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DNA-based stable isotope probing identifies formate-metabolizing methanogenic archaea in paddy soil

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ABSTRACT

Paddy methane (CH_4) production is biologically dominated by methanogenic archaea that metabolize a variety of organic and/or inorganic carbon sources. Though formate is easily dissimilated into H_2/CO_2, formate-metabolizing methanogenic archaea are distinct from CO_2-utilizing methanogen taxa. The identity of formate-metabolizing methanogenic archaea in paddy soil remains elusive. In this investigation, molecular approaches based on stable isotope probing (SIP) technique were conducted to identify the formate-metabolizing methanogenic archaea in paddy soil. CH_4 emission monitor, real-time quantitative PCR (qPCR) and Denaturing Gradient Gel Electrophoresis (DGGE) analyses consistently indicated that some methanogenic archaea metabolized ^{13}C-labeled formate in microcosm and accounted for a large portion of formate-metabolizing archaea in anoxic paddy soil. Phylogenetic identification further found that this guild was affiliated to *Methanobacteriaceae*. Taken together *Methanobacteriaceae* could be the dominant formate-metabolizing methanogenic archaea and play an important role in the CH_4 production in paddy soil. These findings would extend the extant knowledge on paddy methanogenic archaea and microbial-driven paddy CH_4 emission.

1. Introduction

The global warming potential of methane (CH_4) is 25 times greater than that of carbon dioxide (CO_2) on 100-year horizon. IPCC (2007) reported that among global anthropogenic greenhouse gas emissions in 2004, CH_4 accounts for 14.3% in terms of CO_2 equivalent. Rice is the world's most important agronomic plant, with nearly 150 million ha under cultivation globally (Roger, 1996). In about 75% of this land rice grows under flooded conditions. Thus, paddy fields are one of largest anthropogenic sources of global CH_4 emissions (IPCC, 2007).

Biologically, paddy CH_4 production is dominated by methanogenic archaea. Methanogenesis is the final degradation process of organic matter in paddy soil and dependent on intermediary substrates derived from their degradation. Organic matter is first anoxically degraded to small molecules, such as acetate, formate, CO_2 and H_2, by diverse bacteria. Methanogenic archaea metabolize some of these molecules and further convert into CH_4 (Watanabe et al., 2007). Acetate contributes 79–83% to CH_4 production as a carbon substrate for methanogens (Chin and Conrad, 1995). In paddy soil, the CH_4 produced from acetate was between 51- 67% (Chidthaisong et al., 1999), while other substrates, such as H_2/CO_2 (or formate) contribute 17 − 31% to CH_4 production (Rothfuss and Conrad, 1992). Therefore, the identification of specific substrate-metabolizing methanogenic archaea is of great significance toward the knowledge of both the microbial-driven paddy carbon cycle and ecological functions of paddy methanogenic archaeal guild.

The CO_2- and acetate-utilizing methanogens in anoxic paddy soil have been previously documented. For example, Liesack et al. (2000) summarized that the predominant acetate-utilizing methanogens in paddy soil belong to *Methanosarcinaceae* and *Methanosaetaceae*. Members of the *Methanobacteriaceae* are the predominant group of hydrogenotrophic methanogens in rice paddy soils (Tonouchi, 2002). However, the information regarding identity of formate-assimilating methanogenic archaea in paddy soil remains limited. Formate is an important organic acid and a significant driver of methanogenesis in paddy soil (Penning and Conrad, 2006). The concentration of formate can exceed 150 μM in anoxic paddy soil (Rothfuss and Conrad, 1992). In spite of the easy dissimilation of formate to H_2 and CO_2, we propose that formate can be directly metabolized by methanogenic archaea to produce CH_4 in paddy soils, and the formate- and CO_2-utilizing

Abbreviations: DGGE, denaturant gradient gel electrophoresis; DNA-SIP, DNA-based stable isotope probing; FACE, free-air CO_2 Enrichment; qPCR, real-time quantitative PCR; SOC, soil organic C

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methanogen taxa are presumably distinct. Indeed, Hunger et al. (2011) have identified different functional guilds in the actively formate-metabolizing and H_2/CO_2-utilizing methanogens in fen soil. However, there is no unambiguous evidence to identify formate-assimilating methanogens in flooded paddy soil. DNA-based stable isotope probing (DNA-SIP) is a helpful technique to link the microbial metabolic function and their taxonomic identity in complex environment (Radajewski et al., 2003). The technique has been used to identify dominant formate-metabolizing bacteria in paddy soil, phylogenetically related to the bacteria in the classes of Clostridia and α-Proteobacteria (Feng et al., 2012), and to distinguish the difference in actively formate-assimilating and H_2/CO_2-utilizing methanogens in fen soil (Hunger et al., 2011). Therefore, in this investigation a microcosm-based DNA-SIP experiment was employed to identify formate-metabolizing methanogens in a flooded paddy soil.

2. Materials and methods

2.1. Soil sampling

Soil samples were taken from a rice-wheat rotation paddy field in Jiangsu Province, China (31°35′N, 120°30′E). The soil is classified as stagnic anthrosols. The soil properties are shown in Table 1. The station sits in the subtropical climatic zone with a mean annual precipitation of 900–1000 mm, the mean air temperature between June and August of 30 °C, an average daily integral radiation of 12.3 MJ/m^2, a total annual sunshine time of more than 2000 h and a frost-free period of more than 230 days. Soil samples at depth of 0–5 cm were collected from five points along S curve. Then the samples were kept in a cooler and shipped to the lab as quickly as possible. In the lab, the soil samples were pooled, passed through a 2 mm sieve and immediately stored at 4 °C after plant materials, roots, and stones were removed.

2.2. Anoxic microcosms

The DNA-SIP microcosm contained 5 g bulk soil and 0.5 mmol of ^{13}C-labeled formate (99 atom at% ^{13}C) purchased from Sigma-Aldrich (Kalyuzhnaya et al., 2008). As comparisons, the microcosms of ^{12}C-formate addition and the control without formate addition were established. Each treatment had three replicates. All the incubations were performed in sterile 120 ml serum bottle. The serum bottles were closed with rubber stoppers and crimp seals and flushed with sterile N_2 (100%) to make microcosms anaerobic. A 24-day anoxic incubation of microcosms was performed at 60% soil maximum water holding capacity, 30 °C and darkness. Gas samples (10 ml) were taken with a gas-tight syringe from the headspace of the soil microcosms every 3 days. The same volume of N_2 was then injected into the bottle to keep the equitant pressure. Totally, 24 gas samples were collected for each treatment. Production of CH_4 was monitored by a Varian 3380 with FID.

2.3. DNA extraction and SIP gradient fractionation

On the day after final gas sampling, soil samples from each microcosm were collected, mixed and sieved (< 2 mm). Samples were kept at −20 °C for molecular analysis. A half gram of moist soil from each sample was used for DNA extraction using FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions. The extracted DNA was dissolved in 50 μl TE buffer, quantified by spectrophotometer and stored at −20 °C until further use.

DNA-SIP was performed by published protocol (Hunger et al., 2011). Briefly, the gradient fractionation of total DNA extract (3.0 μg) from each SIP microcosm was performed with an initial CsCl buoyant density of 1.720 kg l^{-1} subjected to centrifugation at 177,000*g* for 44 h at 20 °C. The density gradient was divided into 340-μl fractions and the buoyant density of each fraction was determined by the refractive index. Fifteen fractions were generated covering buoyant densities from 1.696 kg l^{-1} to 1.743 kg l^{-1}, and nucleic acids were separated from cesium chloride by PEG 6000 precipitation and the resulting pellets were dissolved in 30 μl of TE buffer.

2.4. Real-time quantitative PCR

The abundances of methanogenic archaeal (primer set 1106F/1378R) and total archaeal (primer set A364aF/A934b) 16S rRNA genes along density gradients were quantified by quantitative PCR (qPCR) following the protocols of Watanabe et al. (2007) and Kemnitz et al. (2005) respectively. Standard curves were obtained using 10-fold serial dilutions of the *Escherichia coli*-derived vector plasmid pMD18-T (TaKaRa) containing a cloned corresponding target gene, using 10^2 to 10^8 gene copies μl^{-1}. The reactions were performed in C1000™ Thermal Cycler equipped with CFX96™ Real-Time system (Bio-Rad, USA). The 25-μl reaction mixture contained 12.5 μl of SYBR® *Premix Ex Taq*™ (TaKaRa), primer set (0.5 μM each), 200 ng BSA μl^{-1}, 1.0 μl template containing approximately 2–9 ng DNA. Negative control was always run with water as the template instead of soil DNA extract. The qPCR program used for methanogenic archaea or total archaea was: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C or 66 °C for 30 s and 72 °C for 90 s or 50 s, and extension and signal reading. The specificity of the amplification products was confirmed by melting curve analysis, and the expected sizes of the amplified fragments were checked in a 1.5% agarose gel. Real-time qPCR was performed in triplicate and amplification efficiencies of 97.4–104% were obtained with R^2 values of 0.966–0.977.

2.5. Denaturant gradient gel electrophoresis (DGGE) analysis

Methanogenic archaeal 16S rRNA gene fragments in 280-bp length were amplified using the primer set 1106F-GC (CGCCCGCCGCGCGCG GCGGGCGGGG CGGGG GCACGGGGGGTTWAGT CAG GCAACGAGC) and 1378R (CCCATGGTCCAGC GCCAGAA) (Watanabe et al., 2007)along buoyant density gradientsfor all treatments. Approximately 150–250 ng PCR amplicons from each sample were loaded onto an 8% (w/v) acrylamide-bisacrylamide gel with 45%–75% denaturant gradient. DGGE was run in 1 × TAE buffer for 10 h at 60 °C and 100 V with a Dcode Universal Mutation Detection System (Bio-Rad, Hercules, Calif.). After electrophoresis, the DGGE gel was stained for 20 min with SYBR Green I nucleic acid staining solution with 1:10000 (v/v) (Invitrogen, Oregon, USA) and photographed. DGGE fingerprinting profiles of methanogenic archaeal 16S rRNA genes were digitalized by using a Gel Doc™ EQ imager (Bio-Rad, USA) combined with Quantity One 4.4.0 (Bio-Rad, Hercules, CA, USA). The dominant and representative bands in DGGE gels were excised, left overnight in 25 μl Milli-Q water, re-amplified and run again on the DGGE system to ensure purity and correct mobility of the excised DGGE bands.

2.6. Cloning, sequencing and phylogenetic analysis

After confirmation, the excised DGGE bands were re-amplified with the primer set of methanogenic archaea without GC clamp, followed by the purification using the QIAquick PCR Purification Kit (QIAGEN). The purified PCR products were inserted into a pMD18-T vector (TaKaRa) in accordance with the manufacturer's instructions and further introduced into *Escherichia coli* DH5α competent cell. Six random clones were sequenced by Invitrogen Sequencing Department in Shanghai, China.

One representative clone sequence of each band with high quality after sequence comparisons using DNASTAR software package was chosen for phylogenetic analysis. The representative sequences of DGGE bands were compared with sequences in BLAST to obtain the three nearest phylogenetic neighbors. Then a phylogenetic tree was built by the neighbor-joining method using the software package of MEGA 4.0 version (Molecular Evolutionary Genetics Analysis) (Tamura et al., 2007). The GenBank accession numbers for methanogenic

Table 1
The physicochemical properties of tested soil.

Properties	Sand	Silt	clay	Bulk density	Soil organic C	total N	total P	Available P	pH
	%			g cm^{-3}	g kg^{-1}			mg kg^{-1}	
content	9.2	65.7	25.1	1.2	15.0	1.59	1.23	10.4	6.8

Diameter: Sand 0.05–1 mm; Silt: 0.001–0.05 mm; Clay: < 0.001 mm.

archaeal 16S rRNA gene fragments sequenced in this investigation are KM104155 to KM104166.

2.7. Statistical analysis

Statistical procedures were carried out with SPSS 13.0 for Windows. Data were expressed as means with standard deviation (SD). Mean separation was conducted based on Tukey's multiple range test. Differences at $P < 0.05$ were considered statistically significant. Principal component analysis (PCA) was performed using the R package (Vegan) software (Version 2.12.1) and all the DGGE bands were used in the calculation.

3. Results and discussion

3.1. Microbial methane production in microcosms

The methanogenic archaeal activity was assessed by monitoring the CH_4 concentrations in the headspace of microcosms during the 24-d incubation period. Under formate treatments (Fig. 1), regardless of the ^{13}C- and the ^{12}C-labeled, CH_4 accumulations reached a maximum of around 46–52 μmol g^{-1} dry weight soil (*d.w.s*). In contrast, the CH_4 concentrations in the controls without formate were significantly lower than those in formate treatments during the entire incubation period (Fig. 1, $P < 0.05$), indicating that formate addition stimulated methanogenic archaeal activity, and some methanogenic archaea produced CH_4 from formate and subsequently increased CH_4 evolution in anoxic paddy soil.

3.2. The abundances of total archaeal and methanogenic archaeal 16S rRNA genes across the isotopically fractionated DNA gradients

To distinguish the "heavy" and "light" DNA fractions, the copy numbers of total archaeal and methanogenic archaeal 16S rRNA genes in each DNA buoyant density fraction were assayed by qPCR (Fig. 2). Analyses of the copy numbers of the total archaeal genes revealed a light fraction peak in the ^{12}C-formate treatments between 1.711–1.725 g ml^{-1} ("light" DNA fractions), and a heavy fraction peak in the ^{13}C-formate treatments between 1.728 and 1.739 g ml^{-1} ("heavy" DNA fractions) (Fig. 2A). The peak numbers, ranging from 2.25×10^4-2.5×10^4 μl^{-1} DNA, were significantly higher than those in the controls with 0.7×10^4 copy numbers μl^{-1} DNA ($P < 0.05$). These differences indicated that a portion of the archaea in anoxic paddy soil might metabolize ^{13}C-labeled formate and subsequently their nucleic acids become "heavier."

For the methanogenic archaeal guild, similar changing patterns of copy numbers between formate-treated microcosms and the controls were observed (Fig. 2B): under formate treatments, the maximal copy numbers of methanogenic archaea significantly increased from 0.26×10^4 up to 0.7×10^4-1.0×10^4 μl^{-1} ($P < 0.05$). There were also a light fraction peak in the ^{12}C-formate treatments and a heavy fraction peak in the ^{13}C-formate treatments. Both results indicated that some methanogenic archaea metabolized ^{13}C-labeled formate. Furthermore, the comparison of the peak copy numbers in "heavy" DNA fraction with buoyant density of 1.735 g ml^{-1} between total archaea (Fig. 2A) and methanogenic archaea (Fig. 2B) in ^{13}C-labeled formate microcosm implied that the methanogenic archaea responsible for formate metabolism accounted for a considerable proportion of total archaeal population.

3.3. DGGE fingerprinting analysis of methanogenic archaeal 16S rRNA genes across the isotopically fractionated DNA gradients

The methanogenic archaea involved in the metabolism of formate-derived C was identified by PCR-DGGE fingerprints in combination with phylogenetic analysis. Based on the qPCR results along DNA density gradients in different microcosms, the "light" fractions in the ^{12}C-formate and control treatments, and both "light" and "heavy" DNA fractions in the ^{13}C-formate treatment were chosen to analyze by DGGE technique (Fig. 3). In general, the fingerprinting profiles of methanogenic archaeal 16S rRNA genes in "heavy" DNA fractions of ^{13}C-labeled formate microcosms were different from those in "light" DNA fractions of ^{13}C- and ^{12}C-labeled formate microcosms as well as the control. For example, DGGE bands 6, 7 and 8 appeared, and the intensity of DGGE band 10 was greatly increased in "heavy" DNA fractions (Fig. 3). For a better visualization, a principal component analysis (PCA) was

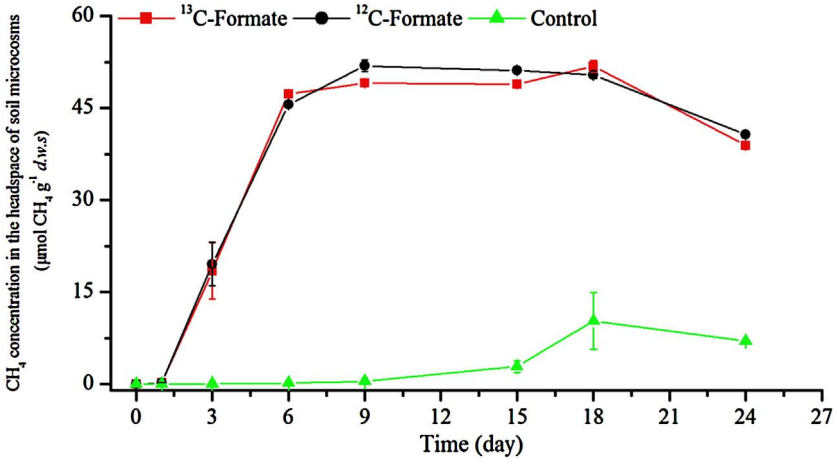

Fig. 1. Methane concentrations in soil microcosms during incubation for 24 d. Control denotes the treatment without formate addition; ^{13}C- and ^{12}C-formate denote the soil treated with ^{13}C- and ^{12}C-formate, respectively; *d.w.s.* is dry weight soil.

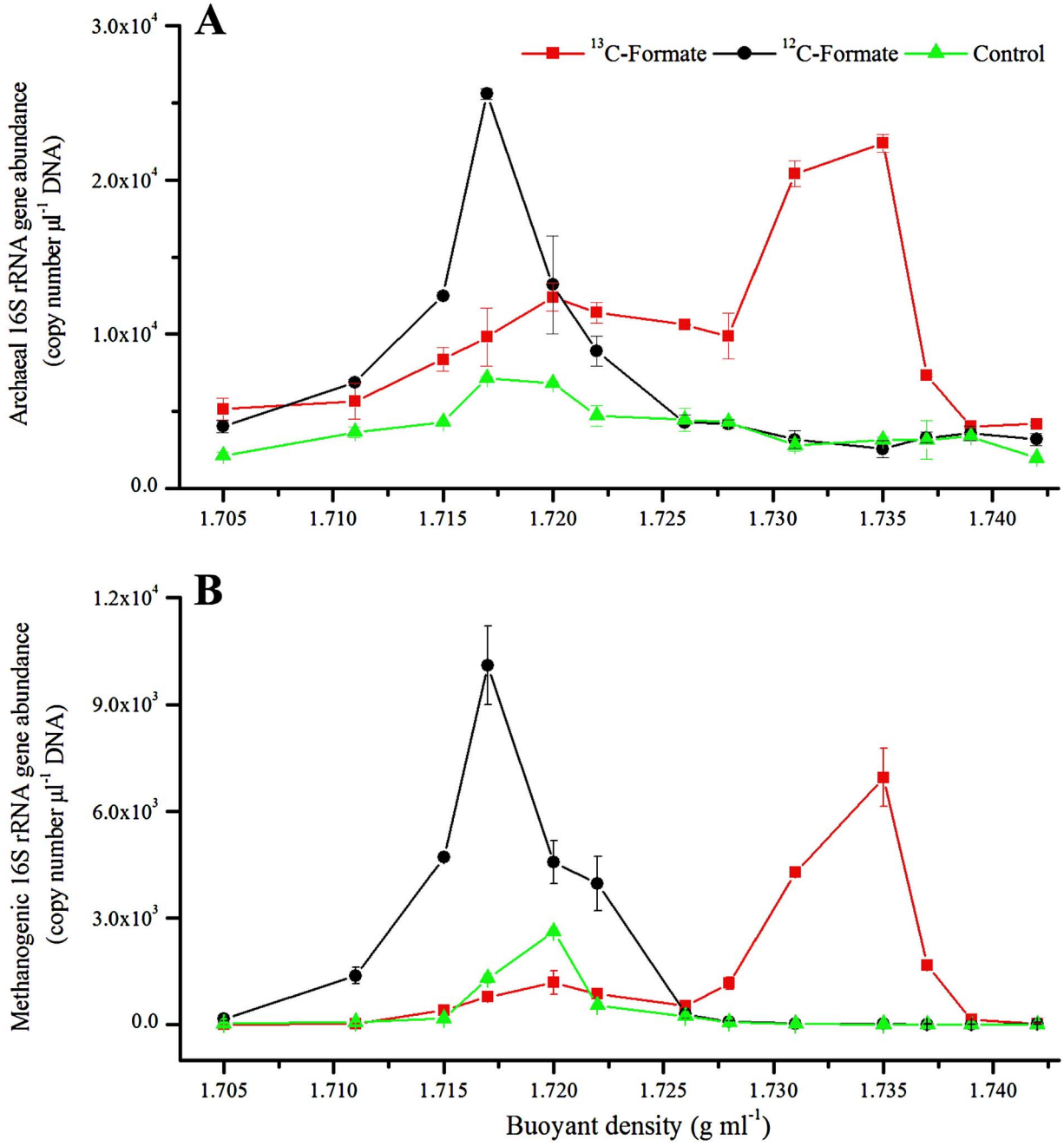

Fig. 2. Distribution of the copy numbers of the archaeal (A) and methanogenic archaeal (B) 16S rRNA genes across the buoyant densities of the DNA density gradients of soil samples treated with ^{13}C- or ^{12}C-formate or the control.

conducted (Fig. 4) to reveal the differences in fingerprinting patterns between the "heavy" and "light" DNA fractions from the soil samples treated with ^{13}C-formate, as well as the differences between "light" DNA fractions from the soil samples treated with formate and the control. The first principal component differentiated the methanogenic archaeal compositions in different DNA buoyant density fractions of different treatments into two groups (43.4% of contribution rate). Specifically, the community compositions in "heavy" DNA fractions of ^{13}C-labeled formate microcosms were clustered together and separated from those of "light" DNA fractions in ^{13}C- and ^{12}C-labeled formate microcosms as well as the negative control. These phenomena were consistent with the qPCR results, indicating that some methanogenic archaea metabolized ^{13}C-labeled formate and they were separated by ultracentrifugation due to itself "heavier" nucleic acids.

Phylogenetic identification further revealed that twelve dominant DGGE bands (Fig. 3) were highly affiliated to *Methanoregulaceae*, *Methanocellaceae*, *Methanobacteriaceae* and the acetoclastic groups, *Methansaetaceae* and *Methanosarcinaceae* (Fig. 5). The formate-metabolizing methanogenic archaea in this investigation, however, were predominantly grouped into *Methanobacteriaceae*. This finding was partly consistent with the reports of the cultivation-dependent and −independent approaches. Zinder (1993) reviews that several *Methanobacterium* species can utilize formate and H_2/CO_2 as electron donors to produce methane. For strain physiological evaluation, Joulian et al. (2000) find that *Methanobacterium oryzae* can directly metabolize formate for growth and produce CH_4; some new species, such as *Methanobacterium movilense* sp. nov. isolated from anoxic sediment of subsurface lake (Schirmack et al., 2014) and *Methanobacterium aggregans* sp. nov isolated from anaerobic digester (Kern et al., 2015), have been reported to have the ability of utilizing formate as substrate. Benstead

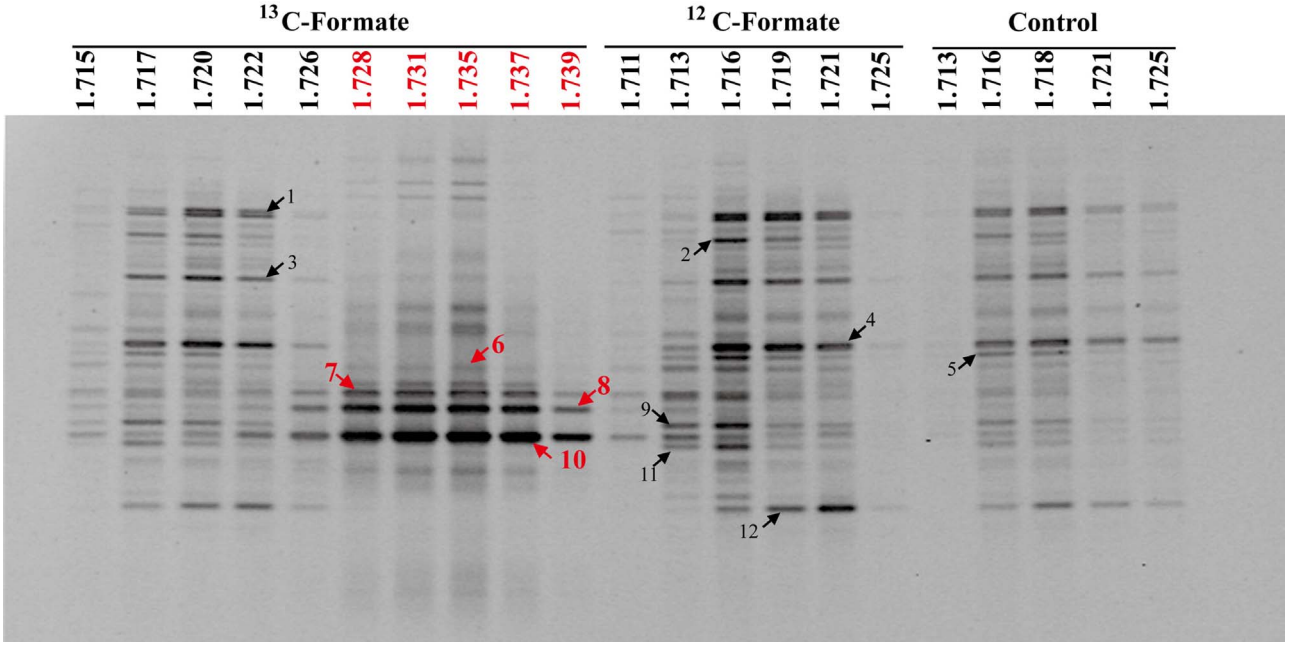

Fig. 3. DGGE fingerprinting profiles of methanogenic archaeal 16S rRNA genes in the "heavy" and/or "light" DNA fractions from ^{13}C- and ^{12}C-formate treatments and the untreated control. The buoyant density of the DNA used for PCR-DGGE analysis is labeled above each DGGE lane. The bands excised for sequencing analysis are indicated by arrows numbered from 1 to 12.

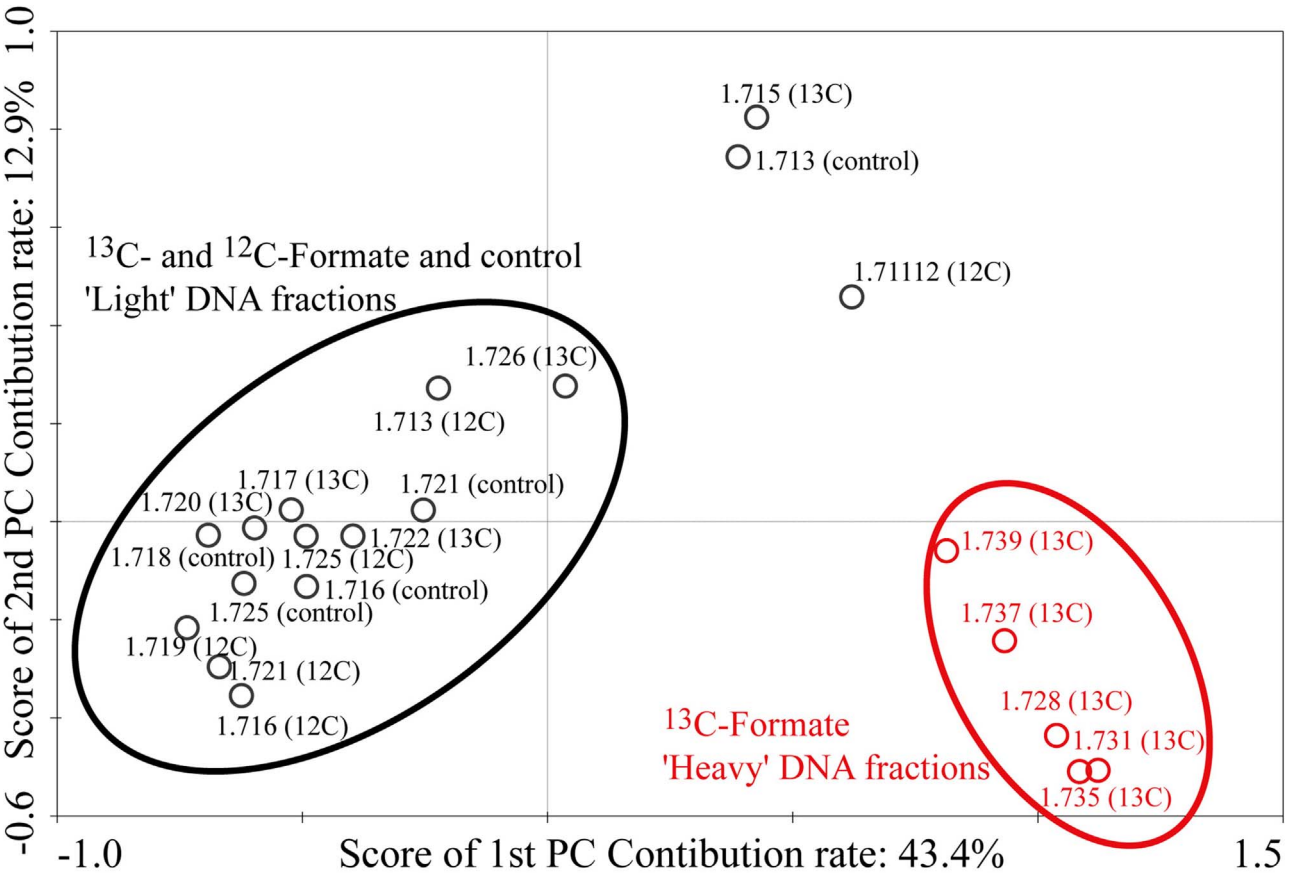

Fig. 4. Principal component analysis of the methanogenic archaeal 16S rRNA gene DGGE band patterns in the "heavy" and/or "light" DNA fractions from ^{13}C- and ^{12}C-formate treatments and the untreated control. The contribution rates of the first and second principal components (PC) are 43.4 and 12.9%, respectively.

et al. (1991) report that *Methanobacterium bryantii* could produce CH_4 from formate. Using SIP technique Hunger et al. (2011) document that both *Methanobacteriaceae* and *Methanocellaceae* could *in situ* assimilate formate-derived carbon in fen soil. Indeed, *Methanobacteriaceae* has been previously defined as hydrogenotrophic methanogens and its members, such as *Methanobacterium*, are the predominant group of hydrogenotrophic archaea in rice paddy (Liesack et al., 2000; Tonouchi, 2002). Though formate is readily dissimilated to CO_2 biotically and

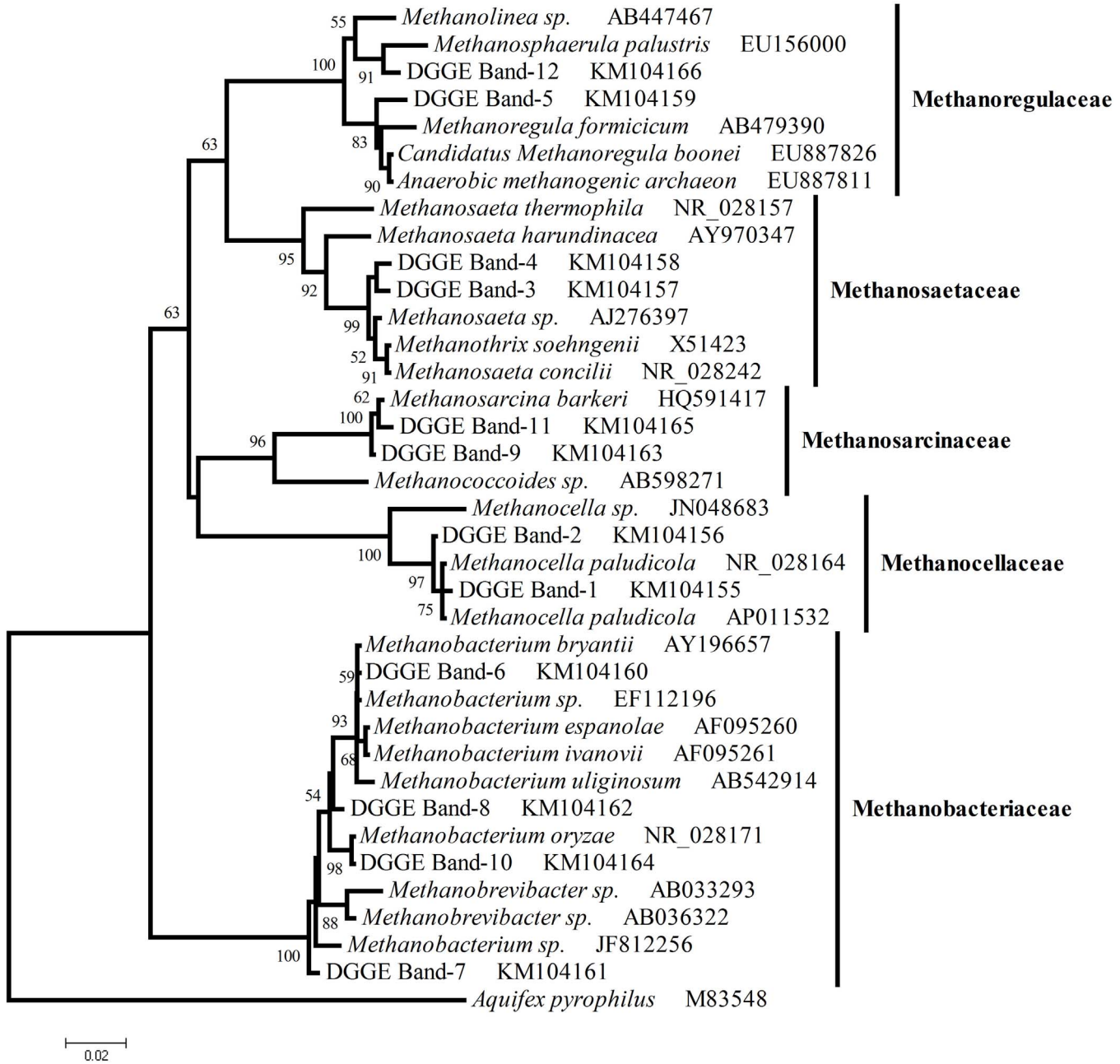

Fig. 5. Phylogenetic identification of methanogenic archaeal 16S rRNA genes in DGGE fingerprinting profiles in Fig. 3. *Aquifex pyrophilus* acts as the outgroup. Bootstrap values of > 50% based on 1000 replicates are indicated at the nodes. The scale bar indicates the number of nucleotide substitutions per site.

abiotically and inevitably *Methanobacterium* is assumed to assimilate $^{13}CO_2$ derived from ^{13}C-formate, the current findings, together with the previous records, suggested that *Methanobacteriaceae* could be the important formate-metabolizing methanogenic archaea in paddy soil.

It is noted that formate can also be syntrophically degraded by *Methanobacterium* (e.g. *Methanothermobacter* sp. and *Methanobrevibacter* sp.) and bacterium *Moorella* sp. (Dolfing et al., 2008). Considering that the species *Moorella* sp. is actively involved in the formate-metabolism in the same soil determined by bacterial SIP experiment (Feng et al., 2012), we speculated that the syntrophical interaction might occur between bacteria and methanogenic archaea, and contribute to CH_4 emission. Another point needed to be mentioned is that formate has a "priming" effect on the utilization of organic carbon and formate-derived H_2 might fuel acetogenesis and methanogenesis under anoxic condition (Hunger et al., 2016). This process could influence carbon and energy flow in paddy soil, and subsequently impact compositions and abundances of syntrophic microbes, and finally those of formate utilizers. More details on this point should be considered in the future work to reveal the formate-metabolizing microorganisms in paddy soils.

Besides the clear identification of *Methanobacteriaceae* genotypes of *Methanosaeta*-like DGGE band 3 with very weak intensity faintly in the "heavy" fraction. This species is generally acknowledged to be a specialist that uses only acetate based on physiological studies (Morita et al., 2011). Although the genome sequencing suggests that *Methanosaeta* might be more metabolically diverse than previously thought (Smith and Ingram-Smith, 2007), there is so far no direct evidence that *Methanosaeta* could use formate. Therefore, we could not guarantee that the weak band of *Methanosaeta* was related to formate-metabolism.

Another phenomenon noteworthy is that *Methanocellaceae*-like phylotypes, linked to the production of formate-derived methane in fen soils (Hunger et al., 2011; Hunger et al., 2015), were not observed in "heavy" DNA fractions of ^{13}C-formate microcosm in this investigation.

We supposed this absence might be ascribed to the differences in the soils and the measuring approaches. Specifically, fen soils in Hunger et al. (2011) and the paddy soil in current investigation could lead to the different findings. Besides, the identifying resolution is relatively lower for DGGE technique than that of T-RFLP used by Hunger et al. (2011).

Taken together, using DNA-SIP technique, we identified roles of some microbial guilds carrying out formate metabolism in paddy soil, which would be conducive to understanding of the specific functional microorganisms and paddy microbial-driven carbon cycling. This information would improve our understanding of the ecophysiology of paddy methanogenic archaea.

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