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Molecular diversity of methanogenic archaea and methane production potential of soil in relation to rice cultivars

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Rice is a staple food of human kind and its demand is ever increasing. Asia accounts for 91% of global rice production of which India contributes 23%. The increasing demand has led to the development of various high yielding varieties and cultivars. Rice cultivation in flooded fields is known to influence the atmospheric methane-budget. Here, we studied the effects of rice cultivars on methane (CH_4) production, methanogenic archaeal diversity and abundance in Indian rice soils using six rice cultivars (IDR 763, HUR 3022, Sahbhagi, Swarna sub 1, MTU 7029 and BPT 5204). Methanogen community size and diversity was analyzed using qPCR and DGGE targeting *mcr*A and 16S rRNA gene fragments, respectively. Methanogenic community size varied among cultivars (1.65-97.6 × 10^5 copies g^{-1} dws). The diversity analysis revealed five methanogenic groups i.e., *Methanocellaceae*, *Methanobacteriaceae*, *Methanomicrobiaceae*, *Methanosaetaceae* and *Methanosarcinaceae* common to all the cultivar samples but with varying composition. Methane production of soil samples was in the order: Sahbhagi <HUR 3022 <IDR 763 <Swarna sub 1 <MTU 7029 <BPT 5204. It has been observed that rice cultivars with different plant biomass could provide favourable niche for the prevalence of methanogenic archaeal community that imparts differential effects on soil CH_4 production.

Keywords: BPT 5204, CH_4 production, HUR 3022, IDR 763, MTU 7029, Paddy soils, Sahbhagi, Swarna sub 1

Rice is the world's second largest cereal crop after maize in terms of yield. The global rice production has increased at the average rate of 154.3 million tones/year over the past ten years. About 91% of rice demand across the world is met by Asian countries. India contributes to about 23% of the rice produced in Asia[1]. Rice productivity is increased either by applying fertilizers or cultivating the high yield varieties. Presently, 489 rice varieties have been developed, introduced and cultivated in different states of India. Use of such varieties has led to increased rice production, and among these, Uttar Pradesh ranks first[2]. Rice cultivation in flooded fields is the main anthropogenic source of methane production that influences the atmospheric methane budget.

During methanogenesis, organic matter is degraded through the involvement of complex microbial communities comprising hydrolytic, fermenting, and methanogenic microorganisms[3]. The methanogenic archaea avail the organic matter to generate methane during the terminal step of decomposition. Methane emission rate varies considerably among different types of rice cultivars. Several studies on methane flux variations with varying cultivars[4-7] are available. However, it is not clear how cultivars respond to the rhizospheric methanogenic archaeal flora which in turn is responsible for methane production in rice fields. Studies on the effect of rice cultivars on methanogenic community structure residing in paddy soils at global level are scanty and nil for Indian paddy soils. Moreover, the available reports provide contradictory observations where Wu *et al*.[8] observed variations in rhizospheric methanogenic community structure as influenced by the rice cultivars. However, no such effect was reported by Ma *et al*.[4] Thus, further studies seem imperative for a better understanding of this aspect. The present study, therefore, has been aimed at assessing the methanogenic community structure and methane production potential of soils in relation to rice cultivars commonly adopted in Uttar Pradesh, India.

Materials and Methods

Study site

Study site was the agriculture farm of the Institute of Agricultural Sciences, Banaras Hindu University,

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Varanasi, Uttar Pradesh, India (83° 59' 8.964" E and 25° 16' 31.468" N). This region experiences a seasonally dry tropical climate followed by typical monsoon. Annual rainfall and average temperature during the experimental year (2012) were 1100 mm and 8-42°C, respectively. In the present study, our emphasis was to evaluate the effect of rice cultivars, and hence, all cultivars were planted in the same type of soil having similar physicochemical properties. Soil was classified as inceptisol, silty loam with sand (32%), silt (65%) and clay (3%) with pH 7.2, water holding capacity (WHC) 44%, organic carbon 0.75% and total-N 0.12%.

Experimental design

The experimental site comprised of 18 plots, each of 5 × 3 m in size. A 0.5 m strip separated each plot. Basal treatment of KCl + P_2O_5 + farmyard manure in the ratio of 60:60:1000 kg/ha was applied to all the plots at the time of plowing[9]. The plots were arranged in a completely randomized block design. Six rice cultivars (IDR 763, HUR 3022, Sahbhagi, Swarna sub 1, MTU 7029 and BPT 5204), were selected for the present study. Each cultivar was planted in three plots. Three seedlings (21 days old) of each cultivar were transplanted at hill-to-hill spacing of 15 cm, and row-to-row spacing of 20 cm.

Soil sampling

Our previous study revealed the flowering stage to be most favourable for methanogenic community proliferation[10-12]. Therefore, the present study was carried out only during mid-flowering stage of the rice cultivars. Soil samples from the rhizosphere of each cultivar in triplicate, were collected as per the method described by Vishwakarma and Dubey[13] from every plot and mixed together to get the composite sample. Soil samples were brought to laboratory and kept at –20 and 4°C for molecular and soil analyses, respectively.

Plant biomass

One rice hill of each cultivar with soil was harvested using a rectangular open-top plastic cylinder. Soil was completely removed by washing through water. Roots and shoots were dried at 60°C to constant weight for biomass determination. Root and shoot weights were pooled to generate plant biomass[9].

DNA extraction from soils and polymerase chain reaction (PCR) amplification

Total genomic DNA was extracted from soils (0.5 g) as per the protocol given in the Fast DNA® Spin Soil Kit (MP Biomedicals, Solon, Ohio, USA) using bead-beating method. DNA concentration and purity was evaluated spectrophotometrically.

The DNA samples were amplified for 16S rRNA gene of methanogenic archaea using specific primer pairs 1106 F-GC (5′-TTW AGT CAG GCA ACG AGC-3′) and 1378 R (5′-TGT GCA AGG AGC AGG GAC-3′)[14]. However, 16S rRNA methanogenic set primers are also known to target non-methanogenic Crenarcheota[11]. The PCR reaction mixture (50 µL) contained 10X reaction buffer (5 µL), 2.5 mM dNTPs (5 µL), 50 µM of each primer (0.5 µL), (5 U) *Taq* DNA polymerase (Bangalore Genei Bangalore, India) (0.25 µL) and 50 ng of the DNA template (1.0 µL). The PCR was performed using a 96-well Thermal Cycler (My Cycler™ Thermal Cycler, Bio Rad Laboratories, Inc., Australia), as under: an initial denaturation at 95°C (90 s) followed by 35 cycles of denaturation at 95°C (30 s), annealing at 55°C (30 s), and elongation at 72°C (90 s). The last cycle was followed by extension at 72°C (6 min). The PCR products were resolved by electrophoresis in 2.0% (w/v) agarose (Amresco) in 1 X TAE buffer stained with ethidium bromide (0.5 µg/mL). The images were digitized with gel documentation system (AlphaImager, NC, DE400-220, MTZ Zoom, Alpha Innotech Corp., San Leandro, CA), and DNA fragment lengths were identified using 100 bp DNA ladder (New England BioLabs, Ipswich, UK) as the molecular weight standard. Amplified PCR products were purified with NucleoSpin®Gel and PCR clean-up Kit (MN GmbH, Duren, Germany) and quantified using NanoDrop Spectrophotometer (ND 1000, NanoDrop Technologies, Inc., Wilmington, DE, USA) and stored at –20°C for further analysis.

DGGE analysis

DGGE analysis was performed with the DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) following the method described by Muyzer *et al.*[15]. The PCR products (approx. 200 ng) were loaded on to 8% (w/v) polyacrylamide gel immersed in 1X TAE buffer, and electrophoresed for 14 h at 60°C under a constant voltage (100 V). The polyacrylamide gel was prepared with denaturing gradients in the range of 32 to 62% (100% denaturation was achieved by using 7 mol/L urea and 40% formamide). After electrophoresis, the gel was stained with the SYBR Green I nucleic acid gel stain (1: 10000 dilution) (Lonza, Molecular Application, Rochland ME, USA), rinsed using

distilled water, and photographed on an UV-trans-illuminator (Vilber Lourmat, Marne-la-Vallee, France) at 302 nm with the SYBR Green gel stain photographic filter (Lonza).

Sequence analysis of DGGE bands

Most of the visible DGGE bands (23) were excised from the gel with sterilized 1.0 mL pipette tips (Fig. 1). Each band was suspended in 30 µL TE buffer containing 1.5 mL tube and incubated for 24 h (4°C) to allow the DNA fragments diffuse into the buffer. The resultant solution (aliquot) was then used as the template for re-amplification of the target gene. To get a single band, the entire DGGE procedure was repeated at least twice (i.e., first and second mobility test). The PCR product of 1st DGGE was used as the template in the first mobility test, and the 2nd mobility test performed with the PCR product of 1st mobility test.

The re-amplified PCR products with oligonucleotides 1106F/1378R (without GC) were used as template for sequencing reactions performed with Automated Sequencer (3730 X L DNA Analyzer, Applied Biosystems, USA) using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Sequencing was done by Avesthagen Ltd., Bangalore, India. Close relatives and phylogenetic affiliation of the sequences obtained were determined by aligning the sequences against known ones in the GenBank database using the BLAST search programme available at the NCBI website (www.ncbi.nlm.nih.gov). Phylogenetic analysis was done using MEGA 4.1 software[16]. A phylogenetic tree was constructed by 1000-fold bootstrap analysis using neighbor-joining method, Clustal W program and NJ plot software[17].

Real time PCR (qPCR)

Quantitative PCR (qPCR) assay was performed (Applied Biosystems 7500 Fast Real Time PCR system) to quantify the copy numbers of *mcrA* genes present in different soil types. The primer sets mcrA-f (GGTGGTGTMGGATTCACACARTAYGCWACA GC), mcrA-r (TTCATTGCRTAGTTWGGRTAGTT)[18] were used to amplify *mcrA* genes, which amplified a fragment of about 410-430 bp (excluding the primer regions).

For the amplification of *mcrA* gene, real-time PCR was performed in 25 µL mixture comprising 12.5 µL SYBR green master mix, 0.1 µL of each primer (50 µM), 1 µL DNA as the template and 11.3 µL of sterile water. The thermal conditions followed were those used by Watanabe *et al.*[19], and comprised initial

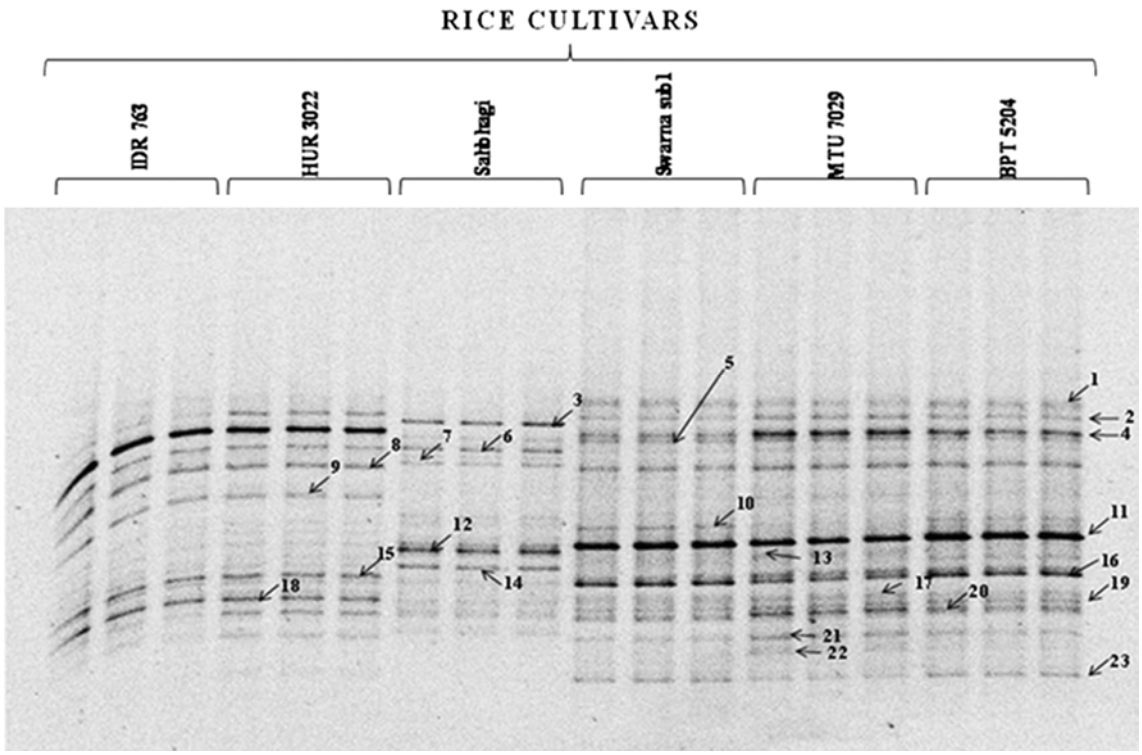

Fig. 1 — DGGE profiles of methanogenic archaeal community showing effect of different rice cultivars in triplicate. Numbers represent excised DGGE bands namely SI 1 to SI 23.

denaturation at 95°C (30 s) followed by 45 cycles with two steps each, denaturation at 95°C (40 s), and annealing at 55°C (30 s), plus the extension at 72°C (1 min). The mixtures of known numbers (10^1-10^7 copies) of *mcrA* gene fragments[19] from three methanogenic archaeal strains were used as the standard. The standard curve revealed a slope of –3.464 corresponding to an efficiency of 94.4% and R^2 of 0.99 similar to those reported in other studies[20].

Nucleotide sequence accession numbers

All sequences determined in this study were deposited with NCBI databases under accession numbers KC756367 to KC756386 and KC777367 to KC777369.

Methane production potential

Methane production potential was determined as per the method described by Singh *et al.*[10]. To monitor methane production, anoxic sterile water was added to 50 g of soil sample in 250 mL flasks to provide a 10 mm standing water layer above the soil surface. The flasks (in triplicate) were dark-incubated statically at 30°C (20 days). Methane in the headspace was measured by injecting 1 mL in gas chromatograph (GC Varian 3800, The Netherlands) equipped with a Flame Ionization Detector (FID) and a Porapak Q Column (3m). The column, injector and detector temperatures were set as 80, 70 and 200°C, respectively. N_2 gas at a flow rate of 30 mL/min, was the carrier.

Statistical analyses

The analysis of variance (one way) (ANOVA) was performed to determine the effect of rice cultivars on the observed variables. Tukey's HSD test (at <0.05) was applied for differences in mean values. Regression analysis was used to determine the relationship between plant biomass, methanogenic community size and CH_4 production potential of soils. All such analyses used SPSS 16.0 statistical package.

Results

Plant biomass

All the six rice cultivars distinctly differed in biomass that varied from 54.37 to 89.05 g/plant (Table 1). One way ANOVA indicated significant difference in biomass due to cultivars ($F_{5,18}$ = 90.17; P = 0.000).

Table 1 — Variation in plant biomass, methanogens community size and methane production potential of soils in relation to rice cultivars

Cultivars	Plant Biomass (g plant^{-1})	Methanogens (X 10^5 copies g^{-1} dws)	CH_4 Production (µg g^{-1} dws)
IDR 763	63.81±1.93^{a}	3.04±0.22^{p}	379.3±58.05^{x}
HUR 3022	59.69±1.56^{a,c}	3.96±0.38^{p,r}	307.9±14.11^{x}
Sahbhagi	54.37±1.69^{a,c}	1.65±0.15^{p,s}	295.8±11.71^{x}
Swarna sub 1	70.98±1.49^{a,d}	60.35±8.64^{p,t}	444.2±16.59^{y,z}
MTU 7029	84.05±2.56^{b}	79.6±7.14^{p,t}	542.0±13.29^{y}
BPT 5204	89.05±2.25^{b}	97.57±2.48^{q,t}	684.3±33.37^{y,v}

*Letters in superscript wherever common indicate in-significant differences

Methanogens community size

The community size of methanogens [×10^5 *mcrA* gene copies per gram dry weight soil (copies/g dws)] varied from 3.04 to 97.57 among different cultivars (Table 1). The methanogenic archaeal community size varied significantly in all the cultivars with lower size in IDR 763, HUR 3022 and Sahbhagi compared to Swarna sub 1, MTU 7029 and BPT 5204 cultivated soil.

Methanogenic community composition

The bands at the same position in the gel are assumed to have the identical nucleotide sequence, thus bands with different gel positions are selected for sequencing. Hence, in the present study, total of 23 bands were excised through DGGE gel for sequencing to assess the diversity of methanogens in rhizospheric soils of six different rice cultivars (Fig. 1). Blast search of the sequenced DGGE bands from the soil samples suggested methanogenic members belong to *Methanocellaceae*, *Methanobacteriaceae*, *Methanomicrobiaceae*, *Methanosaetaceae* and *Methanosarcinaceae* (Fig. 2). Non-methanogenic members of Crenarchaeota were also recovered from the experimental site using 16S rRNA gene primer. The differences among the rhizospheric soil samples are based on the number of bands for rice cultivars i.e. IDR 763, HUR 3022, Sahbhagi, Swarna sub1, MTU 7029 and BPT 5204, representing 14, 14, 11, 16, 17, and 18 bands, respectively.

The DGGE bands SI 04, SI 06, SI 11, SI 15, SI 18 and SI 20 were common to all the cultivars. The presence of bands SI 03, SI 07 and SI 12, and the absence of band SI 21 were unique to only Sahbhagi cultivar, indicating a quite distinct banding pattern. Band SI 09 was unique for HUR 3022 and IDR 763 samples. Band SI 22 was found in MTU 7029 and

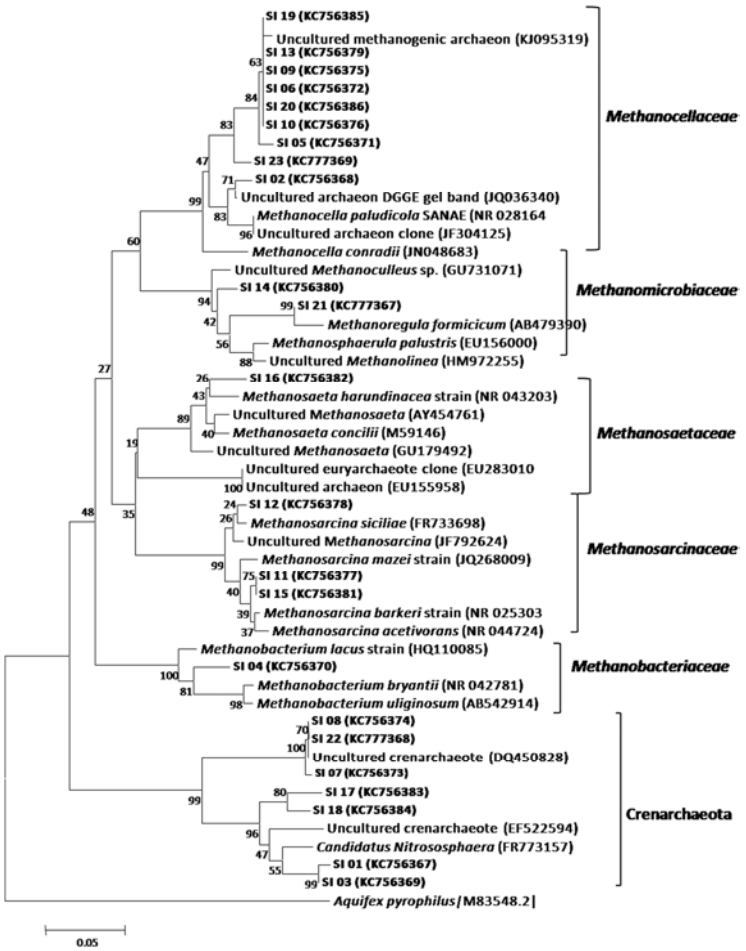

Fig. 2 — Phylogenetic relationship of representative methanogenic archaeal 16S rRNA gene sequence for rhizospheric soil DNA. Uncultured and cultured NCBI databases are showing relationship with representative partially sequenced DGGE bands. The scale bars represent an estimated of 5% sequence divergence. Bootstrap values above 50% are shown at each branch point. GenBank accession numbers are indicated for each sequenced DGGE bands in parentheses.

BPT 5024. Although banding patterns for IDR 763 and HUR 3022 cultivar soil samples seem quite similar, but differed in their intensity, and were distinct among the cultivar samples. Bands SI 11 and SI 16 were very prominent in Swarna sub1, MTU 7029 and BPT 5204 cultivar compared to other three remaining samples where 6 bands namely, SI 03, SI 06, SI 08, SI 09, SI 15 and SI 18, were prominent (Fig. 1).

Out of 23 bands sequenced, 9 DGGE bands were affiliated to *Methanocellaceae*, 3 to *Methanosarcinaceae*, 1 to *Methanosaetaceae*, 1 to *Methanobacteriaceae*, 2 to *Methanomicrobiaceae* and 7 to Crenarchaeota. All groups of methanogens were found in six cultivar soil samples with the relative abundance of closely resembling bands (SI 5, SI 5, SI 3, SI 6, SI 7 and SI 6, respectively in IDR 763, HUR 3022, Sahbhagi, Swarna sub1, MTU 7029 and BPT 5204) of *Methanocellaceae,* being the highest in all cultivars (Fig. 1). Six cultivar samples constituted both hydrogenotrophic and acetotrophic methanogens. Bands representing acetotrophic methanogens were SI 11, SI 12, SI 15 and SI 16, whereas hydrogenotrophic methanogens were represented by bands SI 02, SI 04, SI 05, SI 06, SI 09, SI 10, SI 13, SI 14, SI 19, SI 20, SI 21 and SI 23. The presence of multiple bands in each soil sample represented the diverse methanogenic community.

Methane production

From Table 1, it is evident that the extent of methane production ranged from 295.8 to 684.3 µg CH_4/g dws for the six cultivars. Methane production was the highest for BPT 5204 and the least for Sahbhagi cultivar soil. One way ANOVA revealed significant effect of cultivars on methane production potential of soils ($F_{5,18} = 62.50$; $P = 0.000$).

Discussion

Effect of cultivars on methanogens community size

The results of present study clearly indicate the influence of rice cultivars on methanogenic archaeal community size. From Table 1, it is evident that methanogenic archaeal community size for Swarna sub 1, MTU 7029 and BPT 5204 cultivars was an order of magnitude higher than those for IDR 763, HUR 3022 or Shabhagi cultivar. The cultivars influence the chemical composition of rhizosphere and provide microbial growth substrates by rhizodeposition. Depending on the plant species, between 20 and 50% of plant photosynthate is transported belowground out of which 17% is released to the soil environment[21,22]. Wang and Adachi[23] reported that community size of methanogens increased in soils planted with rice cultivars contributing higher root exudates, and the size range varied from 5.2-110.0 × 10^4 no./g dry soil for the three cultivars studied. The plant biomass has been shown to have a positive effect on root exudation[24]. Thus, the observed variations in methanogenic archaeal community size could probably be due to higher plant biomass. This is also evident from the regression analysis that revealed community size of methanogens to be positively correlated with the plant biomass (R^2 = 0.89; P = 0.000).

Effect on methanogenic community composition

The present results indicate that the highest number of bands obtained for soils of Swarna sub 1, MTU 7029 and BPT 5204 rice cultivars, reflected richness in comparison to IDR 763, HUR 3022 or Sahbhagi rice cultivar. The most dominant band in DGGE profile of Swarna sub1, MTU 7029 and BPT 5204 samples was SI 11, and is closely related to SI 15 band in IDR 763 and HUR 3022 sample. Both of these bands were closely (~98%) related to cultured strain of *Methanosarcina barkeri*. Some bands also had similarity with crenarchaeotal lineages (SI 01, SI 03, SI 07, SI 08, SI 17, SI 18 and SI 22), and formed the largest group in abundance.

Bands SI 11 and SI 15 showed close resemblance with *Methanosarcina barkeri* and band SI 12 with *Methanosarcina siciliae*. The former is a H_2 metabolizing species in addition to using acetate as substrate, but the latter strictly dependent on acetate. Band SI 16 showed relatedness (~98%) with *Methanosaeta harundinacea*, an acetate scavenging methanogens[4]. Two bands (SI 14 and SI 21) were closely related to *Methanomicrobiaceae* while SI 21 showed ~99% resemblance with *Methanoregula formicicum*, the anaerobic methanogens that utilize hydrogen and formate for growth and methane production. This strain is mostly found in anaerobic sludge[25]. The presence of hydrogenotrophic as well as acetotrophic methanogens in all the soil samples indicates the richness of diversity. Among hydrogenotrophic methanogens, *Methanocellaceae*, *Methanomicrobiaceae* and *Methanobacteriaceae* were common to all the samples. Among them *Methanobacteriaceae* group has relatively a higher demand of hydrogen concentrations in paddy soil than *Methanocellaceae* and *Methanomicrobiaceae*[26,27]. The acetotrophic group constitutes *Methanosarcinaceae* and *Methanosaetaceae*, showing difference in abundance with 3 and 1 DGGE bands, respectively (Fig. 2). Relatively higher abundance of *Methanosarcinaceae* group compared to *Methanosaetaceae* is attributed to its ability for faster growth and substrate versatility using acetate, methanol and H_2-CO_2 rather than the obligate dependence of *Methanosaetaceae* on acetate[28,29].

The study on the relationship between six cultivated rice varieties and the root microbiome suggested that rice genotype accounted for a significant amount of variation between microbial communities[6]. Conrad *et al.*[30] demonstrated that the effect of cultivars on methane emission was due to the colonized archaeal community. This could be related to variability in the structure of different cultivars, amount of C-substrate available from the decaying organic matter, death of crop roots, and carbohydrate exudates from the living roots. The relative abundance of methanogens as observed in the present study might have been favoured by the higher plant biomass resulting in higher root exudates. Wang *et al.*[31] also made similar conclusions. The observations of the present study are in agreement with our earlier findings that revealed the influence of substrates on methanogenic community size and structure in paddy soils[9,10]. All the rhizospheric soil samples were colonized by members of *Methanocellaceae* as the major methanogenic archaea community owing to their better adaptations to the active assimilation of plant root derived carbon[32,33], and ability to detoxify partly oxic conditions prevailing in rice roots[26].

Effect of cultivars on methane production

Methane production was in the order: Sahbhagi <HUR 3022 <IDR 763 <Swarna sub 1 <MTU 7029 <BPT 5204. Variations in methane production might be attributed to changes in the methanogenic community

size, structure and plant biomass. A positive correlation between methane production and plant biomass (R^2 = 0.85; P = 0.000) was obtained. Thus, it can be inferred that methane production was strongly correlated with the plant biomass in addition to other factors. This is also supported by the results of Kerdochoechuen[24]. Furthermore, the influence of plants on microbial methane production and consumption through altered substrate availability (such as root exudates) was also reported by King and Reeburgh[34] and Saarnio *et al*.[35]. Methane emissions study from different cultivars showed to be significantly and positively correlated with biomass, soil organic matter, dissolved soil organic carbon and total carbon suggesting that organic source strength provides the substrate for methane production[5,7]. Further, a positive correlation was also observed with the methanogen community size that directly influenced methane production potential of soils (R^2 = 0.78; P = 0.000). Wang and Adachi[23] reported similar observations contrary to that of Ma *et al*.[4] on three rice cultivars. However, the correlation and regression analyses by Ma *et al.*[36] showed that the abundance of *mcrA* genes is positively correlated with methane production potential of soil.

Conclusion

Although, it is difficult to correlate directly the diversity of methanogens with soil methane production, Swarna sub 1, MTU 7029 and BPT 5204 cultivar soils clearly exhibited higher number of bands representing *Methanocellaceae* group indicating these soils to be the prominent source of methane relative to the soils of other 3 rice cultivars. However, the relative abundance of bands representing acetotrophic methanogenic group could not be correlated with methane production in the soils of six cultivars. Thus, it can be concluded that rice cultivars with different plant biomass could possibly offer favourable niche for the prevalence of methanogenic archaeal community that imparts differential effects on methane production from the soil.

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Conflict of interest

The authors declare that they have no conflict of interest.

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