotroph *pmoA* sequences found in tropical soils indicated the presence of *Methylobacter*, *Methylocaldum*, *Methylococcus* and group 501 (an uncultivated group related to *Methylococcus* and *Methylocaldum*). Among type II methanotrophs, *Methylocystis* and *Methylosinus* group-specific probes showed more hybridization. Intermittent flooding and drying are characteristic of a rice field, which make it a wetland ecosystem of its own kind and which directly affects CH_4 oxidation and production by the microbial community[21]. It is, therefore, inevitable that rice fields provide the growth substrate with fluctuation in CH_4 and O_2 turnover and hence lead to the dominance of type II methanotrophic community.

The study of two entirely different soils revealed that the type Ia methanotrophs related to *Methylobacter* (P_Mb_UrC278, Mb267, MbA486, MbA557) were present in the red soil (S2) only, whereas in the black soil (S3) signals were found to be weak. Type Ib genus *Methylocaldum* (Mc 1408) was found in all samples of the black soil (S3 and S4), whereas only one sample of the red soil (S2) exhibited the presence of Mc1408. Group 501 was present in both the soils, whereas probe 501–286 was higher in the red soil. *Methylococcus* showed its presence in all the samples. Generic type Ib probe was more in the red soil than in the black soil. A recent study on the diversity of methanotrophs in tropical rice soils showed the presence of types I and II methanotrophs, where the former was more abundant than the latter[8]. This study indicated that plant age and land-use history play a major role in determining the methanotrophic community in rice soils. In a microcosm-based study it has been reported that type I methanotrophs are found in the rice soil where methane concentration is comparatively lower[22]. It has also been reported that flooded conditions are favourable for type II methanotrophs, whereas type I methanotrophs prefer drained conditions[23]. In our earlier study it has been observed that moisture content of the red soil is lower compared to the black soil, which might have led to aerobicity of the red soil[12]. Hence, the lowering of methane concentration provided a suitable niche for type I methanotrophs in the red soil. Further, the results also exhibited that both the soil types show the presence of type Ib methanotrophs below detection limit. As far as type II methanotrophs are concerned, high abundance of *Methylocystis* and *Methylosinus*-specific probes was detected from the red and black soils. Probes Mcy522 and Mcy304 were detected to be higher in the red soil. MSci 232 and generic type II probes were quantified approximately similar for the two soils. Probe PNMsi, related to genus *Methylosinus*, was typically pre-

Table 2. Probes used for microarray analysis. Column number corresponds to the order in which the probes are arranged for the microarray analysis as shown in Figure 2

Column number	Probe	Column number	Probe	Column number	Probe	Column number	Probe
1	O_BB51-299	36	P_JRC4-432	71	O_Mcy522	106	B2all343
2	Mb292	37	P_MclT272	72	P_Mcy264	107	O_B2all341
3	O_Mb282	38	P_MclG281	73	P_Mcy270	108	pmoAMO3-400
4	P_Mb_URC278	39	P_MclE302	74	P_Mcy459	109	P_ESR-579
5	P_Mb267	40	P_MclS402	75	O_Mcy255	110	P_TUSC409
6	511-436	41	Mcl408	76	P_McyM309	111	P_TUSC502
7	P_MbA486	42	P_501-375	77	P_McyB304	112	mtrof173
8	P_MbA557	43	O_501-286	78	P_MsT214	113	mtrof362-I
9	P_Mb_SL#3-300	44	P_USC3-305	79	P_MsT343	114	mtrof661
10	Mb460	45	Mc396	80	P_MM_MsT343	115	mtrof662-I
11	P_Mb_LW12-211	46	Fw1-639	81	Msi520	116	mtrof656
12	P_Mb_C11-403	47	O_fw1-641	82	O_Msi269	117	NmNc533
13	Mb271	48	P_fw1-286	83	P_MsS314	118	Nsm_eut381
14	PS80-291	49	P_LW21-374	84	P_MsS475	119	PS5-226
15	Est514	50	P_LW21-391	85	P_Msi263	120	PI6-306
16	Mm_pel467	51	P_OSC220	86	P_Msi423	121	NsNu207
17	Mb_SL-299	52	P_OSC300	87	O_Msi294	122	NsNv363
18	O_Mb_SL#1-418	53	P_JRC3-535	88	Msi232	123	P_Nit_rel471
19	P_DS1_401	54	P_LK580	89	Peat264	124	Nit_rel223
20	P_Mm531	55	P_JRC2-447	90	O_II509	125	P_ARC529
21	P_Mm_ES294	56	O_M90-574	91	O_II630	126	Nit_rel470
22	P_Mm_ES543	57	O_M90-253	92	xb6-539	127	Nit_rel351
23	P_Mm_ES546	58	Mth413	93	LP21-190	128	Nit_rel304
24	P_Mm_M430	59	Ib453	94	O_LP21-260	129	M84P105-451
25	P_Mm_MV421	60	Ib559	95	P_NMcy1-247	130	WC306_54-385
26	Mm275	61	P_DS3-446	96	P_NMcy2-262	131	M84_P22-514
27	P_Mm451	62	P_JR2-409	97	P_NMsiT-271	132	gp23-454
28	peat_1_3-287	63	P_JR2-468	98	LP21-232	133	MR1-348
29	Jpn284	64	P_JR3-505	99	O_RA14-594	134	P_gp619
30	Mmb303	65	P_JR3-593	100	P_RA14-591	135	gp391
31	P_Mmb259	66	Nc_oce426	101	P_Wsh1-566	136	gp2-581
32	O_Mmb562	67	P_USCG-225	102	P_Wsh2-491	137	RA21-466
33	LP20-644	68	P_USCG-225b	103	P_Wsh2-450		
34	O_Ia193	69	P_Mcy233	104	O_B2rel251		
35	O_Ia575	70	O_Mcy413	105	B2-400		

sent in each sample. As far as type II methanotrophs are concerned, high abundance of *Methylocystis* and *Methylosinus*-specific probes were detected in both the soils. This is in line with the study of Gilbert *et al.*[24], where it was reported that that type II methanotrophs were in higher abundance in the soil than type I methanotrophs. The present study also shows that the red soil comprised of more diverse methanotrophic community than the black soil. It would be appropriate to point out here that the lower cell ratio in environment samples could restrict hybridization with specific probes, which in turn affects the detection limit of array analysis[17]. In this study higher methane oxidation activity was found in the black soil compared to the red soil. Therefore, it could be concluded that there is no correlation between the methane oxidation potential and methanotroph diversity.

Signals for the peat group (an uncultivated group of type II methanotrophs usually reported from acidic soils, including peat frequently found in temperate soils) were also detected. The probes RA14-591, B2all341 and B2all343 are prone to false positive signal on RA14-594 (for RA14-591) or on B2rel251 or B2-400 (for B2all341 and B2all343). The two generic probes for *Methylocapsa* and related probes are again prone to false positives signals. With regard to the presence of peat associated group in two rice fields, this is probably due to typical Indian rice cultivation patterns. During field preparation, puddling practices avoid movement of water from the fields, which generates anaerobic situation leading to dissolution of nutrients causing lowering of the pH. In addition, organic matter load, intermittent flooding and drying also lower the pH of the soil. Further, rhizospheric soil shows normally lower pH compared to non-rhizospheric soil due to release of H^+ ions from rice roots[25]. These factors may be responsible for the similar observations of the experimental soils of the present study with those of the peat soil.

Microarray hybridization revealed that the growth of type Ia (*Methylobacter*) and type II (*Methylocystis*) methanotrophs was favoured by the NMS medium used in this study. Type II probes have not shown much varia-

tion. It is remarkable to find peat group 264 in enriched culture C4 of the black soil, as the presence of peat group was particularly unexpected in relation to the previous hypothesis that the peat clade groups are believed to be acidophilic and are yet uncultivated[17]. A single genus of *Methylobacter* (type I) was found from the red soil alone. It is known that only certain types of methanotrophs prefer the enrichment condition used in laboratories[26]. From the above it is clear that using both culture-based method and culture independent method, it is possible to define the cultivability of each species. The significance of studying the cultivable fraction is to get a glimpse of the total cultivable fraction of methanotrophs. Further *ex situ* studies to assess the possible beneficial applications of methanotrophic species in the industry, environmental pollution control, etc. need to be explored under controlled laboratory conditions.

In conclusion, the soils showed diverse groups of methanotrophs. The soil and mixed methanotrophic culture from both the sites have shown potential for methane oxidation. Laboratory incubation has favoured the growth of both type I and II methanotrophs. Further extension of the study will help in screening out the potential methanotroph as a bioresource for environmental application, including methane degradation which will eventually help in the formulation of strategies for mitigation of greenhouse effect due to CH_4 emission in the environment.

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