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Methane and Nitrous Oxide Productions and Community Structure of Methanogenic Archaea in Paddy Soil of South Sulawesi, Indonesia

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The potential of methane and nitrous oxide productions and community structure of methanogenic archaea has been characterized by incubation studies and PCR-DGGE approaches, respectively. The results showed that addition of straw into the soil was the most important factor that influenced CH_4 production in this incubation experiment, while N_2O production was observed higher in soil without straw amendment under saturated condition. Most of the clone sequences revealed from DGGE band was associated with a archaea lineage, *Methanocellales* ord. nov (Rice Cluster I). Other sequences belong to *Methanomicrobiales*, *Methanosaetales*, and Rice Cluster 2 (RC-2). The study also showed that the hydrogenotrophic methanogens were the dominant members in methanogenic archaea community obtained from tropical paddy field soil, while acetoclastic methanogens present in relatively minor quantity in the soil.

Key words: CH_4, methanogenic archaea, N_2O, paddy soil

Telah dilakukan karakterisasi struktur komunitas archaea metanogenik dari tanah persawahan dengan metode PCR-DGGE serta potensinya dalam menghasilkan gas metana dan nitrous oksida secara teknik inkubasi. Hasil memperlihatkan bahwa pemberian jerami merupakan faktor yang sangat mempengaruhi dalam produksi gas metana, sementara produksi gas nitrous oksida tertinggi pada tanah berkondisi air jenuh tanpa jerami. Secara umum klon sekuen dari DGGE band teraffiliasi dengan archaea *Methanocellales* ord. nov (Rice Cluster I). Sekuen lainya adalah *Methanomicrobiales*, *Methanosaetales,* dan Rice Cluster 2 (RC-2). Studi ini juga menunjukkan bahwa kelompok metanogen hidrogenotrofik adalah struktur komunitas archaea metanogen yang dominan, sedangkan komunitas metanogen asetoklastik merupakan struktur komunitas yang minor pada tanah persawahan di Maros, Sulawesi Selatan.

Kata kunci: archaea metanogen, CH_4, N_2O, tanah sawah

Global atmospheric concentration of methane (CH_4) and nitrous oxide (N_2O) have increased due to natural process, biological processes and also by anthropogenic activities. CH_4 concentration in the atmosphere has increased from early 90s of about 1732 ppb to 1774 ppb in the 2005, and that concentration has increased up to 40% since pre-industrial time, while the global atmospheric concentration of N_2O increased from 270 ppb in pre-industrial time to about 319 ppb in 2005 (IPPC 2007a).

More than one third of all CH_4 emissions come from soils, as a result of the microbial breakdown of organic compounds in strictly anaerobic conditions. This process occurs in natural wetlands, in flooded rice fields, and in landfills rich in organic matter, as well as in the gut of some species of soil dwelling termites. The rice field and landfill sources make up an important part of anthropogenic methane emissions, which have led to the more than doubled concentration in the atmosphere. Analysis of gas trapped in polar ice shows that in the pre-industrial era it was only *ca*. 0.7 µmol mol^{-1} (IPPC 2007b).

CH_4 is an important component of the global carbon cycle and contributes to enhanced greenhouse effect as well as N_2O. Paddy cultivation is considered as one of the main human-related sources of CH_4, and has been predicted that CH_4 emission from paddy cultivation will increase as rice production increase by 65 % from end 1990s of 460 Mt yr^{-1} to 760 Mt yr^{-1} in 2020 (Neue 1997). CH_4 emission from paddy cultivation contributes about 20 to 150 Tg CH_4 yr^{-1} to the global budget (Crutzen 1995). Larger variation in the magnitude of CH_4 emission from that source is attributed to soil parameters (pH, redox potential, organic matter content, temperature, clay) or agronomic condition (rice cultivars and farming practices) (Fumoto *et al*. 2008).

The magnitude of CH_4 emission from paddy field reflects the balance between methanogenesis and methanotrophy. CH_4 production is the final process in the anoxic microbial degradation pathway in paddy

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field soil, while, methane oxidation occurs at anaerobic-aerobic interface with available oxygen (O_2) and CH_4 concentration. The quantity of CH_4 actually emitted depends on both production and oxidation of CH_4. Therefore, basic knowledge on the underlying processes is required when designing strategies to mitigate and control CH_4 emission from paddy field (Henckel *et al*. 2000; Bodegom *et al*. 2001).

CH_4 is produced by microbial community consisting of various fermenting microorganisms that degrade organic matter and ultimately to acetate, H_2 and CO_2. The most actual production of CH_4 is brought about by methanogenic archaea, which either convert acetate to CH_4 and CO_2 (acetoclastic methanogenesis) or convert H_2 plus CO_2 to CH_4 (hydrogenotrophic methanogenesis) (Conrad *et al*. 2006; Sakai *et al*. 2008). In paddy field soil, the taxonomy of methanogenic archaea members consisted of *Methanosaetaceae* and *Methanosarcinaceae* as acetoclastic methanogens type. While, the type of hydrogenotrophic methanogens are including members of *Methanomicrobiales*, *Methanobacteriales*, and Rice Cluster1 (RC-I) lineage with new name *Methanocellales* ord.nov (Sakai *et al*. 2007 and 2008) .

Not many studies have been conducted to measure the potential of CH_4 and N_2O productions and characterize the responsible microbe of methanogenic archaea from tropical paddy soil. Therefore, the current paper showed the potential of CH_4 and N_2O productions and methanogenic archaea structure from paddy soil in South Sulawesi, Indonesia that approached by incubation experiments and PCR-DGGE, respectively.

MATERIALS AND METHODS

Soil Sampling and Analysis Descriptions. Soil was sampled in Maros district, south Sulawesi province, Indonesia (5m asl., 05°00.419` S 119°31.219` E), which is a rice-growing area (6513 ha). Soil was classified as Typic Haplusterts according to U.S. Soil Taxonomy, 1998. The soil was alluvial type according to Center for Soil and Agro-climate Research, Indonesia, 2000. Soil samples were taken from each micro-plot either before transplanting or after plowing at a depth of 0-15 cm (plowed layer) and sieved as moist condition through 2 mm mesh and stored at 4 °C until soils were used in laboratory incubation experiment. The soil had a pH (H_2O) of 7.22, EC of 0.17 dS m^{-1}, total carbon content of 22.5 g-C kg^{-1}, total N content of 2.30 g-N kg^{-1} dry soil, and C/N ratio of 9.78, measured by electrode methods and a C/N analyzer (MT 700, Yanaco, Kyoto, Japan), respectively.

Determination of Potential Production of CH_4 and N_2O. The Greenhouse gas production potential (CH_4 and N_2O) of paddy field soil sample were determined by incubation method in the laboratory. A composite portion of 100 grams soil were pre incubated at 30 °C for seven days in sealed 1000 mL glass bottle for pre-incubation purpose. The soil samples were then added with inorganic fertilizer (urea) at a rate of 120 mg-N kg^{-1}. Then, these samples were set as two factorial design with 3 replications, and the first factor with organic amendments (with 1.0 g of rice straw or without), and the second factor was water regime (WS=water saturated, WSS=water saturated plus straw). All soil samples were incubated at 30 °C and gas in the headspace of each bottle were taken every five days interval to quantify the concentrations of CH_4 and N_2O. The concentrations of CH_4 and N_2O in the samples were quantified using gas chromatographs (Shimadzu, GC 14B, Kyoto, Japan) equipped with a flame ionization detector (FID) and an electron capture detector (ECD), respectively. After gases were sampled the bottle stopper were opened for 30 min to change the air in side the bottle and soils were incubated continually until 35 d.

Means and standard deviations of the data were calculated. Means were compared using the least significant differences (LSD=0.05) value by SPSS software (Ver.11.0 for windows, SPSS Inc., Chicago, USA).

DNA Extraction and Polymerase Chain Reaction (PCR) Amplification. At the end of incubation (35 d), soil from surface (0.5 cm) and subsurface (1.0 cm below surface) of each treatment was collected carefully and subjected for extraction of DNA from soil. Soil DNA was extracted using a FastDNA SPIN Kit for soil (Bio 101, Inc. Vista, CA, USA) following the manufacturer's instructions. In this procedure, cell lyses was performed by vigorous shaking in a mini-beadbeater (Biospec product, Wakenyaku, Co., Tokyo, Japan) with intense speed of 4.8 for 30 sec. DNA extracts were stored at -20 °C before used as a template for subsequent PCR reaction.

One µL of DNA extract was used as template for PCR amplification with primers 0357F (5`-CCCTAC GGGGCGCAGCAG-3`; *Escherichia coli* position 340-357) and 0691R (5`-GGATTACARGATTTCAC-3`; *E.coli* position 707-691) (Watanabe *et al*. 2004, 2006 and 2007). The primer pair was used for PCR amplification of 16S rRNA gene fragment of methanogenic archaea. PCR amplification was performed in a total volume of 50 µL reaction mixture

containing 5 µL of 10x Ex *Taq* buffer (20 mM Mg^{2+}), 4 µL of dNTP mixture with final concentration of 2.5 mM of each, 0.5 µL of each primers (80 pmol µL) and 0.3 µL of Ex *Taq* DNA polymerase (5 U μL^{-1}, a hot start version) and made to volume using a sterile distilled water (Otsuka Pharm., Japan). Ex *Taq* DNA polymerase, dNTP mixture, and 10x Ex *Taq* buffer were purchased from Takara Bio Inc. Shiga, Japan.

The PCR reaction was run using a DNA thermocycler (Takara Bio Inc. model TP600, Japan). The thermal profile regime was a hot start at 94 °C for 3 min (denaturation) and 35 cycles at 94 °C for 1 min, followed by 53 °C for 1 min, and then 72 °C for 2 min. A final extension step was at 72 °C for 5 min. The quality of the PCR products were tested by running 5µL of it on a 2% agarose for 30 min at 100 V in 1 X TAE and visualized by UV transillumination (ATTO printgraph, model AE-6932) after staining with ethydium bromide for 30 min and washing with distilled water.

Denaturing Gradient Gel Electrophoresis (DGGE), Sequencing, and Phylogenetic Analysis. DGGE analysis was performed on the D-Code system (Bio-Rad, Hercules, Calif.). PCR products were applied directly into 7% (wt/vol) polyacrylamide gels in 1X TAE (40 mM Tris base (pH 7.2), 20 mM acetic acid, 0.5 M EDTA 1 mM) with 35-60% denaturing gradient (urea and formamide). One hundred percent denaturing acrylamide was defined as 7 M urea and 40% formamide. A top gel without denaturant was cast above the denaturing gel before the polymerization started. 25 µL of PCR products were loaded into each lane and run at 60 V for 14 hours at constant temperature of 60 °C and visualized after staining with ethydium bromide for 30 min and washed twice with distilled water.

Prominent DGGE bands were excised from the gel with a sterile razor blade and placed in 1.5 mL plastic tube with 50 µL of sterile distilled water and vortex gently for 5 seconds and then incubated overnight at 4 ˚C. After centrifugation at 2000 rpm for 10 seconds, 1 µL of liquid was used as template for PCR reactions performed under the conditions described above and then separated on DGGE again after only a single band appeared. The single band was excised as mentioned above, and then subjected as template for PCR reactions performed under the conditions described before without GC clamp on forward prime. The quality of DNA recovered from gel acrylamide after PCR reactions were determined on agarose gel electrophoresis as explained above.

The remaining PCR products were purified using Suprex™ (Takara Bio. Inc. Shiga, Japan) according to manufacturer's instructions. One µL of purified PCR product was used as a template for cycle sequencing reaction performed with a DNA sequencing kit-BigDye™ Terminator v3.0 (Applied Biosystem, Foster City, CA, USA) with forward and reserve primers run separately. The sequencing reaction was performed for 30 cycles at 95 °C for 30 sec (denaturation), at 60 °C for 30 sec (annealing) and at 75 °C for 95 sec (extension). Prior to DNA sequence analyses with an ABI 3100 genetic analyzer (Applied Biosytems, USA) the reaction products were purified with Centri-Sep Columns (Princeton Separation, NJ) following manufacturer's instructions.

Phylogenetic relationships of the aligned sequences were inferred using the neighbor-joining (Saitou and Nei 1987). A bootstrap analysis with 1000 replicates was made for all database sets to evaluate the stability of phylogeny (Felsenstein 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980). Phylogenetic analyses were conducted in MEGA 4 (Tamura *et al*. 2007). The nucleotide sequences in this study have been deposited in the DNA databank of Japan (DDBJ, URL: http://www.ddbj.nig.ac.jp/) under accession no. AB 440226 through AB440235.

RESULTS

Change in production of greenhouse gas (CH_4 and N_2O) during soil incubation is shown in Fig 1A and B, respectively. The production of CH_4 with straw amendment under flooded condition was significantly higher than the other treatments (Fig 1A). The straw amended soil under flooded condition had produced 20.6 µg CH_4-C g^{-1} dry $soil^{-1}$ after 5 days of incubation and its almost ten times higher compared with water saturated (2.6 µg CH_4-C g^{-1} dry $soil^{-1}$) and peaked on 10 day incubation (103 µg CH_4-C g^{-1} dry $soil^{-1}$), then gradually declined thereafter. Meanwhile, CH_4 productions in the other treatments were almost negligible.

On the other hand, the N_2O production was observed higher in soil without straw amendment and under unflooded water condition at 5 d incubation compared to the other treatments, and then rapidly decreased on 10 d incubation. After 15 d of incubation small amount of N_2O was produced and then decline after the end of incubation (Fig 1B). Soil incubated with straw amendment and under saturated water

condition produced small amount of N_2O at 5 d of incubation and then declined thereafter. The other treatments were almost not producing N_2O during the incubation period.

The community structure of methanogenic archaea in paddy field soil in tropical region such as Indonesia were investigated with PCR-DGGE method

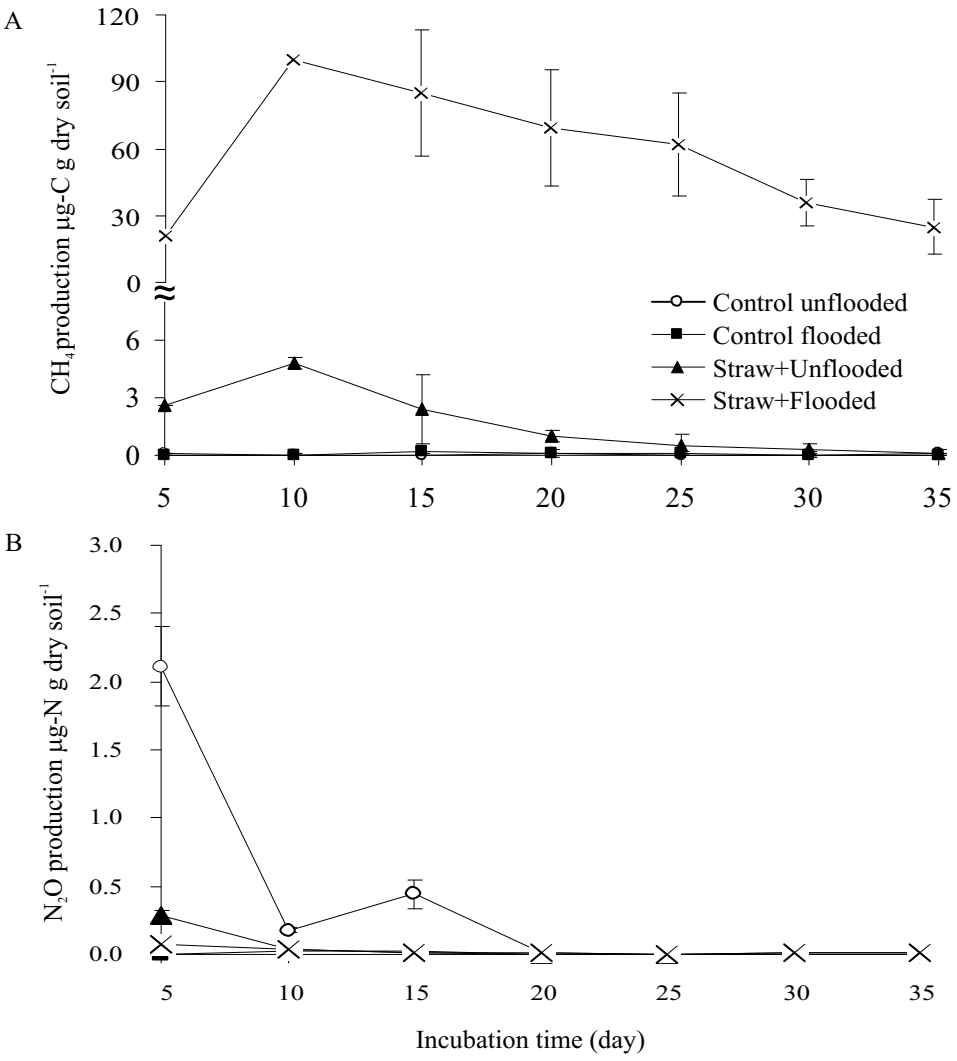

Fig 1 Change in production of (A) CH_4 and (B) N_2O during incubation of soil samples. The error bars indicate the standard deviation of the results.

approaches with a primer set (0357F-GC/0691R). The pair primer is suitable for investigating methanogenic archaeal community in paddy field soil. Extraction of the soil DNA revealed that from soils incubated after 35 d at different layer indicated fine reproducibility of the DGGE banding patterns (Fig 2). The DGGE band showed that no apparent change of archaea community structure in all soil treated, even in the soil amended with straw which had more than 30 times higher CH_4 production potential (Fig 1A).

Most clones were, revealed from the DGGE band, affiliated with a clone lineage of archaea, Rice Cluster I (RC-I). Other sequences belong to *Methanomicrobiales*, *Methanosaetales* and Rice Cluster 2 (RC-2) (Fig 3). All paddy field soil incubation exhibited a high frequency (70%) of sequences characteristic of hydrogenotrophic methanogens (*Methanocellales* (RC-I) and *Methanomicrobiales*), whereas the frequency of acetoclastic methanogenesis (*Methanosaetales* and *Methanosarcinales*) was as low as 10 %.

DISCUSSION

The production of CH_4 during incubation was much higher in flooded condition with straw amendment due to the pathway of CH_4 formation. This CH_4 production pattern is a typical CH_4 kinetics. This is similar to the results found in comparable experimental systems under continuous production for several

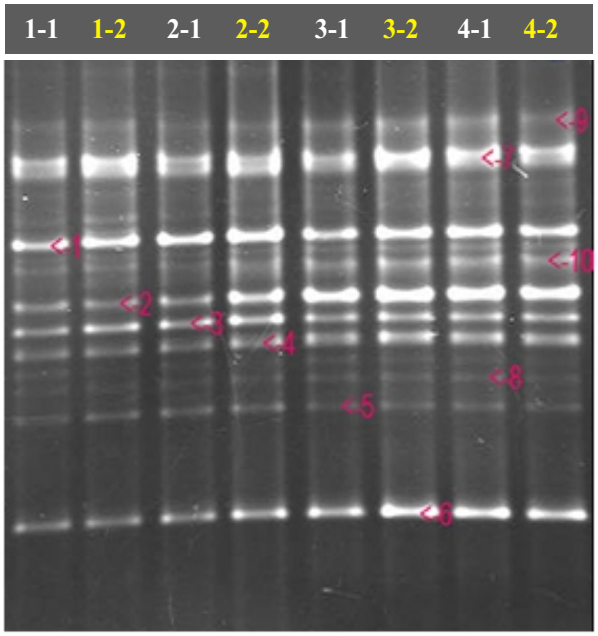

Fig 2 Denaturing Gradient Gel Elecrophoresis (DGGE) band patterns of PCR products with 0357F-GC and 0691R primer. Lane 1-1: Without straw saturated-surface soil; Lane 1-2: Without straw saturated-subsurface soil; Lane 2-1: Without straw flooded-surface soil; Lane 2-2: Without straw saturated-subsurface soil; Lane 3-1: Straw saturated-Surface soil; Lane 3-2: Straw saturated-Subsurface soil; Lane 4-1: Straw flooded -surface soil; Lane 4-2: Straw flooded -subsurface soil.

weeks as described previously (Frenzel *et al*. 1996; Yagi *et al*. 1997; Inubushi *et al*. 1992 and 2002). Flooded condition allows the soil environment to become anoxic, which is the required environmental condition for the methanogens to produce CH_4 by converting substrate acetate or H_2 plus CO_2.

Acetate or H_2 plus CO_2 are sub-products of various fermenting microorganisms that degrade straw. Therefore, the CH_4 production was not detected in soil incubated without straw even under flooded condition, while small amount of CH_4 production occurred during the early stages incubation in saturated soil condition with straw amendment, which then decreased to almost nil value at 35 d (Fig 1A). Hence, the incorporation of straw into the soil was the most important factor that influenced CH_4 production in this incubation experiment.

Conrad and Klose (2006) reported that the addition of straw enhanced the intermediate production of H_2 and acetate that served as the methanogenic substrates. Also some fatty acid was determined increased transiently upon straw addition. They also suggested that the copy number of bacteria and archaea 16S rRNA genes increased at the end of their experiment. In addition, under steady state condition, cellulose would methanogenically be degraded to 50% CO_2 + 50% CH_4, while methanogenesis from acetate and H_2 plus CO_2 theoretically contribute >67% and <33% to total CH_4 production, respectively (Conrad *et al*. 2002). Chin *et al*. (2003) suggested that CH_4 was initially produced from reduction of CO_2 resulting in the accumulation of acetate and at this stage the relative abundance of acetoclastic methanogens was low. Later on, the production of CH_4 was originated from acetate by 40% with relative increase of population of *Methanosarcina* spp.

Lueders and Freidrich (2000) observed that the population structure of methanogenic archaea remained remarkably constant over time after flooding in the paddy field soil even though they observed a shift in the activity of the *Methanosarcina*-like populations. Similar observation by Watanabe *et al*. (2006) showed that the community structure of methanogenic archaea in Japanese paddy field soil did not change throughout a year even at the mid-season drainage and upland cropping time. However, Sugano *et al*. (2005) suggested that methanogenic archaea communities involved in the decomposition of rice straw under flooded condition were affected by mid-season drainage irrespective of plant part on the rice straw (leaf sheath or leaf blade). In addition, using different sources of soil samples, Nicol *et al*. (2003) showed a change of the methanogenic archaea community in the managed grassland rhizosphere soil compared to the natural one after incubated for 28 d in anaerobic condition.

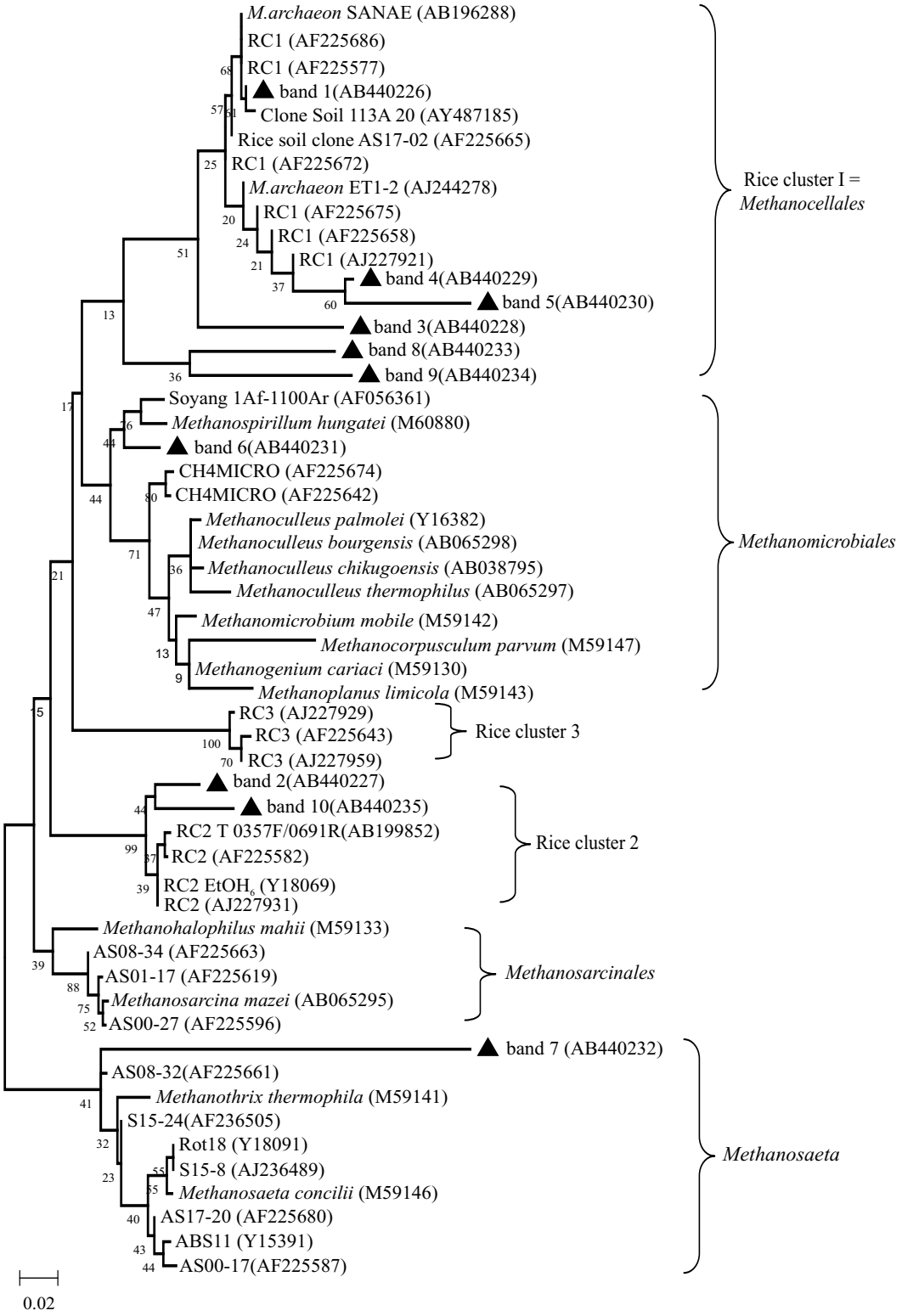

Fig 3 Phylogenetic tree based on partial nucleotide acid retrieved from the DGGE bands. Bands obtained from this study are marked with triangle shape (▲). Values in the figures are bootstrap values, and branching corresponding to partitions reproduced in less than 50% bootstrap replicated are collapsed. The scale of bar indicates 2 changes per 100 nucleotide acid.

The present work indicated that soil incubated at flooded or saturated condition with and without straw did not alter the community structure of methanogenic archaea at different layers of soil sampled. But, in another work that used the same soil DNA extraction, we determined a few changes of bacteria community structure by PCR-DGGE using pairs of universal bacteria primers of 341f/534r (data not shown).

The explanation of these results might be due to the soil chemical properties during incubation that have

favorable environment for the methanogenic archaea communities to survive under all the treatments used. Meanwhile, the fermenting microorganisms (dominantly bacteria) might have a shift of population during the straw degradation. Another possibility was the incubation time was not long enough to induce the appearance of other methanogenic population particularly acetoclastic methanogenesis type as suggested by Kruger *et al*. (2005).

The methanogenic archaea is also known to tolerate higher oxygen and have some enzymes to detoxify oxygen-related toxic compounds, hence, methanogens are proposed to be more resistant to O_2 (Lueders and Friedrich 2000; Erkel *et al*. 2006). The study also suggested that the production of CH_4 by methanogenic archaea seems not correlated with the band patterns but mainly due to the soil environments that enhanced methanogenesis such as in soil-flooded with straw amendments.

Similar to other reports, this study showed that hydrogenotrophic methanogens were dominant members in methanogenic archaea community retrieved from temperate paddy field soil (Lueders and Friedrich 2000; Sugano *et al*. 2005; Watanabe *et al*. 2006), while acetoclastic methanogens were relatively minor in abundance in the paddy field soil. We speculated that the members of community structure of hydrogenotrophic methanogens, *Methanocellales* (RC-I), was dominant in the soil used that took from the tropic region, of Indonesia.

The *Methanocellales* (RC-I) lineage has been found in almost every paddy field soil samples, particularly on rice roots and also detected dominant appeared in the prolonged incubation at 50 °C (Chin *et al*. 2003; Conrad *et al*. 2006; Wu *et al*. 2006). In addition, due to the capacities and a unique set of antioxidant enzyme and DNA repair, *Methanocellales* should have a competitive superiority over the other hydrogenotrophic methanogens in rice rhizosphere and other methanogenic environments with oxic episodes, such as boreal peat lands and tropic soils (Erkel *et al*. 2006).

More over Ramakrishnan *et al*. (2001) also reported that *Methanocellales* or RC-I occurred in such soils from various geographic regions. In addition, Lu and Conrad (2006) reported that *Methanocellales* (RC-I) were found to be highly active and play key role in CH_4 production from plant-derived carbon and, therefore, the group was responsible for microbial CH_4 emission from paddy field (Erkel *et al*. 2006).

Methanocellales (RC-I) has been identified as a different genotypic group from the orders of *Methanosarcinales* and *Methanomicrobiales* due to low sequence similarity value with those of the two aforementioned orders (Großkopf *et al*. 1998). The physiological properties of this members is also not similar with members of the *Methanosarcinales* and *Methanomicrobiales*, which utilize acetate and simple methylated compound, while this member of *Methanocellales* (RC-I) use H_2 plus CO_2 to produce CH_4 (Conrad 1996; Erkel *et al*. 2006; Sakai *et al*. 2008).

Two bands (band 2 and 10) were revealed as rice cluster 2 (RC-2). The RC-2 were placed between the orders *Methanosarcinales* and *Methanomicrobiales* (Fig 3), where it has also been reported by Großkopf *et al*. (1998) based on the level of rDNA similarity to these orders. Only 1 band of each member of *Methanosaetales* and *Methanomicrobiales* was retrieved from DGGE as band 7 and band 6, respectively, while no member of *Methanosarcinales* or Rice cluster 3 were identified from DGGE band (Fig 2), Großkopf *et al*. (1998) and Fey and Conrad (2000) reported that *Methanosaetales* was hardly detected in roots but commonly appeared in anoxic bulk soil, especially at low acetate concentration. Since the paddy field soils used was taken as a bulk soil before transplanting time, it is also possible that member of *Methanosaetales* was detected in soil used.

N_2O gas is emitted in paddy soils via nitrification and denitrification or nitrate accumulated when soils have an aerobic surface layer and anaerobic condition, respectively (Akiyama *et al*. 2005; Inubushi *et al*. 1996; Jumadi *et al*. 2008). The main pathway of N_2O production from paddy-soil system depends on the soil water status. Therefore, current study also shows that N_2O production in almost neglected monitored even in closed system experiment. In addition, at saturated condition the N_2O released by denitrification increase with decreasing O_2 partial pressure. Therefore, after five days incubation N_2O production at control flooded was monitored higher and then decrease thereafter substantially to lower concentration. That might be due to the availability of NO_3^- and reduced O_2 concentration.

The study also showed that N_2O emissions were suppressed in all the treatments and it could be predicted that N_2O emission was almost not existent. The field scale studies in the tropical region of Indonesia showed that N_2O was almost not emitted. However, in incubation experiment the N_2O production by nitrification occurred even in moist soil (50-70% of soil moisture) and it was also influenced by the number of responsible microbe such as ammonium oxidation

bacteria and nitrite oxidation bacteria (Hadi *et al*. 2008; Inubushi *et al*. 2003; Jumadi *et al*. 2005; Jumadi *et al*. 2008b; Jumadi *et al.* 2012).

The study showed that the hydrogenotrophic methanogens were dominant members in methanogenic archaea community obtained from tropical paddy field soil of south Sulawesi Indonesia, while, acetoclastic methanogens were relatively minor presence in the soil sample. Study also suggested that N_2O production almost neglected in paddy soil with saturated condition.

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