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Characterization of the Bacterial and Archaeal Communities in Rice Field Soils Subjected to Long-Term Fertilization Practices[§]

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The bacterial and archaeal communities in rice field soils subjected to different fertilization regimes for 57 years were investigated in two different seasons, a non-planted, drained season (April) and a rice-growing, flooded season (August), by performing soil dehydrogenase assay, real-time PCR assay and pyrosequencing analysis. All fertilization regimes increased the soil dehydrogenase activity while the abundances of bacteria and archaea increased in the plots receiving inorganic fertilizers plus compost and not in those receiving inorganic fertilizers only. Rice-growing and flooding decreased the soil dehydrogenase activity while they increased the bacterial diversity in rice field soils. The bacterial communities were dominated by *Chloroflexi*, *Proteobacteria*, and *Actinobacteria* and the archaeal communities by *Crenarchaeota* at the phylum level. In principal coordinates analysis based on the weighted Fast UniFrac metric, the bacterial and archaeal communities were separated primarily by season, and generally distributed along with soil pH, the variation of which had been caused by long-term fertilization. Variations in the relative abundance according to the season or soil pH were observed for many bacterial and archaeal groups. In conclusion, the microbial activity, prokaryotic abundance and diversity, and prokaryotic community structure in the rice field soils were changed by season and long-term fertilization.

Keywords: rice field, bacteria, archaea, community, long-term fertilization, pyrosequencing

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Introduction

Rice is one of the most important food crops, as more than a billion people depend on its cultivation for their livelihoods and more than 3.5 billion people depend on rice for more than 20% of their daily calories (http://www.irri.org). In 2010, the production of rice amounted to 672 million tons, about 90% of which were produced from Asia (http://faostat.fao.org). Rice field soil is also important as a major source of atmospheric methane (CH_4), greenhouse gas with much higher global warming potential than carbon dioxide (CO_2) (IPCC, 2007).

Microorganisms in rice field soil are important with respect to rice growing and greenhouse gas emission. They influence rice growth directly through symbiosis or indirectly through nutrient cycling and emit greenhouse gases during sequential reduction processes initiated by the flooding of rice field soil (Liesack *et al.*, 2000; Kögel-Knabner *et al.*, 2010).

Rice field soil provides a unique environment for soil microbial residents through two anthropogenic activities: periodic flooding/drainage and fertilizer applications. The redox potential oscillation caused by periodic flooding and drainage has a significant influence on the soil microbial community, their metabolism, and thus short-term biogeochemical processes (Bossio and Scow, 1995; Noll *et al.*, 2005; Kögel-Knabner *et al.*, 2010). Fertilizer applications provide excess nitrogen and organic matter to soil microorganisms, affecting soil microbial abundance, activity, and community structure (Marschner *et al.*, 2003; Chu *et al.*, 2007; Nemergut *et al.*, 2008; Ahamadou *et al.*, 2009; Shen *et al.*, 2010; Islam *et al.*, 2011; Wu *et al.*, 2011; Chen *et al.*, 2012). Recent studies have shown that long-term agricultural practices change the bacterial community structure and their function in rice field soils (Bannert *et al.*, 2011; Cui *et al.*, 2012). Rice root also has significant influences on soil microorganisms via root exudates and the supply of oxygen (Liesack *et al.*, 2000).

Many studies have been conducted to understand the effects of these factors on the soil bacterial communities in rice field soils (Lüdemann *et al.*, 2000; Lu *et al.*, 2006; Kikuchi *et al.*, 2007; Watanabe *et al.*, 2010; Wu *et al.*, 2011). Although these studies have provided useful information about the global changes caused by these factors, the techniques used for bacterial community analyses (16S rRNA gene clone library analysis and fingerprinting methods) are thought to be insufficient for surveying the full extent of soil bacterial diversity (Roesch *et al.*, 2007; Bartram *et al.*, 2011) and unable to provide detailed information about the variation of individual bacterial groups. For archaea communities in rice field soil, there are almost no comprehensive studies on understanding their structures (Lueders and Friedrich,

2000), although there have been studies on specific functional groups such as methanogens (Conrad *et al*., 2009; Wang *et al*., 2010) or nitrifiers (Wang *et al*., 2009).

In this study, we investigated the bacterial and archaeal communities in bulk soil of rice fields that were subjected to different fertilization regimes since 1954. The metabolic activity, abundance, and community structure were examined, and the effects of season and fertilization regimes were analyzed via a soil dehydrogenase assay, real-time PCR assay, and high-throughput pyroseqencing analysis. The results of this study may provide more insight into the effects of water management and fertilization on soil bacterial and archaeal communities as well as subsequent effects on nutrient cycling and climate change.

Materials and Methods

Soil sampling, chemical characterization, and DNA extraction

The sampling site was located at the experimental rice field of the National Institute of Crop Science, Suwon, Korea (37°16′ N, 126°59′ E). The long-term fertilization experiment started in 1954, and 32 different combinations of ammonium sulfate (symbolized by A, 110 kg as N ha^{-1} $year^{-1}$), urea (symbolized by U, 110 kg as N ha^{-1} $year^{-1}$), fused and superphosphate (symbolized by P, 70 kg as P_2O_5 ha^{-1} $year^{-1}$), potassium chloride (symbolized by K, 80 kg as K_2O ha^{-1} $year^{-1}$), calcium hydroxide (symbolized by L, enough to adjust pH to 6.5, 0.39–1.17 Mg ha^{-1} $year^{-1}$), silicate fertilizer (symbolized by W, 2 Mg ha^{-1} $year^{-1}$), and a rice straw compost (symbolized by C, 7.5 Mg ha^{-1} $year^{-1}$) were applied to the experimental plots (6.3 m×8.3 m each), in which rice was cultivated as a single crop (Yeon *et al*., 2007; Suh *et al*., 2009). The following 6 treatments were selected in this study as the representatives of the 32 combinations: inorganic NPK fertilizer (APK), inorganic NPK fertilizer+organic fertilizer (CAPK), inorganic NPK fertilizer+neutralizing agent (LAPK), inorganic NPK fertilizer+silicate fertilizer (WAPK), the combination of all the fertilizers (CLWAPK), and no fertilizer (NF). Soil samples were collected in a non-planted, drained season (April) and a rice-growing, flooded season (August) in 2011. Five soil cores (diameter, 1.8 cm) were collected from the upper layer (0–15 cm) of each plot, mixed thoroughly, passed through a 2-mm sieve, and stored at -70°C until further molecular analyses. For chemical analysis, soil samples were air dried at room temperature in the shade and sieved through a 2-mm screen. Chemical properties of soil samples were analyzed as follows: pH and EC (1:5 water extraction), organic matter (Wakely and Black method) (Allison, 1965), total nitrogen (Kjeldahl method), available P_2O_5 (Lancaster method) (NIAST, 2000), contents of exchangeable Ca^{2+}, Mg^{2+}, and K^+ (1 M NH_4-acetate pH 7.0, ICP-AES; GBC Integra-XMP, Australia). Soil dehydrogenase activities were determined according to the method of Casida *et al.* (1964). Soil DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, USA), according to the manufacturer's instructions.

Quantification of bacterial and archaeal 16S rRNA genes by real-time PCR

Bacterial and archaeal 16S rRNA genes were quantified in duplicate by using the CFX96 Real-Time PCR detection system (Bio-Rad, USA). The reaction mixture (20 μl) contained 1×iQ SYBR green Supermix (Bio-Rad), 400 nM of each primer, 1 mg/ml of bovine serum albumin (Sigma-Aldrich, USA), and 1 μl of 1/100 diluted DNA. Bacterial assays used primers 338f (Muyzer *et al*., 1993)/V3-541R (Chun *et al*., 2010) and the following thermal program: 94°C for 10 min, 35 cycles of denaturing (15 sec at 94°C), annealing (30 sec at 57°C), extension (30 sec at 72°C), and plate reading (10 sec at 80°C). Archaeal assays used primers 931f (Einen *et al*., 2008)/A1041R (Kolganova *et al*., 2002) and the following thermal program: 94°C for 10 min, 40 cycles of denaturing (15 sec at 94°C), annealing (30 sec at 58°C), and extension/plate reading (30 sec at 72°C). A standard curve was constructed with plasmids containing the 16S rRNA gene of either *Bacillus subtilis* KACC 10854^T for bacteria or that of *Halococcus dombrowskii* KACC 16462^T for archaea.

Statistical analysis

To determine whether season and fertilization regimes affected the soil dehydrogenase activity and the abundances of bacteria and archaea in the rice field soils, statistical significance was tested with ANOVA in the SigmaPlot v.11.0 software (Dundas Software LTD, Germany) using the Holm-Sidak method ($p<0.05$).

Bacterial and archaeal 16S rRNA gene PCR and pyrosequencing

The 50-μl reaction mixture contained 1× PCR buffer (Roche, Germany), 0.2 mM of each deoxynucleoside triphosphates (dNTPs), 400 nM of each primer, 1 mg/ml of bovine serum albumin (Sigma-Aldrich), 1.25 units of *taq* DNA polymerase (Roche, Germany), and 1 μl of 1/10 diluted DNA. PCR amplification was performed using the following touchdown PCR protocol: initial denaturation (5 min at 94°C), 10 cycles at 94°C for 30 sec, 60°C for 45 sec, and 72°C for 90 sec (the annealing temperature was reduced by 0.5°C/cycle from the preceding cycle), and 20 cycles at 94°C for 30 sec, 55°C for 45 sec, and 72°C for 90 sec. Bacterial 16S rRNA genes were amplified using the primers V1-9F (5′-X-AC-GAGTT TGATCMTGGCTCAG-3′) and V3-541R (5′-X-AC-WTTA CCGCGGCTGCTGG-3′) (Chun *et al*., 2010), and archaeal 16S rRNA genes were amplified using the primers 4F (5′-X-AG-TCCGGTTGATCCTGCCRG-3′) (Kendall *et al*., 2007) and AV3-519R (5′-X-AG-GGTDTTACCGCGGC KGCTG -3′) (Hur *et al*., 2011), where X denotes a 7–11 nucleotide long barcode followed by a common linker AC for bacteria or AG for archaea. The PCR products were gel-purified with the QIAquick Gel extraction kit (QIAGEN, Germany) and subjected to pyrosequencing, which was performed either by Chunlab, Inc (Seoul, Korea) or National Instrumentation Center for Environmental Management (Seoul, Korea) using a 454 GS FLX Titanium Sequencing System (Roche), according to the manufacturer's instructions.

Processing of pyrosequencing data

Pyrosequencing data were analyzed using the Mothur software package (version 1.23.1) (Schloss *et al.*, 2009). Briefly, pre-filtered flowgrams of the pyrosequencing reads were clustered using the PyroNoise algorithm (Quince *et al.*, 2011), and chimeric sequences were removed using UCHIME (Edgar *et al.*, 2011) and DECIPHER (http://decipher.cee.wisc.edu) (Wright *et al.*, 2012). The resulting sequences were classified up to a genus level using the Bayesian method with a bootstrap cut-off value of 80%. The taxonomic classifications of bacterial and archaeal were performed based on the silva taxonomy (Pruesse *et al.*, 2007) and greengenes 2011 taxonomy (McDonald *et al.*, 2012), respectively. Although the greengenes taxonomy included *Thaumarchaeota* in the *Crenarchaeota* phylum (McDonald *et al.*, 2012), we separated this archaeal group as a new phylum as previously suggested (Brochier-Armanet *et al.*, 2008). Sequences assigned to the mitochondria or chloroplasts were removed from the subsequent analyses. Qualified sequences from each sample were merged, and the pair-wise distances were calculated using the "pairwise.seqs" command in the Mothur package, which aligns two sequences using the Needleman algorithm. Sequences were then clustered into operational taxonomic units (OTUs) at the cut-off of 97% similarity using the furthest-neighbor algorithm.

Principal coordinates analysis (PCoA) based on the Fast UniFrac metric

Qualified sequences were imported into the ARB software package (version 5.2) (Ludwig *et al.*, 2004) and aligned by using the SINA aligner (version 1.1) (Pruesse *et al.*, 2012) and the SILVA 16S rRNA database (SSURef-108) (Pruesse *et al.*, 2007). A phylogenetic tree was constructed using FastTree (Price *et al.*, 2009), and the resulting tree and the abundance data were imported into the Fast UniFrac environment (http://bmf.colorado.edu/Fast UniFrac/) (Hamady *et al.*, 2009). Weighted Fast UniFrac distances between the samples were calculated and principal coordinates analysis (PCoA) was performed on the basis of the distance measures.

Phylogenetic analysis

Representative sequences for dominant bacterial and archaeal OTUs and closely related sequences in GenBank were aligned as mentioned above. Almost full-length sequences (≥ 1,300 bases) were used for the construction of the initial tree via the maximum-likelihood algorithm (Phylip) and positional variability filter provided by the ARB package. Shorter sequences (< 1,300 bases) were added to this tree using the ARB parsimony tool, which allowed the addition of short sequences to phylogenetic trees without changing global tree topologies (Ludwig *et al.*, 1998). The alignments of almost full-length sequences were exported to the MEGA program (version 5.0) (Tamura *et al.*, 2011) and bootstrap values were calculated using the maximum-likelihood algorithm. The relative abundances of dominant OTUs in each sample were represented as a heat map, which was produced with the iTOL tool (http://itol.embl.de) (Letunic and Bork, 2011).

DNA sequence data

The raw pyrosequencing data are available in the NCBI Sequence Read Archive under the accession number SRP-013887.

Results and Discussion

The chemical properties of rice field soils

Long-term fertilization significantly changed the soil chemical properties of rice field soils (Table 1). The soil pH of the non-fertilized plot (NF) was either 6.1 or 6.4, whereas the addition of inorganic NPK fertilizer or inorganic NPK fertilizer + organic fertilizer (APK and CAPK) decreased the soil pH to 5.7–5.8. On the other hand, the addition of lime or silicate fertilizer (LAPK, WAPK, and CLWAPK) increased the soil pH to 6.2–7.4. These observations agreed with those reported in the previous studies on the same site (Yeon *et al.*, 1997). The decrease in soil pH by the long-term addition of inorganic nitrogen was previously reported (Šimek and Hopkins, 1999; Enwall *et al.*, 2005; Yao *et al.*, 2011) and was

Table 1. Characterization of the rice field soils used in this study

Sample[a]	pH (1:5)	Organic matter (g/kg)	Ave. P_2O_5 (mg/kg)	Ex. cations ($cmol_c$/kg)		
				K	Ca	Mg
Apr-NF	6.4	19	23.0	0.30	4.5	0.8
Apr-APK	5.8	22	152	0.32	4.4	0.8
Apr-CAPK	5.7	32	162	0.29	4.9	1.0
Apr-LAPK	6.4	23	127	0.24	5.2	1.3
Apr-WAPK	6.9	22	132	0.30	7.0	1.0
Apr-CLWAPK	7.4	31	163	0.28	9.0	1.5
Aug-NF	6.1	25	15.6	0.10	3.6	0.7
Aug-APK	5.7	28	144	0.04	3.5	0.7
Aug-CAPK	5.7	38	166	0.07	3.7	0.7
Aug-LAPK	6.2	30	126	0.06	3.5	1.0
Aug-WAPK	6.5	29	139	0.05	4.7	0.7
Aug-CLWAPK	7.0	41	192	0.10	5.9	1.0

[a] Symbols: NF, non-fertilized; A, ammonium sulfate; P, fused and superphosphate; K, potassium chloride; C, compost; L, calcium hydroxide; and W, silicate fertilizer.

supposed to occur due to the combined effects of fertilizer application and the increased production of acids by nitrification (van Breemen *et al.*, 1982) and fermenting bacteria. The addition of compost, phosphorus and lime increased the corresponding properties of rice field soils. Rice grain yields were 2.45 Mg/ha (NF), 5.94 Mg/ha (APK), 7.76 Mg/ha (CAPK), 5.97 Mg/ha (LAPK), 6.32 Mg/ha (WAPK), and 7.21 Mg/ha (CLWAPK), indicating that the grain yield was increased by fertilization, particularly by compost.

Microbial activity and prokaryotic abundance in rice field soils

Soil dehydrogenase activity represents the total metabolic activity of soil microorganisms. All fertilization regimes increased soil dehydrogenase activity in April as well as in August (Fig. 1), indicating the prolonged effect of fertilization on soil microbial activity. These results corroborate previously reported results (Chu *et al.*, 2007; Islam *et al.*, 2011). With the exception of the LAPK plot, soil dehyrogenase activity was significantly lower in August compared to that in April for each fertilization treatment (Fig. 1). This is contrary to earlier reports, in which flooding and anaerobiosis increased soil dehydrogenase activity (Ross, 1971; Pedrazzini and McKee, 1984; Subhani *et al.*, 2001), and seems to be due to the decrease in the density of prokaryotes in the rice field soils in August compared to in April, as indicated below.

The numbers of bacterial and archaeal 16S rRNA genes in 1 g of rice field soils ranged from 1.4×10^{10}–2.9×10^{10} and 5.4×10^{8}–1.7×10^{9}, respectively. A significant increase ($P<0.05$) was observed in the plots receiving the compost application (CAPK and CLWAPK) for each season, with the exception of archaea in the CLWAPK plot in April (Figs. 2A and 2B), indicating that organic matter is the most important limiting factor in these rice field soils. The increase in microbial abundance by the addition of organic fertilizer was also observed in previous studies, but the results were variable for the addition of inorganic fertilizer (Bittman *et al.*, 2005; Chu

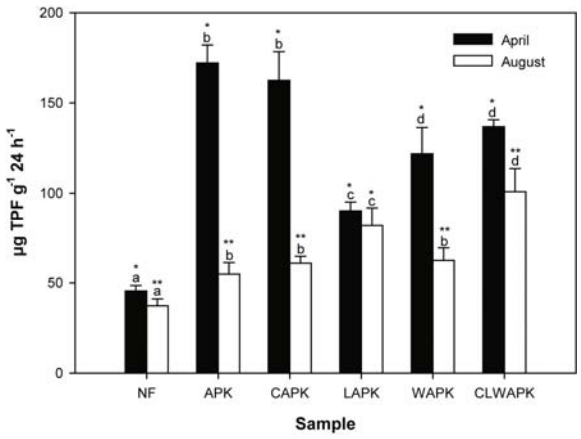

Fig. 1. Dehydrogenase activities in rice field soils. The averages of three or four replicates are presented with standard deviations. For a given season and fertilization regime, significant differences ($p<0.05$) were determined using the Holm-Sidak test and *t* test, respectively, and indicated as different lowercase letters and symbols, respectively. Refer to Table 1 for the symbols used to define each sample.

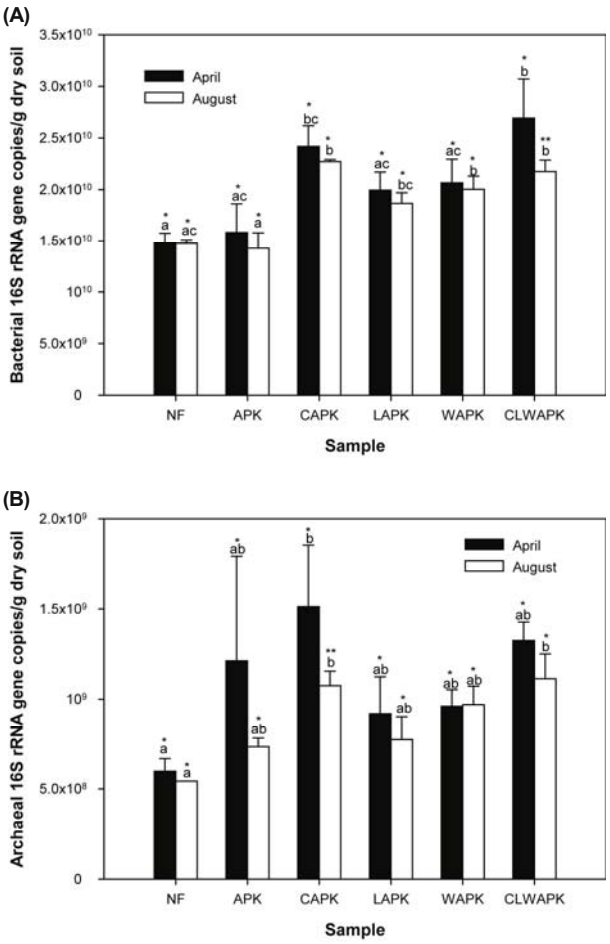

Fig. 2. Copy numbers of bacterial (A) and archaeal (B) 16S rRNA genes in rice field soils. The averages of duplicate measurements are presented with standard deviations. For a given season and fertilization regime, significant differences ($p<0.05$) were determined using the Holm-Sidak test and *t* test, respectively, and indicated as different lowercase letters and symbols, respectively. Refer to Table 1 for the symbols used to define each sample.

et al., 2007; Shen *et al.*, 2010; Wu *et al.*, 2011). The abundances of bacterial and archaeal 16S rRNA genes decreased in August compared to those in April for each fertilization treatment although significant differences ($P<0.05$) were observed only in the CLWAPK plot for bacteria and the CAPK plot for archaea.

The decreases in the dehydrogenase activity and prokaryotic abundance in August compared to in April were unexpected considering the increases in organic matter (Table 1) and temperature in August. Microbial metabolism using organic residue seems to be as active in April as in August in the rice field soils and aerobic soil condition in April might increase the total microbial activity compared to in August.

Summary of pyrosequencing data

On average, 4,112 bacterial and 4,130 archaeal pyrosequencing reads of the 16S rRNA gene were obtained from a soil sample (Table 2). After chimera removal, 2,265 bacterial and 2,954 archaeal reads remained, indicating a high occurrence

Table 2. Summary of the pyrosequencing data obtained from rice field soils. (A) bacteria and (B) archaea

	Sample [a]	Number of reads		Number of OTUs[b]	Good's coverage[b]	Richness estimator[b]		Diversity index[b]	
		Raw	Chimera removed			Chao1	ACE	Shannon	Inverse simpson
(A)	Apr-NF	5,793	3,591	1,634	0.72	3,400	4,898	6.9	533
	Apr-APK	5,709	2,938	1,699	0.58	4,739	8,286	7.1	814
	Apr-CAPK	6,707	3,922	1,737	0.74	3,465	4,720	7.0	521
	Apr-LAPK	3,879	2,495	1,170	0.72	2,270	3,323	6.7	511
	Apr-WAPK	2,900	1,865	895	0.70	1,909	2,705	6.4	427
	Apr-CLWAPK	6,783	3,873	2,095	0.62	5,325	8,979	7.2	641
	Aug-NF	2,912	1,439	1,061	0.41	3,509	6,047	6.8	1,402
	Aug-APK	3,115	1,631	1,165	0.44	3,688	6,114	6.9	1,188
	Aug-CAPK	1,566	681	542	0.34	2,131	2,102	6.2	1,135
	Aug-LAPK	2,029	1,011	780	0.36	3,140	5,771	6.5	1,082
	Aug-WAPK	4,150	1,903	1,397	0.41	4,632	8,365	7.1	1,247
	Aug-CLWAPK	3,917	1,830	1,284	0.45	4,140	7,656	6.9	1,035
(B)	Apr-NF	5,870	4,163	637	0.92	378	470	4.2	34
	Apr-APK	5,737	3,685	728	0.89	378	370	4.4	50
	Apr-CAPK	5,723	3,552	661	0.90	432	412	4.6	56
	Apr-LAPK	1,155	892	241	0.84	403	521	4.2	32
	Apr-WAPK	1,336	1,038	220	0.89	389	454	4.1	28
	Apr-CLWAPK	6,124	5,231	363	0.98	374	424	4.0	23
	Aug-NF	379	282	111	0.77	531	615	4.3	36
	Aug-APK	1,300	1,021	240	0.88	410	385	4.4	42
	Aug-CAPK	3,706	2,665	489	0.89	474	499	4.6	53
	Aug-LAPK	4,933	3,347	526	0.92	350	334	4.1	30
	Aug-WAPK	7,756	5,716	602	0.95	432	732	4.4	32
	Aug-CLWAPK	5,537	3,858	524	0.94	420	520	4.1	24

[a] Refer to Table 1 for symbols.
[b] calculated at a 97% sequence similarity cut-off.

of chimeric sequences under the experimental conditions used (on average 46% and 27% for bacteria and archaea, respectively). Good's coverage values calculated at a 97% similarity cut-off for bacteria indicated that the current numbers of pyrosequencing reads were insufficient to capture the bacterial diversity fully in the rice field soils. They were lower in August (0.34–0.45) than in April (0.58–0.74) due to the lower number of pyrosequencing reads (on average 1,416 reads) from the August soil samples than those from the April soil samples (on average 3,114 reads) and higher bacterial diversity in August than in April (see below). For archaea, Good's coverage values were higher than those for bacteria, ranging between 0.77–0.98.

Bacteria and archaeal diversity in rice field soils

Richness estimators and diversity indices showed that the bacterial richness and diversity of rice field soils were much

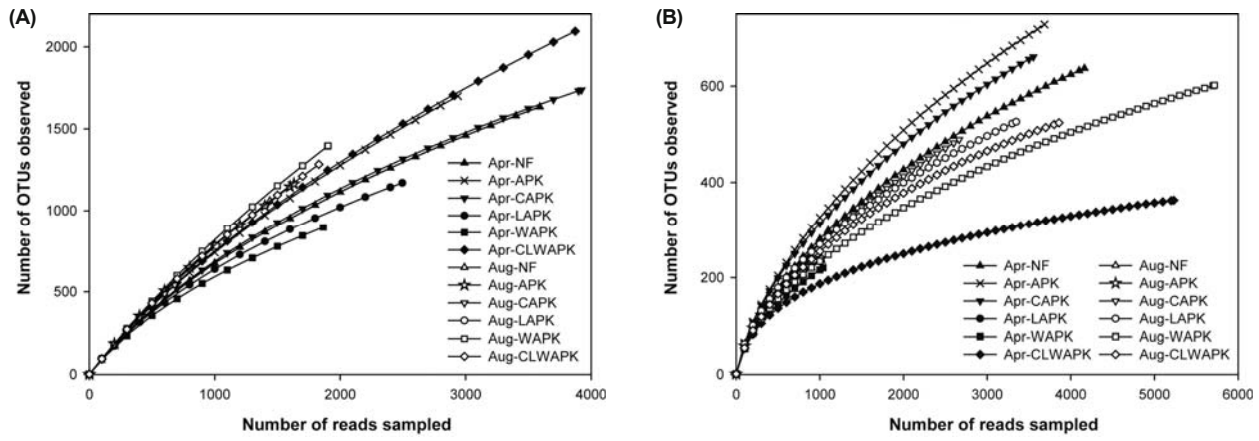

Fig. 3. Rarefaction curves for bacterial (A) and archaeal (B) operational taxonomic units, which were defined at a 97% similarity cut-off. The curves were generated using 1,000-random samplings without replacement. Refer to Table 1 for the symbols used to define each sample.

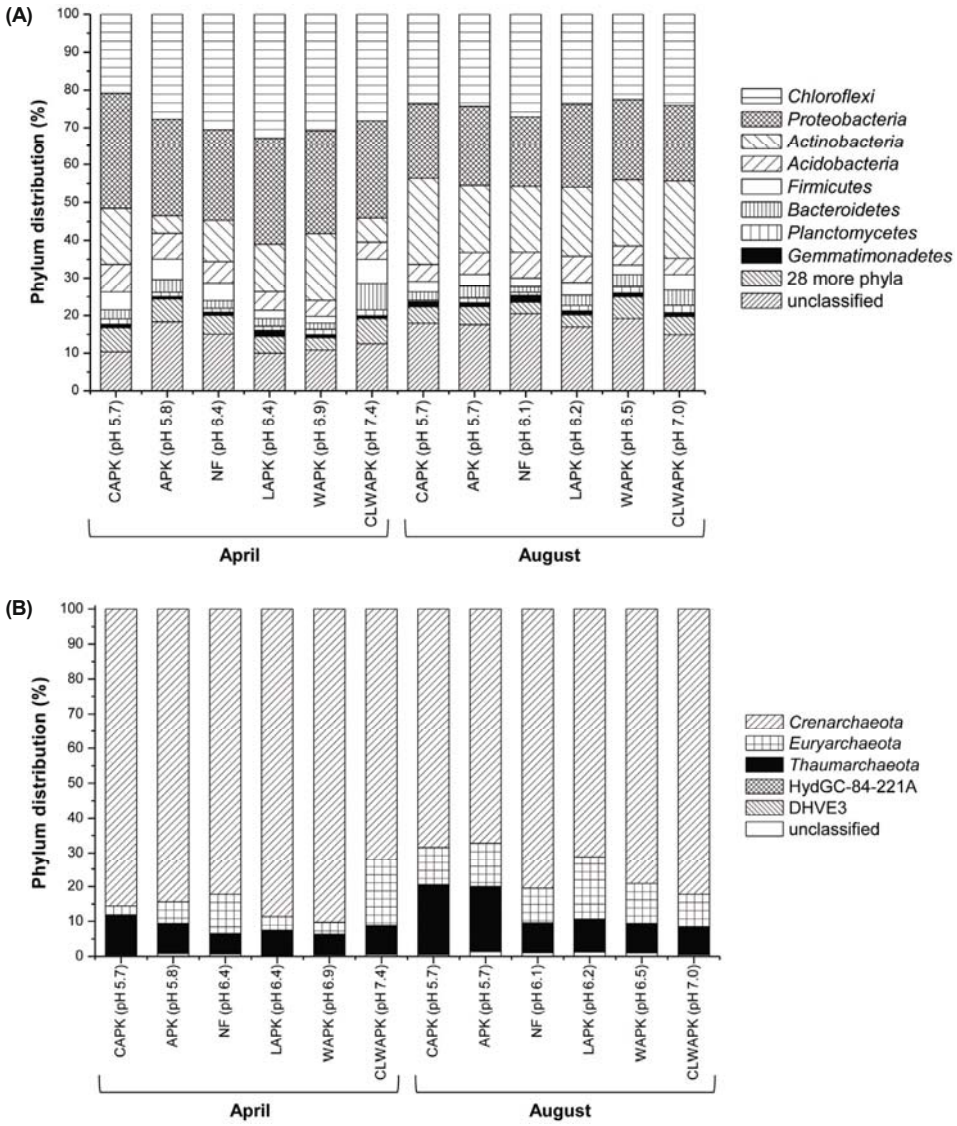

Fig. 4. Phylum distributions of bacteria (A) and archaea (B) in rice field soils. Different fertilization regimes were sorted in the order of increasing pH within each season. Refer to Table 1 for the symbols used to define each sample.

higher than those of archaea (Table 2).

Rarefaction analysis showed that bacterial diversity was high in APK and CLWAPK plots and low in LAPK and WAPK plots compared to the NF plot in April (Fig. 3A). In addition, it showed that bacterial diversity was higher in August than in April, which is supposed to be due to the occurrence of various types of habitats, such as flooded water, oxic-surface soil, anoxic bulk soil and rhizosphere in August (Liesack *et al*., 2000; Lüdemann *et al*., 2000; Asakawa and Kimura, 2008). For archaea, the APK and CAPK treatments increased the archaeal diversity in April, whereas the WAPK and CLWAPK treatments decreased it (Fig. 3B), suggesting that the archaeal community may be sensitive to soil pH. In August, the differences in bacterial and archaeal diversity between different fertilization regimes were reduced compared to those in April, indicating that season had a more significant effect on bacterial and archaeal communities than did fertilization.

Taxonomic distributions of bacteria and archaea in rice field soils

Bacterial phylum distributions in rice field soils are indicated in Fig. 4A (See Supplementary data Tables S1 and S2 for the detailed taxonomic distributions of bacteria and archaea). The *Chloroflexi*, *Proteobacteria*, and *Actinobacteria* phyla occupied 58–76% of the bacterial sequences obtained from rice field soils followed by *Acidobacteria* (4–7%), *Firmicutes* (2–6%), *Bacteroidetes* (2–7%), *Planctomycetes* (0.4–2%), and *Gemmatimonadetes* (1–2%). Only 18–35% of the sequences obtained from rice field soils could be classified up to a genus level (at a bootstrap value ≥80%) based on the silva taxonomy (Pruesse *et al*., 2007), indicating that the bacterial communities in these rice field soils are largely unexplored.

The sequences affiliated with the phylum *Chloroflexi* occupied the highest portions (21–33%) of the bacterial sequences in most soil samples. *Chloroflexi*-affiliated sequences were dominated by *Anaerolineaceae* (54–70%) and *Caldilineacea*

(8–14%) at a class level (Supplementary data Table S1). Most of the species belonging to these classes are strict anaerobes, fermenting sugars and polysaccharides into organic acids and hydrogen (Grégoire *et al.*, 2011; Podosokorskaya *et al.*, 2012). Thus, the *Chloroflexi*-affiliated bacteria are supposed to be the primary bacterial degraders of polysaccharides in the anoxic zones of rice field soils.

The second most abundant phylum was *Proteobacteria*, which occupied 20–31% of the bacterial sequences in rice field soils. Predominant proteobacterial taxa could be divided into the following two functional groups based on their known physiology: chemoorganotrophs utilizing fermentation products such as fatty acids, alcohols or methane and chemolithotrophs utilizing reduced inorganic compounds such as ammonia, sulfur or iron (II) for energy sources. Examples of the former group are *Pseudolabrys*, *Hyphomicrobium*, *Rhodobium*, *Methylocystis*, *Anaeromyxobacter*, *Desulfobacca*, *Geobacter*, and *Methylobacter*, and examples of the latter are *Nitrosomonas*, *Thiobacillus*, and *Sideroxydans* (Supplementary data Table S1). Because these bacterial groups utilize oxygen, nitrite, nitrate, sulfate or iron (III) as an electron acceptor and are generally not fermentative, they are supposed to be active in zones where both external electron acceptors and either organic or inorganic electron donors are present.

The third most abundant phylum was *Actinobacteria*, which occupied 5–23% of the bacterial sequences in rice field soils. The predominant actinobacterial genera in rice field soils were *Arthrobacter*, *Marmoricola*, *Oryzihumus*, *Terrabacter*, *Nocardioides*, *Frankia*, and *Mycobacterium* (Supplementary data Table S1). Because the species belonging to these genera are known to be aerophilic or microaerophilic, they are supposed to be involved in the degradation of organic matter in the oxic zones of rice field soils. *Arthrobacter* was the most abundant actinobacterial genus in rice field soils, occupying 4–27% of *Actinobacteria*-affiliated sequences. In previous studies, *Arthrobacter* was reported to be a predominant actinobacterial clone (Lee *et al.*, 2011) or isolate (Kim *et al.*, 2005) in rice field soils in Korea. The high abundance of *Arthrobacter* appears to be related to their nutritional versatility and high resistance to dryness and starvation (Jones and Keddie, 2006).

Figure 4B shows the phylum distributions of archaeal communities in rice field soils. The pyrosequencing reads affiliated with *Crenarchaeota* occupied 67–90% of the archaeal communities in rice field soils followed by *Thaumarchaeota* (6–20%) and *Euryarchaeota* (3–19%). Only 6–25% of the archaeal sequences obtained from rice field soils could be classified up to a genus level (at a bootstrap value ≥80%) based on greengenes taxonomy (McDonald *et al.*, 2012).

Most of the pyroseqeuncing reads affiliated with *Crenarchaeota* (≥95%) were classified as pGrfC26 at the order level (Supplementary data Table S2), which was reported as Rice Cluster IV in previous studies (Großkopf *et al.*, 1998; Ramakrishnan *et al.*, 2001). Currently, there are no cultured isolates in this taxonomic group, and most of the environmental sequences assigned to this group were obtained from sediment, anaerobic digester or rice field soil (http://greengenes.lbl.gov), suggesting that the archaeal group is anaerobic or facultatively anaerobic.

The phylum *Euryarchaeota* was dominated by *Thermoplasmata* (38–94%) and *Methanomicrobia* (6–56%) (Supplementary data Table S2). Most of the pyrosequencing reads affiliated with *Thermoplasmata* were assigned to WCHD3-02 at a family level, for which there are no cultured isolates. Although the species of *Thermoplasmata* are extreme acidophilic, growing best at pH≤2 (Reysenbach, 2001; Golyshina *et al.*, 2009), environmental sequences classified as WCHD3-02 were obtained from non-acidic sources, such as marine sediment, rumen, anaerobic digester, deep subsurface groundwater and cattle manure compost, in which anaerobic conditions most likely dominate (http://greengenes.lbl.gov).

The *Methanomicrobia*-affiliated pyrosequencing reads were dominated by *Methanosaeta* (30–100%), *Methanosarcina* (0–17%) and *Methanocella* (0–60%) at the genus level (Supplementary data Table S2). The members of the genera *Methanosaeta* and *Methanosarcina* are acetotrophic methanogens

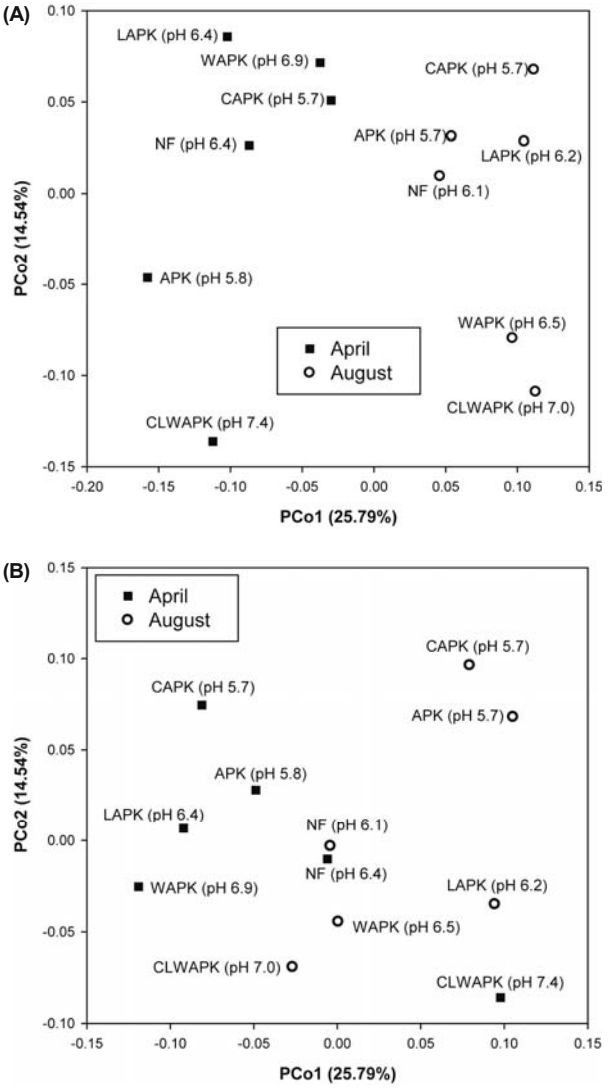

Fig. 5. Principal coordinates analysis (PCoA) of the pyrosequencing reads obtained from rice field soils subjected to different fertilization regimes based on the weighted Fast UniFrac metric. (A) bacteria and (B) archaea.

and cosmopolitan in rice field ecosystems (Conrad, 2007). *Methanosarcina* spp. can also use H_2/CO_2 unlike *Methanosaeta* spp. Another hydrogenotrophic methanogens, *Methanocella* spp. were isolated from rice field soils (Sakai *et al*., 2008, 2010). They were previously known as Rice Cluster I, which was reported to play a key role in methane production in rice paddy fields, particularly on the rhizosphere (Conrad *et al*., 2006).

All isolates or enrichment cultures affiliated with *Thaumarchaeota* were shown to oxidize ammonia aerobically (Pester *et al*., 2011; Brochier-Armanet *et al*., 2012). The *Thaumarchaeota*-affiliated sequences obtained in this study were dominated by *Candidatus* Nitrososphaera-affiliated sequences (63–98%) at the genus level (Supplementary data Table S2). Considering the low abundances of sequences affiliated with *Nitrosomonas* or *Nitorosococcus*, ammonia-oxidizing bacterial groups (0.0–0.1% in bacterial communities, Supplementary data Table S1), we propose that this archaeal group plays the most important role in the nitrification in rice field soils.

Effects of season and long-term fertilization on the prokaryotic community structure

The variations in bacterial and archaeal communities caused by long-term fertilization in two different seasons were investigated using principal coordinates analysis (PCoA) based on the weighted Fast UniFrac metric (Figs. 5A and 5B, respectively). The weighted Fast UniFrac metric measures the fraction of branch lengths of the phylogenetic tree that leads to descendants from either one community or the other but not both. It also accounts for the relative abundances of branches between different communities (Lozupone and Knight, 2008). Similar patterns were observed for both bacterial and archaeal communities. The principal coordinate 1 separated the bacterial and archaeal communities in April from those in August, with the exception of the archaeal communities obtained from the NF and CLWAPK plots. The principal coordinate 2 generally distributed the bacterial and archaeal communities along with the soil pH: the bacterial and archaeal communities obtained from plots with relatively acidic pH (5.7–6.1, APK and CAPK) were generally located in the upper part, whereas those from plots with near-neutral pH (6.5–7.4, WAPK and CLWAPK) were in the lower part. Other chemical properties measured showed no apparent pattern in this analysis. The bacterial and archaeal communities in the plots treated with compost (CAPK and CLWAPK) did not cluster together, although significant increases in bacterial and archaeal abundance were observed only in those plots (Figs. 2A and 2B). These results indicate that the bacterial and archaeal communities were changed by long-term fertilization, especially through the resulting pH change as well as by season.

The effect of season on the prokaryotic communities in the rice field soils is supposed to be induced by at least three factors - flooding, temperature and rice planting. What is the most important can't be said in this field experiment and we supposed that all the three factors had significant effects on the prokaryotic communities in the rice field soils.

The shifts of bacterial communities along with soil pH gradient were also observed in previous studies (Lauber *et al*., 2009; Rousk *et al*., 2010), and the soil pH was suggested to be one of the most important factors shaping the overall bacterial community composition in soil. The narrow pH range for the growth of most bacterial taxa has been suggested to be the reason for the strong influence of pH on the soil bacterial community (Rousk *et al*., 2010).

Phylogenetic analysis of dominant bacterial and archaeal OTUs

The phylogenetic positions of dominant bacterial and archaeal OTUs (clustered at a 97% similarity cut-off) are indicated with heat maps of their relative abundances in each plot in Figs. 6A and 6B, respectively. Because the archaeal OTUs were dominated by *Crenarchaeota*-affiliated OTUs, the dominant *Euryarchaeota*- and *Thaumarchaeota*-affiliated OTUs were also included in the phylogenetic tree.

Most of the dominant *Proteobacteria*- and *Actinobacteria*-affiliated OTUs showed high similarities (partial 16S rRNA gene similarity ≥94%) to the nearest type strains, whereas the *Bacteroidetes*- and *Chloroflexi*-affiliated OTUs showed 16S rRNA gene similarities less than 90% to the nearest type strains.

Among the dominant bacterial OTUs (Fig. 6A), Otu09663 was recovered only from the CLWAPK plot (2.7 and 0.4% in April and August, respectively). This OTU formed a cluster with cultured (*Heliscomenobacter hydrossis*) and uncultured (*Candidatus* H. calcifugiens and *Candidatus* Aquirestis calciphila) representatives of so-called SOL cluster, which are freshwater filamentous bacteria affiliated with the family *Saprospiraceae* of the phylum *Bacteroidetes* (Hahn and Schauer, 2007). Members of this group were observed in freshwater only with pH values >6 (Hahn and Schauer, 2007), and the growth of *H. hydrossis* was much faster at pH 7.5 than 6.4 (Kämpfer, 2010), which is consistent with the soil pH in the CLWAPK plot being the highest (pH 7.4 and 7.0 in April and August, respectively).

Many of the dominant *Proteobacteria*-affiliated OTUs were assigned to *Rhizobiales* at the order level. The relative abundances of the OTUs were generally higher in April than in August. In contrast, the relative abundance of Otu09611, which was the most abundant *Actinobacteria*-affiliated OTU in rice field soils and assigned to *Arthrobacter* at the genus level, was generally higher in August than in April, suggesting that this OTU was better adapted to the rice-growing condition than the *Proteobacteria*-affiliated OTUs.

Among the dominant *Crenarchaeota*- and *Thaumarchaeota*-affiliated OTUs, many showed a strong dependence on soil pH (Fig. 6B). The abundance of Otu1775 and Otu1553 was the highest in CAPK and APK plots and lowest in WAPK and CLWAPK plots. On the other hand, the reverse was observed for Otu1982, Otu1946, and Otu1979.

Among these OTUs, Otu1553 was affiliated with *Thaumarchaeota*, and the same pH dependency was also observed at the phylum level (Fig. 4B). The observed variation in the relative abundance of *Thaumarchaeota*-affiliated sequences based on soil pH is of some relevance to soil fertility and climate change because this archaeal group is supposed to contribute to the loss of nitrogen and emission of nitrous oxide via nitrification (Kim *et al*., 2012). It was reported that soil pH was an important factor in determining the relative

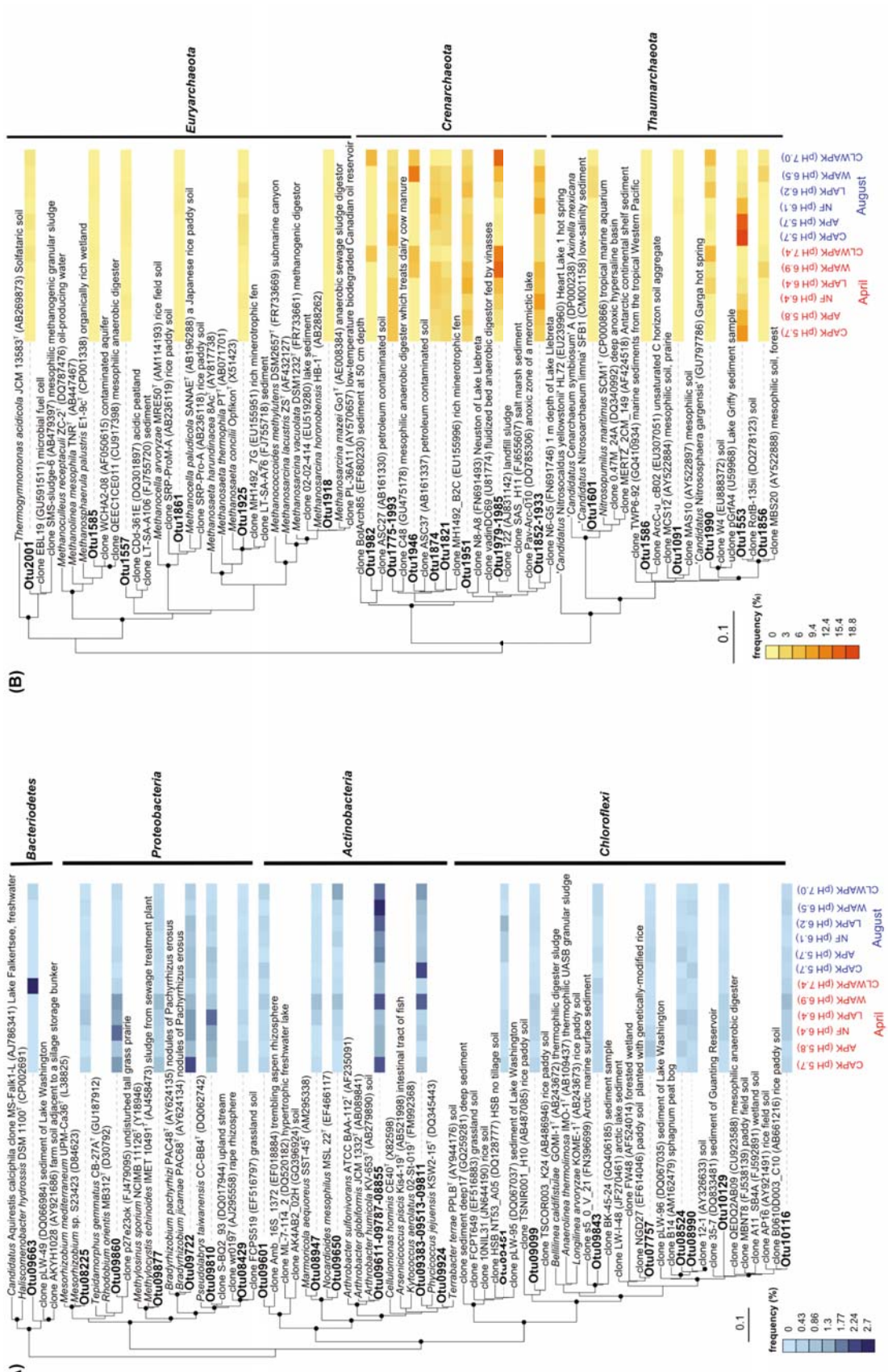

Fig. 6. Phylogenetic positions of the dominant bacterial (A) and archaeal (B) operational taxonomic units (OTUs) obtained from rice field soils (in bold) and their relative abundances in each plot represented by a color gradient (heat map). Only one representative sequence for each OTU was included in the trees. Dominant *Euryarchaeota*- and *Thaumarchaeota*-affiliated OTUs were also included in the archaeal tree. If the pair-wise similarity between the representative sequences exceeded 99%, only one of them was presented in the trees and their relative abundances were combined in the heat map. The black spots on the tree nodes indicate bootstrap support above 90% based on 1,000 iterations. The scale bar indicates 0.10 estimated change per nucleotide. Known isolation sources are indicated for reference sequences. The different fertilization regimes in the heat map were sorted by increasing pH within each season.

abundances of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB), and AOA dominated over AOB in acidic soils (Nicol *et al*., 2008; Gubry-Rangin *et al*., 2010; Yao *et al*., 2011). Additional studies are required to provide further insight on these issues in rice field soils.

In this study, we investigated the bacterial and archaeal communities in rice field soils subjected to long-term fertilization in two different seasons and showed that the microbial activity, prokaryotic abundance and diversity, and prokaryotic community structure in the rice field soils were changed by seasonal variation and long-term fertilization. All fertilization regimes investigated increased the soil microbial activity and organic fertilizer increased the prokaryotic abundance in the rice field soils. The community structure of prokaryotes was shifted mainly by seasonal variation and then, by pH variation induced by fertilizer application. The variations of the relative abundances according to season and fertilization regime were observed for many of the dominant OTUs in the rice field soils.

How these variations will affect nutrient cycling, plant growth and the emission of greenhouse gases in rice field soil will be the next subject. To accomplish this, functional studies on the dominant bacterial and archaeal groups which were sensitive to the variations of season and fertilization regime must be performed. The *Rhizobiales*- and *Arthrobacter*-affiliated bacteria, and *Crenarchaeota*- and *Thaumarchaeota*-affiliated archaea are the examples of such groups (Figs. 6A and 6B). Particularly, the *Crenarchaeota*-affiliated archaea in the rice field soils have no closely related isolate at present despite their predominance among the archaeal communities in the rice field soils. The effort to culture this archaeal group will be needed to investigate its functional roles in the rice field soils. The variations of the OTUs affiliated with *Chloroflexi* and *Methanomicrobia* according to season and fertilization regime were not clear compared to the above mentioned groups (Figs. 4A and 4B; Figs. 6A and 6B; Supplementary data Tables S1 and S2). However, as the most dominant bacterial group and probably the most important source of methane in the rice field soils, respectively, more attention must be given to these groups in future studies.

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