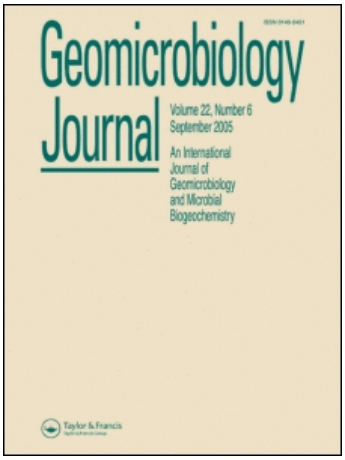

Geomicrobiology Journal
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713722957

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Online publication date: 24 February 2011

To cite this Article Zhao, Weidong , Song, Zhaoqi , Jiang, Hongcheng , Li, Wenjun , Mou, Xiaozhen , Romanek, Christopher S. , Wiegel, Juergen , Dong, Hailiang and Zhang, Chuanlun L.(2011) 'Ammonia-oxidizing Archaea in Kamchatka Hot Springs', Geomicrobiology Journal, 28: 2, 149 — 159
To link to this Article: DOI: 10.1080/01490451003753076
URL: http://dx.doi.org/10.1080/01490451003753076

Geomicrobiology Journal, 28:149–159, 2011

ISSN: 0149-0451 print / 1521-0529 online
DOI: 10.1080/01490451003753076

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Ammonia-oxidizing Archaea in Kamchatka Hot Springs

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Mounting evidence suggests that ammonia-oxidizing archaea (AOA) may play important roles in nitrogen cycling in geothermal environments. In this study, the diversity, distribution and ecological significance of AOA in terrestrial hot springs in Kamchatka (Far East Russia) were explored using *amoA* genes complemented by analysis of glycerol dialkyl glycerol tetraethers (GDGTs) of archaea. PCR amplification of functional genes (*amoA*) from AOA and ammonia-oxidizing bacteria (AOB) was performed on microbial mats/streamers and sediments collected from three hot springs (42°C to 87°C and pH 5.5-7.0). No *amoA* genes of AOB were detected. The *amoA* genes of AOA formed three distinct phylogenetic clusters with Cluster 3 representing the majority (∼59%) of OTUs. Some of the sequences from Cluster 3 were closely related to those from acidic soil environments, which is consistent with the predominance of low pH (<7.0) in these hot springs. Species richness (estimated by Chao1) was more frequently higher at temperatures below 75°C than above it, indicating that AOA may be favored in the moderately high temperature environments. Quantitative PCR of 16S rRNA genes showed that crenarchaeota counted for up to 80% of total archaea. S-LIBSHUFF separated all samples into two phylogenetic groups. The profiles of GDGTs were well separated among the studied springs, suggesting a spatial patterning of archaeal lipid biomarkers. However, this patterning did not correlate significantly with variation in archaeal *amoA*, suggesting that AOA are not the predominant archaeal group in these springs producing the observed GDGTs.

Keywords ammonia-oxidizing archaea, *amo*A, GDGT, hot springs, Kamchatka

INTRODUCTION

Ammonia oxidizing bacteria (AOB) oxidize ammonia to nitrite through hydroxylamine (Kowalchuk and Stephen 2001), which is the first step of nitrification and a key component of the global nitrogen cycle. AOB are exclusive members of β- and γ-subgroups of *Proteobacteria*, and were previously considered as the most important contributors to the ammonia oxidation process (Purkhold et al. 2000; Prosser and Embley 2002).

Genes encoding putative homologues of ammonia monoxygenase subunits A and B have been identified in association with the 16S rRNA gene of crenarchaeota through metagenomic studies of marine and soil samples (Venter et al. 2004; Schleper et al. 2005), and subsequently confirmed by whole genome studies of "*Candidatus* Nitrosopumilus maritimus" (Könneke et al. 2005) and a sponge symbiont *Cenarchaeum symbiosum* (Hallam et al. 2006). PCR-based surveys using primers specific to putative archaeal *amoA*-like genes have indicated widespread occurrence of ammonia-oxidizing archaea (AOA) in soil (Leininger et al. 2006), marine (Francis et al. 2005; Wuchter et al. 2006) and more recently, high temperature environments (Spear et al. 2007; Weidler et al. 2007; Reigstad et al. 2008; Zhang et al. 2008; Wang et al. 2009). The latter finding is supported by the enrichment of thermophilic AOA, "*Candidatus* Nitrosocaldus yellowstonii" (T_{opt} = 65–72°C) from a hot spring in Yellowstone National Park (de la Torre et al. 2008) and moderately thermophilic "*Candidatus* Nitrososphaera gargensis" (T_{opt} = 46°C) from the Siberian Garga hot spring (Hatzenpichler et al. 2008).

Advances in phylogenetic and genomic studies of archaea, especially crenarchaeota, are paralleled by advances in biogeochemical studies of archaeal membrane lipids, i.e., glycerol dialkyl glycerol tetraethers (GDGTs), using high performance liquid chromatography-mass spectrometry (LC-MS) (Hopmans et al. 2000; Schouten et al. 2000). GDGTs usually contain zero to four cyclopentyl rings (Koga et al. 1993; Koga and Morii 2005) but up to eight rings have been reported in some hyperthermophilic archaea (Derosa and Gambacorta 1988). The presence of cyclopentyl rings in GDGTs is known to increase the

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thermal tolerance of archaeal membranes, and studies of cultures and the marine crenarchaeota show that the average number of rings correlates positively with temperature (Uda et al. 2001; Schouten et al. 2002). A unique crenarchaeotal GDGT, crenarchaeol, was discovered to contain one cyclohexyl-ring and four cyclopentyl rings. It was originally observed to be specifically associated with marine nonthermophilic crenarchaeota (DeLong et al. 1998; Schouten et al. 2000; Schouten et al. 2002), but recently it has been found in lakes, soil, hot spring environments and a thermophilic AOA culture (Pearson et al. 2004; Weijers et al. 2006; Zhang et al. 2006; Schouten et al. 2007; de la Torre et al. 2008).

The majority studies of AOA have focused on marine (e.g., Francis et al. 2005; Könneke et al. 2005; Lam et al. 2007) and soil (e.g., Schleper et al. 2005; Leininger et al. 2006; He et al. 2007) systems and only a few on geothermal springs (see reviews by Francis et al. 2007 and Prosser and Nicol 2008). It remains unclear to what extent thermophilic ammonia-oxidizing microorganisms are involved in nitrification in geothermal systems.

Kamchatka (Far East Russia) is one of the most active and pristine geothermal areas in the world. Hot springs in the Uzon Caldera of Kamchatka are mostly acidic and contain relatively high concentrations of ammonium (10s to 100s ppm), which may provide an energy source for the growth of AOA. In this study, we examined AOA in three hot springs in Kamchatka using archaeal *amoA* gene and GDGT biomarkers. Relationships between these biomarkers and environmental variables including pH and temperature were evaluated to determine the factors that control microbial diversity in these geothermal environments.

MATERIALS AND METHODS

Sites, Sample Collection, and Physicochemical Analysis

Three hot springs in the East Thermal Field of the Uzon Caldera, Kamchatka were investigated: 1) Thermophile (54°29′55.18″N, 160°00′42.42″E), 2) Cascadnaya (54°29′59.68″N, 160°00′46.00″E), and 3) Burlyashi (54°29′58.79″N, 160°00′08.09″E). At each spring, water samples were collected along a temperature gradient in the outflow channel. Water chemistry (alkalinity, sulfate, total sulfide, nitrite, and ammonium) was measured using Hach kits in the field. Temperature, pH, total dissolved solids (TDS), and oxidation-reduction-potential (ORP) were measured *in situ* with a portable pH meter prior to sample collection. The pH meter was calibrated at 25°C and pH values were measured at *in situ* temperature. Microbial mats/streamers and underlying sediments were collected directly below where water samples were collected.

Samples were placed in sterile plastic bags and stored in cold water (4–7°C) in the field for up to 10 days before transporting to the University of Georgia on ice. Samples were then stored at −80°C until analysis. At the source of each spring, bubbles from vent gases were collected below the water surface using an inverted bell jar and transferred immediately to Vacutainers (BD Inc., Franklin Lakes, NJ, USA) through a gas-tight port. Gaseous H_2S was removed in the laboratory by the addition of up to 1 ml of 0.25 mol L^{-1} lead acetate in the Vacutainer. Concentrations of H_2, CH_4 and CO were determined using a SENTE methane gas analyzer (model GS-19S, Sente Inc., USA) under isothermal conditions at 54°C. CO_2 concentration was determined using a Trace GC C-IRMS (Finnigan MAT GmbH, Bremen, Germany) equipped with a Carboxen 1010 plot column (Supelco, Bellefonte, PA, USA) under isothermal condition at 100°C.

DNA Extraction, *amoA* Gene Amplification and Cloning Libraries

DNA was extracted from sediment and mat/steamer materials using the MoBio PowerSoil™ DNA extraction kit (MoBio Laboratories, Carlsbad, CA, USA). Putative archaeal *amoA* genes were amplified by PCR with a primer set Arch-*amoA*F (5′-STAATGGTCTGGCTTAGACG) and Arch-*amoA*R (5′-GCGGCCATCCATCTGTATGT) using previously published conditions (Francis et al. 2005). Presence of the bacterial *amoA* genes was also tested using the bacterial primer set *amoA*_1F and *amoA*_2R (Rotthauwe et al. 1997); however, none of the samples contained detectable bacterial *amoA* genes after PCR amplification.

Triplicate archaeal *amoA* gene PCR products were pooled and purified using QIAquick® gel extraction kit (QIAGEN, Valencia, CA, USA), followed by cloning and transformation using the TOPO TA Cloning® kit (Invitrogen Inc., Carlsbad, CA, USA) with kanamycin as the selection agent. Clones with positive inserts were re-grown in secondary agar plates and transferred to Luria-Bertani (LB) freezing media containing 50 μg/ml kanamycin in 96-well-plates. The plates were then placed in an oven of 37°C overnight and then frozen at −80°C until sequencing.

Sequencing, Phylogenetic and Statistical Analyses

From each clone library, 40 to 60 randomly selected clones were sequenced using either Arch-*amoA*F or Arch-*amoA*R primers on an ABI3730xl capillary sequencer in the Laboratory for Genomics and Bioinformatics at the University of Georgia. Chimeric sequences were excluded by manual BLAST examination following Bellerophon screening (http://foo.maths.uq.edu.au/∼huber/bellerophon.pl). Deduced amino acid sequences were aligned using ClustalX. A total of 323 archaeal *amoA* sequences containing 176 aligned positions were chosen for phylogenetic analysis using MEGA3.1 (Kumar et al. 2004). DOTUR software was used for calculating rarefaction, Chao1 phylotype richness estimators and Shannon's diversity index (Schloss and Handelsman 2005). A threshold of 5%, representing *ca.* 9 amino acid substitutions was used to define the operational taxonomic unit (OTU) due to the high diversity of AOA in the hot springs studied. S-LIBSHUFF was used to compare the similarity between clone libraries (Schloss et al. 2004). The Jones, Taylor and Thornton (JTT) substitution

method was used for calculating distance matrices using applicable programs.

Quantitative PCR

For each sample, 5 to 10 ng of extracted DNA templates were added with iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) in triplicate. Universal primers A364aF (5′-CGGGGYGCASCAGGCGCGAA)/A934b (5′-GTGCTCCCCCGCCAATTCCT; Kemnitz et al. 2005) and Cren-28F (5′-AATCCGGTTGATCCTGCCGGACC)/Cren-457R (5′-TTGCCCCCCGCTTATTCSCCCG; Schleper et al. 1997) were used to quantify the copy numbers of 16S rRNA genes of total archaea and crenarchaeota, respectively. Quantitative PCR was performed using an iCycler system (Bio-Rad, Hercules, CA, USA) using the following program: initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 50 s. Calibration of samples was performed using known copies of *Sulfolobus acidocaldarius* (ATCC 33909) 16S rRNA gene (10^2 and 10^7 copies), amplified with the universal primer set 7F (5′-TTCCGGTTGATCCYGCCGGA) and 1492R (5′-TACGGYTACCTTGTTACGACTT). Data are reported using gene copies per nanogram of DNA. Q-PCR of archaeal- and bacterial *amoA* genes were performed using primers and protocols as previously described (Wutcher et al. 2006).

Lipid Extraction and LC-MS Analysis

Freeze-dried samples (∼5 g) were extracted using the modified Bligh and Dyer method (White et al. 1979) for total lipid extracts (TLE). TLE were evaporated under high-purity nitrogen gas and subsequently hydrolyzed in 5% HCl in methanol (transesterification) for 2 h at 70°C to cleave polar head-groups. The transesterified products were extracted using CH_2Cl_2, dried under nitrogen gas, and loaded on to a C18 Bond-Elut® solid phase extraction (SPE) column (Varian Inc., Palo Alto, CA, USA). Three fractions were sequentially eluted using 1:1 acetonitrile: ethyl acetate for the polar fraction, 1:3 ethyl acetate: hexane for the GDGD fraction, and 1:10 ethyl acetate: hexane for the nonpolar fraction. The GDGT fractions were dried under pure nitrogen gas and dissolved in 1.3% isopropanol in hexane for analysis by HPLC-MS (Pearson et al. 2004). Intact GDGTs were identified with an Agilent 1100 series high performance liquid chromatograph (HPLC)/atmospheric pressure chemical ionization-MS using a Zorbax NH_2 (4.6 by 250 mm, 5 μm) column. GDGTs were eluted isocratically in 1.3% isopropanol in hexane at 30°C. Spectra were scanned over the m/z range from 1,000 to 1,350.

Nucleotide Sequence Accession Numbers

Sequence data have been deposited in GenBank (accession numbers EF654133-EF654511).

RESULTS

Physicochemical characteristics of the hot springs

The geochemical conditions varied considerably among the three hot springs (Table 1). The pH of Thermophile spring increased from 6.0 at the source (70°C) to 7.0 in the outflow

TABLE 1
Physicochemistry of sampling locations at Burlyashi (BLS), Thermophile (TM), and Cascadnaya (CAC) springs; suffix of spring name represents different sampling locations in the spring or outflow channel

	BLS-A	BLS-B	BLS-C	BLS-D	TM-A	TM-B	TM-C	TM-D	CAC-S	CAC-A	CAC-B	CAC-C	CAC-D
Sample description	S/Md	S/Md	S/Md	Md	S, St	Mt, St	Mt	Mt	Md	Md	St	Mt	Mt
T°C	87	70	58	51	70	64	53	42	74	85	71	59	50
pH25°C	6.0	6.5	6.5	6.0	6.0	6.5	6.5	7.0	6.0	6.0	6.5	6.0	5.5
TDS[a] (mg L^{-1})	730	461	586	733	493	499	499	497	NA[d]	1471	1332	1242	904
ORP[b] (mV)	−305	−153	−163	−170	−301	−365	−336	−288	NA	−323	−141	−62	−271
Alk [c] (mmol $L^{-1}CaCO_3$)	1.18	1.20	1.23	1.20	2.65	3.30	3.20	3.25	1.10	0.90	0.75	0.65	0.50
SO_4^{2-} (mmol L^{-1})	2.3	2.2	2.2	2.2	0.1	0.1	0.3	0.3	0.7	1.1	1.8	1.4	1.9
S^{2-} (μmol L^{-1})	43.8	BD[e]	6.3	BD	43.1	1.3	0.6	0.6	12.5	5.6	5.0	4.1	1.6
NO_2^- (μmol L^{-1}N)	0.1	0.1	0.1	0.3	0.6	0.3	0.2	0.4	0.4	1.4	1.1	0.3	0.4
NH_4^+ (mmol L^{-1} N)	1.1	1.5	1.5	1.5	0.3	0.3	4.0	0.2	1.5	2.2	2.4	2.7	2.7
Vent Gases:													
H_2 (μmol L^{-1})	45				89					89			
CH_4 (mmol L^{-1})	1.0				0.18					0.63			
CO_2 (mmol L^{-1})	26				16					32			
CO (μmol L^{-1})	6.3				1.7					BD			

Abbreviations used in table for sample descriptions: S = sand, Md = mud, Mt = mat, and St = streamer.
[a]Total dissolved solids.
[b]Oxidation-reduction potential.
[c]Alkalinity.
[d]Not applicable.
[e]Below detection limit.

TABLE 2
Statistics of *amoA* gene clone libraries

	BLS-D	TM-A	TM-B	TM-D	CAC-S	CAC-A	CAC-B	CAC-D
Temperature (°C)	51	70	64	42	74	85	71	50
pH	6	6	6.5	7	6	6	6.5	5.5
No. of clones	40	37	43	40	44	44	35	40
No. of singletons[a]	3	1	3	4	2	1	1	1
No. of total OTUs (N_{OTU})	7	4	11	15	12	10	6	10
N_{OTU} (N_{clone})								
Cluster1	6(35)	3(34)	1(17)	1(7)	1(11)	1(14)	4(31)	1(11)
Cluster2	1(5)	1(3)	2(2)	2(2)	1(2)	1(1)	2(4)	2(2)
Cluster3	0	0	8(24)	12(31)	10(31)	8(29)	0	7(27)
Chao1 estimator[a]	7.5	4.0	14.0	24.0	15.0	15.0	5.5	25.0
Shannon's index H'[a]	0.91	0.90	1.93	2.25	2.01	1.79	0.76	1.84

[a]Singletons were defined as clones phylogenetically distinct from other clones in the same sample. Chao1 phylotype richness estimator and Shannon's diversity index H' were calculated using individual numbers of clones and OTUs from each sample.

channel (42°C). This spring had relatively low TDS (∼490 mg L^{-1}), high alkalinity (>2.6 mmol L^{-1} $CaCO_3$), low sulfate (≤0.3 mmol L^{-1}), relatively high nitrite (0.6 μmol L^{-1} N) and relatively low ammonium (0.2 mmol L^{-1} N) except for TM-C (Table 1). Sulfide concentration was high (43.1 μmol L^{-1}) at the source but dropped sharply to 0.6 μmol L^{-1} in the outflow channel (TM-C and TM-D; Table 1). The pH of Cascadnaya spring varied from 6.5 at the source (74°C) to 5.5 (50°C) in the distal outflow channel. TDS (>900 mg L^{-1}), sulfate (≥0.7 mmol L^{-1}), and ammonium (≥1.5 mmol L^{-1} N) were higher than those at the source of Thermophile spring, while sulfide was lower (12.5 μmol L^{-1}) (Table 1).

The Burlyashi spring at the source had pH and temperature similar to those at Cascadnaya, and overall had intermediate values for TDS (461-733 mg L^{-1}), alkalinity (∼1.2 mmol L^{-1} $CaCO_3$) and ammonium (1.1–1.5 mmol L^{-1} N) compared with the other two springs. Sulfide concentration was high at the source (43.8 μmol L^{-1}), but low (6.3 μmol L^{-1}) to non-detectable in the outflow channel (Table 1). The ORP for all the springs varied between -365 mV and -62 mV (Table 1). Gas compositions differed greatly among the three pools. Carbon dioxide was predominant at all source sites (16-32 mmol L^{-1}), followed by CH_4 (< 1.0 mmole L^{-1}) and H_2 (< 89 μmole L^{-1}), while CO ranged from below detection limit to 6.3 μmole L^{-1} (Table 1). Considered collectively, variations in gas and water chemistry were generally greater between springs than within a single spring.

Diversity and Distribution of the Archaeal *amoA* Genes

Archaeal *amoA* genes were detected in eight out of the thirteen sampling sites in the three springs. At Burlyashi, only sample BLS-D (T = 51°C, pH = 6.0) contained the archaeal *amoA* gene (Table 2), while at the Thermophile spring the archaeal *amoA* gene was detected at 3 locations (-A, -B, -D) and at Cascadnaya spring it was detected at four locations (-S, -A, -B, -D). Bacterial *amoA* gene was not detected in any of the thirteen samples while it was always detected in a positive control DNA extracted from *Nitrosomonas europaea*. The results suggest that AOB did not exist or it may be undetectable using the primers selected for this study.

A total of 323 archaeal *amoA* gene fragments were obtained from the thirteen sites in this study (Table 2). Deduced amino acid sequences were grouped into 34 OTUs based on 95% similarities. They formed three distinct clusters (Figure 1). Cluster 1 contained 9 OTUs (26.5% of total OTUs) with 160 sequences (49.5% of the total sequences). Closely related sequences in this cluster included characteristic crenarchaeotal group 1.1a sequences, e.g., from "*Candidatus* Nitrosopumilus maritimus" and *Cenarchaeum symbiosum*.

Cluster 2 had five OTUs (14.7% of total OTUs) with only 21 sequences (6.5% of total sequences). Sequences in Cluster 2 were closely related to non-thermophilic soil and sediment sequences with 95-98% similarities and they were distantly related to the fosmid clone 54d9 (88-92% homologous), suggesting that Cluster 2 mainly represents previously described crenarchaeotal group 1.1b (Schleper *et al*., 2005). Notably, the recently reported sequence from thermophilic "*Candidatus* Nitrosocaldus yellowstonii" is also included in this group but it was less than 80% homologous with Kamchatka sequences (Figure 1).

Cluster 3 contained 20 OTUs (58.8% of total OTUs) with 142 sequences (44% of total sequences). This cluster had two major clades that were about 91% similar. One clade contained a single OTU CAC-SF-G11, which was closely related to sequences from acidic fertilized soil (He et al. 2007). The other clade contained 124 sequences in 19 OTUs, and was closely related to sequences from either fertilized soil (He et al. 2007) or estuary sediment (Francis et al. 2005).

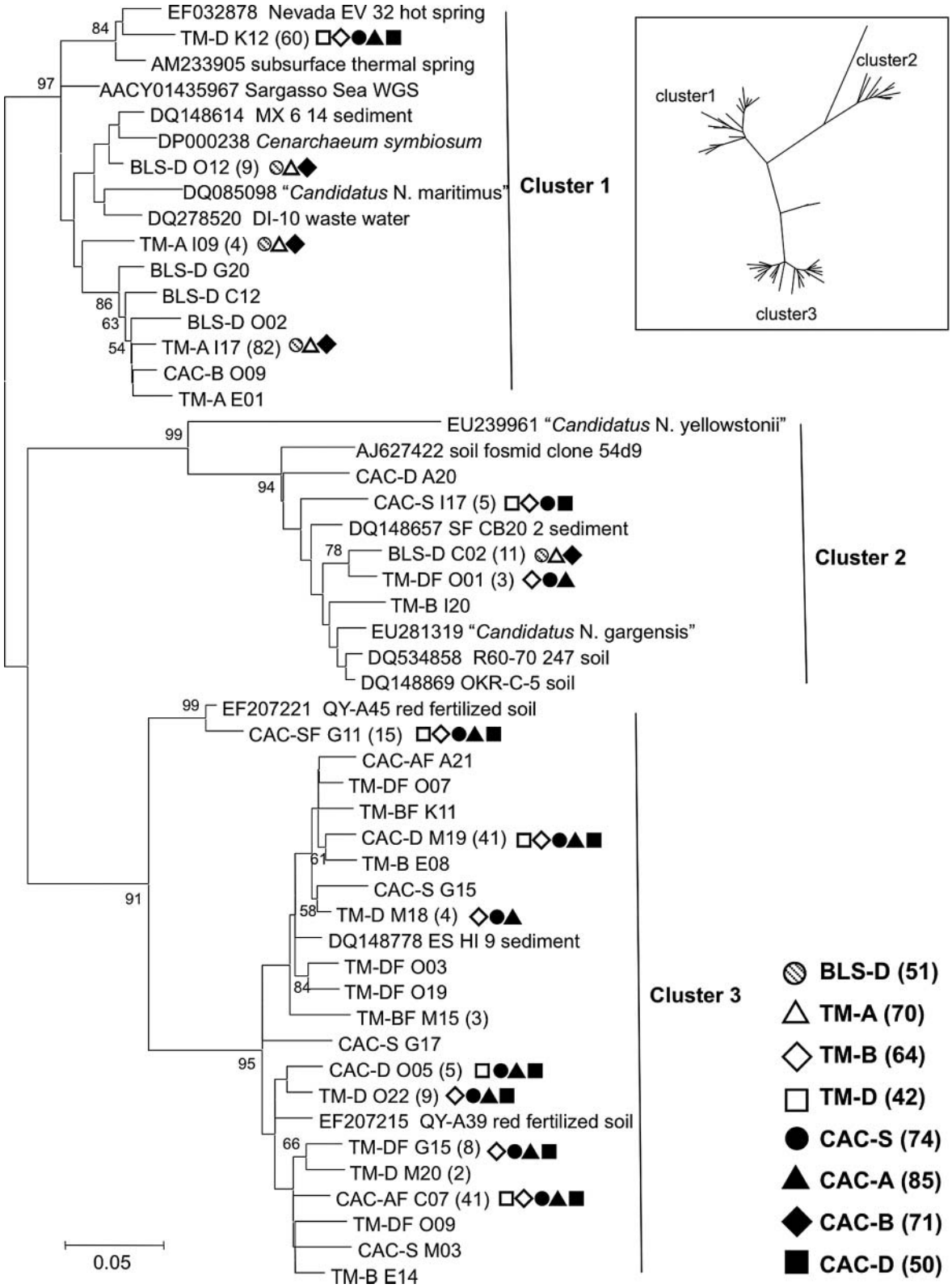

FIG. 1. Deduced amino acid trees of archaeal *amoA* gene obtained from hot springs in the Uzon Caldera. Only representative OTUs (<95% sequence identity) are shown. Neighbor-joining distance was calculated using the Jones, Taylor and Thornton (JTT) substitution method, and greater than 50% support in bootstrapping values from 1000 replicates are shown at the major nodes. A radiation tree illustrating the three clusters is also shown on the top right. Maximum Parsimony (1000 replicates) and Minimum Evolution (1000 replicates) were also computed and showed nearly identical tree structures. Numbers of clones greater than 1 within each OTU are shown in parentheses. Symbols indicate sample sources (Table 1). *In situ* temperatures in °C are shown in parentheses after sample name in legend.

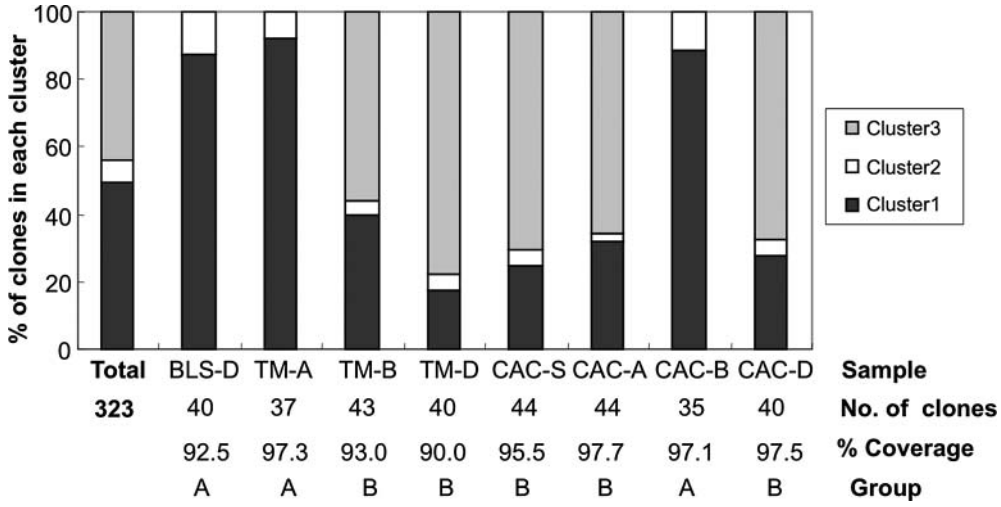

FIG. 2. Relative distribution of *amoA* gene clones in the three phylogenetic clusters. Numbers of clones sequenced and Good's estimator of coverage for each sample are shown at the bottom panel. Good's coverage was calculated based on formula (1-n/N)*100, where n is the number of singletons and N is the total number of clones in that sample.

Good's sampling coverage estimator (Kemp and Aller 2004), nonparametric Chao1 estimator of species (OTUs) richness, and Shannon's diversity index H' were evaluated using the numbers of sequences and OTUs in each clone library (Table 2, Figure 2). Good's sampling coverage estimated 90 to 97% coverage (Figure 2). At Thermophile, AOA phylotype richness (Chao 1) and diversity (Shannon) increased with decreasing temperature from source to the outflow channel (Table 2).

In contrast, AOA Shannon's index at Cascadnaya was similar (1.8–2.0) at 3 of the 4 locations (S, A and D), while Chao1 richness estimators were intermediate at locations S and A, low at B and highest at D. Most samples from the three hot springs appeared to positively co-vary between Chao1 richness estimator and the Shannon's index, suggesting that the increase in diversity corresponded to an increase in richness of AOA at those sites.

Comparison of AOA community structures using S-LIBSHUFF separated samples (clone libraries) into two groups: Group A comprising BLS-D, CAC-B and TM-A and Group B containing the remaining samples (Table 3). A detailed examination of the Group A sequences showed they did not contain any sequences from Cluster 3 of the *amoA* gene clone library, whereas Group B contained sequences from all three clusters (Figure 2). In addition, all Group A samples were dominated by Cluster 1 sequences (Figure 2). Also they had lower values for both Chao1 estimator and Shannon's diversity index H' than

TABLE 3
S-LIBSHUFF comparison of *amoA* gene clone libraries

		Y							
	—	BLS-D[a]	TM-A[a]	TM-B[b]	TM-D[b]	CAC-S[b]	CAC-A[b]	CAC-B[a]	CAC-D[b]
	BLS-D[a]	—	0.3011	*0.0000*	*0.0000*	*0.0000*	*0.0000*	0.5575	*0.00001*
	TM-A[a]	0.9919	—	*0.0000*	*0.0000*	*0.0000*	*0.0000*	0.4229	*0.0000*
	TM-B[b]	*0.0000*	*0.0000*	—	0.0685	0.0011	0.0021	*0.0000*	0.0103
X	TM-D[b]	*0.0000*	*0.0000*	0.0540	—	0.2852	0.8383	*0.0000*	0.7860
	CAC-S[b]	*0.0000*	*0.0000*	0.0610	0.4449	—	0.3464	*0.0000*	0.2854
	CAC-A[b]	*0.0000*	*0.0000*	0.1713	0.7617	0.5593	—	*0.0000*	0.6880
	CAC-B[a]	0.4175	0.4133	*0.0000*	*0.0000*	*0.0000*	*0.0000*	—	*0.0000*
	CAC-D[b]	*0.0000*	*0.0000*	0.0567	0.5354	0.1030	0.6665	*0.0000*	—

Values in the table represent P-values for ΔCXY of homologous library X and heterologous library Y (below the diagonal), and ΔCYX of homologous library Y and heterologous library X (above the diagonal). Minimum P-value is 0.0009 and 0.0000 for family-wise and Monte Carlo Error at 95% margin of error. Significant P values for 56 comparisons are italicized. Two libraries are distinct if both ΔCXY and ΔCYX are statistically significant. [a]Group A samples. [b]Group B samples.

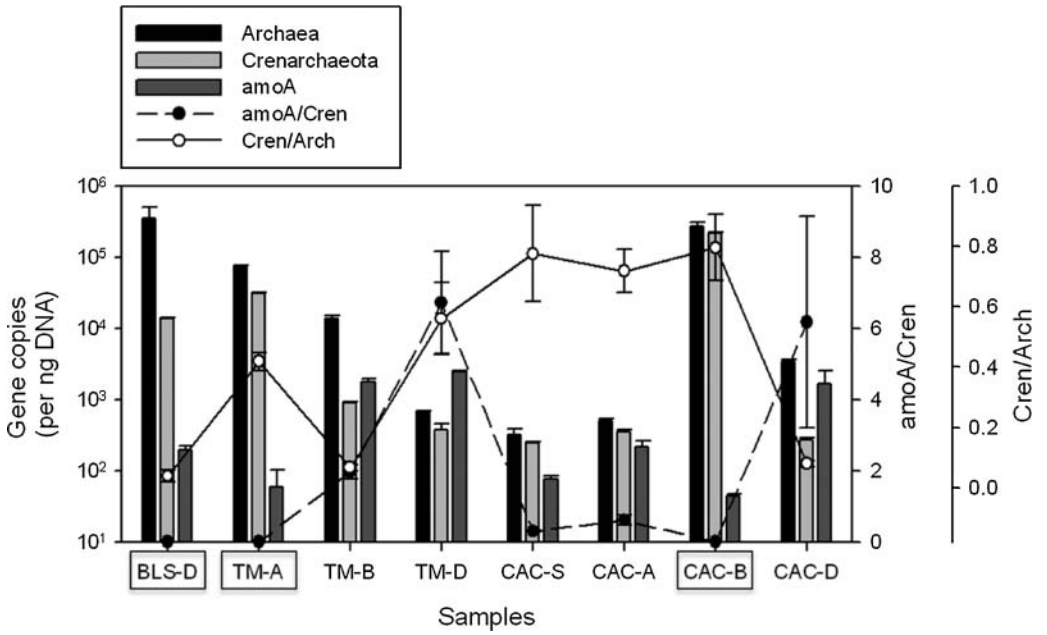

FIG. 3. Abundances of total archaea, crenarchaeota and AOA in each sample determined by quantitative PCR of corresponding genes. Total archaea and crenarchaeota were based on the 16S rRNA gene, and AOA was based on the *amoA* gene. The solid and dashed lines illustrate the abundance ratio of 16S rRNA genes of crenarchaeota to archaea and ratio of *amoA* gene to crenarchaeotal 16S rRNA gene, respectively. Names of Group A samples are within squares.

Group B samples, indicating lower AOA-phylotype richness and diversity.

Quantitative PCR

Relative abundance of 16S rRNA genes varied by 3 orders of magnitude among the eight samples (10^2-10^5 copies/ng template DNA) (Figure 3). Group A samples (BLS-D, TM-A and CAC-B) had more abundant archaeal and crenarchaeotal 16S rRNA genes ($>10^4$ copies/ng DNA) than group B samples ($<1.3\times10^4$copies/ng DNA). The percentage of crenarchaeota in the total archaeal pool also varied dramatically from 4% to 80% and did not correlate with the total archaea (Figure 3). Copies of *amoA* gene varied greatly from less than 10^2 to $\sim3 \times 10^3$copies/ng DNA and did not co-vary with either archaeal or crenarchaeal 16S rRNA gene copies (Figure 3).

However, there appeared to be an inverse correlation between the *amoA* Shannon's index and abundances of both archaeal ($r^2 = 0.81$) and crenarchaeotal ($r^2 = 0.88$) 16S rRNA gene copies (Figure 4), indicating that a decrease in *amoA* gene diversity corresponded with an increase in total archaeal or crenarchaeotal abundance.

Glycerol Dialkyl Glycerol Tetraethers (GDGTs)

GDGTs were identified in 8 samples from the 3 hot springs (Figure 5a). GDGT-0 (acyclic caldarchaeol) was predominant (48–55%) in all samples from Thermophile spring (-A, -B, -D). GDGTs 1–4 had similar relative abundances in these samples, with each contributing 7–16% to the total GDGTs. Samples from Cascadnaya spring (-S, -A, -B, -D) contained variable GDGT-0 (1–45%) and elevated GDGT-4 up to 35% (CAC-A). At Burlyashi spring, GDGTs 2–4 had similar abundances and counted for 76% of total GDGTs; whereas GDGT-0 and GDGT-1 had lower abundances and together counted for 20% of total GDGTs. Other GDGT components including GDGT-5, -6, -7 and crenarchaeol were all less than 5% in each sample (Figure 5a).

Cluster analysis of the GDGT compositions revealed spatial separation of archaeal communities among the springs (Figure 5b). Samples from a single spring tended to be similar except

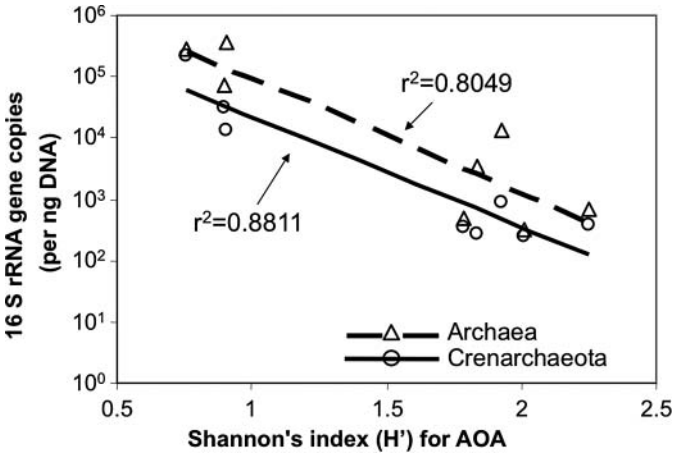

FIG. 4. Correlation between the Shannon's diversity index H' of AOA phylotypes and 16S rRNA gene copies of total archaea and crenarchaeota.

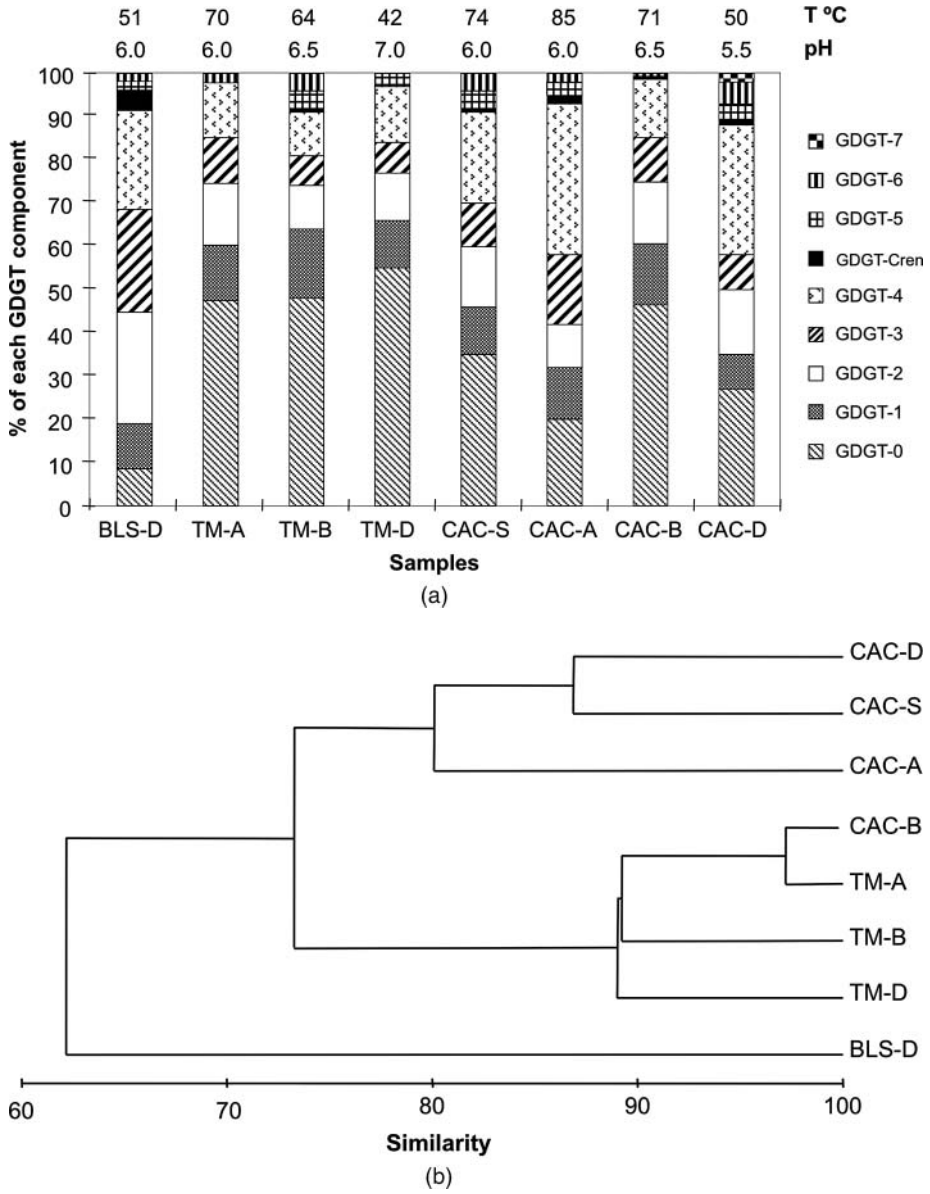

FIG. 5. a) Normalized GDGT compositions of 8 bulk environmental samples. GDGT compounds are identified as: GDGT-0, m/z1,302, no rings; GDGT-1, m/z1,300, one cyclopentyl ring; GDGT-2, m/z1,298, 2 cyclopentyl rings; GDGT-3, m/z1,296, 3 cyclopentyl rings; GDGT-4, m/z1,294, 4 cyclopentyl rings; GDGT-Cren, crenarchaeol, m/z1,292, 4 cyclopentyl rings, 1 cyclohexyl ring; GDGT-5, m/z1,292, crenarchaeol regioisomer, 5 cyclopentyl rings; GDGT-6, m/z1,290, 6 cyclopentyl ring; GDGT-7, m/z 1288, 7 cyclopentyl ring (Schouten et al. 2002). b) Cluster analysis of the GDGT composition. Percentage of GDGT components was transformed [log(x+1)] before the Bury-Curtis similarity was computed between samples with nine GDGT components using PRIMER5 (Clarke 1993).

for CAC-B, which was more similar to TM-A at Thermophile spring. This high similarity in GDGT profiles between CAC-B and TM-A was reflected in the similarities of their AOA community structure (Table 3) and abundance of total archaea (Figure 3). BLS-D, although grouped with CAC-B and TM-A in *amoA* gene (Table 3), was distinct in GDGT from CAC and TM samples (Figure 5b).

To examine if any relationships existed between temperature, pH and membrane lipid characteristics, a ring index (RI) was calculated for each GDGT cluster using the equation

$$RI = \sum_{i=1} (n_i \times C_i) / \sum_{i=1} C_i$$

where n_i is the number of rings in each GDGT molecule and C_i is the percentage of each GDGT peak area over the total GDGT area. Average ring indices were 1.4 ± 0.1 (n = 4), 2.4 ± 0.3 (n = 3) and 2.4 ± 0.2 (n = 3) for the Thermophile (including

CAC-B), Cascadnaya and Burlyashi springs, respectively. No correlations were found between RI and pH or temperature, indicating that other chemical, physiological, or genetic conditions may influence ring structure of the archaeal lipids. Furthermore, the low abundance or lack of crenarchaeol biomarker in some of the samples indicated that not all of the AOA produced this biomarker. As such, it may not be appropriate to use this biomarker for evidence of archaeal ammonia oxidation in certain environments.

DISCUSSION

Occurrence of AOA in Kamchatka Hot Springs

Hot springs in Kamchatka appear to contain very different microbial communities from lower temperature soil and marine sediments, where, for instance, AOB usually co-occur with AOA. This result is consistent with a recent study of high temperature hot springs in Iceland and Kamchatka (Reigstad et al. 2008). In addition, a monophylotype of AOA "*Candidatus* Nitrososphaera gargensis" was found in a moderately thermophilic enrichment culture (46°C) of microbial mats collected from the Siberian Garga hot spring, in which no AOB were observed (Hatzenpichler et al. 2008) even though a *Nitrosospira*-like bacterial species was originally reported from the same enrichment culture (Lebedeva et al. 2005). It is suggested that elevated temperatures (>40°C) do not favor AOB. This is consistent with the observation that hot spring AOB enrichments at 40–55°C are difficult to maintain (Golovacheva 1976; Lebedeva et al. 2005).

In contrast, AOA occur in a wide temperature range (Nicol and Schleper 2006; Francis et al. 2007; de la Torre et al. 2008; Hatzenpichler et al. 2008; Zhang et al. 2008), and a wide pH range such as acidic or alkaline soils (Leininger et al. 2006; He et al. 2007; Shen et al. 2008), and geothermal springs of different pHs (Weidler et al. 2007; Reigstad et al. 2008; Zhang et al. 2008). This study shows that AOA in Kamchatka hot springs occur over a range of 42°C (e.g., TM-D) to 85°C (e.g., CAC-A; Figure 1) and neutral to acidic pH. Some of the sequences from Cluster 3 (e.g., CAC-AF A07, CAC-AF A15, and CAC-AF A17) are closely related to those from acidic soil environments in China (QY-A39, QY-A45), which is consistent with the predominance of relatively acidic chemistry of the hot springs.

Distribution and Diversity of AOA

Physicochemical factors including pH, TDS, and ammonium concentration usually show weak and inconsistent relationships to AOA diversity and distribution (Francis et al. 2005; Wuchter et al. 2006; He et al. 2007; Urakawa et al. 2008). Kamchatka samples also show such inconsistency (Table 1). For example, many major OTUs of AOA are frequently present at multiple locations of the hot springs that have great variations in temperature and chemistry.

Similar to the distribution of AOA, diversity index and phylotype richness estimator of AOA do not vary consistently with temperature or water chemistry. Chao1 richness estimator on the other hand, is consistently the highest at the lowest temperature locations in both Thermophile and Cascadnaya springs, suggesting that AOA in Kamchakta hot springs may favorably adapt to the moderately high temperature environment. On the other hand, it has been demonstrated that lower temperature decreases the phylogenetic diversity of AOA in <19°C aquarium systems (Urakawa et al. 2008).

Patterns of AOA diversity and richness are different between the two sample groups. Group A, which contained BLS-D, TM-A and CAC-B, had a lower AOA diversity and Chao1 phylotype richness than Group B samples (Table 2). Q-PCR showed that the abundance of total archaea and crenarchaeota are one order of magnitude higher in Group A than Group B, and they inversely correlate with the AOA Shannon's diversity indices (Figure 4). TM-A and CAC-B in Group A are also dominated by samples containing streamers (TM-A, TM-B, CAC-B; Table 1), whereas Group B samples are mostly dark green mats at lower temperature locations and black mud at higher temperature locations. Moreover, the GDGT patterns showed that the two streamer-containing samples, CAC-B and TM-A (Table 1), had the highest similarity in lipid structure (Figure 5). These results suggest that the low diversity AOA community in Group A samples may preferably coexist with high abundance crenarchaeota in the streamer-containing samples.

Abundance of AOA

Based on grouping from the S-LIBSHUFF analysis of *amoA* gene clone libraries (Table 3), archaea are more abundant in group A samples than group B samples. Except for CAC-B, however, the ratios of 16S rRNA genes for crenarchaeota/archaea were less than 10%. This also results in low abundance of AOA given the lower ratios of *amoA* gene copies/crenarchaeotal 16S rRNA gene copies in most samples (Figure 3).

Archaeal core membrane lipid GDGT has also been used to estimate the AOA contribution to total archaea. Leininger et al. (2006) showed that both total GDGTs and crenarchaeol correlated with *amoA* gene copies in soil. They suggested that AOA constituted a major proportion of crenarchaeota in their samples and that the crenarchaeol they detected originated from AOA. de la Torre et al. (2008) proposed that crenarchaeol may be a characteristic biomarker for AOA because crenarchaeol was abundant in the ammonia-oxidizing archaea "*Candidatus* Nitrosopumilus maritimus" and "*Candidatus* Nitrosocaldus yellowstonii" (Könneke et al. 2005; de la Torre et al. 2008).

Our data suggest that both crenarchaeol and AOA abundance (estimated using qPCR) are relatively low in all our samples. This is consistent with estimates of AOA abundance in hot springs of Iceland and Kamchatka (Reigstad et al. 2008). However, the relatively low pH (5–7) of the sampling locations may

also depress crenarchaeol production because low pH may inhibit the synthesis of crenarchaeol (Macalady et al. 2004; Pearson et al. 2004; Zhang et al. 2006; Schouten et al. 2007).

It is well established that relatively high crenarchaeol abundance occurs in alkaline environments over a wide range of temperatures from cold marine sediments to hot springs (DeLong et al. 1998; Schouten et al. 2000; Pearson et al. 2004; Könneke et al. 2005; Weidler et al. 2007; de la Torre et al. 2008; Pearson et al. 2008). Therefore, it is unclear whether the relatively low abundance of AOA in our samples is related to lower pH (<7). On the other hand, the archaeal ring index in our samples does not correlate with either temperature or pH, suggesting that other environmental and biological variables may affect archaeal community structure in Uzon hot springs.

In summary, diverse AOA, potentially including thermophilic members, are widespread in high temperature terrestrial hot springs of the Uzon Caldera in Kamchatka. They form three major phylogenetic clusters representing crenarchaeotal group 1.1a as Cluster 1, crenarchaeotal group 1.1b as Cluster 2, and a presumable acidic hot spring/soil clade as Cluster 3. Cluster 3 is particular abundant and diverse in Kamchatka hot springs. Two AOA groups with physiological similarities were identified: 1) Group A, which contains less diverse *amoA* sequences but high archaeal abundance, and 2) Group B, which comprises highly diverse *amoA* sequences and relatively low archaeal abundance.

The highest AOA phylotype richness occurred at the lower temperature end of both Thermophile and Cascadnaya springs, suggesting that moderately high temperature conditions are probably more favorable for AOA than hyperthermal (>75°C) conditions. Although AOA abundance is low in most samples, the wide occurrence of archaeal *amoA* genes in contrast to the absence of bacterial counterparts suggests that AOA play a primary role in nitrification in Kamchatka hot springs.

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation Microbial Observatory Program (to J.W., C.S.R., and C.L.Z.). We thank Dr. Ann Pearson and Yundan Pi (Harvard University) for assistance in performing HPLC analysis of GDGT, and Qi Ye for technical assistance in the construction of clone libraries.

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