Nitrification of archaeal ammonia oxidizers in a high temperature hot spring

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Abstract

The oxidation of ammonia by microbes has been shown to occur in diverse natural environments. However, it remains poorly understood about the link of *in situ* nitrification activity to taxonomic identities of ammonia oxidizers in high-temperature environments. Here, we studied *in situ* ammonia oxidation rates and the diversity of ammonia-oxidizing archaea (AOA) in surface and bottom sediments at 77°C in the Gongxiaoshe hot spring, Tengchong, Yunnan, China. The *in situ* ammonia oxidation rates measured by the $^{15}N\text{-}NO_3^-$ pool dilution technique in the surface and bottom sediments were 4.80 and 5.30 nmol N $g^{-1}h^{-1}$, respectively. Real-time quantitative PCR (qPCR) indicated that the archaeal 16S rRNA genes and *amoA* genes were present in the range of 0.128 to 1.96 × 10^8 and 2.75 to 9.80 × 10^5 gene copies g^{-1} sediment, respectively, while bacterial *amoA* was not detected. Phylogenetic analysis of 16S rRNA genes showed high sequence similarity to thermophilic '*Candidatus* Nitrosocaldus yellowstonii', which represented the most abundant operational taxonomic units (OTU) in both surface and bottom sediments. The archaeal predominance was further supported by fluorescence in situ hybridization (FISH) visualization. The cell-specific rate of ammonia oxidation was estimated to range from 0.410 to 0.790 fmol N archaeal $cell^{-1}$ h^{-1}, higher than those in the two US Great Basin hot springs. These results suggest the importance of archaeal rather than bacterial ammonia oxidation in driving the nitrogen cycle in terrestrial geothermal environments.

1 Introduction

Nitrogen is a key element controlling the species composition, diversity, dynamics, and functioning of many ecosystems (Vitousek et al., 1997). Despite of recent processes in our understanding of nitrogen cycling activities in soils, fresh and marine waters, and sediments (Francis et al., 2005; He et al., 2007; Beman et al., 2008; Jia and Conrad., 2009; Konneke et al., 2005; Nicol and Schleper, 2006), gaps in knowledge associated with high-temperature ecosystems have prevailed (Zhang et al., 2008a). Recently, some studies have elucidated nitrogen metabolism and cycling in high-temperature hot spring ecosystems (Dodsworth et al., 2011b; Nishizawa et al., 2013; Gerbl et al., 2014). In such systems, there has been evidence of microbial communities oxidizing ammonia, the first and rate-limiting step of nitrification (Reigstad et al., 2008; Hatzenpichler et al., 2008). Since the occurrence of a putative archaeal *amoA* gene in hot spring environments was first reported by Weidler et al. (2007) and Spear et al. (2007), thaumarchaeota possessing ammonia monooxygenase (AMO) have been obtained from some terrestrial hot springs in the USA, China and Russia (Pearson et al., 2008; Zhang et al., 2008a).

Previous studies targeting ammonia oxidation in hot springs mainly focused on archaeal *amoA* gene (AOA) via a variety of culture-independent approaches (e.g. 16S rRNA clone library, biomarkers) (Weidler et al., 2007; Francis et al., 2007; Zhang et al., 2008a; Jiang et al., 2010; Xie et al., 2014). The results from these studies suggested that ammonia-oxidizing archaea (AOA) may be ubiquitous in high-temperature environments and even more abundant than their bacterial counterparts, which has led to a hypothesis that Archaea rather than Bacteria drive ammonia oxidation in high-temperature hot spring environments. This hypothesis, however, still needs to be verified. Currently, our knowledge about the activity of AOA in such high-temperature environments is largely constrained, especially due to the data deficiency of ammonia oxidation rates (Reigstad et al., 2008; Dodsworth et al., 2011b; Li et al., 2015). *In situ* incubation experiments are urgently required to verify the potential activity of AOA and their contribution to ammonia oxidation in such high-temperature environments.

In this study, we selected the Gongxiaoshe hot spring at Tengchong Geothermal Field as a representative site to test the hypothesis that Archaea rather than Bacteria drive ammonia oxidation in high-temperature hot spring environments. The reasons for choosing the

Gongxiaoshe hot spring as the research site are: 1) Ammonia concentration in the Gongxiaoshe hot spring water is 102.61 μg L^{-1}, thermodynamically favorable to ammonia oxidation (Shock et al., 2005); 2) Ammonia-oxidizing archaea "*Candidatus* Nitrosocaldus yellowstonii" were dominant in the hot spring water and no AOB *amoA* genes were detected in the hot spring (Hou et al., 2013), indicating that the ammonia oxidation driven by Archaea might be active. Here, in combination of culture-independent (fluorescence *in situ* hybridization, quantitative PCR and clone library) and culture-dependent (^{15}N pool dilution technique) approaches, we provide direct evidences that AOA are indeed responsible for the major portion of ammonia oxidation in high-temperature hot spring environments.

2 Materials and methods

2.1 Site description and chemical measurements

Gongxiaoshe hot spring is a small pool with a diameter of ~300 cm and a depth of ~130 cm (Fig. 1). Hot spring water in the pool is well mixed and water chemistry shows no difference in different areas of the pool (Zhang et al., 2008b). Sediments of Gongxiaoshe hot spring are found to be only present at the margin of the pool and at the bottom of the pool, representing two typically sedimentary environments in this pool. The samples from the pool margins and sediments from the bottom of the spring, designated SS (Surface Sediments) and BS (Bottom Sediments), respectively, were collected using sterile equipment in April 2013. During transportation, all of the samples were packed with dry ice. They were then stored in a freezer at –80 °C in lab for further analysis.

Temperature and pH were measured *in situ* in the hot water spring. Temperature was determined with an iButton thermometer (DS1922T, Dallas Semiconductor, USA). The pH was measured using a pH Meter (SevenGo™ pH meter SG2, Mettler Toledo, USA). Water samples for cation and anion analysis were filtered through a syringe filter with a 0.22 μm filtration membrane; these samples were diluted 10 times with deionized water and stored in 100 mL polypropylene bottles in the field because an analysis was carried out after two days. The cation concentrations were determined using an IRIS Advantage ICP-AES, whereas the

anion (F^-, SO_4^{2-}, Cl^-) concentrations were determined using the Ion Chromatography System (DIONEX ICS-1500, Thermo Scientific, USA). The HCO_3^- concentration was measured using the Gran titration method (Appelo and Postma, 1996). The NH_4^+-N and NO_3^--N concentrations were determined using a Nutrient Analyzer (Micromac 1000, Partech, UK).

2.2 ^{15}N stable isotope tracing of nitrification activity

Gross N nitrification rates were determined *in situ* by the ^{15}N pool dilution technique. All of the nitrification measurements were conducted in 500 mL polycarbonate culture flasks (Nalgene) with a silicone plug that contained 400 mL of mud (~1/3 sediment by volume). Two subsamples were collected from the bottom and surface sediments with 350 μL of $K^{15}NO_3$ (485 μmol L^{-1}, at 10% ^{15}N). For each sample, two experiments were conducted to measure the *in situ* nitrification activity: A1 (SS slurry + $^{15}NO_3^-$) and A2 (BS slurry + $^{15}NO_3^-$). Meanwhile, potential nitrification activity was determined in the presence of high ammonium concentration: B1 (SS slurry + $^{15}NO_3^-$ + $^{14}NH_4^+$) and B2 (BS slurry + $^{15}NO_3^-$ + $^{14}NH_4^+$). Two pairs of duplicate reactors were set up in four experiments. The reactors were incubated near the *in situ* conditions of the hot spring water at 77 °C for 30 and 120 min. At certain time intervals (e.g., 30 min, 120 min), 80 mL aliquots were collected from the experimental reactors with sterile serological pipettes and transferred to acid-cleaned 250 mL polypropylene bottles. Prior to filtration, 40 mL of KCl (3 M) was added to each sample bottle, and the samples were shaken at 120 rpm for 1 h and then centrifuged at 1600 ×*g* for 10 min (Reigstad et al., 2008). The supernatant was filtered through a syringe filter containing a 0.22 μm filtration membrane; the supernatant was subsequently stored in acid-cleaned 60 mL polypropylene bottles at 4 °C, and analysis was performed after 2 days.

In the laboratory, the concentrations of NH_4^+ and NO_3^- in the filtrate were determined by a Nutrient analyzer (Micromac-1000, UK). The NO_3^- ($^{15}NO_3^-$ and $^{14}NO_3^-$) ions of the filtrates were converted to N_2O by denitrifying bacteria (*Pseudomonas aureofaciens*) lacking N_2O reductase activity, and N_2O was quantified by coupled gas chromatography isotope ratio mass spectrometry (GC-IRMS, Thermo Scientific, USA) (Dodsworth et al., 2011a). The ammonia oxidation rates were calculated using the equations of Barraclough. D. (1991) as were the concentrations and N isotope ratios of NO_3^- in the samples incubated for 30 and 120 min,

117 respectively.

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119 2.3 DNA extraction and purification

120 DNA was extracted by the SDS-based extraction method described by Zhou et al. (1996), with
121 some modifications. Briefly, approximately 5 g samples were frozen with liquid nitrogen and
122 milled three times. Then the powdered samples were mixed with 13.5 mL of DNA extraction
123 buffer and 100 μL of proteinase K (10 mg ml^{-1}) in tubes; these tubes were horizontally shaken
124 at 225 rpm for 30 min at 37 °C. After shaking, 1.5 mL of 20% SDS was added, and the
125 samples were incubated in a water bath; the temperature of the water bath was maintained at
126 65 °C for 2 h. During this period, the tubes were subjected to gentle end-over-end inversions
127 every 15 to 20 min. The supernatant fluids were collected after subjecting the tubes to
128 centrifugation at 6000 ×*g* for 10 min at room temperature; the collected supernatant tubes
129 were subsequently transferred into 50 mL centrifuge tubes. The supernatant fluids were mixed
130 with an equal volume of chloroform: isoamyl alcohol solution (24:1, vol/vol). The aqueous
131 phase was recovered by centrifugation and precipitated with a 0.6 volume of isopropanol at
132 room temperature; this process was carried out for at least 1 h. Crude nucleic acids were
133 obtained by centrifugation at 16,000 × *g* for 20 min at room temperature; these crude nucleic
134 acids were washed with cold 70% ethanol and resuspended in sterile deionized water; the final
135 volume of this solution was 100 μL. The crude nucleic acids were purified with a Cycle-Pure
136 Kit (Omega, USA). These crude nucleic acids were then resuspended in the elution buffer, and
137 the final volume of the solution mixture was 50 μL; this solution was stored at –80 °C.

138 2.4 PCR and clone library construction

139 16S rRNA gene was amplified with purified genomic DNA as templates using universal
140 primers. The primer pairs A21F (5′-TTC CGG TTG ATC CYG CCG GA-3′) and A958R
141 (5′-YCC GGC GTT GAM TCC AAT T-3′) were chosen for Archaea (Delong, 1992) and
142 Eubac27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and Eubac1492R (5′-GGT TAC CTT
143 GTT ACG ACT T-3′) were chosen for bacteria (Lane, 1991). In a total volume of 50 μL, the

reactions were performed using 1.25 U of Taq DNA polymerase (Takara, Japan). The amplification conditions were as follows: an initial denaturation was carried out at 94 °C for 4 min, and then, the same denaturation was continued at 94 °C for 1 min. Thereafter, annealing was carried out at 55 °C for 45 s, while extension was conducted at 72 °C for 60 s; the process was repeated for 30 cycles, followed by a final extension step at 72 °C for 10 min. The PCR products were excised after being separated by gel electrophoresis; a gel-extraction kit (Omega, USA) was used to purify the products in accordance with the manufacturer's instructions. The purified PCR products were cloned into pMD20-T vectors (Takara, Japan) and transformed into competent *Escherichia coli* DH5α cells. To select the positive clones, colony PCR was used to determine the presence of correctly sized inserts containing vector-specific primers M13f (5′-GTA AAA CGA CGG CCA G-3′) and M13r (5′-CAG GAA ACA GCT ATG AC-3′).

2.5 Sequencing and phylogenetic analysis

All of the clones were sequenced by the dideoxynucleotide chain-termination method. In this procedure, an ABI 3730 capillary electrophoresis sequencer (Applied Biosystem, Inc., USA) was coupled with the T-vector universal primers M13f and M13r. The whole sequence of each clone was spliced using DNAMAN software (version 6.0), and the vector sequences were deleted; the presence of chimeras was checked using the Greengenes chimera check tool (Bellerophon server) (Huber et al., 2004). The program DOTUR was used to determine the operation taxonomic units (OTU) for each sequence; 97% similarity was considered as the cut-off for the chimeric sequences. To find closely related sequences in the GenBank and EMBL databases for phylogenetic analysis, none of the chimeric sequences were submitted to the Advanced BLAST search program. Phylogenetic trees were constructed using the neighbor-joining method and the software MEGA (version 5.05). A bootstrap analysis was used to provide confidence estimates of the tree topologies.

2.6 Amplification of *amoA* (ammonia monooxygenase subunit A)-related sequences.

Archaeal *amoA* gene fragments were amplified using the primer pair Arch-*amo*AF (5´-STA ATG GTC TGG CTT AGA CG-3´) and Arch-*amo*AR (5´-GCG GCC ATC CAT CTG TAT GT-3´) (Francis et al., 2005). Bacterial *amoA* genes were also tested using the bacterial primer sets *amoA* 1F (5´-GGG GTT TCT ACT GGT GGT-3´) and *amoA* 2R (5´-CCC CTC KGS AAA GCC TTC TTC-3´) (Rotthauwe et al. 1997). PCR cycling was performed by the method of Francis et al. (2005). In this method, PCR products from SS and BS were recovered from the gel slices using a gel-extraction kit (Omega, USA) in accordance with the manufacturer's instructions. The purified PCR products from each type of sample were cloned into the pMD20-T vectors (Takara, Japan) and transformed into competent *Escherichia coli* DH5α cells. Cloning and sequencing were performed according to the above-mentioned process. Forty to fifty randomly selected colonies per sample were analyzed for the presence of insert archaeal *amoA* gene sequences.

2.7 Quantification of 16S rRNA genes and *amoA* genes

Archaeal and bacterial populations were determined by quantify their 16S rRNA genes with 344F-518R (Øvreas et al., 1998) and 518F-786R primer pairs (Muyzer et al., 1993), respectively. In addition, the abundance of AOA and AOB were quantified using amo196F-amo277R (Treusch et al., 2005) and amoA-1F and amoA-2R (Rotthauwe et al., 1997) primers, respectively. All sample and standard reactions were performed in triplicate. The SYBR Green I method was used for this analysis. The 20 μL reaction mixture contained 1 μL of template DNA (10 ng), a 0.15 μM concentration of each primer, and 10 μL of Power SYBR Green PCR master mix (Applied Biosystems Inc., USA); this reaction mixture was analyzed with ROX and SYBR Green I. The PCR conditions were as follows: 10 min at 50 °C, 2 min at 95 °C; 40 cycles consisting of 15 s at 95 °C and 1 min at 60 °C; 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C to make the melting curve (Wang et al., 2009). Melting curve analysis was performed after amplification, and the cycle threshold was set automatically using system 7500 software v2.0 Patch 6. The efficiencies of the qPCR runs were 87.8-105.6%

(R^2=0.992-0.999) for 16S rRNA genes and 102% (R^2=0.998) for AOA. Primers targeting different genes are listed in Table 1.

2.8 Sample processing for FISH

To visualize Crenarchaea cells *in situ*, FISH was performed according to the procedure described by Orphan et al. (2002, 2009). Small aliquots of sediment were fixed overnight at 4 °C using 2 % formaldehyde in 1×PBS [145 mM NaCl, 1.4 mM NaH_2PO_4, 8 mM Na_2HPO_4 (pH =7.4)]; these aliquots of sediments were washed twice with 1×PBS and stored at –20 °C in ethanol: PBS (1:1, vol/vol) medium. The total supernatant was filtered through a polycarbonate filter (Millipore) under low vacuum (<5 psi; 1psi=6.89 kPa). Filters were cut into suitably sized pieces and transferred onto untreated, round, 1 in glass slides. The transfer of filters onto glass slides was performed according to the procedure described by Murray et al. (1998). In this process, 5 μL of a 1×PBS solution was spotted onto a glass slide that was scored with a diamond pen prior to mapping, and half of the freshly prepared filter was used to invert the sample onto the slide; this inverted sample was then air-dried. Prior to FISH, the samples on the glass slides were treated with an EtOH dehydration series (50, 75, and 100% EtOH), dried, and stored at –20 °C. Hybridization and wash buffers were prepared according to the procedure described by Pernthaler et al., 2001. Here, 20 μL of hybridization buffer containing 35% or 20% formamide was added to the samples on the glass slides. FITC-labeled oligonucleotide Cren679 probe described by Labrenz et al. (2010), was added to the hybridization buffer so that the final solution had a concentration of 5 ng μL^{-1}.

The hybridization mixtures on the slides were incubated for 1.5 h at 46 °C in a pre-moistened chamber. After hybridization, the slides were transferred into a preheated wash buffer and incubated for an additional 15 min at 48 °C. The samples were rinsed in distilled water and air-dried in the dark. The microscopic images of the hybridized samples were recorded on a Leica Imager (Leica, DMI 4000B, Germany).

2.9 Nucleotide sequence accession numbers

The clone libraries for archaeal communities (21F-958R), bacterial communities (27F-1492R),

and archaeal amoA genes(amoAF-amoAR) were constructed. All of the small-subunit rRNA gene sequences and the *amoA* sequences were deposited in the GenBank/EMBL nucleotide sequence database under the following accession numbers: KP784719 to KP784760 for partial 16S rRNA gene sequences and KP994442 to KP994448 for the *amoA* sequences.

3 Results

3.1 Water chemistry

The hot spring water (pH = 7.7) contained Ca (20.25 mg L^{-1}), K (41.97 mg L^{-1}), Mg (3.986 mg L^{-1}), Na (313.3 mg L^{-1}), SiO_2 (130.3 mg L^{-1}), HCO_3^- (963 mg L^{-1}), NH_4^+-N (102.61 μg L^{-1}), NO_3^--N (7.68 μg L^{-1}), F^- (9.158 mg L^{-1}), Cl^- (418.9 mg L^{-1}) and SO_4^{2-} (24.96 mg L^{-1}). The bottom water had a temperature of 77 °C, higher than the surface water that had a temperature of 55 °C. This hot spring was previously categorized as a Na-HCO_3 spring due to the high concentration of alkaline metal ions (K, Na, and Ca) (Zhang et al., 2008b).

3.2 Ammonia oxidation rates

In the surface and bottom sediments (without NH_4^+ stimulation), the near *in situ* rates of ammonia oxidation were estimated to be 4.80 ± 0.2 and 5.30 ± 0.5 nmol N $g^{-1}h^{-1}$ using ^{15}N-NO_3^- pool dilution technique, respectively. In the meantime, the nitrate concentration increased from 2.84 ± 2 μM to 3.25 ± 2 μM in the surface sediments and from 2.33 ± 3 μM to 2.62 ± 3 μM in the bottom sediments, further providing evidences for strong nitrification activity under *in situ* conditions in the hot springs. Furthermore, the potential activity of ammonia oxidation was determined with ammonium amendments. The nitrate concentration increased significantly upon the addition of NH_4^+, and the ammonia oxidation rates recorded in the surface sediments and bottom sediments (with NH_4^+) were 5.70 ± 0.6 and 7.10 ± 0.8 nmol N $g^{-1}h^{-1}$, respectively.

3.3 Archaeal community composition and phylogenetic analysis.

A total of 152 archaeal clone sequences of 16S rRNA genes were obtained in this study. Phylogenetic analysis showed the distribution of the clone sequences into three monophyletic groups: Thaumarchaeota, Crenarchaeota, and Euryarchaeota (Fig 4). In this study, the most abundant archaeal phylum was Thaumarchaeota. Among them, two phylotypes (SS-A19 and BS-A1) were the most dominant archaeal lineage, representing 89% and 86% of the cloned archaeal sequences in surface and bottom sediments, respectively. These sequences were closely related to the thermophilic, autotrophic, ammonia-oxidizing archaeal "*Ca.* N. yellowstonii" (de la Tarre et al., 2008). The seven archaeal OTUs found here belonged to Crenarchaeota, which contains sequences recovered from hydrothermal vents and hot spring environments. In addition, two phylotypes (BS-A47 and BS-A8) that were branched with uncultured sequences belonged to *Desulfurococcales*, which was also recovered from sediments of the hot spring. Euryarchaeota also occurred in both the sediments, but with relatively low abundances. Phylotype BS-A80 is associated with *Geoglobus ahangari*, which belongs to *Archaeoglobales* and is capable of oxidizing organic acids (Kashefi, et al., 2002). SS-A12, which represents four clones recovered from the surface sediments, showed 93% similarity to an uncultured archaeal clone that was recovered from the Spring River. SS-A47 belonged to the *Thermoplasmatales* that were 96% similar to their nearest neighbor sequence, which were collected from the Spring River. The other euryarchaeotal sequences BS-14 and BS-A80 were similar to their uncultured counterparts (from 96 to 99% identity), which were mostly recovered from high-temperature geothermal environments.

3.4 Community analysis of AOA

A total of 113 archaeal *amoA* gene fragments were obtained from the two samples. They were all branched within the four distinct clusters of archaeal *amoA* sequences: Cluster *Nitrosopumilus, Nitrososphaera, Nitrosotalea, Nitrosocaldus* (Fig 5). *Nitrosopumilus* Cluster contained phylotypes SS-AOA-4 and BS-AOA-22, which branched with large numbers of sequences recovered from the sediments and water samples in the marine or fresh environments. The other clade, Cluster *Nitrososphaera*, has two phylotypes representing 44

sequences. OTU BS-AOA-62 contained 18 sequences, which was closely related to sequences from soil. The clone SS-AOA-76 clustered within clade *Nitrososphaera* and showed up to 99% sequence identity to an uncultured archaeon clone GHL2_S_AOA_19 (JX488447) obtained from lake sediment.

Cluster *Nitrosotalea* had 1 phylotype (SS-AOA-65) with 11 sequences (12% of the total sequences). The closely related sequences in this cluster included characteristic crenarchaeotal group sequences that were obtained from alpine soil (with 98% identity). Another clone, MX_3_OCT_18 (DQ501052), from estuary sediment was 96% similar.

Cluster *Nitrosocaldus* contained two phylotypes (BS-AOA-15 and SS-AOA-50) with 34 sequences (30% of the total sequences). They were closely related to the geothermal water sequences, with 95-99% similarity. Furthermore, Cluster *Nitrosocaldus* mainly represented previously described ThAOA/HWCG III (Prosser and Nicol, 2008). Notably, the recently reported *amoA* gene sequence of "*Ca.* N. yellowstonii" (EU239961) (De la Torre et al., 2008) showed 85% sequence identity to clones BS-AOA-15 and SS-AOA-50.

3.5 Quantitative PCR

The qPCR results (Fig. 2b) indicated that the abundance of the archaeal 16S rRNA gene in the two samples was similar, ranging from 1.28 to 1.96×10^{7} gene copies g^{-1} of dry weight of sediments. However, the abundance of the bacterial 16S rRNA gene varied greatly, ranging from 6.86×10^{6} to 4.25×10^{8} gene copies g^{-1} of dry weight of sediments (Fig. S2 in the Supplement). The copy numbers of archaeal *amoA* genes in the surface and bottom sediments are 2.75×10^{5} and 9.80×10^{5} gene copies g^{-1} sediment, respectively. The copy numbers of the archaeal 16S rRNA genes in the bottom sediments were significantly higher than those of the bacterial 16S rRNA genes, with a ratio of 28.57. However, in surface sediments, the ratio of bacterial 16S rRNA genes to archaeal 16S rRNA genes is 3.32.

3.6 FISH

FISH was used to analyze the relative abundance of Crenarchaea in two samples. As expected, most metabolically active Crenarchaea cells and aggregated cells were detected by FISH

probes (Cren679) (Fig 3). Based on the qPCR results, a high abundance of crenarchaea in the hot spring sediments harbored *amoA* genes, providing strong evidence supporting the important role of Crenarchaea in the oxidation of ammonia.

4 Discussion

4.1 Environmental factors affecting the occurrence of ammonia-oxidizing microorganisms

Temperature is likely a very important factor influencing microbial community structure. This interpretation is supported by the results of qPCR (Fig. 2b and Fig. S2). The sediment samples from the bottom of pool (T=77 °C) are dominated by Archaea, whereas the sediment samples from the margin of pool (T=55 °C) are dominated by Bacteria. In addition, no AOB were detected in both bottom and margin samples, indicating that it might be difficult for AOB to inhabit in high-temperature hot spring environments (Lebedeva et al., 2005; Hatzenpichler et al., 2008). Additionally, the abundance of AOA *amoA* gene in bottom sediments is slightly higher than that in margin sediments, reflecting that although AOA can adapt to a wide range of temperature, higher temperature could be more favorable to the growth of AOA (de la Torre, et al., 2008; Hatzenpichler et al., 2008; Jiang et al., 2010).

Ammonia concentration may be another factor that influences the potential activity of AOA and AOB in hot springs. Because AMO in AOA has a much higher affinity for the substrate compared to a similar process in AOB, the ability of AOA to compete for ammonia in oligotrophic hot spring environments is also substantially higher than that of AOB (Hatzenpichler et al., 2008). In Gongxiaoshe hot spring, the ammonia concentration of 102.61 μg L^{-1} is lower compared to other hot springs with high ammonia concentrations. This relatively low ammonia concentration may possibly be responsible for the absence of AOB in Gongxiaoshe hot spring.

4.2 Composition and abundance of AOA

The rarefaction curves (Fig. S3) for archaeal 16S rRNA genes and *amoA* genes in the surface and bottom sediment samples reached a plateau, and their coverage values were relatively high (89-99%). This result indicated that a large part of the archaeal/*amoA* diversity at this

spring was probably included in the archaeal/*amoA* clone libraries. The majority of archaeal sequences were closely related to '*Ca.* N. yellowstonii', a known AOA, which may be responsible for the oxidation of ammonia in this spring.

In this study, phylogenetic analyses of archaea *amoA* genes showed that *Candidatus* Nitrosocaldus yellowstonii dominated in both of the samples. This result also agreed with previous hot spring observations reported by Dodsworth et al. (2011b) and Hou et al. (2013). According to the sequences retrieved from NCBI, *Nitrosotalea and Nitrososphaera* clusters were closely related to the cluster soil. One possibility is that some of the *amoA* genes obtained in this study may derive from soil AOA, particularly those sequences in cluster *Nitrosotalea* and cluster *Nitrososphaera*, which have been widely found in sediments and soils. Those AOA from soil might have evolved multiple times and have adapted to high-temperature environments. Based on the analysis of the real-time PCR and FISH methods, our data indicate that the abundance of AOA is relatively high in both samples. The archaeal *amoA* gene copy numbers ranged from 2.75 to 9.80×10^5 per gram dry weight of sediments in this study. This is comparable to the abundance in other hot water springs [10^4-10^5 copies g^{-1} (Dodsworth et al., 2011b)], but is lower than the abundance of the archaeal *amoA* gene in non-thermal environments, such as paddy rhizosphere soil [10^6-10^7 copies g^{-1} (Chen et al., 2008)] and marine sediments [10^7-10^8 copies g^{-1} (Park et al., 2008)]. The bacterial *amoA* genes were not detected, indicating that AOB is absent or is a minority in this hot spring ecosystem. A predominance of archaeal *amoA* genes versus bacterial *amoA* genes indicated that ammonia oxidation may be due to the activity of archaea in the Gongxiaoshe hot spring.

4.3 The role of AOA in the nitrification of terrestrial geothermal environments

In the surface and bottom sediments (without NH_4^+), the ammonia oxidation rates calculated from the ^{15}N-NO_3^- pool dilution data were 4.80 ± 0.2 and 5.30 ± 0.5 nmol N $g^{-1}h^{-1}$, respectively. The ammonia oxidation rates recorded in the surface sediments and bottom sediments (with NH_4^+) were 5.70 ± 0.6 and 7.10 ± 0.8 nmol N $g^{-1}h^{-1}$, respectively. Moreover, the rates reported here were comparable with those observed in the two US Great Basin (GB) hot springs [5.50-8.60 nmol N $g^{-1}h^{-1}$(Dodsworth et al., 2011b)] and in two acidic (pH = 3, T = 85 °C) Iceland hot springs [2.80-7.00 nmol NO_3^- $g^{-1}h^{-1}$ (Reigstad et al., 2008)]. However, the

rates reported in this study were lower than those observed in some wetland sediments and agricultural soils [85-180 nmol N $g^{-1}h^{-1}$ (White and Reddy, 2003; Booth et al., 2005)].

The ammonia oxidation rates in bottom sediments (without NH_4^+) were slightly higher than those observed in surface sediments (without NH_4^+). This result agrees with the distribution of archaeal *amoA* genes, which were found to be in higher abundance in the bottom sediment than in the surface sediment. High abundance of ammonia-oxidizing archaea corresponds to high ammonia oxidation rates, which were consistent with the results reported by Isobe et al. (2012). Compared with the incubation experiments unamended with NH_4^+, the ammonia oxidation rate appeared to be stimulated after amendment with NH_4^+ (1 M). There are indications that the ammonia concentration is an important factor affecting the rates of nitrification (Hatzenpichler et al., 2008).

To understand the relationship between the ammonia oxidation rates and abundances of *amoA* in the two samples, we specifically estimated the contribution of archaeal cells to nitrification. By assuming two *amoA* copies per cell (Bernander and Poplawski, 1997) and by comparing the ammonia oxidation rates with the qPCR results of AOA *amoA* per gram (however, some uncertainties of this method may still exist, with respect to the stage of cell cycle and the diversity of archaea), the cell-specific nitrification rates were estimated to be 0.410 fmol N $cell^{-1}h^{-1}$ and 0.790 fmol N $cell^{-1}h^{-1}$ in the surface and bottom sediments of the hot spring, respectively. These results are much higher than those for AOA in US hot springs [0.008-0.01 fmol N $cell^{-1}h^{-1}$(Dodsworth et al., 2011b)]. It is interesting that although the GBS hot spring possesses higher *amoA* gene copies (3.50-3.90 × 10^8 gene copies g^{-1} of dry weight) and higher NH_4^+ concentration (663 μg L^{-1}), it exhibits a lower cell-specific nitrification rate than Gongxiaoshe hot spring. This may imply that both the abundance of AOA and the NH_4^+ concentration are not important factors that control the cell-specific nitrification rates in high-temperature hot spring environments. The difference in cell-specific nitrification rates between the Gongxiaoshe hot spring and the GBS hot spring may reflect the difference of AOA population structure in those two hot springs (Gubry-Rangin et al., 2011; Pester et al., 2012). In line with this AOA heterogeneity, cell-specific nitrification rates do not reflect the overall AOA abundance or NH_4^+ concentration in these AOA-dominated hot springs. Alves et al. (2013) reported a similar case where soil dominated by AOA (clade A) exhibited the lowest

nitrification rates, in spite of harboring the largest AOA populations. These results also suggest the importance of cultivation studies for comparative analysis of environmentally representative AOA in a wide variety of hot springs.

5 Conclusions

Combination of $^{15}N\text{-}NO_3^-$ pool dilution and molecular analyses demonstrate that the oxidation of ammonia by AOA occurs actively in the high-temperature Gongxiaoshe geothermal system. The presence of considerable *in situ* nitrification rates in the hot spring is likely due to two dominant groups that include phylotypes that are closely related to the autotrophic AOA *'Ca.* N. yellowstonii'. The detection of archaeal *amoA* genes and the absence of AOB indicate that archaeal ammonia oxidizers, rather than AOB, significantly contribute to the nitrification in the Gongxiaoshe geothermal systems. Due to the AOA heterogeneity, cell-specific nitrification rates may not reflect the overall AOA abundance or NH_4^+ concentration in the AOA-dominated hot springs. Our results shed light on the importance of AOA in driving the oxidation of ammonia in high-temperature environments, which may be ubiquitous in other terrestrial hot springs on Earth.

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572 **Table 1.** FISH probe and PCR primer pairs used in this study

Application	Probe/ Primer set	Specificity	sequence(5'-3')	FA(%)/ AT(℃)[a]	Reference
FISH	Cren679	Crenarchaeota	TTTTACCCCTTCCTTCCG	35	Labrenz M, et al. 2010
qPCR	518F	Bacteria	CCAGCAGCCGCGGTAAT	57	Muyzer et al. 1993
	786R		GATTAGATACCCTGGTAG		
	344F	Archaea	ACGGGGCGCAGCAGGCGCGA	60	Øvreas et al., 1998
	518R		ATTACCGCGGCTGCTGG		
	*amo*196F	Archaeal	GGWGTKCCRGGRACWGCMAC	60	Treusch et al., 2005
	*amo*277R	*amoA*	CRATGAAGTCRTAHGGRTADCC		
Clone library	A21F	Archaea	TTCCGGTTGATCCYGCCGGA	55	Delong, 1992
	A958R		YCCGGCGTTGAMTCCAATT		
	Eubac27F	Bacteria	AGAGTTTGATCCTGGCTCAG	55	Lane, 1991
	Eubac1492R		GGTTACCTTGTTACGACTT		
	Arch-*amo*AF	Archaeal	STAATGGTCTGGCTTAGACG	53	Francis et al., 2005
	Arch-*amo*AR	*amoA*	GCGGCCATCCATCTGTATGT		
	amoA 1F	Bacterial	GGGGTTTCTACTGGTGGT	60	Rotthauwe et al. 1997
	amoA 2R	*amoA*	CCCCTCKGSAAAGCCTTCTTC		

573 [a]FA, Formamide; AT, Annealing Temperature

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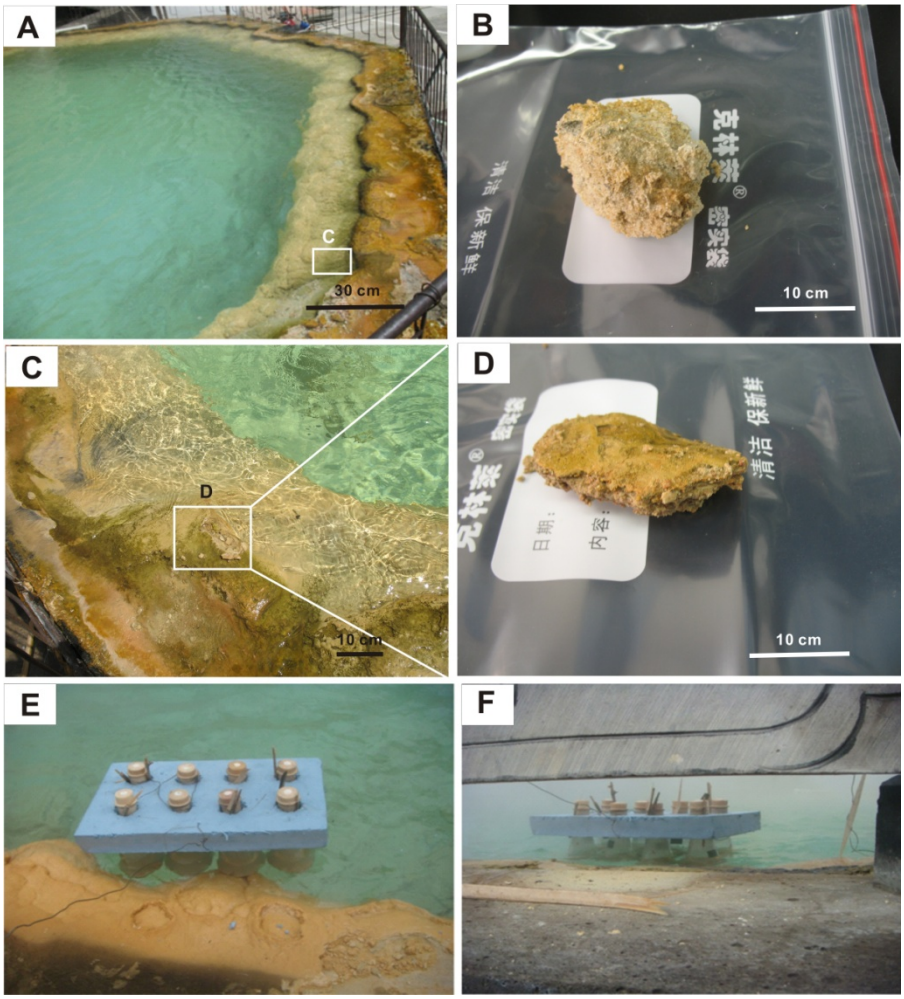
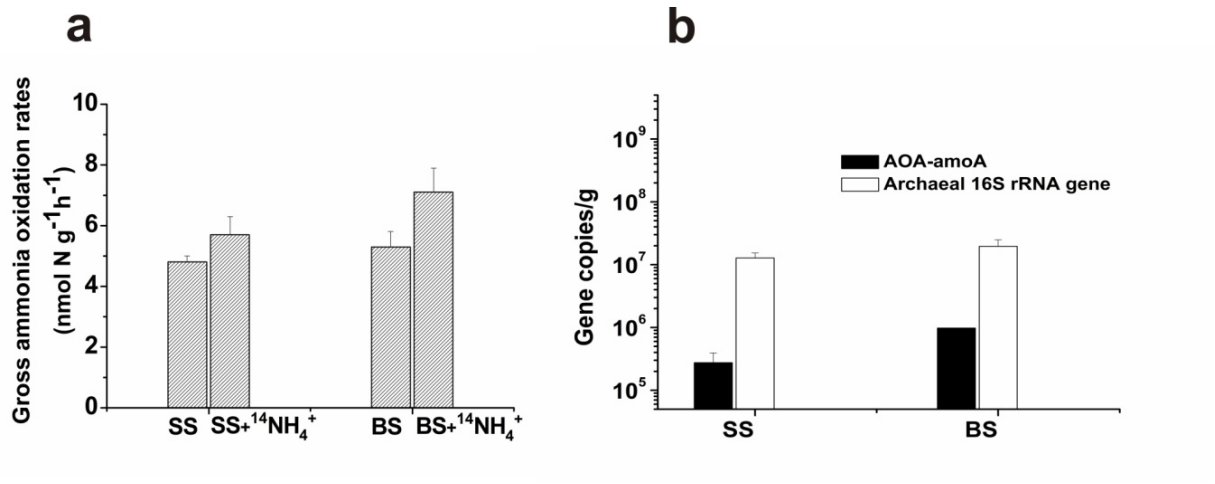


Figure 1. The Gongxiaoshe hot spring, located in the Ruidian geothermal area. (**a**). A full view of the spring; (**b**). Bottom sediments of the hot spring, designated as BS; (**c**). An enlarged view of the white box from Fig 1a, surface sediments of the hot spring; (**d**). Surface sediments of the hot spring designated as SS; and (**e, f**), *In situ* nitrification activity and potential nitrification activity experiments in the field.



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589 **Figure 2.** (**a**) Gross ammonia oxidation rates calculated from $^{15}N\text{-}NO_3^-$ pool dilution
590 experiments on amended (add $^{14}NH_4^+$) or unamended SS and BS sediment slurries. It defines
591 that the amendment with "$^{15}NO_3$" represents *in situ* nitrification activity, while $^{15}NO_3$ plus
592 $^{14}NH_4$ is considered as potential nitrification activity. Bars represent the mean and standard
593 error of the mean (n = 3) for 30 and 120 min incubation. (**b**) Abundance of archaeal 16S rRNA
594 genes and archaeal amoA genes for SS and BS samples collected from Gongxiaoshe hot
595 spring. Data are expressed as gene copies per gram of sediment (dry weight). Error bars
596 represent the standard deviation of the mean (n=3).

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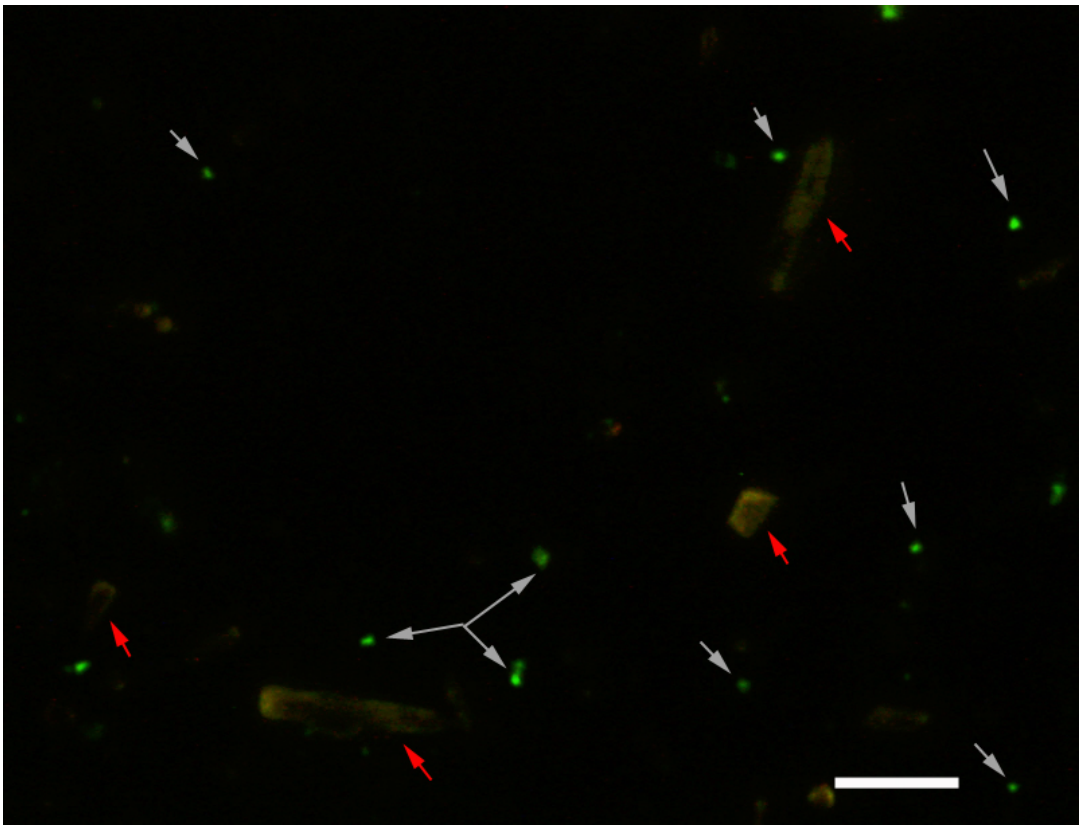

Figure 3. Epifluorescence photomicrograph of Crenarchaeota cells and cell aggregates. (White and red arrows show the cells and carbonate crystals, respectively. Scale bar corresponds to 20 μm)

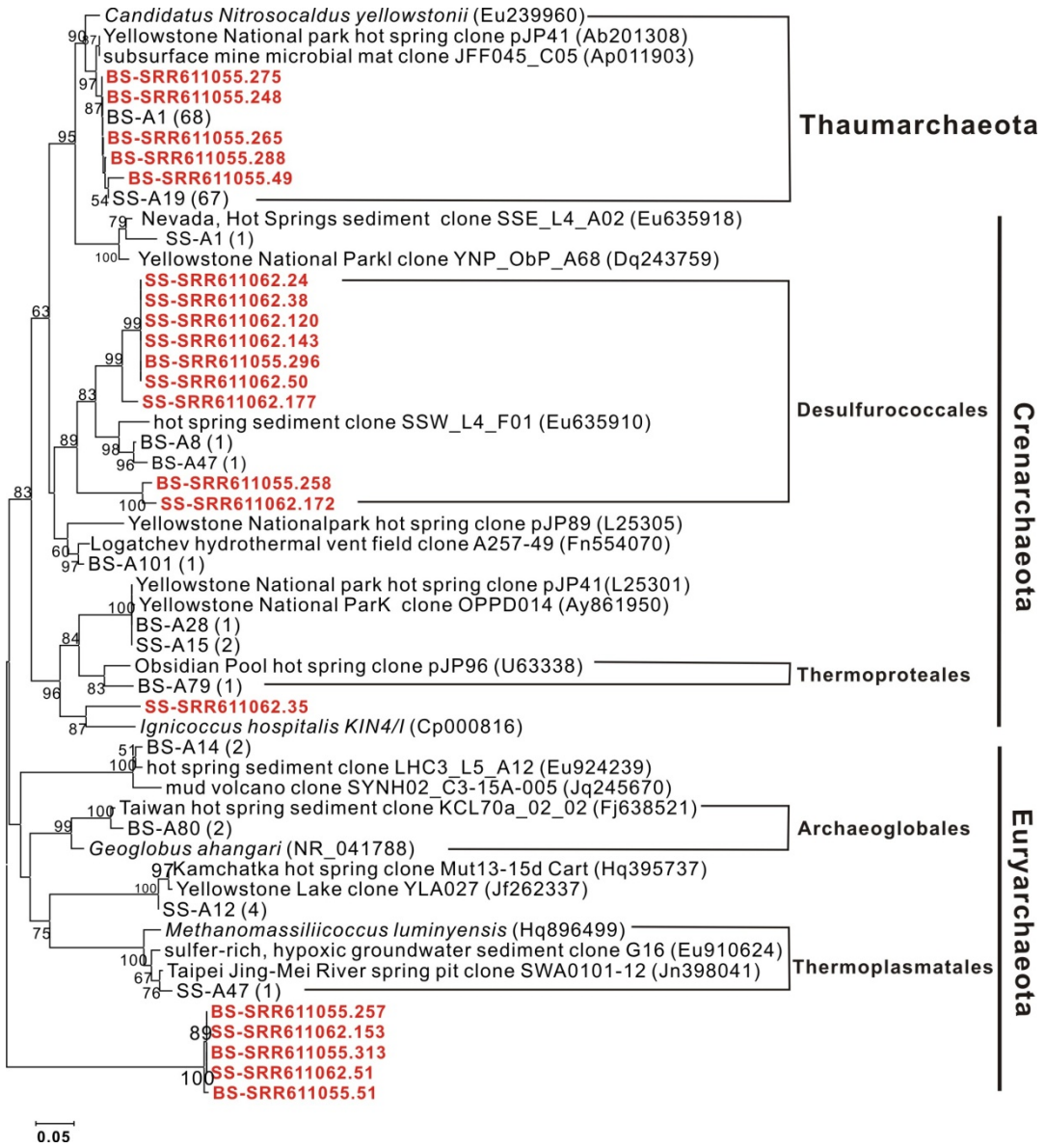

Figure 4. Archaeal phylogenetic tree based on 16S rRNA gene sequences, including various 16S rRNA gene clones obtained from the Gongxiaoshe hot spring sediments (SS and BS) and cited some sequences from Hou et al. (2013) (stained by red). The tree is constructed using the neighbor-joining method, and bootstrap confidence values over 50% (1000 replicates) are shown. The scale bar represents the expected number of changes per nucleotide position.

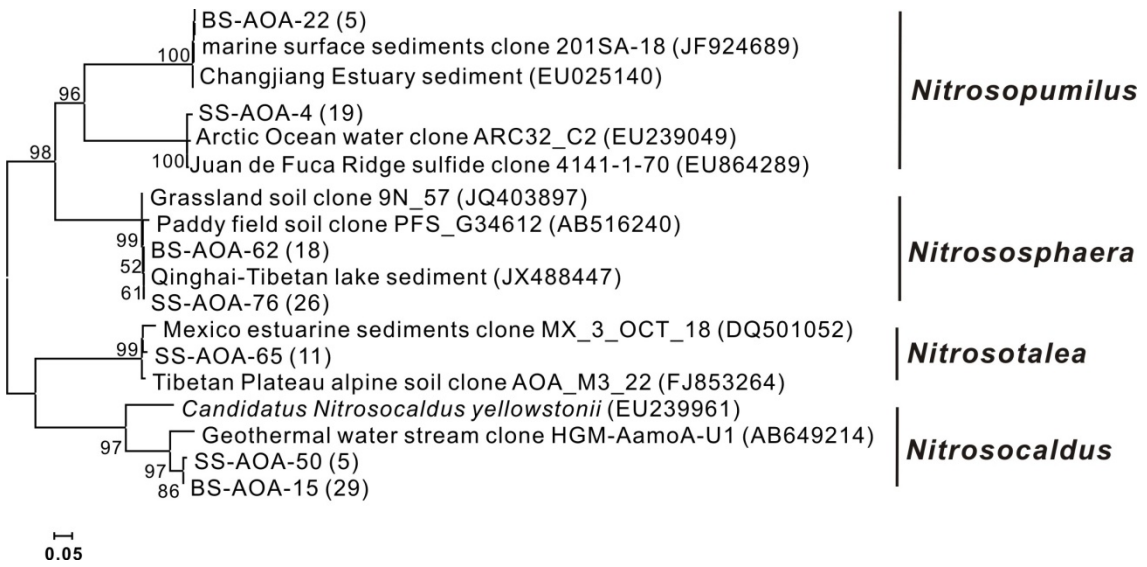

Figure 5. The phylogenetic tree of archaeal amoA genes is cloned from the Gongxiaoshe hot spring sediments (SS and BS). The tree is constructed using the neighbor-joining method, and bootstrap confidence values over 50% (1000 replicates) are shown. The scale bar represents the expected number of changes per nucleotide position.