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16S rRNA phylogenetic analysis and quantification of *Korarchaeota* indigenous to the hot springs of Kamchatka, Russia

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Abstract The candidate archaeal division *Korarchaeota* is known primarily from deeply branching sequences of 16S rRNA genes PCR-amplified from hydrothermal springs. Parallels between the phylogeny of these genes and the geographic locations where they were identified suggested that *Korarchaeota* exhibit a high level of endemism. In this study, the influence of geographic isolation and select environmental factors on the diversification of the *Korarchaeota* was investigated. Fourteen hot springs from three different regions of Kamchatka, Russia were screened by PCR using *Korarchaeota*-specific and general *Archaea* 16S rRNA gene-targeting primers, cloning, and sequencing. Phylogenetic analyses of these sequences with *Korarchaeota* 16S rRNA sequences previously identified from around the world suggested that all Kamchatka sequences cluster together in a unique clade that subdivides by region within the peninsula. Consistent with endemism, 16S rRNA gene group-specific quantitative PCR of all Kamchatka samples detected only the single clade of *Korarchaeota* that was found by the non-quantitative PCR screening. In addition, their genes were measured in only low numbers; small *Korarchaeota* populations would present fewer chances for dispersal to and colonization of other sites. Across the entire division of *Korarchaeota*, common geographic locations, temperatures, or salinities of identification sites united sequence clusters at different phylogenetic levels, suggesting varied roles of these factors in the diversification of *Korarchaeota*.

Keywords 16S rRNA gene · Abundance · Hydrothermal · Kamchatka · Korarchaeota · Phylogeny

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Introduction

Korarchaeota is a proposed archaeal division that has been characterized primarily by analysis of small subunit ribosomal RNA (16S rRNA) gene sequences detected exclusively in high temperature hydrothermal environments on continents, in shallow water, and in the deep ocean (Barns et al. 1996; Auchtung et al. 2006). *Korarchaeota* are of particular interest because of their deeply rooted position in phylogenetic analyses (Barns et al. 1996). 16S rRNA gene sequence analyses strongly suggest that they are a monophyletic group; however, their position relative to other *Archaea* is weakly supported. The only reported genome sequence of a *Korarchaeota* is from *Korarchaeum cryptofilum*, a putative peptide-fermentor that was enriched in 85°C, pH 6.5, low salinity anaerobic mixed culture (Elkins et al. 2008). *K. cryptofilum* was found to have a blend of crenarchaeal and euryarchaeal-like genes whose analyses support the deep-branching position of *Korarchaeota* in the archaeal lineage.

Previous phylogenetic analyses of *Korarchaeota* 16S rRNA genes indicated that the sequences divided into five groups (I–V), each of which, except for Group IV, were well supported by high bootstrap values (Auchtung et al. 2006). Groups I and II sequences clustered together with high bootstrap support; however, all other relationships between groups were largely unresolved. Sequences within each group were detected in the same general geographic location, suggesting that *Korarchaeota* may exhibit endemicity (groups existing exclusively in a given region), with sequences of Group I from Iceland, Groups II and III from Yellowstone National Park, Group IV from the Pacific Ocean, and Group V from the Indian Ocean and Aegean Sea (Auchtung et al. 2006).

Geography may be an important factor in the diversification of *Korarchaeota* groups, as demonstrated for *Sulfolobus* (Whitaker et al. 2003); however, environmental factors may also play a role. Hydrothermal fluid can have a wide temperature and pH range and come from either freshwater, seawater, or a mix of the two (Van Dover 2000; Fournier 2005). Terrestrial hot springs generally have a freshwater source that becomes hydrothermal fluid containing low concentrations (~0.1%) of dissolved ions (i.e., salinity), whereas marine hydrothermal vents typically have seawater sources with salinities of ~3.5% (mostly Na^+ and Cl^-), much of which remains in the vent fluid. The fluid of submarine and terrestrial springs that are located near the coast of a marine body of water may originate from a mix of the two sources (e.g., Takai and Sako 1999). In analyses of the cyanobacteria *Synechococcus* in North American hot springs, the authors found that phylogenetic divergence in the A/B lineage is correlated with temperature, and within thermal ecotypes, with geography (Papke et al. 2003; Allewalt et al. 2006). For the bacterial division *Aquificales*, geography correlates with phylogenetic divergence at the subgeneric level, but pH and/or salinity appear to have had a greater role in the diversification of higher phylogenetic groupings (Huber and Eder 2006; Ferrera et al. 2007; Takacs-Vesbach et al. 2008).

Here, the phylogeny of *Korarchaeota* indigenous to the hot springs of Kamchatka, Russia was investigated by examining 16S rRNA genes from multiple sites across the peninsula, and using quantitative PCR to assess whether PCR-amplified *Korarchaeota* genes represented the only phylogenetic groups present. Few studies have examined microbial diversity within Kamchatka (Hohn et al. 2002; Whitaker et al. 2003; Reigstad et al. 2010), and prior to the completion of this investigation, *Korarchaeota* 16S rRNA genes had not yet been reported from this location. *Korarchaeota* 16S rRNA gene sequences PCR-amplified from Kamchatka and from previous studies obtained around the world were phylogenetically analyzed; inferred relationships were compared to the geography, temperature, pH, and water source of collection sites to investigate factors potentially impacting *Korarchaeota* diversification.

Materials and methods

Sampling areas and collection

Kamchatka is a volcanically active peninsula in northeastern Russia (~50–62°N, 155–164°E). Multiple hot springs of three areas of Kamchatka were sampled in August 2005 for *Korarchaeota*. Filament, sediment, mud, powder, mat, and/or sinter samples were collected from two areas that are eight km apart, Valley of the Geysers (54.5°N, 160.1°E; three sites) and Uzon Caldera (54.5°N, 160.0°E; seven samples from six sites), and a third area 260 km away, Mutnovsky Volcano (Dachnye springs; 52.5°N, 158.2°E; five sites) (Table 1). Sites were between 1 m and 1 km apart within the areas, and ranged in temperature from 52 to 98°C, pH from 2.0 to 8.0, and salinity from 0 to 0.2%. Temperature and pH at the sampling sites were measured in the field using digital thermometers and pH meters. Fluid samples were taken for later analysis of salinity using an optical refractometer. Using sterile syringes or spatulas, samples (~1 ml) for microbial DNA extraction and microscopy were added to 1 ml of sucrose-lysis buffer (Gordon and Giovannoni 1996). Due to the remoteness of the sampled sites, tubes were stored as cold as possible (4–20°C) until frozen at −80°C after 7–9 days.

DNA extraction

DNA was extracted from Kamchatka samples using a bead-beating kit or a chemical/enzymatic lysis procedure. The DNA in ~100 mg of sediment, mud, and sinter samples was extracted using a Bio 101 FastDNA SPIN kit for soil according to manufacturer's instructions. The DNA in 100 µl of filaments, powder (on bottom of runoff channel), and microbial mats was extracted by the addition of 200 µl CTAB buffer (1% cetyltrimethylammonium bromide, 0.75 M NaCl, 50 mM Tris, 10 mM EDTA, pH 8.0), then according to Gordon and Giovannoni (1996), lysozyme, proteinase K, sodium dodecyl sulfate, phenol, and chloroform were added. Additionally, ribonuclease A was added to a concentration of 100 µg/ml when lysozyme was added. The DNA was ethanol-precipitated, resuspended in 1:100 TE, quantified by spectrophotometry (NanoDrop ND-1000; 4–540 ng/µl; Table 1), and stored at −80°C.

16S rRNA gene PCR and phylogenetic analyses

Korarchaeota 16S rRNA genes were PCR-amplified using combinations of four primers that were previously designed

Table 1 Kamchatka site and sample characteristics, concentrations of DNA extracted, *Korarchaeota* and *Archaea* detection summary, and Group I/II *Korarchaeota* 16S rRNA gene copies/estimated 10^6 organisms as determined by quantitative PCR

Site	Site characteristics[a]			Sample	DNA[b] (ng/μl)	PCR primer set[c]				Group I/II *Korarchaeota* 16S rRNA gene copies/10^6 organisms[d]
	Temp (°C)	pH	Salinity (%)			Kor236F Kor1236R	Kor236F Univ1492R	Arc26F Kor1236R	Arc26F Univ1492R	
Valley of the Geysers										
VG1	62	8.0	0.1	Orange/brown filaments	7	–	–	–	*11* (*2*)	nd
VG2	78	5.0	0.1	Tan powdery sediment	20	–	x	x	x	nd
VG3	98	7.0	n/a	Light brown mud	170	–	–	–	–	nd
Uzon Caldera										
Uzon4	52	3.0	0.2	Gray powder	70	–	–	–	*10* (*7*)	160 ± 15
Uzon5	78	5.5	0.1	White filaments	17	+	–	10 (9)	*12* (*2*)	220 ± 260
Uzon6	77	6.0	0.05	Black sediment with white filaments	200	–	–	–	*12* (*2*)	nd
Uzon7	80	5.5	0.05	Gray compact mat	50	–	–	–	*10* (*2*)	3 ± 4
Uzon8f	75	7.0	0.05	Mostly gray with some white filaments	140	2 (2)	–	+	*11* (*4*)	1,090 ± 460
Uzon8s	75	7.0	0.05	Black sediment	4	–	–	–	–	20 ± 30
Uzon9	55	7.0	0.1	Black filaments	50	2 (2)	10 (1)	+	2 (1) *10* (*5*)	290 ± 40
Mutnovsky Volcano										
Mut10	88	2.0	0.2	Gray mud	540	–	–	–	–	nd
Mut11	78	5.0	0.05	Black filaments with some sediment	120	1 (1)	1 (1)	+	*10* (*1*)	14 ± 18
Mut12	86	4.5	n/a	Brown sediment	210	–	–	–	–	nd
Mut13	75	6.3	0.0	White filaments	120	3 (3)	10 (1)	+	*11* (*6*)	7 ± 7
Mut14	74	6.0	0.1	Glowing peach sinter	230	–	–	–	*1* (*1*)	nd

[a] Temperature was measured in the field using digital thermometers, pH was measured in the field using pH meters (when possible) or in the laboratory using litmus paper, and except for the non-transparent samples (n/a), salinity was measured in the laboratory using an optical refractometer

[b] Only in Uzon4 were large, presumably high DNA content eukaryotic, cells observed

[c] Primers were developed previously (Auchtung et al. 2006). Number, *Korarchaeota* (not italicized) or non-korarchaeotal *Archaea* (italicized) clones, respectively, obtained as identified by BLAST after partial sequencing with M13F; (number), clones of each type that were completely sequenced; +, faint product detected on agarose gel but not cloned; −, product not detected; *x*, no amplification (even of positive controls)

[d] Assuming 2 Mb average genome size in the community and 100% DNA extraction efficiencies. *nd Korarchaeota* were not detected. The limit of detection for an individual reaction was 2–50 copies/estimated 10^6 organisms. Notation = number of copies ± standard deviation

(Auchtung et al. 2006) but also accurately target recently available sequences. Universal *Archaea* Arc26F or *Korarchaeota*-specific Kor236F was used with *Korarchaeota*-specific Kor1236R or universal *Archaea*/*Bacteria* Univ1492R (Table 1; *Escherichia coli* numbering for this and all subsequent primers and nt positions). To detect PCR inhibition or technical problems, a control replicate of every reaction was spiked with 10^5 copies of the positive control, the M13F-M13R PCR-amplified product of plasmid KorY38, containing nt 27–1,491 of a Group III *Korarchaeota* 16S rRNA gene (Auchtung et al. 2006). PCR conditions were as described previously (Auchtung et al. 2006), except with 400 nM primers and optimized Mg^{2+} concentrations (2.5, 3.5, and 4.0 mM for Kor236F-Kor1236R and Kor236F-Univ1492R, Arc26F-Kor1236R, and Arc26F-Univ1492R, respectively). Various amounts of each DNA sample were included as template. A portion of each completed reaction was visualized on a 1% agarose gel. If a relatively bright band of the appropriate size was present, it was gel-extracted using a QIAquick kit (Qiagen, Valencia, CA), and ligated into pDrive (Qiagen). However, if the band was faint, to increase the number of clones generated, the remaining volume of the reaction was used as template in four concurrent five-cycle PCRs that were pooled, dried, resuspended in TE, and the DNA gel-extracted and ligated. Ligation reactions were ethanol-precipitated, resuspended in water, and mixed with Invitrogen One Shot TOP10 electrocompetent cells in 0.1 cm electrode gap cuvettes (Bio-Rad, Hercules, CA). An electric current of 1.8 kV was applied for 5 ms, then 250 μL

SOC medium (Sambrook and Russell 2001) was added. Electroporated cells were incubated at 37°C for 2 h before plating onto selective agar.

Colonies were screened by PCR with primers M13F and M13R and run on 1% agarose gels to identify inserts of the correct size. Up to 12 inserts/sample were partially sequenced using M13F and initially classified using BLAST. Sequencing was performed with an ABI BigDye terminator v3.1 cycle sequencing kit and either an ABI 3700 or 3730xl Genetic Analyzer. Complete sequencing was accomplished using M13R, Arc907R (CCGCCAA TTCCTTTAAGTTT), Arc924F (GAAACTTAAAGG AATTGGC), Arc1086F (GCTCGTGCCGTGAGGTGTC), Arc1390R (GACGGGCGGTGTGTGCAA), SK213-1252F (CTACAATGGCTGGGACAGC), and the four primers used in PCR. Partial sequences >99% identical to others from a sample were not completely sequenced, since much of the diversity at this level is due to Taq errors (Acinas et al. 2005). All sequences were examined for chimeras using the program Mallard (Ashelford et al. 2005), and submitted to GenBank under accession numbers HQ395686–HQ395745.

The phylogeny of all Kamchatka *Archaea* 16S rRNA gene sequences (amplified with Arc26F-Univ1492R) was examined by compiling a 1,226 nt alignment in myRDP (Cole et al. 2009; nt 95–1,341) with all 32 full-length sequences (including one *Korarchaeota*) that were non-chimeric and <99% identical to others from each sample, plus additional public sequences from representatives of every major archaeal group. Alignments were exported to PAUP, where trees were constructed and bootstrap resampled (500 replicates) using maximum parsimony with 500 heuristic random-addition sequence searches, the tree bisection reconnection branch-swapping option, and treating gaps as new states (Swofford 2003).

To specifically examine the phylogeny of *Korarchaeota*, a previous alignment of 28 *Korarchaeota* 16S rRNA gene sequences (Auchtung et al. 2006) was updated in BioEdit (http://www.mbio.ncsu.edu/BioEdit/BioEdit.html) to include 21 Kamchatka *Korarchaeota* sequences from this study and 33 recent non-chimeric *Korarchaeota* sequences in GenBank that were identified by BLAST (as of 11 April 2009): a87R6, a87Y34, and a87R58 from 9°N EPR (Ehrhardt et al. 2007), CH1a42, CH2a50, CH1a4, OUTa12, OUTa24, OUTa13, F99a11, F99a19, F99a109, and F99a104 from the Juan de Fuca Ridge in the northeast Pacific Ocean (Schrenk 2005), Fhm3A25, Fhm3A35, Fhm2A91, Fhm2A74, and Fhm2A25 from the southern Mariana Trough south of Japan (unpublished), VulcPIw.226 from Vulcano Island, Italy (Rogers and Amend 2005), YNP_ObP_A42 from Yellowstone National Park (Meyer-Dombard et al. 2005), IAN1-1 from the Okinawa Trough southwest of Japan (Nakagawa et al. 2005), 179A5 and 179A8 from Guaymas Basin in the Gulf of California (Pagé et al. 2008), *Candidatus Korarchaeum cryptofilum* OPF8 from Yellowstone National Park (Elkins et al. 2008), SSW_L4_D06 from northwest Nevada (Costa et al. 2009), LHC1_L5_G08 from eastern central California (unpublished), 854e_Kor1 from the Kermadec Arc northeast of New Zealand (Stott et al. 2008), 1A-5 and 4135A1S3 from the Juan de Fuca Ridge (unpublished), pIta-vmat-44 from just off southwest Japan (Hirayama et al. 2007), and T065-G2, T065-C3, and T066-E7 from the Eastern Lau Spreading Center northeast of New Zealand (unpublished). OUTa16, OPPE037, and WB3D011 were identified as chimeras, and since not from unique sites, were not included in analyses. T065-A6 (579 nt) was 100% identical to T065-G2 (605 nt) and therefore also not analyzed.

Since many *Korarchaeota* 16S rRNA gene sequences obtained from databases were not full length, in order to examine the interrelationships of all *Korarchaeota*, alignments of various lengths that included different subsets of sequences were analyzed (438 nt, 975–1,390, $n = 42$ sequences, 447 nt, 284–770 nt, 57 sequences; 532 nt, 873–1,377, $n = 42$ sequences; 622 nt, 342–975, $n = 56$ sequences; 635 nt, 119–770, $n = 50$ sequences; 745 nt, 669–1,377; 40 sequences; 912 nt, 361–1,235, $n = 47$ sequences; 946 nt, 29–975, $n = 42$ sequences; 1,016 nt, 237–1,235, $n = 44$ sequences; 1,073 nt, 361–1,390, $n = 39$ sequences; 1,361 nt, 29–1,390, $n = 30$ sequences). Since all Kamchatka sequences shared high identity, only three representative sequences (to which all others were ≥99.3% across 969 nt) were included in these analyses. Alignments were exported to PAUP, as described above. A comprehensive *Korarchaeota* phylogram was constructed by adding shorter sequences (italicized in figure) from their positions in trees generated from variable length sequence analyses to the phylogram generated from the longest length (1,361 nt) alignment. Bootstrap values from the shorter length analyses are not reported since tree topologies varied. Sequence identities between representatives of different groups were determined in BioEdit using the 1,361 nt alignment. For resolving the relationships among Kamchatka *Korarchaeota,* one alignment that included 21 Kamchatka sequences plus one Group I (Iceland) and five Group II (Yellowstone) *Korarchaeota* outgroups (969 nt, positions 237–1,235) was analyzed as described above.

Korarchaeota group-specific quantitative PCR

Primers were designed to target specific groups of *Korarchaeota* by visual comparison of an alignment of their 16S rRNA genes (Table 2). The IDT program PrimerQuest (http://scitools.idtdna.com/Primerquest/) was also used to identify candidate primers that had similar melting temperatures and limited secondary structure. Primers were

Table 2 Quantitative PCR primers

Target	Primers[a]	Sequence	Minimum mismatches to non-targets sequences (nt)[b]	Target sequences with perfect match	Annealing temp (°C)	Mg^{2+} (mM)[c]	Product melting temp (°C)[d]	Sensitivity (copies)
Group I/II	KorGI/II-518F	GGGCAAGGCTGGTGG	0	45/45	63	1.25 or 1.50	87.5	10^1 or 10^2
	KorGI/II-601R	GTCTACAGGCGGATTTAACTGC	3	45/45				
Group III	KorGIII-188F	GGATAGGGGTGGATTCCTGG	4[e]	7/7	55	1.50 or 1.90	89.0	10^1 or 10^2
	KorGIII-444R	CTACCCCCTGCTTTTTACACAG	5[f]	8/8				
Group IV	KorGIV-494F	GGGTGTAAACAGCCCGGGGT	2	31/33	60	1.50 or 1.60	88.5	10^1
	KorGIV-686R	CCGGGGATCTACGCATTTTACCGCTAT	2	26/29				

[a] Numbering corresponds to relative *E. coli* position of primer 3′ nt

[b] According to our alignments, and as of 11 April 2009, Ribosomal Database Project-II release 8.1 Probe Match and BLAST

[c] Optimal Mg^{2+} concentration varied, depending on SYBR Green mix

[d] Determined by melting curve following positive control QPCR

[e] Except Group IV *Korarchaeota*, to which it perfectly matched 23/27 sequences

[f] Except Group I/II *Korarchaeota*, to which it perfectly matched 9/14 sequences

Table 3 Primer efficiency in quantitative PCR of 10^6 copies of control templates

Primers	Copies detected from:					
	Korarchaeota Group I/II[a]	*Korarchaeota* Group III	*Korarchaeota* Group IV	*Korarchaeota* Group V	*Escherichia coli* Genomic DNA[b]	DNA from River Sediment
Group I/II	10^6 (KorY01)	$<10^3$ (KorY38)	$<10^2$ (Kor9NEPR)	$<10^2$ (Kor9NEPR)[c]	$<10^2$	$<10^2$
Group III	$<10^2$ (KorY01)	10^6 (KorY38)	$<10^2$ (pOWA54-short)	$<10^2$ (pCIRA-R-short)	$<10^2$	$<10^3$
Group IV	$<10^2$ (KorY01)	$<10^2$ (KorY38)	10^6 (Kor9NEPR)	Not determined	$<10^2$	$<10^2$

[a] *Korarchaeota* 16S rRNA gene controls were created by PCR of clones with primers M13F and M13R

[b] *E. coli* genomic and river sediment DNA were present in an amount equivalent to 10^6 genomes the size of *E. coli*

[c] For the Group I/II-specific reaction, Group V targets would not be expected to amplify more efficiently than Group IV targets such as Kor9NEPR because in the Group I/II primer regions, the Group V sequence contains the same mismatches as Group IV, plus more

chosen that contained multiple 3′ mismatches to other *Korarchaeota* groups (corroborated using BLAST and Ribosomal Database Project-II release 8.1 Probe Match (Cole et al. 2009). Because Group I and Group II sequences were so similar (>98% identical across 1,361 nt), we designed primers specific to the entire Group I/II clade. Group V *Korarchaeota* 16S rRNA genes were not quantified, as a positive control was not available. The specificity of each primer set was compared between target and non-target DNA controls (Table 3). The cycle threshold (C_T) of QPCR of 10^6 copies of each negative control was greater than or equal to that of 10^3 positive control.

DNA for positive and negative controls was generated by PCR of plasmids KorY01, KorY38, and Kor9NEPR (Auchtung et al. 2006), pOWA54-short (Group IV; construction described below), and pCIRA-R-short (Group V; construction described below) using primers M13F and M13R. The products were run on an agarose gel, excised, purified using a QIAquick Gel Extraction kit (Qiagen), eluted in TE, and spectrophotometrically quantified. To preserve the DNA, but prevent QPCR inhibition from EDTA, working stocks of controls were stored in 1:100 TE at −80°C. River sediment DNA (Auchtung et al. 2006) and *E. coli* genomic DNA served as additional negative controls. For Group III QPCR, where no Group IV or Group V clones with the relevant region were available to determine specificity, splice overlap extension (Horton et al. 1989) was used to produce DNA containing the necessary primer regions, albeit missing

some of the intervening sequence. Long primers were used to create DNA containing 50 nt from positions 177 to 209 and 50 nt from positions 404 to 496, but missing nt 210–403, of published clone sequences pOWA54 and pCIRA-R, respectively (Takai and Sako 1999; Takai et al. 2004). This DNA was subsequently cloned (as described above), creating pOWA54-short and pCIRA-R-short. The correct insert sequences were confirmed by sequencing.

QPCR was performed in an Opticon 2 thermal cycler, using white 200 µL thin-wall tubes and ultra clear caps (all MJ Research/Bio-Rad). The 25 µl reactions contained 1× SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, St. Louis, MO), 600 nM primers, 1.25–1.90 mM $MgCl_2$ (Table 2; concentrations depended on primer set and SYBR Green mix used), and sample and/or positive control. The cycling parameters were: 94°C, 2 min, 45 cycles of (94°C, 10 s; 55, 60, or 63°C, 10 s; 72°C, 20 s; 81, 82, or 84°C, 2 s; plate read), followed by a melting curve from 60 to 95°C. Melting temperatures of products were between 87.5 and 89.0°C, and each reaction could detect as low as 10 or 100 copies of target template (Table 2).

Group-specific QPCR was performed on each sample at least twice. One reaction contained 2.2 ng of environmental DNA [the amount in 10^6 microorganisms, assuming an average genome size of 2 Mb (the approximate size of sequenced hyperthermophiles' genomes); Liolios et al. 2006], and a second contained higher amounts of the DNA in an effort to detect *Korarchaeota* present at very low levels. Ten or 100 copies of positive control were added to replicates of every QPCR performed. The abundance of *Korarchaeota* 16S rRNA genes was standardized to the amount present per 2.2 ng DNA template ($\sim 10^6$ organisms). All *Korarchaeota*-positive samples were observed at ×100 and ×400 on a Zeiss Axioskop optical microscope to determine whether a high number of eukaryotes (with significantly >2 Mb genomes) were present that may cause an overestimation of the total number of organisms, and therefore an underestimate of the relative amount of *Korarchaeota*. Large, presumably eukaryotic, cells were only visualized in Uzon4. The detection limit of an individual reaction was 10 or 100 copies (for 2–50 copies/10^6 organisms), significantly more sensitive than the 10^5 copies/reaction standard of non-quantitative PCR. Amplification of intended targets was corroborated by analysis of amplicon melting temperatures, sizes, and from at least one reaction/sample, sequences.

Results

Kamchatka *Korarchaeota* 16S rRNA gene sequences

Fifteen samples from 14 hydrothermal sites in three different areas of Kamchatka were screened for *Korarchaeota* 16S rRNA genes (Table 1). *Korarchaeota* 16S rRNA genes were PCR-amplified using various primer combinations and cloned from five sites (Uzon 5, 8f, 9, Mut 11, 13), while only other *Archaea* 16S rRNA genes (10 *Euryarchaeota*, 20 *Crenarchaeota*, and 1 forming a unique clade; Fig. 1), were amplified and cloned from another five (VG1, Uzon 4, 6, 7, Mut14). No products were amplified from the remaining five samples, except in positive control reactions (Table 1). A total of 41 clones, with up to 10 clones/sample, were partially sequenced (resulting in typically >500 nt), and at least one representative (>99% identical to other partial sequences) from each reaction was fully sequenced (Table 1). Complete sequences ($n = 21$) from the five sites were ≥98.4% identical across 969 nt. Chimeras were not detected and indels and nucleotide changes were non-consecutive in all sequences, except at nt 193, where CT was present in all 11 Kamchatka sequences that contained that region, instead of the TC that is in all other known *Korarchaeota* sequences.

To examine phylogenetic relationships within the *Korarchaeota*, including the position of those from Kamchatka, analyses were conducted using alignments of different lengths that contained various numbers of the sequences (Fig. 2). Sequences <1,361 nt were analyzed and reported for completeness, however, bootstrap values are only from the longest length (1,361 nt) alignment analysis. *Korarchaeota* groups are defined according to the specificity of QPCR primers and as previously established (Auchtung et al. 2006).

Kamchatka *Korarchaeota* sequences grouped within the clade containing Group I (Iceland) and Group II (Yellowstone) sequences (100% bootstrap support). Due to the high similarity between all of these sequences (>98% identical across 1,361 nt), they are referred to collectively as Group I/II. Group III (Yellowstone) sequences grouped together (97%), sister to two Group IV sequences from California and Nevada (100%). This western U.S. group clustered with sequences identified from east and west Pacific Ocean vents and from Japan. The Japan group within this cluster had strong bootstrap support (98%), however, the entire western U.S./Pacific/Japan grouping was poorly supported (62%). Lying sister to the western U.S./Pacific/Japan cluster were a number of unresolved Group IV sequences from various Pacific Ocean vents (59%). A separate clade of Group IV East Pacific Rise sequences (100%) branched sister to the western U.S./Pacific/Japan/unresolved Pacific clade (75% for the entire Group III/IV clade). Group V (99%) was sister to Group III/IV (94% for the entire Group III/IV/V clade). Overall, sequences from *Korarcheota* Groups I/II, III, IV, or V were 89–95% identical to those in other groups.

To examine differences between the *Korarchaeota* 16S rRNA genes detected in different regions of Kamchatka, all

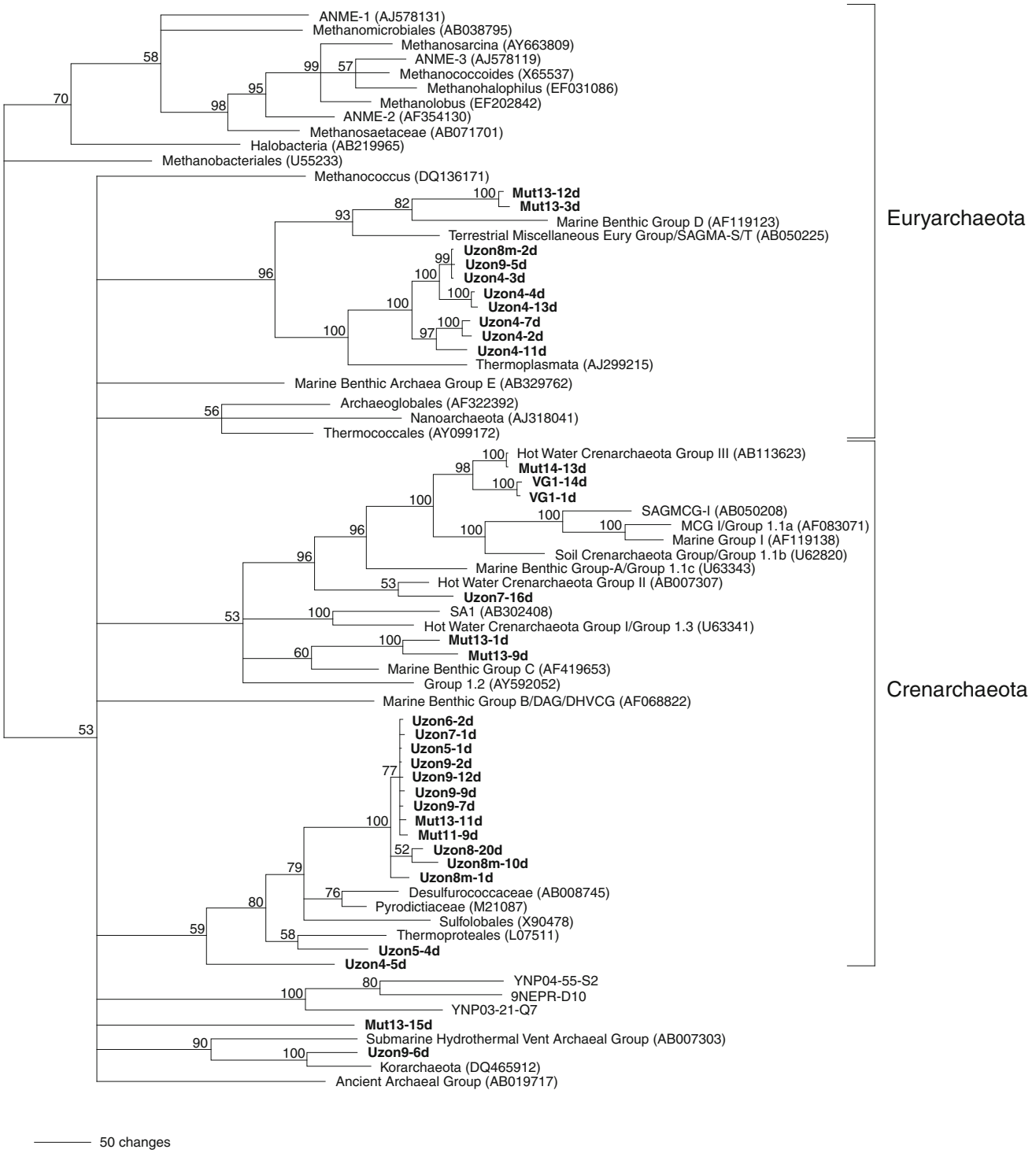
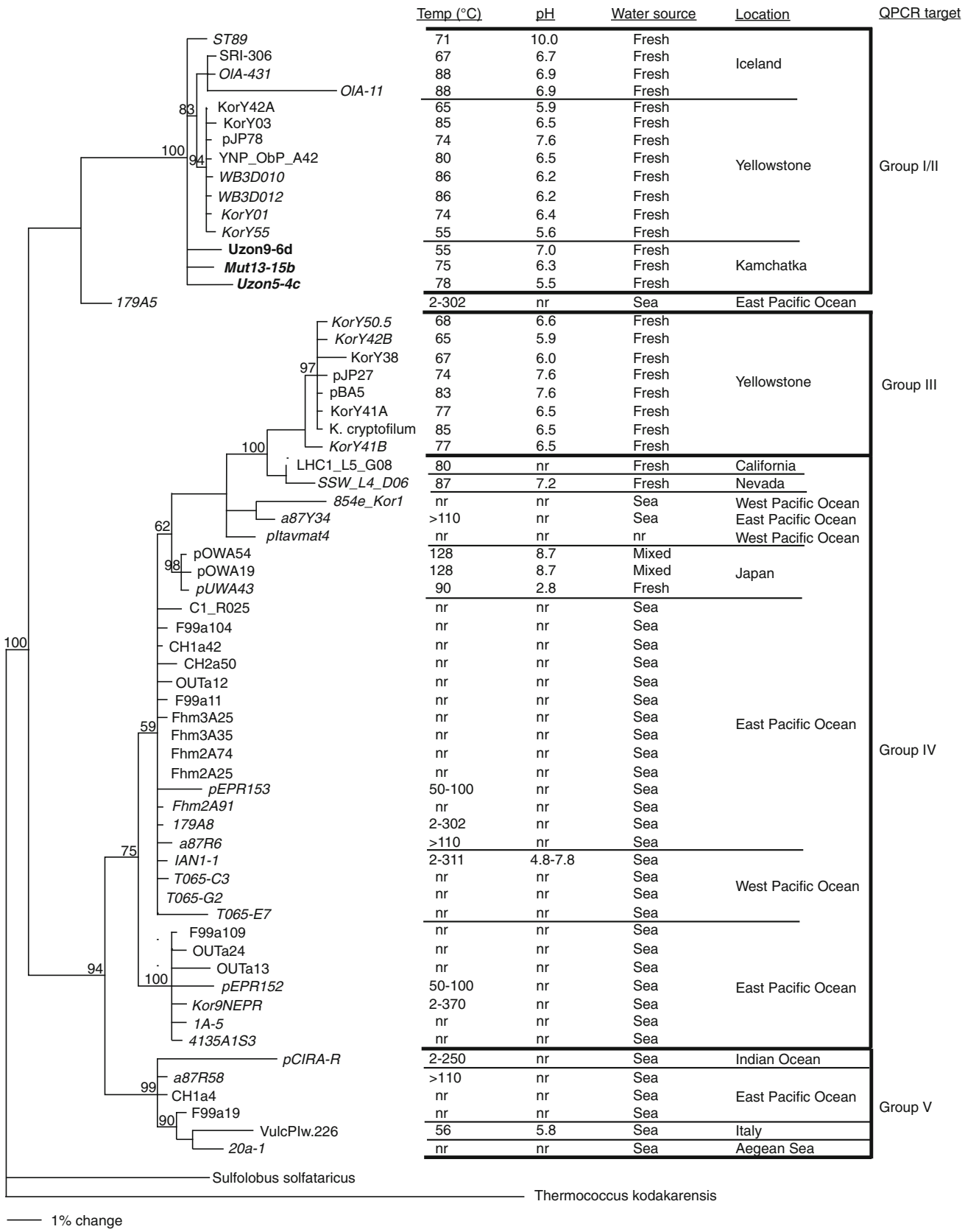


Fig. 1 Phylogram of *Archaea* 16S rRNA gene sequences amplified from Kamchatka hot spring samples with Arc26F-Univ1492R. A 1,226 nt alignment (*E. coli* positions 95–1,341) of all 32 Kamchatka clone sequences that were non-chimeric and <99% identical to others from each sample, plus sequences from representatives of every major archaeal group, were compiled using myRDP (Cole et al. 2009). The tree topology and bootstrap values were generated in PAUP by maximum parsimony analysis (500 replicates of 500 heuristic random-addition sequence searches). Kamchatka sequences are shown in bold and have the following notation: site region (*VG* Valley of the Geysers, *Uzon* Uzon Caldera, *Mut* Mutnovsky Volcano), site number—clone number, primer set designation (*d* Arc26F-Univ1492R)


Temp (°C)
pH
Water source
Location
QPCR target
ST89 71 10.0 Fresh
SRI-306 67 6.7 Fresh
OlA-431 88 6.9 Fresh
Iceland
OlA-11 88 6.9 Fresh
KorY42A 65 5.9 Fresh
KorY03 85 6.5 Fresh
pJP78 74 7.6 Fresh
YNP_ObP_A42 80 6.5 Fresh
WB3D010 86 6.2 Fresh
WB3D012 86 6.2 Fresh
KorY01 74 6.4 Fresh
KorY55 55 5.6 Fresh
Yellowstone
Group I/II
Uzon9-6d 55 7.0 Fresh
Mut13-15b 75 6.3 Fresh
Uzon5-4c 78 5.5 Fresh
Kamchatka
179A5 2-302 nr Sea East Pacific Ocean
KorY50.5 68 6.6 Fresh
KorY42B 65 5.9 Fresh
KorY38 67 6.0 Fresh
pJP27 74 7.6 Fresh
pBA5 83 7.6 Fresh
KorY41A 77 6.5 Fresh
K. cryptofilum 85 6.5 Fresh
KorY41B 77 6.5 Fresh
Yellowstone
Group III
LHC1_L5_G08 80 nr Fresh California
SSW_L4_D06 87 7.2 Fresh Nevada
854e_Kor1 nr nr Sea West Pacific Ocean
a87Y34 >110 nr Sea East Pacific Ocean
pItavmat4 nr nr nr West Pacific Ocean
pOWA54 128 8.7 Mixed
pOWA19 128 8.7 Mixed
pUWA43 90 2.8 Fresh
Japan
C1_R025 nr nr Sea
F99a104 nr nr Sea
CH1a42 nr nr Sea
CH2a50 nr nr Sea
OUTa12 nr nr Sea
F99a11 nr nr Sea
Fhm3A25 nr nr Sea
Fhm3A35 nr nr Sea
Fhm2A74 nr nr Sea
Fhm2A25 nr nr Sea
East Pacific Ocean
Group IV
pEPR153 50-100 nr Sea
Fhm2A91 nr nr Sea
179A8 2-302 nr Sea
a87R6 >110 nr Sea
IAN1-1 2-311 4.8-7.8 Sea
T065-C3 nr nr Sea
T065-G2 nr nr Sea
T065-E7 nr nr Sea
West Pacific Ocean
F99a109 nr nr Sea
OUTa24 nr nr Sea
OUTa13 nr nr Sea
pEPR152 50-100 nr Sea
Kor9NEPR 2-370 nr Sea
1A-5 nr nr Sea
4135A1S3 nr nr Sea
East Pacific Ocean
pCIRA-R 2-250 nr Sea Indian Ocean
a87R58 >110 nr Sea
CH1a4 nr nr Sea
F99a19 nr nr Sea
East Pacific Ocean
Group V
VulcPIw.226 56 5.8 Sea Italy
20a-1 nr nr Sea Aegean Sea
Sulfolobus solfataricus
Thermococcus kodakarensis
1% change

◀ **Fig. 2** Phylogram of *Korarchaeota* based on 16S rRNA gene sequences. The tree topology and bootstrap values were generated in PAUP by parsimony analysis of the 1,361 nt alignment of positions 29–1,390. A *Crenarchaeota* (*Sulfolobus solfataricus*) and *Euryarchaeota* (*Thermococcus kodakarensis*) served as outgroups. Sequences <1,361 nt (italicized) were added from their positions in trees generated from shorter alignment analyses, and thus have no bootstrap values. Since all Kamchatka sequences were almost identical, only three were analyzed (to which all others were ≥99.3% across 969 nt) and are shown in bold. Notation for the Kamchatka *Korarchaeota* sequences is: site region (*Uzon* Uzon Caldera, *Mut* Mutnovsky Volcano), site number—clone number, primer set designation (*b* Kor236F-Univ1492R, *c* Arc26F-Kor1236F, *d* Arc26F-Univ1492R). PCR amplification failed to detect *Korarchaeota* from Valley of the Geysers. The temperature, pH, water source, and geographic location of the sites from which the *Korarchaeota* sequences were amplified, and major phylogenetic groupings as previously defined (Auchtung et al. 2006) that are the targets of the quantitative PCR primers used in this study, are shown to the right of the tree. Samples with range of values were obtained from sulfide chimneys where cold basic ambient seawater mixes with hot acidic hydrothermal fluid. *nr* not reported

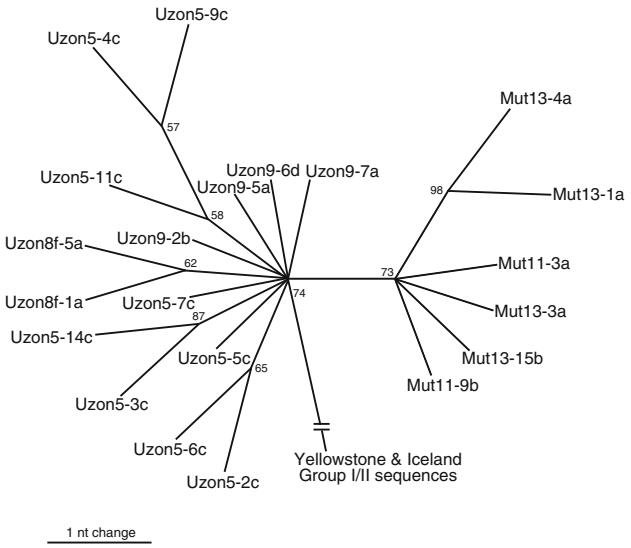


Fig. 3 Unrooted phylogram of *Korarchaeota* 16S rRNA genes amplified from Kamchatka samples. The tree topology and bootstrap values were generated by parsimony analysis of the 969 nt alignment of positions 237–1,235. Clone notation is: site region (*Mut* Mutnovsky Volcano, *Uzon* Uzon Caldera), site number—clone number, primer set designation (*a* Kor236F-Kor1236R, *b* Kor236F-Univ1492R, *c* Arc26F-Kor1236F, *d* Arc26F-Univ1492R). Other Group I/II sequences from Iceland (SRI-306) and Yellowstone (KorY42A, KorY03, KorY01, pJP78, YNP_ObP_A42) served as outgroups

21 Kamchatka clones that contained the region from nt 237 to 1,235 (969 nt) and six other sequences from Group I/II (outgroups) were aligned and analyzed (Fig. 3). As suggested by the analyses that used only representative sequences (Fig. 2), all Kamchatka sequences grouped together (bootstrap value = 74). Within this clade, the six sequences from Mutnovsky (sites 11 and 13) clustered (bootstrap value = 73) to the exclusion of the fifteen sequences from Uzon (sites 5, 8, and 9). The basis of the region-specific grouping is a single nucleotide (nt 494) that is a C in Mutnovsky *Korarchaeota* (present in all 12 Mutnovsky sequences that contained the region) and a T in Uzon *Korarchaeota* (present in all 22 Uzon sequences that contained the region).

Temperature, pH, water source, and geography of *Korarchaeota* habitats

Korarchaeota phylogeny was compared with temperature, pH, water source, and geography using all reported or inferable environmental data (Fig. 2). Temperature and geography united some phylogenetic groups, such as the Group III/IV clade sequences from 78 ± 8°C springs of Yellowstone, California, and Nevada, but were not consistent within other clades [e.g., the two reported temperatures from Group V sites were 56 and >110°C, and Group I/II sequences were from three vastly distant areas (Iceland, Yellowstone, and Kamchatka)]. pH was similar across most sites (6.4 ± 1.4), however, the tightly clustered Japan sequences were from sites of pH 2.8 and 8.7, and one Group I/II sequence was identified from a pH 10.0 spring. Sequences clustered based on salinity at a high phylogenetic level, with all Group I/II and Group III/IV western U.S. clade sequences coming from freshwater (low salinity) sites, while all other Group IV (except the Japan clade) and V sequences were from seawater (high salinity) sites.

Kamchatka *Korarchaeota* abundance

To examine whether the Group I/II *Korarchaeota* identified by 16S rRNA phylogenetic analysis was the major *Korarchaeota* group present, and to look for the presence of other, possibly rare, *Korarchaeota* groups, the abundance of Group I/II, III, and IV *Korarchaeota* 16S rRNA genes in each Kamchatka sample was determined using quantitative PCR. Group I/II *Korarchaeota* were detected by QPCR in eight of the fifteen samples examined (3–1,090 copies/estimated 10^6 organisms; Table 1). Consistent with the products being true Group I/II *Korarchaeota* genes, the melting point of reaction products matched the positive control (~87.5°C), and a single band of the correct size (~120 bp) was observed when products were analyzed on agarose gels. Direct sequencing confirmed the specific amplification of Group I/II *Korarchaeota*. Group I/II *Korarchaeota* were not detected in the remaining 7 samples (<3 to <50 copies), and Group III and IV *Korarchaeota* were not detected in any of the 15 samples examined (<2 to <100 and <0.5 to <10 copies, respectively).

Discussion

This study presented quantitative 16S rRNA phylogenetic evidence that a single clade of Group I/II *Korarchaeota* are indigenous to the hot springs of Kamchatka. Though observed in low abundance, these 16S rRNA genes are unlikely to be from transient *Korarchaeota* since they were identified from seven different springs in two different regions of the peninsula. Although *Korarchaeota* 16S–23S rRNA gene internal transcribed spacer (ITS) sequences were too divergent (unpublished data) and there is not yet genomic data available from multiple *Korarchaeota* groups to more fully determine their relationships, analysis of 16S rRNA gene sequences identified from Kamchatka and around the world provided sufficient phylogenetic resolution for consideration of the possible roles of geography and environmental factors in *Korarchaeota* diversification.

Korarchaeota diversification

Within most 16S rRNA phylogenetic clades, *Korarchaeota* sequences have been identified from a single geographic region (Fig. 2), suggesting that they may be unique to these regions. Even within Kamchatka, while the salinity, temperature, and pH of *Korarchaeota*-positive sites was similar between Uzon and Mutnovsky, *Korarchaeota* 16S rRNA genes differed (Fig. 3). Similar local geographic divergence is also present in *Sulfolobus* and *Aquificales* (Whitaker et al. 2003; Takacs-Vesbach et al. 2008). *Korarchaeota* endemism is also supported by the available quantitative data: quantitative PCR of Kamchatka samples reported here measured detectable levels of only the *Korarchaeota* clade (Group I/II) identified by PCR and sequencing. Using clone libraries, Reigstad and coworkers also only detected *Korarchaeota* from this phylogenetic group in Kamchatka (Reigstad et al. 2010). However, geographic isolation of *Korarchaeota* from limited dispersal and colonization does not currently appear to be caused strictly by the distance between habitats. For example, *Korarchaeota* detected in Kamchatka belong to Group I/II, only previously detected in Yellowstone (6,100 km away) and Iceland (6,800 km away). Yet, in the closer locations of southern Japan (3,200 km away) and the Juan de Fuca Ridge (5,100 km away), only Group IV and V *Korarchaeota* have been identified.

A comparatively slow rate of physiological adaptation to different temperatures and salinities may also have been important in the diversification of *Korarchaeota*, assuming that the environmental conditions recorded where *Korarchaeota* sequences were identified were representative of their habitats. If so, similar environmental conditions at the identification sites of sequences in a given phylogenetic cluster may have been key in the divergence of that clade (Fig. 2). Indeed, the Group I/II *Korarchaeota* may not have been living in the sampled regions of Japanese or Juan de Fuca springs because the temperatures and salinities, respectively, were too high. The importance of salinity in the diversification of *Korarchaeota* may be representative of a larger trend; global analyses suggest salinity is the most significant environmental variable in shaping the phylogenetic structure of archaeal and bacterial communities (Lozupone and Knight 2007; Auguet et al. 2010). As with other microorganisms (e.g., *Aquificales*; Ferrera et al. 2007), a confident understanding of the importance of geographic and environmental factors in the diversification of *Korarchaeota* will only come when more quantitative phylogenetic information from diverse geographic sites and data on the conditions in which cultured representatives can survive and grow is obtained.

Low population sizes

Group I/II *Korarchaeota* were measured at relatively low abundance (3–1,090 cells in 10^6; Table 1) in the eight Kamchatka filament, powder, mat, and sediment samples (seven sites) they were detected. It is possible that the DNA extraction efficiency of *Korarchaeota* was poor compared to other organisms, however, two different DNA extraction procedures were used and *Korarchaeota* were determined to be present in relatively low numbers from DNA of each. Numerous eukaryotes were only observed in one sample (Uzon4); their large genomes may have caused an underestimation of the number of organisms present in the DNA, and therefore an artificially low abundance of *Korarchaeota*. It is also possible that the samples from which *Korarchaeota* were quantified were not their preferred environments or just not collected at a time when they were abundant. A low abundance was also observed for Group I/II and III *Korarchaeota* in Yellowstone samples [0.2–500 cells in 10^6 (12 sites) and 0.3–150 cells in 10^6 (6 sites) (Auchtung 2007) and by Reigstad and co-workers (2010) in Iceland and Kamchatka samples [20–6,000 cells in 10^6 (12 sites)]. If *Korarchaeota* do exist at a relatively low natural abundance in all microhabitats and at all times, they may be an example of the "rare biosphere" (Sogin et al. 2006), the extreme diversity of rare microorganisms that survive, even in the face of intense competition or predation (Curtis and Sloan 2005; Gillespie and Emerson 2007). It has been hypothesized that a dependence upon other microorganisms, such as the *Aquificales*, for metabolic requirements may constrain *Korarchaeota* population sizes (e.g., Reysenbach et al. 2000; Reigstad et al. 2010). Low cell numbers would also present fewer chances for dispersal to and colonization of other sites, thereby contributing to the endemism of *Korarchaeota*.

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