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ORIGINAL PAPER

Abundance and community structure of ammonia-oxidizing archaea and bacteria in an acid paddy soil

Xin Chen · Li-Mei Zhang · Ju-Pei Shen · Wen-Xue Wei · Ji-Zheng He

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Abstract Nitrification is essential to the nitrogen cycle in paddy soils. However, it is still not clear which group of ammonia-oxidizing microorganisms plays more important roles in nitrification in the paddy soils. The changes in the abundance and composition of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) were investigated by real-time PCR, terminal restriction fragment length polymorphism, and clone library approaches in an acid red paddy soil subjected to long-term fertilization treatments, including treatment without fertilizers (CT); chemical fertilizer nitrogen (N); N and potassium (NK); N and phosphorus (NP); N, P, and K (NPK); and NPK plus recycled crop residues (NPK+C). The AOA population size in NPK+C was higher than those in CT, while minor changes in AOB population sizes were detected among the treatments. There were also some changes in AOA community composition responding to different fertilization treatments. Still few differences were detected in AOB community composition among the treatments. Phylogenetic analysis showed that the AOA sequences fell into two main clusters: cluster A and cluster soil/sediment. The AOB composition in this paddy soil was dominated by *Nitrosospira* cluster 12. These results suggested that the AOA were more sensitive than AOB to different fertilization treatments in the acid red paddy soil.

Keywords Nitrification · Ammonia-oxidizing archaea · Ammonia-oxidizing bacteria · Acid paddy soil · Fertilization · Real-time PCR · T-RFLP · Clone library

Introduction

Irrigated rice fields are subjected to temporal or permanent flooding during the rice growth. The paddy soils are predominantly anaerobic. However, a thin oxic layer is formed in the surface soil due to gradual diffusion through the flooding water (Conrad and Rothfuss 1991). In addition, microaerobic niches are also present in the field due to oxygen leakage from rice roots (Revsbech et al. 1999). Thus, aerobic nitrification can occur in the flooding paddy soils. Nitrification is essential to the N cycle in paddy soils. It couples with denitrification and has been suggested as a significant source of N loss in the paddy soils.

Nitrification is the microbial oxidation of ammonia, first to nitrite and subsequently to nitrate. Traditionally, the first step of nitrification is mainly carried out by ammonia-oxidizing bacteria (AOB). All known AOB occurring in soils belong to β-*proteobacteria* represented by the genera *Nitrosospira* and *Nitrosomonas* (Purkhold et al. 2000). For a long time, it was believed that only AOB possess the *amoA* gene for ammonia monooxygenase, the key enzyme of ammonia oxidation (Rotthauwe et al. 1997). The occurrence of AOB has been investigated in the flooding

X. Chen · L.-M. Zhang · J.-P. Shen · J.-Z. He (✉)
State Key Laboratory of Urban and Regional Ecology,
Research Center for Eco-Environmental Sciences,
Chinese Academy of Sciences,
Beijing 100085, China
e-mail: jzhe@rcees.ac.cn

X. Chen
Graduate University, Chinese Academy of Sciences,
Beijing 100049, China

W.-X. Wei
Key Laboratory of Agro-ecological Processes in Subtropical Region, Institute of Subtropical Agriculture,
Chinese Academy of Sciences,
Changsha 410125, China

paddy soils, including surface soil, rhizosphere, and bulk soil, based on the *amoA* gene (Briones et al. 2002; Nicolaisen et al. 2004; Bowatte et al. 2006, 2007; Chen et al. 2008; Wang et al. 2009; Chu et al. 2010; Fujii et al. 2010). Many studies indicate that *Nitrosospira* are predominant in the paddy soils (Bowatte et al. 2006; Chen et al. 2008; Chu et al. 2010) while *Nitrosomonas* are prevalent in high-N fertilizer paddy soils or in the surface of rice roots (Briones et al. 2002; Nicolaisen et al. 2004).

In the last few years, the traditional view of ammonia oxidation has been changed by the discovery of the *amoA* gene associated with the domain archaea (Venter et al. 2004; Könneke et al. 2005; Treusch et al. 2005), suggesting that ammonia-oxidizing archaea (AOA) may be another group of ammonia oxidizers. The AOA are widespread in marine and terrestrial environments with high abundance values (Francis et al. 2005; Leininger et al. 2006; Wuchter et al. 2006; He et al. 2007; Shen et al. 2008; Schauss et al. 2009). Several studies also indicated that AOA outnumbered AOB in paddy soils (Chen et al. 2008; Wang et al. 2009; Fujii et al. 2010), but the contribution of AOA to nitrification in the paddy soil is still unclear. Recently, some researchers found that archaea were not important for nitrification in their tested soils (Di et al. 2009; Jia and Conrad 2009). However, other studies provided evidences for the important role of archaea in soil ammonia oxidation (Offre et al. 2009; Zhang et al. 2010). Especially, the AOA may be important actors within the N cycle in unfavorable environmental conditions, e.g., low nutrient availability, low pH or sulfide-containing environments (Valentine 2007; Erguder et al. 2009). It seems possible that the AOA could be an important player for nitrification in flooding acid paddy soils.

Some previous studies have shown that the abundance and community structure of ammonia-oxidizing microorganisms could change following different fertilization practices in upland soils (He et al. 2007; Shen et al. 2008). In this study, we hypothesized that long-term fertilization practices could result in the shifts of AOB and AOA population size and community structure in flooding acid paddy soils. Real-time PCR and terminal restriction fragment length polymorphism (T-RFLP) combined with clone library approaches based on *amoA* genes were used to characterize the abundance and community compositions of AOA and AOB in this study.

Materials and methods

Site description and soil sampling

The site was located at the Taoyuan Long-Term Experimental Station of Agro-ecosystem Observation, Chinese Academy of Sciences (28°55′ N, 111°26′ E), Hunan Province, China. It has a subtropical monsoon moist climate with a mean annual temperature of 16.5°C and precipitation of 1,447.9 mm. The paddy soil was derived from quaternary red soil and classified as a waterlogged paddy soil. The fertilization experiments started in 1990 and received fertilizers every year since then, including six treatments with three replicates for each treatment in a random plot design. The treatments were control without fertilizer (CT); chemical fertilizer nitrogen (N); N and potassium (NK); N and phosphorus (NP); N, P, and K (NPK); and NPK plus recycled crop residues (NPK+C). The annual input amounts of N, P, K, and C were reported in a previous publication (Zheng et al. 2008). The rotation of early rice and late rice was employed, and the early rice season was from April to July and the late rice season from July to October. The soil was flooded during most periods of the rice growth. Top soil samples (0–20 cm) were collected from each replicate plot in May 2009 when the rice was in the tillering stage and the soil was waterlogged. Five soil cores (approx. 5 cm diameter) were taken from each plot and were mixed to form one composite sample. All samples were divided into two parts: one was stored at 4°C for chemical analysis and another stored at −80°C for DNA extraction.

Chemical analysis

Soil pH was measured by a pH meter using a soil-to-water ratio of 1:2.5. Soil total N content was determined with an Elemental Analyzer (Vario EL III, Elementar, Germany). Soil ammonium and nitrate contents were extracted from fresh soil samples with 2 M KCl and determined by a Continuous Flow Analyser (SAN++, Skalar, Holland; Shen et al. 2008). Soil organic matter was measured using the $K_2Cr_2O_7$ oxidation–reduction titration method. Particle size distribution was measured using the rapid sieving procedure of Kettler et al. (2001).

Soil DNA extraction

DNA was extracted from 0.5 g (fresh weight) paddy soil using MoBio UltraClean™ Soil DNA isolation kit (San Diego, CA, USA). The extracted DNA was checked on 1% agarose gel and the concentration was determined with Nanodrop® ND-1000 UV–vis spectrophotometry (USA).

Quantification of *amoA* gene by real-time PCR

Abundances of AOA and AOB were determined by real-time PCR using an iCycler iQ5 Thermocycler (Bio-Rad, USA) with the fluorescent dye SYBR-Green I. Amplification was performed in 25-μl reaction mixtures, including

12.5 μl SYBR® Premix Ex Taq™ (Takara Biotechnology, Japan), 1 μl bovine serum albumin (25 mg ml^{-1}), 0.5 μl each primer (10 μM) listed in Table 1, and 2 μl DNA template (1–10 ng), as described by Zhang et al. (2009). The standards and the DNA samples were performed on the same plate. The results were analyzed with the iQ™ 5 software (Bio-Rad).

Terminal restriction fragment length polymorphism

For T-RFLP analysis, the PCR amplification was performed using primer pairs Arch-amoAF/Arch-amoAR and amoA1F/amoA2R (Table 1), with each forward primer labeled with 6-carboxyfluorescein (FAM; Ying et al. 2010). PCR reactions (50 μl) contained 5 μl 10× PCR buffer (Mg^{2+} plus), 4 μl 2.5 mM dNTPs, 0.5 μl *Ex Taq* HS polymerase (5U μl^{-1}, Takara Biotechnology), 1 μl of each primer, and 2 μl DNA template (1–10 ng). A touchdown PCR strategy was employed for the amplification, consisting of an initial denaturation step at 95°C for 3 min, ten cycles of 95°C for 30 s, 60°C (for AOA) or 62°C (for AOB, decreasing by 0.5°C per cycle) for 45 s, 72°C for 1 min, followed by 30 cycles of 95°C for 30 s, 55°C (for AOA) or 57°C (for AOB) for 45 s, 72°C for 1 min, and a final elongation step of 72°C for 10 min. The labeled PCR products were gel-purified with the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and then digested with the restriction enzyme *Mbo*I (Takara Biotechnology) at 37°C for 3 h. The digestion products were precipitated with 0.1 volume of sodium acetate (3 M, pH 5.2) and 3 volumes of ice-cold ethanol at −20°C overnight. After centrifugation at 16,000×*g* for 30 min, the pellets were washed with 70% ice-cold ethanol and then air dried. The pellets were then dissolved in 10 μl of sterile MilliQ water. The purified digestion products were mixed with deionized formamide and internal standard GeneScan™-500 LIZ (Applied Biosystems) at 95°C for 3 min and then determined with an ABI PRISM® 3130XL Genetic Analyzer (Applied Biosystems; Wang et al. 2009). The electropherograms were analyzed using Genescan analysis software v3.7 (Applied Biosystems). The relative abundance of the individual terminal restriction fragment (T-RF) was calculated as the percentage of total peak area in a given T-RFLP profile. Only those T-RFs with a relative abundance >1% and fragment lengths in the range of 30–500 bp were considered in the analyses.

Cloning and sequence analysis

To identify the T-RFs, three AOA clone libraries and three AOB clone libraries were constructed from CT, NK, and NPK+C by the primer pairs Arch-amoAF/Arch-amoAR and amoA1F/amoA2R (Table 1) without FAM labeling. The PCR conditions, reaction mixtures, and purification were the same as T-RFLP analysis. The purified PCR products were ligated into the pGEM-T Easy Vector (Promega) and then transformed into *Escherichia coli* JM109 (Takara Biotechnology) according to the manufacturer's instructions. Random positive clones (about 40–60) were selected from these clone libraries and sequenced.

The obtained sequences were subjected to homology analysis with the software DNAMAN, version 6.0.3.48 (Lynnon Biosoft, USA). The sequences displaying more than 97% identity with each other were grouped into the same operational taxonomic units (OTUs). Only one representative sequence of each OTU was used for phylogenetic tree construction. The GenBank sequences most similar to representative sequences in this study and reference sequences for defining clusters were included in phylogenetic tree construction. Phylogenetic analysis was performed using MEGA, version 4.0, and the neighbor-joining tree was constructed using *p* distance with 1,000 replicates to produce bootstrap values (Tamura et al. 2007).

The sequences determined in this study have been deposited in GenBank nucleotide sequence database with accession numbers HQ215911–HQ215930.

Statistical analysis

The copy numbers of *amoA* gene were log-transformed prior to statistical analysis. Principle component analysis (PCA) was performed using CANOCO for Windows, version 4.5, to determine the variation of T-RFLP profiles among the treatments. The relative abundances of T-RFs were directly used in PCA. The statistical analyses were performed using ANOVA and paired-sample *t* test with SPSS for Windows. If significant differences were detected

Table 1 Primers used for molecular analyses

Target group	Primer	Sequence(5′–3′)	Length of amplicon (bp)	Reference
AOB	amoA1F amoA2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	491	Rotthauwe et al. (1997)
AOA	Arch-amoAF Arch-amoAR	STAATGGTCTGGCTTAGACG GCGGCCATCCATCTGTATGT	635	Francis et al. (2005)

($P<0.05$), the Student–Newman–Keuls test was used to check for quantitative differences between treatments.

Results

Soil chemical properties

There were some changes in soil pH values among the different fertilization treatments. The pH values in all treatments varied between 5.09 and 5.39 (Table 2). No significant differences in soil total N and organic matter were found among the treatments, except for NPK+C which had significantly higher contents than the other treatments (Table 2). Soil NH_4^+–N content in these treatments ranged from 27.08 to 85.91 mg kg^{-1} (Table 2). The highest NH_4^+–N content was in NPK+C and the lowest in CT. In addition, significant differences in soil NO_3^-–N contents were detected among all treatments (Table 2).

Abundance of AOA and AOB in the paddy soil

The abundance of AOB and AOA based on *amoA* genes were detected using real-time PCR. Some variations in the AOA abundance were observed in different fertilization treatments (Fig. 1). The archaeal *amoA* gene copy numbers varied from 8.31×10^7 to 2.12×10^8 copies per gram dry soil (Fig. 1). The AOA population size in NPK+C was higher than that in CT. No significant differences in the AOB abundance were observed among the treatments (Fig. 1). The average bacterial *amoA* gene copy numbers was 1.90×10^5 copies per gram dry soil in the paddy soil (Fig. 1). The ratios of AOA to AOB *amoA* gene copy numbers ranged from 417 to 2,383 in all treatments, indicating that the AOA abundance among the treatments was significantly higher than that of AOB (paired-sample *t* test, $P<0.05$). In addition, the correlation analysis indicated that the AOA abundance had a positive correlation with soil total N ($r=0.519$, $n=18$, $P<0.05$), organic matter ($r=0.486$, $n=18$, $P<0.05$), and NH_4^+–N content ($r=0.576$, $n=18$, $P<0.05$), while the AOB abundance had a positive correlation with soil pH ($r=0.521$, $n=18$, $P<0.05$) and NH_4^+–N content ($r=0.514$, $n=18$, $P<0.05$).

Community structure and phylogeny of AOA in the paddy soil

The AOA community structures from different fertilization treatments in the paddy soil were determined by T-RFLP. Four main T-RFs (330, 371, 422, and 444 bp) were present in all treatments, but they showed significant differences ($P<0.05$) in their relative abundance among fertilization treatments (Fig. 2). The T-RF of 371 bp was detected with the highest relative abundance (35%) in NPK+C treatment, while in other treatments, 444-bp T-RF was the dominating peak (31–40%). The T-RF of 330 bp was the lowest relative abundance in NPK and NPK+C treatments, while in other treatments, 330- and 371-bp T-RFs were both the lowest relative abundance. These community shifts were further investigated by PCA (Fig. 3). The first PCA axis, which is related to the main compositional variation, separated the communities of the different treatments. The main variation was mainly caused by changes in the relative abundance of the 371- and 444-bp T-RFs.

In order to identify the individual T-RF, clone libraries of archaeal *amoA* gene were constructed from CT, NK, and NPK+C treatments. Randomly selected clones were sequenced and these clones (with 50 clones for CT, 50 clones for NK, and 47 clones for NPK+C) characterized with T-RFLP analysis. The sequence data are shown in a phylogenetic tree, also indicating the length of the T-RF obtained for individual clones (Fig. 4). All AOA sequences fell into two main clusters: 80% are in cluster A and others are in soil/sediment. The clones with T-RFs of 371 and 422 bp were only assigned to cluster A, while those with T-RFs of 330 and 444 bp were detected in the two clusters.

Table 2 Basic properties of the paddy soil under different fertilization treatments

Treatment[a]	Sand (%)	Silt (%)	Clay (%)	pH (H_2O)	Total nitrogen (mg kg^{-1})	Organic matter (g kg^{-1})	NH_4^+–N (mg kg^{-1})	NO_3^-–N (mg kg^{-1})
CT	11	65	24	5.39±0.08b	180±10a	29.2±1.3a	27.1±1.3a	0.58±0.03a
N	11	66	23	5.26±0.10ab	188±23a	29.8±4.1a	53.5±3.6b	0.55±0.04a
NK	11	64	25	5.34±0.04b	190±29a	30.4±5.4a	55.8±6.6b	0.65±0.09ab
NP	13	65	24	5.23±0.03ab	198±39a	32.0±6.3a	46.3±5.5b	0.76±0.01bc
NPK	12	70	18	5.09±0.05a	200±28a	32.1±4.7a	46.6±1.5b	0.80±0.04c
NPK+C	12	69	19	5.30±0.14b	267±04b	41.6±0.8b	85.9±8.12c	0.93±0.11d

[a] Treatment: control without fertilizers (CT), fertilizer N (N), fertilizers NK (NK), fertilizers NP (NP), fertilizers NPK (NPK), fertilizers NPK plus recycled crop residues (NPK+C). Values are mean or mean±standard deviation ($n=3$). Values within the same column followed by the different letters indicate significant difference ($P<0.05$)

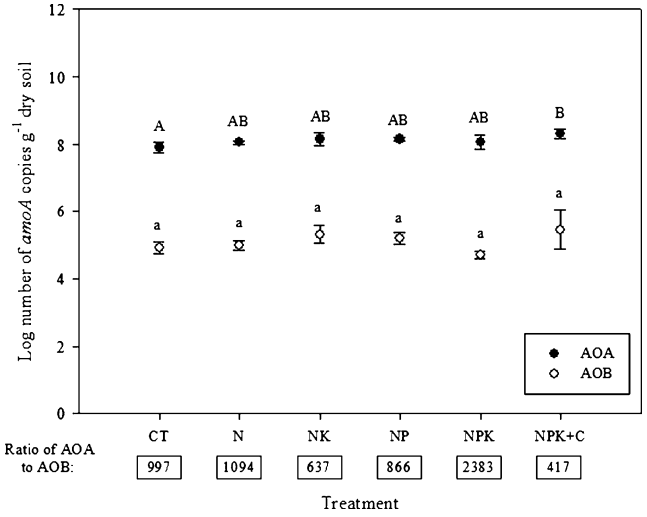

Fig. 1 Abundance of AOA and AOB based on *amoA* genes in the paddy soil under different fertilization treatments. *Different letters on the points* indicate significant difference ($P<0.05$). Ratios of AOA to AOB *amoA* gene copy numbers are shown at the *bottom of each treatment*. *CT* control without fertilizers, *N* fertilizer N, *NK* fertilizers NK, *NP* fertilizers NP, *NPK* fertilizers NPK, *NPK+C* fertilizers NPK plus recycled crop residues

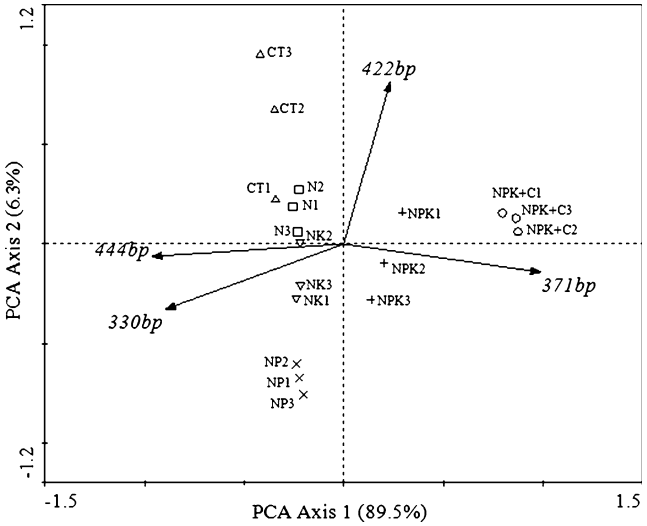

Fig. 3 Principle component analysis (*PCA*) of T-RFLP profiles using archaeal *amoA* gene T-RF relative abundance data obtained from the different fertilization treatments in the paddy soil. The percentages indicate the proportions of variation explained by the first and second ordination axes. *Symbols* indicate different treatments: CT treatment (*up-triangle*), N treatment (*square*), NK treatment (*down-triangle*), NP treatment (*X mark*), NPK treatment (*cross*), NPK+C treatment (*circle*). The *numbers in the labels* denote three replicates of each treatment. *Arrows* indicate the distribution of T-RFs. *CT* control without fertilizers, *N* fertilizer N, *NK* fertilizers NK, *NP* fertilizers NP, *NPK* fertilizers NPK, *NPK+C* fertilizers NPK plus recycled crop residues

Community structure and phylogeny of AOB in the paddy soil

Using T-RFLP to investigate the AOB community structures, only one dominant T-RF of 109 bp was detected in all treatments. Three AOB clone libraries with 127 clones from CT, NK, and NPK+C treatments were screened for the T-RF. Phylogenetic analysis revealed that most sequences with T-RF of 109 bp belonged to *Nitrosospira amoA* cluster 12 (Fig. 5). A few remaining sequences with the restriction site that was not found in the T-RFLP analysis were affiliated with *Nitrosospira amoA* cluster 11 (Fig. 5).

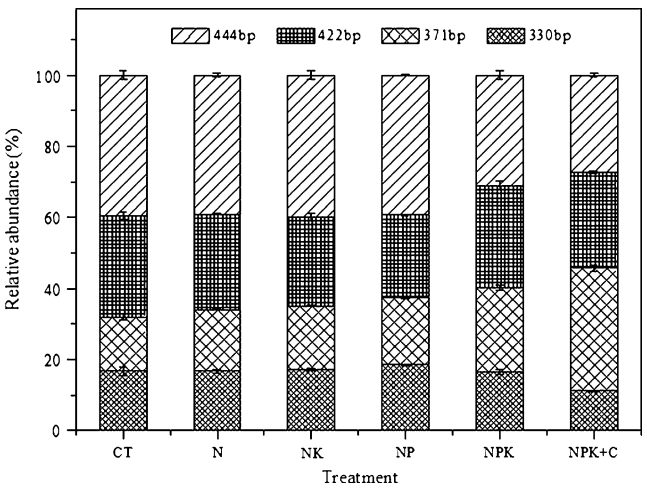

Fig. 2 Relative abundance of T-RFs for AOA in the paddy soil under different fertilization treatments. *Bars* represent the mean of three replicates with standard deviation. *CT* control without fertilizers, *N* fertilizer N, *NK* fertilizers NK, *NP* fertilizers NP, *NPK* fertilizers NPK, *NPK+C* fertilizers NPK plus recycled crop residues

Discussion

Effects of different fertilization treatments on soil AOA and AOB abundance

Although flooding paddy soil is predominantly anaerobic, we detected large numbers of AOA (8.31×10^7–2.12×10^8 copies per gram dry soil) in this acid paddy soil, similar to some upland soils (Shen et al. 2008; Ying et al. 2010). Previous studies also found high abundance of AOA in low-oxygen environments (Park et al. 2006; Lam et al. 2007; Santoro et al. 2008). In addition, long-term fertilizer application seemed to stimulate the AOA growth, especially the NPK+C. A previous study also found that the AOA population size changed greatly in respond to the different fertilization treatments (He et al. 2007). In that study, the changes of soil pH caused by long-term mineral fertilization played a main role in affecting AOA population size. However, in this study, the fertilizer itself as a substrate supply for AOA could play a more important role than the

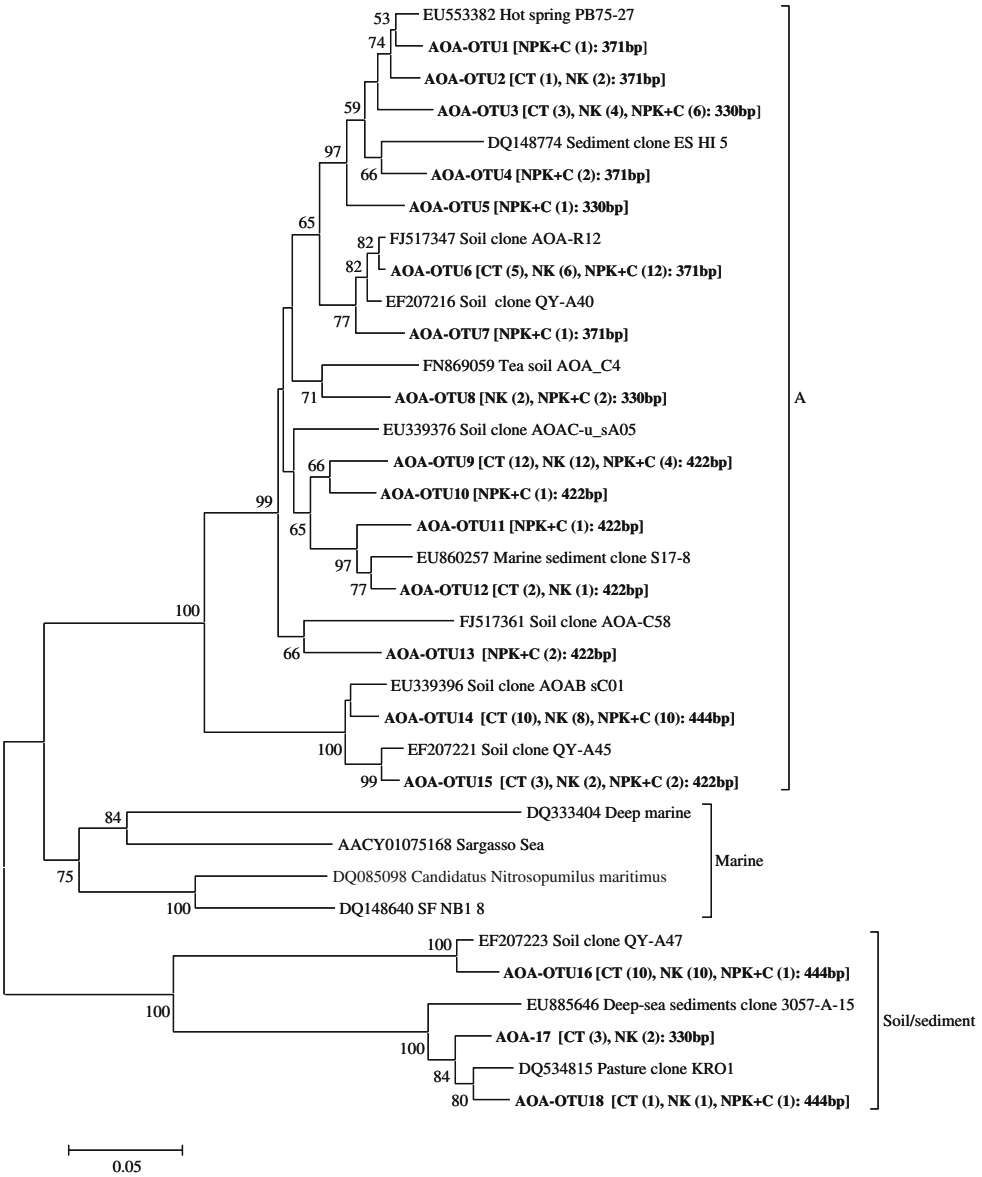

Fig. 4 Neighbor-joining phylogenetic tree of archaeal *amoA* sequences retrieved from CT, NK, and NPK+C fertilization treatments of the paddy soil. Sequences from this study are shown in *bold*. *Between brackets* Sequence number belonging to the OTU in each treatment and the corresponding T-RF size. Bootstrap values (>50) are indicated at *branch points*. The *scale bar* represents 5% estimated sequence divergence. *CT* control without fertilizers, *NK* fertilizers NK, *NPK+C* fertilizers NPK plus recycled crop residues

soil pH. Statistical analyses showed that the AOA abundance has a positive correlation with soil total N (r=0.519, n=18, P<0.05), organic matter (r=0.486, n=18, P<0.05), and NH_4^+–N content (r=0.576, n=18, P<0.05), further confirming the major effect of the fertilizer itself as a substrate.

In our study, the bacterial *amoA* copy numbers were on average 1.90×10^5 copies per gram dry soil in different fertilization treatments, similar to a recent report which found that the bacterial *amoA* gene copy numbers ranged from 1.0×10^5 to 9.3×10^5 copies per gram dry soil in the upland and flooded bulk soils of a Japanese rice paddy field over 2 years (Fujii et al. 2010). However, compared with some results of upland soils (He et al. 2007), the AOB abundance was lower, indicating that aerobic conditions may be an important factor determining the AOB population size in soil (Chu et al. 2010). Though the correlation analysis showed the AOB abundance to have a positive correlation with soil pH (r=0.521, n=18, P<0.05) and NH_4^+–N content (r=0.514, n=18, P<0.05), no significant differences in the AOB abundance were detected among the different fertilization treatments. This indicates that the pH and the substrate (NH_4^+–N) content could have some influence on the AOB abundance. However, as the low pH values and oxygen environment in all treatments were not suitable for the AOB growth (Prosser 1989), the effects of fertilizer application were not significant. In addition, compared with the AOA copy numbers, the AOB were also much lower, which were consistent with many studies of agricultural soils (Leininger et al. 2006; He et al. 2007; Shen et al. 2008). Therefore, these results may imply that the AOB growth was inhibited.

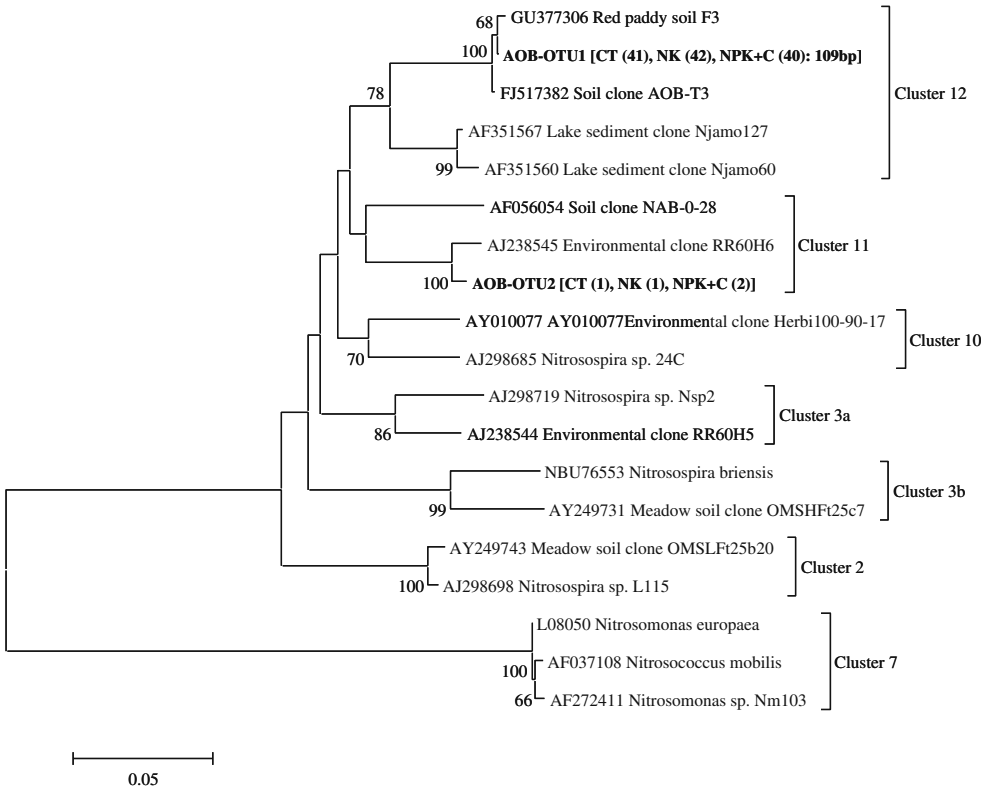

Fig. 5 Neighbor-joining phylogenetic tree of bacterial *amoA* sequences retrieved from CT, NK, and NPK+C fertilization treatments of the paddy soil. Sequences from this study are shown in *bold*. *Between brackets* Sequence number belonging to the OTU in each treatment and the corresponding T-RF size. Bootstrap values (>50) are indicated at *branch points*. The *scale bar* represents 5% estimated sequence divergence. *CT* control without fertilizers, *NK* fertilizers NK, *NPK+C* fertilizers NPK plus recycled crop residues

Effects of different fertilization treatments on soil AOA and AOB community structure

By using T-RFLP analysis and PCA, we found that there were some changes in AOA community composition responding to different fertilization treatments in the paddy soil, while few differences were detected in the AOB community. These results were consistent with those of He et al. (2007) in upland of acid red soil. Ying et al. (2010) also found the shift of AOA to cultivation in acid red soils. Thus, it could be hypothesized that AOA were more sensitive than AOB in acid red soils, irrespective of paddy soil and upland soil.

Phylogeny of AOA and AOB in the paddy soil

The sequences of AOA obtained from this study were associated with two distinct clusters (cluster A and cluster soil/sediment), and the majority of sequences were placed within cluster A. In a previous study, cluster A was defined as sediment and soil V, which was distinct from soil and marine clusters (Nicol et al. 2008). It was also interesting to note that the sequences (this study and references) which were placed in cluster A were all cloned from a low pH environment. Other sequences in our study fell into soil/sediment cluster, which were closely related to the sequences commonly obtained from soil and marine sediments.

Phylogenetic analyses of AOB identified that *Nitrosospira*-like species dominated in this study, as already shown in paddy soils (Bowatte et al. 2006; Chen et al. 2008; Chu et al. 2010). It has been reported that the AOB in the Japanese paddy field was dominated by *Nitrosospira amoA* cluster 1 (Bowatte et al. 2006; Chu et al. 2010). Other researches also detected *Nitrosospira amoA* cluster 3 in the paddy soils (Chen et al. 2008; Wang et al. 2009). However, in this study, most AOB sequences contained T-RF of 109 bp, which belongs to *Nitrosospira amoA* cluster 12, and few sequences grouped in *Nitrosospira amoA* cluster 11, which were not commonly reported in previous reports. Although *Nitrosospira amoA* clusters 1, 2, 3, and 4 have been identified in acidic forest soils or fertilized upland soils (Laverman et al. 2001; Mintie et al. 2003; Compton et al. 2004; Nugroho et al. 2005; He et al. 2007; Boyle-Yarwood et al. 2008), we did not detect those clusters, suggesting that acid red paddy soil may have the unique *amoA* lineage.

Conclusions

In conclusion, this study found that the AOA abundance and community composition had some changes among the fertilization treatments in the paddy soil, but few differences were observed in the AOB, suggesting that AOA

were more sensitive to different fertilization treatments in the acid red paddy soil than AOB.

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