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Ammonia-oxidizing archaea: important players in paddy rhizosphere soil?

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Summary

The diversity (richness and community composition) of ammonia-oxidizing archaea (AOA) and bacteria (AOB) in paddy soil with different nitrogen (N) fertilizer amendments for 5 weeks were investigated using quantitative real-time polymerase chain reaction, denaturing gradient gel electrophoresis (DGGE) jand clone library analysis based on the ammonia monooxygenase α-subunit (*amoA*) gene. Ammonia-oxidizing archaea predominated among ammonia-oxidizing prokaryotes in the paddy soil, and the AOA:AOB DNA-targeted *amoA* gene ratios ranged from 1.2 to 69.3. Ammonia-oxidizing archaea were more abundant in the rhizosphere than in bulk soil. Rice cultivation led to greater abundance of AOA than AOB *amoA* gene copies and to differences in AOA and AOB community composition. These results show that AOA is dominant in the rhizosphere paddy soil in this study, and we assume that AOA were influenced more by exudation from rice root (e.g. oxygen, carbon dioxide) than AOB.

Introduction

The supply of nitrogen (N) fertilizer to paddy fields is recognized as a major factor determining rice yields (Cassman *et al*., 1993). Although ammonium is considered as the major form to which rice is especially adapted (Shen, 1969; Wang *et al*., 1993), it has been suggested that rice may absorb significant amounts of nitrate formed by nitrification of ammonium in the rhizosphere (Kronzucker *et al*., 2000). The ammonia monooxygenase α-subunit (*amoA*) gene, which is responsible for catalysing the rate-limiting step in autotrophic bacterial ammonia oxidation, has been used extensively as a molecular marker for cultivation-independent studies of ammonia-oxidizing bacteria (AOB) communities. Some archaea may carry *amoA* genes, as demonstrated by Venter and colleagues (2004) using whole genome shotgun analysis of DNA sequences derived from the Sargasso Sea. Further, Schleper and colleagues (2005) using fosmids derived from soil *Crenarchaeota* libraries identified a Sargasso Sea-like *amoA* homologue, suggesting that archaea capable of ammonia oxidation may be present in soils. Recently, a definitive link between this novel *amoA* and archaeal ammonia oxidation was established by isolation of an ammonia-oxidizing member of the marine group 1 *Crenarchaeota* (Könneke *et al*., 2005). Thus it is likely that ammonia oxidation is carried out by both ammonia-oxidizing archaea (AOA) and AOB. Considering the ubiquity of AOA in multiplex habitats, such as oxic and suboxic marine water bodies, estuarine sediments and soils (Francis *et al*., 2005), it seemed possible that AOA could also be present in paddy soil. Ammonia-oxidizing archaea have been demonstrated to be the dominant group among ammonia-oxidizing prokaryotes in the ocean (Wuchter *et al*., 2006) and in soils (Leininger *et al*., 2006), but the relative abundance of AOA and AOB in paddy soil has not been investigated so far.

The rice paddy microcosm is a useful system for the description and prediction of microbiological and biogeochemical processes in wetland habitats. Oxygen deplete rapidly in a few millimetres beneath the soil surface, leaving the bulk soil anoxic. However, oxygen is considered to leak from aerenchymatous tissue in rice creating an oxic rhizosphere within the anoxic bulk soil (Revsbech *et al*., 1999). Aerenchyma, a porous tissue which may be an adaptation to anoxic root environment in rice plants, establishes a gas channel between the atmosphere and the root tissue. It has been suggested that oxygen released from rice roots may support aerobic microbial processes (e.g. nitrification) (Arth *et al*., 1998; Brune *et al*., 2000). However, a less stereotype information is available regarding the abundance, composition and activity of populations of AOA and AOB occurring in the rice rhizosphere.

Environmental functional genes based on mRNA are related to activity, and sequence heterogeneity may be related to phylogenetic distance (Wawer and Muyzer, 1995). Aoi and colleagues (2004) has demonstrated that *amoA* mRNA showed sensitive response to ammonia

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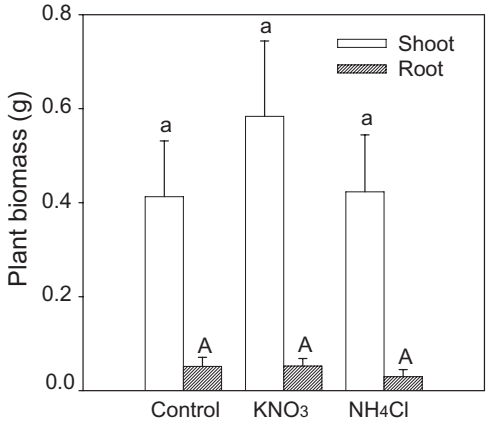

Fig. 1. Plant biomass after 5 weeks of growing in a greenhouse of potting soil with different nitrogen fertilizer amendments. Error bars indicated standard deviation. Within shoot and root biomass, means (mean ± SE, $n = 4$) followed by the same letter are not significantly different at $P < 0.05$.

oxidation activity, which can be used as a biomarker of ammonia oxidation activity in wastewater treatment processes where many bacterial species exist. Therefore, we assumed that *amoA* mRNA level was also correlated with ammonia oxidation activity in paddy soil. In addition, recently unequivocal data have indicated the strong rice root-promoted nitrification and active AOB in rice rhizosphere (Li *et al.*, 2008). In this study, we analysed the relative abundance of AOA and AOB in the rice rhizosphere and bulk soil using quantitative real-time polymerase chain reaction (PCR) based on DNA and cDNA reverse transcribed from RNA. The community structures of AOA and AOB from soil were also detected using PCR-denaturing gradient gel electrophoresis (DGGE) approach based on the *amoA* gene.

Results

Shoot and root dry weights of rice plants grown with nitrate addition appeared only slightly higher than those from the control and ammonia treatments. However, differences between these treatments were not significant (Fig. 1).

amoA-*targeted quantitative real-time PCR assay*

The DNA-targeted archaeal *amoA* gene copy numbers in the paddy soil ranged from 1.0×10^6 to 1.8×10^7 per gram dry weight of soil, and the DNA-targeted bacterial *amoA* gene ranged from 1.6×10^5 to 9.8×10^6 per gram dry weight of soil (Fig. 2A). The AOA:AOB *amoA* gene ratios ranged from 1.2 to 69.3. According to paired-sample *t*-test, both cDNA-targeted archaeal and bacterial *amoA* gene copy numbers were significantly higher than those from DNA-targeted in each soil sample (Fig. 2B). Interestingly, cDNA-targeted *amoA* gene ratio of AOA to AOB was correlated well with DNA-targeted quantitative measurements, and the maximal AOA:AOB *amoA* gene ratio was 22.9.

The DNA-targeted archaeal *amoA* gene copy numbers in the soil samples without rice were significantly lower than those in soil with rice except for the control (Fig. 2A). Furthermore, DNA-targeted archaeal *amoA* copy numbers in the rhizosphere soil were much higher than those in the bulk soil, irrespective of N treatments. In addition, the DNA-targeted archeal *amoA* gene copy numbers in the rhizosphere soil with the addition of

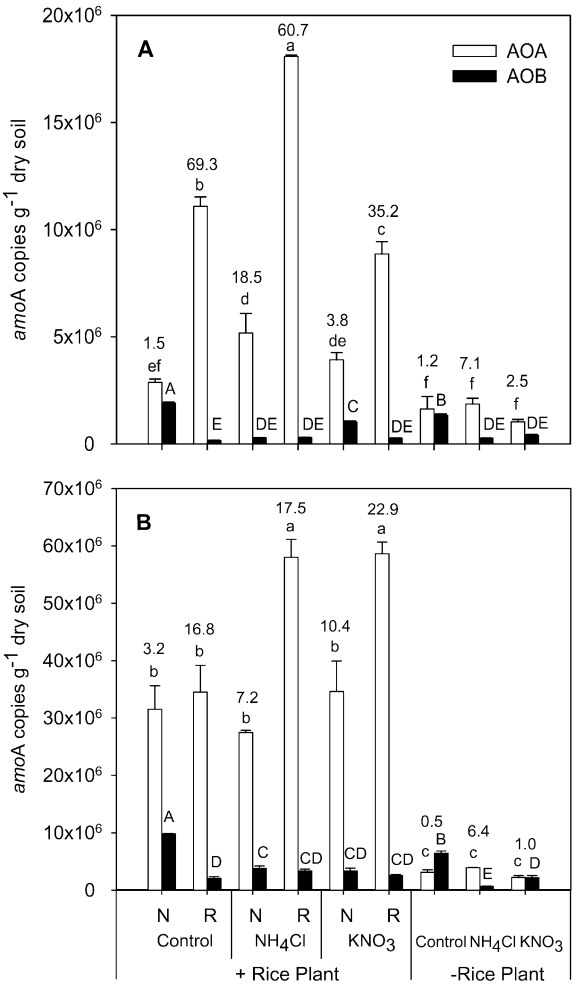

Fig. 2. Abundance of AOA and AOB in paddy soil with different nitrogen fertilizer amendments expressed as DNA-targeted (A) and cDNA-targeted (B) *amoA* copy numbers per gram dry weight of soil respectively. Ratios of AOA to AOB *amoA* copies are shown at the top columns with each treatment. Error bars indicate standard deviation. N, bulk; R, rhizosphere. Within DNA-targeted (A) and cDNA-targeted (B) archaeal and bacterial *amoA* copy numbers, means (mean ± SE, $n = 4$) followed by the same letter are not significantly different at $P < 0.05$.

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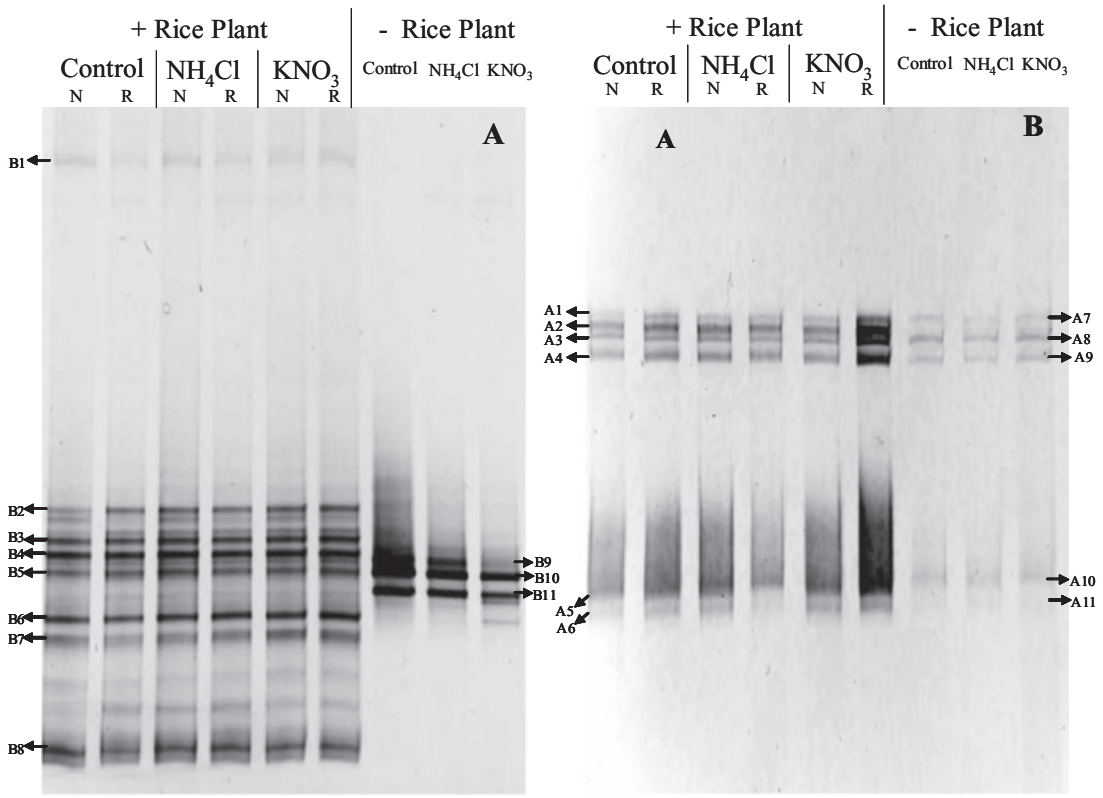

Fig. 3. Denaturing gradient gel electrophoresis (DGGE) analysis of the predominant PCR-amplified partial cDNA-targeted *amoA* of AOB (A) and AOA (B) obtained from the paddy soil with different N fertilizer amendments. Respective 11 major AOA and AOB DGGE bands that showed unique positions in the gels were observed and excised. N, bulk; R, rhizosphere.

ammonia were significantly higher than the control. Interestingly, archaeal *amoA* gene copy numbers varied little in the soil with different N amendments without rice plants. However, there was no significant difference in DNA-targeted bacterial *amoA* gene copy numbers in all soil samples except the control with and without rice. The variation in DNA-targeted *amoA* gene copy numbers was confirmed by cDNA-targeted analysis. The cDNA-targeted archaeal *amoA* gene copy numbers in the soil samples with rice were significantly higher than those in soil without rice (Fig. 2B). cDNA-targeted archaeal *amoA* copy numbers in the rhizosphere soil were significantly higher than those in the bulk soil with ammonia and nitrate treatments. The cDNA-targeted archeal *amoA* gene copy numbers in the rhizosphere soil with the addition of ammonia and nitrate were significantly higher than the control. There was no significant difference in the soils without rice. Except for the control with and without rice, the DNA-targeted bacterial *amoA* gene abundance did not vary significantly.

DGGE profiles of archaeal and bacterial communities

Denaturing gradient gel electrophoresis analysis was performed on archaeal and bacterial *amoA* PCR products obtained from cDNA generated using reverse transcription (RT)-PCR products (Fig. 3). Reproducible profiles were obtained from both templates for all soil treatments, and DNA- and cDNA-derived profiles of both AOA and AOB were indistinguishable, and DNA-profile data were not shown. The numbered bands with two replicates with the same mobility in the DGGE gels were excised for sequencing, and cloned sequences obtained from different bands of the same mobility had high homology (> 97%). One representative sequence from cDNA PCR-DGGE pattern was chosen to do phylogenetic analysis (Fig. 4). Both AOA and AOB community profiles in the rhizosphere soil with different N amendments were indistinguishable with those in bulk soil, while AOB communities in soil without rice plants were clearly different from those with planted rice.

AOA and AOB phylogeny

Phylogenetic analysis of archaeal *amoA* gene showed that all sequences were distributed in several subclusters of soil and sediment clusters (Fig. 5). All the sequences detected in the paddy soil without rice plants fell into cluster 1 except for band 7, which belonged to cluster 7. Interestingly, cluster 7 contained most sequences of archaeal

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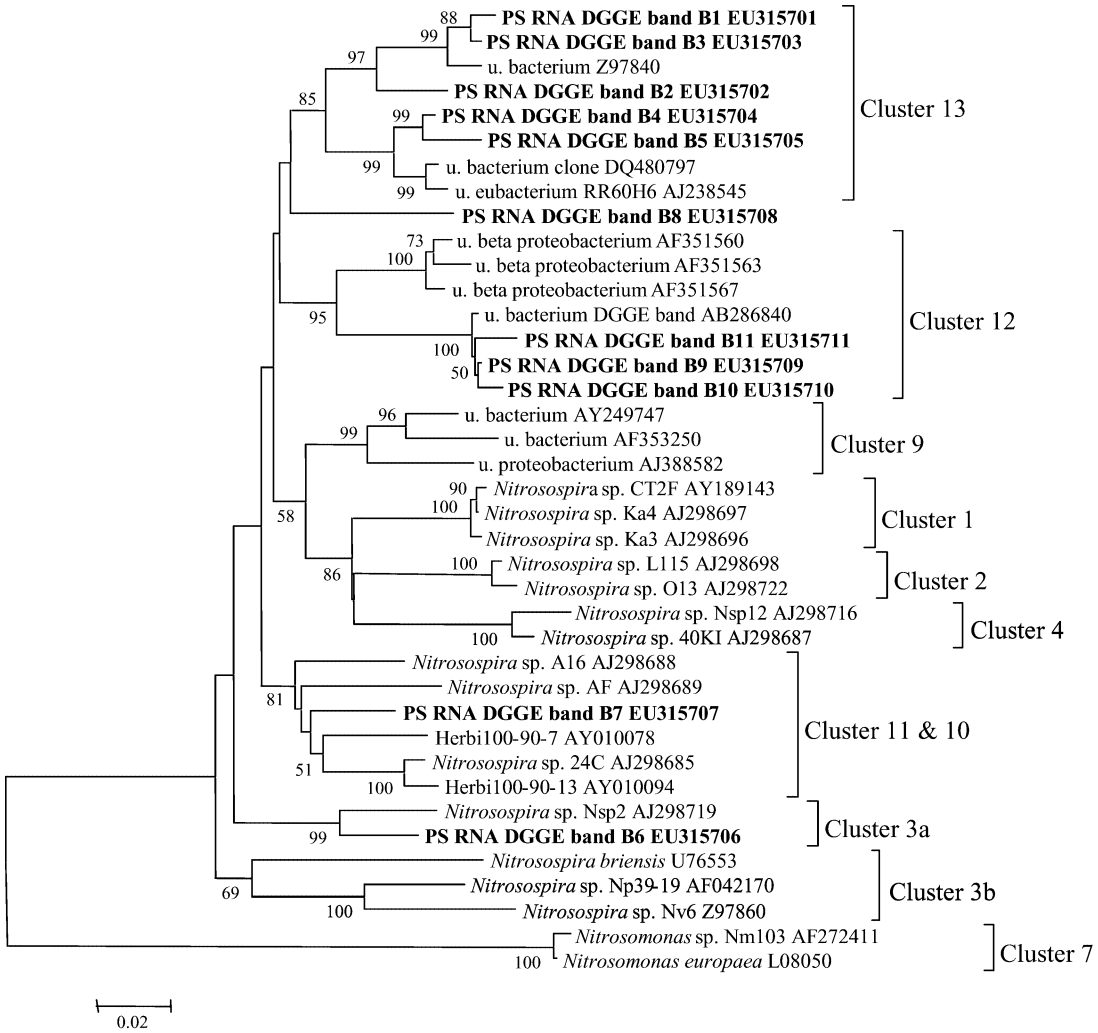

Fig. 4. Phylogenetic analyses of cDNA-targeted bacterial *amoA* recovered from DGGE bands of paddy soil with different nitrogen fertilizer amendments (shown in bold). The sequences of DNA bacterial *amoA* DGGE bands that are not mentioned in the tree due to > 99% amino acid identity to sequences obtained from the RNA bacterial *amoA* DGGE bands. The scale bar indicates two changes per 100 amino acid positions.

amoA gene recovered from the soil with rice. While bands 5 and 6 were grouped in cluster 1 and 8 respectively.

Polymerase chain reaction amplification of a 635 bp archaeal *amoA* fragment was obtained from DNA extracts of the control soil. Approximately 100 clones were selected from the library and re-amplified successfully. A total of 20 archael *amoA* gene agarose gel patterns of restriction digestion (data not shown), using restriction endonuclease HaeIII and RsaI, were obtained. For sequence types that exhibited at least 99% amino acid similarity to each other, only 12 representative sequences were selected for the construction of trees. Based on those *amoA* gene sequences and primers cut-off standard with sequences obtained from DGGE bands, 12 representative clone sequences were distributed in eight subclusters in soil/sediment cluster. Sequences from the archaeal *amoA* clone library of paddy soil in the control were correlated with the recovered DGGE bands.

A neighbour-joining tree was constructed using sequences of bacterial *amoA* DGGE bands and the related sequences deposited in GenBank (Fig. 5). According to the nomenclature for *Nitrosospira amoA* clusters as defined by Avrahami and colleagues (2002) and Avrahami and Conrad (2003), all the band sequences appeared to be the genus *Nitrosospira*, without *Nitrosomonas* species in the paddy soil. All the sequences in the paddy soil without rice were grouped within cluster 12. Bands 1–5 recovered from the paddy soil with rice were distributed solely in cluster 13. Band 8, one of the common bands in the soil with rice, was not grouped into any defined cluster. Bands 6 and 7 fell in cluster 3a and cluster 11 and 10 respectively.

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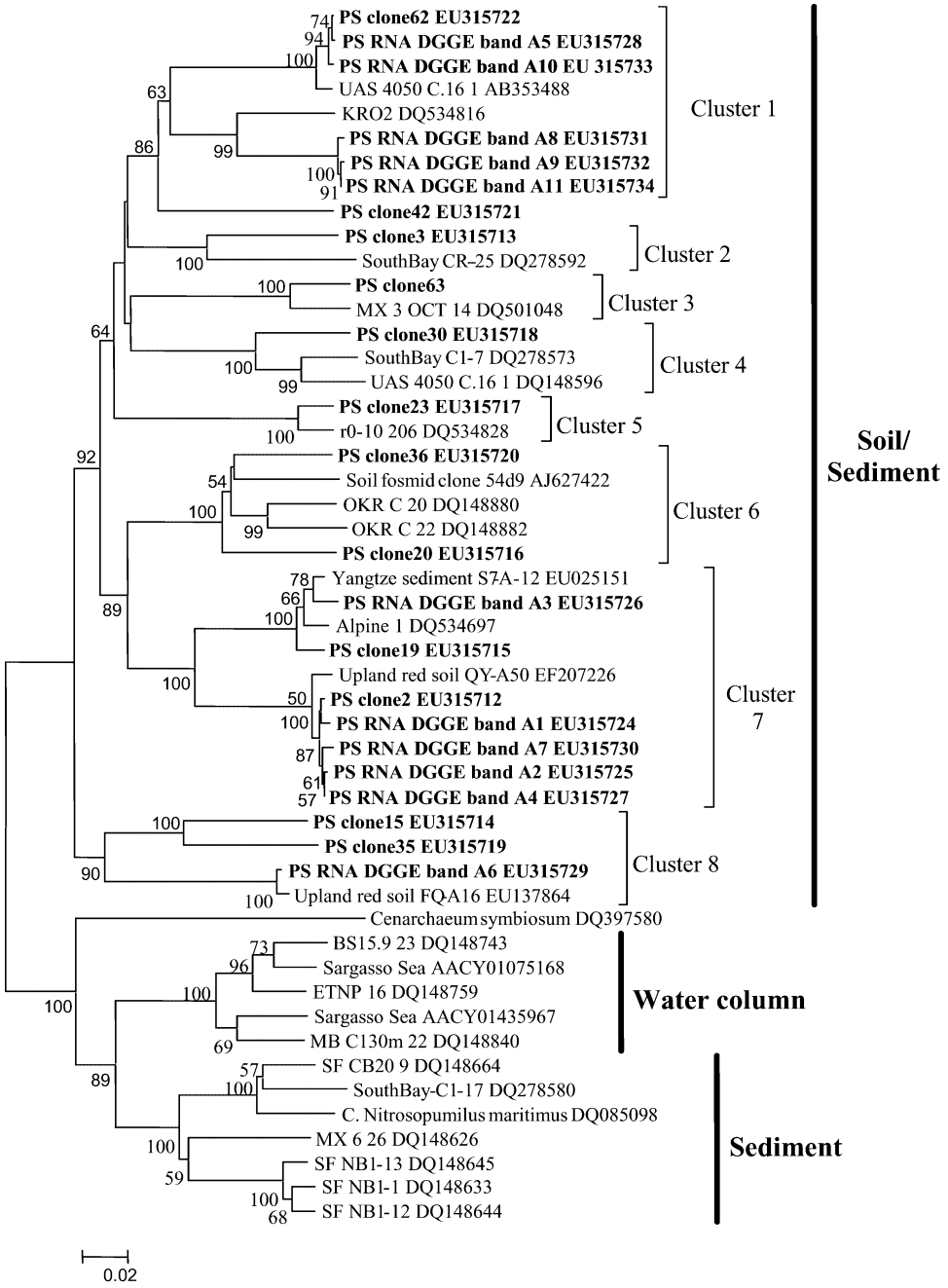

Fig. 5. Phylogenetic relationships among RNA archaeal *amoA* recovered from DGGE bands of paddy soil with different nitrogen fertilizer amendments and archaeal *amoA* gene clone library from untreated paddy soil (shown in bold) and previously reported environmental sequences. The sequences of DNA archaeal *amoA* DGGE bands and partial clone sequences that are not mentioned in the tree due to > 99% amino acid identity to sequences obtained from the RNA archaeal *amoA* DGGE bands or another clone sequence. The scale bar indicates two changes per 100 amino acid positions.

Discussion

Both AOA and AOB were highly abundant in paddy soil, based on *amoA* genes using quantitative real-time PCR methods. The archaeal *amoA* gene copy numbers ranged from 1.0×10^6 to 5.9×10^7 per gram dry weight of soil, similar to the numbers determined in an upland red soil using the same primers as in this study, a soil that is commonly considered to be more oxic than paddy soil (He *et al.*, 2007). The emergent rice plants had a strong stimulating effect on the numbers of AOA in the paddy soil. The numbers of DNA archaeal *amoA* gene copies in

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the rhizosphere were significantly higher than in the bulk soil. It is well known that rice roots release oxygen through aerenchymatous tissue at rates sufficient to support non-specific aerobic microbial processes (Bedford *et al.*, 1991) and aerobic nitrification (Mosier *et al.*, 1990; Arth *et al.*, 1998). In addition, carbon dioxide, which elevated in the rhizosphere due to both root and microbial respiration, would be the carbon source for chemolithoautotrophs. On the contrary, in the bulk soil there would only be microbial respiration but lower oxygen concentrations. Our results suggested that AOA were significantly influenced by exudation from rice roots (e.g. oxygen, carbon dioxide). Although little information of AOA has been published (Könneke *et al.*, 2005; De La Torre *et al.*, 2008), we assumed that carbon source exuded from root would influence AOA if they are heterotrophic. Bowatte and colleagues (2007) investigated the effect of elevated atmospheric CO_2 on AOB communities associated with rice roots; it was expected that AOB community would be influenced by CO_2 exudation from rice root. However, there was no significant difference in bacterial *amoA* gene copy number in the rhizosphere and the bulk soil. Compared with AOA, the bacterial DNA *amoA* gene copy numbers were much lower, and the ratio of AOA to AOB ranged from 1.2 to 69.3, which indicated that archaea dominated in paddy soil. Briones and colleagues (2002) did not obtain significant products of bacterial *amoA* gene in the rhizosphere soil with rice. Taking into account the extremely low yields of AOB in the bulk soil with different N treatments, amounts of AOB increased when AOA was relatively lower in the bulk soil of the control and the treatment with nitrate amendment. It is very likely that AOA competed with AOB in the ammonification, and that AOA may be more competitive than AOB for ammonification in rice rhizosphere microenvironment. This may imply that AOA may have a higher affinity for oxygen or carbon dioxide, which may explain their predominance in the rhizosphere.

Despite the great differences in the abundances of AOA between rhizosphere and bulk soils, little difference in both the archaeal and bacterial *amoA* DGGE patterns was detected between the rhizosphere and the bulk soils with nitrate and ammonia fertilizers. Phylogenetic analysis in the present study based on bacterial *amoA* identified that *Nitrosopira*-like species dominated in paddy soil, which was in agreement with a previous study in paddy soil (Bowatte *et al.*, 2006a) or associated weeds in paddy soil (Bowatte *et al.*, 2006b). However, Bowatte and colleagues (2007) also found that both *Nitrosospira*-like and *Nitrosomonas*-like AOB sequences were detected on rice roots. Briones and colleagues (2002) further demonstrated that the levels of abundance of *Nitrosomonas* spp. were different between different cultivars of rice. This might be due to the ability of *Nitrosomonas* to outcompete *Nitrosospira*-like AOB at high substrate and oxygen concentrations. The phylogenetic tree also revealed that most of the AOB detected here in the paddy soil belong to an undescribed cluster 13 in the rhizosphere and the bulk soil. Previous studies have demonstrated that AOB in the surface soil were dominated by cluster 1 in a Japanese paddy field (Murase *et al.*, 2003), and cluster 7 in a Philippine paddy field (Nicolaisen *et al.*, 2004). These studies indicated that paddy soil can harbour a limited AOB community in comparison with other soils (grassland, meadow, forest soils) (Avrahami *et al.*, 2002; Mintie *et al.*, 2003; Horz *et al.*, 2004). Similar to AOB, all archaeal *amoA* gene sequences belonged to soil and sediment clusters. In addition, the AOA in the paddy soil with rice plants belonged to one subcluster (cluster 7). It appears that limiting numbers of both AOA and AOB, which predominate for the ammonia-oxidizing community in paddy soil, are adapted to life in low-oxygen or periodically anoxic habitats. Bodelier and colleagues (1996) demonstrated that the ammonia-oxidizing communities of grassland soil lost more than 90% of initial nitrifying capacity upon anoxic incubation, while the ammonia-oxidizing communities in lake sediment started to nitrify within 1 h upon exposure to oxygen at the level of the initial capacity. From our results, it seems that AOA were more capable to react rapidly to the presence of oxygen in fluctuating oxic and anoxic rhizosphere of rice plants.

To explore more thoroughly the AOA community in paddy soil, an archaeal *amoA* clone library was generated from the control paddy soil. From the phylogenetic analysis of representative *amoA* clone sequences, it was observed that clone library satisfactorily covered the archaeal *amoA* DGGE patterns. However, DGGE separated the predominant species in the community. All of the clone sequences were distributed in different multiplex subclusters in soil sediment cluster, which indicated that AOA community was diverse in paddy soil.

The results based on quantitative real-time PCR and PCR-DGGE showed great differences in the abundances and exactly the same community composition of AOB and AOA in the rhizosphere and bulk soil, which prompted our investigation into the abundance and composition of AOB and AOA populations in the paddy soil without rice plants with different N amendment. Interestingly, both AOA and AOB abundance were kept at a relatively low level in the soil without rice plants, and the phylogenetic analysis also revealed that both AOA and AOB detected in the paddy soil without rice belonged to a different cluster from that from the paddy soil with rice. Upon anoxic incubation, the AOA community lost nitrifying capacity and an extreme low *amoA* gene level was detected by quantitative real-time PCR compared with that in soil with rice. Flooded paddy soil without rice plant is generally anoxic except for the upper few millimetres.

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The oxidation of ammonia to nitrite by ammonia-oxidizing bacteria, which are strict aerobic chemolithoautotrophic microorganisms, can only occur in the presence of oxygen and thus would be restricted to the oxic upper few millimetres of flooded soils. However, from the DGGE fingerprints, several species of both AOA and AOB, which were grouped within different clusters from that in the paddy soil with rice plants, survived in the paddy soil without rice plants. Molina and colleagues (2007) explored the community structure of β class AOB in an oxygen gradient-associated zone with minimum oxygen, and found a clear community shift at the *amoA* level along the strong oxygen gradient. Our results also provided strong evidence that the presence or absence of oxygen and carbon dioxide (leaked through rice roots) was the major factor determining the changes in AOA and AOB community structure.

To obtain further evidence for our conclusion, the archaeal populations detected on the DNA level were the metabolically active groups, and an RNA-based approach followed by RT and quantitative real-time PCR and PCR-DGGE analysis was applied. The results based on RNA for both rhizosphere and bulk soil with and without rice plants were similar to those obtained based on the DNA analysis. This suggested that the microbial groups detected on the DNA level were the metabolically active populations in the paddy soil. From the result of quantitative real-time PCR based on DNA and RNA, archaeal cDNA-targeted *amoA* gene copy numbers in the rhizosphere soil with nitrate addition significantly increased, which indicates that nitrate could be denitrified to ammonia in flooding soil and increase archaeal *amoA* gene expression on the RNA level. Ammonia addition could stimulate both AOA growth and activity compared with the control. From our results, AOA is predominant in the paddy soil; it is therefore necessary to understand the role of AOA in the rhizosphere, and to further elucidate their interactions with denitrifiers, to better manipulate N cycling in paddy soil.

Experimental procedures

Pot experiment

The paddy soil was obtained from a field located in Shangyu, Zhejiang province (China). Before the experiment, the soil was air-dried and sieved through a 2 mm mesh. The characteristics of the soil were list as follows: pH (H_2O), 6.8; organic matter, 9.32 g kg^{-1}; alkali-hydrolysable N, 49.9 mg kg^{-1}; available phosphorus, 16.5 mg kg^{-1}. Plants were allowed to grow with different nitrogen treatments: (i) untreated soil (control), (ii) addition of KNO_3 and (iii) addition of NH_4Cl, and equimolal potassium as KCl with treatment of KNO_3. Nitrogen (either 1 mM kg^{-1} soil nitrate or ammonium) was added every week. Rice plants were harvested 1 week after the last fertilization, when soil samples were collected for analyses.

Rice seeds (*Oryza sativa* L.) cv. Jiahua-1 were disinfected in 30% H_2O_2 (w:w) solution for 10 min, followed by thorough washing with de-ionized water. The seeds were germinated in moist perlite. After 3 weeks, uniform seedlings were selected and transplanted into bags (37 μm nylon mesh, 7.5 cm diameter, 10 cm height, one plant per bag) filled with 0.2 kg of sieved soil, which were placed in the centre of 1.5 kg pots, and the gap between nylon bag and PVC pot was filled with 0.8 kg of soil, the same as the soil into nylon bag. This allowed a separation of root/rhizosphere compartment from a soil compartment. In order to evaluate the influence of the rice plants on the rice paddy soil characteristics, control experiments without rice were incubated with ammonia and nitrate amendments as described above. To eliminate effects of additional N during the experiment, through N_2 fixation by cyanobacteria, for example, the top of the pot was covered by black nylon with a gap to allow the rice to grow.

After transplanting into the PVC pots, the rice plants were grown for 5 weeks in a greenhouse with a 14/10 h light/dark cycle, and the temperature was kept at 25°C during the day and 16°C during the night, with 70% relative humidity.

At harvest, the rhizosphere and bulk soil were collected and immediately frozen in liquid N_2 and stored at –80°C until required. Analysis was carried out on samples from four separate PVC pots of the same treatment.

DNA and RNA extraction

DNA was extracted using a FastDNA SPIN Kit for soil (Bio 101, Vista, CA) following the manufacturer's instructions.

RNA was extracted using the bead-beating method developed by Noll and colleagues (2005). The raw extracts of nucleic acids were purified by Quick-clean SPIN filters (Cat. No. 6070-057, Bio 101, Vista, CA) and digested with DNase I (Takara Shuzo, Shiga, Japan) to remove DNA. Performing PCR on RNA samples after DNase treatment confirmed the amplification of RNA templates free of DNA contamination. The first-strand cDNA was synthesized using PrimeScriptTM 1st Strand cDNA Synthesis kit (Takara Shuzo, Shiga, Japan) according to the manufacturer's instruction. Prior to RT, the RNA secondary structure was melted by incubating the RNA samples with random 6 mers primer at 0.25 μM at 65°C for 5 min. Samples of annealed primer template were snapped on ice, and 10 μl of RT reaction mixture (containing maximum of 500 ng of template RNA, 2 μl of 5× PrimeScript buffer and 100 U of PrimeScript RTase supplied by the Kit) was added. Reverse transcription was carried out at 30°C for 10 min, 42°C for 30 min and 70°C for 15 min according to instruction.

Construction of archaeal amoA *gene fragment libraries*

A clone library of archaeal *amoA* amplicons obtained from the control soil sample was constructed to analyse the AOA community structure according to Weidner and colleagues (1996). Briefly, after PCR amplification of the archaeal *amoA* gene of the total DNA extracted from soil samples, gel slices of an agarose gel containing the PCR products were excised, and purified using Agarose Gel DNA Purification Kit (Ver. 2.0, TaKaRa). The purified PCR products were ligated into p-GEM T easy vector (Promega, Madison, WI) and then cloned into

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Escherichia coli JM109 (TaKaRa) in accordance with the manufacturer's instructions. The 635 bp amplicons from re-amplification of the randomly chosen colonies using the primers Arch-amoAF and Arch-amoAR were analysed with restriction endonuclease HaeIII and RsaI (Bio Basci, Canada). Restriction digestion was carried out in a total volume of 20 ml including 5 U of each restriction enzyme and 4 ml of PCR products, and incubated for 1 h at 50°C and 2 h at 37°C respectively. Digested DNA fragments were analysed by separation of fragments on a 2% agarose gel and visualized with a GBOX/HR-E-M (Syngene, UK). *amoA* gene ARDRA patterns were estimated for both of the restriction enzymes and cluster analyses were performed. About 20 representative digestion patterns were observed. And representative clones from each pattern were randomly selected and sequenced with an ABI PRISM 3730 sequencer.

Quantitative real-time PCR

The copy numbers of archaeal *amoA*, as well as bacterial *amoA* gene copy numbers in all samples, were determined in triplicate using an iCycler IQ5 Thermocycler (Bio-Rad, Hercules, CA). The quantification was based on the fluorescent dye SYBR-Green I, which binds to double-stranded DNA during PCR amplification. A total of 40 cycles were run with annealing temperatures and primers as listed in Table 1. Each reaction was performed in a 25 μl volume containing 1–10 ng of DNA or cDNA, 0.2 mg ml^{-1} BSA, 0.2 μM of each primer and 12.5 μl of SYBR Premix Ex Taq™ (Takara Shuzo, Shiga, Japan). Product specificity was confirmed by melting curve analysis (65–98°C, 0.2°C per read, 6 s hold) and visualization in agarose gels, which showed specific product bands at the expected size of *c*. 491 bp and 635 bp for the bacterial and archaeal *amoA* gene respectively.

Archaeal and bacterial *amoA* gene fragments were cloned as described above, and clones that had the right *amoA* gene inserts were chosen as the standards for real-time PCR. Plasmid DNA was extracted with Plasmid Kit (TaKaRa), and the plasmid concentration was measured with a spectrophotometer (Nanodrop). As the sequences of the vector and PCR inserts were known, the copy numbers of *amoA* were calculated directly from the concentration of extracted plasmid DNA (Okano *et al*., 2004). Ten-fold serial dilutions of a known copy number of plasmid of the *amoA* gene clone from the soil were generated to produce the standard curve over seven orders of magnitude (6×10^2 to 6×10^8 copies of template for archaeal *amoA* and 1.37×10^2 to 1.37×10^8 copies of template for bacterial *amoA*) per assay respectively. High amplification efficiencies of 100.7–104.5% were obtained for archaeal and bacterial *amoA* quantification with R^2 values between 0.98 and 0.99 and slopes from −3.2 to −3.3. Data analysis was carried out with iCycler software (version 1.0.1384.0 CR). The parameter Ct (threshold cycle) was determined as the cycle number at which a statistically significant increase in the reporter fluorescence was detected.

PCR and DGGE analysis of amoA *fragments*

For DGGE analysis, DNA and cDNA from RT reactions were amplified with the amoA-1F* and amoA-2R primers set spe-

Table 1. Primers and PCR conditions used for the PCR-DGGE and real-time PCR.

Target group	Primer	Sequence (5′–3′)	Amplicon length (bp)	Thermal profile for real-time PCR	Thermal profile for PCR-DGGE	Reference
AOA	Arch-amoAF Arch-amoAF-GC[a] Arch-amoAR	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	635	3 min at 94°C, followed by 40 cycles of 30 s at 94°C, 1 min at 53°C, and 1 min at 72°C. Fluorescence was read during each cycle at 83°C	5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72 °C, and 5 min at 72°C	Francis *et al.* (2005)
AOB	amoA-1F* amoA-1F*-GC[a] amoA-2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	491	3 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 55°C, and 45 s at 72°C. Fluorescence was read during each cycle at 83°C	5 min at 94°C, followed by 20 cycles of 1 min at 94°C, 1 min at 65–55°C touchdown (−0.5°C per cycle), and 1 min at 72°C, and 14 cycles of 94°C, 1 min at 55°C, and 1 min at 72°C, and 5 min at 72°C	McTavish *et al.* (1993)

a. A GC clamp (5′-CCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGG-3′) (Muyzer *et al*., 1997) was attached to the 5′ end of primers Arch-amoAF and amo-1F.

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cific for the bacterial *amoA* gene, and the ArchamoAF and ArchamoAR set specific for archaeal *amoA* gene. The primer sequence and PCR protocols are listed in Table 1.

Denaturing gradient gel electrophoresis was carried out using a D-Code universal mutation detection system (Bio-Rad Laboratories) according to the instruction manual, and 6% (w/v) polyacrylamide [acrylamide-bisacrylamide (37.5:1)] gels containing denaturing gradients of 40–60% (100% denaturant containing 7 M urea and 40% formamide) for separation of PCR products. The gels were stained for 20 min in ethidium bromide and destained twice for 10 min in 1× TAE buffer [48.22 g of Tris base, 2.05 g of anhydrous sodium acetate, and 1.86 g of disodium EDTA·$2H_2O$ (pH 8) in 1 l of distilled H_2O] prior to UV transillumination.

The dominant bands in the DGGE gels were excised, and the acrylamide slices were crushed and re-suspended overnight at 4°C in 30 μl of sterile water to elute the DNA. Re-amplified PCR products were cloned to *E. coli* described as above and white colonies were selected for sequencing.

Sequences were compared with GenBank database sequences using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), and the closest matches were included in the alignment. DNA–DNA sequence similarity percentages were calculated using the sequence similarity matrix function as implemented in BioEdit (freeware by Tom Hall, Department of Microbiology, University of North Carolina). The neighbour-joining trees were constructed using MEGA version 3.1 [Molecular Evolutionary Genetics Analysis (http://megasoftware.net)] and bootstrapped 1000 times to calculate linear distances.

Sequences of DGGE bands have been deposited with Accession No. EU315701 to EU315711 (bacteria) and EU315724 to EU315734 (archaea). The archaeal *amoA* gene sequences of the clone library reported here have been deposited in GenBank under Accession No. EU315712 to EU315723.

Data analysis

Analysis of variance (one-way ANOVA) on plant biomass and archaeal and bacterial *amoA* gene copy numbers was performed using SPSS 11.5 (SPSS, Chicago, IL). If significant differences were detected ($P < 0.05$), comparisons of means were made using LSD (Least Significant Difference) test.

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