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Archaeal diversity along a soil salinity gradient prone to disturbance

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Summary

We employed a cultivation-independent approach to examine archaeal diversity along a transient soil salinity gradient at Salt Spring in British Columbia, Canada that is routinely eroded due to heavy, recurrent rainfall. Archaeal 16S rRNA gene libraries were created using DNA extracted from three soil samples collected along this gradient. Statistical comparisons indicated similar archaeal richness across sites but, a significant shift in archaeal community composition along the salinity gradient. Seven distinct phylogenetic groups were represented in soil libraries. Haloarchaea were the most commonly sampled group. Other 16S rRNA sequences were related to uncultured *Euryarchaeota* and *Crenarchaeota* or halophilic methanogens. Haloarchaeal diversity was remarkably high in soil of elevated salinity compared with previously characterized haloarchaeal communities. Salt Spring haloarchaea were not closely related to known low-salt adapted/tolerant species, suggesting they may be frequently faced with local mortality as a result of frequent declines in soil salinity. We speculate that ecosystem disturbance – in the form of salinity fluctuations – is one mechanism for maintaining a diverse community of haloarchaea at Salt Spring.

Introduction

Competition between species plays a central role in regulating biological diversity. Under a restricted set of environmental conditions, strong competitive interactions can lead to a loss of diversity because selection eradicates all but the most fit or best-adapted types from the community (Hardin, 1960; Begon *et al*., 1986). However, mechanisms for generating and maintaining diversity by reducing competition exist and in fact most natural communities, especially microbial communities, are highly diverse. Application of molecular techniques has revealed an astonishing level of prokaryotic diversity in the biosphere, only a small proportion of which has been cultivated (DeLong and Pace, 2001; Rappé and Giovannoni, 2003). Soil and sediments are among the most diverse microbial ecosystems and are estimated to contain 6400–38 000 prokaryotic 'species' per gram, an order of magnitude more than in aquatic environments (Curtis *et al*., 2002; Torsvik *et al*., 2002); perhaps the result of higher spatial heterogeneity in soil environments. The high structural and chemical complexity of soil may lead to partitioning of resources and the generation of many niches capable of supporting many species. Analyses of laboratory bacterial populations in microcosms have shown that the maintenance of diversity can be attributed to spatial heterogeneity (Korona *et al*., 1994; Rainey and Travisano, 1998). Empirical analyses (Zhou *et al*., 2002; Treves *et al*., 2003) have suggested that spatial isolation provided by the soil matrix may also control diversity by restricting the free flow of resources and microbes, which in turn may limit the competition between individuals.

A second important mechanism thought to be responsible for maintaining species diversity in the presence of strong competitive interactions is ecosystem disturbance, defined as mortality caused by fluctuations in environmental conditions (Connell, 1978). According to the 'Intermediate Disturbance Hypothesis', the greatest number of species is predicted to occur at an intermediate level of disturbance (Petraitis *et al*., 1989). If disturbance is too infrequent or gentle, the ecosystem may approach equilibrium and be dominated by a few species able to outcompete all others. If too frequent or severe, only a few species resistant to the disruption may persist. Several studies have demonstrated shifts in bacterial community structure as a result of disturbance (Ferris *et al*., 1997; Nusslein and Tiedje, 1999; Ibekwe *et al*., 2002; Norris *et al*., 2002; Fierer *et al*., 2003) and intermediate disturbance can explain the maintenance of the highest biological diversity in experimental bacterial populations (Buckling *et al*., 2000). However, whether intermediate disturbance maintains the highest natural prokaryotic

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diversity has yet to be clearly demonstrated and still remains uncertain.

The Archaea are a prokaryotic domain often associated with habitats of extreme temperature, salinity or pH (Madigan *et al*., 2003). However, archaeal 16S rRNA community analysis has demonstrated that novel groups of archaea are also abundant in the open ocean (DeLong *et al*., 1994; Massana *et al*., 1997; Vetriani *et al*., 1999; Karner *et al*., 2001), soil (Bintrim *et al*., 1997; Buckley *et al*., 1998; Großkopf *et al*., 1998; Zepp Falz *et al*., 1999) and freshwater (MacGregor *et al*., 1997; Schleper *et al*., 1997) ecosystems. In hypersaline environments such as solar salterns, hypersaline lakes, the Dead Sea and underground salt deposits, halobacteria (also known as haloarchaea) are present in high abundance and are often the dominant prokaryotes (Oren, 2002).

The haloarchaea are a monophyletic group comprised of all known aerobic, obligate halophilic archaea. All are chemo-organotrophs; most utilize carbohydrates or amino acids and grow optimally between 20% and 26% NaCl (Grant *et al*., 2001). In addition, haloarchaea generally require a minimum of 9% NaCl (Grant *et al*., 2001). In this respect, many haloarchaea appear ecologically equivalent in terms of resource and physiological requirements and therefore exhibit considerable overlap in their fundamental niches, defined as the combination of conditions and resources which allow a species to maintain a viable population (Begon *et al*., 1986). However, there are notable exceptions to this observation. For example, while most haloarchaea exhibit optimal growth at near neutral pH, many are alkaliphilic and require at least pH 8.5 for growth (Grant *et al*., 2001). Recent molecular studies have detected haloarchaea in several low-salinity environments (Jurgens *et al*., 1997; Munson *et al*., 1997; Takai *et al*., 2001; Elshahed *et al*., 2004). In addition, Purdy and colleagues (2004) isolated haloarchaea from a coastal salt marsh that exhibited optimal growth at 10% NaCl but could grow slowly at 2.5% NaCl.

In this study we explore the 16S rRNA gene diversity of archaea present in hypersaline soil prone to frequent disturbance in the form of salinity fluctuations. The soil habitat under investigation is adjacent to a saline aquifer named Salt Spring, located on Saltspring Island, off the south-west coast of British Columbia, Canada (48°51′N, 123°30′W). Water slowly percolates from the spring source which has a diameter of ~5 cm. Salt Spring is associated with a major fault and the saline water probably originates from deep within (> 1000 m) the sedimentary basin (Dakin *et al*., 1983). In the summer, drought conditions produce a steep salinity gradient in the surrounding soil as moderately hypersaline water (7% NaCl) seeps from the source and evaporates within several metres. This salinity gradient is transient and the salinity at Salt Spring routinely drops below 7% NaCl due to heavy, recurrent rainfall. Analysis of soil salinity on several occasions during the winter failed to identify salt concentrations > 7% (D.A. Walsh, pers. obs.). Such a shift in environmental salinity represents a significant disturbance for haloarchaeal communities. It is possible that the decrease in salinity due to precipitation may be so severe that only a few low-salt adapted/tolerant types, perhaps similar to those reported from other low salinities (Jurgens *et al*., 1997; Munson *et al*., 1997; Takai *et al*., 2001; Elshahed *et al*., 2004), are able to persist. In contrast, fluctuations in salinity may maintain a relatively high diversity of haloarchaea as predicted under the intermediate disturbance hypothesis.

Results

Characteristics of Salt Spring soil and salinity gradient

Soil saturated with saline water emanating from Salt Spring ranged from 7% NaCl at the spring source to 18% NaCl at a distance of approximately 225 cm from the spring source. Past this range, the soil was dry and infused with salt crystals. Soil samples were collected from three sites along the salinity gradient and were labelled as 0 cm, 115 cm and 300 cm to reflect the distance of the sample from the spring source. The soil samples saturated with saline water (0 cm and 115 cm) were characterized by a top layer of soil (2–3 cm) appearing orange to brown in colour. Below this crust the soil was black in colour and had a strong sulfidic odour. Sample site 300 cm was located outside of the present spring effluent and the soil was brown in colour and lacked a sulfidic odour. The chemical composition of Salt Spring source water is reported in Table 1 and the physiochemical data for the soil at sample sites is presented in Table 2. Coinciding with the salinity gradient was a steep

Table 1. Chemical composition of Salt Spring source water.[a]

Parameter	Value[b]
Major ions	
Na^+	24 500
K^+	566
Ca^{2+}	718
Mg^{2+}	148
Cl^-	28 000
SO_4^{2-}	18 000
Minor components	
NO_3^{1-}	< 0.05
PO_4^{3-}	< 0.01
TOC	< 0.5
pH	7.2
Conductance	151 000

a. A water sample was analysed by a commercial water chemistry laboratory.
b. Major ions and Minor components are in mg l^{-1}. Conductance is in $\mu S\ cm^{-1}$. The total salinity was 71.93 g l^{-1}.
TOC, total organic carbon.

Table 2. Physiochemical data and summary of cloned archaeal 16S rRNA gene sequences from Salt Spring soil.

Soil[b]	NaCl (% w/v)	pH	No. of clones	No. of unique sequences	Phylogenetic group[a]						
					UEG-1	UEG-2	UEG-3	TpRG	Halo	Methan	Cren
0 cm	7	7	70	47	7 (12.3)	19 (39.7)	1 (1.4)	11 (30.1)	3 (7)	3 (4)	3 (5.5)
115 cm	13	9–10	130	79	0	11 (15.6)	1 (0.8)	0	63 (79.6)	0	4 (4)
300 cm	NA[c]	NA[c]	114	82	0	0	0	0	82 (100)	0	0
		Total	314	191[d]	7 (2.8)	29 (15.6)	2 (0.6)	11 (7)	133 (70)	3 (1)	6 (3)

a. Number of unique 16S rRNA gene sequences detected in the soil sample (percentage of clones). Halo, haloarchaea; Methan, *Methanosarcinales*; Cren, *Crenarchaeota*.
b. Sample name reflects distance of soil sample from spring source.
c. Soil 300 cm was dry and infused with salt crystals. The salinity and pH of the soil were not determined.
d. The number of unique sequences in each soil sample does not sum to the totals because identical unique 16S rRNA gene sequence were obtained from multiple soil samples.

pH gradient, ranging from near neutral at the spring source to highly alkaline at a distance of 225 cm from the spring. This increase in pH possibly reflects the release into the atmosphere of dissolved CO_2, initially introduced into Salt Spring water by the dissolution of carbonate (J. Thom, pers. comm.).

Construction of 16S rRNA gene libraries and identification of polymerase chain reaction (PCR)-generated chimeric sequences

Archaeal 16S rRNA gene libraries were constructed using DNA extracted from soil at 0 cm, 115 cm and 300 cm. To reduce biases associated with 16S rRNA gene community analysis (Baker *et al*., 2003), two libraries were constructed from each soil sample using different archaea-specific polymerase chain reaction (PCR) primer sets. A total of 419 clones containing 16S rRNA gene fragment inserts were sequenced. From soil samples 0 cm, 115 cm and 300 cm, we sequenced 135, 138 and 146 clones respectively. From all six libraries we identified 105 clones (~25%) as possible chimeric sequences using a combination of chimera detection software. This is consistent with the observed frequency of chimera formation reported by Wang and Wang (1997).

Estimation of soil archaeal richness

The Chao1 richness estimator (Chao, 1984; Chao *et al*., 1993) was used to estimate the total archaeal richness in Salt Spring soils. When the sample size is plotted against the estimated operational taxonomic units (OTU) for Chao1, it is clear that additional sampling would reveal additional richness at all sites (*Supplementary material*, Figure 3). Chao1 estimates that each soil sample harbours between 104 and 177 unique archaeal 16S rRNA gene sequences and ~31–52 archaeal ribogroups (Table 3). The considerable overlap of the confidence intervals for the Chao1 estimates from soils 0 cm, 115 cm and 300 cm indicates there is no significant difference in archaeal richness between soils. On the other hand, soil library diversity estimated by the Shannon-Weaver Index indicated a positive correlation between 16S rRNA sequence diversity and salinity.

Phylogenetic groups recovered from Salt Spring soil libraries

Comparison of the overall composition of 16S rRNA gene libraries from soils 0 cm, 115 cm and 300 cm by ∫-LIBSUFF (Singleton *et al*., 2001; Schloss *et al*., 2004) demonstrated the libraries from separate soil samples were

Table 3. Estimation of archaeal diversity in saline Salt Spring soil.[a]

	No. of OTUs[b]		Shannon–Weaver Index	
	16S rRNA gene sequences	Ribogroups	16S rRNA gene sequences	Ribogroups
0 cm	104 (66, 206)[c]	36 (30, 63)	3.48 (3.26, 3.71)	3.00 (2.80, 3.21)
115 cm	142 (95, 256)	31 (25, 55)	3.70 (3.52, 3.88)	2.39 (2.19, 2.58)
300 cm	177 (124, 289)	52 (43, 83)	4.13 (3.97, 4.29)	3.29 (3.11, 3.45)

a. Estimation of archaeal diversity was performed using DOTUR (Schloss and Handelsman, 2005). Columns headed with '16S rRNA gene sequences' correspond to OTUs defined as unique sequences and columns headed with 'Ribogroup' correspond to OTUs as groups of sequences exhibiting ≥ 97% identity with one another.
b. Number of total OTUs in Salt Spring soil estimated by Chao1.
c. The values in parentheses are the 95% confidence intervals.

Table 4. Comparison of 16S rRNA gene libraries.[a]

Library composition	Homologous library (X)	*P*-value of ΔC_{XY} heterologous library (Y)				
		Complete			Haloarchaea	
		0 cm	115 cm	300 cm	115 cm	300 cm
Complete[b]	0 cm	–	< 0.0001	< 0.0001	–	–
	115 cm	< 0.0001	–	< 0.0001	–	–
	300 cm	< 0.0001	< 0.0001	–	–	–
Haloarchaea[c]	115 cm	–	–	–	–	0.2885
	300 cm	–	–	–	< 0.0001	–

a. Comparisons between libraries were made using ∫-LIBSHUFF (Schloss *et al*., 2004) with 10 000 randomizations and an upper integration bound of infinity.
b. The complete libraries consisted of all the clones recovered from each of 0 cm ($n = 70$), 115 cm ($n = 130$) and 300 cm ($n = 114$).
c. The haloarchaeal communities at 115 cm ($n = 106$) and 300 cm ($n = 114$) were compared by removal of all non-haloarchaeal clones from 115 cm followed by comparison to 300 cm.

significantly ($P < 0.0001$) different from one another (Table 4). The nature of this compositional difference becomes clear upon phylogenetic analysis of the 16S rRNA gene fragments. From the 314 putatively non-chimeric 16S rRNA gene clones we found 191 unique sequences. Forty-seven, 79 and 82 unique sequences were obtained from 0 cm, 115 cm and 300 cm respectively (Table 2). We delineated groups of sequences exhibiting ≥ 97% identity as ribogroups, resulting in the identification of 68 unique ribogroups. From 0 cm, 115 cm and 300 cm we recovered 28, 21 and 35 ribogroups respectively. For each ribogroup, a single representative 16S rRNA gene sequence was included in our phylogenetic analyses. Representatives of the 68 Salt Spring ribogroups clustered within seven distinct archaeal groups. The haloarchaea were the most commonly sampled group in this study. Within the haloarchaea, we recovered 38 ribogroups (Fig. 1), encompassing 133 unique 16S rRNA gene sequences and 70% of the total clones (Table 2). We obtained 25 ribogroups (26% of the clones) that were most closely related to uncultured members of the *Euryarchaeota*. Nineteen of these ribogroups were associated with one of three archaeal groups solely comprised of environmental *Euryarchaeota* 16S rRNA gene sequences. These 'uncultured *Euryarchaeota* groups' (UEG) were distantly related to cultivated archaeal groups and have been designated as UEG-1, UEG-2 and UEG-3 in Fig. 2. We also recovered environmental 16S rRNA gene sequences representing a '*Thermoplasmatales*-related Group' (TpRG) (Fig. 2). Six ribogroups encompassing 7% of total clones clustered within TpRG (Table 2). Two ribogroups (1% of clones) were associated within the methanogenic euryarchaeal order *Methanosarcinales* and three ribogroups (3% of clones) were affiliated with the phylum *Crenarchaeota* (Fig. 1; Table 2).

Haloarchaea. There was a clear difference in the abundance and diversity of haloarchaeal sequences represented in libraries from soils 0 cm, 115 cm and 300 cm. Only 7% of clones from 0 cm were phylogenetically affiliated with the haloarchaea, while the percentage of haloarchaeal clones from 115 cm and 300 cm were 79.6% and 100% respectively (Table 2). With respect to the 38 haloarchaeal ribogroups, 3, 13 and 35 were represented at 0 cm, 115 cm and 300 cm respectively. Of the 133 unique haloarchaeal sequences, 67 and 48 were unique to the 300 cm and 115 cm sites, respectively, while 15 sequences were common to both. In fact, ∫-LIBSHUFF comparison of the haloarchaeal communities from 115 cm and 300 cm resulted in $P = 0.2885$ for $\Delta C_{(115\ cm)(300\ cm)}$ and $P < 0.001$ for $\Delta C_{(300\ cm)(115\ cm)}$ (Table 4), indicating the haloarchaeal community detected at 115 cm is a subcommunity of the haloarchaeal community detected at 300 cm (Singleton *et al*., 2001; Schloss *et al*., 2004).

The haloarchaeal 16S rRNA gene sequences recovered from Salt Spring soil were all unique (i.e. not recovered from any previously published studies) and were comprised of many lineages distributed throughout the haloarchaea. One ribogroup (ss057) was found to be distributed among the three soil samples; however, no single unique sequence was found in all three sites. Phylogenetic analysis placed ss057 within the genus *Halorubrum* (Fig. 1) and ss057 is 97–99% identical to *Halorubrum lacusprofundi*, an archaeon isolated from a 21–28% salt lake in Antarctica (Franzmann *et al*., 1988) and 99% identical to Haloarchaeon sp. DW-7, which was isolated from Salt Spring the previous winter (January, 2003). A second *Halorubrum*-related ribogroup (ss035) was detected at 0 cm and 300 cm and fell into a distantly related *Halorubrum* clade that also contained *Halorubrum coriense* (Fig. 1). The representative 16S rRNA gene sequence of ss035 was 99% identical to Haloarchaeon sp. DW-16, a second archaeon isolated from Salt Spring in January 2003.

Ten haloarchaeal ribogroups were affiliated within the Clade I haloarchaea (Fig. 1), a well-supported

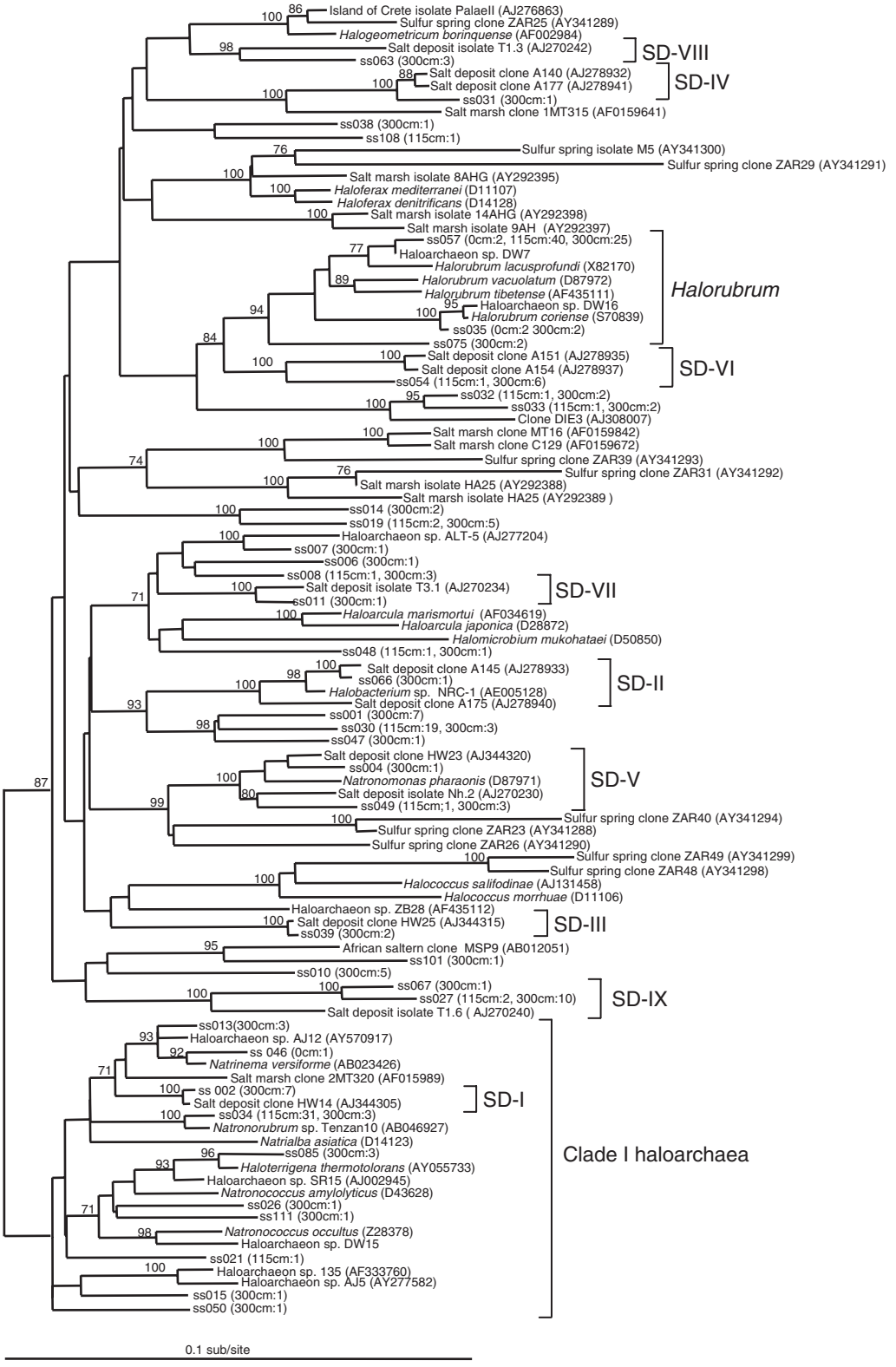

Fig. 1. Phylogenetic tree of the haloarchaea inferred from a fragment of the 16S rRNA gene, including representative sequences of the ribogroups detected in Salt Spring soil. The tree was inferred from a 16S rRNA gene sequence alignment of 893 nt positions with PAUP*4.b10 using a LogDet/paralinear distance method. Support for nodes in the tree corresponds to bootstrap values for 100 pseudoreplicates. Only bootstrap values greater than 70% are displayed. The soil sample in which representatives of the ribogroup were detected and the number of clones within the ribogroup are included in brackets within the taxon name. The tree has been arbitrarily rooted on the Clade I haloarchaea (Walsh *et al.*, 2004).

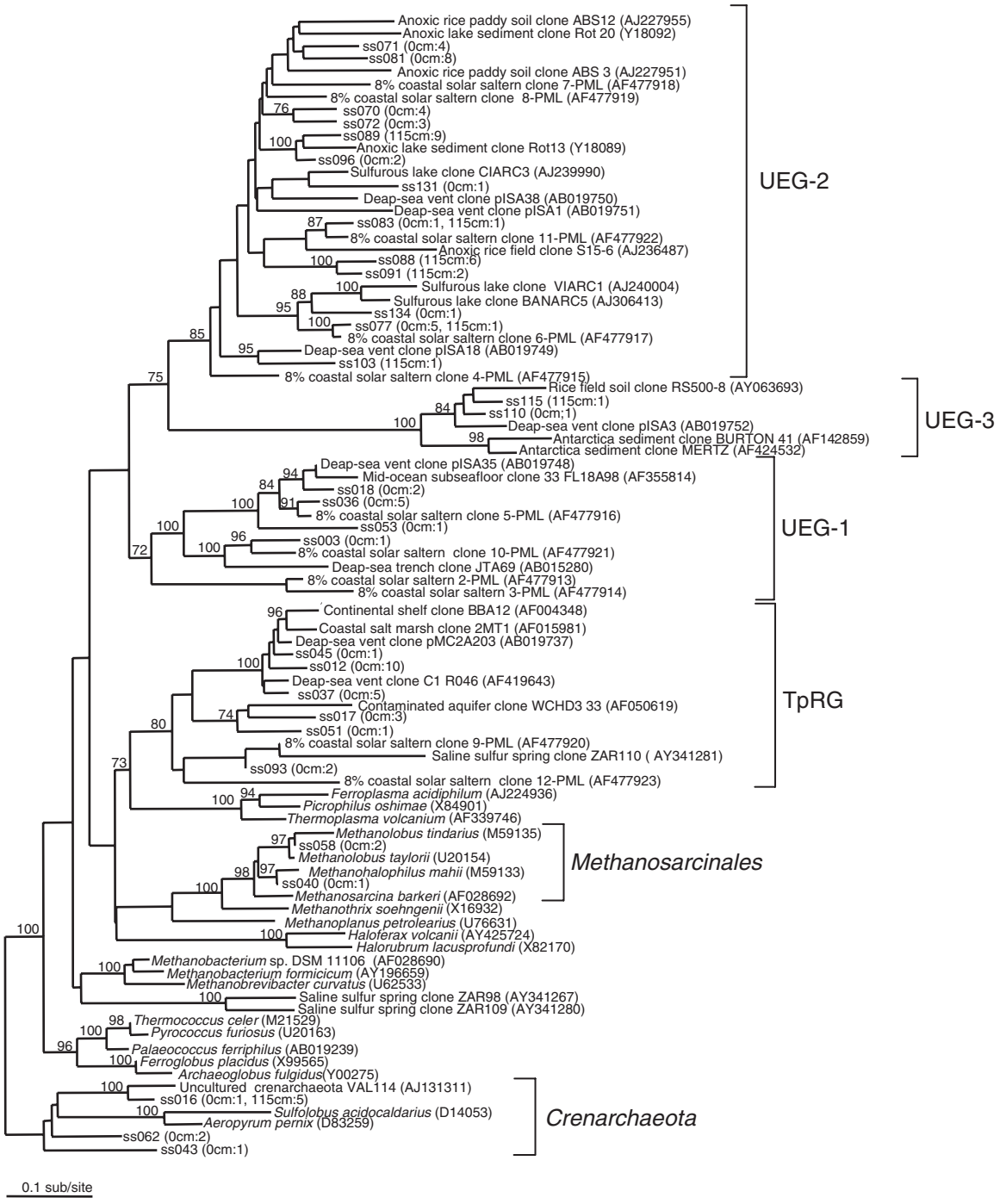

Fig. 2. Phylogenetic tree of archaea inferred from a fragment of the 16S rRNA gene, including representative sequences of the ribogroups detected in Salt Spring soil. The tree was inferred from a 16S rRNA gene sequence alignment of 868 nt positions with PAUP*4.b10 using a maximum-likelihood distance method. Support for nodes in the tree corresponds to bootstrap values for 100 pseudoreplicates. Only bootstrap values greater than 70% are displayed. The soil sample in which representatives of the ribogroup were detected and the number of clones within the ribogroup are included in brackets within the taxon name. The tree has been arbitrarily rooted on the *Crenarchaeota*.

monophyletic group that contains most of the alkaliphilic haloarchaea species (Walsh *et al*., 2004). Within Clade I, representatives of the genus *Natrinema* were obtained from 0 cm and 300 cm. A singleton sequence recovered from 0 cm (ss046) exhibited 98% identity to *Natrinema versiforme* (Xin *et al*., 2000) and a sequence from 300 cm (ss013) was 98% identical to Haloarchaeon sp. AJ12. Only a single Clade I ribogroup (ss034) was represented

at both 115 cm and 300 cm and it exhibited 98–99% identity with *Natronorubrum* sp. Tenzan-10 and 97% identity with two alkaliphilic species, *Natronorubrum bangense* and *Natronorubrum tibetense* (Xu *et al*., 1999). An additional Clade I sequence detected at 300 cm (ss085) was 98% identical to *Haloterrigena thermotolorans*, while four sequences (ss015, ss026, ss050 and ss111) from 300 cm and one from 115 cm (ss021) were 94–95% similar to various *Natronococcus* species (McGenity and Grant, 1993; Kanal *et al*., 1995).

With the exception of the genus *Halorubrum* and the Clade I haloarchaea, very few of the sequences recovered from 115 cm and 300 cm were closely related to any known genera. Eleven sequences were most closely related to 16S rRNA genes cloned previously from ancient salt deposits. Salt Spring and ancient salt deposit sequences formed nine well-supported clusters that were broadly distributed within the haloarchaeal phylogeny. These clusters are labelled as SD-I through SD-IX in Fig. 1. A comparison of the Salt Spring sequences within the nine clusters and their closest relatives in the public database is presented in Table 5. Salt Spring sequences within SD-I to SD-IV were only recovered from 300 cm and were 99–98% identical to 16S rRNA gene sequences obtained from salt deposits dating from the Permo-Triassic period (Radax *et al*., 2001; Wieland, 2001). SD-VII to SD-IX contained Salt Spring sequences that displayed 92–97% identity to 16S rRNA gene sequences of haloarchaea isolated from salt deposits dating from the late Cretaceous (McGenity *et al*., 2000).

Uncultured Euryarchaeota *groups: UEG-1, UEG-2, UEG-3 and TpRG*

From the 0 cm site we recovered 38 unique 16S rRNA gene sequences consisting of 83% of the clones that were most closely related to uncultured members of the *Euryarchaeota* (Table 2). Likewise, 12 unique 16S rRNA gene sequences recovered from 115 cm (16.4%) were most similar to uncultured *Euryarchaeota*. These 0 cm and 115 cm sequences formed 25 ribogroups, representatives of which grouped within UEG-1, UEG-2, UEG-3 and TpRG (Fig. 2). UEG-1 and TpRG were specific to 0 cm and were not detected in libraries from 115 cm or 300 cm. Within UEG-1, four ribogroups (ss003, ss018, ss036 and ss053) were retrieved from 0 cm and they exhibited 85–97% identity with 16S rRNA gene sequences obtained from an 8% NaCl coastal solar saltern (Benlloch *et al*., 2002) and a deep-sea hydrothermal vent (Takai and Horikoshi, 1999). Six ribogroups (ss012, ss017, ss037, ss045, ss051 and ss093) were grouped within TpRG and were phylogenetically affiliated with sequences obtained from marine (Vetriani *et al*., 1998; Takai and Horikoshi, 1999), terrestrial (Dojka *et al*., 1998) and moderately hypersaline habitats (Munson *et al*., 1997; Benlloch *et al*., 2002; Elshahed *et al*., 2004). UEG-2 sequences were the most abundant sequences in 0 cm libraries, and were also a minor, but significant component of 115 cm libraries (Table 2). We defined a total of 13 ribogroups that were affiliated with UEG-2. Seven (ss070, ss071, ss072, ss081, ss096, ss131 and ss134) were represented only in 0 cm libraries and four (ss088, ss089, ss091 and ss103) were represented only in 115 cm libraries. Sequences representing these 11 ribogroups shared 84–92% identity to 16S rRNA gene sequences retrieved from either anoxic rice paddy soil (Großkopf *et al*., 1998), anoxic lake sediment (Zepp Falz *et al*., 1999) or a sulfurous lake (Casamayor *et al*., 2000). UEG-2 sequences belonging to ribogroups ss077 and ss083 were recovered from both 0 cm and 115 cm and exhibited the highest similarity to environmental 16S rRNA gene sequence recovered from an 8% coastal solar saltern. Representatives of ss077 were ~96% identical to clone 6-PML and representatives of ss083 were ~92% identical to clone 11-PML (Benlloch *et al*., 2002). Only two UEG-3 clones were detected at Salt Spring, one from each of the 0 cm and 115 cm libraries. These two sequences, ss115 and ss110, shared 94% identity with each other and both were 90% identical to pISA3, a clone obtained from a deep-sea hydrothermal vent (Takai and Horikoshi, 1999).

Table 5. Sequence similarities between cloned 16S rRNA gene sequences from Salt Spring soil and sequences retrieved from ancient salt deposits.

Cluster	Ribogroup	Site	Closest match (accession No.)	Identity (%)	Salt deposit period	Reference
I	ss002	300 cm	Clone HW14 (AJ344305)	99	Permo-Triassic	Wieland (2001)
II	ss066	300 cm	Clone A174 (AJ278939)	99	Permo-Triassic	Radax *et al*. (2001)
III	ss039	300 cm	Clone HW25 (AJ344315)	99	Permo-Triassic	Wieland (2001)
IV	ss031	300 cm	Clone HW11 (AJ344308)	98	Permo-Triassic	Wieland (2001)
V	ss004	300 cm	Clone HW23 (AJ344320)	98	Permo-Triassic	Wieland (2001)
	ss049	115 cm, 300 cm	Isolate Nh.2 (AJ270230)	95–97	Permo-Triassic	McGenity *et al*. (2000)
VI	ss054	115 cm, 300 cm	Clone A151 (AJ278935)	94	Permo-Triassic	McGenity *et al*. (2000)
VII	ss011	300 cm	Isolate T3.1 (AJ270234)	97	Late Cretaceous	McGenity *et al*. (2000)
VIII	ss063	300 cm	Isolate T1.3 (AJ270242)	95–96	Late Cretaceous	McGenity *et al*. (2000)
IX	ss067	300 cm	Strain T1.6 (270240)	94	Late Cretaceous	McGenity *et al*. (2000)
	ss027	115 cm, 300 cm	Strain T1.6 (270240)	92–93	Late Cretaceous	McGenity *et al*. (2000)

Methanosarcinales. Methanogenic archaea were not detected at 115 cm or 300 cm; however, a minor fraction of clones (4%) recovered from 0 cm were most closely related to methanogens (Table 2). These sequences corresponded to two ribogroups (ss040 and ss058) that grouped within the order *Methanosarcinales* (Fig. 2), a metabolically versatile group capable of using methanol and methylamines as substrates (Boone *et al*., 2001). Both ribogroups were related to methanogens previously isolated from saline environments. The 16S rRNA gene sequence representing ribogroup ss058 was 96% identical to *Methanolobus oregonensis*, isolated from a saline, alkaline spring in Oregon (Liu *et al*., 1990), while the sequence representing ss040 was 98% identical to *Methanohalophilus mahii*, a moderate halophile capable of growth at pH 9 isolated from sediment of the Great Salt Lake (Paterek and Smith, 1988).

Crenarchaeota. Three crenarchaeal ribogroups were represented in 0 cm libraries (ss016, ss043 and ss062). Sequences from ribogroups ss043 and ss062 were 95–96% identical to sequences BBA6 (Vetriani et al., 1998) and 19C-51 (Reed et al., 2002), both of marine sediment origin. Sequences within ribogroup ss016 were obtained from 0 cm and 115 cm and exhibited ~91% identity to clone HTA-B10 recovered from a freshwater reservoir (Stein et al., 2002).

Discussion

In this study, we provide a preliminary description of a complex community of archaea associated with a transient soil salinity gradient generated by evaporation of saline spring water. Community analysis was performed on three soil samples representing the range in salinity detected at Salt Spring. The Chao1 estimation of total archaeal richness appeared to be similar between soil sites. However, the Chao1 estimate was dependent on sample size suggesting we have undersampled, and therefore underestimated the true diversity at all sites. Although the soil sites may display a similar degree of archaeal richness, statistical comparison between soil libraries using the program ∫-LIBSHUFF (Singleton *et al*., 2001; Schloss *et al*., 2004) demonstrated the genetic composition of this richness is significantly different ($P < 0.001$) between all three sites (Table 4). These results indicate a clear shift in archaeal community structure along the salinity gradient.

Comparison between soil libraries demonstrated that an increase in soil salinity was accompanied by an increase in haloarchaeal diversity and a corresponding decrease in other archaeal groups. The 300 cm libraries, which were composed solely of haloarchaeal sequences, contained the highest diversity (Table 3). However, haloarchaeal species diversity can potentially be overestimated by community analysis of the 16S rRNA gene as three haloarchaeal genera contain species that exhibit ≥ 5% divergence between 16S rRNA gene copies (Mylvaganam and Dennis, 1992; Boucher *et al*., 2004). With this caveat in mind, the recovery of over 130 unique haloarchaeal sequences representing 38 ribogroups suggests the haloarchaeal community at Salt Spring may be exceptionally diverse in comparison with other described haloarchaeal communities. For example, the Shannon–Weaver Indices of haloarchaeal diversity calculated for 22% and 32% NaCl Spanish solar salterns were between 1.003 and 1.673 (Benlloch *et al*., 2002) while our calculations for soil 300 cm at Salt Spring were between 4.13 and 3.29 depending on the degree of sequence similarity used to define an OTU (Table 3).

Many haloarchaeal ribogroups were recovered from Salt Spring soil; however, two ribogroups (ss034 and ss057) appeared to dominate the 115 cm and 300 cm libraries. Because of inherent PCR biases, the abundance of a sequence type in a 16S rRNA gene library may not accurately reflect the abundance of the organism in the environment (Suzuki and Giovannoni, 1996; Polz and Cavanaugh, 1998). However, ss034 and ss057 sequences were retrieved from multiple libraries that differed in PCR primer sets, giving us a measure of confidence that abundance in the environment may be accurate. Ribogroups ss034 and ss057 also exhibited high similarity to cultured haloarchaea and thus, the general physiology of these organisms may be tentatively inferred.

Ribogroup ss057 dominated the 300 cm site (35% of clones) and was the second most abundant ribogroup at site 115 cm (22% of clones). *Halorubrum lacusprofundi* and Haloarchaeon sp. DW-7 both fall within the ss057 ribogroup. *Halorubrum* species have never been isolated from salinities as low as 7%, yet the detection of ss057 sequences at 0 cm combined with the isolation of a strain with an almost identical 16S rRNA gene sequence during the winter indicates this ribogroup likely remains viable at Salt Spring throughout the year. In addition, the success of this ribogroup at Salt Spring may in part be a result of a broad range in growth temperature. Most haloarchaea have optimal growth temperatures between 35 and 45°C. However, *H. lacusprofundi* is cold-adapted and capable of growth at 4°C. Given the average monthly temperatures at Salt Spring for January and July are 7°C and 24°C, respectively, a haloarchaeon capable of growth at these low temperatures would have a selective advantage over its warm-adapted relatives.

In addition to salinity, pH may play an important role in shaping community structure at Salt Spring. There are currently six haloarchaeal genera containing alkaliphilic species: *Halorubrum*, *Natrialba*, *Natronobacterium*, *Natrononococcus*, *Natronomonas* and *Natronorubrum.* The

second most abundant ribogroup at Salt Spring and the dominant ribogroup at 115 cm (24% of clones) was ss034. Ribogroup ss034 was 97–98% identical with *Natronorubrum* species and is likely to be alkaliphilic itself. In addition, multiple ribogroups recovered from 300 cm and 115 cm were most closely related to *Natronococcus* species and may also represent alkaliphilic haloarchaea. Although we detected several *Halorubrum*-related species, these 16S rRNA gene sequences grouped within the neutrophilic species and are unlikely to represent alkaliphiles. The heterogeneous soil pH appears to increase haloarchaeal diversity at Salt Spring by increasing the number of realized niches, allowing the coexistence of neutrophilic and alkaliphilic haloarchaea.

The recovery of such a diversity of haloarchaea from Salt Spring was unanticipated as the majority of known haloarchaea undergo lysis at NaCl concentrations below 9% NaCl (Grant *et al.*, 2001) and the salinity of Salt Spring appears to be below this requirement for a considerable amount of the year. Perhaps, several lineages of haloarchaea at Salt Spring are adapted to or can tolerate low salinity. Purdy and colleagues (2004) have isolated three novel haloarchaeal lineages (HA Gp1, HA Gp2 and HA Gp3) from a coastal salt marsh and representatives grow at salinities as low as 3.5%. Five lineages of haloarchaea 16S rRNA gene sequences (Groups I–V; Elshahed *et al.*, 2004) have been recovered from a <1% NaCl sulfur-rich spring and Munson and colleagues (1997) have detected additional haloarchaeal 16S rRNA gene sequences in a coastal salt marsh (Fig. 2). Only a single 16S rRNA gene sequence (clone 1MT315) from either the salt marsh or the sulfur-rich spring grouped with a Salt Spring ribogroup and it formed a deep branch at the base of SD-IV (Fig. 2). In fact, the haloarchaeal communities reported by Munson and colleagues (1997), Elshahed and colleagues (2004) and Purdy and colleagues (2004) are more similar phylogenetically than either is to the community residing at Salt Spring. For example, sulfur spring 16S rRNA gene sequences reported by Elshahed and colleagues (2004) and coastal salt marsh sequences reported by Purdy and colleagues (2004) formed a well-supported group with *Haloferax* (Fig. 1). *Haloferax* species have the lowest known salt optimum and are capable of prolonged survival at 4% NaCl (Elshahed *et al.*, 2004). In addition to *Haloferax*, members of the genus *Halococcus* are known to remain intact at low salt concentrations (Rodriguez-Valera *et al.*, 1979). Although *Halococcus*-related sequences were recovered from a < 1% NaCl sulfur spring (Elshahed *et al.*, 2004), we did not detect *Halococcus* sequences in this study.

The recurrent fluctuation in soil salinity does not appear to be so severe that only a few low-salt adapted/tolerant haloarchaea are able to survive at Salt Spring. In contrast, our results suggest fluctuation in both salinity and pH may be a mechanism for generating and maintaining a diverse community of haloarchaea at Salt Spring by producing a dynamic, spatially heterogeneous environment. Assuming the majority of haloarchaeal lineages at Salt Spring can not tolerate salt concentrations ≤ 7%, disruption of the soil salinity gradient due to rainfall potentially presents a bottleneck for the haloarchaeal community, leading to mass mortality or even extinction of local haloarchael populations. In this regard, the haloarchaea present at Salt Spring can be considered a component of a metapopulation, defined as a population of unstable subpopulations inhabiting a patch of suitable habitat bordered by unsuitable habitat (Hanski, 1998). The decline in salinity is unlikely to result in extinction of the entire haloarchaea community and many haloarchaea may survive periods of reduced salinity in microniches of higher salinity maintained by the heterogeneous nature of the soil environment. However, we speculate that migratory haloarchaea readily recolonize Salt Spring if the indigenous haloarchaeal population size is reduced after the yearly rainy season. The high sequence similarity between haloarchaea at Salt Spring and haloarchaea from some ancient salt deposits raises the intriguing possibility that one source of colonizers may be the subsurface salt reservoir through which Salt Spring water permeates.

Experimental procedures

DNA extraction

DNA was extracted from sediment samples by a CTAB/SDS-based freeze–thaw method as described by Zhou and colleagues (1996). The nucleic acid was finally precipitated by addition of 2.5 volumes of absolute ethanol and 0.1 volumes 3 M sodium acetate and resuspended in 10 mM TE buffer. A 30 μl aliquot was electrophoretically separated on an agarose gel (1% low melt agarose) and high-molecular-weight (> 40 kb) DNA was recovered from the agarose using GELase (EPICENTRE) according to manufacturer's instructions.

Clone library construction, screening and sequencing

A fragment of the 16S rRNA gene was amplified by PCR from the purified environmental DNA in a 25 μl reaction volume containing 2.5 μl of 10× *Taq* PCR buffer, 0.2 mM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 0.5 μM each primer, 0.6 U *Taq* DNA polymerase and 1 μl of DNA. Two separate PCR reactions, differing in primer sets, were performed for each DNA sample. The thermal profile for amplification using forward primer 344F (5′-ACG GGG YGC AGC AGG CGC GA-3′) and reverse primer 915R (5′-GTG CTC CCC CGC CAA TTC CT-3′) (Benlloch *et al.*, 2002) was: one cycle of 94°C for 5 min, 25 cycles of 94°C for 20 s, 52°C for 45 s and 72°C for 2 min, followed by a final extension at 72°C for 10 min. The thermal cycling profile for forward primer 21F (5′-TTC CGG TTG ATC CTG CCG GA-3′) and 958R (5′-YCC GGC GTT GAM TCC AAT T-3′) (Schrenk *et al.*, 2003) was the same as

above except the annealing temperature was 55°C. Aliquots (3 μl) of the PCR reaction were observed by agarose (1%) gel electrophoresis to confirm primer specificity.

The PCR products were cloned directly using a TOPO TA Cloning Kit (Invitrogen) using pCR 2.1-TOPO as a vector. Transformants were analysed for insert by colony PCR using primer set M13F/M13R as described in the TOPO TA Cloning Manual. Positive transformants were sequenced using MegaBase Technology and BigDye chemistry using M13F/M13R. These sequence data were submitted to the EMBL nucleotide database as accession# AJ969741 to AJ969931.

16S rRNA gene library analysis

The RDP CHIMERA-CHECK (Maidak *et al*., 2001) and BELLEROPHON (Huber *et al*., 2004) programs were used to detect PCR-generated chimerical sequences. Suspected chimerae were not included in the final phylogenetic analysis. 16S rRNA gene sequences were compared with the nucleotide database using GeneMatcher from the Canadian Bioinformatics Resource (http://cbr-rbc.nrc-cnrc.gc.ca/services/genematcher_e.php) to determine their approximate phylogenetic position.

The Chao1 and Shannon–Weaver Index diversity estimators were calculated using the software program DOTUR (Schloss and Handelsman, 2005) using the default settings. Statistical comparison of 16S rRNA gene libraries was performed using the software program ∫-LIBSHUFF (Singleton *et al*., 2001; Schloss *et al*., 2004) with 10 000 Monte Carlo permutations and a significance of $P < 0.001$. The distance matrices required for these programs were calculated from 16S rRNA gene alignments (~500 nt) using DNADIST with a Jukes–Cantor distance correction implemented in PHYLIP3.6a (Felsenstein, 2000).

Sequence representatives of the ribogroups were aligned with sequences retrieved from GenBank using CLUSTALW (Thompson *et al*., 1994) and edited manually to remove gaps and ambiguously aligned characters. Phylogenetic analysis was performed with PAUP*4.b10 (Swofford, 1998) using either LogDet/paralinear distance (Fig. 1) or maximum-likelihood distance (Fig. 2) and the tree-bisection-reconnection branch-swapping algorithm. The support for each node was determined by assembling a consensus tree of 100 bootstrap replicates using the same phylogenetic settings.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Chao1 estimated OTU richness of archaea in Salt Spring soils in relation to sample size plotted for 0 cm (circles), 115 cm (triangles) and 300 cm (squares) when (A) OTUs are defined as unique 16S rRNA gene sequences and (B) OTUs are defined as clusters of sequences exhibiting ~97% identity.