

**Microbial ammonia
oxidation in acid soil**

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Ecosystem-specific selection of microbial ammonia oxidizers in an acid soil

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Abstract

The function of ammonia-oxidizing archaea (AOA) and bacteria (AOB) depends on the availability of ammonia substrate and the supply of oxygen. The interactions and evolutions of AOA and AOB communities along ecological gradients of substrate availability in 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 in response to long-term field fertilization and flooding management in an acid soil. Real-time quantitative PCR of *amoA* genes demonstrated that the abundance of AOA was significantly stimulated after conversion of upland to paddy soils, while slight decline of AOB populations was observed. DGGE fingerprints of *amoA* genes further revealed remarkable changes in community compositions of AOA in paddy soil when compared to upland soil. Sequencing analysis revealed that upland soil was dominated by AOA within the soil group 1.1b lineage, while the marine group 1.1a lineage predominated AOA communities in paddy soils. Irrespective of upland and paddy soils, long-term field fertilizations led to higher abundance of *amoA* genes of AOA and AOB than control treatment that received no fertilization, whereas archaeal *amoA* gene abundances outnumbered their bacterial counterpart 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 treatments. The results of this study suggest that the marine group 1.1a AOA could be better adapted to low-oxygen environment than AOA ecotypes of the soil group 1.1b lineage, and implicate that long-term flooding as the dominant selective force driving the community diversification of AOA populations in the acid soil tested.

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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 then to nitrate by nitrite oxidizers. Ammonia oxidation is the first and rate-limiting step of nitrification that sustains global nitrogen cycles (Kowalchuk and Stephen, 2001). It was long time believed that microbial ammonia oxidation is solely performed by bacteria and that only bacteria possess the *amoA* gene for the 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), and suggested that relative contributions of AOA and AOB to nitrification varied greatly in physiochemically 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 soil environment (Leininger et al., 2006) and both of which derive energy from ammonia oxidation in support of their growth (Lehtovirta-Morley et al., 2011; Tourna et al., 2011). Comparative genomic analysis has indicated that AOA and AOB may greatly differ 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). Intensive studies demonstrate that environmental factors have shaped the communities structures of AOA (Erguder et al., 2009). For instance, recent discovery has suggested that the remarkably low concentration of ammonia in acid soil could afford AOA greater ecological advantages than AOB communities (Lehtovirta-Morley et al., 2011; Yao et al., 2011; Levičnik-Höfferle et al., 2012). However, it remains largely unclear about the ecological force that drives diversification of AOA and/or AOB, particularly in natural environments including anthropogenically disturbed ecosystems. The dynamic changes in community structure of AOA and AOB along ecological gradients that may provide strong hints regarding the divergence of the niche and their relative role in nitrification.

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

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substrate availability in complex environment under in situ conditions. Previous investigations showed that long-term fertilization significantly altered community structure of AOB rather than AOA in alkaline soil (Chu et al., 2007; Shen et al., 2008; Wu et al., 2011), in addition to apparent shifts in the diversity of total bacteria and fungi (Ge et al., 2008; He et al., 2008). Contrasting results were also reported that AOA and AOB respond to long-term field fertilizations in different manners (He et al., 2007; Wessén et al., 2010), and no consistent patterns could be generalized. As for 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). Molecular survey indeed indicated that AOA were 30 times less abundant than AOB in the oxic zone, and 10 times more abundant in the low-oxygen environment (Santoro et al., 2008). Moreover, the existence of archaeal *amoA* genes was demonstrated in activated sludge with low dissolved oxygen levels under low oxygen concentrations (Park et al., 2006), and in the water bodies of pelagic oxygen minimum zones (Beman et al., 2008). It is likely that AOA might be more suited to the low-oxygen environment than AOB. But it remains unclear to what extent oxygen availability could have shaped the diversification of AOA and/or AOB communities in natural environment.

Increasing line of evidences has suggested important roles of AOA in acid soil (Prosser and Nicol, 2012). This could be well explained by the exceptionally high affinity of AOA to ammonia substrate that is too low in acid soil to support the growth of AOB (Lehtovirta-Morley et al., 2011; Lu et al., 2012). Yet, very little information is available demonstrating the chronic effect of oxygen supply and nitrogen fertilizations on community changes of AOA and AOB in natural environment. Therefore, two agroecosystems of maize and paddy rice crops under long-term different fertilizations of > 25 yr were selected to investigate community diversification of AOA and AOB in acid soils. The paddy field was converted from upland soil which is about 150 m away maize field. Both paddy and maize soils are originated from the same parent material and meteorological conditions. Conversion of upland acid soil to paddy field can result in the

depletion of oxygen in soil, leading to ecological pressure for the evolution of obligate aerobic AOA and AOB.

2 Materials and methods

2.1 Site description and soil sampling

5 The soil samples used in this study were collected from the Ecological Experimental Station of Red Soil, Chinese Academy of Sciences, located at 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, annual evaporation of 1318 mm and a mean annual temperature of 18.1 °C with about 262 days without frost per year.

10 Both paddy and upland soils are originated from quaternary red clay with dominant kaolintic mineral, and classified as a Haplic Stagnic Anthrosol according to USDA soil taxonomy. The long-term fertilization experiment of upland soil was established in 1986, approximately 150 m from the paddy field experiment site. The paddy field was converted from upland soil and has a history of rice cultivation (*Oryza sativa* L.) more

15 than 100 yr before construction of 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 (November to April of next year). As for paddy field, it consists of early rice (April to July), late rice (July to November) and winter fallow without floodwater layer (November to April of next year).

20 Both long-term experimental fields received 9 different treatments of fertilizations. It consists of (1) no fertilization control (CK); (2) chemical nitrogen fertilization (N); (3) chemical phosphorus fertilization (P); (4) chemical potassium fertilization (K); (5) combination of chemical N and P (NP); (6) combination of chemical N and K (NK); (7) combination of chemical N, P and K (NPK); (8) double dose of chemical N, P and K (2NPK); (9) combination of chemical N, P and K fertilizer plus organic ma-

25 nure (NPKOM). Each treatment was conducted in triplicate plots with a randomized

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design while, the application rate of chemical fertilizers and of organic manures were slightly different between paddy field and upland maize experiments. The chemical fertilizers of N, P and K were applied to paddy field at the rates of 90, 45, and 75 kg ha⁻¹ both for early and late rice cultivations, respectively. Milk vetch and pig manure with 22.5 Mg ha⁻¹ (fresh weight) were used as organic manure for early and late rice, respectively. Approximately 66 % of N, 100 % of P, 100 % of K, and 100 % of organic manure are applied as the basal fertilizers prior to rice transplantation. The rest of nitrogen fertilizers are applied as top-dressing at the panicle initiation stage and 10 days before the flowering stage. As for upland maize cropping system, the chemical fertilizers of N, P and K were applied at the rates of 60, 30, and 60 kg ha⁻¹ for each season, respectively. One additional treatment of organic manure fertilization was established only for upland field. Pig manure was used exclusively as organic fertilizers for upland fields of maize cropping with 15 Mg ha⁻¹ (fresh weight) for each season, and applied as the basal fertilizer before maize seeding. For both paddy and maize fields, N, P and K are provided in the form of urea, calcium-magnesium phosphate and potassium chloride, respectively.

Sampling was performed in April of 2010 for upland soil at 0–20 cm depth shortly before maize cultivation, and paddy soil at 0–15 cm depth was collected in November of 2010 after harvesting of late rice. Five cores were randomly taken from each of the three replicate field plots for each treatment. Soil samples were placed in a sterile plastic bag, sealed, and brought back to laboratory on ice within a day, and sieved through 8 mm mesh. The composite sample was obtained by homogenization of five cores from each of the three replicate field plots. The total of 27 and 30 soil composite samples were subsequently analyzed from long-term fertilization fields of paddy and maize crop, respectively. Soil properties such as 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 dichromate oxidation method (Mebius, 1960) and Kjeldahl digestion method (Bremner,

1965), respectively. Soil samples used for molecular analysis were kept at -80°C until further use.

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

DNA was extracted from approximately 0.5 g of fresh soil using bead-beating method as previous reported (Griffiths et al., 2000) with slight modifications. Cell lysis was performed through vigorous shaking in a bead beater at a rate of 6.0 for 40 s. Three consecutive extractions were repeated for each soil sample in order 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 humic substance contamination. The purified DNA was eluted with 100 μL of TE buffer. DNA quality and quantity were checked using a NanoDrop spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE). The soil DNA was stored at -20°C before 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 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. The blank was always run with water as the template instead of the soil DNA extract. The 20 μL reaction mixture contained 10.0 μL of SYBR Premix Ex Taq (TaKaRa Biotech, Dalian, China), 0.25 μM of each primer, and 2 μL of DNA template. The PCR conditions used for the archaeal and bacterial *amoA* genes were the same as previously described (Lu et al., 2012). PCR amplification efficiencies for AOA and AOB in upland soils were 99.2% with a R^2 value of 0.987 and 96.7% with a R^2 value of 0.990, respectively. As for paddy soil, PCR amplification efficiencies of 100.8% and 95.8% were obtained

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for *amoA* genes of AOA and AOB with R^2 values of 0.998 and 0.994, respectively. The specific amplifications of *amoA* genes were also determined using a melting curve analysis, which always resulted in a single peak.

2.3 Polymerase chain reaction – denaturing gradient gel electrophoresis (DGGE)

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 μ L volume containing 2.5 μ L 10 \times PCR buffer, 0.25 μ M of each primer, 200 μ M (each) deoxyribonucleoside triphosphate, 1.5 U of Taq DNA polymerase, and 1 μ L 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 amount of PCR 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 PCR primer is attached with GC clamp that is prerequisite for subsequent DGGE analysis. Therefore, the composition of AOA was analyzed by DGGE fingerprinting and clone library was constructed for AOB analysis.

Approximately 150 ng of PCR amplicons of archaeal *amoA* genes from each sample was 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 a 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 peristaltic pump 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 to insert a comb to make wells. The gels were run

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in 1× TAE at 75 V for 17 h and 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 the Quantity One software (BIO-RAD laboratories, Hercules, CA).

The distinct DGGE bands of archaeal *amoA* genes of AOA were excised and re-amplified for sequencing analysis. PCR conditions were described as mentioned above for re-amplification 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 and phylogenetic analysis

For AOB, PCR products of *amoA* genes were obtained directly from different treatments for clone library construction. For upland soil, NPK, NPK + OM, 2NPK and OM treatments and for paddy soil, N, NPK, NPK + OM and 2NPK treatments were used to construct the clone library. PCR amplification of the bacterial *amoA* gene was performed using the primer sets *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 10× PCR buffer, 0.25 µM of each primer, 200 µM (each) 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 Ram-sing, 2002). The purified PCR products were cloned and the clones that contained the correct insert were selected and sequenced using the same procedures as described for AOA. At least 10 clones were randomly selected for each treatment. The obtained sequences were subjected to homology analysis with the Mothur software (Schloss et al., 2009). The sequences displaying more than 97 % identity with each other were grouped into the same operational taxonomic units (OTUs).

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The sequences for the DGGE bands of AOA and only 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 value was calculated based on 1000 replications.

2.5 Statistics

Spearman's correlation analyses were performed to assess the relationships among soil properties and the abundance of the AOA and AOB (SPSS 11.5 package, SPSS, Chicago, IL). One-Way ANOVA with Duncan's post hoc tests was performed to evaluate the differences within the datasets, with a *P* value of 0.05 selected for significance.

3 Results

3.1 Soil properties

Long-term application of chemical N fertilizers resulted in apparent decrease of pH values from 3.98 in control plots down to 3.64 in N treatments of upland soils. Organic matter amended treatments of NPK + OM and of OM 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. SOC appeared to be elevated in fertilizer-amended plots, in particular for organic manure treatment. Soil total nitrogen yet remained largely unchanged among all treatments. Similar results were obtained for paddy soils, despite the fact that long-term field fertilization affected soil properties to a much less extent than upland soil. It is noteworthy that conversion of upland to paddy field significantly stimulated both pH and SOC, irrespective of fertilization treatments.

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3.2 Abundance change of soil AOA and AOB under long-term field flooding

The abundances of AOA and AOB were determined by quantitative PCR (qPCR) targeting the *amoA* genes (Fig. 1). Long-term flooding appeared to have strong selective advantage for AOA than AOB (Table 1). The copy number of archaeal *amoA* genes was significantly higher than their bacterial counterparts in all treatment, particularly for paddy soil. For instance, the ratio of AOA to AOB varied from 7.13 to 16.1 in upland soil, while significant elevation was observed for paddy soil ranging from 50.9 to 75.6. The ratio of upland to paddy soil AOA ranges from 0.18 to 0.32. The N amendment holds the lowest value, while the highest ratio was observed in P treatment. In stark contrast, AOB abundance was generally higher in upland than that in paddy soil. The mean number bacterial *amoA* gene copies was 1.73×10^7 per gram dry weight soil (g^{-1} d.w.s.) for the upland soil, while $1.10 \times 10^7 \text{ g}^{-1}$ d.w.s. was found in the paddy soil. It thus led to an average ratio of upland to paddy soil AOB as 1.37 ± 0.49 for all 9 treatments (Table 1). The abundance of AOB stayed largely constant for both upland and paddy soil in field plots of CK, P and K treatments, while AOB was stimulated to a greater extent upon nitrogen fertilizations in upland than paddy soils.

3.3 Abundance change of soil AOA and AOB under long-term field fertilization

Population sizes of AOA and AOB varied greatly among different long-term fertilization regimes. The archaeal *amoA* gene copy numbers varied from 8.79×10^7 to $2.80 \times 10^8 \text{ g}^{-1}$ d.w.s. for upland soil, while it ranges from 4.62×10^8 to 9.93×10^8 for paddy soil. Regardless of upland and paddy soils, the NPK + OM treatment had the significantly highest copy numbers of archaeal *amoA* genes among the treatments. The lowest gene copy number was detected in CK ($8.79 \times 10^7 \text{ g}^{-1}$ d.w.s.) and P treatment ($4.62 \times 10^8 \text{ g}^{-1}$ d.w.s.) for upland and paddy soil, respectively. Compared to control field amended without fertilizers, *amoA* gene abundances of AOA showed an increasing trend in fertilized plots of both upland (Fig. 1a) and paddy soils (Fig. 1b), despite not being statistically significant different for most fertilized soils.

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Similar results were observed for bacterial *amoA* genes in response to long-term field fertilizations regardless of paddy and upland soils. For upland soil, the CK treatment showed the lowest copy numbers of $6.69 \times 10^6 \text{ g}^{-1} \text{ d.w.s.}$, and the highest number was found in the NPK + OM treatment with $3.32 \times 10^7 \text{ g}^{-1} \text{ d.w.s.}$ (Fig. 1c). Long-term field fertilization led to stimulation of bacterial *amoA* gene copies to different extents. As for paddy soil, the CK soil had the lowest bacterial *amoA* gene copy number of $7.23 \times 10^6 \text{ g}^{-1} \text{ d.w.s.}$, and being statistically similar with P ($9.08 \times 10^6 \text{ g}^{-1} \text{ d.w.s.}$) treatment (Fig. 1d). On the contrary, the highest bacterial *amoA* gene copy number ($1.46 \times 10^7 \text{ g}^{-1} \text{ d.w.s.}$) was detected in the NPK + OM treatment but statistically similar with NPK ($1.27 \times 10^7 \text{ g}^{-1} \text{ d.w.s.}$) and 2NPK ($1.30 \times 10^7 \text{ g}^{-1} \text{ d.w.s.}$) 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 ($\rho = 0.83$, $n = 30$, $P < 0.01$) for upland soil. As for paddy soil, strong positive correlation was obtained between AOA abundance, soil total N ($\rho = 0.47$, $n = 27$, $P < 0.05$) and soil organic carbon ($\rho = 0.57$, $n = 27$, $P < 0.01$) while, AOB abundance showed a positive correlation with soil organic carbon ($\rho = 0.54$, $n = 27$, $P < 0.01$).

3.4 Composition changes of soil AOA and AOB under long-term field flooding

The composition of AOA communities for both upland and paddy soils was revealed by fingerprinting analysis of archaeal *amoA* genes in the triplicate field plots of all treatments. In addition, three pseudoreplicate soil samples from each of the triplicate plots were used for DGGE analysis. Highly reproducible DGGE fingerprints were obtained, and only one representative replicate from each of the triplicate field plots was shown.

Conversion of aerobic upland to flooding paddy soils significantly altered the compositions of AOA communities by pairwise comparison of DGGE fingerprints of archaeal *amoA* genes in soil (Fig. 2). AOA communities in upland soil were dominated by DGGE band UP-1 and UP-2 that were affiliated with the soil group 1.1b lineage (Fig. 3). Compared to upland soil, long-term field flooding stimulated the diversity of

AOA communities as more dominant DGGE bands were demonstrated in paddy soil. DGGE band PS-1, PS-3 and PS-4 could be placed within the marine group 1.1a lineage whereas; band PS-2 and band PS-5 fell well within the soil group 1.1b lineage (Fig. 3).

5 PCR amplicons of bacterial *amoA* genes could not be obtained when GC clamp was attached to primers for subsequent DGGE analysis. Clone library was then constructed to reveal the community structures of AOB in the upland and paddy soils. Furthermore, phylogenetic analyses of bacterial *amoA* genes showed that *Nitrosospira* cluster 3-like species dominated AOB communities in the different treatments irrespective of upland and paddy soils (Fig. 4). However, there was a slight change in AOB compositions between paddy and upland soils. For instance, bacterial *amoA* genes affiliated with cluster 7 and 8 was retrieved from paddy soils, while upland soil recognized the presence of bacterial *amoA* genes belonging to cluster 2 and cluster 0.

15 3.5 Composition changes of soil AOA and AOB under long-term field fertilization

Distinctly different DGGE fingerprints of archaeal *amoA* genes were observed among different treatments for both upland (Fig. 5a) and paddy soils (Fig. 5b), and long-term nitrogen fertilizations appeared to affect DGGE banding patterns. For instance, the intensities of band U-3 and U-6 were significantly higher in field plots that received chemical N fertilizers than the CK treatment without nitrogen fertilizations in upland soils. These two DGGE bands could be placed within the soil group 1.1b lineage, in addition to band U-2, U-5 and U-8 occurring exclusively in field plots amended with organic matters (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 indicated a pronounced difference in the community composition of AOA in response to the long-term fertilization treatments and soil group 1.1b lineage AOA might be the dominant ammonia oxidizers in upland soil.

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field represents a niche specialization of oxygen as a constraint on the evolution of soil AOA and AOB. Long-term field flooding of > 100 yr significantly stimulated the growth of AOA communities in paddy soils, leading to the predominance of the marine group 1.1a-associated lineage that was hardly detected in upland soils. Long-term nitrogen fertilization of > 25 yr in field further enhanced the abundance of both AOA and AOB in paddy and upland soils. These findings implicate that the availability of oxygen and ammonia as the dominant selective forces driving divergence between soil AOA ecotypes, and demonstrate the importance of long-term field experiment for revealing the ecology and evolution of complex microbial systems in nature.

Long-term field flooding led to significant shift of soil AOA communities. Both paddy and upland fields of long-term experiments are originated from the same parental material of quaternary red clay origin, and only 150 m away from one another. The flooding leads to depletion of oxygen, the supply of which could likely act as one of the key selective agents shaping the structure of obligate aerobes of AOA and AOB communities in complex soil. The AOA ratio of upland soil AOA to paddy soil AOA ranges from 0.18 to 0.32 (Table 1). It indicates that chronic pressure of oxygen at low concentration in paddy soil appeared to favor the growth of AOA communities, when compared to upland soils. Our results agreed well with recent findings of ammonia oxidizer dynamics in paddy (Chen et al., 2011) and upland soils (He et al., 2007). The fields of these two acid soils are 487 km away from one another but originated from the same quaternary red clay as used in this study. The copy number of archaeal *amoA* gene was generally one order of magnitude higher in paddy (Chen et al., 2011) than upland soils (He et al., 2007), while the reverse trend was observed for bacterial *amoA* genes. The predominance of AOA communities was indeed frequently observed in microaerophilic niches such as oxygen minimum zones (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 AOB (Wells et al., 2009). It seems plausible that AOA could have been better adapted to low-oxygen environment than AOB.

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DGGE fingerprinting analysis further demonstrated that long-term field flooding lead to remarkable changes of AOA compositions in acid soils. The dominant AOA communities in upland soils were represented by DGGE bands UP-1 and UP-2 (Fig. 2) that 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 PS-1 in paddy soil 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 with the most dominant band UP-1 in upland soil, and formed phylogenetically distinct clusters within the soil group 1.1b. It remains obscure about the mechanisms that regulate the maintenance and emergence of phylogenetically different AOA communities. It appears that 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). In the meantime, physiological studies have demonstrated the higher oxygen affinity of marine group 1.1a lineage than that of soil group 1.1b (Park et al., 2010; Kim et al., 2012). The interspecies competition experiments with cultures have not yet been performed in an attempt to decipher the divergence patterns of AOA communities. Our results provide strong hint that AOA might be excellently suited to thrive in oxygen-limited environments, and oxygen might play a key role in the divergence of AOA communities in natural habitats.

Our results agreed well with previous findings for the predominant AOA communities of the marine group 1.1a-associated lineage in paddy soil (Chen et al., 2011) and of the soil group 1.1b in upland soil (He et al., 2007). Phylogenetic analyses further demonstrated the impact of long-term field flooding on AOB communities. The *Nitrosospira* cluster 3-like AOB dominated the communities of bacterial ammonia oxidizers in upland soil, being consistent with previous studies (He et al., 2007). In the meantime, bacterial *amoA* genes affiliated with *Nitrosomonas europaea* cluster 7 and *Nitrosomonas communis* cluster 8 were exclusively observed in paddy soil, while the existence of AOB

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members within *Nitrosospira* cluster 2 and cluster 0 was demonstrated only in upland soils. It indicated that long-term field flooding did not result in significant shift of AOB composition since 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 cluster 11 and 12 (Chen et al., 2011), cluster 1 and 2 (Bowatte et al., 2007), cluster 0, cluster 3 and 8 (Wang et al., 2009; Ke and Lu, 2012). The oxygen fluctuation has been shown to affect the growth 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 AOB communities could be readily reactivated even after severe starvation of oxygen deprivation, and it remains poorly understood about the relationship of oxygen status to AOA and/or AOB in natural habitats (Kowalchuk et al., 1998; Geets et al., 2006). Current study has suggested that community shifts of AOA and AOB in complex environment could hardly be related to a single factor (Schleper, 2010). Soil heterogeneity such as variations of ammonia, oxygen and temperature may have co-shaped the diversity of ammonia oxidizers in nature (Hatzenpichler, 2012).

The availability of ammonia in natural environments could be a key factor shaping the community structures of AOA and AOB. Long-term field fertilization enhanced soil ammonia concentration, promoted the growth of ammonia oxidizers, and resulted in increasing trends of both AOA and AOB abundances in fertilized field soils when compared to those in unfertilized controls regardless of paddy and upland ecosystems (Fig. 1). The highest abundances of both AOA and AOB were observed in field plots with amended with organic manure and/or balanced application of chemical NPK fertilizers. These results agreed well with previous findings with similar long-term field fertilization treatments of acid paddy (Chen et al., 2010) and upland soils (He et al., 2007) in southern China. It is noteworthy that in alkaline agricultural soils positive correlation was often observed between field fertilization and AOB rather than AOA communities in 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). Furthermore, DGGE fingerprints

of *amoA* genes indicated that long-term fertilization alters the community structure of AOA, while clone library construction suggested no significant shift of AOB communities. These results provide strong hints that AOA dominates ammonia oxidation in acidic soils. 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). ¹⁵N isotope tracing and DNA/RNA-based stable isotope probing provide powerful tools to investigate kinetic changes of nitrification activities in field and of ecophysiology of active AOA and/or AOB in response to long-term field flooding fertilization treatments (Lu et al., 2012; Lu and Jia, 2012).

5 Conclusion

Taken together, our results demonstrated that long-term field flooding is the primary selective force driving the community divergence of archaeal ammonia oxidizers in an acid soil. Ecosystem-specific selection of AOA affiliated with the marine group 1.1a is likely attributed to oxygen limitation in paddy soils due to flooding, while soil group 1.1b AOA dominated the communities of archaeal ammonia oxidizers in upland soils. *Nitrosospira* cluster 3 AOB predominated the communities of bacterial ammonia oxidizers in both paddy and upland soils, and AOA were far more abundant than AOB in soils for all fertilization and flooding regimes. The results of this study provide strong hints for ecotype divergence of archaeal ammonia oxidizers in complex soil systems, and highlight the importance of long-term field experiment for revealing the fundamental evolutionary processes of ecologically important microbial guilds in natural habitat.

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Table 1. Soil properties and ratios of AOA and AOB under different fertilization treatments.

Treatments	pH (H ₂ O)		SOC (gkg ⁻¹)		TN (gkg ⁻¹)		Ratios of AOA to AOB		AOA Ratio Upland to Paddy	AOB Ratio Upland to Paddy
	Upland	Paddy	Upland	Paddy	Upland	Paddy	Upland	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 designation of treatments represents control without fertilizers (CK), and different fertilization regimes of chemical fertilizers and organic manure (OM) including (N, P, K, NP, NK, NPK, NPK + OM, and OM). The designation of 2NPK indicates that application rate was twice the treatment of NPK in field plots. Values are the mean of the three replicated filed plots. The different letters following the values within the same column indicate significant differences ($P < 0.05$).

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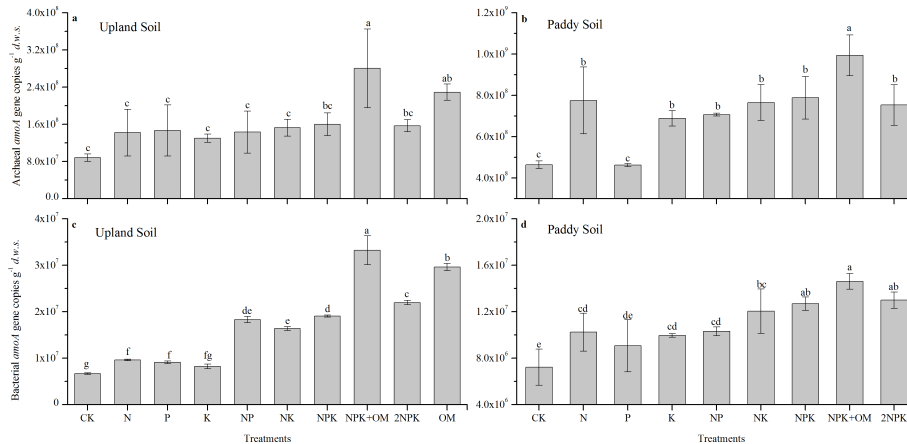


Fig. 1. The *amoA* gene abundance of archaea (a, b) and of bacteria (c, d) in upland and paddy soils. The designation of treatments represents control without fertilizers (CK), and different fertilization regimes of chemical fertilizers and organic manure (OM) including (N, P, K, NP, NK, NPK, NPK + OM,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

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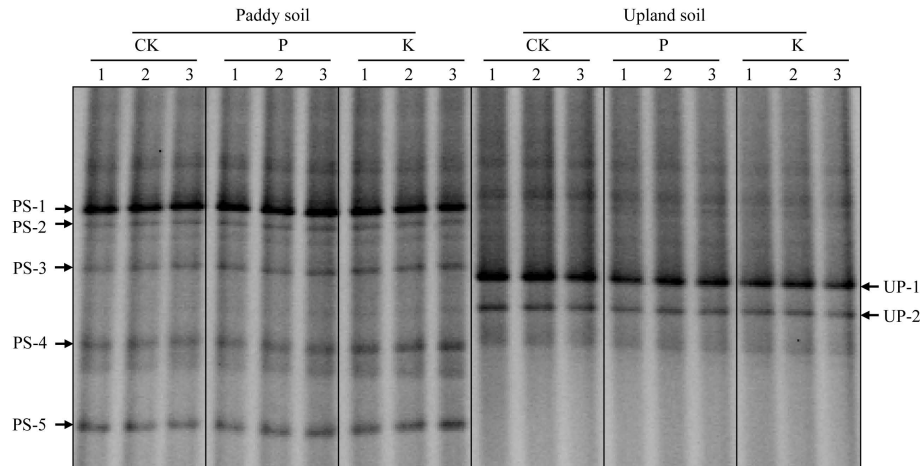


Fig. 2. DGGE fingerprints of archaeal *amoA* genes derived from CK, P and K treatments of upland and paddy soils. The arrows indicate DGGE bands excised for sequencing. Lane 1, 2 and 3 represent three replicated plots for each treatment.

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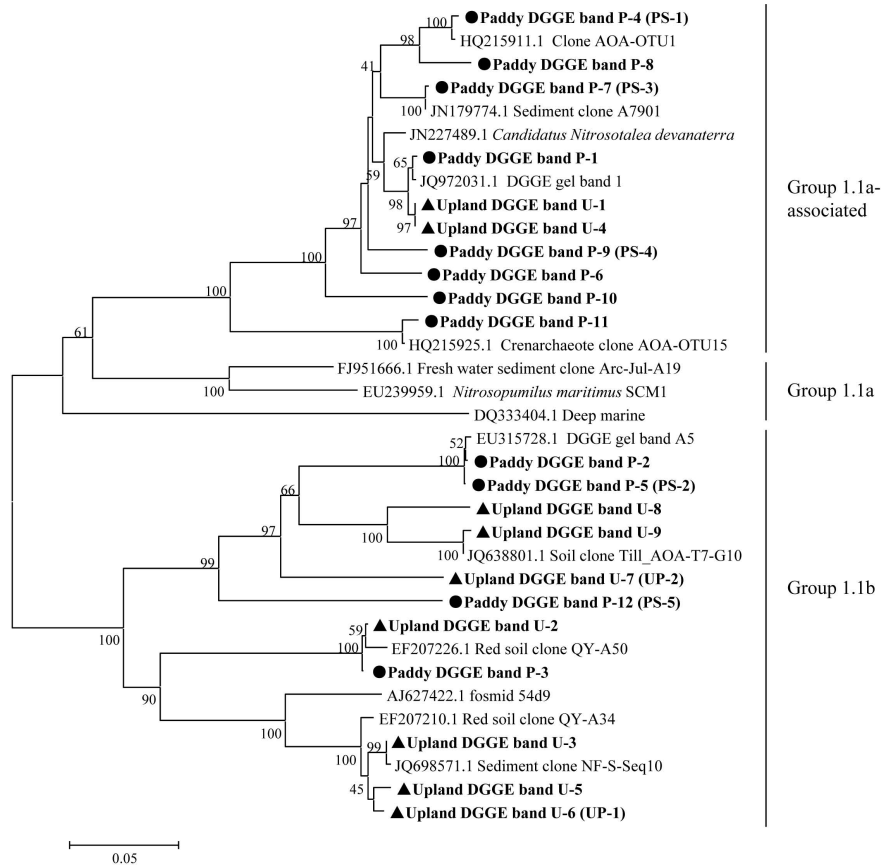


Fig. 3. Neighbor-joining tree showing the relationships of the archaeal *amoA* genes retrieved from the DGGE bands of upland and paddy soils to those in the GenBank. The scale bar indicates 5 changes per 100 nucleotide acid positions. Bootstrap values (> 40 %) are indicated at branch points. Sequences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

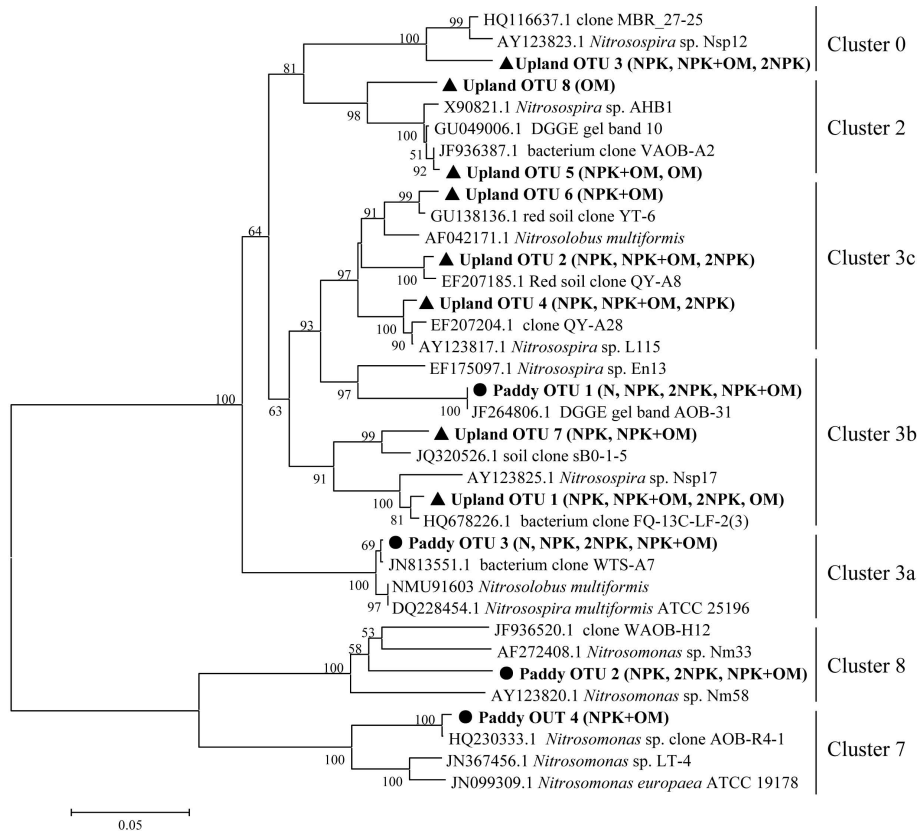


Fig. 4. Neighbor-joining tree showing the relationship of bacterial *amoA* genes retrieved from the clone library of upland and paddy soils to those in the GenBank. The scale bar indicates 5 changes per 100 nucleotide acid positions. Bootstrap values (> 40%) are indicated at branch points. Sequences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

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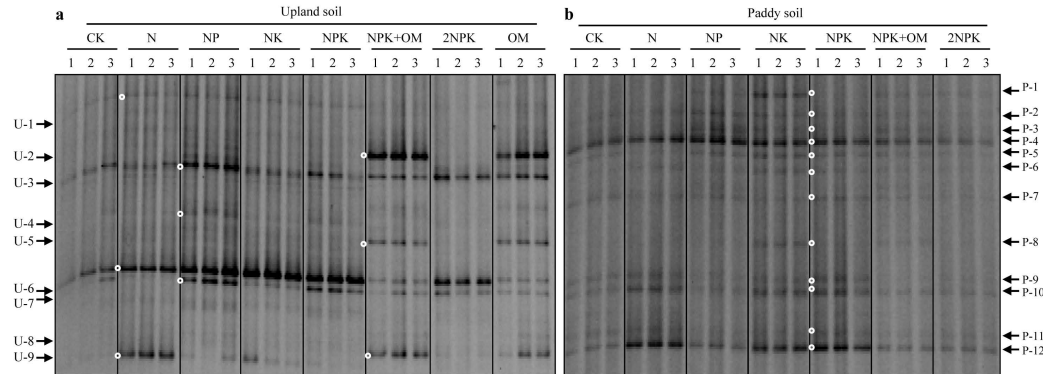


Fig. 5. DGGE fingerprints of archaeal *amoA* genes derived from upland soil **(a)** and from paddy soil **(b)**. Designations for treatments are the same as those in Fig. 1. The arrows indicate DGGE bands excised for sequencing. Lane 1, 2 and 3 represent three replicated plots for each treatment.

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