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Molecular Phylogeny of Uncultivated *Crenarchaeota* in Great Basin Hot Springs of Moderately Elevated Temperature

Zhiyong Huang
Savannah River Ecology Laboratory, University of Georgia, Aiken, South Carolina 29802, USA

Brian P. Hedlund
School of Life Sciences, University of Nevada Las Vegas, Las Vegas, Nevada 89154, USA

Juergen Wiegel
Department of Microbiology, University of Georgia, Athens, Georgia 30605, USA

Jizhong Zhou
Stephenson Research & Technology Center, University of Oklahoma, Norman, Oklahoma 73019, USA

Chuanlun L. Zhang
Savannah River Ecology Laboratory, University of Georgia, Aiken, South Carolina 29802, USA

Uncultivated *Crenarchaeota* are distributed widely in low temperature (<30°C) environments, and it has been hypothesized that they evolved from (hyper)thermophilic species thriving in marine hydrothermal vents or terrestrial hot springs. To further our understanding of the environmental distributions of *Crenarchaeota*, we studied mat samples collected from hot springs of moderately elevated temperature (∼49–67°C) in California and Nevada, USA. Clone libraries of archaeal 16S rRNA genes were constructed for selected samples using a PCR-based approach. Sequences from the Nevada hot springs (Rick's Hot Creek and Hard to Find) were closely related to uncultivated *Crenarchaeota* found near deep sea hydrothermal vents or from the subsurface geothermal system; sequences from the California hot spring (Surprise Valley), on the other hand, were closely related to sequences from freshwater sediments. Statistical analysis showed that the community structure of *Archaea* was significantly different between any two springs with greater differences occurring between the Nevada and California hot springs ($P = 0.002$). To determine whether these sequences represent indigenous microorganisms of geothermal springs, and not soil contaminants, archaeal 16S rRNA gene clone libraries were also constructed from soil samples taken from around Rick's Hot Creek and Surprise Valley hot springs. None of the hot spring sequences was closely related to those from the surrounding soil in Nevada or California or to the predominant soil *Crenarchaeota* in other locations, indicating that soil contamination to the hot spring environment was insignificant. Results of this study expand the distribution of *Crenarchaeota* into the moderately thermobiotic environment, which has been much less intensively studied than high temperature (>70°C) or low temperature natural habitats, and demonstrates that thermophiles inhabiting moderate temperature portions of Great Basin hot springs are phylogenetically distinct from both cultivated hyperthermophilic *Crenarchaeota* and sympatric soil *Crenarchaeota*.

Keywords *Crenarchaeota*, diversity, thermophiles, hot springs, soils, Nevada, California

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Address correspondence to Chuanlun L. Zhang, Savannah River Ecology Laboratory, University of Georgia, Aiken, SC 29802, USA. E-mail: zhang@srel.edu.

INTRODUCTION

Nonthermophilic *Crenarchaeota* represent a fast-evolving branch of the crenarchaeal lineage within the Domain *Archaea*. Due to the advancement of culture-independent molecular approaches, these organisms have been identified from soils (e.g., Bintrim et al. 1997; Jurgens et al. 1997; Jurgens and Saano 1999; Ochsenreiter et al. 2003), freshwater and marine systems (e.g., DeLong 1992; Fuhrman et al. 1992; Hershberger et al. 1996; MacGregor et al. 1997; Vetriani et al. 1999; Jurgens

et al. 2000; Abreu et al. 2001; Karner et al. 2001; Stein et al. 2001, 2002; Beja et al. 2002), deep subsurface mines (Inagaki et al. 2001; Takai et al. 2001) and deep subsurface paleosol (Chandler et al. 1998), terrestrial hot springs (Barns et al. 1996; Kanokratana et al. 2004; Kvist et al. 2005) and marine hydrothermal vents (Takai and Horikoshi 1999; Teske et al. 2002), and invertebrate bodies (Preston et al. 1996; Margot et al. 2002; Lee et al. 2003). The great abundance of *Crenarchaeota* in the world's oceans (Karner et al. 2001; Damsté et al. 2002a; Herndl et al. 2005; Mincer et al. 2007) suggests that they play a globally significant role in the marine ecosystem. Most recently, molecular evidence, cultivation, and in situ studies have collectively demonstrated that *Crenarchaeota* may play an important role in global carbon and nitrogen cycles by mediating the chemolithoautotrophic oxidation of ammonia to nitrite (Francis et al. 2005; Herndl et al. 2005; Könneke et al. 2005; Treusch et al. 2005; Ingalls et al. 2006; Leninger et al. 2006).

So far, only one nonthermophilic crenarchaeal species, Candidatus *Nitrosopumilus maritimus*, has been obtained in pure culture. It is a chemolithoautotroph that uses ammonia as an electron donor for aerobic respiration (Könneke et al. 2005); however, the other physiological and biochemical properties of this culture are largely unknown, including the lipid composition (Könneke et al. 2005). Prior to the isolation of *N. maritimus*, a nonthermophilic culture, Candidatus *Cenarchaeum symbiosum,* had been found to be symbiotically associated with a marine sponge (Preston et al. 1996). The DNA polymerase of *C. symbiosum* shows thermal sensitivity, which is consistent with the low temperature environment in which this species was found (Schleper et al. 1997). Overall, however, the information on physiological properties of these *Archaea* remains limited (Hallam et al. 2006a; Leininger et al. 2006). As a result, the phylogenetic and phenotypic relationships among different species remain to be solved (Schleper et al. 1997; DeLong 1998; Dawson et al. 2001; Ochsenreiter et al. 2003; López-García et al. 2004). Ochsenreiter et al. (2003) did observe, however, that a specific group of *Crenarchaeota*, group 1.1b, is found in all soil environments tested. These may be the only organisms among mesophilic *Crenarchaeota* to compete effectively with soil bacteria.

Based on the phylogenetic relationships among crenarchaeal sequences, it has been proposed that the ancestors of modern-day, cold-adapted marine *Crenarchaeota* once lived in anoxic, high-temperature habitats. For instance, 16S rRNA genes from uncultivated *Crenarchaeota* recovered from low-temperature ecosystems have lower G+C content and form longer branches in phylogenetic trees than thermophilic species (Barns et al. 1996; Dawson et al. 2001). Long branching is viewed as an indication of fast evolution; whereas low G+C content corresponds with low growth temperatures (Galtier et al. 1999). Thus, the low temperature lineages of *Crenarchaeota* may have evolved from (hyper)thermophilic species (Barns et al. 1996; Hershberger et al. 1996). The topology of the crenarchaeal tree shows close relationships between low-temperature and high-temperature sequences in several lineages, suggesting that adaptation of (hyper)thermophilic species to low temperature happened multiple times (Hershberger et al. 1996; Vetriani et al. 1999; Dawson et al. 2001). López-García et al. (2004), on the other hand, proposed that adaptation of (hyper)thermophilic *Crenarchaeota* to mesophilic conditions occurred only once (López-García et al. 2004).

Most studies on *Crenarchaeota* focus on either very high temperature (>70°C) geothermal or low temperature (<30°C) marine/freshwater or soil environments and consequently, much less attention has been paid to the moderately thermobiotic (40–70°C) environments. Recently, Pearson et al. (2004) and Zhang et al. (2006) observed the widespread occurrence of a unique archaeal lipid biomarker, crenachaeol, in Nevada and California hot springs, with a maximum enrichment relative to other archaeal lipids at a temperature of ∼45°C. In the geologic past, this lipid may have facilitated the radiation of thermophilic *Crenarchaeota* into lower temperature environments because the lipid structure allows the otherwise rigid membrane of the hyperthermophilic Archaea to maintain fluidity at lower temperatures (Damsté et al. 2002b). In the current study, novel crenarchaeal 16S rRNA gene sequences were obtained from California and Nevada hot springs that ranged in temperature from 49–67°C. Phylotypes in the California hot spring were different from those in the Nevada hot springs, and none of the sequences were closely related to soil *Crenarchaeota*, indicating that the archaeal communities in these hot springs are indigenous to the moderately thermobiotic environments.

MATERIALS AND METHODS

Water Chemistry and Sample Collection

The three hot springs selected for this study were Hard to Find (4540865.0 N and 330711.7 E) and Rick's Hot Creek (4504013.6 N and 299900.7 E) in Nevada and Surprise Valley (4602596.3 N and 243657.9 E) in California. At each location, water pH, temperature, and total dissolved solids (TDS) were determined using a Hach pH-meter equipped with a pH and temperature probe and a TDS probe. Calibration of the pH meter was performed at ambient temperature (∼25°C) and measurements of pH were expressed as $pH^{25^\circ}C$ in reporting. Alkalinity was determined in the laboratory in $HgCl_2$-poisoned water using an automated alkalinity titrator (Apollo SciTech., Inc., Bogart, GA) with a precision of 0.2%. Mat samples were collected into sterile plastic tubes or plastic bags and immediately cooled on ice or frozen on dry ice. Samples were subsequently stored at −80°C until analyses of DNA.

To compare archaeal community from hot springs to that of the surrounding soil, two soil samples were collected, one near Rick's Hot Creek and the others near the Surprise Valley spring, including an interplant sample and a sample from the rhizosphere of a common local plant, *Salicornia virginica*, commonly known as pickleweed. Sites were within 10 meters of the hot spring sampling locations but were deliberately chosen to be up elevation from the spring to minimize the possibility of spring to soil contamination.

DNA Extraction and PCR Amplification of Archaeal 16S rRNA Genes

Genomic DNA was extracted from mat or soil samples using the UltraClean Mega soil extraction kit (MoBio Lab Inc., Solana Beach, CA) according to Zhou et al. (1996) or the QBiogene FastDNA SPIN Kit for soil. The DNA was purified using the Wizard DNA Clean-up System (Promega, CA), or used directly as a template for PCR amplification. For the mat samples from hot springs, approximately 900 bp rDNA fragments were obtained from samples by using the universal archaeal primers Arch21F and Arch958R (DeLong et al. 1999). For the soil samples nearly full-length fragments were obtained by using the archaeal primer 8aF and the universal primer 1512uR (Eder et al. 1999). Standard protocols for 16S rRNA gene PCR were used (Eder et al. 1999). PCR products were cloned using a T/A cloning kit (Invitrogen) and colonies were randomly picked and sequenced using vector primers or PCR primers using an ABI3700 Sequencer (Perkin-Elmer, Wellesley, MA).

Phylogenetic Analyses

Sequences from the environmental DNA clones were edited using the SEQUENCHER program (v.4.0, Gene Codes, Ann Arbor, MI) and manually aligned to reference sequences obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov). Aligned sequences were checked for chimeric artifacts using the CHECK_CHIMERA program through the Ribosomal Database Project (http://rdp.cme.msu.edu/html) and Bellerophon (Huber et al. 2004). Moreover, the secondary-structures of all the sequences were analyzed by using the RNAfold program described by Hofacker (Hofacker 2003) to verify that all bases were unambiguous. Final sequences (about 550 nt) were manually assembled and aligned using the BIOEDIT (Hall 1999) and CLUSTALX1.83 software package.

Phylogenetic analyses were performed using the neighbor-joining and parsimony methods and PAUP software (version 10). Additional software packages, including Phylip, MrBayes, and Treecon, were also used for phylogenetic analyses. The phylogenetic relationships were inferred by distance (Kimura 2-parameter correction), maximum likelihood, and maximum parsimony algorithms. Bootstrap values determined from 1,000 replicates were >50% for most nodes. The statistical program LIBSHUFF (http://libshuff.mib.uga.edu/) was used to determine the significance of differences between two clone libraries (Singleton et al. 2001).

Nucleotide Sequence Accession Numbers

All 16S rRNA gene sequences determined in this study have been deposited in the GenBank/EMBL/DDBJ databases. The accession numbers of sequences are DQ397641 to DQ397662 and EF503693 to EF503710 for hot spring sequence and soil sequences, respectively.

RESULTS

Water Chemistry

Rick's Hot Creek (RHC) is a fast flowing ~1 m wide stream located near the town of Gerlach, NV. Water issues from the subsurface at 95.5°C, which is boiling at that altitude, and the stream cools to ambient temperature over a ~0.5 km distance. Samples were taken at a location about 50 m down stream from the source of spring, which had a temperature of 67°C and a pH of 7.7. Hard to Find (HTF) had a temperature of about 58°C and a pH of 6.2, and Surprise Valley (SV) had a temperature of about 56°C and a pH of 5.5. Total dissolved solids were similar in HTF (791 mg/L) and SV (684 mg/L) but were significantly higher in RHC (3600–3900 mg/L). The alkalinity was around 1.0 mM for RHC and SV and not determined for HTF.

Hot Spring *Crenarchaeota* Community Structure and Phylogenetic Analysis

A total of 61 archaeal clones were obtained from the hot spring samples: 23 from Rick's Hot Creek (RHC), 20 from Hard to Find (HTF), and 18 from Surprise Valley (SV). Each clone was sequenced using the forward primer (ca. 550 to 600 nucleotides), and then the sequences were grouped into operational taxonomic units (OTUs) at a 97% similarity value using DOTUR (Schloss and Handelsman 2005). Although each library had a moderate number of clones, rarefaction analysis suggested that they represented the majority of archaeal diversity in these habitats (data not shown), which is consistent with the general observation of low archaeal diversity in terrestrial hot springs (Barns et al. 1996). The number of OTUs in each clone library ranged from 5 in SV to 10 in RHC (Table 1).

To determine whether the crenarchaeal communities were similar in the different springs, pairwise sequence comparisons were made. OTUs from the SV clone library were 100% unique, meaning no SV clones were detected at either of the other two sites. HTF and RHC, on the other hand, shared three OTUs. In addition, HTF had four unique OTUs and RHC had six unique OTUs (Table 1).

Statistical analysis of clone libraries using the LIBSHUFF program gave ΔCxy values of 0.94 between HTF and RHC, 7.24 between HTF and SV, and 17.34 between RHC and SV hot springs (Table 2). The ΔCxy values corresponded to p values that were all less than 0.05 (Table 2), suggesting that the archaeal community structure was significantly different between any two sites. However, the difference was smaller between the two Nevada hot springs ($p = 0.038$) than between either pair of Nevada and California springs ($p = 0.002$) (Table 2).

To determine the phylogenetic affiliations of the new *Crenarchaeota* sequences, representatives of each OTU were compared with the sequences in the GenBank database by using BLAST (http://www.ncbi.nlm.nih.gov/) and a phylogenetic tree was generated (Figure 1). BLAST revealed that the hot spring OTUs did not have close relationships to any known hyperthermophilic archaea (Figure 1). Three OTUs clustered in the methanogenic

TABLE 1
Characteristics of archaeal clones from Rick's Hot Creek (RHC) and Hard to Find (HTF) of Nevada and Surprise Valley (SV) of California and nearby soil communities

	Hot Spring Clones			Soil Clones		
	HTF	RHC	SV	SoilSV2	SoilSV2R	SoilRHC
Total clones screened	20	23	18	18	14	11
No. of OTUs[a]	7	10	5	14	12	9
Unique OTUs[b]	4	6	5	4	3	4

[a]Operational taxonomic units (OTU) based on the similarities between sequences; sequences having or above 97% identity were defined as one OTU.
[b]OTU that is only present in a particular sample.

Euryarchaeota (NV_RA-G01, CA_SA-B03 and CA_SA-D02), and were closely related to organisms from a tidal flat (BS1-1-20; Kim et al. 2005) or rice-fields (HrhA48; Lu and Conrad 2005; *Methanobacterium* sp.; Joulian et al. 1998) (Figure 1). The remaining OTUs were clearly affiliated with *Crenarchaeota* but did not belong to the *Thermoprotei* (Figure 1), which circumscribes all cultivated hyperthermophilic *Crenarchaeota*.

Many of the new crenarchaeal sequences, particularly those from Nevada springs, formed a well defined cluster in the *Crenarchaeota* tree, which we refer to as Great Basin Hot Spring *Crenarchaeota* Cluster I (GBHSC I; Figure 1). This cluster contained 15 OTUs, 8 from RHC and 7 from HTF (Figure 1), and 2 sequences that were isolated from deep-sea sulfide chimney rocks, FZ3aA119 and FZ3bA142 (Schrenk et al. 2003). Basal to this cluster is a sequence from terrestrial subsurface geothermal water (Subt_14; Marteinsson et al. 2001). A related but distinct phylogenetic cluster included sequences from hot springs (AK29; Kanokratana et al. 2004), soils (SCA1166 and SCA1158; Bintrim et al. 1997), and the Marine Group I *Archaea*, including Candidatus *Cenarchaeum symbiosum* (DeLong 1992; Preston et al. 1996) and Candidatus *Nitrosopumilus maritimus* (Könneke et al. 2005). Other *Crenarchaeota* sequences, particularly those from SV, were scattered among a group of sequences from a variety of habitats including a deep-sea sulfide chimney (Schrenk et al. 2003), freshwater environments (Hershberger et al. 1996) (Jurgens et al. 2000), and a Yellowstone hot spring (pSL- and pJP-sequences; Barns et al. 1996; Figure 1).

In summary, all crenarchaeal sequences from Nevada except NV_RAF05 grouped in GBHSC I, whereas those from

TABLE 2
Difference in community structure among Hard to Find (HTF) and Rick's Hot Creek (RHC) of Nevada and Surprise Valley (SV) of California and sympatric soil Crenarchaeota communities

	OTUs (x)	OTUs (y)	ΔCxy[†]	p-value
Hot spring vs. hot spring comparisons				
HTF (x) vs. SV (y)	7	5	7.24	0.002
RHC (x) vs. SV (y)	10	5	17.34	0.002
HTF (x) vs. RHC (y)	7	10	0.94	0.038
Soil vs. soil comparisons				
SoilSV2 (x) vs. SoilSV2R (y)	14	12	6.85	0.001
SoilRHC (x) vs. SoilSV2 (y)	9	14	2.51	0.001
SoilRHC (x) vs. SoilSV2R (y)	9	12	7.037	0.001
Hot spring vs. soil comparisons				
SV(x) vs. SoilSV2 (y)	5	14	25.74	0.001
SV (x) vs. SoilSV2R (y)	5	12	25.82	0.001
RHC (x) vs. SoilRHC (y)	10	9	12.05	0.001

[†] ΔCxy is the difference between a homologous coverage curve, *CX(D)*, and a heterologous coverage curve, *CXY(D)*, which is calculated using a Cramér-von Mises-type statistic. Each ΔCxy is accompanied by a p value. Two libraries are considered significantly different when p is less than 0.05 and the bigger the ΔCxy, the smaller the p value.

Statistical analysis was performed using the LIBSHUFF program according to Singleton et al. (2001).

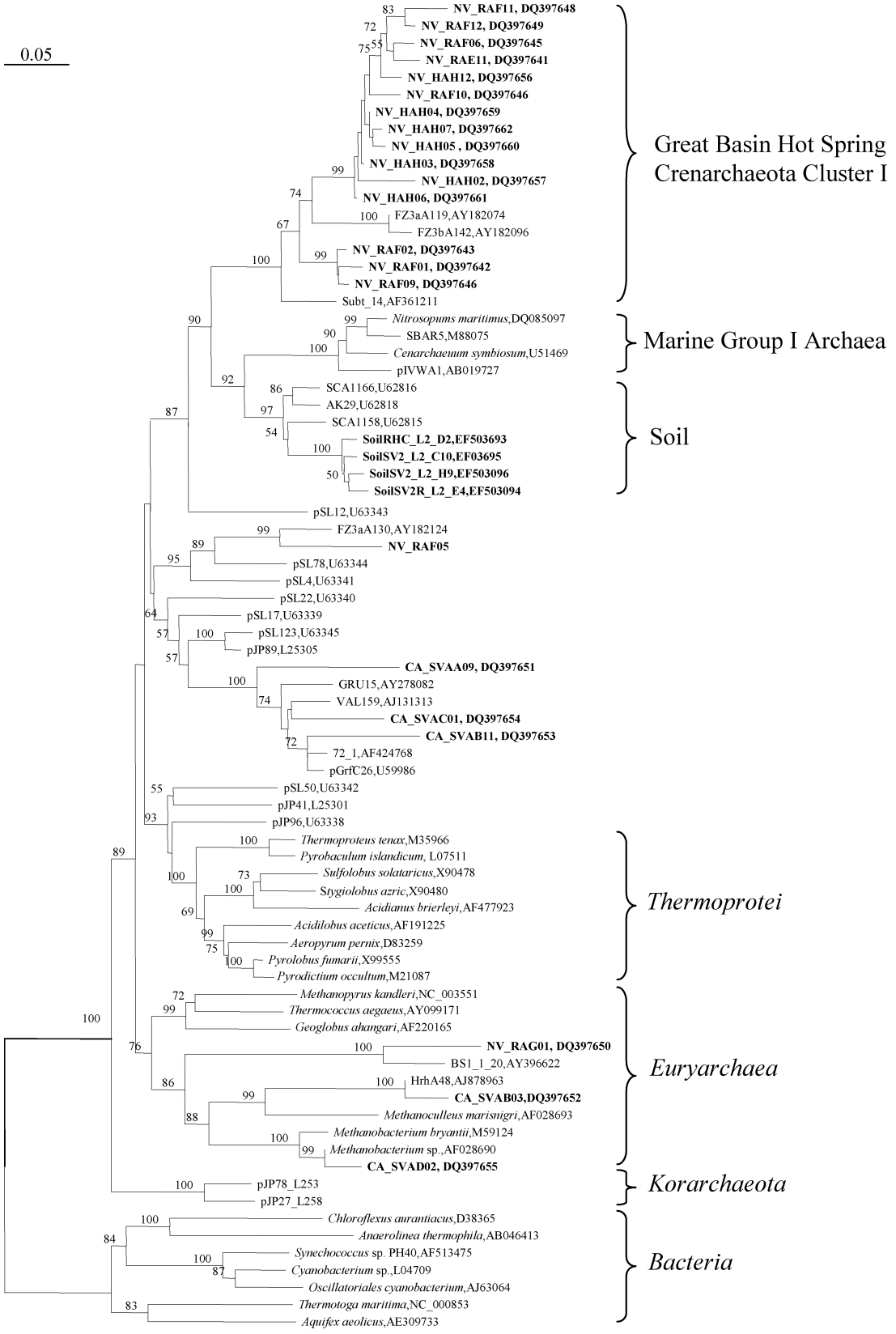

FIG. 1. Phylogenetic neighbor-joining tree of representatives of each OTU from the archaeal clone libraries obtained from Rick's Hot Creek (designated as NV_R-sequences), Hard To Find (designated as NV_H-sequences), and Surprise Valley (designated as CA_SV-sequences). Representative soil sequences from Rick's Hot Creek (designated as SoilRHC) and Surprise Valley (designated as SoilSV2) were also included. Bacterial species were used as the outgroup.

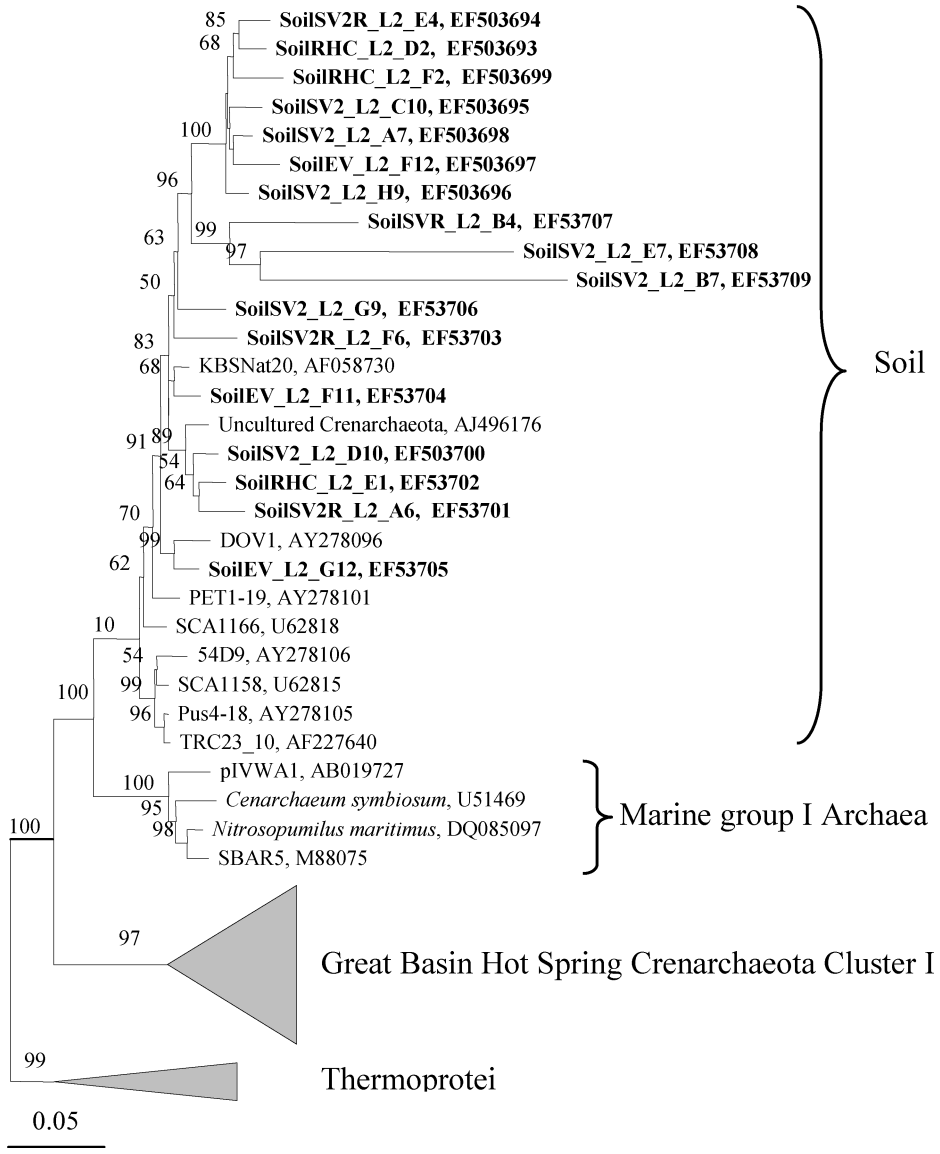

FIG. 2. Phylogenetic neighbor-joining tree of representatives of each OTU from the archaeal clone libraries obtained from soils near Rick's Hot Creek (SoilRHC-sequences) and Surprise Valley (SoilSV2-sequences). Species of *Thermoprotei* (hyperthermophilic *Crenarchaeota*) were used as the outgroup.

California (CA_SVAA09, CA_SVAC01, and CA_SVAB11) branched in a separate lineage. This observation is consistent with the statistical analysis of the community structures between springs (Table 2). No sequence from Nevada or California was closely related to known hyperthermophilic crenarchaeal species in the *Thermoprotei* (Figure 1).

Sympatric Soil *Crenarchaeota* are Distinct from Hot Spring *Crenarchaeota*

It has been suggested that *Crenarchaeota* sequences recovered in cultivation-independent censuses of terrestrial hot springs may not be indigenous to the springs and may instead represent contamination from nearby soil *Crenarchaeota* populations (DeLong 1998). To account for this possibility, topsoil was collected from the vicinity of SV and RHC, and used as a substrate for DNA extraction and archaeal 16S rRNA gene PCR and clone library construction. At SV, soil was collected from an inter-plant space and also from the rhizosphere of an abundant local plant, *Salicornia virginica*, to account for the possibility that these samples represent distinct populations. The sequences that were recovered were distinct from both the sympatric hot spring sequences and other sequences from geothermal habitats; instead they formed a well-defined clade with *Crenarchaeota* from other soil habitats (Figure 2). Furthermore, comparisons of the soil *Crenarchaeota* sequences with the hot spring *Crenarchaeota* sequences using LIBSHOFF confirm that the hot spring *Crenarchaeota* populations are distinct from those in local soils (Table 2).

DISCUSSION

Nonthermophilic *Crenarchaeota* have only been recognized recently as an abundant and widely distributed fraction of the prokaryotic microorganisms in low-temperature environments. However, because of the difficulty in cultivating them, a comprehensive understanding of their roles in nature and their classification is still unavailable (DeLong 1992, 1998; Buckley et al. 1998; Jurgens et al. 2000; Dawson et al. 2001; Ochsenreiter et al. 2003). Most of the cultivated *Crenarchaeota* are hyperthermophiles that branch within the *Thermoprotei*, whereas only one species of marine nonthermophilic *Crenarchaeota* has been isolated (Könneke et al. 2005). The majority of presumably nonthermophilic *Crenarchaeota* are known only from 16S rRNA gene sequences from cultivation independent censuses (Ochsenreiter et al. 2003; Schleper et al. 2005).

This study extends our knowledge of the environmental distribution of *Crenarchaeota* into terrestrial springs of moderately elevated temperatures. The majority of sequences from Nevada springs formed a well-defined clade, GBHSC I, which was monophyletic except for a pair of sequences recovered from a black smoker chimney.

Based on rDNA phylogenetic reconstructions, most *Crenarchaeota* from moderately high temperature environments seem to be taxonomically distinct from nonthermophilic and hyperthermophilic species, but there are exceptions (Figure 1). In particular, the close relationship of hot spring sequences (e.g., pSL12, Figure 1) with nonthermophilic groups has been interpreted as evidence for the thermophilic origin of nonthermophilic *Crenarchaeota* (Barns et al. 1996). DeLong (1998), on the other hand, provided an alternative view that some of hot-spring-derived *Crenarchaeota* sequences that branch outside the *Thermoprotei* (e.g., pSL and pJP sequences; Figure 1) might actually originate from terrestrial runoff into the spring.

To address this possibility as it relates to our study, we prepared clone libraries from soils that were within 10 meters of the exact locations from which the hot spring samples were taken. Soil sampling locations were chosen to represent "typical" local soils, which have a low water content, high salinity, and high carbonate alkalinity. To account for both the possibility of the hot spring *Crenarchaeota* serving as a source of soil contamination during flooding events, sample sites were higher in elevation than the hot springs. In addition, a sample from the rhizosphere of a common plant in the vicinity of Great Basin hot springs, *Salicornia virginica*, was included. The resulting phylogenetic analyses and community profile comparisons rule out the possibility of significant reciprocal contamination of *Crenarchaeota* between sympatric arid soils and hot springs, at least at the temperatures that were examined. Our study does not rule out the possibility that *Crenarchaeota* from local soils may inhabit cooler parts of the springs nor does it specifically rule out that the hot spring *Crenarchaeota* derive from aeolian transport from distant sources.

However, given the phylogenetic coherence of the hot spring *Crenarchaeota*, particularly GBHSC I, and in the absence of a specific hypothetical source for such contamination, the simplest explanation is that the hot spring *Crenarchaeota* described in this publication are indigenous to Great Basin hot springs. It is also significant that the Nevada hot spring sequences cluster with sequences from marine hydrothermal vents (FZ sequences, Figure 1) and a sequence from a deep subsurface geothermal system in Iceland (Subt_14; Marteinsson et al. 2001), and not with sequences recovered from soil studies.

In summary, results of this study and others demonstrate that environmental sequences from geothermal environments in Nevada and California are unlikely to be derived from soil contamination. Instead, because of their phylogenetic independence from the hyperthermophiles, environmental sequences in GBHSC I may have a moderately thermophilic origin. This hypothesis will need to be tested using genomic and evolutionary biological approaches. The moderately thermobiotic springs in Nevada and California will provide unique opportunities for testing such a hypothesis.

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