

Compositional Shifts in Ammonia-Oxidizing Microorganism Communities of Eight Geographically Different Paddy Soils

—Biogeographical Distribution of Ammonia-Oxidizing Microorganisms

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Abstract

Soil nitrification is mediated by ammonia-oxidizing archaea (AOA) and bacteria (AOB), which occupy different specialized ecological niches. However, little is known about the diversification of AOA and AOB communities in a large geographical scale. Here, eight paddy soils collected from different geographic regions in China were selected to investigate the spatial distribution of AOA and AOB, and their potential nitrification activity (PNA). The result showed that the abundance of AOA was predominant over AOB, indicating that the rice fields favor the growth of AOA. PNA highly varied from 0.43 to 3.57 $\mu\text{g NO}_x\text{-N}\cdot\text{g}\cdot\text{dry}\cdot\text{soil}\cdot\text{h}^{-1}$, and was positively related with soil NH_3 content, the abundance of AOA community, and negatively related with the diversity of AOB community ($P < 0.01$), which indicating that AOA might be the more dominant ammonia oxidizers in the collected paddy soils. Denaturing gradient gel electrophoresis fingerprints of *amoA* genes revealed remarkable differences in the compositions of AOA and AOB community. Phylogenetic analyses of *amoA* genes showed that *Nitrospira* cluster-3-like and *Nitrosomonas* cluster 7-like AOB extensively dominated the AOB communities, and 54d9-like AOA within the soil group 1.1b predominated in AOA communities in paddy soils. Redundancy analysis suggested that the spatial variations of AOA community structure were influenced by soil TN content ($P < 0.01$), while no significant correlation between AOB community structure and soil properties was found. Findings highlight that ammonia oxidizers exhibit spatial variations in complex paddy fields due to the joint influence of soil

#These authors contributed equally to this work.

variables associated with N availability.

Keywords

Paddy Soil, Ammonia-Oxidizing Microorganism, Nitrification Activity, Large Geographical Scale, Diversification

1. Introduction

Microbial ammonia oxidation is the first and rate-limiting step of nitrification, which is the only oxidative process that connects the oxidized and reduced state of inorganic nitrogen to maintain the global nitrogen cycling [1]. The discovery of ammonia-oxidizing archaea (AOA) has fundamentally expanded our perceptions of nitrification, which has long been thought to be restricted to ammonia-oxidizing bacteria (AOB), which are phylogenetically belonging to *Proteobacteria* [2]. Although recent evidence demonstrated *Nitrospira* bacteria also has the complete nitrification system [3], the ubiquity of AOA and AOB across a wide variety of environment and habitats implies that metabolically distinct ammonia oxidation microorganisms play critical roles in nitrification and may be adapted to live under physicochemical contrasting niches [4]. Numerous researchers have recognized that the functionally relevant diversity of AOA has greatly outnumbered their bacteria counterparts [5] [6]. AOA has been proved to be the dominant group in nitrification in some soils [7] [8], and in some acidic soils which only AOA are detectable [6] [9], while the converse has also been demonstrated in agricultural soils [10] and N-rich soils [11]. These inconsistent results raise continuous debate about the contribution of AOA and AOB to nitrification [12]. So far, the environmental factors that drive diversification of ammonia-oxidizing microorganism remain largely unexplored, especially anthropogenically disturbed ecosystems. The large-scale geographic analysis of AOA and AOB population may provide intriguing hints regarding the niche differentiation of ammonia oxidizer communities and their contribution in nitrification.

Rice fields occupy a large portion of global cropland and nearly account for 26% of China's total croplands [13]. Nitrification in paddy soils contributes a lot to the nitrogen cycling in the terrestrial ecosystem. The paddy soils are subjected to temporal or permanent flooding during the growth of rice, which makes them model systems to investigate the divergence of ammonia oxidizers communities in microaerobic niches of the surface soil. Cultured-based assays indicated that AOA can be enriched under such circumstances [14]. AOA which exhibit the capacity of detoxification of reactive oxygen species (ROS) may support them to be active in the microaerobic environment [15]. *amoA*-based quantification showed that AOA was more abundant in the paddy soils [16] [17]. In consideration of those data, it seems that AOA might be more active to the paddy soils rather than AOB.

In addition to oxygen fluctuations, much is now known about the multiple environmental factors which were reported to regulate the community structures of AOA and AOB, including ammonia substrate availability [18], pH [19], temperature [20], moisture [21], and soil organic carbon [22]. Among these factors, many studies have stated that pH might be the most dominant variable to govern the niche differentiation of AOA and AOB [12]. Acidophilic AOA isolate *Nitrosotalea devanaterra* showed extraordinary affinity to ammonia substrate and grow at an extremely low ammonia concentration of 0.18 nM [23], while the activity of AOB was found to decrease with pH and could only grow on ammonia at μM level [24]. It has also been demonstrated that long-term crop rotation, fertilization, and tillage can impact the activity and structure of ammonia-oxidizing communities [25]. Nevertheless, little is known about the extent of paddy soil heterogeneity could have shaped the differentiation of AOA and AOB communities.

Large geographical scale experiment may provide comprehensive insights into how different types of soils with various chemical properties lead to the diversification of ammonia-oxidizing communities. The present study was conducted on eight paddy soils which geographically distribute in different temperature zone, from cold temperate zone to subtropical zone, and also have a rice cultivation history of >15 years with a wide pH range of 5.37 - 7.99. We employed quantitative real-time PCR and DGGE combined with clone library approaches based on the *amoA* gene to explore the correlation between variable soil properties and activity and community structure of ammonia-oxidizing microorganisms.

2. Material and Methods

2.1. Site Description and Soil Sampling

A total of eight paddy soil samples representing eight parent material types from different provinces were collected from northern China to southern China during September, 2016, to exclude seasonal variation (Figure 1 and Table S1). These paddy soil samples were obtained from Heilongjiang (HLJ), Beijing (BJ), Shanxi (SX), Henan (HeN), Anhui (AH), Jiangxi (JX), Chongqing (CQ) and Hunan (HuN), respectively. The types of soils were indicated in Figure 1. The paddy field all had a history of rice cultivation for more than 15 years. At each site, triplicate surface soil samples (depth, 0 to 15 cm) were randomly obtained by using a sterile hand trowel. Collected samples were placed in sterile plastic bags, sealed, and transported to the laboratory on ice. Plant residues and other materials, such as stones and obvious macrofauna, were removed before each replicate from each field was homogenized through a 2 mm mesh. Aliquots (5 g wet weight) of soil from each site were stored at -80°C prior to subsequent DNA extraction and molecular analysis. The subsamples were stored at 4°C for chemical determination and potential nitrification activity (PNA). PNA was processed immediately after arrival.

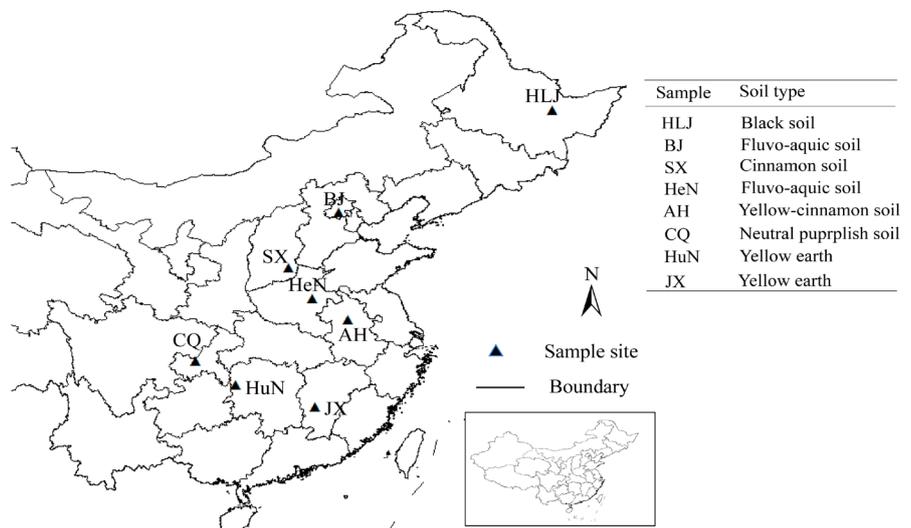


Figure 1. Geographic map of sampling sites.

2.2. Soil Physiochemical Properties Analysis

Chemical characteristics including pH, soil water content (SWC), NH_4^+ -N, NO_3^- -N, soil organic matter (SOM) and total nitrogen (TN) were determined in this study. The soil pH was determined using a Mettler Toledo 320-S pH meter (Mettler-Toledo Instruments Co.Ltd., Shanghai, China) with a water-to-soil ratio of 2.5. Water content was determined gravimetrically by drying the soil at 105°C for 6 h. NH_4^+ -N and NO_3^- -N were extracted from soil samples with 2 M KCl, and the contents were determined using a Skalar San Plus segmented flow analyzer (Skalar Inc., Breda, The Netherlands). SOM was detected using a total carbon analyzer (TOC-V CPH, Shimadzu, Japan). TN was determined by the Kjeldahl digestion method. All analyses were performed in triplicate for each soil sample. These parameters were then calculated based on oven-dried soil weight.

2.3. Nitrification Potential Measurements

The PNA is the maximum capacity of nitrifying microorganisms to transform NH_4^+ -N to NO_3^- -N in a soil population. Changes in nitrification potential provide quantitative information on how nitrifying communities behave in various environmental conditions and reflect the *in situ* nitrification rates. Nitrification potential was determined according to the shaken-slurry method [26]. PNAs were measured with fresh soils from each site by mixing each of triplicate. 15 g (wet weight) of soil from each sample was mixed with 100 ml of 1.5 mM ammonium sulfate. After incubation for 0, 2, 6, 12 and 24 h at 25°C , 10 ml slurry samples were centrifuged and the supernatant was filtered through a $0.45\ \mu\text{m}$ pore size membrane and stored at -20°C until analysis. NO_x^- -N content in the supernatant was immediately analyzed. NO_x^- -N concentration increased linearly, and nitrification potential ($\mu\text{g}\ \text{NO}_x^-$ -N $\cdot\text{g}^{-1}\cdot\text{h}^{-1}$) was calculated from the rate of increase in NO_x^- -N concentration over time in the slurry using linear regression.

2.4. Soil Genomic DNA Extraction

The total DNA in soil samples was extracted and purified from 0.5 g (dry weight) of soil using a FastDNA spin kit for soil (MP Biomedicals, Cleveland, OH, USA), according to the manufacturer's instructions. The quantity and quality of DNA extracts were assayed using a NanoDrop ND-1000 UV-visible light spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The soil DNA was stored at -20°C for further analysis.

2.5. Real-Time qPCR of *amoA* and 16S rRNA Genes

Real-time quantitative PCR (qPCR) was performed in triplicate on a CFX96 Optical RealTime detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to determine the copy number of *amoA* and 16S rRNA genes in soil DNA extracts. The number of *amoA* gene copies per g of soil (dry weight) was measured using primers *amoA*-1F and *amoA*-2R [27] targeting *amoA* gene of AOB, and primers *amoA*-AF and *amoA*-AR [28] targeting *amoA* gene of AOA. A universal 16S rRNA gene qPCR was conducted to assess the total copy number of bacterial communities by using the primer 515/907. The primers and PCR/qPCR thermal conditions were detailed in Table S2 in the supplemental material.

The real-time quantitative PCR standard was generated using plasmid DNA from one representative clone containing bacterial or archaeal *amoA*, and 16S rRNA gene. A dilution series of standard template over eight orders of magnitude per assay was used. In addition, a series dilution of soil DNA extract was also used to assess whether a PCR inhibition by humic substance co-extraction occurred in the paddy soils. The *amoA* gene copies decreased proportionally with the diluted soil DNA template concentrations. A 10-fold diluted soil DNA was used for subsequent analysis. qPCR was performed in a reaction mixture containing $1 \times$ SYBR[®] Premix Ex Taq[™] (TaKaRa Biotech, Dalian, China), 0.5 mM of each primer, and approximately 1.0 - 5.0 ng DNA template. Amplification efficiencies ranged from 91% to 103%, with R^2 values of 0.993 to 0.999. Melting curve analysis and standard agarose gel electrophoresis were always performed at the end of a PCR run to verify the amplification specificity.

2.6. DGGE Analysis of *amoA* Gene

AOB and AOA composition were analyzed by a DGGE of *amoA* genes using the D-Code system (Bio-Rad Laboratories, Hercules, CA), as described previously [6]. PCR reaction was carried out in a thermal cycler (Bio-Rad Laboratories, Hercules, CA). The thermal condition was listed in Table S2. The PCR products were checked using 1.2% agarose gel to ascertain the specificity of bacterial *amoA* and archaeal *amoA* gene amplification with a size of ~489 bp and ~629 bp, respectively. The 150 to 200 ng PCR amplicons of bacterial *amoA* were separated using 8% (wt/vol) polyacrylamide [acrylamide-bisacrylamide (37.5:1)] gels with a denaturing gradient of 30% - 60% (100% denaturant contains 7 M urea

and 40% formamide), and the 150 to 200 ng PCR amplicons of archaeal *amoA* were separated using 6% gels with a denaturing gradient of 30% - 50%. A 1 mm thick gel 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 24 wells. An electrophoresis separation of bacterial *amoA* and archaeal *amoA* gene amplicons was carried out using a 0.5 × Tris-acetate-EDTA buffer at 80 V for 17 h at 60°C. The gels were stained with 1:10000 (v/v) SYBR Green I for 30 min, and then scanned with a Molecular Imager FX using Quantity One software package (Bio-Rad Laboratories, Hercules, CA, USA). Dominant bands in the DGGE fingerprints were excised and reamplified. The reamplified PCR products of the DGGE bands were cloned using pEASY-T3 vector (TransGen-Biotech, Beijing, China) according to the instructions of the manufacturer. The clones containing the exact bacterial *amoA* and archaeal *amoA* gene insert from each DGGE band were sequenced (Invitrogen, Shanghai, China). DNAMAN software was used to check the clone sequences manually for subsequent analyses. Phylogenetic analysis was performed using the Molecular Evolutionary Genetics Analysis (MEGA 7.0) software package. The sequences of basic tree were from known AOB and AOA cultures, and fosmid clones of the *amoA* genes.

2.7. Statistical Analysis

The concentration of NO₃⁻-N, NH₄⁺, SOM, TN, PNA and *amoA* gene copy numbers were compared through multiple sample comparisons using one-way ANOVA analysis followed by Student-Newman-Keulstest to check for quantitative variance between different samples. The two-way ANOVA analysis was performed to examine the effect of different soil characteristics and soil types on PNA. Pairwise comparisons between treatments were determined with two-tailed Student's t-tests. All analyses were conducted using SPSS version 20.0 (IBM, Armonk, NY, USA). Pearson's correlation analyses were performed to assess the relationships among PNA, soil properties and the abundances of AOA and AOB. Redundancy analysis (RDA) with the Monte Carlo permutation's test (499 permutations) was carried out to determine if the AOA and AOB community structures were correlated with PNA and soil properties using Canoco (version 4.5).

2.8. Accession Numbers of Nucleotide Sequences

The nucleotide sequences were deposited at the GenBank with Accession Numbers MG000601-MG000613 and MG000592-MG000600 for the bacterial *amoA* and archaeal *amoA* genes in this study, respectively.

3. Results

3.1. Soil Properties and Nitrification Activity

Soil properties including SOM, TN, inorganic nitrogen concentration, soil water

content, pH were listed in **Table 1**. The soil samples were collected from different latitude from 21 m to 931 m. Soil samples from BJ, SX, HeN, AH, and CQ had similar pH values from 7.72 to 7.99, which was classified as alkaline soil. Acidic soil samples from HLJ, JX, and HuN had relatively lower pH values of 5.37, 5.38 and 6.10, respectively. The water content of the soil samples ranged from 10.82% to 29.37%. There was significantly difference in SOM and TN content of these soil samples from different sampling sites ($P < 0.05$), ranging from 21.34 to 49.11 $\text{g}\cdot\text{kg}^{-1}$ and 0.68 to 1.23 $\text{g}\cdot\text{kg}^{-1}$, respectively, with the highest content in HLJ sample. SX sample had the highest NO_3^- -N and NH_4^+ -N content, while CQ sample had the lowest NO_3^- -N content and JX had the lowest NH_4^+ -N content. Soil NH_3 concentrations were estimated to be varied 0.41 to 553.18 μM . The NH_3 content was lowest in the acidic soil sample JX and was highest in the alkaline soil sample AH. This is consistent with the principle that NH_3 is ionized exponentially to NO_3^- with decreasing pH [29]. The PNA ranged from 0.43 to 3.57 $\mu\text{g NO}_3^-$ -N $\cdot\text{g}^{-1}\cdot\text{h}^{-1}$ (**Table 1**) across all paddy soils. Sample from AH was found to have the high PNA. As revealed by Pearson correlation analysis, PNA had significant relationship with soil NH_3 concentration ($r = 0.82$, $P < 0.01$) in **Table 2**.

3.2. Abundance of Bacterial and Archaeal 16S rRNA Gene and *amoA* Genes

Bacterial 16S rRNA and *amoA* gene were quantified by qPCR to investigate the variation in population sizes of microbial and nitrifying communities (**Figure 1S** and **Figure 2**). As shown in **Figure 2**, the copy numbers of archaeal and bacterial *amoA* genes ranged from 3.58×10^9 to 6.54×10^{10} , and from 3.35×10^6 to 5.09×10^8 copies $\cdot\text{g}^{-1}$ *d.w.s* in these paddy soils, respectively. AOA abundance was

Table 1. Sampling site and soil characteristics^a.

Soil sample	Latitude (m)	Soil Water Content (%)	pH (1:2.5H ₂ O) ^c	SOM ^b (g $\cdot\text{kg}^{-1}$)	TN ^b (g $\cdot\text{kg}^{-1}$)	NO_3^- -N ($\mu\text{g}\cdot\text{g}^{-1}$)	NH_4^+ -N ($\mu\text{g}\cdot\text{g}^{-1}$)	Predicted soil NH_3 concentration (μM) ^d	PNA ($\mu\text{g NO}_3^-$ -N $\text{g}^{-1}\cdot\text{h}^{-1}$) ^e
HLJ	82	18.60 \pm 1.71d	5.37 \pm 0.04d	49.11 \pm 0.13a	1.23 \pm 0.01a	30.56 \pm 4.92d	19.29 \pm 0.52b	0.95	0.64 \pm 0.36e
BJ	57	10.82 \pm 0.18f	7.98 \pm 0.04a	32.78 \pm 0.17b	0.87 \pm 0.01b	39.01 \pm 2.49c	14.81 \pm 1.13d	511.72	1.60 \pm 0.38c
SX	931	18.71 \pm 0.32d	7.83 \pm 0.02ab	28.56 \pm 0.15d	0.79 \pm 0.01c	140.27 \pm 7.17a	26.33 \pm 1.16a	372.88	2.59 \pm 0.36b
HeN	60	14.30 \pm 0.28e	7.87 \pm 0.03a	23.61 \pm 0.36g	0.71 \pm 0.01e	40.56 \pm 2.90c	16.96 \pm 0.18c	294.34	1.27 \pm 0.24cd
AH	21	13.10 \pm 0.11e	7.99 \pm 0.17a	27.12 \pm 0.02e	0.80 \pm 0.01c	36.78 \pm 3.28c	18.49 \pm 0.35b	553.18	3.57 \pm 0.05a
JX	72	29.37 \pm 0.57a	5.38 \pm 0.020d	31.38 \pm 0.37c	0.83 \pm 0.01c	62.64 \pm 3.61b	12.38 \pm 0.32e	0.41	0.86 \pm 0.19
CQ	320	27.76 \pm 0.72b	7.72 \pm 0.03b	21.34 \pm 0.04h	0.68 \pm 0.01f	3.85 \pm 0.46f	15.38 \pm 1.01d	114.73	0.43 \pm 0.16e
HuN	195	23.00 \pm 1.00c	6.10 \pm 0.02c	24.56 \pm 0.19f	0.76 \pm 0.01d	23.64 \pm 2.09e	16.99 \pm 0.58c	3.70	0.93 \pm 0.09de

^aMeans of three replicates per treatment are presented with standard deviation; ^bSOM and TN denote soil organic matter and total nitrogen, respectively. ^cpH was measured with water-soil ratio of 2.5. ^dSoil NH_3 concentration was estimated on the basis of soil water content, ammonium concentration, pH and adjusted for temperature (pKa of 9.245 at 25°C); ^ePNA, potential nitrification activity.

Table 2. Pearson correlation of chemical properties, PNA, abundance or ratio of bacteria, AOB and AOA.

Item	pH	SOM	TN	NO ₃ ⁻ -N	NH ₄ ⁺ -N	Soil Water Content	NH ₃	PNA
PNA	0.43	-0.20	-0.24	0.33	0.26	-0.28	0.82**	1
AOA	0.22	-0.23	-0.26	0.26	0.16	-0.07	0.39	0.83**
AOB	-0.32	0.65*	0.64*	-0.23	-0.13	0.23	-0.01	-0.16
16S rRNA Bacterial	0.06	0.29	0.36	-0.65*	-0.07	-0.20	0.21	0.09
AOA/AOB	0.18	-0.44	-0.36	0.22	0.03	-0.28	-0.14	-0.08
AOB/16S rRNA Bacterial	-0.37	0.24	0.12	0.49	-0.13	0.46	-0.14	-0.03

*Correlation is significant at the 0.05 level; **Correlation is significant at the 0.01 level.

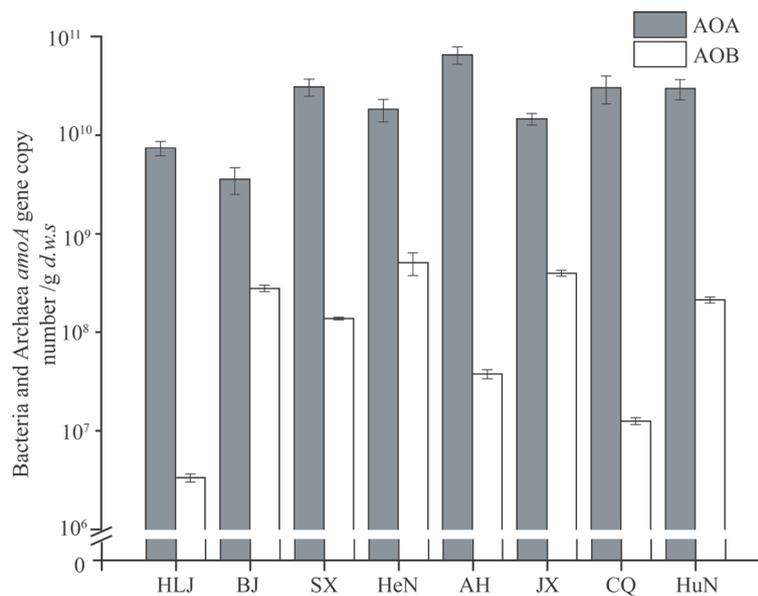


Figure 2. The abundances of ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacteria (AOB) *amoA* genes (represented by black and white bars, respectively). The error bars represent the standard error of the means of the triplicates. All other designations are the same as those in **Table 1**.

generally higher than AOB abundance in paddy soils. The ratio of archaeal to bacterial *amoA* gene copy numbers varied from 12.8 to 2400.0. The abundance of bacterial 16S rRNA genes ranged from 3.67×10^{12} to 2.45×10^{13} copies $\cdot g^{-1}$ *d.w.s* in different paddy soil samples (**Figure S1**).

Correlation analysis was performed to reveal the influence of environmental parameters on the nitrifying communities and PNA (**Table 2**). There was a significant positive correlation between PNA and AOA abundance ($r = 0.83$, $P < 0.01$), whereas no relationship was observed between PNA and AOB abundance ($r = -0.16$, $P > 0.05$). The PNA was also significantly correlated with soil NH₃ content ($r = 0.82$, $P < 0.01$). A significant positive correlation was found between AOB population size and SOM ($r = 0.65$, $P < 0.05$) and TN ($r = 0.64$, $P < 0.05$). In addition, the abundance of bacterial 16S rRNA gene was positively correlated

with soil NO_3^- -N content.

3.3. Diversity and Structure of Ammonia-Oxidizing Bacteria and Ammonia-Oxidizing Archaea Communities

The structures of AOA and AOB communities were revealed by fingerprinting analysis of bacterial and archaeal *amoA* genes in all paddy soils (Figure 3(a) and Figure 4(a)). Highly reproducible DGGE fingerprints were obtained in the triplicate of each soil sample. The diversity of AOA and AOB were indicated by Shannon's diversity index (*H*), richness (*S*) and Evenness (*Eq*), which were calculated by the relative abundance of various DGGE fingerprints in different soil samples (Table 3). The *H* and *S* index of AOA communities in HLJ, BJ and SX samples were clearly higher, when compared with the corresponding index of AOB, whereas the opposite trend was observed for the remaining soil samples. The highest *H*, *S* and *Eq* index of AOA communities was found in BJ soil, while the lowest was found in AH soil.

Distinct different DGGE fingerprints of archaeal *amoA* genes were observed among the different paddy soils. AOA communities in paddy soils were dominated by the DGGE band-A3, which were affiliated with the 54d9-like cluster of the soil group 1.1b lineage (Figure 3 and Figure 5). The majority of the DGGE bands for AOA were affiliated with the soil group 1.1b lineage except for the DGGE band-A2, which was placed within the *Nitrosotalea devanattera*-like cluster of the marine group 1.1a-associated lineage. The DGGE band-A5 and A6 showed higher intensities in the SX, HuN and HLJ samples, although they were faint in other soils. The DGGE band-A8 and A9 were observed in six soil samples except for AH and JX. The result was confirmed by cluster analysis which revealed that AH and JX samples were clearly separate from other samples with an 89% similarity (Figure 3(b)). DGGE band-A7 was only detected in HLJ.

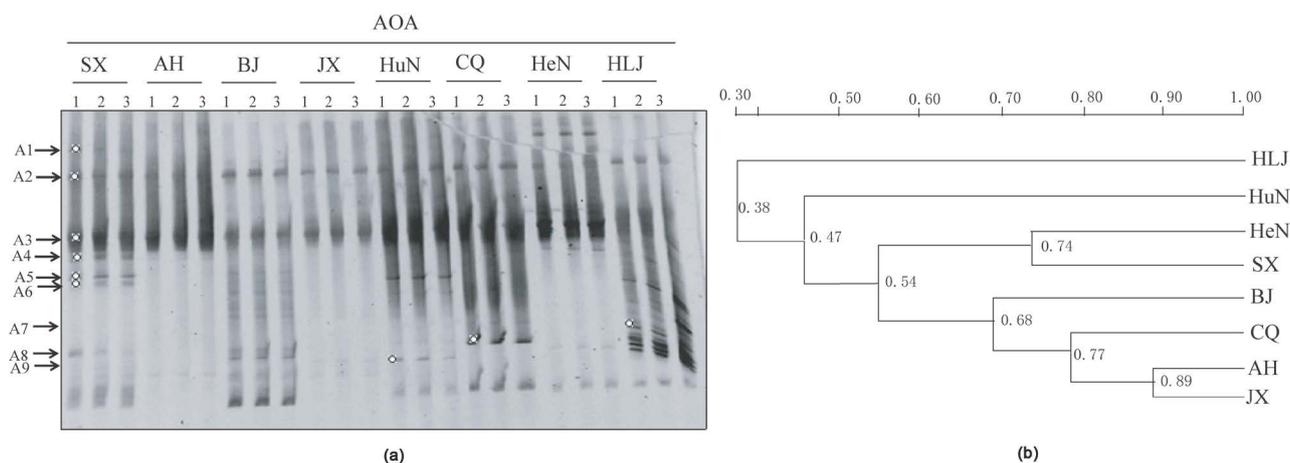


Figure 3. DGGE fingerprints of the archaeal *amoA* genes in different paddy soils (a), and similarity dendrograms (UPGMA, Dice coefficient of similarity) of AOA band patterns calculated from DGGE fingerprints in different paddy soils (b). The numbers 1, 2 and 3 represent the soil triplicates of each paddy soil. The arrows indicate the DGGE bands excised for sequencing. All other designations are the same as those in Table 1.

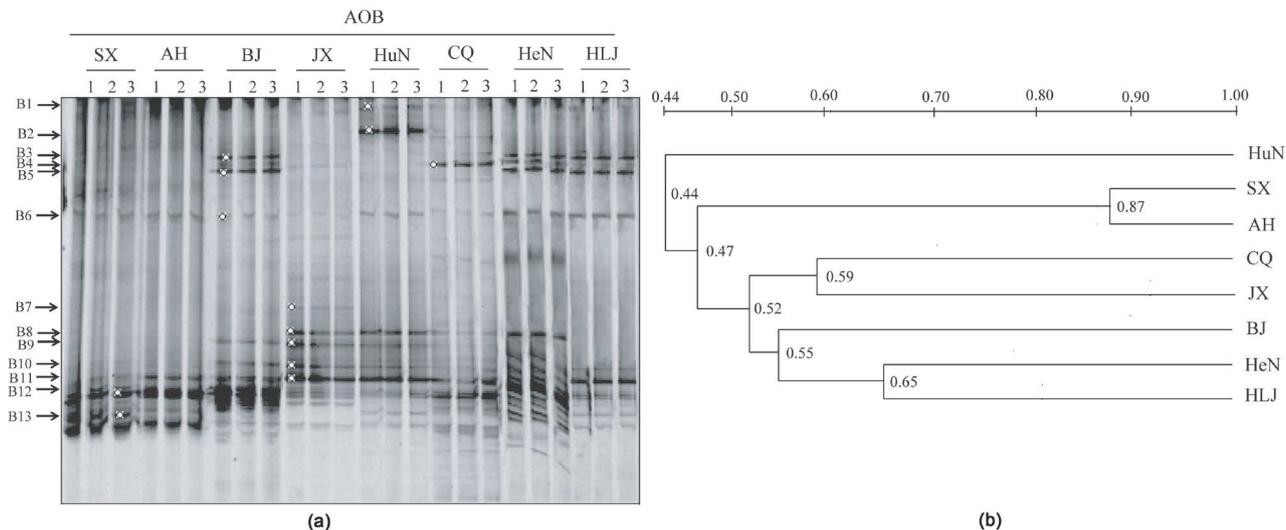


Figure 4. DGGE fingerprints of the bacterial *amoA* genes in different paddy soils (a), and similarity dendrograms (UPGMA, Dice coefficient of similarity) of AOB band patterns calculated from DGGE fingerprints in different paddy soils (b). The numbers 1, 2 and 3 represent the soil triplicates of each paddy soil. The arrows indicate the DGGE bands excised for sequencing. All other designations are the same as those in **Table 1**.

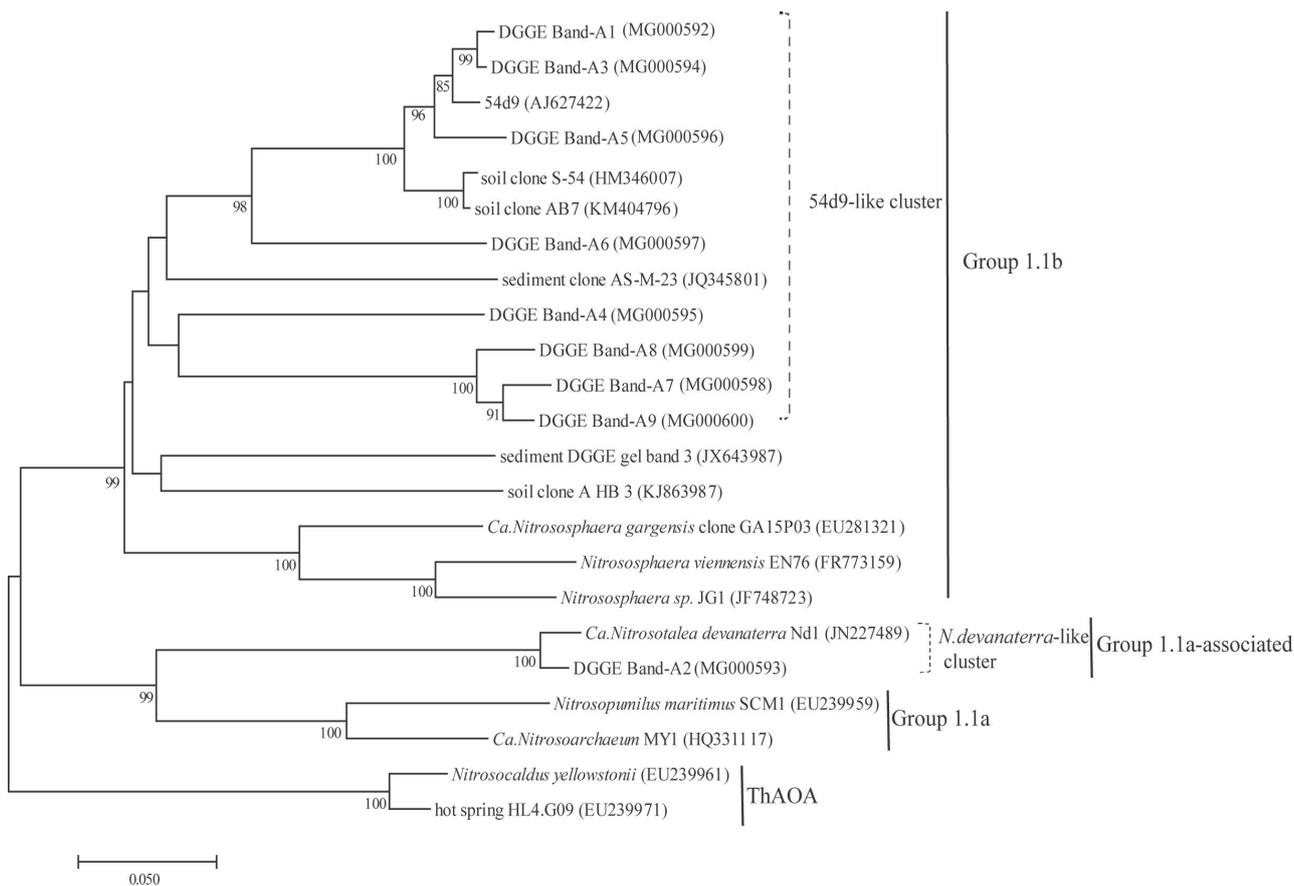


Figure 5. Phylogenetic tree showing the relationship of the archaeal *amoA* gene in different paddy soil. Bootstrap values higher than 80% are indicated at the branch nodes. The scale bar represents five changes per 100 nucleotide positions. GenBank accession numbers are given in parentheses.

Table 3. Shannon index richness obtained by DGGE profiles of bacterial and archaeal *amoA* gene in different paddy soils.

Sample	Shannon's diversity index(<i>H</i>)		Richness(<i>E</i>)		Evenness(<i>Eh</i>)	
	AOA	AOB	AOA	AOB	AOA	AOB
HLJ	2.278 ± 0.134b	2.039 ± 0.004d	10 ± 1ab	8 ± 0d	0.977 ± 0.009bc	0.981 ± 0.002a
BJ	2.391 ± 0.002a	2.148 ± 0.004c	11 ± 0a	9 ± 0c	0.997 ± 0.001a	0.978 ± 0.002bc
SX	2.152 ± 0.005cd	1.586 ± 0.010e	9 ± 0c	5 ± 0e	0.979 ± 0.002bc	0.985 ± 0.006a
HeN	2.229 ± 0.012ab	2.500 ± 0.044a	11 ± 0a	13 ± 1a	0.959 ± 0.005d	0.985 ± 0.002a
AH	1.312 ± 0.005f	1.588 ± 0.004e	4 ± 0e	5 ± 0e	0.946 ± 0.004e	0.987 ± 0.002a
JX	1.749 ± 0.003e	2.241 ± 0.016b	6 ± 0d	10 ± 0b	0.976 ± 0.002bc	0.973 ± 0.007c
CQ	2.078 ± 0.069d	2.270 ± 0.003b	8 ± 1c	10 ± 0b	0.981 ± 0.002b	0.986 ± 0.001a
HuN	2.240 ± 0.005bc	2.264 ± 0.005b	10 ± 0b	10 ± 0b	0.973 ± 0.002c	0.983 ± 0.002ab

Note: Different lowercase letters in the same column mean significant difference ($P < 0.05$)

Distinct AOB communities were also observed in the paddy soils. Phylogenetic analysis showed that eight of the thirteen DGGE bands were related with the *Nitrosospira* cluster lineage, whereas the remaining 5 DGGE bands were placed within *Nitrosomonas* cluster 7 (Figure 4(a) and Figure 6). The dominant AOB DGGE bands B11, B12 and B13 with high-intensity were all clearly present in all soil samples, which were affiliated with the *Nitrosospira* cluster 3. Additionally, the DGGE band B3 and B5 belonging to *Nitrosomonas europaea*-like cluster seemed obviously higher in BJ, HeN and HLJ samples, which were also grouped into one cluster with a similarity of 55% (Figure 4 and Figure 6). The AOB communities in SX and AH samples reached 87% of the similarity. The high intensity of DGGE band-B2 affiliated with the *Nitrosomonas* cluster 7 was only detected in HuN sample.

3.4. Correlations of Soil Properties and PNA with Community Structures of Ammonia-Oxidizing Bacteria and Archaea

Pearson correlation analyses were performed to reveal the impact of soil properties on the diversity index of nitrifying communities and PNA (Table 4). The PNA was significantly negatively correlated with the diversity of AOB ($r = 0.81$, $P < 0.01$). The Richness *E* of AOA and AOB communities was positively correlated with soil SOM (AOA, $r = 0.66$, $P < 0.05$ and AOB, $r = 0.64$, $P < 0.05$) and TN (AOA, $r = 0.63$, $P < 0.05$ and AOB, $r = 0.66$, $P < 0.05$). All other diversity indexes did not show a significant correlation with the soil properties in paddy soils.

RDA was conducted to determine the correlation of soil properties with community structures of AOA and AOB (Figure 7). Partial RDAs based on Monte Carlo permutation ($n = 499$) only retained the significant parameters in the models. The first and second axes accounted for 49.0% and 18.0% of the total variation in AOA community structure, respectively (Figure 7(a)). Regarding

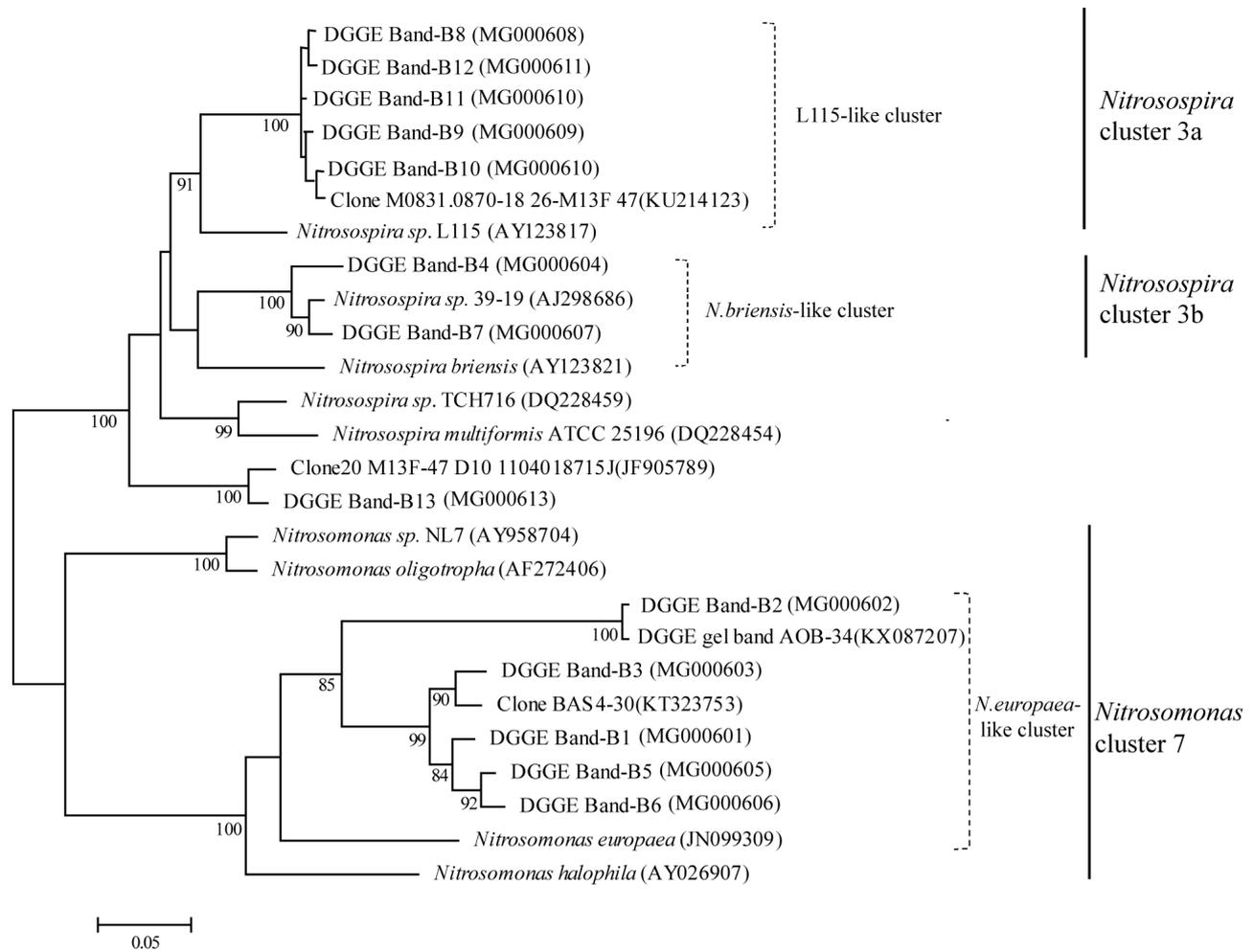


Figure 6. Phylogenetic tree showing the relationship of the bacterial *amoA* gene in different paddy soil. Bootstrap values higher than 80% are indicated at the branch nodes. The scale bar represents five changes per 100 nucleotide positions. GenBank accession numbers are given in parentheses.

Table 4. Pearson's correlation coefficients of soil properties, PNA with community diversity of ammonia-oxidizing bacteria and archaea.

Sample	Shannon's diversity index(<i>H</i>)		Richness(<i>E</i>)		Evenness(<i>Eh</i>)	
	AOA	AOB	AOA	AOB	AOA	AOB
PNA	-0.58	-0.81**	-0.48	-0.73*	-0.50	0.40
SOM	0.49	0.53	0.66*	0.64*	-0.12	0.29
TN	0.46	0.56	0.63*	0.66*	-0.14	0.28
NO ₃ ⁻ -N	0.19	-0.58	0.16	-0.55	0.11	0.45
NH ₄ ⁺ -N	0.03	-0.48	0.01	-0.40	-0.05	0.42
WC	-0.08	0.16	-0.09	0.15	-0.21	-0.37
pH	-0.41	-0.51	-0.54	-0.54	0.27	0.13
NH ₃	-0.25	-0.51	-0.14	-0.44	-0.18	0.35

*Indicate significant level at $p < 0.05$ level. **Indicate significant level at $p < 0.01$ level.

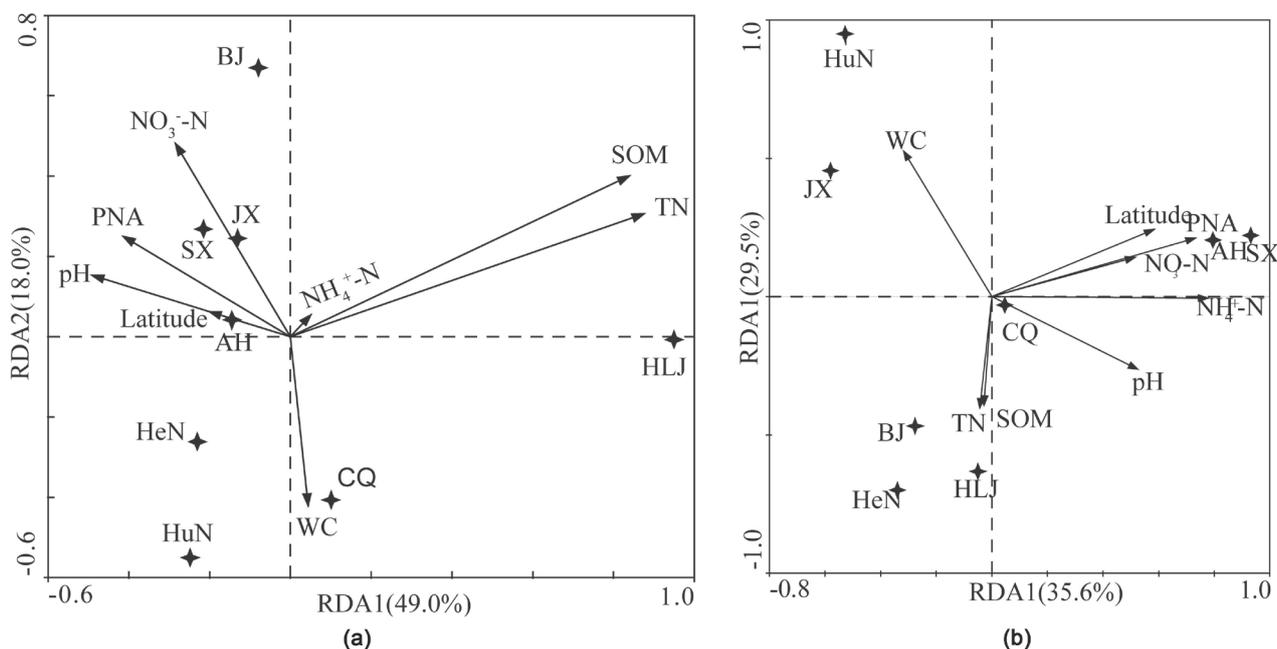


Figure 7. Correlation of soil properties with community structure of AOA (a) and AOB (b) determined by redundancy analysis (RDA). The four-pointed star represents the soil sample. Arrows indicate the direction and magnitude of variables.

the AOA community, the most influential constraining variable 40% was TN ($F = 4.04$, $P = 0.002$) (Figure 7(a) and Table 5). The first and second axes accounted for 35.6% and 29.5% of the total variation in AOB community structure (Figure 7(b)). No significant correlation between AOB community structure and soil properties was found.

4. Discussion

The great demand for rice in Asia has prompted the intensified application of synthetic nitrogen chemical fertilizers to increase the rice productivity [30]. This agricultural intervention combined with flooding management is the major ecological force to drive the diversification of AOA and AOB communities. Our results exhibited the overview of phylogenetically and functionally distinct AOA and AOB communities in the 8 different paddy soils of China. This study suggests the strong adaptation of the versatile and diverse ammonia oxidizers to markedly different soil environmental conditions in the collected paddy soils.

The ratio of AOA to AOB in this study ranged from 12.8 to 2400.0. It appears that the paddy soils with agricultural interventions, such as frequent flooding and fertilizations, favored the growth of AOA communities. Our results agreed well with recent findings regarding ammonia oxidizer dynamics in a red paddy soil with the AOA/AOB ratio of 36.0 - 1686.0 [31] and other paddy soil in China [32]. In our study, the abundance of AOA had negative correlation with SOM and TN, which was also observed previously in surface soil [33]. This might be due to the increase of bioavailability of carbon and nitrogen that stimulate the

Table 5. Eigen values, F values and P values obtained from the partial RDAs testing the influence of the significant parameters on the AOA and AOB community composition^a.

Parameters included in the model	Eigen value ^b	Variation explains solely	Fvalue ^c	Pvalue ^{c,d}
AOA				
TN	0.40	40	4.04	0.002
SOM	0.14	14	1.51	0.208
NH ₄ ⁺ -N	0.10	10	1.13	0.416
NO ₃ ⁻ -N	0.09	9	1.00	0.480
PH	0.09	9	1.04	0.448
Latitude	0.09	9	1.15	0.488
AOB				
NH ₄ ⁺ -N	0.23	23	1.84	0.106
SWC	0.14	14	1.09	0.412
PNA	0.18	18	1.57	0.240
SOM	0.08	8	0.70	0.644
pH	0.16	16	1.57	0.250
Latitude	0.09	9	0.76	0.550

^aPartial RDA based on Monte Carlo permutation (n = 499) kept only the significant parameters in the models; ^bSum of all Eigen values for both partial RDA were 1.000. ^cF and P values were estimated using Monte Carlo permutations. ^dP < 0.05 indicates the significant effect.

growth of heterotrophic bacteria, and thus inhibit the chemoautotrophic ammonia oxidizers [34]. Although AOA might be capable of mixotrophic or heterotrophic growth as previously suggested [12] [35], the ecological significance of a heterotrophic lifestyle for AOA in complex environments remains poorly understood. In contrast, positive correlations were found between SOM, TN and AOB abundance in the test soils. This nutrient-induced stimulation of AOB abundance further supports the idea that AOB could play an important role in environments with high fertilizer and nitrogen loads [36] [37]. The abundance of AOA rather than AOB was positively significantly correlated with PNA, demonstrating that AOA might be the more dominant nitrifiers in the collected paddy soils, which was also consistent with previous studies [38].

DGGE fingerprinting analysis showed that the community structures of AOA were remarkable variable in the test soils. The dominant composition of AOA fell within 54d9-like cluster belong to soil group 1.1b (Figure 5). Pester et al. [39] also indicated that the *amoA* gene of soil metagenome fragment 54d9 was widely distributed, which was detected in 75% of the 146 soils covering different biomes, like forest, grasslands, deserts and agricultural soils. The existence of AOA DGGE band-A2 affiliated with *N. devanattera*-like cluster indicated the AOA belonging to Group 1.1a could adapt and survive in more diverse conditions [40]. Flooding management in rice field leads to rapid depletion of oxygen

beneath the soil surface. A number of studies have shown that AOA may have higher substrate affinity for oxygen than AOB [41] [42], and be prone to adapt to life under low oxygen [43]. A marine AOA even sustains high ammonia oxidation activity at $<10 \mu\text{M O}_2$ concentrations in the suboxic region [44]. RDA revealed that soil TN content significantly affected AOA community ($P < 0.01$) (Table 5), which is consistent with previous study [45]. Studies stated that AOA within the marine group 1.1a and 1.1a-associated lineages have higher ammonia affinity [8] [46], whereas AOA within the soil group 1.1b lineage could tolerate high ammonium concentrations [47]. These findings indicate that the N available in the paddy soil could be a key parameter to shape the diversification of AOA community.

DGGE fingerprinting analysis combined with phylogenetic analyses demonstrated that AOB affiliated with the *Nitrospira* cluster-3a and 3b dominated the communities of AOB in the test soils, which was consistent with the previous studies in paddy soils [48] [49]. It was previously proved that *Nitrospira* cluster 3 - like AOB performed most bacterial ammonia oxidation in some Chinese paddy soils [49]. AOB belonging to *Nitrospira* cluster 3 often outcompete other *Nitrospira* species under high ammonia conditions [50]. The highly enrichment of *Nitrospira* cluster 3 in these paddy soil could be due to the anthropogenic management such as irrigation and fertilization. Moreover, AOB affiliated with *Nitrosomonas europaea* (cluster 7) was also observed in some of the test paddy soils except AH and SX samples. The detection of *Nitrosomonas*-like AOB in paddy soils was in agreement with previous studies [48] [51], whereas in some studies only *Nitrospira*-like AOB was detected [45] [52]. The rice paddy ecosystems with regular flooding, which could lead to strong fluctuations in available oxygen. Some members of *Nitrosomonas* has high affinity for oxygen, supporting them to be dominant in the micor-oxic environment [53], and the extensive present of *Nitrosomonas*-like AOB in the test paddy soils. Moreover, the different pattern of AOB community structures in these paddy soils might be due to the different ecological niches of *Nitrosomonas*-like and *Nitrospira*-like AOB shaped by environmental factors. It was supported by the positive correlation between soil SOM ($r = 0.64$, $P < 0.05$), TN ($r = 0.66$, $P < 0.05$) contents, and AOB community richness (Table 4). However, a higher resolution of the AOB communities and their major influence factors are required to be further studied with more powerful analysis techniques.

PNA representing the soil ammonia oxidation activity varied among different paddy soils. The positive correlation between PNA and NH_3 content, the abundance of AOA community, the diversity of AOB community. This finding could be attributed to the dependence of monooxygenase on NH_3 , which has been thought to be the actual substrate for ammonia oxidizers [29] [54]. The positive correlation between PNA and NH_3 content indicated that soil NH_4^+ might be the limiting factor during nitrification in the test paddy soils. However, rice fields are generally subjected to drastic anthropogenic disturbances with fertili-

zation and crop cultivation. The intensified fertilization was found to stimulate soil nitrification and the activity of ammonia oxidizers in neutral soil [55] and acidic soil [56], which thus promoting the demand of NH_4^+ and NH_3 substrate. Therefore, the changes of AOA and AOB population, and these soil physico-chemical variables associated with N availability together determine the PNA in different paddy soils.

5. Conclusion

Taken together, our study demonstrates the differential abundance and community structure of AOA and AOB in paddy soils from northern China to southern China. The abundance of AOA was significantly predominant over AOB. A positive correlation was observed between the PNA and soil NH_3 content, AOA abundance, while a negative correlation was detected between PNA and the diversity of AOB community ($r = -0.81$, $P < 0.01$). The results imply that AOA might play a more important role in controlling nitrification activity in these alkaline and acidic paddy soils. The coexistence of diverse AOA and AOB population further suggested that nitrification might be performed by different ammonia-oxidizing phylotypes combinations. This study also demonstrated that the contrasting soil physicochemical parameters of the geographically different paddy soils could synthetically determine the spatial variation of AOA and AOB communities.

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Supplementary

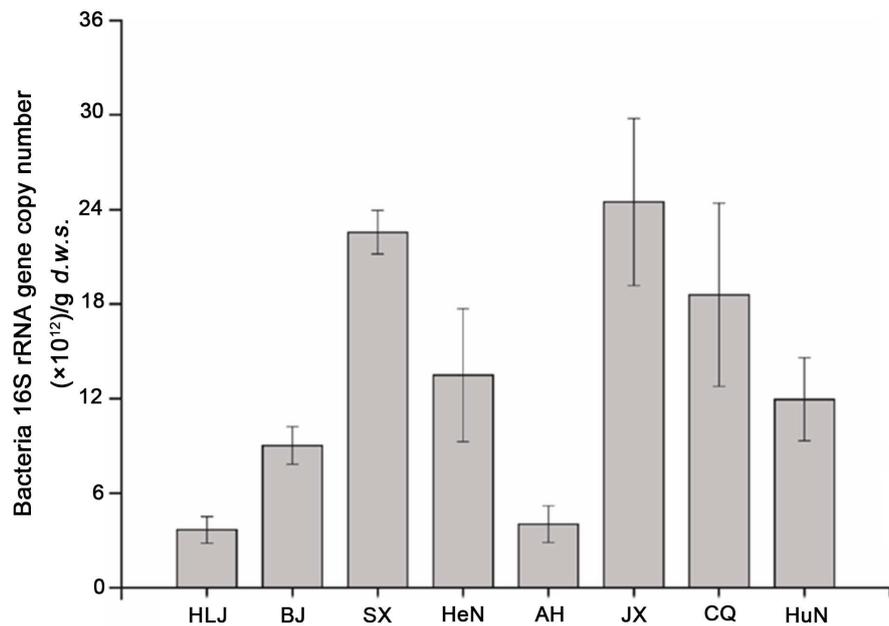


Figure 1S. The copy number of bacterial 16S rRNA genes in the eight different paddy soils. The error bars represent the standard errors of the means of the triplicates. All the designations are the same as those in **Table 1**.

Table S1. Sampling site characteristics.

Soil samples	Latitude (m)	Lat. (N)	Lon. (E)	Soil taxonomy
HLJ	82	46°48'29.96"	130°20'59.39"	Black soils
BJ	57	39°54'11.78"	116°24'3.31'E	Fluvo-aquic soils
SX	931	36°11'44.27"	113°06'35.33'E	Cinnamon soils
HeN	60	34°04'53.19"	114°38'57.14'E	Fluvo-aquic soils
AH	21	32°40'51.68"	117°1'19.62"	Yellow-cinnamon soils
JX	72	26°46'1.46"	114°52'41.53"	Yellow earths
CQ	320	29°51'37.15"	107°04'35.65"	Neutral purplish soil
HuN	195	28°16'11.85"	109°40'42.41"	Yellow earths

Table S2. Primers and conditions used in this study.

Primer Name	Primer sequence (5'-3')	Target gene	Molecular analysis	Thermal Profile	Reference
515F	GTG CCA GCM GCC GCG G	universal 16S rRNA bacterial gene	qPCR	95°C, 3 min; 40 × (95°C, 30s; 55°C, 30 s; 72°C, 30 s with plate read); Melt curve 65.0°C to 95.0°C, increment 0.5°C, 0:05 + plate read	(Stubner 2002) [1]
907R	CCG TCA ATT CMT TTR AGT TT				
CrenamoA23f	ATGGTCTGGCTWAGACG	crenarchaeal <i>amoA</i>	DGGE	94°C, 5 min; 35 × (94°C, 30 s; 56°C, 45 s; 72°C, 45 s); 72°C, 8 min; hold at 4°C	(Tourna <i>et al.</i> 2008) [2]
CrenamoA616r	GCCATCCATCTGTATGTCCA				
<i>amoA</i> -1F [#]	GGG GTT TCT ACT GGTGGT	bacterial <i>amoA</i>	qPCR/DGGE	95°C, 3 min; 35 × (95°C, 30s; 55°C, 30 s; 72°C, 45 s with plate read); Melt curve 65.0°C to 95.0°C, increment 0.5°C, 0:05 + plate read	(Avrahami <i>et al.</i> 2003) [3] Avrahami, Liesack & Conrad 2003)
<i>amoA</i> -2R	CCC CTC GGG AAA GCC TTC TTC				

The forward primer of bacterial *amoA* *amoA*-1F was attached with a 27 bp GC clamp (Nicolaisen and Ramsing 2002 [4]).

Supplementary Reference

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