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Conversion of upland to paddy field specifically alters the community structure of archaeal ammonia oxidizers in an acid soil

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Abstract. The function of ammonia-oxidizing archaea (AOA) and bacteria (AOB) depends on the major energy-generating compounds (i.e., ammonia and oxygen). The diversification of AOA and AOB communities along ecological gradients of substrate availability in a complex environment have been much debated but rarely tested. In this study, two ecosystems of maize and rice crops under different fertilization regimes were selected to investigate the community diversification of soil AOA and AOB upon conversion of an upland field to a paddy field and long-term field fertilization in an acid soil. Real-time quantitative polymerase chain reaction of ammonia monooxygenase (*amoA*) genes demonstrated that the abundance of AOA was significantly stimulated after conversion of upland to paddy soils for more than 100 yr, whereas a slight decline in AOB numbers was observed. Denaturing gradient gel electrophoresis fingerprints of *amoA* genes further revealed remarkable changes in the community compositions of AOA after conversion of aerobic upland to flooded paddy field. Sequencing analysis revealed that upland soil was dominated by AOA within the soil group 1.1b lineage, whereas the marine group 1.1a-associated lineage predominated in AOA communities in paddy soils. Irrespective of whether the soil was upland or paddy soil, long-term field fertilization led to increased abundance of *amoA* genes in AOA and AOB compared with control treatments (no fertilization), whereas archaeal *amoA* gene abundances outnumbered their bacterial counterparts in all samples. Phylogenetic analyses of *amoA* genes showed that *Nitrosospira* cluster-3-like AOB dominated bacterial ammonia oxidizers in both paddy and upland soils, regardless of fertilization treatment. The results of this study suggest that the marine group 1.1a-associated AOA will be better adapted to the flooded paddy field than AOA ecotypes of the soil group 1.1b lineage, and indicate that long-term flooding is the dominant selective force driving the community diversification of AOA populations in the acid soil tested.

1 Introduction

Ammonia-oxidizing archaea (AOA) and bacteria (AOB) play central roles in the biogeochemical cycle of nitrogen. Ammonia is converted to nitrite by AOA and/or AOB and subsequently to nitrate by nitrite oxidizers. Ammonia oxidation is the first and rate-limiting step of nitrification that plays an important role in the global nitrogen cycle (Kowalchuk and Stephen, 2001). For many years, it was believed that microbial ammonia oxidation was solely performed by bacteria and that only bacteria possess the *amoA* gene, which encodes the alpha subunit of ammonia monooxygenase, the key enzyme of nitrification. Recent discoveries have expanded the known ammonia oxidizers from the domain Bacteria to Archaea (Könneke et al., 2005; Treusch et al., 2005). Comparative genomic analysis has indicated that, although AOA and AOB differ greatly in metabolic pathways (Walker et al., 2010; Spang et al., 2012) and physiology (Martens-Habbena et al., 2009; Park et al., 2010; Tourna et al., 2011), they are functionally closely related. Both AOA and AOB derive energy from ammonia oxidation to support their growth (Lehtovirta-Morley et al., 2011; Tourna et al., 2011).

Previous research has indicated that the relative contributions of AOA and AOB to nitrification vary greatly in physicochemically distinct soils (Jia and Conrad, 2009; Zhang et al., 2010). It is generally recognized that AOA outcompete AOB in abundance up to 3000-fold in the soil environment (Leininger et al., 2006). Intensive studies have demonstrated that environmental factors have shaped the AOA community structures (Erguder et al., 2009). For example, a recent discovery has suggested that the remarkably low concentration of ammonia in acid soil could provide greater ecological advantages to AOA versus AOB communities (Lehtovirta-Morley et al., 2011; Yao et al., 2011; Levičnik-Höfferle et al., 2012). However, the ecological force that drives diversification of AOA and/or AOB remains largely unclear, particularly in complex environments, including anthropogenically disturbed ecosystems. The dynamic changes in AOA and AOB community structures along ecological gradients may provide strong hints regarding the divergence of ammonia oxidizer communities and their relative roles in nitrification.

A long-term field fertilization experiment can serve as a model system to investigate niche specialization of AOA and/or AOB communities in response to ammonia substrate availability in a complex environment under in situ conditions. A number of studies have shown that long-term fertilization significantly alters the community structure of AOB rather than AOA in alkaline soil (Chu et al., 2007; Shen et al., 2008; Wu et al., 2011), whereas in acid soils, AOA rather than AOB communities respond positively to field fertilizations (He et al., 2007; Wessén et al., 2010b). Regarding oxygen stress, it is interesting to note that shaking abolished the growth of AOA in pure culture, suggesting that AOA may be better adapted to life under low oxygen (Könneke et al., 2005). Indeed, a molecular survey indicated that AOA were 30 times less abundant than AOB in the oxic zone and 10 times more abundant in low-oxygen environments (Santoro et al., 2008). Moreover, the existence of archaeal *amoA* genes was demonstrated in activated sludge with low levels of dissolved oxygen (Park et al., 2006), and in the water bodies of pelagic oxygen minimum zones (Beman et al., 2008). It appears that AOA might be more suited to low-oxygen environments than AOB. However, the extent to which oxygen availability could have shaped the diversification of AOA and/or AOB communities in the natural environment remains unclear.

In addition to ammonia and oxygen fluctuations, a wide variety of environmental factors are thought to govern the community structures of AOA and AOB, including temperature (Andert et al., 2011; Tourna et al., 2008), moisture (Horz et al., 2004; Gleeson et al., 2010), soil organic carbon (Wessén et al., 2010b; Innerebner et al., 2006) and pH (He et al., 2007; Nicol et al., 2008; Wessén et al., 2010a). It has also been demonstrated that plant species can alter the soil microbial community (Garbeva et al., 2004) and the ammonia oxidizers (Mao et al. 2011; Chu et al., 2009). Moreover, it has been reported that bacterial ammonia oxidizers must compete with heterotrophic bacteria for limiting amounts of ammonium (Van Niel et al., 1993; Verhagen and Laanbroek, 1991) in natural environments. However, contradictory findings are often reported regarding the responses of ammonia oxidizers to environmental changes. It remains largely unknown to what extent soil heterogeneity may determine the community diversification of AOA and AOB in a complex environment.

Increasing lines of evidence have suggested important roles for AOA in acid soil (Prosser and Nicol, 2012). This could be explained by the exceptionally high affinity of AOA for ammonia substrate, levels of which are too low in acid soil to support the growth of AOB (Lehtovirta-Morley et al., 2011; Lu et al., 2012). However, very little information is available regarding the effect of oxygen supply on AOA community changes in a complex environment. Therefore, two agroecosystems of maize and paddy rice crops under different long-term field fertilizations of > 25 yr were selected to investigate the community diversification of AOA and AOB in acid soils. The paddy field has a cultivation history of > 100 yr and was converted from upland soil that is approximately 150 m away from the maize field. Both paddy and maize soils originated from the same parent material and are exposed to similar meteorological conditions. Conversion of upland acid soil to paddy field can result in oxygen depletion and changes in many other soil properties, including levels of redox-sensitive nutrients such as iron and soil organic carbon, which may alter the community structure of ammonia oxidizers in soil.

2 Materials and methods

2.1 Site description and soil sampling

The soil samples used in this study were collected from the Ecological Experimental Station of Red Soil, Chinese Academy of Sciences, which is located in Yingtan, Jiangxi Province, China (28°15′ N, 116°55′ E). The region has a typical subtropical monsoon climate with a mean annual precipitation of 1727 mm, mean annual evaporation of 1318 mm and a mean annual temperature of 18.1 °C with approximately 262 days without frost per year. Both paddy and upland soils originated from quaternary red clay with dominant kaolinitic minerals and were classified as a Haplic Stangnic Anthrosol according to USDA soil taxonomy. The long-term field fertilization experiment of upland soil was initiated in 1986 approximately 150 m from the paddy field experiment site. The paddy field was converted from upland soil and had a history of rice (*Oryza sativa* L.) cultivation for more than 100 yr before the initiation of the long-term field fertilization experiment in 1981. The crop rotation system for upland soils includes early maize (*Zea mays* L.) from April to July, late maize from July to October and winter fallow from

November to April of the subsequent year. The paddy field is used for early rice (April to July), late rice (July to November) and winter fallow without a floodwater layer (November to April of the subsequent year).

Both long-term experimental fields received 9 different fertilization treatments consisting of (1) a no fertilization control (CK); (2) chemical nitrogen fertilization (N); (3) chemical phosphorus fertilization (P); (4) chemical potassium fertilization (K); (5) a combination of chemical N and P (NP); (6) a combination of chemical N and K (NK); (7) a combination of chemical N, P and K (NPK); (8) a double dose of chemical N, P and K (2NPK); and (9) a combination of chemical N, P and K fertilizer plus organic manure (NPK + OM). Each treatment was conducted in triplicate plots with a randomized design, and the application rates of chemical fertilizers and organic manures were slightly different between the paddy field and upland maize experiments. The chemical fertilizers N, P and K were applied to the paddy field at rates of 90, 45 and 75 kg ha^{-1} for both early and late rice cultivations, respectively. Milk vetch and pig manure were used as organic manure for early and late rice, respectively, at 22.5 Mg ha^{-1} (fresh weight). Approximately 66 % of N, 100 % of P, 100 % of K, and 100 % of organic manure were applied as basal fertilizers prior to rice transplantation. The remaining nitrogen fertilizer was applied as a top-dressing at the panicle initiation stage and 10 days before the flowering stage. In the upland maize cropping system, the chemical fertilizers N, P and K were applied at the rates of 60, 30, and 60 kg ha^{-1}, respectively, for each season. One additional organic manure fertilization treatment was established only for the upland field. Pig manure was used exclusively as an organic fertilizer for the upland maize fields at 15 Mg ha^{-1} (fresh weight) for each season and was applied as the basal fertilizer before maize seeding. For both paddy and maize fields, N, P and K were provided in the forms of urea, calcium-magnesium phosphate and potassium chloride, respectively.

Sampling was performed in April of 2010 for upland soil at a 0–20 cm depth shortly before maize cultivation, and paddy soil was collected at a 0–15 cm depth in November of 2010 after harvesting of late rice. Five cores were randomly obtained from each of the three replicate field plots for each treatment. Soil samples were placed in a sterile plastic bag, sealed, brought back to the laboratory on ice within 1 day, and sieved through 8 mm mesh. A composite sample was obtained via homogenization of five cores from each of the three replicate field plots. A total of 27 and 30 soil composite samples were subsequently analyzed from long-term fertilized paddy fields and maize crops, respectively. Soil pH was determined with a Mettler Toledo 320-S pH meter (Mettler-Toledo Instruments Co. Ltd., Shanghai, China) using a soil-to-water ratio of 1 : 5. Soil organic carbon and total N were determined by the dichromate oxidation method (Mebius, 1960) and the Kjeldahl digestion method (Bremner, 1965), respectively. Soil samples used for molecular analysis were stored at −80 °C until further use.

2.2 Soil DNA extraction and real-time quantitative polymerase chain reaction (PCR)

DNA was extracted from approximately 0.5 g of soil using the bead-beating method as previously reported (Griffiths et al., 2000) with slight modifications. Cell lysis was accomplished via vigorous shaking in a bead beater at speed 6.0 for 40 s. Three consecutive extractions were performed for each soil sample to maximize the recovery efficiency of microbial genomic DNA. Microbial genomic DNA was obtained from triplicate soil samples, and DNA pellets were washed using 5.5 M guanidine thiocyanate solution to remove contaminating humic substances. The purified DNA was eluted with 100 µL of TE buffer. The DNA quality and quantity were determined using a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). The soil DNA was stored at −20 °C until use.

Real-time quantitative PCR (qPCR) was performed to determine the copy numbers of the *amoA* genes using the primer sets Arch-amoAF/Arch-amoAR for AOA (Francis et al., 2005) and amoA-1F/amoA-2R-GG for AOB (Rotthauwe et al., 1997) with a CFX96 Optical Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA). The qPCR standard was generated using plasmid DNA (pEASY-T1 plasmid vector; EU233623) from representative clones containing the bacterial or archaeal *amoA* gene. A dilution series of a standard template across six orders of magnitude (1.32×10^2 to 1.32×10^8 for AOB and 1.51×10^2 to 1.51×10^8 for AOA) per assay was used to optimize the qPCR conditions. A blank was always run with water as a template. Each 20 µL reaction mixture contained 10.0 µL of SYBR Premix Ex Taq (TaKaRa Biotech, Dalian, China), 0.25 μM each primer, and 2 µL of DNA template. The PCR conditions used to amplify the archaeal and bacterial *amoA* genes were the same as previously described (Lu et al., 2012). The PCR amplification efficiencies for AOA and AOB in upland soils were 99.2 % with an R^2 value of 0.987 and 96.7 % with an R^2 value of 0.990, respectively. In paddy soil, PCR amplification efficiencies of 100.8 % and 95.8 % were obtained for the *amoA* genes of AOA and AOB with R^2 values of 0.998 and 0.994, respectively. The specific amplification of *amoA* was also determined using melting curve analyses, which always resulted in a single peak.

2.3 Polymerase chain reaction-denaturing gradient gel electrophoresis

For the denaturing gradient gel electrophoresis (DGGE) analysis, PCR amplification of the archaeal *amoA* gene was performed using the primer sets CrenamoA23f and CrenamoA616r (Nicol et al., 2008; Lehtovirta-Morley et al., 2011). The PCR reaction was performed in a 25 µL

volume containing 2.5 µL of 10 × PCR buffer, 0.25 µM each primer, 200 µM deoxyribonucleoside triphosphate, 1.5 U of Taq DNA polymerase, and 1 µL of soil DNA. The PCR was performed in a Thermal Cycler Dice (Takara Bio, Shiga, Japan) as previously described (Lu et al., 2012). The PCR products were run on a 1.5 % agarose gel to determine the amplification specificity, and the quantity of amplicons was spectrophotometrically determined. Despite the use of a range of degenerate primers and PCR conditions (Stephen et al., 1999; Nicolaisen and Ramsing, 2002), bacterial *amoA* genes could not be amplified when the PCR primer contained a GC clamp, which is a prerequisite for subsequent DGGE analysis. Therefore, the composition of AOA was analyzed by DGGE fingerprinting, and a clone library was constructed for analysis of AOB communities.

Approximately 150 ng of PCR amplicons of archaeal *amoA* genes from each sample were separated by DGGE gels using the D-Code System (Bio-Rad Laboratories, Hercules, CA) as described previously (Lu et al., 2012). PCR products were run on 6 % acrylamide gels with a denaturing gradient of 20–50 % (100 % denaturant corresponds to 7 M urea and 40 % deionized formamide). A 1 mm-thick gel with 30 wells was poured from bottom to top using a gradient former and a peristaltic pump run at a speed of 4.5 mL min^{-1}. A 5.0 mL stacking gel containing no denaturants was subsequently added on top before polymerization, and a comb was inserted to form wells before the addition of gel. The gels were run in 1 × TAE at 75 V for 17 h, stained with SYBR Green I dye for 30 min and scanned with a Molecular Imager (Gel Doc System, BIO-RAD Laboratories, Hercules, CA). The DGGE images were analyzed using Quantity One software (BIO-RAD Laboratories, Hercules, CA).

The distinct DGGE bands of archaeal *amoA* genes of AOA were excised and reamplified for sequencing analysis. The PCR conditions were the same as those mentioned above for reamplification of 9 *amoA* bands from upland soil and of 12 *amoA* bands from paddy soil. The purified PCR products were cloned using the pEasy-T1 cloning kit (TransGen Biotech Co., Beijing). The clones that contained the correct insert were selected and sequenced using an ABI 3730 XL DNA analyzer (Beijing Genomics Institute, Shenzhen, China).

2.4 Clone library of bacterial *amoA* genes

For AOB, the PCR products of *amoA* genes were obtained directly from the different treatments for clone library construction. For upland soil, NPK, NPK + OM, 2NPK and OM treatments were used to construct the clone library; for paddy soil, N, NPK, NPK + OM and 2NPK treatments were used. PCR amplification of the bacterial *amoA* gene was performed using the primer set amoA-1F/amoA-2R-GG for AOB (Rotthauwe et al., 1997). The PCR reaction was performed in a 25 µL volume containing 2.5 µL of 10 × PCR buffer, 0.25 µM each primer, 200 µM deoxyribonucleoside triphosphate, 1.5 U of Taq DNA polymerase, and 1 µL of soil DNA. The PCR was performed in a Thermal Cycler Dice (Takara Bio, Shiga, Japan) as previously described for AOB (Nicolaisen and Ramsing, 2002). The purified PCR products were cloned, and the clones that contained the correct insert were selected for sequencing as described for AOA. At least 10 clones were randomly selected for each treatment. The obtained sequences were subjected to homology analysis using mothur software (Schloss et al., 2009). Sequences displaying more than 97 % identity were grouped into the same operational taxonomic unit (OTU).

2.5 Phylogenetic analysis

The sequences of the DGGE bands of AOA and one representative sequence of each OTU for AOB as well as their closest relatives (obtained by BLAST analyses; http://blast.ncbi.nlm.nih.gov/Blast.cgi) were aligned using CLUSTAL X 1.83 (Thompson et al., 1997). Phylogenetic trees were constructed using the neighbor-joining method based on the Jukes–Cantor correction algorithm of the software package MEGA (Molecular Evolutionary Genetics Analysis) version 4 (Tamura et al., 2007). Bootstrap values were calculated based on 1000 replications.

2.6 Statistics

Spearman's correlation analyses were performed to assess the relationships among soil properties and the abundance of AOA and AOB (SPSS 11.5 package, SPSS, Chicago, IL). One-way ANOVA with Duncan's post-hoc test was performed to evaluate the differences within the datasets, and a P value of 0.05 was used to determine significance. Cluster analysis of DGGE fingerprints of archaeal *amoA* genes was performed via the unweighted pair group method with an arithmetic mean clustering method using Quantity One software (BIO-RAD). This software was also used to perform a density profile of the DGGE bands in different lanes. The diversity of the microbial communities was then analyzed by richness (S), Shannon indices (H), and evenness (E_H) methods according to the following equations (Zak et al., 1994):

$$H = -\sum_{i=1}^{s} p_{\mathrm{i}} \ln p_{\mathrm{i}}$$

and

$$E_H = H/H_{\max} = H/\ln S.$$

Here, p_{i} is the intensity ratio of a specific DGGE band to the total DGGE bands in a single lane, and S is the total number of bands in each lane sample.

2.7 Nucleotide sequence accession numbers

The sequences obtained in this study were deposited in GenBank and assigned the accession numbers KC568412 to

KC568432 for the archaeal *amoA* genes and KC568433 to KC568444 for the bacterial *amoA* genes

3 Results

3.1 Soil properties

Long-term application of chemical N fertilizers resulted in an apparent decrease in pH values from 3.98 in control plots down to 3.64 in N treatments of upland soils. The treatment amended with organic matter (NPK + OM) and OM alone had the highest pH, whereas soil pH showed a decreasing trend in the field plots that only received chemical N fertilizers (Table 1). The stimulation of soil pH was observed in both field plots amended only with P and K fertilizers. Soil organic carbon (SOC) appeared to be elevated in fertilizer-amended plots, particularly in those receiving organic manure treatments. Soil total nitrogen remained largely unchanged among all treatments. Similar results were obtained for paddy soils, although long-term field fertilization affected soil properties to a lesser extent in paddy soils compared with upland soils. It is noteworthy that the conversion of upland soil to paddy field significantly stimulated both pH and SOC irrespective of fertilization treatments.

3.2 Change in the abundance of ammonia oxidizers upon long-term field flooding

The abundances of AOA and AOB were determined by qPCR targeting the *amoA* genes (Fig. 1). Long-term field flooding appeared to have strong selective advantage for AOA compared with AOB (Table 1). The copy number of archaeal *amoA* genes was significantly higher than their bacterial counterparts in all paddy soil treatments. For example, the ratio of AOA to AOB varied from 7.13 to 16.1 in upland soil, whereas this ratio was significantly elevated from 50.9 to 75.6 in paddy soil. The ratio of upland to paddy soil AOA ranged from 0.18 to 0.32. In contrast, AOB abundance was generally higher in upland versus paddy soil. The mean number of bacterial *amoA* gene copies was 1.73×10^7 per gram of dry weight soil (g^{-1} d.w.s) for the upland soil, whereas 1.10×10^7 g^{-1} d.w.s was observed in the paddy soil. Thus, the average ratio of upland to paddy soil AOB was 1.37 ± 0.49 for all 9 treatments (Table 1).

3.3 Change in the abundance of ammonia oxidizers upon long-term field fertilization

The population sizes of AOA and AOB varied greatly among different long-term field fertilization regimes. The archaeal *amoA* gene copy numbers varied from 8.79×10^7 to 2.80×10^8 g^{-1} d.w.s for upland soil, whereas these numbers ranged from 4.62×10^8 to 9.93×10^8 for paddy soil. Regardless of whether the soil was upland or paddy soil, the NPK + OM treatment had the highest copy numbers of archaeal *amoA* genes among the treatments. The lowest gene copy numbers were detected in the CK (8.79×10^7 g^{-1} d.w.s) and P treatments (4.62×10^8 g^{-1} d.w.s) in upland and paddy soil, respectively. Compared to the control field, which was amended without fertilizers, the *amoA* gene abundances of AOA showed an increasing trend in fertilized plots of both upland (Fig. 1a) and paddy soils (Fig. 1b); however, no statistically significant differences were noted among most fertilized soils.

Similar results were observed for bacterial *amoA* genes in response to long-term field fertilization regardless of whether the soil was paddy or upland soil. For upland soil, the CK treatment showed the lowest copy number (6.69×10^6 g^{-1} d.w.s), and the highest number was observed in the NPK + OM treatment (3.32×10^7 g^{-1} d.w.s.) (Fig. 1c). Long-term field fertilization increased bacterial *amoA* gene copies to different extents. In paddy soil, the CK treatment had the lowest bacterial *amoA* gene copy number (7.23×10^6 g^{-1} d.w.s), which was statistically similar to that of the P treatment (9.08×10^6 g^{-1} d.w.s) (Fig. 1d). The highest bacterial *amoA* gene copy number (1.46×10^7 g^{-1} d.w.s) was detected in the NPK + OM treatment, which was statistically similar to those of the NPK (1.27×10^7 g^{-1} d.w.s) and 2NPK (1.30×10^7 g^{-1} d.w.s) treatments.

Statistical analysis revealed a positive correlation between AOA abundance and soil organic carbon ($\rho = 0.45$, $n = 30$, $P < 0.05$). The population size of AOB was positively related to soil total N ($\rho = 0.51$, $n = 30$, $P < 0.01$), and soil organic carbon ($\rho = 0.83$, $n = 30$, $P < 0.01$) in the upland soil. In paddy soil, a strong positive correlation was obtained between AOA abundance, soil total N ($\rho = 0.47$, $n = 27$, $P < 0.05$) and soil organic carbon ($\rho = 0.57$, $n = 27$, $P < 0.01$), whereas AOB abundance was positively correlated with soil organic carbon ($\rho = 0.54$, $n = 27$, $P < 0.01$).

3.4 Changes in the composition of ammonia oxidizers upon long-term field flooding

The composition of AOA communities in both upland and paddy soils was revealed by fingerprinting analysis of archaeal *amoA* genes in the triplicate field plots of all treatments. The conversion of aerobic upland to flooded paddy soils significantly altered the compositions of AOA communities based on pairwise comparisons of DGGE fingerprints of archaeal *amoA* genes in soil (Fig. 2). AOA communities in upland soil were dominated by the DGGE bands UP-1 and UP-2, which were affiliated with the soil group 1.1b lineage (Fig. 3). More DGGE bands were observed in paddy versus upland soils, suggesting the stimulation of AOA stability via long-term field flooding and rice cultivation. The DGGE bands PS-1, PS-3 and PS-4 were placed within the marine group 1.1a-associated lineage, whereas band PS-2 and band PS-5 fell well within the soil group 1.1b lineage (Fig. 3).

PCR amplicons of bacterial *amoA* genes could not be obtained when a GC clamp was attached to the primers for

Table 1. Soil properties and ratios of AOA and AOB under different long-term field fertilization treatments.

Treatments	pH (H_2O)		SOC ($g\,kg^{-1}$)		TN ($g\,kg^{-1}$)		Ratios of AOA to AOB		AOA ratio upland	AOB ratio upland
	Upland	Paddy	Upland	Paddy	Upland	Paddy	Upland	Paddy	to paddy	to paddy
CK	3.98cd	5.2ab	7.98d	20.1bc	1.03ab	2.05b	13.2	64.1	0.19	0.92
N	3.64e	5.16ab	8.82bcd	20.3bc	1.06ab	2.02b	14.7	75.6	0.18	0.94
P	4.21b	5.29ab	8.54cd	19.9c	0.85b	1.96b	16.1	50.9	0.32	1.01
K	4.04c	5.44a	8.58cd	20.0c	0.88b	1.93b	15.8	69.1	0.19	0.83
NP	3.89d	5.07b	8.93bcd	21.0bc	0.91b	2.06b	7.8	68.5	0.20	1.77
NK	3.68e	5.20ab	9.56bc	20.5bc	0.95ab	2.02b	9.3	63.5	0.20	1.36
NPK	3.90d	5.14ab	9.87b	20.1bc	1.04ab	2.05b	8.38	62.1	0.20	1.50
NPK + OM	4.83a	5.38ab	12.1a	25.7a	1.17a	2.51a	8.44	68.0	0.28	2.27
2NPK	3.70e	5.09b	9.76b	21.1b	0.97ab	1.96b	7.13	57.9	0.21	1.69
OM	5.07a	–	10.72ab	–	1.14a	–	7.73	–	–	–

Note: The treatments employed were control without fertilizers (CK) and various fertilization regimes including chemical fertilizers and/or organic manure (OM) as follows: N, P, K, NP, NK, NPK, NPK + OM, and OM. The designation "2NPK" indicates that the application rate was twice that of NPK in the field plots. Values are the means of the three replicated field plots. Different letters following values within the same column indicate significant differences among the treatments ($P < 0.05$).

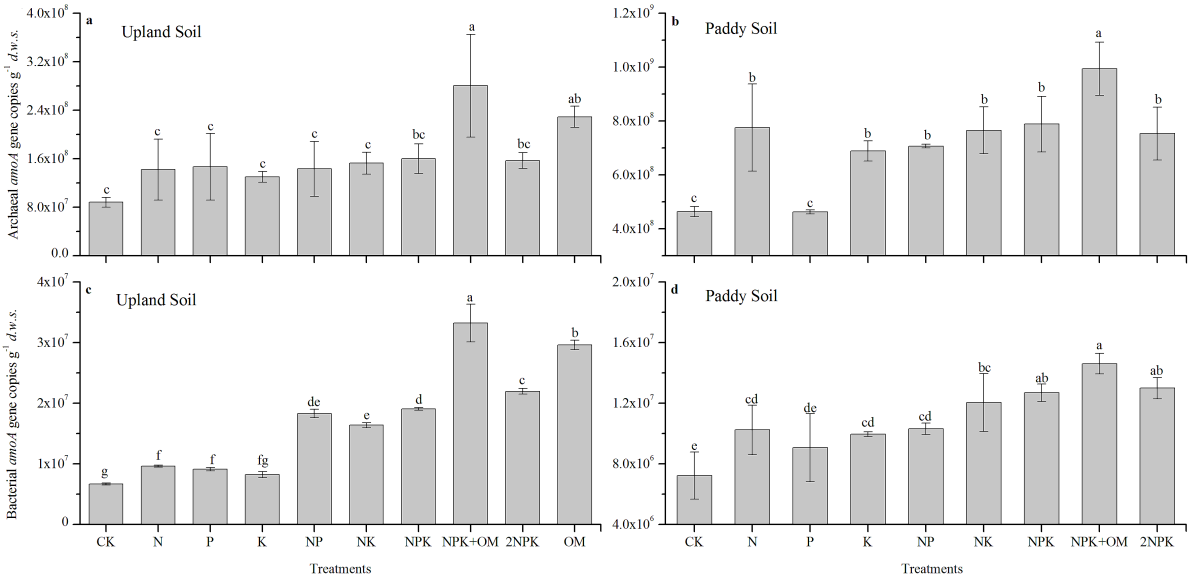

Fig. 1. The *amoA* gene abundance of Archaea **(a, b)** and of Bacteria **(c, d)** in upland and paddy soils. The treatments were control without fertilizers (CK) and various fertilization regimes including chemical fertilizers and/or organic manure (OM) as follows: N, P, K, NP, NK, NPK, NPK + OM, and OM. The designation "2NPK" indicates that the application rate was twice that of NPK in the field plots. Error bars represent the standard deviation of the three field replicate field plots examined for each treatment. The same letter above the columns indicates no statistically significant difference among the treatments ($P > 0.05$).

subsequent DGGE analysis. Clone libraries were thus constructed to reveal the community structures of AOB in the upland and paddy soils. Phylogenetic analyses of bacterial *amoA* genes showed that *Nitrosospira* cluster-3-like species dominated AOB communities in the different treatments regardless of whether the soil was upland or paddy soil (Fig. 4). However, there was a slight change in AOB compositions between paddy and upland soils. For example, bacterial *amoA* genes affiliated with clusters 7 and 8 were retrieved from paddy soils, whereas upland soil contained bacterial *amoA* genes belonging to cluster 2 and cluster 0.

3.5 Changes in the composition of ammonia oxidizers upon long-term field fertilization

Distinctly different DGGE fingerprints of archaeal *amoA* genes were observed among the different treatments for both upland (Fig. 5a) and paddy soils (Fig. 5b), and long-term nitrogen fertilization appeared to affect the DGGE banding

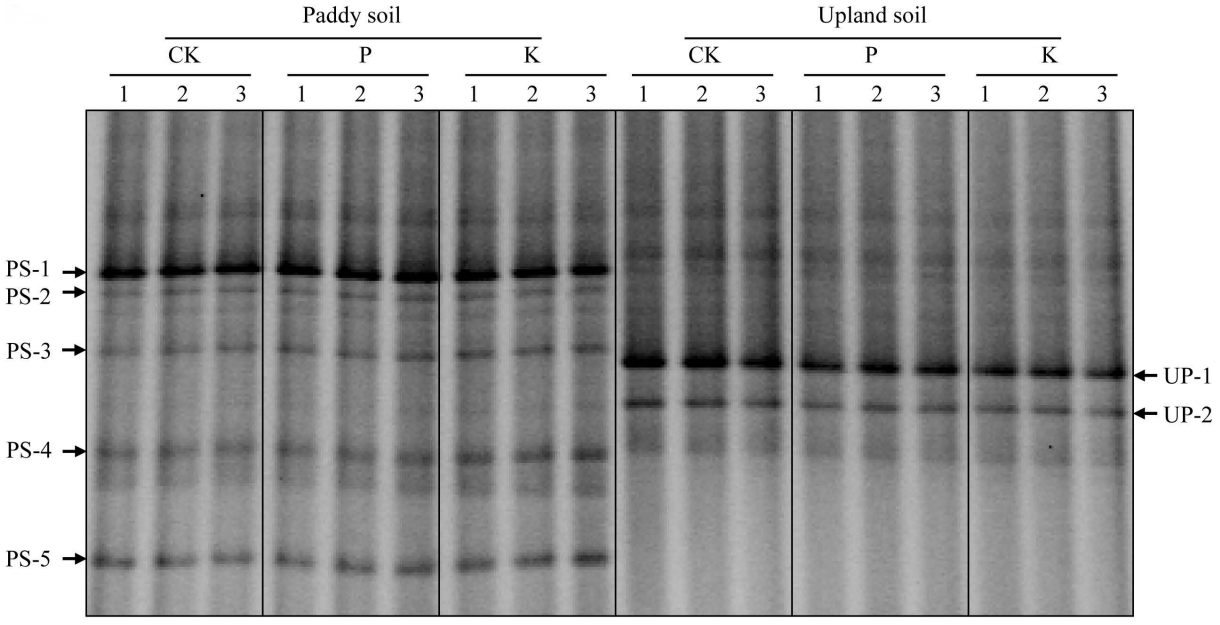

Fig. 2. DGGE fingerprints of archaeal *amoA* genes derived from CK, P and K treatments of upland and paddy soils. The arrows indicate DGGE bands excised for sequencing. Lanes 1, 2 and 3 represent three replicated plots for each treatment.

patterns. For example, the intensities of bands U-3 and U-6 were significantly higher in field plots that received chemical N fertilizers compared with the CK treatment (without nitrogen fertilization) in upland soils. These two DGGE bands could be placed within the soil group 1.1b lineage, in addition to bands U-2, U-5 and U-8, which occurred exclusively in field plots amended with organic matter (Fig. 5a). The majority of the DGGE bands for AOA in upland soils were affiliated with the soil group 1.1b lineage (Fig. 3). These results indicate a pronounced difference in the community composition of AOA in response to long-term fertilization treatments, and AOA of the soil group 1.1b lineage might be the dominant ammonia oxidizers in upland soil.

It is interesting to note that distinct AOA communities were observed in the paddy soil. Eight of the 12 DGGE bands were placed within the marine group 1.1a-associated lineage, whereas the remaining 4 DGGE bands fell within the soil group 1.1b lineage (Fig. 3). Furthermore, a comparison of the DGGE patterns between different treatments indicated distinct variations in *amoA* genes in field plots, particularly in soils amended with N fertilizer. The intensities of the DGGE bands P-4 and P-12 were higher in mineral N fertilizer-amended treatments. The DGGE band P-4 could be placed within the marine group 1.1a lineage, whereas the DGGE band P-12 could be placed within the soil group 1.1b lineage. Surprisingly, the 2NPK treatment showed bands with lower intensity compared with those in the NPK treatment. The DGGE bands P-1 and P-8 showed higher intensities in the NK treatment, although they were faint in the other treatments.

Cluster analysis of PCR-DGGE band patterns of archaeal *amoA* genes in upland soil indicated that organic-matter-amended treatments grouped into one cluster (Fig. 6a) that was distinctly different from the other treatments. In paddy soil, the CK, N and NPK treatments grouped together, whereas NK and 2 replicates of NPK + OM formed a separate cluster (Fig. 6b). It is noteworthy that very narrow Euclidean distances were observed among the paddy soil treatments, although different clusters were formed. The Shannon diversity index (H) and richness (S) were clearly higher in organic-matter-amended treatments when compared with all other treatments for upland soil (Table 2). Fertilizer-amended plots showed comparatively higher diversity indices (H) and richness (S) compared with the CK treatment in both types of soil. Regarding species evenness, all upland soil treatments showed similar values, and no consistent patterns were observed (Table 2). The NPK + OM and NK treatments of the paddy field showed the highest Shannon index (H) and richness (S) compared with all the other treatments (Table 2). The lowest Shannon index (H) and richness (S) values were observed in the CK treatments; however, these values were statistically similar among field plots that received only N fertilizer. Evenness was not significantly different among the paddy soil treatments.

Phylogenetic analyses of the bacterial *amoA* gene fragments from paddy soil showed that two out of four OTUs that were affiliated with the *Nitrosospira* cluster 3 lineage were present in all treatments; one OTU placed within cluster 7 was exclusively detected in the NPK + OM treatment, and the remaining OTUs were most closely related to cluster 8 in

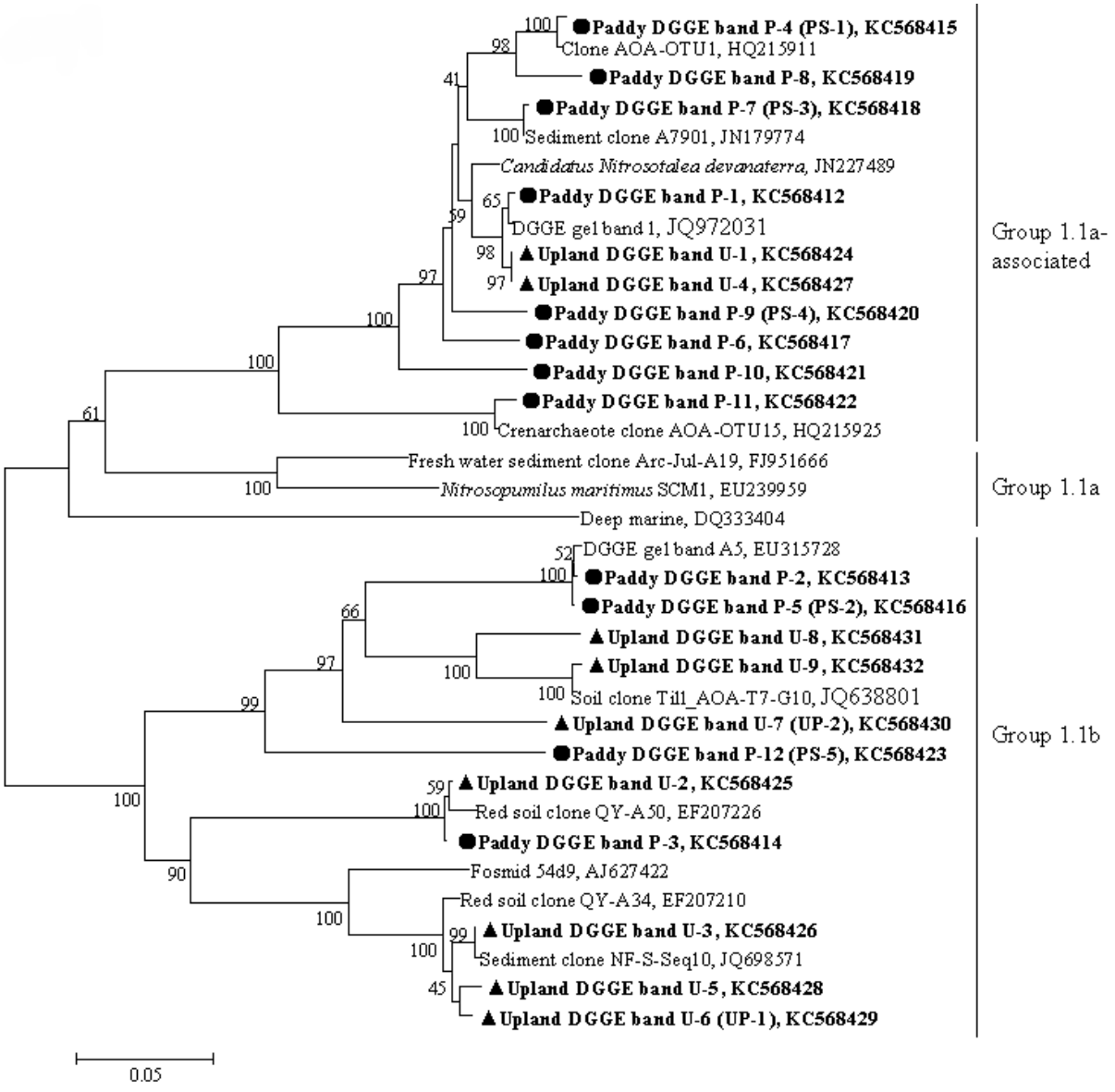

Fig. 3. Neighbor-joining tree showing the relationships of the archaeal *amoA* genes retrieved from the DGGE bands of upland and paddy soils to those in GenBank. The scale bar indicates 5 changes per 100 nucleotide acid positions. Bootstrap values (> 40 %) are indicated at branch points. Sequences from this study are shown in bold. ▲ represents the sequences derived from upland soil, and • represents the sequences derived from paddy soil.

the field plots under NPK, 2NPK, and NPK+OM treatments (Fig. 4). In upland soil, most of the OTUs were also affiliated with *Nitrosospira* cluster 3 (Fig. 4). In addition, two out of eight OTUs were affiliated with cluster 2 (NPK + OM, OM treatments) and one OTU was affiliated with cluster 0 (NPK, NPK + OM, and 2NPK treatments).

4 Discussion

Microorganisms live in complex multispecies communities and drive biogeochemical cycles of soil nutrients that maintain the sustainability of the terrestrial ecosystem. The ecological force driving the emergence and diversification of microbial communities is poorly understood. The phylogenetically distinct (but functionally closely related) ammonia oxidizers of AOA and AOB provide an ideal model to investigate the driving forces that lead to the diversification of microbial communities at the ecosystem level. Conversion of aerobic upland soil to a flooded paddy field may represent niche specialization with oxygen as a constraint on the community divergence of soil AOA and AOB. Long-term field flooding of > 100 yr significantly stimulated the growth of

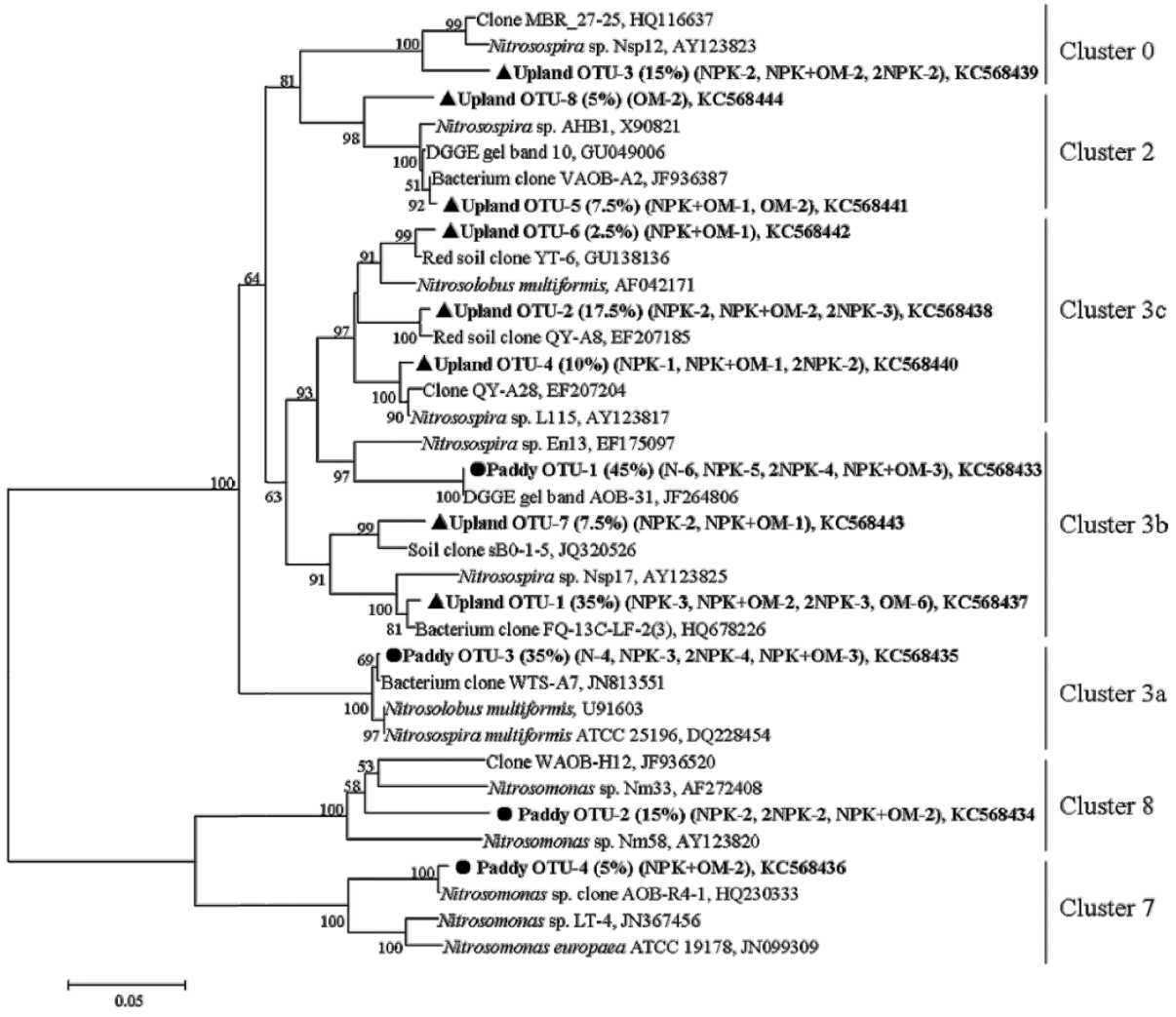

Fig. 4. Neighbor-joining tree showing the relationship of bacterial *amoA* genes retrieved from the clone library of upland and paddy soils to those in GenBank. The scale bar indicates 5 changes per 100 nucleotide acid positions. Bootstrap values (> 40 %) are indicated at branch points. Sequences from this study are shown in bold. ▲ represents the OTU derived from upland soil, and ● represents the OTU derived from paddy soil.

Table 2. Diversity indices based on DGGE profiles of *amoA* gene fragments.

Treatment	Shannon's diversity index (H)		Richness (S)		Evenness (E_H)	
	Upland	Paddy	Upland	Paddy	Upland	Paddy
CK	1.59 ± 0.20c	2.19 ± 0.0003d	5 ± 1c	9 ± 0d	0.999 ± 0.0004a	0.999 ± 0.0001a
N	1.79 ± 0.001b	2.23 ± 0.06d	6 ± 0b	9 ± 0.6d	0.998 ± 0.0003a	0.999 ± 0.0003a
NP	1.78 ± 0.002b	2.39 ± 0.0003b	6 ± 0b	11 ± 0b	0.994 ± 0.001c	0.998 ± 0.0001a
NK	1.78 ± 0.002b	2.48 ± 0.0003a	6 ± 0b	12 ± 0a	0.994 ± 0.001c	0.999 ± 0.0001a
NPK	1.78 ± 0.001b	2.30 ± 0.0001c	6 ± 0b	10 ± 0c	0.995 ± 0.001bc	0.999 ± 0.0001a
NPK + OM	1.94 ± 0.003a	2.48 ± 0.001a	7 ± 0a	12 ± 0a	0.997 ± 0.001ab	0.998 ± 0.0004a
2NPK	1.60 ± 0.001c	2.39 ± 0.0001b	5 ± 0c	11 ± 0b	0.998 ± 0.001a	0.999 ± 0.0001a
OM	1.94 ± 0.004a	–	7 ± 0a	–	0.996 ± 0.002bc	–

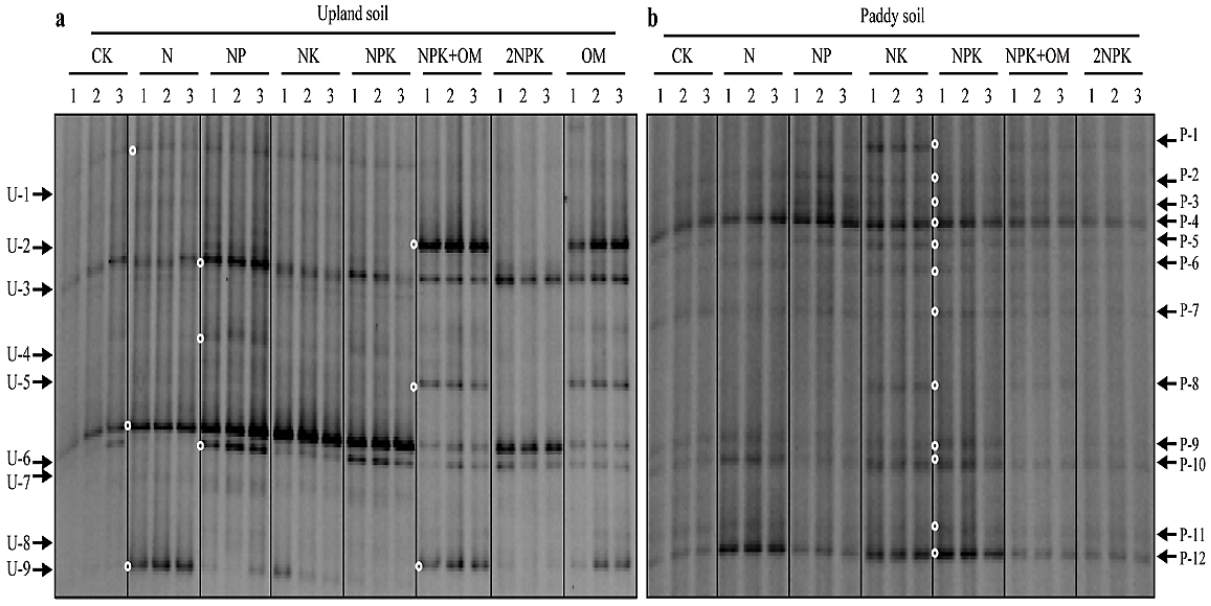

Fig. 5. DGGE fingerprints of archaeal *amoA* genes derived from upland soil **(a)** and from paddy soil **(b)**. Designations for the treatments are the same as those stated in Fig. 1. The arrows indicate DGGE bands excised for sequencing. Lanes 1, 2 and 3 represent three replicated plots for each treatment.

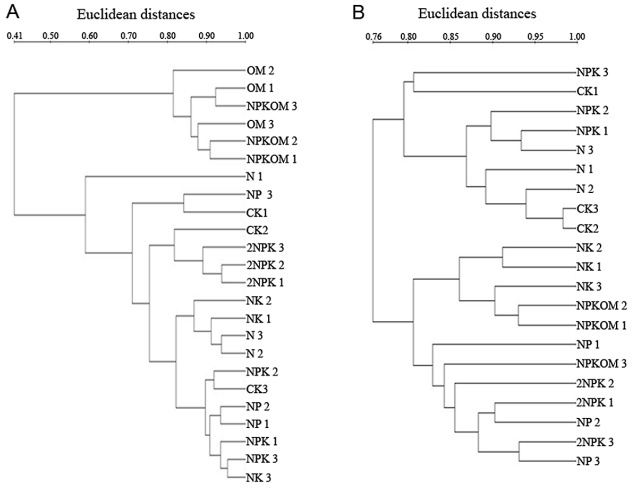

Fig. 6. Clustering analysis of PCR-DGGE banding patterns of archaeal *amoA* genes as influenced by long-term field fertilization treatments in upland soil **(a)** and paddy soil **(b)**. The dendrogram was generated by Pearson's product-moment coefficient and an unweighted pair group method with an arithmetic mean clustering algorithm. The scale indicates the Euclidean distance.

AOA communities in paddy soils, leading to the predominance of the marine group 1.1a-associated lineage that was rarely detected in upland soils. Long-term nitrogen fertilization of > 25 yr further enhanced the abundance of both AOA and AOB in paddy and upland soils. These findings implicate that long-term flooding and ammonia exposure act as the dominant selective forces altering soil AOA ecotypes and demonstrate the importance of long-term field experiments for revealing the ecology and divergence of microbial communities in nature.

Conversion of upland field to flooded paddy soils led to a significant shift of soil AOA communities. Both paddy and upland fields originate from the same parental material of quaternary red clay origin, and the fields are separated by only 150 m. Flooding leads to depletion of oxygen, the supply of which could likely act as one of the key selective agents shaping the community structure of obligate aerobic AOA and AOB in complex soil. The ratio of upland to paddy soil AOA ranges from 0.18 to 0.32 (Table 1). It appears that the flooded condition of paddy soil favored the growth of AOA communities when compared with upland soils. Our results agreed well with recent findings regarding ammonia oxidizer dynamics in paddy (Chen et al., 2011) and upland soils (He et al., 2007). The fields of these two acid soils are separated by 487 km but originated from the same quaternary red clay described in this study. The copy number of the archaeal *amoA* gene was generally one order of magnitude higher in paddy (Chen et al., 2011) versus upland soils (He et al., 2007), whereas the reverse trend was observed for bacterial *amoA* genes. It is noteworthy that AOA members of the marine group 1.1a-associated lineage dominated the paddy soil (Chen et al., 2011), whereas the AOA within the soil group 1.1b lineage were more abundant in upland soil (He et al., 2007). This finding agrees well with our results, indicating that AOA community diversification might be attributed to variations in oxygen availability and other environmental

factors upon conversion of upland to flooded paddy soil. The predominance of AOA communities was indeed frequently observed in oxygen minimum zones of marine environments (Beman et al., 2008; Bouskill et al., 2012) and oligotrophic lake waters (Herrmann et al., 2008; Auguet et al., 2012). In a highly aerated sludge with high concentrations of dissolved oxygen, AOA abundance was at least three orders of magnitude lower than that of AOB (Wells et al., 2009). Batch culture studies indicated that AOA has a higher affinity for oxygen compared with AOB (French et al., 2012; Kim et al., 2012), suggesting that AOA could be better adapted to low-oxygen environments.

DGGE fingerprinting analysis further demonstrated that long-term field flooding led to remarkable changes in the AOA compositions of acid soils. The dominant AOA communities in upland soils were represented by the DGGE bands UP-1 and UP-2 (Fig. 2), which were affiliated with the soil group 1.1b (Fig. 3). Long-term field flooding appeared to completely eliminate these two DGGE bands in paddy soil, while the most dominant DGGE band in paddy soil, PS-1, was affiliated with the marine group 1.1a-associated lineage. The DGGE banding patterns of PS-2 and PS-5 in paddy soils were different from the most dominant band in upland soil, UP-1, and formed phylogenetically distinct clusters within the soil group 1.1b. The environmental factors that determined the emergence and maintenance of phylogenetically distinct AOA remain unclear. Exceptionally high substrate affinity was frequently observed in AOA within the marine group 1.1a (Martens-Habbena et al., 2009) and the marine group 1.1a-associated lineages (Jung et al., 2011; Lehtovirta-Morley et al., 2011), while AOA within the soil group 1.1b lineage could tolerate high ammonium concentrations (Tourna et al., 2011) up to 20 mM (Kim et al., 2012). Physiological studies have demonstrated a higher oxygen affinity in the marine group 1.1a lineage compared with soil group 1.1b (Park et al., 2010; Kim et al., 2012). Interspecies competition experiments have not been performed with cultures in an attempt to decipher the divergence patterns of AOA communities. Our results indicated that AOA within the marine 1.1a-associated lineage might be better adapted to flooded paddy soils than soil group 1.1b AOA, and conversion of an upland field to a flooded paddy field plays a key role in the divergence of AOA communities in acid soil.

Phylogenetic analyses demonstrated that *Nitrosospira* cluster-3-like AOB dominated the communities of bacterial ammonia oxidizers in upland soil, which was consistent with previous studies (He et al., 2007). Bacterial *amoA* genes affiliated with *Nitrosomonas europaea* (cluster 7) and *Nitrosomonas communis* (cluster 8) were exclusively observed in paddy soil, whereas the existence of AOB members within *Nitrosospira* cluster 2 and cluster 0 was demonstrated only in upland soils. Long-term field flooding did not result in a significant shift in AOB composition because both paddy and upland soils were dominated by bacterial ammonia oxidizers within the *Nitrosospira* cluster 3 lineage. The phylogenetically distinct clusters were often detected in paddy soil, including clusters 11 and 12 (Chen et al., 2011), clusters 1 and 2 (Bowatte et al., 2007), cluster 0, cluster 3 and cluster 8 (Wang et al., 2009; Ke and Lu, 2012). Oxygen fluctuation has been shown to affect the kinetic parameters of nitrifying communities in a mixed culture (Laanbroek and Gerards, 1993) and nitrification kinetics in root-oxygenated sediments (Bodelier et al., 1996). However, it appears that AOB communities could be readily reactivated even after severe starvation for oxygen, and the relationship between oxygen status and AOA and AOB in natural habitats remains poorly understood (Kowalchuk et al., 1998; Geets et al., 2006). Meanwhile, it is noteworthy that the AOA and AOB community compositions were determined by DGGE and clone library, respectively. The kinetic changes in AOA and AOB communities and their relative contributions to nitrification in acid soils warrant further studies with greater detail.

The availability of ammonia in natural environments could also be a key factor in shaping the community structures of AOA and AOB. Long-term field fertilization enhanced the soil ammonia concentration, promoted the growth of ammonia oxidizers, and resulted in increasing trends of AOA and AOB abundance in fertilized field soils when compared with unfertilized controls regardless of the type of ecosystem (paddy or upland) (Fig. 1). The largest populations of AOA and AOB were observed in field plots amended with organic manure and/or a balanced application of chemical NPK fertilizers. Furthermore, DGGE fingerprints of *amoA* genes indicated that long-term fertilization alters the community structure of AOA communities. qPCR analysis demonstrated that archaeal ammonia oxidizers are much more abundant than bacterial ammonia oxidizers in both upland and paddy soils. These results imply that AOA dominates ammonia oxidation in acidic soils. Our results agreed well with previous studies employing similar long-term field fertilization treatments in acid paddy (Chen et al., 2010) and upland soils (He et al., 2007) in southern China. Statistical analysis further indicated that AOA abundance was positively related to total nitrogen in paddy soil, suggesting that ammonia generated from soil nitrogen mineralization might fuel archaeal nitrification in acid soil, as previously reported (Zhang et al., 2012). It should be noted that the opposite trend was observed in alkaline agricultural soil. Several studies have shown that long-term field fertilization altered the community structure of AOB rather than AOA in alkaline agricultural soils such as black soil (Fan et al., 2011), paddy soil (Wu et al., 2011), grassland soil (Shen et al., 2011) and sandy loam soils (Shen et al., 2008). ^{15}N isotope tracing and DNA/RNA-based stable isotope probing provide powerful tools for investigating kinetic changes in nitrification activities in fields and ecophysiology of active AOA and/or AOB in response to long-term field flooding and fertilization treatments (Lu et al., 2012; Lu and Jia, 2013).

The community shift of AOA in this study could not be related to a single factor because soil heterogeneity, such

as variations in ammonia, soil pH, oxygen and temperature, might influence the diversity of ammonia oxidizers in nature. It must be emphasized that the resolution is generally low for fingerprinting techniques such as DGGE and clone libraries, which were used in this study. For example, DGGE fingerprints of archaeal *amoA* genes remained largely unchanged in upland soils between the CK and 2NPK treatments (Fig. 5a). Long-term field fertilization with NPK could likely alter the community structure of ammonia oxidizers in acid soils, and this effect might be better analyzed by pyrosequencing analysis of *amoA* genes with unprecedented coverage. Furthermore, the community shift in AOA observed in this study could not be conclusively related to any environmental factor in association with land-use change in this study due to the enormous soil heterogeneity under field conditions as previously reported (Pett-Ridge et al., 2013). Therefore, a controlled laboratory experiment is required to provide direct evidence for community diversification of ammonia oxidizers in response to environmental variations.

Conversion of an upland field to a paddy field significantly stimulated soil pH irrespective of the fertilization treatments, indicating that soil pH might play important roles in altering the community structure of archaeal ammonia oxidizers. Numerous studies have demonstrated that soil pH determines the availability of ammonia, which plays a key role in shaping the community structures of soil ammonia oxidizers (Nicol et al., 2008; Lu and Jia, 2013). However, statistical analysis indicated no significant correlation between AOA/AOB abundance and soil pH, regardless of whether the soil was upland or paddy soil in this study. Other factors, including soil organic matter content, plant species, and interaction with other microbes might also play vital roles in shaping the community of ammonia oxidizers in the soil. For example, correlation analysis indicated a positive correlation between the copy number of *amoA* genes and soil organic carbon, irrespective of whether the soil was paddy or upland soil, which was consistent with previous observations (He et al., 2007; Chen et al., 2011). A physiological study has indeed suggested that AOA could grow on organic substrates (Tourna et al., 2011); however, the ecological significance of a heterotrophic lifestyle for AOA in complex environments remains poorly understood. Furthermore, it has been shown that the community structures of ammonia oxidizers might be affected by plant species (Mao et al. 2011; Chu et. al., 2009). In this study, maize and rice plants were cultivated in upland and flooded fields, respectively. It seems plausible that community diversification of AOA might be related to changes in soil properties under different crop cultivation systems; however, further studies are required to assess how the root exudates entering the soils can affect the community structures of soil ammonia oxidizers. Additionally, ammonium limitation induced by root uptake might affect the ammonia oxidizers due to a greater affinity for ammonium. The distinct biogeography of AOA in marine water is a strong indication of the adaptive diversification of archaeal ammonia oxidizers in response to ammonia constraint (Sintes et al., 2013). Our results indicated that oxygen fluctuation and/or other environmental changes could have acted as selective forces leading to community shifts of soil group 1.1b in upland soil to the marine group 1.1a-associated AOA in paddy soil upon land-use changes.

5 Conclusions

Taken together, our results demonstrate that the conversion of aerobic upland to a flooded paddy field significantly altered the community structure of archaeal ammonia oxidizers in an acid soil. AOA members of the soil group 1.1b lineage dominated the communities of archaeal ammonia oxidizers in upland soils, whereas the predominance of AOA affiliated with the marine group 1.1a in paddy soil is likely attributed to oxygen limitation and other soil physiochemical changes associated with long-term field flooding and rice cultivation. *Nitrosospira* cluster-3-like AOB predominated the communities of bacterial ammonia oxidizers in both paddy and upland soils, and AOA were far more abundant than AOB in soils across all fertilization and flooding regimes. The results of this study provide strong evidence for ecotype divergence of archaeal ammonia oxidizers in complex soil systems and highlight the importance of long-term field experiments for revealing the community succession and diversification of ecologically important microbial guilds in natural habitats.

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