Changes in community structure and transcriptional activity of methanogenic archaea in a paddy field soil brought about by a water-saving practice – Estimation by PCR-DGGE and qPCR of 16S rDNA and 16S rRNA

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Abstract
Effect of a water-saving practice on the methanogenic archaeal community in a paddy field soil of the International Rice Research Institute (IRRI) Farm was investigated by PCR-DGGE and qPCR targeting 16S rDNA and 16S rRNA. Plow-layer soil samples were periodically collected from field plots under the treatments of continuously flooding (Control) and an alternate wetting and drying (AWD) water-saving during a rice cultivation period in 2008. Both DGGE band patterns of 16S rDNA and 16S rRNA were relatively stable throughout the rice cultivation period irrespective of the treatments. Cluster analysis showed a tendency that the patterns of the AWD plots were separated from those of the Control plots. Principal component analysis and sequencing analysis of 16S rDNA indicated members of *Methanosarcinales* and *Methanocellales* mainly characterized the Control and AWD plots, respectively. Numbers of 16S rDNAs significantly fluctuated during the rice cultivation period and differed between the Control and AWD plots. Those of 16S rRNAs decreased in the AWD plots in the last half of the rice cultivation period although the fluctuations were not significant. These results suggest that the water-saving management brings about changes in both community structures and transcriptional activities of methanogenic archaea in paddy field soil.

Key Words
Methanogenic archaea, Paddy field soil, Water-saving irrigation, PCR-DGGE, qPCR

Introduction
Water scarcity caused by climate changes, growth of population, etc. is one of the serious problems in the world. In the International Rice Research Institute (IRRI), alternate wetting and drying (AWD) irrigation technique (Bouman *et al.* 2007) has been developed to reduce water use without any adverse effects on rice production. The AWD technique is a kind of intermittent irrigation management and enables reduction by 15-20% of irrigation water (Tabbal *et al.* 2002; Belder *et al.* 2004) and 35-45 % of annual methane emission (Hosen *et al.* unpublished data) from paddy fields, compared with a conventional continuous flooding water management. Methane is one of the greenhouse gases and paddy fields are known as a major source of atmospheric methane. Methanogenic archaea play a unique role for biological methane production in paddy fields. In relation to the reduction of methane emission by the AWD managements, it is conceivable that changes in community structure and metabolic activity of methanogenic archaea influenced the methane emission from AWD paddy fields. In the present study, therefore, the effect of AWD management on the methanogenic archaeal community in a paddy field soil was evaluated by molecular ecological techniques (PCR-DGGE and qPCR) targeting their 16S rDNA and 16S rRNA.

Materials and methods
Investigated field
The experiment was conducted at the IRRI Farm, Los Baños, Philippines (14°30'N, 121°1'E). The soil (organic C, 1.68 %; total N, 0.17 %; pH 7.0) was a clayey loam Aquandic Epiaquoll (Dobermann *et al.* 2000). Two treatments with three replication plots were chosen: continuous flooding (Control) plots and AWD water-saving plots. The Control plots were basically kept under flooded condition during the rice cultivation period. Irrigation of the AWD plots was basically carried out when soil water potential at 15 cm depth reached –20 kPa except for the early growth and heading periods of rice when the fields were kept under a flooded condition.

Soil sampling
Soil samples were collected four times during the rice cultivation period in the wet season in 2008: 15 July (1st, 15 days after transplanting [DAT]), 12 August (2nd, 33 DAT), 8 September (3rd, 60 DAT) and 9 October (4th, 91 DAT). The 1st sampling was carried out when the both Control and AWD plots were under a flooded condition. The 2nd-4th samplings were carried out when the depth of water decreased to 0 cm (i.e. soil surface) in two of three replicated plots as it was predicted that methanogenic activities in the AWD paddy soils would reach a peak after soil submergence. Soil samples were collected from 3-13 cm depth of the plow-layer soil. In total, 600 ml of soil sample was collected from three spots in each replication and composited in a clean plastic bag. The collected soils were immediately brought back to the laboratory and mixed well in the plastic bags. The soil samples were stored in -20 ºC and -80 ºC freezers for DNA and RNA extraction, respectively.

Molecular ecological analysis of methanogenic archaeal community
DNA and RNA were separately extracted from the soil samples by the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH USA) and following the procedure described by Watanabe *et al.* (2007), respectively. cDNA was synthesized from extracted RNA by reverse transcription reaction using SYBR PrimeScript RT reagent kit (TaKaRa, Shiga, Japan). PCR-DGGE using the primers 1106F-GC/1378R and subsequent multivariate analyses (cluster analysis and principal component analysis [PCA]) were carried out, as described by Watanabe *et al.* (2006). Numbers of methanogenic archaeal 16S rDNAs and 16S rRNAs were determined by qPCR analysis using the LightCycler (Roche Diagnostics, Basel, Switzerland), as described by Watanabe *et al.* (2007). Nucleotide sequences of 16S rDNA fragments recovered from the DGGE bands were determined by the direct sequencing method as described Watanabe *et al*. (2004). Phylogenetic affiliations of the sequences were determined by the BLAST program on the DDBJ web site.

Results

PCR-DGGE analysis of methanogenic archaeal 16S rDNA and 16S rRNA
Figure 1 shows DGGE band patterns of methanogenic archaeal 16S rDNA and 16S rRNA retrieved from the soils in the Control and AWD plots. Twenty-seven bands were observed at different positions in the both DGGE band patterns. The numbers of bands fluctuated between 17 and 24 and 17 and 22 among the patterns of 16S rDNA and 16S rRNA, respectively. However, major bands with strong intensity were commonly observed in both DGGE band patterns irrespective of the water management.

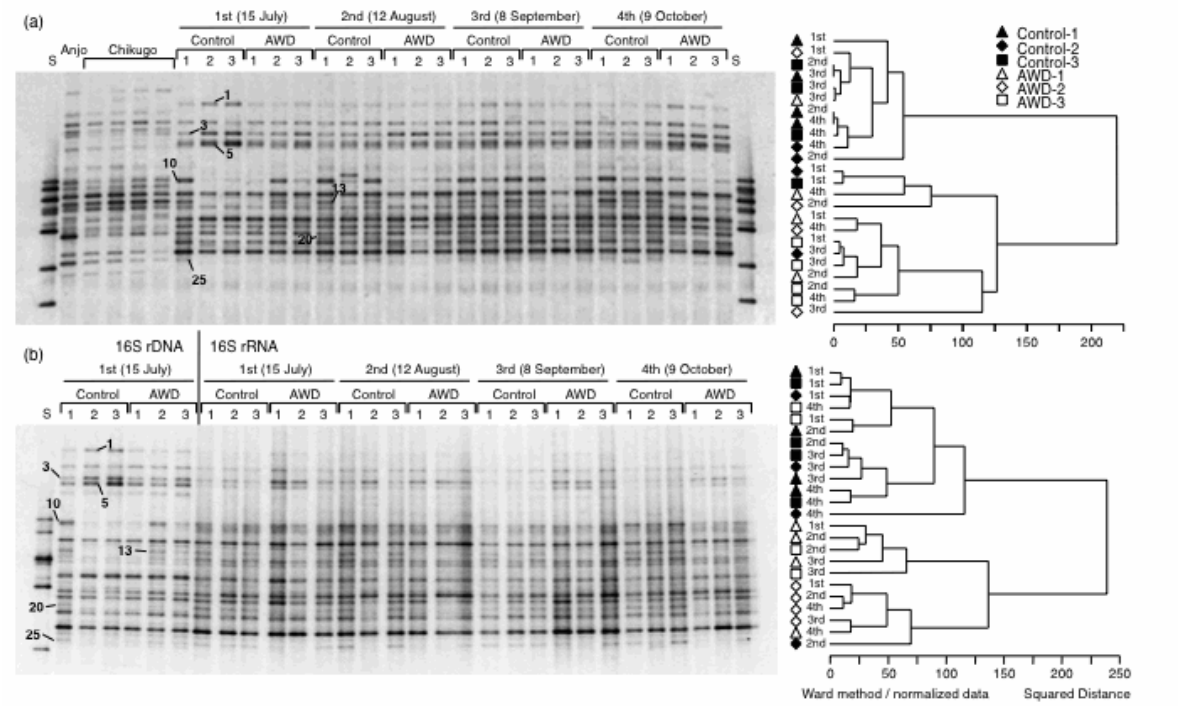

Figure 1. DGGE band patterns and cluster analysis of methanogenic arcaheal 16S DNA (a) and 16S rRNA (b) obtained from the Control and AWD paddy field soils at IRRI. Anjo and Chikugo show the patterns obtained from Japanese paddy field soils, where a previous investigation was performed (Watanabe *et al.* 2006). S is a mixture of PCR amplicons derived from 13 strains of methanogenic archaea. The denaturant gradient range was 32 to 62 %.

Cluster analysis and PCA were carried out, based on relative intensity and mobility of the DGGE bands. Both analyses of the DGGE band patterns of 16S rDNA and 16S rRNA showed a tendency for the Control and AWD samples to cluster separately although the patterns obtained from the 1st sampling did not show uniformity. These findings suggest that the community structures and transcriptional activities of methanogenic archaea in the both Control and AWD plots changed during the rice cultivation period, but the changes were different between the Control and AWD plots. The DGGE bands characterizing the Control and AWD plots were determined from the coefficient of PCA (Figure 1; the bands 10, 13, 20 and 25 for the Control and 1, 3, and 5 for the AWD plots).

Quantification of methanogenic archaeal 16S rDNA and 16S rRNA

Numbers of methanogenic archaeal 16S rDNAs in the Control and AWD plots fluctuated between 1.1×10^8 and 5.6×10^8 and 6.8×10^7 and 4.5×10^8/g dry soil, respectively (Figure 2). Those of 16S rRNAs in the Control and AWD plots ranged from 4.4×10^8 to 8.4×10^8 and 7.7×10^7 to 9.5×10^8/g dry soil, respectively. Although the maximum numbers of 16S rDNAs were almost same between the Control and AWD plots, the numbers in the AWD plots gradually increased and rapidly decreased. The numbers of 16S rDNAs were significantly different between the sampling dates ($P < 0.001$) and between the Control and AWD plots ($P < 0.05$). A mutual influence between the sampling date and water management was also observed ($P < 0.05$). The numbers of 16S rRNAs in the Control plots did not fluctuate throughout the rice cultivation period, while those in the AWD plots tended to decrease in the last half of the rice cultivation period although the differences were not significant. These results indicate that the AWD management repressed proliferation and metabolic activity of methanogenic archaea, compared with the Control plots. The repressions may partly contribute to the reduction of methane emission from the AWD paddy fields.

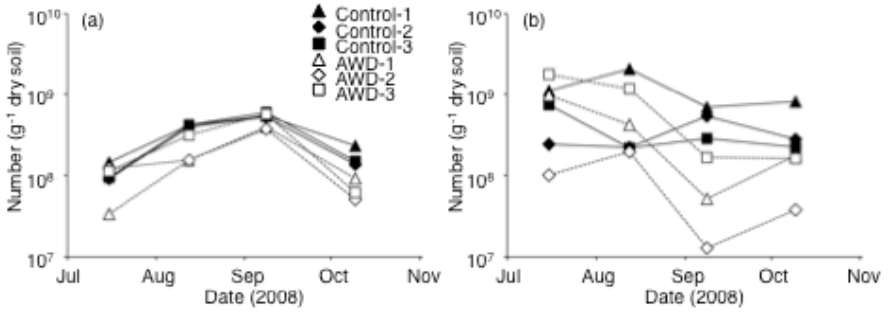

Figure 2. Number of methanogenic archaeal 16S rDNAs and 16S rRNAs in the Control and AWD paddy field soils during the rice cultivation period of wet season 2008 (means of each replication plot, n = 3).

Phylogenetic affiliation of methanogenic archaeal 16S rDNA

In total, 26 DGGE bands were successfully sequenced from the band patterns of 16S rDNA. Those sequences were affiliated with *Methanobacterium* spp., *Methanosarcina* spp., *Methanosaeta* spp., uncultured group of ZC-I in *Methanosarcinales*, uncultured group of *Methanomicrobiales*, *Methanocellales* (formerly, Rice cluster I) and Crenarchaeota. Sequences of the DGGE bands, which characterized the Control plots (bands 10, 13, 20 and 25 in Figure 1), were closely related to members of *Methanosaeta* spp. (band 10) and the ZC-I cluster (bands 13, 20 and 25). It is known that *Methanosaeta* spp. use only acetate for methanogenesis (Garcia *et al.* 2000). The members in ZC-I cluster are enriched in the Zoige wetland of the Tibetan plateau (Zhang *et al.* 2008) and are assumed to use acetate, H_2/CO_2, methanol and trimethylamine as substrates for methanogenesis. These findings suggest that acetoclastic methanogenic archaea and methanogenesis became more dominant in the Control paddy fields during the rice cultivation period. Previous studies investigating *mcrA* genes and their transcripts in a Japanese paddy field soil also showed uncultured members of *Methanosarcinales* actively transcribed *mcrA* genes under a flooded condition (Watanabe *et al.* 2009). On the other hand, sequences characterizing the AWD plots (bands 1, 3 and 5 in Figure 1) were affiliated with members of *Methanocellales* (band 3) and uncultured Crenarchaeota (bands 1 and 5). Although all methanogenic archaea hitherto isolated are strictly anaerobic microorganisms, it has been estimated from genome information that a member of *Methanocellales* possess multiple sets of genes encoding antioxidant enzymes (Erkel *et al.* 2006). Previous study showed that *mcrA* transcripts derived from *Methanocellales* were preferentially recovered from a Japanese paddy field soil under unflooded condition (Watanabe *et al.* 2009). Therefore, these members might be relatively resistant to the oxic condition in the AWD paddy field. Members of *Methanocellales* are known as hydrogenotrophic methanogenic archaea. Population and transcription activity of methanogenic archaea increased gradually in the AWD plots,

compared with the Control plots, suggesting acetoclastic methanogenic archaea could not actively proliferate and produce methane in the AWD plots. These findings indicated that the AWD management changed the methanogenic pathway (acetate vs. H_2/CO_2) in the paddy field soil.

Conclusion

DGGE band patterns of methanogenic archaeal 16S rDNA and 16S rRNA obtained from the AWD paddy field soil were relatively stable through the rice cultivation period, but multivariate analyses showed a tendency for patterns differ between the Control and AWD plots. The numbers of methanogenic archaeal 16S rDNAs fluctuated during the rice cultivation period and differed between the Control and AWD plots. Those of 16S rRNAs in the AWD plots tended to decrease in the last half of the rice cultivation. Phylogenetic analysis indicated that methanogenic pathway differently changed depending on the Control and AWD plots. These results suggest that AWD water-saving management moderately brings about changes in community structure (composition and population) and transcriptional activities of methanogenic archaea and the changes partly contribute to the reduction of methane emitted from the paddy field.

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