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Community structures of ammonia-oxidising archaea and bacteria in high-altitude lakes on the Tibetan Plateau

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SUMMARY

1. Community structures of planktonic ammonia-oxidising archaea (AOA) and bacteria (AOB) were investigated for five high-altitude Tibetan lakes, which could be classified as freshwater, oligosaline or mesosaline, to develop a general view of the AOA and AOB in lakes on the Tibetan Plateau.

2. Based on PCR screening of the ammonia monooxygenase α-subunit (*amoA*) gene, AOA were present in 14 out of 17 samples, whereas AOB were detected in only four samples. Phylogenetic analyses indicated that the AOB communities were dominated by a unique monophylogenetic lineage within *Nitrosomonas*, which may represent a novel cluster of AOB. AOA, on the other hand, were distinct among lakes with different salinities.

3. Multivariate statistical analyses indicated a heterogeneous distribution of the AOA communities among lakes largely caused by lake salinity, whereas the uniform chemical properties within lakes and their geographical isolation may favour relatively homogeneous AOA communities within lakes.

4. Our results suggest a wide occurrence of AOA in Tibetan lakes and provide the first evidence of salinity-related differentiation of AOA community composition as well as potential geographical isolation of AOA in inland aquatic environments.

Keywords: ammonia-oxidising archaea, ammonia-oxidising bacteria, *amoA* gene, multivariate statistical analysis, salinity, Tibetan lakes

Introduction

Nitrification, which connects nitrogen fixation and denitrification, is an essential process in the global nitrogen cycle. Ammonia oxidation, the first rate-limiting step of nitrification, was long thought to be mainly performed by ammonia-oxidising bacteria (AOB), which form monophylogenetic lineages within the *Betaproteobacteria* and the *Gammaproteobacteria*, including *Nitrosomonas* spp. (*Beta*-), *Nitrosospira* spp. (*Beta*-) and *Nitrosococcus* spp. (*Gamma*-), and comprise <0.1% of microbial assemblages (Bothe *et al*., 2000).

Recently, a breakthrough in our understanding of a functional group of mesophilic Crenarchaeota – the ammonia-oxidising archaea (AOA) – was made based on studies of metagenomics (Venter *et al*., 2004; Treusch *et al*., 2005) and confirmed by the isolation of the mesophilic crenarchaeon '*Nitrosopumilus maritimus*' (Könneke *et al*., 2005). This has revolutionised our knowledge of the nitrogen cycle and especially of nitrification. For both the AOA and AOB, the *amoA* gene encoding for the α-subunit of ammonia monooxygenase has been broadly applied as a functional marker for investigation of their distribution and abundance in natural environments (Purkhold *et al*., 2000; Francis *et al*., 2005), since it has great advantages over the 16S rRNA gene in resolving the genetic

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diversity of microbial ammonia oxidisers (Purkhold *et al.*, 2000; O'Mullan & Ward, 2005). Recent studies have demonstrated the ubiquitous occurrence of AOA in a broad range of environments (Erguder *et al.*, 2009 and references therein), where some quantitative studies suggest that AOA generally outnumber AOB in most of these environments (e.g. Leininger *et al.*, 2006; Wuchter *et al.*, 2006; Agogué *et al.*, 2008; Nicol *et al.*, 2008; Herrmann, Saunders & Schramm, 2009). Mounting evidence suggests that the mesophilic Crenarchaeota are an important component of natural ecosystems and that AOA in particular play an important role in energy flow and element cycling (Nicol & Schleper, 2006; Francis, Beman & Kuypers, 2007).

Many studies have shown that a number of environmental factors may define the ecological niches of AOA in natural environments, including ammonium, organic carbon, temperature, salinity, dissolved oxygen (DO), pH, sulphide, phosphate and the phytoplankton community as well as primary production (Erguder *et al.*, 2009 and references therein). However, the link between AOA community structure and environmental factors in inland water systems is still unclear. Moreover, reports of AOA and AOB communities in high-altitude aquatic environments [more than 4000 m above sea level (a.s.l)] are rare.

The Tibetan Plateau (TP) is the largest and highest plateau on earth, with an area of ca. 2.5×10^6 km^2 and an average elevation of 4200 m. Lakes are widespread on the TP with little anthropogenic pollution, thus representing pristine environments. The different climate systems and elevation on the TP produce different annual precipitation and evaporation around the plateau and have formed thousands of lakes with different salinities. Two-thirds of these lakes are freshwater, and the remainder are saline or hypersaline (Zheng *et al.*, 1993). Tibetan lakes can serve as ideal ecosystems for exploring the responses of microorganisms to environmental factors, as a result of their own wide gradient of a variety of environmental factors, such as salinity, pH, temperature, light and geographical distance. Previous studies based on the 16S rRNA gene showed that the microbial communities in Tibetan lakes are mainly controlled by salinity (Wu *et al.*, 2006, 2009; Jiang *et al.*, 2007, 2009a). Up to now, only the diversity, abundance and activity of ammonia oxidisers in Lake Qinghai on the northeast TP have been reported by Jiang *et al.* (2009b), who showed that both AOA and AOB are primarily responsible for nitrification in that lake. Which kinds of ammonia oxidisers exist and how these functional groups respond to environmental changes in high-altitude Tibetan lakes is still poorly understood.

We investigated the community structures of AOA and AOB in five Tibetan lakes across salinity and latitude gradients by integrating *amoA* gene analysis and multivariate statistical analysis. The main goal of the study was to test whether (i) ammonia oxidisers are ubiquitous in high-altitude Tibetan lakes (more than 4000 m a.s.l); (ii) the same or similar ammonia oxidisers occur among Tibetan lakes; and (iii) salinity, pH or geographical distance have an effect on AOA and AOB community compositions in Tibetan lakes.

Methods

Study sites and field sampling

The five lakes investigated are located in the eastern TP at altitudes ranging from 4540 to 5030 m a.s.l (Fig. 1). They can be classified into three salinity types: freshwater (Lakes Beng Co and Puma Yumco), oligosaline (Lakes Nam Co and Yamdrok) and mesosaline (Lake Peng Co) according to the salinity classification of Gasse *et al.* (1987). Water temperature, pH, conductivity, DO, oxygen saturation, total dissolved solids and photosynthetically active radiation (PAR) were measured at each site using a Hydrolab DS5 Water Quality Multiprobe (Hach, Loveland, CO, U.S.A.). Water samples were collected using 7-L Niskin bottles in October, 2008. For molecular analyses, about 1-L water samples from various depths were filtered through 0.22-μm-pore-size polycarbonate filters (Millipore, Bedford, MA, U.S.A.) at a pressure of <0.03 MP. Water samples for prokaryotic cell counts were collected in sterile bottles and fixed with glutaraldehyde (1% final concentration). Additional 500-mL water samples were filtered through glass fibre filters (Whatman, Clifton, NJ, U.S.A.) for the determination of chlorophyll *a* (chl *a*) concentration. All samples were frozen at −20 °C in the field and during transportation and then at −80 °C in the laboratory until further analysis.

Flow cytometry and Chl a measurement

Total microbes were stained with SYBR Green I (Molecular Probes, Eugene, OR, U.S.A.), and the

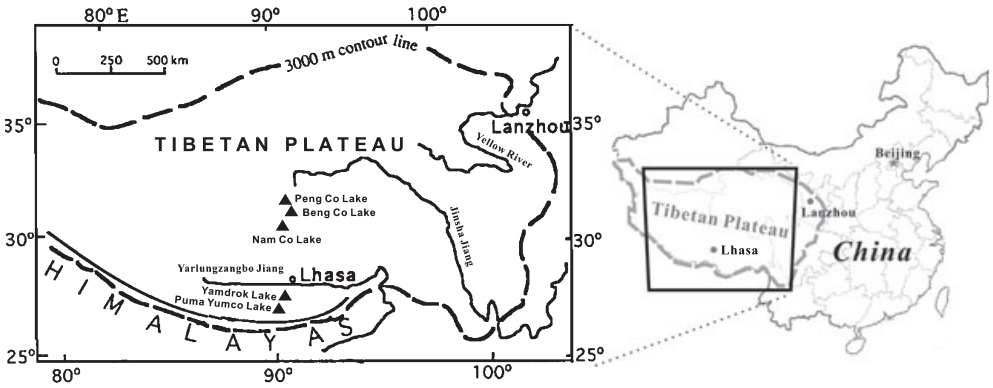

Fig. 1 Location of the five study lakes on the Tibetan Plateau.

abundance was determined using flow cytometry (Beckman Coulter, Epics, Altra II, Miami, FL, U.S.A.) (Jiao *et al*., 2005). Duplicate samples were measured. Chl *a* was measured using a fluorospectrophotometer (Shimadzu Corp., Tokyo, Japan) following an overnight freeze-thaw extraction in 90% acetone. Each sample was measured three times with a standard deviation lower than 10%.

DNA extraction, PCR, cloning and sequencing

DNA was extracted using the UltraClean Soil DNA kit (MoBio, San Diego, CA, U.S.A.) according to the manufacturer's instructions. DNA size and integrity were checked in a 0.8% agarose gel stained with SYBR Green I, after which the concentration and the purity of DNA were measured using a Bio-Rad SmartSpec Plus spectrophotometer (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The archaeal *amoA* gene was amplified with Arch-amoAF/Arch-amoAR (Francis *et al*., 2005); the β-proteobacterial *amoA* gene was amplified with amoA-34F/amoA-2R (Rotthauwe, Witzel & Liesack, 1997; Kim *et al*., 2008); and the γ–proteobacterial *amoA* gene was amplified with amoA-3F/amoB-4R (Purkhold *et al*., 2000). The PCR mixture (50 μL) consisted of 25 μL Failsafe Premix F (Epicentre Biotechnologies, Madison, WI, U.S.A.), 0.5 μM of each primer, 1 U of Plantium Taq DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.) and 1 μL (*c*. 10 ng DNA) of template. The PCRs were run for 30 cycles, following the PCR conditions described in the literature listed previously.

For the construction of clone libraries, three independent PCR products from each sample were pooled and purified with a TaKaRa Agarose Gel DNA Purification Kit (Takara Bio, Dalian, China), ligated into the pMD18-T vector (TaKaRa) and then transformed into competent *Escherichia coli* DH5α (TaKaRa). Positive colonies were randomly chosen for PCR re-amplification with vector primers M-13F/M-13R and selected for sequencing using an ABI 3730 XL sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

Phylogenetic analysis

The microbial *amoA* gene sequences, along with their closest relatives retrieved from GenBank (http://www.ncbi.nlm.nih.gov/), were imported into ARB (Ludwig *et al*., 2004). The sequences were first translated and aligned using Clustal W in ARB, and then the nucleotides were realigned according to their protein alignment. Ambiguously and incorrectly aligned positions were corrected manually using the ARB-edit tool. The sequence bases frequency filters were used to exclude ambiguous positions and columns containing gaps. Prior to tree construction, evolutionary models and model parameters were evaluated using Posada and Crandall's Modeltest v. 3.7 (http://darwin.uvigo.es/software/modeltest.html). The maximum likelihood tree was then constructed using the tree-bisection-reconnection branch-swapping algorithm, with randomised stepwise addition of taxa using the heuristic search method (ten random taxon additions) with PAUP*4.0 (Swofford, 2003). The topology of the phylogenetic tree was also evaluated using neighbour joining (Jukes–Cantor correction) and maximum parsimony with a heuristic search method.

Diversity indices and statistical analyses

Operational taxonomic units (OTUs) were defined using the furthest neighbour algorithm in DOTUR (Schloss & Handelsman, 2005) using a cut-off of ≤2%. Rarefaction richness and diversity indices were also calculated using DOTUR (Schloss & Handelsman, 2005), including the nonparametric richness estimators Chao1, as well as the Shannon diversity index and Simpson's index. AREQUIN v3.1 software (Excoffier, Laval & Schneider, 2005) was applied to calculate mean number of pairwise differences (π) within (π_w) and between (π_B) samples. Values of π provide an estimate of the genetic variation observed when each sequence is compared with all other sequences in a sample of sequences (Martin, 2002; Excoffier *et al*., 2005). Coverage value was calculated using the formula:

Coverage (C) = 1–(n/N)*100

where n is the number of unique clones detected in the sample, and N is the total number of clones analysed (Good, 1953).

To classify the community of ammonia oxidisers, the *F*st dissimilarity indices were used as tests of genetic variation between all pairs of samples. The value of *F*st ranges from 1 (if all of the variation occurs between samples) to 0 (if the variation within samples is equal to the variation between samples). Sample-by-sample matrices of pairwise *F*st 'distances' were calculated using ARLEQUIN v3.1 (Excoffier *et al*., 2005), and those matrices were imported into PAST v1.92 (Hammer, Harper & Ryan, 2001) to perform the cluster analysis with the user distance algorithm. For comparison with the distance-based diversity measurements, the phylogeny-based weighted UniFrac environmental clustering was also applied using the online UniFrac programme (Lozupone, Hamady & Knight, 2006). The relationship between the community structure of the ammonia oxidisers and environmental factors was analysed using canonical correspondence analysis (CCA) with CANOCO v4.53, (Microcomputer Power) (ter Braak & Šmilauer, 2002). Briefly, CCA was chosen to determine any correlations between community structure and local environmental variables, since the maximum gradient length of Detrended Correspondence Analysis is larger than 4.0 (Lepš & Šmilauer, 2003). The percentage frequency of the OTUs (defined at ≤2% cut-off) was used as the species input, and the environmental variables (except pH) were log-transformed [(log(x+1)]. The environmental factors best describing the most influential gradients were identified by forward selection. Explanatory variables were added until the addition of further variables did not result in significant ($P < 0.05$) improvements to the model's explanatory power. The significance of the testable fractions was determined using Monte Carlo tests (999 permutations). A Mantel test was performed to explore the potential relationship between the microbial communities and geographical distance.

Nucleotide sequence accession number

The non-redundant sequences reported in this work were deposited in the GenBank database under accession numbers GQ342628 to GQ342680 (Archaeal *amoA*) and GQ342681 to GQ342686 (Bacterial *amoA*).

Results

Lake characteristics

The main geographical and biogeochemical characteristics of the lakes investigated in this study are summarised in Table 1 and in Fig. S1 in the electronic supplementary material. Briefly, these lakes are generally oligotrophic and slightly alkaline as indicated by Chl *a* concentrations and pH, respectively. All were thermally stratified at the time samples were taken (Fig. S1), but there were no significant variations in pH and conductivity through the water column in each lake. (Environmental data from the sublayer water of Lake Peng Co were unavailable). There was a pronounced salinity gradient among the lakes (Table 1 and Fig. S1).

Occurrence and diversity of archaeal and bacterial amoA genes in Tibetan lakes

To explore the occurrence of ammonia oxidisers in Tibetan lakes, 17 water samples were collected from the five lakes for PCR amplification and clone library construction of archaeal and bacterial *amoA* genes. While archaeal *amoA* genes were successfully amplified from 14 out of 17 samples, *β*-bacterial *amoA* genes could be amplified from only four samples and no

Table 1 Locations and physical and chemical properties of the lakes investigated on the Tibetan Plateau

Lakes	Longitude (E)	Latitude (N)	Altitude (m)	Area (km^2)	Max depth (m)	Sampling depth (m)	Prokaryotic abundance (10^4 ml^{-1})	Chl *a* (μg L^{-1})	Temperature (°C)	pH	Conductivity (ms m^{-3})	DO (mg L^{-1})	DO (%)	PAR (μmol m^{-2} s^{-1})	Lake Types
Peng Co	90.99	31.544	4540	135.7	42	1	9.98	0.08	9.54	10.24	15.46	8.98	106	1668	Mesosaline
						37	4.94	0.44	ND	ND	ND	ND	ND	ND	
Beng Co	91.121	31.254	4691	141.3	73	1	16.78	0.08	8.24	8.86	0.32	10.31	118	1666	Freshwater
						35	11.79	0.09	8.51	8.9	0.32	9.79	113	4	
						55	7.09	0.03	5.12	8.37	0.33	8.85	93.9	1	
						70	11.26	0.15	4.4	8.32	0.33	7.94	82.7	0	
Nam Co	90.76	30.784	4718	1982	95	1	15.13	0.06	9.63	9.45	1.82	6.51	78.2	1034	Oligosaline
						8	10.94	0.06	9.63	9.46	1.82	5.98	71.7	650	
						24	9.68	0.05	6.73	9.48	1.85	6.47	72.3	68	
						40	13.56	0.10	4.24	9.42	1.85	7.09	74.4	7	
						80	7.56	0.07	3.53	9.41	1.85	6.4	65.9	0	
Yamdrok	90.672	29.138	4448	638	59	1	14.1	0.07	10.9	9.45	2.37	10.14	123.7	1351	Oligosaline
						34	11.47	0.40	8.49	9.45	2.44	8.48	97.7	1	
Puma Yumco	90.465	28.579	5030	281	67	1	16.14	0.09	7.41	9.09	0.49	9.88	110.7	2200	Freshwater
						10	13.23	0.08	7.5	9.11	0.49	9.77	109.8	329	
						40	9.37	0.07	7.58	9.1	0.49	9.55	107.5	5	
						65	9.46	0.14	5.82	8.94	0.49	8.43	90.8	0	

ND, not determined.

positive amplicons of γ-bacterial *amoA* gene were obtained (Table 2).

Fourteen archaeal *amoA* libraries were constructed to assess the AOA diversity in these lakes (Table 2). Twenty to 55 clones were sequenced per library to achieve high coverage (81.8–100%), resulting in a total of 510 archaeal *amoA* gene sequences. Fifty-three or 20 OTUs were identified based on the ≤2% or 5% cut-off at the DNA level, respectively. The numbers of OTUs varied widely among these libraries, with only 1 OTU recovered in the NMC80m and Y34m and up to 17 in the NMC40m. These were close to the numbers of OTUs predicted by the Chao1 richness estimator, suggesting that these libraries might have captured the majority of AOA 'species' in these lakes (Table 2). These results were also supported by rarefaction curves (Fig. S2). The mean number of pairwise differences within a sample (π_w) was calculated as a measure of genetic variation within the sample, and it showed that the Pe37m library contained the highest π_w value and the NMC80m contained the lowest π_w value. Most of the archaeal *amoA* libraries displayed lower π_w values, indicating an excess of closely related lineages (Martin, 2002). These results were consistent with our comparisons among the archaeal *amoA* gene from various environments using rarefaction analysis, which showed that the Tibetan lakes harbour a lower diversity of AOA in comparison with other environments such as sediments, soils, seawaters, marine animal tissues and thermal environments (Fig. 2).

For the β-bacterial *amoA* gene, four clone libraries were generated and a total of 88 clones were sequenced, ranging from 14 to 37 from each library (Table 2). In total, 6 OTUs were recovered, based on 2% nucleic acid cut-off, ranging from 5 OTUs from the Pe37m library to only 1 OTU from the other three libraries (Table 2). Likewise, the Shannon–Wiener (H′) and reciprocal Simpson's (1/D) indices were highest for the Pe37m and lowest for the other three libraries (Table 2). The Chao1 richness estimators indicated that the observed OTUs well represented the diversity of AOB in the investigated lakes. Compared to AOA, AOB diversity and genetic variation were both very low in all the libraries examined, as showed by the diversity indices and π_w, respectively (Table 2).

Phylogenetic analysis of archaeal amoA genes

In the phylogenetic analysis, all archaeal *amoA* sequences from this study appeared to fall into two primary clusters as proposed previously (Francis *et al.*, 2005; Beman & Francis, 2006): Cluster A (water column/sediment cluster) and Cluster B (soil/sediment

Table 2 Diversity indices of archaeal and bacterial *amoA* clone libraries from Tibetan lakes

	Archaeal *amoA*							Bacterial *amoA*						
Samples*	*n*†	No. of OTUs‡	C(%)§	H′	1/D	Chao1	π_w	*n*	No. of OTUs	C(%)	H′	1/D	Chao1	π_w
Pe1m	20	2	100	0.69	2.13	2	80.45	14	1	100	0	1	1	5.24
Pe37m	50	14	94	2.41	11.45	15	118.07	37	5	94.6	1.07	2.57	6	41.7
B1m	26	4	96.2	0.89	2	4	9.82	–						
B35m	31	4	96.8	1.12	2.94	4	18.26	–						
B55m	33	6	90.9	0.99	1.89	9	7.31	17	1	100	0	1	1	8.56
B70m	30	5	100	1.35	3.45	5	12.53	–						
NMC40m	44	17	81.8	2.52	11.97	23	22.78	–						
NMC80m	23	1	100	0	1	1	2.08	–						
Y1m	28	2	96.4	0.15	1.08	2	3.45	–						
Y34m	29	1	100	0	1	1	2.27	–						
Pu1m	55	3	100	0.92	2.24	3	11.83	–						
Pu10m	41	12	85.4	2.03	6.25	20	25.3	–						
Pu40m	49	11	93.9	1.93	5.26	12	28.07	20	1	100	0	1	1	11.12
Pu65m	51	6	98	1.4	3.56	6	17.11	–						

*Samples from Tibetan lakes. Pe, Peng Co; B, Beng Co; NMC, Nam Co; Y, Yamdrok; Pu, Puma Yumco; OTUs, Operational taxonomic units. The number following each abbreviation of lake names indicates the depth of lake from which the samples were collected.
†*n*, number of clones sequenced. Minus sign indicates unsuccessful amplication for bacterial *amoA* genes.
‡OTUs were defined as 2% divergence at the DNA level.
§C, coverage.

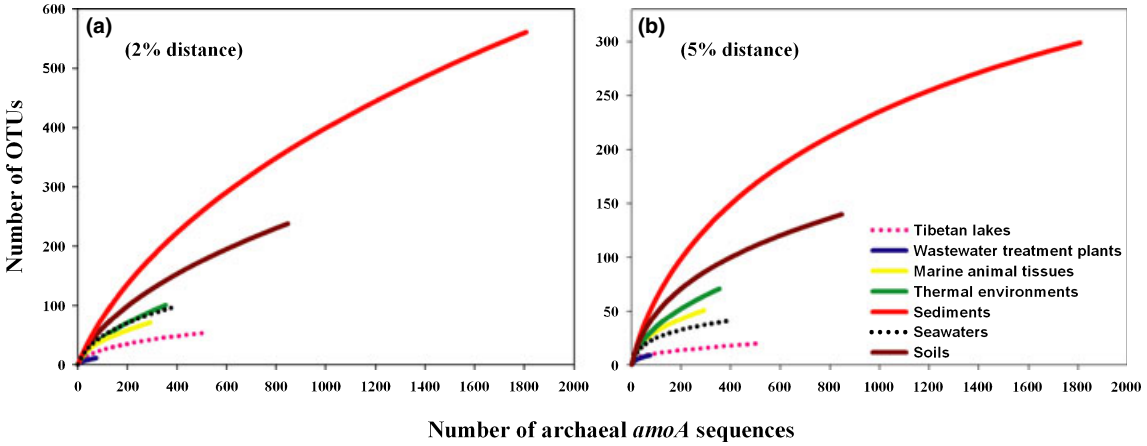

Fig. 2 Rarefaction analysis of the archaeal *amoA* sequences from different environments using an evolutionary distance threshold of (a) 2% (i.e. 98% similarity) and (b) 5% (i.e. 95% similarity) for operational taxonomic units definition. The analysis was performed using DOTUR (Schloss & Handelsman, 2005). Colours in the figures represent the corresponding environments. Only sequences from publications before July 2009 were included in this analysis. Short DGGE archaeal *amoA* sequences, sequences containing terminator codons and ≥ 0.5% degenerate bases were excluded from these analyses owing to a relatively low sequence quality which would create microdiversity artifacts.

cluster). The majority of the sequences (442 out of 510, 86.7%) fell into Cluster A and could be further classified into five subclusters based on the prevailing environmental characteristics including Clade I, II, III, the estuarine sediment clade and the wastewater treatment plant clade (Fig. 3). Clade I contained archaeal *amoA* sequences mainly obtained from oligosaline Lakes Nam Co and Yamdrok, whereas a few sequences (17 out of 130) from freshwater Lakes Puma Yumco and Beng Co were also affiliated with this cluster (Fig. 3). Most of the sequences obtained from freshwater Lakes Puma Yumco and Beng Co were widely dispersed throughout Clades II and III, which also included a few clones (16 out of 176) from the NMC40m library (Fig. 3). Several archaeal *amoA* OTUs dominated these three clades. For instance, within Clade I, one OTU alone (NMC80m-AOA-17) accounted for 66.9% of the sequences of this clade and was recovered from each of the libraries that were obtained from oligosaline lakes in the study. NMC40m-AOA-57 accounted for 77.3% of all sequences from Clade II, while B1m-AOA-22 in Clade III made up 51.1% of all sequences (Fig. 3).

For the estuarine sediment clade, one OTU from the Pu40m library formed a monophylogenetic lineage with sequences recovered from estuarine sediments (Mosier & Francis, 2008; Santoro *et al.*, 2008). The sequence B35m-AOA-14, which was closely related to archaeal *amoA* sequences retrieved from wastewater treatment plants (Park *et al.*, 2006), occurred only once in the B35m library (Fig. 3).

Sequences obtained from mesosaline Lake Peng Co were closely related to those retrieved from polysaline Lake Qinghai (Jiang *et al.*, 2009b), a Chilean saline lake (Clone Hua0-w51, FJ839434), diverse soils (Leininger *et al.*, 2006; Zhang *et al.*, 2008) and estuarine sediments (Beman & Francis, 2006; Dang *et al.*, 2008) and fell into Cluster B, which branched from within large numbers of sequences recovered from terrestrial environments (Fig. 3). Interestingly, few sequences from freshwater and oligosaline lakes were affiliated with Cluster B with the exception of Pu40m-AOA-26 and Pu40m-AOA-5 (Fig. 3).

Phylogenetic analysis of bacterial amoA genes

The bacterial *amoA* sequences recovered in this study were exclusively within the *Nitrosomonas* lineage and formed a monophylogenetic cluster with a large number of sequences recovered from a variety of inland water systems including Lake Qinghai (Jiang *et al.*, 2009b), a high-altitude saline wetland in Chile (Dorador *et al.*, 2008), a soda lake in Austria (Hornek *et al.*, 2006), which were all distantly related to *Nitrosomonas halopilia* (Fig. 4). Therefore, we designated it here as an Inland water cluster, with any two

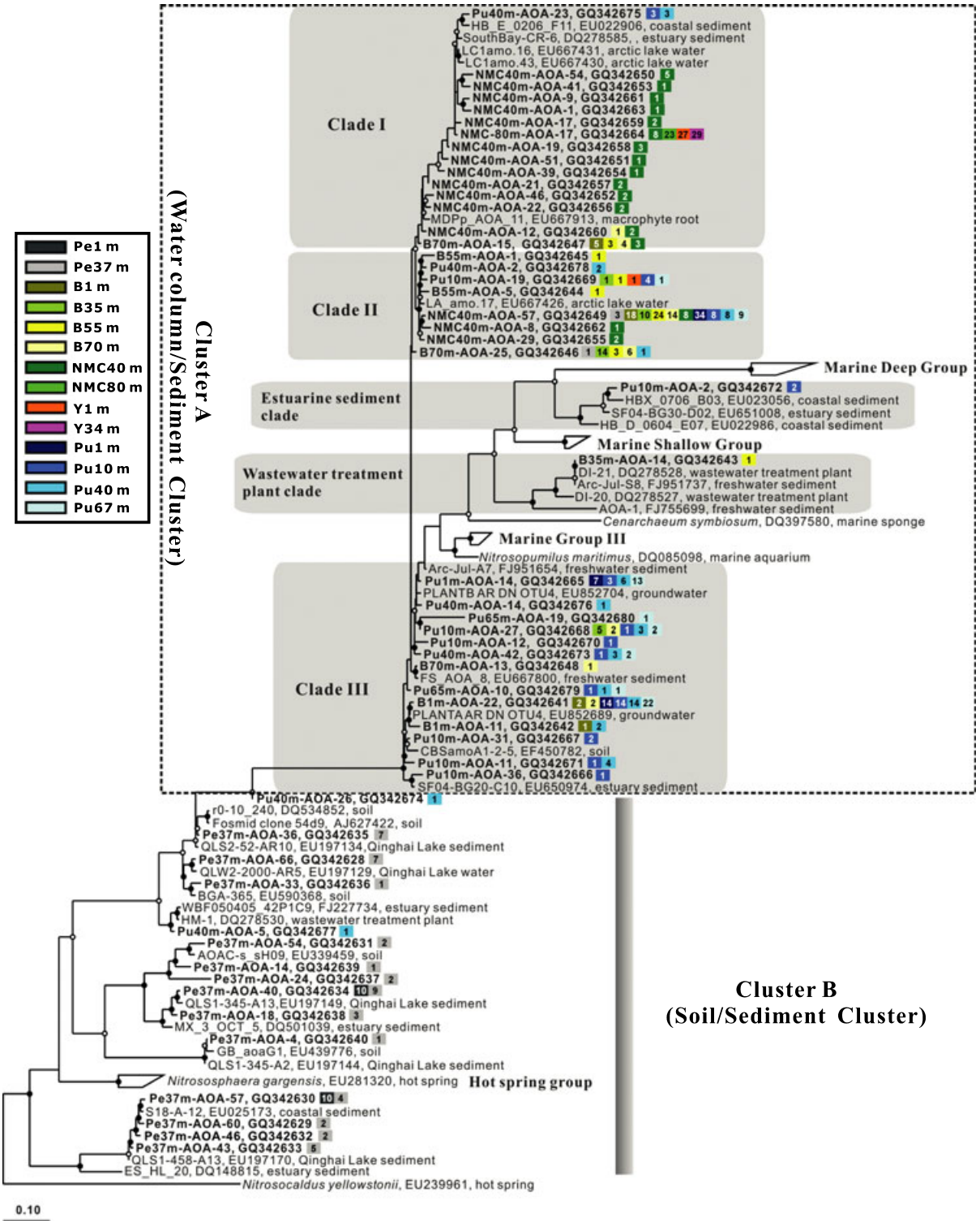

Fig. 3 Maximum likelihood tree of archaeal *amoA* sequences. *Nitrosocaldus yellowstonii* (EU239961) was used as the outgroup to root the tree. Solid circles were supported by maximum-likelihood, maximum-parsimony and neighbor-joining analyses, and open circles involved two of the three methods. All sequences from all libraries that had ≤ 2% distance cutoff were removed and represented by one sequence only. Clone sequences recovered in this study are in boldface. For the definitions of abbreviations see Table 2. The number in the box indicates the number of clones in the operational taxonomic units found in that library. Boxes are colour-coded for samples from different clone libraries. Scale bar indicates 0.1 nucleotide substitution per site.

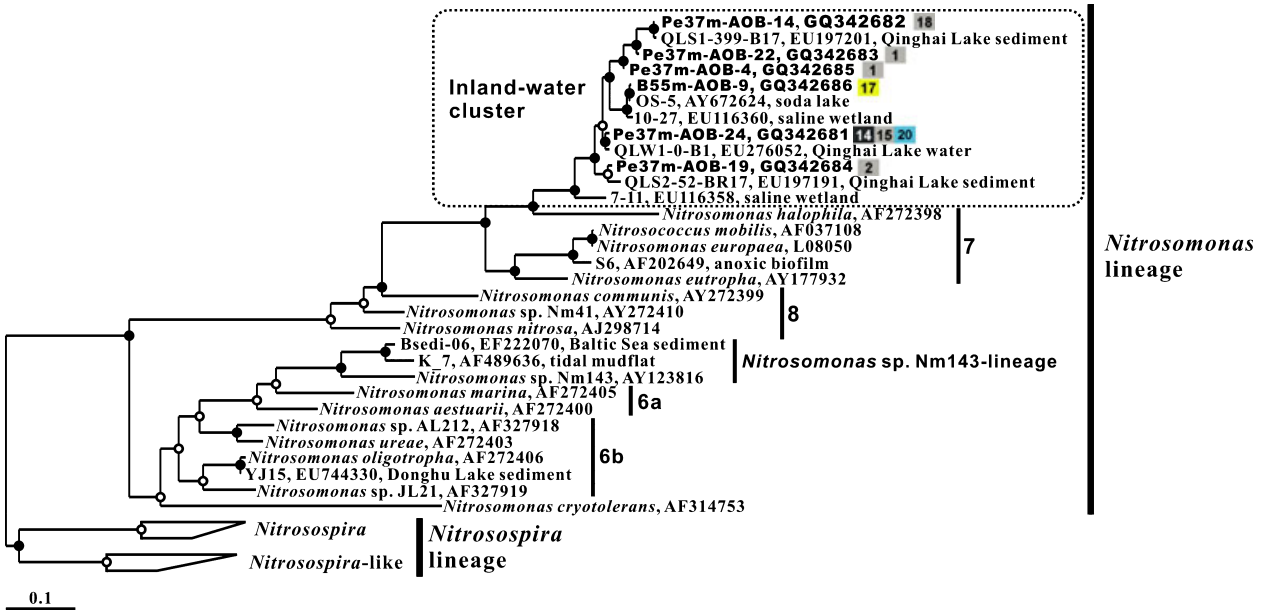

Fig. 4 Maximum likelihood tree of bacterial *amoA* sequences. *Nitrosospira* lineage sequences were used as the outgroup to root the tree. Designations of clusters according to Purkhold *et al.* (2000) and Kim *et al.* (2008). Clade confidence, symbols, and colors are the same as those described in the legend of Fig. 3.

sequences in this cluster sharing 90–100% nucleic acid similarity and 94.3–100% amino acid similarity. Moreover, these sequences were distantly related to all other environmental or cultured AOB (≤86% nucleic acid similarity, 94% amino acid similarity).

Community classification of AOA

Genetic differentiation among the archaeal *amoA* gene libraries obtained from the Tibetan lakes investigated was assessed using the dissimilarity indices (*F*st), which measure 'genetic distance' between pairs of samples. The cluster analysis combined with '*F*st distance' indicated that the AOA libraries from these lakes could be divided into three major groups, which were consistent with variations in salinity (Fig. 5a). Clearly, the libraries from freshwater Lakes Beng Co and Puma Yumco were grouped together, while the second group consisted of the libraries from oligosaline Lakes Nam Co and Yamdrok (except the NMC40m library that was distantly branched with the freshwater lakes group). Furthermore, two libraries from mesosaline Lake Peng Co formed a monophylogenetic clade (Fig. 5a). The phylogeny-based weighted UniFrac clustering analysis further confirmed this correlation (Fig. 5b). However, there were not enough positive samples of AOB for such a statistical analysis.

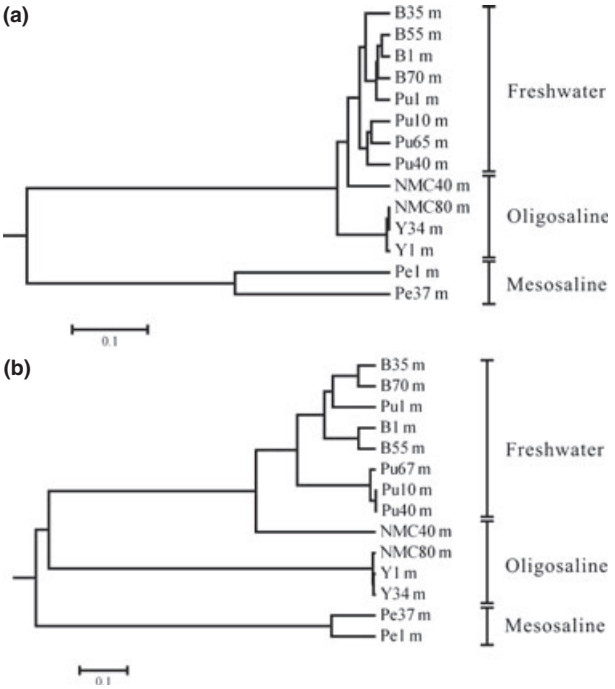

Fig. 5 Clustering of the different archaeal *amoA* clone libraries based on '*F*st distance' (a) and the weighted UniFrac algorithm (b). Scale bar indicates 0.1 *F*st distance (a) or UniFrac distance (b).

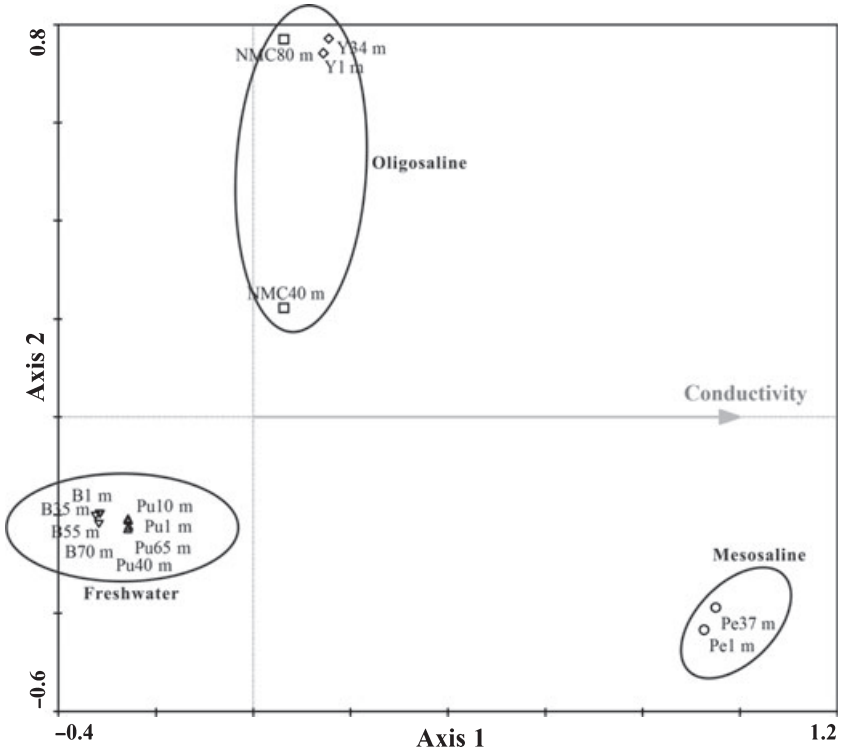

Fig. 6 Canonical correspondence analysis of archaeal *amoA* clone libraries obtained from the lakes investigated in this study based on local environmental variables. The eigenvalues of the first two axes were 0.93 and 0.95, repectively. Conductivity was strongly correlated with the first axis.

Environmental factors explaining AOA community structures

To distinguish between the effects of deterministic (environmental) and stochastic (dispersal) processes on the spatial distribution of AOA in the lakes investigated (Martiny *et al.*, 2006), the CCA analysis using local environmental factors as explanatory variables and the Mantel test in combination with geographical distance were performed, respectively. (Fig. 6 and Table S1). The CCA analysis yielded a high similarity pattern with cluster analyses, which were produced using either a distance- or phylogeny-based algorithm (Figs 5 & 6). For example, three groups in the CCA analysis could be separated according to conductivity ($P = 0.001$, $F = 3.35$, 999 Monte Carlo permutations), while there was no significant relationship between AOA communities and other local environmental factors (Fig. 6). The first group only contained mesosaline Lake Peng Co, the second contained oligosaline Lakes Nam Co and Yamdrok and the third contained freshwater Lakes Beng Co and Puma Yumco. In general, the constrained CCA axis explained 44% of the total variance in the archaeal *amoA* community composition. When polysaline Lake Qinghai data were incorporated into CCA analysis, conductivity and pH were only found to be significant (Fig. S3).

On the other hand, the Mantel test indicated a weak but significant positive correlation between the AOA communities and geographical distance ($r = 0.276$, $P = 0.017$; Table S1). When Lake Qinghai was included in the analysis, a significant positive correlation still existed between the AOA community compositions and geographical distance ($r = 0.43$, $P < 0.001$; Table S1).

Discussion

Diversity and distribution of AOA and AOB in Tibetan lakes

AOA were widespread within the Tibetan lakes with lower diversity, except that they were absent in three samples from the upper water column of Lake Nam Co, which may be a result of incomplete coverage of general AOA primers (Francis *et al.*, 2005) or of extremely low AOA abundance in those samples. Recent experiments with *N. maritimus* have shown the unique ability of AOA to survive under oligotrophic environments that may help them keep their competitive predominance over AOB in oligotrophic Tibetan

lakes (Martens-Habbena *et al.*, 2009). The archaeal *amoA* ecotypes per sample (1–17 OTUs) are comparable to the AOA species richness retrieved from Lake Qinghai (1–12 OTUs) (Jiang *et al.*, 2009b), arctic lakes (3 OTUs) (Pouliot *et al.*, 2009), groundwater and drinking water systems (1–10 OTUs) (Van Der Wielen, Voost & Van Der Kooij, 2009), the Black Sea (4–7 OTUs) (Francis *et al.*, 2005) and wastewater treatment plants (2–5 OTUs) (Park *et al.*, 2006). Bryant *et al.* (2008) have revealed a monotonic decrease in bacterial taxon richness with increased elevation in the Rocky Mountains. A recent study also reported lower bacterial richness in Tibetan lakes than those from low-altitude lakes (Xing, Hahn & Wu, 2009). Similarly, AOA communities in Tibetan lakes had a lower diversity than those from other environments at low altitudes at either 2 or 5% distance DNA level (Fig. 2), suggesting the deterioration of various environmental conditions with increasing elevation may result in a general decrease in microbial richness (Xing *et al.*, 2009). Interestingly, temperature has been identified as a critical factor influencing the diversity of ammonia oxidisers in aquarium biofilm systems (Urakawa *et al.*, 2008). However, further studies are needed to better evalute the influence of elevation on microbial diversity in inland aquatic systems.

In contrast, amplification of the β-bacterial *amoA* gene was successful in fewer samples and no γ-bacterial *amoA* genes were obtained, despite repeated screening using sensitive nested amplification with several different primers (Kim *et al.*, 2008), The low positive AOB amplicons presented here were unsurprising and may be ascribed to two reasons. (i) AOB are only a minor component in Tibetan lakes (Bothe *et al.*, 2000); this is supported by our bacterial 16S rRNA libraries that AOB contributed little biomass to total microbial assemblages (Liu, pers. comm); and (ii) AOB occur at certain times of the year and at specific locations within lake environments. Kim *et al.* (2008) argued that AOB are not evenly distributed in stratified water bodies and have to find their ecological niche in a counter gradient of oxygen and ammonia that occurs immediately below the euphotic zone in stratified lakes during summer (usually the epilimnion). This observation may hold true for our lakes, because two positive amplicons were obtained from the epilimnion of Lake Beng Co and Lake Puma Yumco (Fig. S1). The limited diversity of AOB in this study (Table 2) is consistent with low bacterial *amoA* richness (H') at a 97% DNA identity reported for Lake Qinghai (0–1.42) (Jiang *et al.*, 2009b).

Phylogeny of AOA and AOB

The archaeal *amoA* sequences obtained in this study were closely related to the sequences derived from lake and estuarine habitats, wastewater treatment plants, soils and drinking water systems. These relationships indicate that the AOA phylotypes are not restricted to general habitats (Herrmann *et al.*, 2009). However, the results obtained in this study suggest that salinity may play a critical role in the phylogenetic affiliation of inland aquatic AOA. While Cluster A contained sequences retrieved from the freshwater, oligosaline lakes investigated in this study, affiliation with Cluster B was largely restricted to archaeal *amoA* sequences recovered from a mesosaline lake. Furthermore, the archaeal *amoA* within Cluster A could be subsequently separated into habitat-specific subclusters according to lake salinity types (Fig. 3). The sequences obtained from freshwater lakes are preferentially affiliated within Clades II and III, and the majority of archaeal *amoA* sequences obtained from oligosaline lakes fell into Clade I. These results suggested a specific adaptation of corresponding AOA phylotypes to different salinity. The Clade I, to some extent, corresponded to the low-salinity cluster recovered from the San Francisco Bay estuary (Mosier & Francis, 2008), confirming that the AOA phylotypes within this clade may be widely distributed in the low-salinity habitat. Since Cluster B might be a mirror of the Crenarchaeota group 1.1b based on the archaeal 16S rRNA gene (Beman & Francis, 2006; Nicol *et al.*, 2008), it is possible that the archaeal *amoA* sequences obtained from mesosaline Lake Peng Co might be derived from the 'SCA clones' group, which are widespread in the polysaline and hypersaline lakes on the TP (Jiang *et al.*, 2008, 2009a). A recent observation revealed that archaeal *amoA* genes are also widespread in 12 geographically distant Tibetan soils at altitudes ranging from 4000 to 6550 m a.s.l. (Zhang *et al.*, 2009). Contrary to our results, however, Zhang *et al.* (2009) found almost all AOA in Tibetan soils belong to Cluster B, except for one AOA sequence, and there was no significant shift in phylogenetic structures across altitude gradient, suggesting that different environmental factors are

responsible for controlling the AOA populations in soils and lakes on the TP.

The bacterial *amoA* sequences obtained from the Tibetan lakes were exclusively related to *Nitrosomonas*-like AOB, in sharp contrast to the habitat specificity in the archaeal *amoA* phylogeny, forming a unique monophylogenetic cluster with some sequences previously retrieved from various inland saline waters (Hornek *et al*., 2006; Dorador *et al*., 2008; Jiang *et al*., 2009b). This cluster was not closely related to any of the cultured AOB (Fig. 4). Based on the threshold definition for new clusters proposed by Kim *et al*. (2008), this cluster potentially represents a novel AOB cluster which can tolerate a wide range of salinity (Dorador *et al*., 2008).

Response of the AOA community to environmental changes

In inland aquatic ecosystems, variations in bacterioplankton community composition often correlate with physical, chemical, biological and geographical factors (Yannarell & Triplett, 2004, 2005; Lindstrom, Kamst-Van Agterveld & Zwart, 2005; Crump *et al*., 2007; Van Der Gucht *et al*., 2007), but little attention has been paid to the archaea (Keough, Schmidt & Hicks, 2003). Two recent studies focused on salt lakes and characterisation with 16S rRNA gene-based culture-independent analysis indicated that salinity and water chemistry play important roles in controlling archaeal community structure, which is mainly comprised of haloarchaea (Jiang *et al*., 2009a; Pagaling *et al*., 2009), thus providing some clues to the response of archaeal community to environmental factors. Herrmann *et al*. (2009) showed that AOA community structures are influenced by lake trophic level, rather than by plant species-specific interactions in freshwater sediments. In the current study, the most important environmental variables among the lakes investigated were salinity and latitude (Table 1, Fig. 1). Multivariate statistical analyses showed that the conductivity (salinity) was the most important environmental factor shaping AOA community compositions in the lakes investigated (Figs 6 & S3; Table S1), providing first evidence of salinity-related differentiation of planktonic AOA community composition in inland water environments. This pattern was also supported by phylogenetic analysis, which showed that archaeal *amoA* sequences retrieved from these lakes formed salinity-specific clusters (Fig. 3). Our results are in agreement with the view that mesophilic Crenarchaeota have a cosmopolitan distribution in freshwater lakes (Keough *et al*., 2003) as well as with two previous reports which suggest that haloarchaea biogeography is influenced by local water chemistry rather than historical events (Jiang *et al*., 2009a; Pagaling *et al*., 2009). This is contrary to the distribution of the hyperthermophilic archaea *Sulfolobus islandicus* and also the thermophilic AOA, which show a tendency for endemism in isolated hot springs (Whitaker, Grogan & Taylor, 2003; Zhang *et al*., 2008), implying that mesophilic archaea may have a more robust ability for viability and immigration to disperse over long distances, while thermophilic archaea are less likely to survive at ambient temperatures (Pagaling *et al*., 2009). Nevertheless, it is hard to exclude the impact of geographical factors on the AOA community since a weak positive correlation was found between AOA communities and geographical distance (Table S1). These results suggest that spatial distribution of AOA communities in Tibetan lakes is the result of the determinant effect of local environmental factors (salinity) but that geographical isolation may facilitate the co-evolution of AOA within lakes. Therefore, studies involving long geographical distances (such as inter-continental distances) on AOA need to be conducted in the future.

In contrast to the clear co-variations of AOA communities with lake salinity, AOA community compositions at different depths within each lake were relatively homogeneous (Figs 5 & 6), despite the fact that several environmental factors such as solar radiation and temperature varied sharply within the lake (Fig. S1). Most marine planktonic AOA are affiliated within two depth-specific groups – the Marine Shallow and Marine Deep groups (Hallam *et al*., 2006; Mincer *et al*., 2007; Beman, Popp & Francis, 2008); Agogué *et al*. (2008) have described these shallow and deep groups. Unlike the tendency for phototrophic bacteria to utilise light (Beja *et al*., 2001; Johnson *et al*., 2006), the mechanism of depth-related phylogeny of the marine AOA may be attributed to ammonia monooxygenase protein (AMO) resisting photoinhibition, since the membrane spanning characteristics of AMO experience significant exposure to light (Mincer *et al*., 2007). By contrast, multivariate statistical analyses indicated little influence of light gradient on AOA community compositions

throughout the water column in the lakes investigated (Figs 5 & 6; Table S1), indicating that light was not a pivotal determinant of differences in AOA community structure across the water depth of Tibetan lakes. The relatively homogeneity in the vertical distribution of the AOA communities within lakes might be because of two reasons. First, unlike the deep ocean, periodic mixing of lakes in temperate zones may minimise differences in chemical characteristics between different water masses. Therefore, the relatively homogeneous composition of AOA communities might be determined by the uniform chemical characteristics of these lakes (Fig. S1) (Liu *et al*., 2009). Secondly, geographical isolation may facilitate convergence of the AOA community structures within lakes (Whitaker *et al*., 2003; Martiny *et al*., 2006).

Despite the importance of microbial biogeography that has been documented extensively in recent years, microbial β-diversity patterns, especially for archaea in natural environments, are largely unknown (see review by Martiny *et al*., 2006). Our study is the first to compare the community structures of AOA among high-altitude lakes. Our results revealed lower α-diversity of AOA in Tibetan lakes in comparison with other natural environments, which may be an indirectly altitude-dependent effect. On the other hand, salinity might be a key factor that results in higher β-diversity of AOA among Tibetan lakes. The diversity (α or β) patterns of AOA in Tibetan lakes, together with those of previous studies (e.g. Keough *et al*., 2003; Jiang *et al*., 2009a; Pagaling *et al*., 2009), shed light on the essential influence of modern environments on AOA or mesophilic archaea, whereas the spatial variation of thermophilic archaea is because of the influence of evolutionary events. However, it is important to emphasise that the diversity (α- or β-) patterns of AOA in high-altitude lakes presented here should be treated with caution since only a few lakes were sampled in our study (5 lakes, 14 samples). Thus, more comparative investigations with greater sampling effort are needed to gain in-depth insights into the biogeography of ammonia oxidisers in inland aquatic environments.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Main characteristics of the investigated lakes in this work.

Fig. S2 Rarefaction analysis of the archaeal *amoA* sequences from 14 clone libraries prepared from five Tibetan lakes using an evolutionary distance threshold of 2% (i.e. 98% similarity) for OTU definition.

Fig. S3 Canonical correspondence analysis of archaeal *amoA* libraries obtained from the lakes investigated in this study and two libraries (QLW1-0 and QLW1-1200) recovered from the Lake Qinghai (Jiang *et al.*, 2009b) using local environmental variables.

Table S1 Simple and partial Mantel test for the AOA community[a].

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