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ORIGINAL PAPER

Differential distribution patterns of ammonia-oxidizing archaea and bacteria in acidic soils of Nanling National Nature Reserve forests in subtropical China

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Abstract In addition to ammonia-oxidizing bacteria (AOB) the more recently discovered ammonia-oxidizing archaea (AOA) can also oxidize ammonia, but little is known about AOA community structure and abundance in subtropical forest soils. In this study, both AOA and AOB were investigated with molecular techniques in eight types of forests at surface soils (0–2 cm) and deep layers (18–20 cm) in Nanling National Nature Reserve in subtropical China. The results showed that the forest soils, all acidic (pH 4.24–5.10), harbored a wide range of AOA phylotypes, including the genera *Nitrosotalea*, *Nitrososphaera*, and another 6 clusters, one of which was reported for the first time. For AOB, only members of *Nitrosospira* were retrieved. Moreover, the abundance of the ammonia monooxygenase gene (*amoA*) from AOA dominated over AOB in most soil samples (13/16). Soil depth, rather than forest type, was an important factor shaping the community structure of AOA and AOB. The distribution patterns of AOA and AOB in soil layers were reversed: AOA diversity and abundances in the deep layers were higher than those in the surface layers; on the contrary, AOB diversity and abundances in the deep layers were lower than those in the surface layers. Interestingly, the diversity of AOA was positively correlated with pH, but negatively correlated with organic carbon, total nitrogen and total phosphorus, and the abundance of AOA was negatively correlated with available phosphorus. Our results demonstrated that AOA and AOB were differentially distributed in acidic soils in subtropical forests and affected differently by soil characteristics.

Xian-Hua Gan and Fang-Qiu Zhang have contributed equally to this work.

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Introduction

As the first and rate-limiting step of the two-step nitrification process, ammonia oxidation is a pivotal process in the global nitrogen biogeochemical cycle (Kowalchuk and Stephen 2001). Ammonia-oxidizing bacteria (AOB) were long thought to be the only microbial players that perform this biochemical function (Kowalchuk and Stephen 2001; Winogradsky 1890) until the recent discovery of ammonia-oxidizing archaea (AOA) in the phylum *Thaumarchaeota* (Brochier-Armanet et al. 2008; Francis et al. 2005; Könneke et al. 2005). Both groups of ammonia-oxidizing microorganisms (AOM) can oxidize ammonia to nitrite aerobically, making them share similar ecological function in the environment (Schleper and Nicol 2010). AOB have been well studied for more than 100 years, but the newly discovered AOA and their relatively poorly understood characteristics and functioning in ecosystems has revived research on nitrification in the last decade.

Since the discovery of AOA, many studies have focused on the relative importance of AOA and AOB (Stahl and de la Torre 2012). A widely used approach is to compare their abundances in the environment through quantification of the *amoA* gene, which encodes the α-subunit of the ammonia monooxygenase, the catalytic subunit in both AOA and AOB. Through this approach, AOA have been demonstrated to be widely distributed and outnumber AOB in various environments, including marine sediments and water column, estuary sediments, hot springs, terrestrial soils and freshwater, and even arctic soils (Agogue et al. 2008; Alonso-Sáez et al. 2012; Alves et al. 2013; Beam et al. 2014; Ke et al. 2013; Lee et al. 2014a, b; Leininger et al. 2006; Wang et al. 2013; Wang and Gu 2013). Numerical dominance suggests that AOA play a more important role than AOB in nitrogen biogeochemistry, though this is still in debate because numerical dominance does not necessarily translate to higher activities in nature (Schleper 2010). Eco-physiological studies showed that although both AOA and AOB share the same broad function, they seem to have different niche requirements (Wessén et al. 2011). One of the differences is that AOA favor environments with low ammonium while AOB dominate habitats with high ammonium, which is due to the higher affinity of AOA for ammonia (Auguet et al. 2011, 2012). For example, studies have shown that "*Cadidatus* Nitrosopumilus maritimus" SCM1 has high affinity for ammonia, whereas AOB have lower affinities for ammonia. (Koper et al. 2010; Martens-Habbena et al. 2009).

Recent studies have shown that diverse and abundant AOA can exist in acidic habitats that are adverse to the survival of AOB (Hernández et al. 2014; Lehtovirta-Morley et al. 2011; Qin et al. 2013; Stopnisek et al. 2010; Zhang et al. 2012; Zhou et al. 2014). The concentration and availability of ammonia (NH_3) are both very low in acidic soils because the concentration of NH_3 decreases exponentially with decreasing pH ($NH_3 + H^+ \rightarrow NH_4^+$; $p\mathrm{Ka} = 9.25$). Because ammonia rather than ammonium (NH_4^+) is the substrate for AOA and AOB, the high affinity of AOA for ammonia is recognized as the main mechanism permitting AOA survival in acidic environments (Martens-Habbena et al. 2009; Tourna et al. 2011; Verhamme et al. 2011). The investigated acidic environments included agriculture soils (Gubry-Rangin et al. 2010; Jiang et al. 2014; Lehtovirta-Morley et al. 2011; Qin et al. 2013), tea orchards and forests (Isobe et al. 2012; Lu et al. 2012; Yao et al. 2011; Zhang et al. 2012), forest peat soils (Stopnisek et al. 2010), acidic fens (Herrmann et al. 2012), and acidic geothermal springs (Beam et al. 2014). Investigation on AOA in subtropical forests in China is relatively poorly studied (Isobe et al. 2012).

Forests are an important ecosystem on Earth, accounting for more than 90 % biomass of terrestrial ecosystems (FAOSTAT 2011). Subtropical forests, however, are continuously fragmented because of economic development and have been extensively transformed into commercial plantations (FAOSTAT 2011). Guangdong Province is mainly located in the subtropics, with a forest area of 9.849×10^4 km^2 in 2013 (FDGP 2014). Nanling National Nature Reserve, the largest reserve in Guangdong Province, protects various typical subtropical forests (Chen 2012). In this study, we initiated a molecular study on AOA and AOB in acidic soils of different types of forests in Nanling National Nature Reserve, aiming to uncover the distribution patterns of AOA and AOB in the soils

and the effects of soil characteristics and forest type on their distribution.

Materials and methods

Description of sites and sample collection

Soil samples were collected on October 15, 2013 from Guangdong Nanling National Nature Reserve, which is located in South China (24°37′–24°57′N, 112°30′–113°04′E) with an area of 58,368.4 hm^2. This nature reserve has a subtropical monsoon climate with an annual precipitation of 2108.4 mm. The average temperature in the hottest month (July) is 26.2 °C, while in the coldest month (January) 7.1 °C. The soils in this nature reserve are basically classified into subtropical yellow soil (above 700 m) and red soil (below 700 m), with the inorganic fraction dominated by granite, metamorphic rock or sandstone (NNNR 2015). Vegetation consists of evergreen broadleaf forest, bamboo forest, needle and broadleaf mixing forest, mountain dwarf forest, and mountain grassland. In this study, eight types of forests were investigated, including one type of mountainous dwarf forest, two types of bamboo forests at different elevations, two types of evergreen broadleaf forests at different elevations, and three types of coniferous and broadleaf mixing forests consisting of different tree species (Table 1).

For each of the eight forests, one composite sample of surface soil (0–2 cm deep) and one composite sample of deep layer soil (18–20 cm deep) were collected as follows. At each forest, three soil profiles (at least 10 m apart) were made in a 30 × 30 m plot. Before soil collection, the organic matter horizon was removed. Then one kg of surface and deep layer soils were collected from each of the three profiles, and the three soils of the same layer (surface or deep layer) were pooled and mixed thoroughly to form a composite sample. For each composite sample, 100 g of soil was kept in a small plastic bag for molecular analysis and 900 g of soil was kept in a larger plastic bag for physicochemical analysis; the rest was then discarded. The soil samples were immediately put onto ice bags in a heat-insulated cooler after collection and carried back to the laboratory on the same day of sampling. In the laboratory, samples for subsequent molecular studies were kept at −80 °C and those for physicochemical analysis were processed immediately.

Physicochemical analysis

Physicochemical analysis was carried out in Guangdong Institute of Eco-environment and Soil Sciences according to Methods of Agriculture Chemical Analysis (Lu 2000). Briefly, pH was measured with a pH meter (Starter 3C, OHAUS). Organic carbon was measured using the sulfuric acid dichromate digestion method. Total nitrogen was measured using the Kjeldahl method. Available nitrogen was measured using the KOH diffusion method. Ammonium-N and nitrate–N were extracted with KCl first and then analyzed with Nessler's reagent colorimetry and ultraviolet spectrophotometry, respectively. Total phosphorus was digested with $HClO_4 + H_2SO_4$ first and then measured with the colorimetry method. Available phosphorus was extracted with $NaHCO_3$ first and then measured with the colorimetry method. All the spectrophotometry was done with UV–Vis spectrophotometer (752 N type, Shanghai Jingke Co.).

Soil DNA extraction

Total DNA from each composite soil sample was extracted in duplicate using the SoilMaster DNA Extraction kit (Epicentre Biotechnologies, Madison, WI) according to the manual of the manufacturer. The duplicate DNA extracts from the same composite soil sample were then pooled and stored at −20 °C for subsequent molecular analysis.

Polymerase chain reaction amplification

The archaeal *amoA* genes were amplified using the primers Arch-*amoA*F (5′—STAATGGTCTGGCTTAGACG—3′) and Arch-*amoA*R (5′—GCGGCCATCCATCTGTATGT—3′) (Francis et al. 2005). Based on the standard procedures in the manufacturer's instructions (Promega) and results of previous studies (Francis et al. 2005), the optimized polymerase chain reaction (PCR) mixture contained in a final volume of 50 µl and consisted of the followings: GoTaq Flexi buffer (1× , Promega), $MgCl_2$ (1.5 mM, Promega), dNTPs (0.2 mM of each, Promega), GoTaq Flexi polymerase (0.025 U μl^{-1}, Promega), DNA template (~0.5 ng μl^{-1}), forward and reverse primers (each 0.4 µM), and BSA (0.01 %). PCR conditions were set as follows: 95 °C for 5 min; 30 cycles of 94 °C for 45 s, 53 °C for 1 min, and 72 °C for 1 min; and finally

Table 1 Locations and description of sites, tree species of the forests investigated in this study

Forest type	Sub-type	Forest type code	Location	Elevation (m)	Representative tree species
Mountainous dwarf forest	Mountainous dwarf forest	MD	24.943°N; 113.025°E	1427	*Rhododendron simiarum, Cyclobalanopsis stewardiana, Schima superba*
Coniferous and broad-leaved mixed forest	Coniferous and broad-leaved mixed forest-1	CB1	24.909°N; 113.042°E	966	*Pinus massoniana, Schima kwangtungensis, Paulownia fortunei*
	Coniferous and broad-leaved mixed forest-2	CB2	24.929°N; 113.016°E	1012	*Cunninghamia lanceolata, Liquidambar formosana, Choerospondias axillaris*
	Coniferous and broad-leaved mixed forest-3	CB3	24.928°N; 113.016°E	1032	*Cryptomeria fortunei, Camellia sinensis, Michelia maudiae*
Mountainous bamboo forest	Mountainous bamboo forest-high elevation	MBH	24.942°N; 113.025°E	1360	*Sinobambusa tootsik, Manglietia yuyuanensis, Liquidambar formosana*
	Mountainous bamboo forest-low elevation	MBL	24.921°N; 113.026°E	895	*Phyllostachys heterocycla, Photinia benthamiana, Elaeocarpus sylvestris*
Evergreen broad-leaved forest	Evergreen broad-leaved forest-middle elevation	EBM	24.929°N; 113.019°E	1021	*Schima superba, Machilus thunbergii, Acer davidii*
	Evergreen broad-leaved forest-low elevation	EBL	24.906°N; 113.050°E	672	*Castanopsis fabri, Sloanea sinensis, Cyclobalanopsis bella*

72 °C for 15 min. PCR products were checked by electrophoresis in a 1 % agarose gel stained with GelRed (1:10000, v/v).

The bacterial *amoA* genes were amplified using the primers *amoA*-1F (5′—GGGGGTTTCTACTGGTGGT—3′) and *amoA*-2R (5′—CCCCTCKGSAAAGCCTTCTTC—3′) (Rotthauwe et al. 1997). The optimized PCR reaction mixture contained in a final volume of 50 μl and consisted of the followings: GoTaq Flexi buffer (1 × , Promega), $MgCl_2$ (1.25 mM, Promega), dNTPs (0.2 mM of each, Promega), GoTaq Flexi polymerase (0.025 U μl^{-1}, Promega), DNA template ($\sim$0.5 ng μl^{-1}), forward and reverse primers (each 0.4 μM), and BSA (0.01 %). PCR conditions were set as follows: 94 °C for 3 min; 30 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 50 s; and finally 72 °C for 10 min. PCR products were checked by electrophoresis in a 1 % agarose gel stained with GelRed (1:10000, v/v).

Cloning and sequencing

Clone libraries were established from the PCR products as described by Friedrich et al. (2001). Briefly, the PCR-amplified products were purified using the Gel Advanced^{TM} Gel Extraction System (Viogene-Bio Tek Co., Taiwan, ROC) according to the manufacturer's instructions, and cloned into the PMD18 T-vector (Takara, Japan). The insertion of an appropriate-sized DNA fragment was determined by PCR amplification with the primer set M13F and M13R. About 30 clones in each library were randomly selected for sequencing. Sequencing was performed with ABI 3730xl DNA analyzer (Applied Biosystems).

Phylogenetic analysis and biodiversity calculation

The retrieved sequences were blasted on the web (http://blast.ncbi.nlm.nih.gov) (Altschul et al. 1990) and the most similar sequences were downloaded to rebuild the phylogenetic trees. For each sample, clones with more than 97 % putative protein sequence identity were grouped into the same OTU using the furthest neighbor algorithm in DOTUR (Distance-Based OTU and Richness) program (Schloss and Handelsman 2005), and their representative putative protein sequences (198 and 150 amino acids for AOA and AOB, respectively) were used for phylogenetic analysis using the software MEGA 6 (Tamura et al. 2013). Phylogenetic trees were constructed with the neighbor-joining method with 1000 bootstraps to estimate the confidence of the tree topologies.

Besides OTU assignments, the DOTUR program was also used to generate biodiversity indices such as

Chao1, Shannon index (*H*), and numbers of OTU for each sample.

Real-time quantitative PCR analysis

The abundances of archaeal and bacterial *amoA* genes were determined in triplicate with real-time quantitative PCR amplification using a FastStart Universal SYBR Green Master (Rox) Kit (Roche, Germany). Real-time qPCR was performed in 96-well optical plates placed in the ABI PRISM® 7000 Sequence Detection System (applied biosystems). The primer sets composed of Arch-*amoA*F and Arch-*amoA*R, and *amoA*-1F and *amoA*-2R were used for the amplification of the *amoA* genes of AOA and AOB, respectively. The final reaction volume was 20 µl and the reaction composition and cycling conditions were in accordance with the manual.

The specificity of the PCR amplification was determined by the melting curve and gel electrophoresis. Cycle thresholds were determined by comparing with the standard curves constructed using a 10 fold serial dilution ($10^2 \times 10^7$ gene copies μl^{-1}) of newly extracted plasmids containing corresponding gene fragments. Relative copy numbers among target groups were evaluated and some replicates of large deviation ($|x_i - u| > 2.41\sigma$) were excluded in order to decrease standard error. The correlation coefficient R^2 values were higher than 0.97 for all of the standard curves.

Principal coordinates analysis of AOM community structure

Fast UniFrac provides a suite of tools for the comparison of microbial communities using phylogenetic information (Goecks et al. 2010). To compare microbial communities in different environments, the phylogenetic trees of AOA and AOB were analyzed online using principal coordinates analysis (PCoA) on the website of Fast UniFrac (http://unifrac.colorado.edu).

Nucleic acid sequence accession numbers

The AOA and AOB *amoA* gene sequences determined in this study are available in GenBank under accession numbers KP736255 to KP736365 and KP736366 to KP736411, respectively.

Results

Physicochemical characteristics of forest soils

A total of eight parameters, including pH, organic carbon, ammonium-N, nitrate–N, available nitrogen, total nitrogen, available phosphorus and total phosphorus were investigated for the 16 forest soil samples (Table 2). All the soils were acidic with pH between 4.24 and 5.10. Soil nutrient concentrations varied considerably among different forest types. For example, organic carbon in the surface of one evergreen broadleaf forest (middle-elevation) was very high (151.8 g kg^{-1}), but that in the surface of another evergreen broadleaf forest (low-elevation) was relatively low (69.2 g kg^{-1}). Soil depth was a significant factor affecting nutrient levels. For each nutrient investigated, the average concentration of the surface soils was significantly higher than that of the deep layer soils (Table 2). For example, NH_4^+ concentration of the surface soils was 74.7 ± 25.0 mg kg^{-1} dry soil ($n = 8$), whereas that of the deep layers was only 35.6 ± 7.0 mg kg^{-1} dry soil ($n = 8$).

Phylogeny of ammonia-oxidizing microorganisms

The phylogenetic tree of AOA in the eight types of forests analyzed using putative protein sequences of amplified *amoA* genes is shown in Fig. 1. A total of 488 archaeal *amoA* clones were acquired from the soil samples, representing the eight types of forests. The phylogenetic tree showed that Nanling National Nature Reserve harbored a wide range of AOA phylotypes, which fell into *Nitrosotalea*-like, *Nitrososphaera*-like, and six unknown clusters that have not been retrieved elsewhere. No sequences fell into the *Nitrosopumilus*-like cluster, which is mainly found in oceans (Bertagnolli et al. 2015). *Nitrosotalea*-like AOA detected in this study were also frequently detected in other acid soils (Lehtovirta-Morley et al. 2011). *Nitrososphaera*-like AOA were demonstrated to be widely distributed in various soils (Tourna et al. 2011). Cluster 1 and Cluster 4 included sequences from acidic red soils. Cluster 2 and Cluster 6 included sequences from tributary sediments. Cluster 3 included sequences from forest soils, tributary sediments and sea sediments. Cluster 5 appears to be a new cluster of AOA.

Table 2 Physicochemical characteristics of the forest soils[a]

Forest type[b]	Soil depth[c]	pH	Organic C (g kg^{-1})	NH_4^+-N (mg kg^{-1})	NO_3^--N (mg kg^{-1})	Available N (mg kg^{-1})	Total N (g kg^{-1})	Available P (mg kg^{-1})	Total P (g kg^{-1})
MD	Surface	4.61	124.5	124.2	41.7	800	7.94	3.10	0.436
	Deep layer	4.45	26.7	40.1	14.0	214	2.55	0.04	0.279
CB1	Surface	4.24	95.3	65.8	29.2	411	5.09	6.49	0.256
	Deep layer	4.34	41.1	32.7	10.8	221	2.26	3.20	0.234
CB2	Surface	4.50	83.4	89.3	25.2	546	5.66	4.51	0.290
	Deep layer	4.72	28.2	43.6	10.9	224	1.93	0.18	0.239
CB3	Surface	4.56	64.5	59.5	66.7	405	4.48	0.23	0.228
	Deep layer	4.65	39.9	40.4	12.5	216	2.48	0.04	0.217
MBH	Surface	5.09	71.7	83.8	32.2	618	5.61	21.90	0.341
	Deep layer	4.94	23.7	41.1	14.3	222	1.93	1.36	0.194
MBL	Surface	4.55	21.8	34.9	8.1	159	1.31	0.70	0.251
	Deep layer	5.10	14.2	28.2	7.0	99	0.86	0.18	0.217
EBM	Surface	4.28	151.8	82.3	25.1	630	8.77	16.10	0.459
	Deep layer	4.70	48.9	36.6	11.9	210	3.26	2.49	0.256
EBL	Surface	4.47	62.9	57.8	16.6	390	4.18	4.66	0.251
	Deep layer	4.68	26.5	21.9	10.4	184	2.09	0.75	0.284
Mean ± SD[d]	Surface	4.54 ± 0.24^m	84.5 ± 37.4^m	74.7 ± 25.0^m	30.6 ± 16.6^m	495 ± 183^m	5.38 ± 2.15^m	7.21 ± 7.22^m	0.314 ± 0.084^m
	Deep layer	4.70 ± 0.23^m	31.2 ± 10.5^n	35.6 ± 7.0^n	11.5 ± 2.2^n	199 ± 40^n	2.17 ± 0.64^n	1.03 ± 1.14^n	0.240 ± 0.029^n

[a] All the nutrient units were calculated by dry soil weights

[b] Refer to Table 1 for the detailed information of the forests

[c] Surface represents the layer at 0–2 cm depth; while deep layer represents the layer at 18–20 cm depth

[d] Statistics: values in the same column followed by different letters (m and n) indicate significant differences ($p < 0.05$, $n = 8$), in which one-way analysis of variance (ANOVA) followed by two-tails paired Student's t test was applied

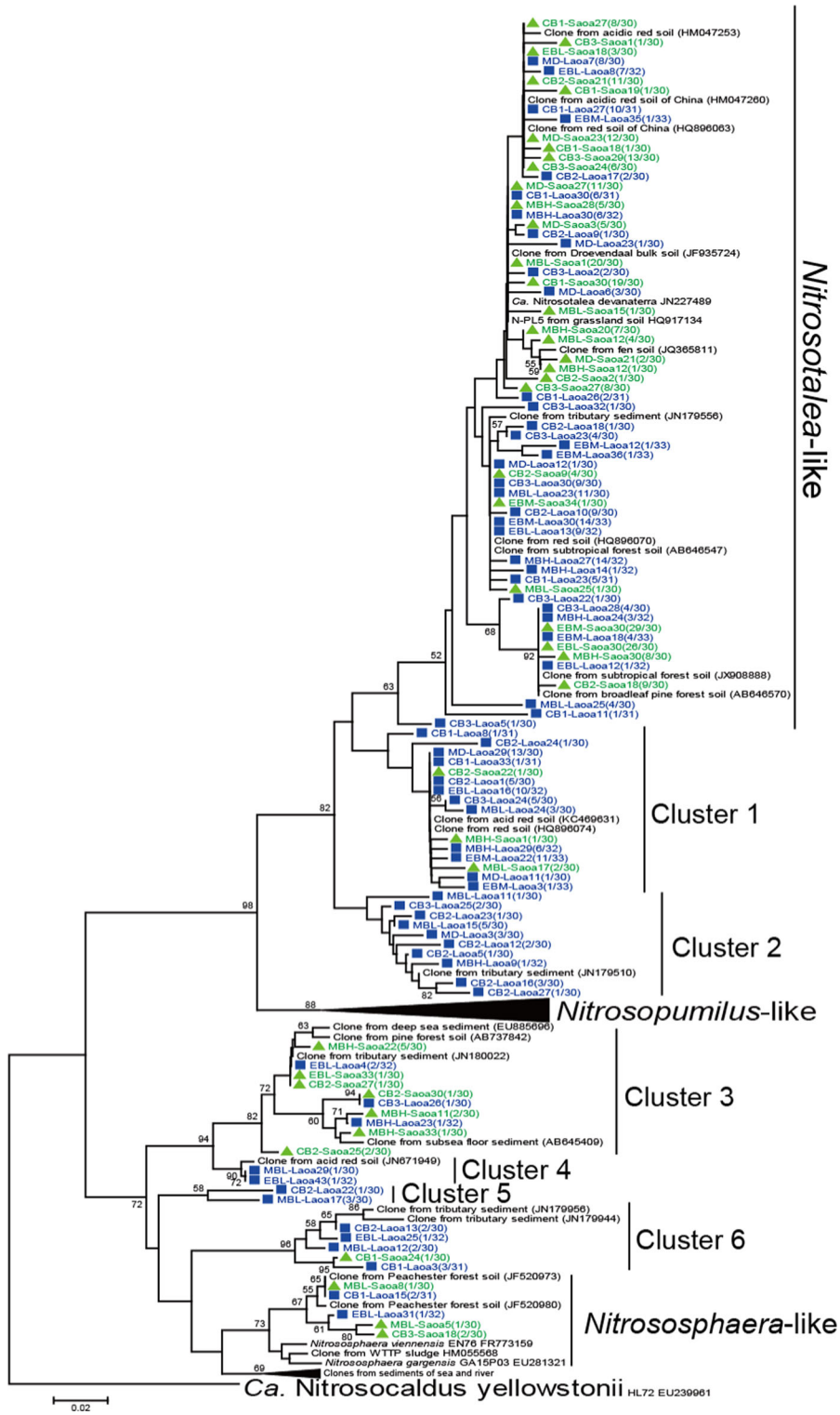

Fig. 1 Phylogenetic tree based on deduced protein sequences of the *amoA* gene sequences of ammonia-oxidizing archaea (198 amino acids). The phylogenetic tree was constructed with the neighbor-joining method with 1000 bootstrapping to estimate the confidence of the tree topologies. Bootstrap values (>50 %) are indicated at the branch points. The *scale bar* represents 0.02 sequence divergence. The solid squares in blue represent the clone sequences from surface soils (0–2 cm deep). The *solid triangles* in green represent the clone sequences from deep soils (18–20 cm deep)

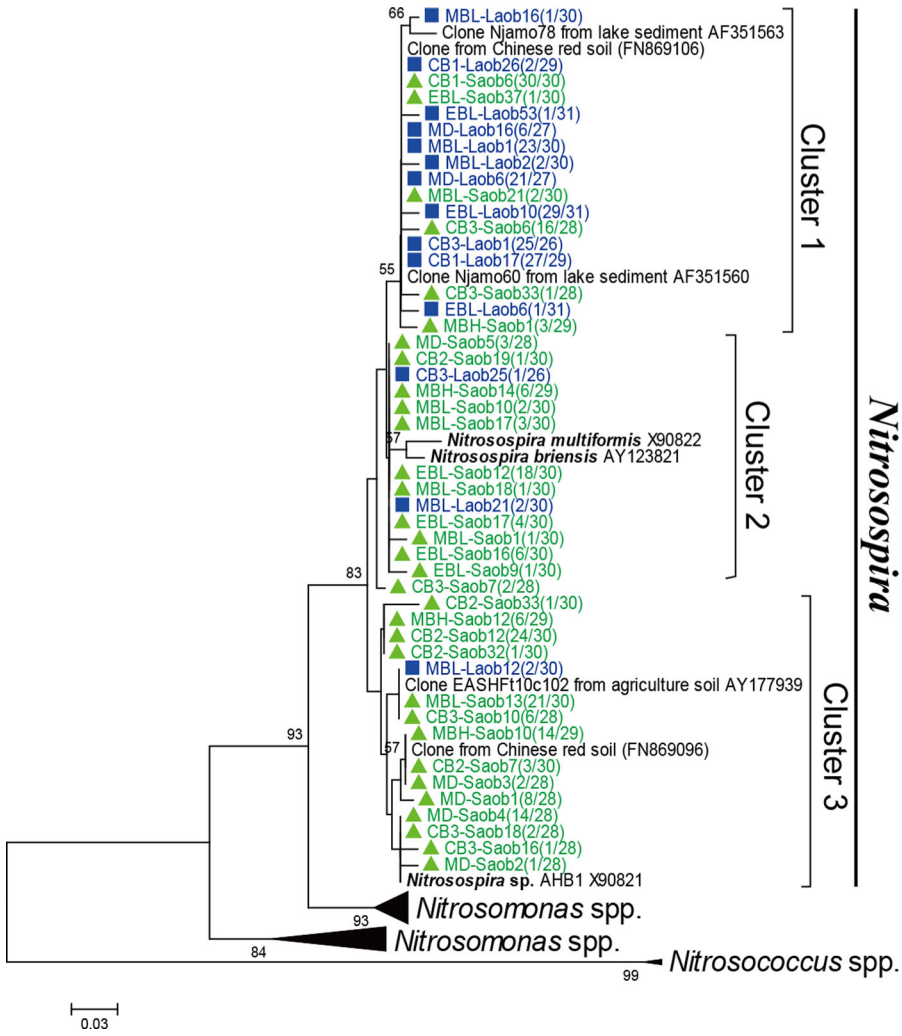

Fig. 2 Phylogenetic tree based on deduced protein sequences of the *amoA* gene sequences of ammonia-oxidizing bacteria (150 amino acids). The phylogenetic tree was constructed with the neighbor-joining method with 1000 bootstraps to estimate the confidence of the tree topologies. Bootstrap values (>50 %) are indicated at the branch points. The *scale bar* represents 0.03 sequence divergence. The *solid squares* in *blue* represent the clone sequences from surface soils (0–2 cm deep). The *solid triangles* in green represent the clone sequences from deep soils (18–20 cm deep)

The phylogenetic tree of AOB in the eight types of forests analyzed using putative protein sequences of amplified *amoA* genes is shown in Fig. 2. A total of 348 bacterial *amoA* gene clone sequences were acquired from the soils of most forests, except the surface soils of the evergreen broadleaf forest at middle elevation, the deep layers of the coniferous and broad-leaved mixed forest, the mountainous bamboo forest and the evergreen broad-leaved forest. Previous studies have shown that AOB in terrestrial environments are mainly classified into two genera: *Nitrosomonas*-like and *Nitrosospira*-like (Kowalchuk and Stephen 2001). In this study, only *Nitrosospira*-like AOB were retrieved from the forests. These sequences can be further classified into three clusters. The three clusters were closely related, and members of the three clusters were also reported elsewhere (Fig. 2). Most of them were found in acidic environments. Cluster 1 included phylotypes from acidic red soil and lake sediment. Cluster 2 included *Nitrosospira multiformis* and *Nitrosospira briensis*. Cluster 3 included sequences from acidic red soils and agriculture soils.

Comparison of AOM community structures in different soils

For each forest, AOA communities of the surface soil and the deep layer were not clustered together (Fig. 3a), indicating AOA community structures in surface and deep layers in the same forest were different. However, the surface soils and deep soils

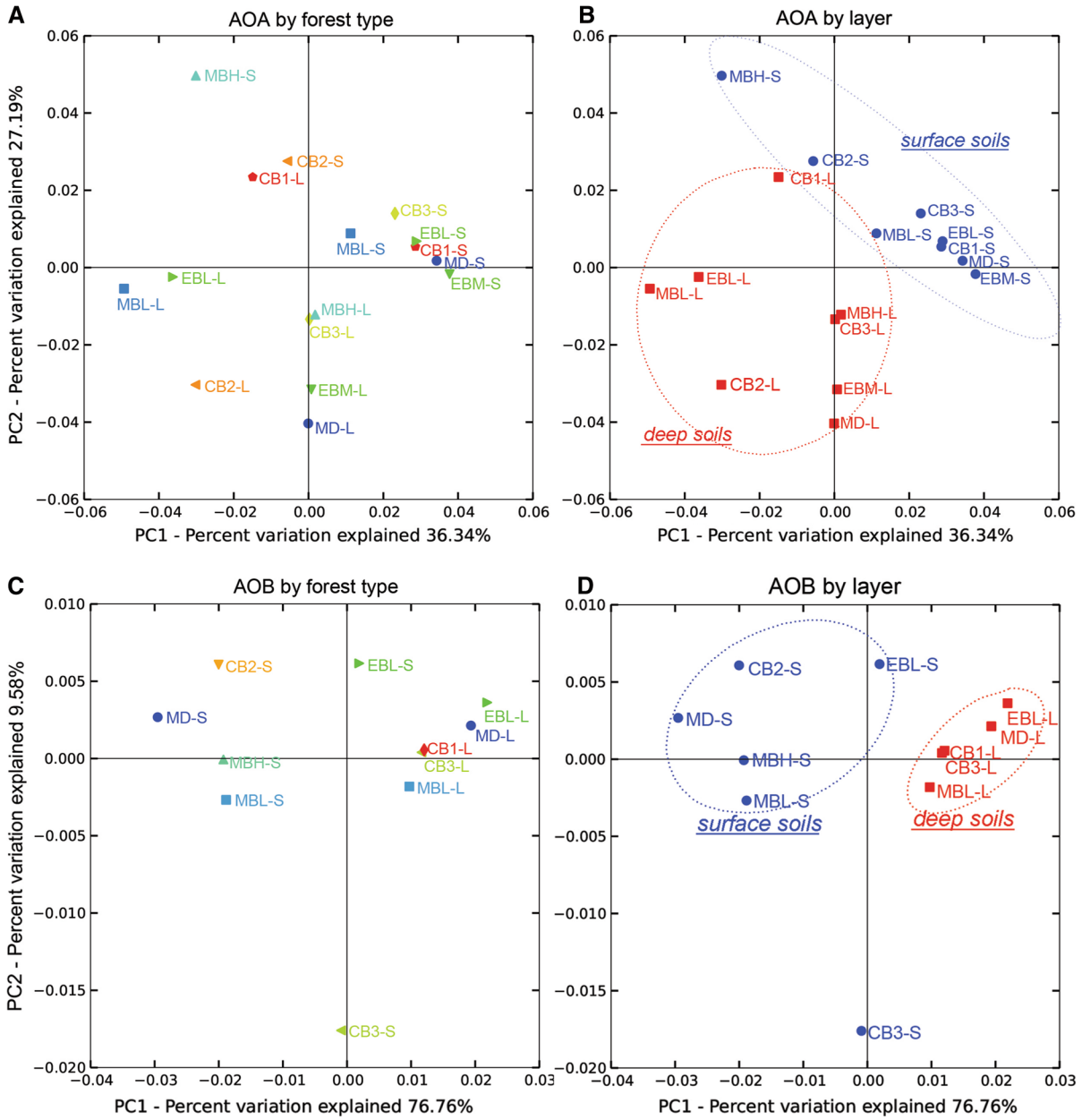

Fig. 3 Principal coordinates analysis (PCoA) based on the Unifrac distance metric of deduced archaeal AmoA protein (**a**, **b**) and deduced bacterial AmoA protein (**c**, **d**), refer to Table 1 for detailed information of soil sample IDs

each clustered together, regardless of forest type. (Figure 3b). This phenomenon indicated that soil depth or a factor associated with soil depth was pivotal in shaping AOA community structure, while forest type did not affect AOA community structure very much.

Similar to AOA, AOB communities of the surface and deep layer in the same forest were not clustered together (Fig. 3c). However, AOB communities in the surface soils of all the investigated forests were clustered together; and those in the deep layers of all investigated forests were clustered together (Fig. 3d). Because of that, soil depth or a factor associated with soil depth is important in shaping AOB community structure, while forest type had less effect on shaping AOB community structure compared to soil depth.

Table 3 Observed and estimated richnesses of archaeal and bacterial *amoA* gene libraries

Soil code[a]	AOA				AOB			
	No. of clones	No. of OTU (3 %)	Chao1	Shannon (3 %)	No. of clones	No. of OTU (3 %)	Chao1	Shannon (3 %)
MD-S	30	4	4	1.21	28	5	5	1.25
MD-L	30	7	10	1.52	27	2	2	0.53
CB1-S	30	5	8	0.98	30	1	1	0.00
CB1-L	31	9	10	1.89	29	2	2	0.25
CB2-S	30	8	11	1.63	30	5	8	0.75
CB2-L	30	13	18.3	2.23				
CB3-S	30	5	5	1.33	28	6	6.3	1.26
CB3-L	30	10	12	2.01	26	2	2	0.16
MBH-S	30	8	9.5	1.81	29	4	4	1.24
MBH-L	32	7	10	1.54				
MBL-S	30	7	10	1.17	30	6	6.3	1.07
MBL-L	30	8	8.5	1.80	30	5	5	0.86
EBM-S	30	2	2	0.15				
EBM-L	33	7	13	1.41				
EBL-S	30	3	3	0.47	30	5	6	1.12
EBL-L	32	8	11	1.66	31	3	4	0.28
Mean ± SE	30.5 ± 1.0	6.9 ± 2.7	9.1 ± 4.1	1.43 ± 0.55	29.0 ± 1.5	3.8 ± 1.7	4.3 ± 2.2	0.73 ± 0.47

OTUs were defined as 3 % difference in protein sequence alignment for AOA and AOB, determined using DOTUR; Chao-estimated richness and Shannon index were also calculated using DOTUR

[a] See Table 1 for the explanation of soil codes

Diversity of ammonia-oxidizing microorganisms

Shannon indices (H'), indicating the diversity of AOA and bacteria, are shown in Table 3. The diversity of AOA ($H' = 1.43 \pm 0.55$) in most forest soils was higher than that of AOB ($H' = 0.73 \pm 0.47$). When observed by depth, the AOA diversity of the deep layer was higher than that of the surface layer in most individual forests. For AOB, on the contrary, the surface soil generally had higher diversity than that of the deep layer in most individual forests, indicating that the diversity pattern of AOA in the layers was opposite to that of AOB in this study.

Abundances of ammonia-oxidizing archaea and bacteria

In these subtropical forests, the abundance of AOA in 13 out of 16 soil samples was higher than AOB (except the surface soils of a coniferous and broad-leaved mixed forest, mountainous bamboo forest at high elevation, and evergreen broad-leaved forest at low elevation) (Fig. 4), suggesting AOA numerically dominated over AOB in those forests. When observed by depth, in 6 out of 8 forests, AOA abundances in the deep layers were higher than those in the surface layers (Fig. 4). On the contrary, for most forests (6 out of 7), AOB abundances in the deep layers were lower than those in the surface layers.

Correlation of characteristics of soils and biodiversity and abundance of AOM

The correlation between the eight physicochemical parameters of soils and biodiversity and abundance of AOA and AOB is shown in Table 4. The results showed that the biodiversity of AOA was significantly correlated with *p*H, organic carbon, total nitrogen and total phosphorus ($p < 0.05$, $n = 16$). The abundance of AOA was significantly correlated with available phosphorus ($p < 0.05$, $n = 16$). However, the biodiversity and abundance of AOB were not significantly correlated with any of the physicochemical parameters, although some correlations were high (Table 4).

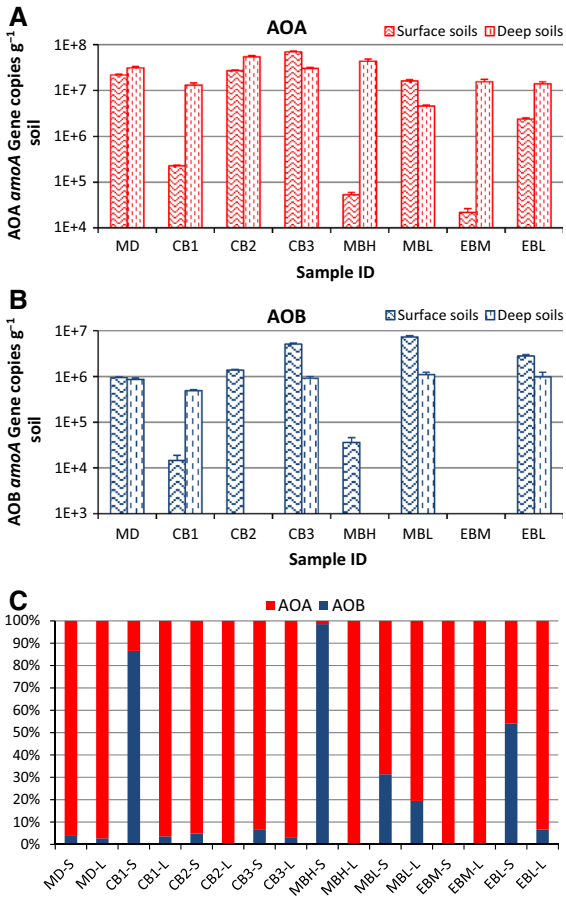

Fig. 4 Quantification of archaeal and bacterial *amoA* genes in the surfaces and deep layers of subtropical forests by qPCR. See Table 1 for the explanation of the sample IDs

Discussion

The abundant occurrence of AOA and AOB in the acidic forest soils

AOB were long thought to be the only ammonia oxidizers in the microbial N cycle (Kowalchuk and Stephen 2001), but recent studies showed that the newly discovered AOA are ubiquitous and numerically dominant over AOB in various habitats (reviewed by Stahl and de la Torre 2012). Moreover, some studies demonstrated that AOA were responsible for the observed nitrification in those environments (Dodsworth et al. 2011; Gubry-Rangin et al. 2010; Levičnik-Höfferle et al. 2012; Nicol et al. 2008; Stopnisek et al. 2010; Verhamme et al. 2011; Yao et al. 2011). These findings indicated that AOA might play a more important role than AOB in the global N cycle. More interestingly, a number of acidic soils, which were believed to be an adverse habitat to AOB, harbored diverse and abundant AOA (Lu et al. 2012; Tripathi et al. 2013; Zhang et al. 2012). The successful survival of AOA in acidic soils was attributed to the high affinity of AOA for ammonia, because ammonia concentrations are usually very low in acidic soils and the high affinity of AOA for ammonia helps them acquire ammonia in environments with low ammonia concentration (Prosser and Nicol 2012; Stahl and de la Torre 2012; Verhamme et al. 2011).

Subtropical forest soils are usually acidic (Zhang et al. 2013). The *p*H values of Nanling forest soils in this study ranged between 4.24 and 5.10 (Table 2). In these acidic forest soils, we observed diverse and abundant AOA. Moreover, the diversity of AOA observed in this study was apparently higher than that observed in Dinghushan Nature Reserve (Isobe et al. 2012), another reserve in Guangdong Province more than two hundred km south from Nanling Reserve, but receiving high N deposition (32–34 kg N ha^{-1} $year^{-1}$ NH_4^+ and NO_3^- in 2004 and 2005) as it was located in Pearl River Delta which is an important industrial manufacturing region in China (Fang et al. 2008).

A total of eight clusters were found in the present study, including *Nitrosotalea*-like, *Nitrososphaera*-like and six unclassified clusters. Most sequences fell into the *Nitrosotalea*-like cluster, in which the cultivated *Nitrosotalea devanaterra* is considered to be a model acidic AOA (Lehtovirta-Morley et al. 2011). Of all the retrieved sequences, none belonged to the Group I.1a *Nitrosopumilus* cluster that are thought to exist mainly in oceans despite one strain, *Nitrosopumilus* sp., which has been enriched from agriculture soil (Jung et al. 2011). The high diversity of AOA observed in Nanling Nature Reserve indicated that a wide range of AOA phylotypes can adapt to acidic conditions, rather than the former speculation that only a small subset of AOA were specialized to adapt to acidic environments (Gubry-Rangin et al. 2011). The ability of AOA to adapt to acidic environments probably is a shared characteristic for most AOA.

Consistent with the findings in many other acidic environments (Tripathi et al. 2013; Zhang et al. 2012), AOA in Nanling forest soils numerically dominated over AOB, suggesting AOA might play a more important role than AOB in nitrification in acidic soils. Isobe et al. (2012) found that AOA were

Table 4 Correlation analyses of environmental parameters and AOM diversity and abundance[a]

	pH	Organic C	NH_4^+-N	NO_3^--N	Available N	Total N	Available P	Total P	Elevation
AOA Shannon	**0.528**	**−0.646**	−0.366	−0.248	−0.435	**−0.594**	−0.341	**−0.502**	0.183
AOA abundance	0.092	−0.292	−0.107	0.357	−0.174	−0.237	**−0.534**	−0.372	0.252
AOB Shannon	0.432	0.236	0.470	0.470	0.469	0.362	0.266	0.402	0.249
AOB abundance	−0.110	−0.254	−0.185	0.153	−0.224	−0.255	−0.347	−0.265	−0.286

[a] Pearson product-moment correlation coefficient (r) was calculated with the following equation: $r = \frac{n(\sum XY)-(\sum X)(\sum Y)}{\sqrt{[n\sum X^2-(\sum X)^2][n\sum Y^2-(\sum Y)^2]}}$. Boldface numbers denote $p < 0.05$ (for AOA, $n = 16$; for AOB, $n = 12$), which is typically regarded as significant, as determined by Excel function TDIST from the t value

responsible for the observed ammonia oxidation in Dinghushan Nature Reserve (more than 200 km south Nanling), where no AOB were detected. However, in Nanling Reserve AOB were also abundant in these acidic forest soils, though not as high abundance as AOA. All detected AOB in Nanling fell into the genus *Nitrosospira*, which was consistent with the fact that cultivated AOB from acidic environments mostly belonged to *Nitrosospira* (De Boer and Kowalchuk 2001). Since both AOA and AOB exist in these acidic soils, future work may focus on determining which group is responsible for nitrification activity in these soils.

Influence of factors on AOA abundance and diversity

Environmental factors are important drivers shaping the community structures and abundance of microorganisms in soils. Studies showed that the community structures and abundances of AOA and AOB were differently affected by some factors such as pH (Nicol et al. 2008), salinity (Wang and Gu 2014), organic carbon (Wang et al. 2014), and elevation (Zhang et al. 2009). Before this study, we had hypothesized that forest type was an important factor shaping AOM communities based on two reasons. Firstly, different forests have litters containing different natural inhibitors such as phenolic acids, flavonoids and terpenoids, which may influence AOM differently (De Boer and Kowalchuk 2001). Secondly, AOA and AOB respond to ammonia sources differently. In addition, in other studies AOA seemed to better utilize ammonia released from organic matter, while AOB tended to favor inorganic ammonia such as fertilizer (Yamamoto et al. 2011). In our study, soil depth, rather than forest type, had a significant influence on community structures of both AOA and AOB. This may be because soils of different layers had distinct characteristics (Table 2), and soil parameters can shape AOM community structures. Compared to soil parameters, forest type had relatively little effect on AOM communities. To our knowledge, this is the first report of AOM community composition being determined by soil depth rather than by forest type (Fig. 3).

Plenty of studies have shown that AOA can occur in habitats with a wide range of pH values (pH 3.5–8.7) and observed relationships between pH and AOA abundance include negative, positive or no correlation depending on the study system (Gubry-Rangin et al. 2011; Prosser and Nicol 2012). In this study, *p*H and seven nutrients (organic carbon, ammonium-N, nitrate–N, available nitrogen, total nitrogen, available phosphorus and total phosphorus) were investigated along with the molecular quantification of putative AOM. AOA abundance showed no significant correlation with pH ($p > 0.05$, $n = 16$; Table 2). However, the diversity of AOA was significantly correlated with pH ($p < 0.05$, $n = 16$; Table 2), indicating more phylotypes of AOA favored habitats of higher pH values. Our finding that AOA diversity decreases with decreasing pH was consistent with the result of Gubry-Rangin et al. (2011), but opposite to that of Tripathi et al. (2013). This result suggests that survival in acidic environments is only an adaptive strategy for AOA, and that acidic environments themselves are not a favorable condition for AOA.

Studies have demonstrated that AOA tend to exist in oligotrophic environments such as low ammonium habitats (Sauder et al. 2012; Verhamme et al. 2011). To our knowledge, the relationship between AOA abundance and phosphorus has not been studied in

soils. In this study AOA abundance was negatively correlated with available phosphorus ($p < 0.05$, $n = 16$; Table 4), suggesting that the acidic forest soils with higher available phosphorus were adverse to AOA. Besides available phosphorus, AOA diversity was negatively correlated with organic carbon, total nitrogen and total phosphorus ($p < 0.05$, $n = 16$). These phenomena again suggested that AOA favored oligotrophic environments of low nitrogen and phosphorus. Former studies mainly focused on the effect of ammonia on AOA as ammonia is a substrate of AOA. Our study for the first time revealed that AOA favored oligotrophic habitats in terms of other nutrients.

The mechanism explaining why phosphorus inhibited AOA in these acidic forest soils might be as follows. Phosphorus is usually a limiting factor for tree growth in many ecosystems, especially in acidic subtropical forests (Porder et al. 2005; Wardle et al. 2004). In subtropical China, available P is generally low in the acidic forest soils (Hou et al. 2015; Zheng et al. 2015), thus it is a factor limiting the growth of plants and microorganisms (Elser et al. 2007). When more available P can be obtained, plants and heterotrophic microorganisms will utilize them quickly and grow much faster than the autotrophic AOA. The fast-growing plants and copiotrophic, heterotrophic microorganisms will then enhance the absorption of other elements, which would in turn inhibit AOA.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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