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# Methane production potential and methanogenic archaeal community structure in tropical irrigated Indian paddy soils

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**Abstract** Soil characteristics regulate various belowground microbial processes including methanogenesis and, consequently, affect the structure and function of methanogenic archaeal communities due to change in soil type which in turn influences the $CH_4$ production potential of soils. Thus, five different soil orders (Alfisol, Entisol, Inceptisol, Podzol and Vertisol) were studied to assess their $CH_4$ production potential and also the methanogenic archaeal community structure in dryland irrigated Indian paddy soils. Soil incubation experiments revealed $CH_4$ production to range from 178.4 to 431.2 µg $CH_4$ $g^{-1}$ dws in all soil orders as: Vertisol<Inceptisol<Entisol<Podzol<Alfisol. The numbers of methanogens as quantified using real-time quantitative polymerase chain reaction (qPCR) targeting *mcrA* genes varied between 0.06 and 72.97 ($\times 10^6$ copies $g^{-1}$ dws) and were the highest in Vertisol soil and the least in Alfisol soil. PCR-denaturing gradient gel electrophoresis (DGGE)-based approach targeting 16S rRNA genes revealed diverse methanogenic archaeal communities across all soils. A total of 43 DGGE bands sequenced showed the closely related groups to Methanomicrobiaceae, Methanobacteriaceae, Methanocellales, Methanosarcinaceae, Methanosaetaceae and Crenarchaeota. The composition of methanogenic groups differed among all soils and only the Methanocellales group was common and dominant in all types of soils. The highest diversity of methanogens was found in Inceptisol and Vertisol soils. Methane production potential varied significantly in different soil orders with a positive relationship ($p < 0.05$) with methanogens population size, permanganate oxidizable C (POXC) and $CO_2$ production. The present study suggested that $CH_4$ production potential of different soils depends on physicochemical properties, methanogenic archaeal community composition and the population size.

**Keywords** $CH_4$ production · DGGE · *mcrA* genes · Methanogens · Paddy soil · Soil characteristics

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## Introduction

Rice ecosystems are considered as one of the most suitable habitat for biogenic methanogenesis due to the presence of all the essential precursors and methanogens, members of Euryarchaeota. According to USEPA (2006) report, rice fields contribute about 10 % to global $CH_4$ budget. Earlier studies showed that $CH_4$ production from paddy fields increased with the intensification of rice cultivation (IRRI 2006; Zhang et al. 2011). Therefore, characterizing rice ecosystems in relation to the $CH_4$ production potential of soils has attracted scientific investigators to address the issue and suggest possible mitigation strategies. Methane emission depends on microbial activities of flooded rice field soils (Mer and Roger 2001). The groups of methanogens studied in different fields, are Methanomicrobiales, Methanosarcinales, Methanobacteriales

and Methanocellales (previously named as Rice cluster I), wherein most of the groups are hydrogenotrophs, i.e., utilize $H_2$ and $CO_2$ as the main source for $CH_4$ production. Only *Methanosarcina* spp. and *Methanosaeta* spp. can utilize acetate for $CH_4$ production and therefore belong to acetoclastic group (Weber et al. 2001; Conrad and Klose 2006).

Factors that affect $CH_4$ production from paddy field soils are temperature (Chin and Conrad 1995), soil type including physicochemical properties (Wachinger et al. 2000), rice variety (Jia et al. 2006), fertilizer application (Hou et al. 2000; Liou et al. 2003; Akiyama et al. 2013), water relations (Ma et al. 2012; Kern et al. 2012), etc. Spatiotemporal variability of $CH_4$ production has also been observed in different studies (Wachinger et al. 2000; Mitra et al. 2002; Kravchenko and Yu 2006). A considerable diversity of methanogenic archaeal community has been observed in different soil types of Japan, China, Italy and Thailand using "culture-independent" techniques, such as terminal restriction fragment length polymorphism or T-RFLP, deoxyribonucleic acid stable isotope probing or DNA-SIP and denaturing gradient gel electrophoresis or DGGE (Watanabe et al. 2009; Wang et al. 2010; Conrad et al. 2012), and different reasons are responsible for diversity in methanogenic archaeal community structure in different soil types.

Among the rice-growing countries in the world, India has the largest cultivated area with a variety of soil types and climatic conditions (about 43.7 million ha.) and ranks next to China in rice production (USDA FAS 2009). The statewise analysis indicated that the trend of $CH_4$ budget contributed by Indian paddy fields during 1979–2006 varied between 3.62 and 4.09 Tg year$^{-1}$ (Gupta et al. 2009). Soil characteristics along with different environmental conditions regulate many belowground microbe-mediated processes including methanogens that ultimately influenced $CH_4$ production potential of soils. Few studies have been undertaken to explain the mechanisms of $CH_4$ emission from tropical rice soils of India (Sethunathan et al. 2000; Gogoi et al. 2005). Most of these studies concentrated on explaining the physicochemical basis including organic C content, soil pH, redox conditions and content of electron donors in the soils. Almost no studies have been conducted to intricately compare the methanogenic diversity and methane production potential in tropical rice soils. Thus, investigating the soil potential for $CH_4$ production vis-à-vis methanogenic archaeal community structure prevailing therein across spatiotemporal divergence would be critical. On the basis of available literature, these aspects have not yet been fully elucidated in tropical dryland-irrigated paddy fields soils globally including India. Recently, Singh and Dubey (2012) and Singh et al. (2012) demonstrated variations in methanogenic diversity in relation to plant age for Indian paddy soils. In the present study, we examined irrigated paddy soils of different characteristics for their $CH_4$ production potential along with associated methanogenic community structure (diversity and density) therein.

## Materials and methods

### Experimental site, soil sampling and analyses

Soil samples (non-rhizosphere) were collected from farmers' irrigated rice fields located at four different places of Eastern Uttar Pradesh (U.P.) and one from Jammu and Kashmir (J&K), India, during September 2012. The sampling was done from flooded fields. The elevations of sites were 65–165 m except J&K (700–900 m) above mean sea level. The experimental areas had a seasonally dry semitropical climate with monsoonal character. Annual rainfall was around 1,100 mm (U.P. zone) and 960 mm (J&K zone); temperature ranged from 8 to 42 °C (U.P.) and 9 to 32 °C (J&K), respectively. As per farmer's practice, fields were flooded, and N-fertilizer (urea) was also applied at 30 kg N ha$^{-1}$ 1 week before sampling. Treatments (cultivation practices and fertilizer application) were common, but the fields located at different geographical locations had different soil orders and characteristics as given in Table 1. At each sampling site, five soil samples were collected randomly at five different locations and pooled together to make a composite sample to represent the overall characteristics of the site studied (Vishwakarma et al. 2010). Composite samples were further divided into two subsamples, one of fresh samples stored at 4 °C for molecular biological analyses within a week, and the other air dried for physicochemical characteristics of soils. All analyses were done in triplicate. All the soil samples were collected (from 0 to 10-cm depth) using a 5-cm-diameter soil corer within a week, sieved through 2-mm mesh. Soil characteristics, such as texture, organic C, total N, $NH_4^+$–N, $NO_3^-$–N, etc., were determined as per the standard procedure (APHA 1985). Permanganate oxidizable C (POXC) was measured as described by Weil et al. (2003).

### Methane production potential

Methane production potential was determined as described by Singh et al. (2012). In brief, to monitor $CH_4$ 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. Before incubation, the head space was purged with $O_2$-free $N_2$ gas for 3 min with constant shaking of flasks to ensure anaerobic environment. The flasks (in triplicate) were dark-incubated statically at 30 °C (20 days). Methane in the headspace was measured by gas chromatograph (Shimadzu GC 14B, Kyoto, Japan) equipped with a Flame Ionization Detector and a Porapak Q Column (3 m). Methane production potential of each soil sample was estimated from the $CH_4$ concentration in the head space at intervals of 0, 60, 120, 180, 240, 300, 360, 420 and 480 h. The methane volume as measured at different time intervals for different soil samples, as used to evaluate the kinetic constants of $CH_4$

**Table 1** Sampling sites, soil characteristics and gene copy number of methanogens (*mcrA* gene) and cumulative $CH_4$ and $CO_2$ concentrations across the different soil orders

| Parameters | Soil orders | | | | |
|---|---|---|---|---|---|
| | Inceptisol | Podzol | Alfisol | Entisol | Vertisol |
| Sampling site | Banaras Hindu University Campus | Jammu and Kashmir | Mirzapur | Maharajganj | Chandaulli |
| Latitude | 25°16′N | 32°5′–33°3′N | 25°09′N | 27°10′N | 25°14′N |
| Longitude | 83°03′E | 70°0′–74°4′E | 83°34′E | 83°32′E | 83°15′E |
| Texture (%) | Sand 32; Silt 65; Clay 3 | Sand 33; Silt 65; Clay 6 | Sand 34; Silt 64; Clay 5 | Sand 31; Silt 64; Clay 7 | Sand 6; Silt 82; Clay 12 |
| pH | 7.2 | 6.3 | 6.2 | 7.8 | 7.5 |
| Organic C (%) | $0.75 \pm 0.02^a$ | $0.64 \pm 0.03^c$ | $0.56 \pm 0.02^c$ | $0.72 \pm 0.04^{ab}$ | $0.84 \pm 0.04^b$ |
| Total N (%) | $0.13 \pm 0.01^a$ | $0.12 \pm 0.01^a$ | $0.12 \pm 0.01^a$ | $0.14 \pm 0.02^a$ | $0.17 \pm 0.03^a$ |
| $NH_4^+$–N ($\mu g\ g^{-1}$) | $7.6 \pm 0.26^{ab}$ | $5.8 \pm 0.09^c$ | $4.4 \pm 0.23^d$ | $7.0 \pm 0.29^b$ | $8.4 \pm 0.29^a$ |
| $NO_3^-$–N ($\mu g\ g^{-1}$) | $4.1 \pm 0.23^{ab}$ | $4.4 \pm 0.23^b$ | $4.0 \pm 0.21^{ab}$ | $4.5 \pm 0.15^b$ | $3.3 \pm 0.26^a$ |
| POXC ($\mu g\ g^{-1}$ dws) | $452.9 \pm 38.0^a$ | $326.2 \pm 12.2^c$ | $215.3 \pm 22.0^d$ | $327.7 \pm 9.0^c$ | $587.5 \pm 17.9^b$ |
| *mcrA* gene ($\times 10^6$ copies $g^{-1}$ dws) | $12.21 \pm 1.7^a$ | $7.31 \pm 0.9^c$ | $0.06 \pm 0.003^e$ | $1.93 \pm 0.3^d$ | $72.97 \pm 6.4^b$ |
| $CH_4$ production ($\mu g\ g^{-1}$ dws) | $287.0 \pm 8.1^a$ | $204.0 \pm 7.8^{cd}$ | $178.4 \pm 6.9^d$ | $242.3 \pm 11.4^c$ | $431.2 \pm 9.3^b$ |
| $CO_2$ production ($\mu g\ g^{-1}$ dws) | $838.5 \pm 21.3^a$ | $327.7 \pm 19.0^c$ | $293.59 \pm 40.5^c$ | $398.34 \pm 36.2^c$ | $1489.2 \pm 153.5^b$ |

Values are means of three replicates ± 1 SE. Different letters in rows indicate significant differences ($p < 0.05$; Tukey's HSD test)

production with the rate constant ($K$) calculated using following equation (Singh et al. 2012):

$$K = \left( \frac{\mu_{max}(X)}{K_s} \right) \quad (i)$$

where $\mu$ is specific growth rate, $K_s$ is substrate constant, and $X$ is the methanogenic population number.

Carbon dioxide concentration in the head space of all samples was also analyzed using GC as above with thermal conductivity detector or TCD and Porapak Q (80–100 mesh) column. Column, injector and detector temperatures were operated at 40, 50 and 50 °C, respectively. Concentrations were estimated by comparing the peak area with those of reference gases (Koatsu Gas Kogyo, Osaka, Japan).

DNA extraction from soils and PCR amplification

Total genomic DNA was extracted from soils (0.5 g) as per the protocol given in Fast DNA® Spin Soil Kit (MP Biomedicals, Solon, OH, USA) using bead-beating method. The DNA concentration and purity were evaluated spectrophotometrically. 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 1378R (5′-TGT GCA AGG AGC AGG GAC-3) (Watanabe et al. 2006). The polymerase chain reaction (PCR) reaction mixture (50 µl) contained 5 µL 10× reaction buffer , 5 µL dNTPs (each 2.5 mM), 0.5 µL 50 µM of each primer, 0.25 µL (5 U µL$^{-1}$) Ex *Taq* polymerase (TaKaRa, Otsu, Japan) and 1.0 µL of 20-fold diluted DNA template.

The PCR was performed using a 96-well Thermal Cycler (PCR Thermal Cycler Dice, TaKaRa, Otsu, Japan), under the following conditions: an initial denaturation time of 90 s at 95 °C 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). At completion, the PCR products were resolved by electrophoresis in 2.0 % (w/v) agarose (Funakoshi, Tokyo, Japan) in 1× TAE buffer stained with ethidium bromide (0.5 µg mL$^{-1}$). The images were digitized with FAS-III (Toyobo, Osaka, Japan) and the DNA fragment lengths 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, Duren, Germany) and quantified using UV-2450 (Shimadzu) and stored at -20 °C for further analysis.

DGGE analysis

DGGE was performed with the DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA) as described by Muyzer et al. (1993). PCR products (approx. 200 ng) were loaded on to 8 % (w/v) polyacrylamide gel immersed in 1× TAE buffer, and electrophoresed for 14 h at 60 °C under a constant voltage (100 V). Polyacrylamide gel was prepared with denaturing gradients in the range of 32 % to 62 % (100 % denaturant was achieved by using 7 mol L$^{-1}$ urea and 40 % formamide). After electrophoresis, the gel was stained with the SYBR Green I nucleic acid gel stain (1:10,000 dilution; Lonza, Molecular Application, Rochland, ME, USA), rinsed using distilled water and photographed on an UV-trans-

illuminator (Printgraph, ATTO, Type-GX 430251, Tokyo, Japan) at 312 nm with the SYBR Green gel stain photographic filter (Lonza).

### Sequence analysis of DGGE bands

Most of the visible DGGE bands (43) were excised from DGGE gel with sterilized 1.0 mL pipette tips. Each band was suspended in 30 μL TE buffer containing 1.5 mL tube and incubated for 24 h (4 °C) in order to allow the diffusion of DNA fragments into the buffer. The resultant solution (aliquot) was then used as a template for re-amplification of the target gene. In order to get the single band, entire DGGE procedure was repeated at least twice and this was named as mobility test. PCR product of the first DGGE result was used as template in first mobility test, and the second mobility test performed with the PCR product of first mobility test.

Re-amplified PCR products with oligonucleotides1106F/ 1378R (without GC) were used as template for sequencing reactions performed with Automated Sequencer (3730XL DNA Analyzer, Applied Biosystems, USA) using BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing was done by outsourcing (TaKaRa Bio, Dragon Genomics, Yokkaichi, Japan). Close relatives and phylogenetic affiliation of the sequences obtained were determined by using the BLAST search programme at the NCBI website (http://www.ncbi.nlm.nih.gov). A phylogenetic tree was constructed by 1,000-fold bootstrap analysis using neighbor-joining method, Clustal W program and NJ plot software (Higgins et al. 1994). Phylogenetic analysis was done using MEGA 4.1 software (Tamura et al. 2007).

### Real-time qPCR

Quantitative PCR (qPCR) assays were performed (Thermal Cycler Dice Real Time System, TaKaRa, Shiga, Japan) to quantify gene copy numbers of *mcrA* genes present in different soil orders. The primers sets mcrA-f (GGTGGTGTM GGATTCACACARTAYGCWACAGC) and mcrA-r (TTCATTGCRTAGTTWGGRTAGTT) (Luton et al. 2002) were used to amplify *mcrA* genes and they amplified a fragment of about 410–430 bp (excluding the primer regions).

For the amplification of *mcrA* gene, real-time PCR reactions were performed in 25 μL mixtures containing 12.5 μL SYBR green master mix, 0.1 μL each primer (50 μM), 1 μL DNA as a template and 11.3 μL sterile water. The thermal conditions were those of Watanabe et al. (2010) with the initial denaturation at 95 °C (30 s) followed by 45 cycles with two steps each, denaturation at 95 °C (40 s), and a step at 55 °C for annealing (30 s), plus the extension step of 72 °C (1 min). Standards were the mixtures of known numbers ($10^1$–$10^7$ copies) of *mcrA* gene fragments (Watanabe et al. 2010) amplified by the primer set ME1/ME2 (Hales et al. 1996) from three methanogenic

archaeal strains, *Methanobrevibacter arboriphilus* SA (accession no. AB300777), *Methanosarcina mazei* TMA (accession no. AB300778) and *Methanoculleus chikugoensis* MG62 (accession no. AB300779). 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 (Zhang and Fang 2006).

### Nucleotide sequence accession numbers

All sequences determined in this study are deposited to the NCBI databases under accession numbers KC136769 to KC136811.

### Statistical analyses

Data were subjected to analysis of variance (ANOVA) in order to determine the effect of sampling site. Tukey's honestly significant difference (HSD) test (at P <0.05) was applied for the differences in mean values. Simple regression analysis was used to determine the relationship between methanogenic archaeal population and $CH_4$ production potential of soils and other variables. All analyses were done using the SPSS 16.0 statistical package.

## Results

### Physicochemical properties of soils

Soil parameters such as soil texture and pH were different for all soils (Table 1). Organic C recorded in Vertisol soil (0.84 %), was significantly higher than that of Podzolic and Alfisol soil ($F_{4,10}$=11.593, $P$=0.001). The $NH_4^+$–N content also significantly varied depending on the sampling site ($F_{4,10}$=42.981, $P$=0.000). However, insignificant differences were observed between Inceptisol and Vertisol soils (Table 1). $NO_3^-$–N concentration although varied across the soils, however, ANOVA did not indicate significant variation.

In each soil, the production of $CO_2$ was measured up to the end of incubation, i.e., 480 h. It ranged from 293.59 to 1,489.16 μg $g^{-1}$ dws. Highest cumulative $CO_2$ concentration was in Vertisol and the least in Alfisol soil (Table 1). ANOVA revealed significant differences in cumulative $CO_2$ concentration due to sampling sites ($F_{4,10}$=64.46, $P$=0.000). Further, Tukey's HSD test suggested that Inceptisol and Vertisol soils were significantly ($p$<0.05) different among each other as well as from the remaining soil orders for $CO_2$ production.

The concentration of POXC varied from 215.28 to 587.52 (μg $g^{-1}$ dws) being highest in Vertisol and the least in Alfisol soil. ANOVA indicated significant differences in POXC values across soils ($F_{4,10}$=54.26, $P$=0.000). Further, Tukey's HSD suggested that POXC in Inceptisol soil was significantly lower than in Vertisol soil and significantly higher than in the other three soils (Table 1).

Population size of methanogens

The copy numbers of methanogenic archaeal *mcrA* genes ranged from 0.06 to 72.97 ($\times 10^6$ copies $g^{-1}$ dws) across the studied soil samples, with the highest value in Vertisol and the lowest in Alfisol soils (Table 1). ANOVA indicated significant effect of sampling sites on methanogens population size ($F_{4,10}=106.05$, $P=0.000$). Further, Tukey's HSD test suggested that the *mcrA* genes copy number varied significantly for all the soil orders (Table 1).

Methane production potential

Figure 1 shows variation in the $CH_4$ production potential. The cumulative values of $CH_4$ production ($\mu g\ CH_4\ g^{-1}$ dws) after 480 h of incubation were 431.2, 278.0, 242.3, 204.0 and 178.4 in Vertisol, Inceptisol, Entisol, Podzol and Alfisol soils, respectively. There was significant difference in $CH_4$ production in five soil orders as indicated by ANOVA ($F_{4,10}=126.65$, $P=0.000$). Tukey's HSD test suggested that $CH_4$ production in the five soils differed significantly except between Podzol and Entisol, and between Podzol and Alfisol soils. For every 60 h interval, the $CH_4$ production rate showed different rate for each soil, which has been fitted to a kinetic model (Fig. 2).

Kinetics of $CH_4$ production

According to Monod kinetics, the rate of $CH_4$ production can be expressed as:

$$\left(\frac{dM}{dt}\right) = \frac{\mu_{max}(X)(M)}{K_s + (M)} \tag{ii}$$

Under the given situation ($M$) is small, ($X$) is nearly constant and $K_s \gg (M)$, hence:

$$\left(\frac{dM}{dt}\right) = \frac{\mu_{max}(X)(M)}{K_s} \tag{iii}$$

or

$$\left(\frac{dM}{dt}\right) = K(M) \tag{iv}$$

where

$$K = \left(\frac{\mu_{max}(X)}{K_s}\right) \tag{v}$$

By integrating Eq. iv, we obtain the first-order rate expression:

$$\ln\left(\frac{M_m}{M_m - M}\right) = Kt \tag{vi}$$

where $M$ is $CH_4$ produced at $t=t$ and $M_m$ is the maximum amount of $CH_4$ produced.

Thus, a plot of $\ln\left(\frac{M_m}{M_m - M}\right)$ vs. $t$ will give a straight line with slope equal to $K$, the pseudo first-order rate constant. The $\ln\left(\frac{M_m}{M_m - M}\right)$ vs. $t$ plots for the different soil orders are given in Fig. 2. Least squares analyses gave the best fit lines as indicated therein. The corresponding values of $K$ and $R^2$ were: $K=0.0060$, $R^2=0.92$ (Vertisol); $K=0.0049$, $R^2=0.92$ (Inceptisol); $K=0.0042$, $R^2=0.94$ (Entisol); $K=0.0036$, $R^2=0.96$ (Podzol) and $K=0.0033$, $R^2=0.95$ (Alfisol).



Fig. 1 Variations in cumulative $CH_4$ production potential of different soil orders. Values are means of three replicates ± 1 SE
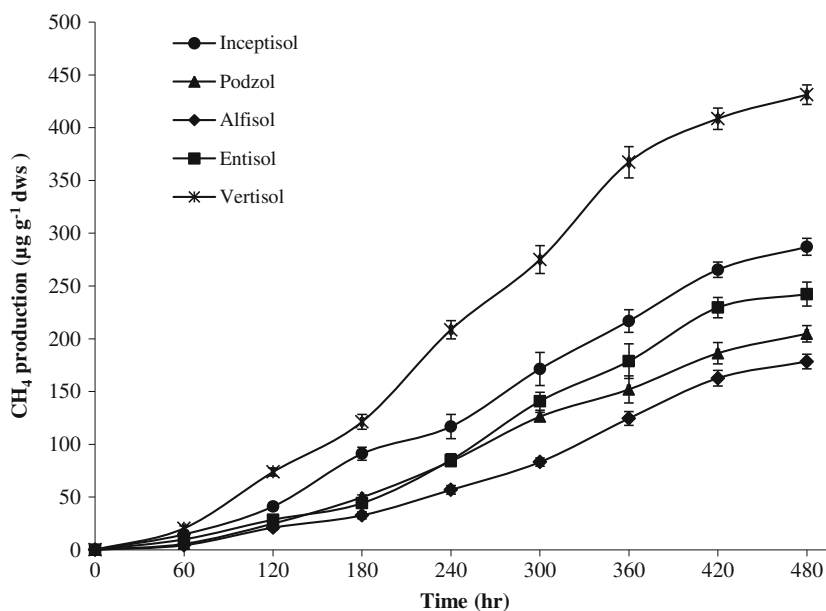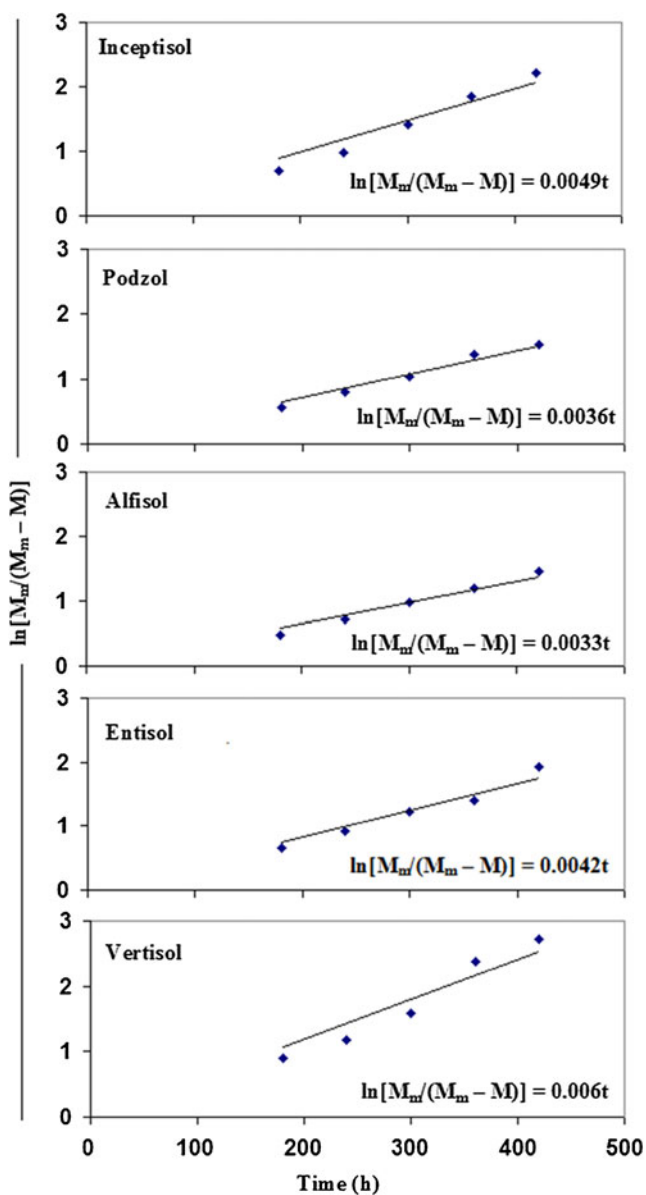
**Fig. 2** First-order kinetic model for cumulative CH$_4$ production in rice fields of five soil orders

DGGE analysis

As indicated in Fig. 3, the clear difference is attributed due to banding pattern and their intensity. The number of bands in each soil varied from 5 to 13. A high range of common bands (bands 1, 13, 18, 30 and 38; bands 7 and 15; bands 10 and 39) was found in all the five soils but some distinct bands, i.e., 31, 32, 34 and 36 in Vertisol, band 9 in Inceptisol and band 27 in Entisol soil are unique among the five soils along with their varying intensities depending on the soil order. According to Fig. 4, the banding pattern of five different soils is grouped into two clusters, and further, one cluster into four sub-clusters. It is here that Vertisol soil form a distinct group while Inceptisol soil and Podzol soil had close similarity.

In total, 43 bands sequenced from all soils order, 1–11 bands belong to Inceptisol (except band 6), 12–17 to Podzol, 18–22 to Alfisol, 23–30 to Entisol and 31–43 to Vertisol soil (Fig. 3). According to the data in Fig. 5, 19 out of 42 bands sequenced (45 %) as observed in DGGE gel could be affiliated to Methanocellales (bands 1, 2, 3, 11, 12, 13, 16, 17, 18, 19, 20, 29, 30, 31, 37, 38, 40, 41, 42). Methanomicrobiaceae (bands 8, 9), Methanobacteriaceae (bands 33, 35, 43), Methanosarcinaceae (bands 10, 39), Methanosaetaceae (bands 7, 14, 15), Haloarchaeon (band 36) and Crenarchaeota (bands 4, 5, 21, 22, 23, 24, 25, 26, 27, 28, 32, 34 ) were also present. Crenarchaeota was represented by the second largest number of eluted bands (27 %). In the present study, the sequence similarity of such different bands with NCBI database varied from 97 % to 100 %.

The methanogenic group (Methanocellales) was common to all soil samples. The bands diversity of Methanocellales was shown by the number of distinct bands in each sample, i.e., 4 out of 11 bands in Inceptisol, 4 out of 6 bands in Podzol, 3 out of 5 bands in Alfisol, 2 out of 8 bands in Entisol, and 6 out of 13 bands in Vertisol soil. In addition, Methanobacteriales (bands 33, 35 and 43) of Vertisol soil, Methanosaetaceae (bands 14 and 15) of Podzol soil and Methanomicrobiaceae (bands 8 and 9) of Inceptisol soil revealed more than one type of band for a single group. Other groups showed their differential occurrence, and thus distinct diversity pattern was found in the five soils. Furthermore, band 26 of Entisol corresponded most (99 %) to Alpine Tundra wet meadow soils of USA. *Methanosphaerula palustris* isolated from minerotrophic fen of USA was closely similar (99 %) to bands 8 and 9 of Inceptisol soil. A strain of Methanocellales group, *Methanocella paludicola* SANAE isolated from a Japanese paddy field, showed close similarity (99 %) to bands 3 and 38 of Inceptisol and Vertisol soil, respectively. The DGGE bands related to Methanosaetaceae in Inceptisol and Podzol soils showed close similarity. In Inceptisol and Vertisol soils, the bands showing close resemblance with Methanosarcinaceae, are found at different positions.

## Discussion

### Distribution of methanogenic archaeal population size in different soils

The assessment of methanogenic archaeal population size via *mcrA* genes is advantageous as: (a) *mcrA* gene is common to all the CH$_4$-producing organisms, and (b) it encodes α subunit of methyl-coenzyme M reductase, the key enzyme in methanogenesis (Luton et al. 2002). The population size reported in the present study agrees well with the previous estimates, i.e., copy number ($\times 10^6$ g$^{-1}$ dws) recorded by Singh et al. (2012) in Alfisol soil of India and (*mcrA* gene copy number as $10^5$ to $10^7$ g$^{-1}$ dry soil) recorded by Wang et al. (2010) in different soil types of China. Watanabe et al.

**Fig. 3** DGGE fingerprinting profiles of methanogenic archaeal communities belonging to non-rhizospheric soils of five different paddy soils. Sample designations are given on top of each DGGE lane. The bands excised for sequence analysis is numbered 1 to 43
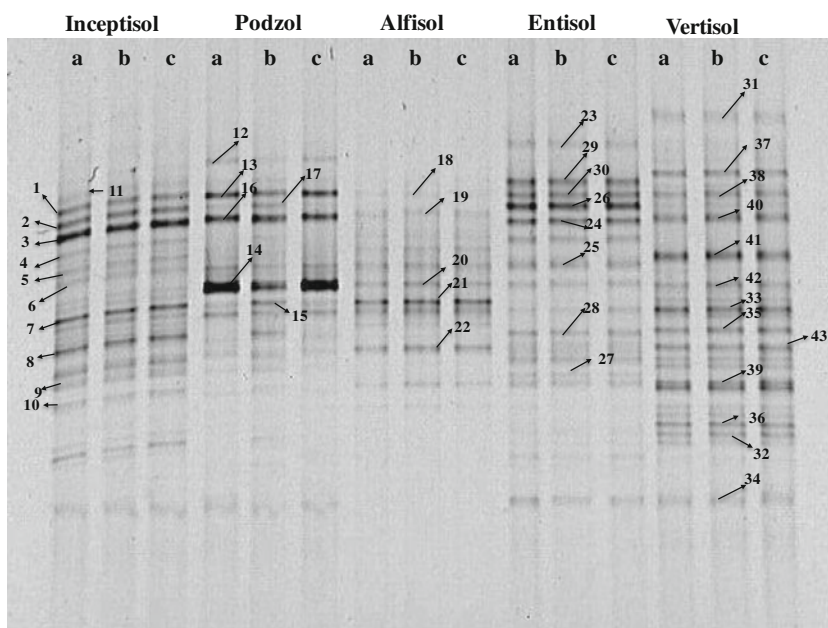
(2010) estimated the number of *mcrA* genes to be between $10^3$ and $10^7$ g$^{-1}$ dry soil in the plowed layer and sub-soils of different Japanese paddy fields. In the present study, *mcrA* gene copy number varied across the soil orders and could be ranked as gene with the trend: Vertisol>Inceptisol>Podzol>Entisol>Alfisol (Table 1). It indicates that variations in soil physicochemical properties influenced the distributional pattern of methanogenic population.

Variation in diversity of methanogenic archaeal community

Presenting a conclusive remark on the relative importance for soil orders in microbial diversity and abundance is still a difficult task, and yet to be resolved, and no general principles emerged yet. The comparison of different soils for a true picture of microbial community structure needs a range of variables including physicochemical, microbiological, field management history, etc. All of these factors may affect the microbial community structure in soils (Chaparro et al. 2012).

Species that represent a low population density and rare species are not shown in the community profile due to low sensitivity of PCR-DGGE (Gelsomino et al. 1999; Muyzer 1999), and thus the community profile data may contribute to analyze the effect of different environmental factors on the distribution of dominant methanogenic archaeal community.

DGGE-banding patterns of the methanogenic communities inhabiting five different paddy field soils clearly indicates variation in methanogenic diversity (Fig. 3). Although large number of bands appeared in each soil, we selected only the major bands from each soil for sequencing. Out of the total 43 bands sequenced, the highest number belongs to Vertisol, i.e., 13, and the least to Alfisol, i.e., 5. The remaining soils (Inceptisol, Entisol and Podzol) comprised 10, 8 and 6 bands, respectively. Such community level variations are possibly due to variations in the physicochemical properties of soils. Hoshino et al. (2011) suggested that soil properties can affect the archaeal communities in agricultural soils. The effect of soil properties like pH on the soil archaeal community at various soil profiles was also

**Fig. 4** Cluster analysis based on the intensity of bands (*0* invisible, *1* weak, *2* moderate and *3* strong) in DGGE gels of 15 banding patterns from PCR results obtained the target genes, showing each soil gets clustered separately. Letters at the left indicate soil orders: *AS* Inceptisol, *BS* Podzol, *CS* Alfisol, *DS* Entisol, *ES* Vertisol in triplicates
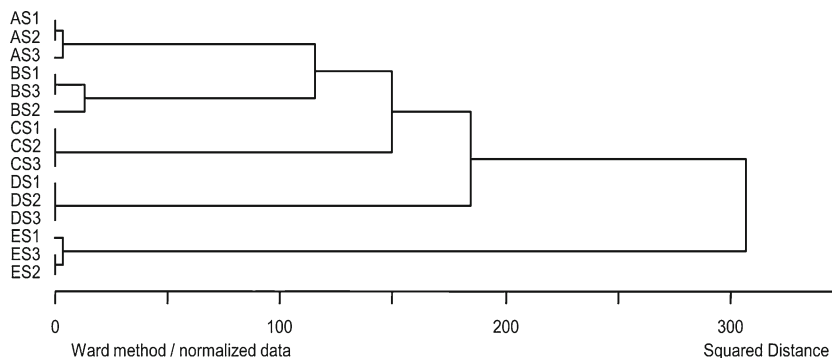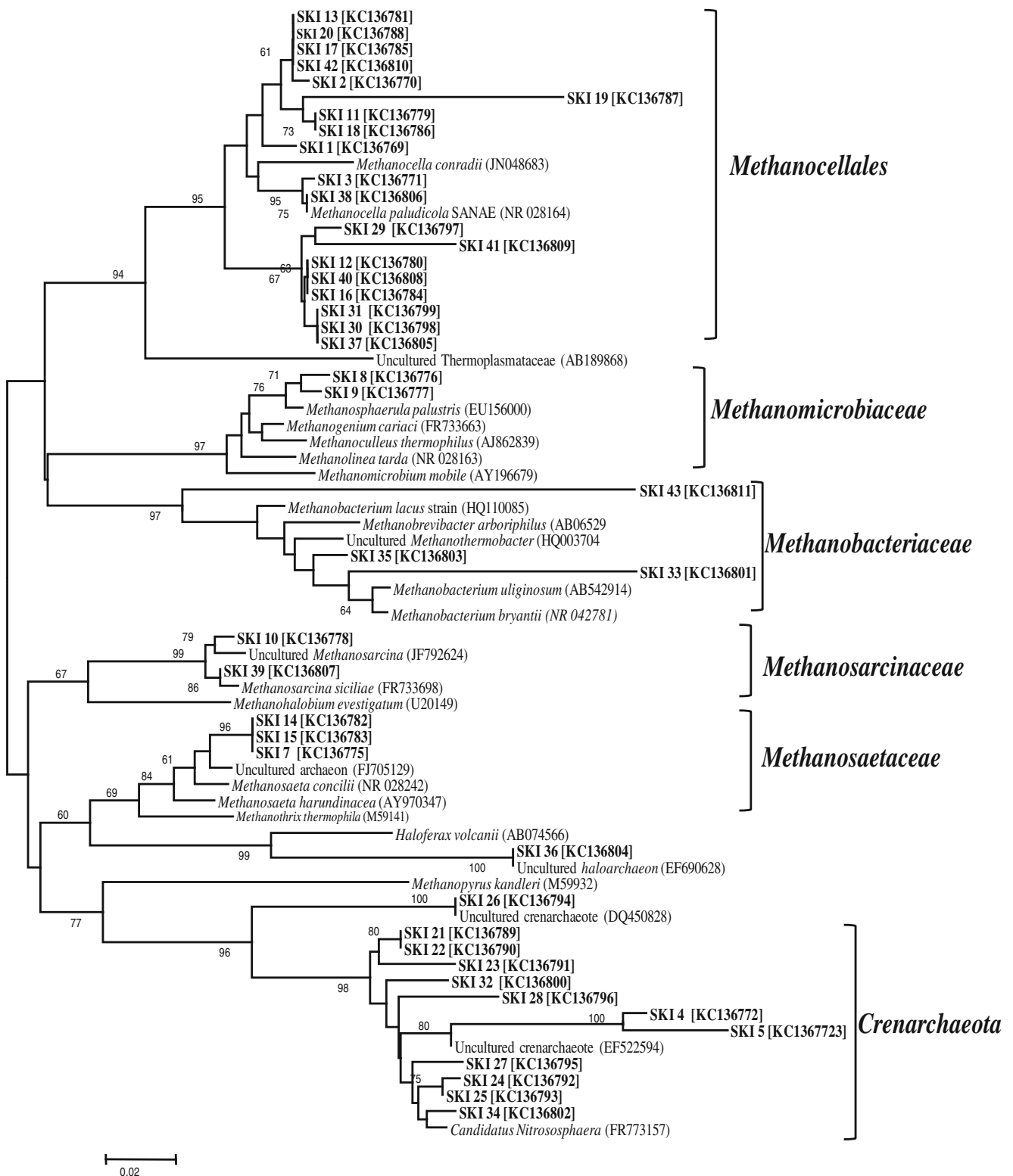
**Fig. 5** Phylogenetic relationship of the representative methanogenic 16S rRNA gene sequence of eluted bands retrieved from DGGE using 1106 F with GC clamp and 1378R primer pairs for non-rhizospheric soil DNA in different rice fields with following soil orders: Vertisol, Inceptisol, Entisol, Podzol and Alfisol. Uncultured and cultured NCBI databases show relationship with the representative clones. The scale bars represent an estimated of 2 % sequence divergence. GenBank accession numbers for each sequenced clone are indicated in parentheses

determined by Cao et al. (2012). Therefore, the present study strengthens the knowledge about the prevalent diverse groups of methanogenic community as evident from the banding pattern of DGGE analyses applied in different soil orders of tropical dryland irrigated paddy soils. A detailed study in this regard will certainly offer a still better insight.

The cluster analysis divided the soil orders into two groups and has branched Vertisol into the distinct cluster (Fig. 4). This is attributed to the presence of distinct types of bands in Vertisol soil showing resemblance to Methanobacteriaceae and *Methanosarcina siciliae* of Methanosarcinaceae.

Phylogenetic tree representation showed the common occurrence of only one methanogenic group, i.e., Methanocellales in all the soil orders as denoted by more than one band for each sample (Fig. 5). This indicates the dominance as well as diversity of Methanocellales in soils with differential ecological niche. According to Conrad et al. (2006), this group is ubiquitous as it prevails in the rice fields of different eco-zones of the world. Being the potential hydrogenotrophic methanogens, $H_2$ and $CO_2$, the chief source of $CH_4$ production, are common to all the experimental fields irrespective of adverse conditions prevailing.

According to Hoshino et al. (2011), soil chemical properties and soil type mostly overlapped in their effects on the archaeal community. Our study also confirms interactions between soil physicochemical properties and methanogenic archaeal community inhabiting the experimental fields. It may be due to the similar type of substrate demand for microbial growth and proliferations at different locations or the diverse type of substrate requirement as applicable to different methanogens.

Methane production potential

Laboratory incubation experiments indicate that the extent of $CH_4$ production potential ranged from 178.43 to 431.23 μg $CH_4$ $g^{-1}$ dws after 20 days of incubation for all soils (Fig. 1). Many studies on Indian paddy field soils revealed a close but varying range of $CH_4$ production potential depending on the soil type (Wang et al. 1993; Ramakrishnan et al. 1998; Mitra et al. 2002; Singh et al. 2012). Differences of such values with those of this study may be attributed due to differences in factors such as climatic conditions, soil properties, composition of methanogenic communities, etc. Our results indicate variability in $CH_4$ production potential of all soils with highest values in Vertisol and the least in Alfisol soil. This may depend on differential ecological conditions that prevailed in the respective soil in terms of their physicochemical and microbiological characteristics (Wachinger et al. 2000; Mitra et al. 2002; Jia et al. 2006). The organic C content and $CH_4$ production potential observed were significantly and positively correlated ($R^2=0.664$; $P=0.000$). It indicates that about 66 % variability in $CH_4$ production occurs due to variation in organic C content. Since $CO_2$ present in the soil is also utilized for $CH_4$ production, there was a significant positive correlation between these two properties ($R^2=0.891$; $P=0.000$). This is in accordance with the observation of Yao et al. (1999) and Glatzel et al. (2004).

Concentration of POXC in all soils varied between 215.3 and 587.5 μg $g^{-1}$ dws, which was under the range of variations from 378 to 814 μg $g^{-1}$ dws (Culman et al. 2012) and from 315.2 to 427.7 μg $g^{-1}$ dws (Bhattacharyya et al. 2012) reported in different agricultural soils. POXC indicates the availability of active carbon in the soil and is therefore considered as the early indicator of carbon change in soils (Culman et al. 2012). The availability of labile C in soil can influence methanogenesis directly due to being the sole C source for methanogens. A positive relation was observed between cumulative $CH_4$ production and POXC among five orders of soils ($R^2=0.883$; $P=0.000$), suggesting that around 88 % variability in $CH_4$ production was probably due to variations in POXC concentrations. Apart from the above possibilities, differences in methanogenic archaeal population size might be the other effective factor responsible for $CH_4$ production variability among different soil orders. There was a positive relationship between $CH_4$ production ($Y$, μg $CH_4$ $g^{-1}$ dws) and methanogenic archaeal population size ($X$, copy number $g^{-1}$ dws) for five soils ($R^2=0.855$; $P=0.000$). The rate constant ($K$) values (Fig. 2) showed the following increasing order for different soil orders: Alfisol<Podzol<Entisol<Inceptisol <Vertisol, a trend also observed in $CH_4$ production. This also showed the significance of kinetic model in determining $CH_4$ production where the rate of release was not constant.

Methanosarcinales, the only acetoclastic group comprising of Methanosarcinaceae and Methanosaetaceae, were detected in this study. Methanosarcinaceae group was present in Vertisol and Inceptisol, but only Methanosaetaceae was present in Inceptisol soil. Methane production by such acetoclastic methanogens using acetate reached about 50–83 % of the whole production in the bulk soil (Joulian et al. 1998). This is also supported here by the maximum $CH_4$ production in the Vertisol and Inceptisol soils that comprise these groups as compared to the other three soils having lower potential for $CH_4$ production possibly due to absence of such highly efficient $CH_4$-producing groups. *Methanosarcina* species dominate at high acetate concentration; thus, *Methanosaeta* is outcompeted by *Methanosarcina* as it can grow at low acetate availability (Chin et al. 2004). From this, it can be inferred that Vertisol soil was rich in acetate (not measured in this study but can be speculated on the basis of organic C) due to the presence of Methanosarcinaceae group.

## Conclusions

The present study demonstrates the prevalence of a diverse group of methanogenic archaeal community (Methanocellales,

Methanomicrobiaceae, Methnaobacteriaceae, Methanosae taceae and Methanosarcinaceae) in the studied soils. Population size and diversity of methanogens and $CH_4$ production potential of soils varied with respect to the soil locations probably due to different soil characteristics. The relative abundance of methanogenic archaeal group did not directly depend on any of the soil characteristics. The current observations contribute to the understanding of habitat-wise distributional pattern of methanogenic archaeal community and its $CH_4$ production potential across different soil types.

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