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Culture-dependent and -independent methods reveal dominance of halophilic *Euryarchaeota* in high-altitude Andean lakes

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ABSTRACT: The hypersaline and extreme environments of the so-called high-altitude Andean lakes in the dry central region of the Andes are considered pristine ecosystems of biotechnological interest. By using culture-dependent and -independent methods, we aimed to describe the phylogenetic affiliation and ecological importance of *Archaea* thriving in extreme, hypersaline lakes and salt flats in the Argentinean and Chilean Puna. For this purpose, water and sediment samples were collected from 14 lakes and salt flats. Denaturing gradient gel electrophoresis (DGGE) indicated that representatives of the phylum *Euryarchaeota* dominated the microbial community in all samples. Most of the DGGE bands were assigned to the family *Halobacteriaceae*, while the rest corresponded to *Methanocaldococcaceae*, *Methanobacteriaceae*, *Methanococcaceae*, *Methanosarcinaceae*, and *Methanothermaceae*. In addition, isolation procedures yielded single colonies of 53 pure isolates belonging to the following genera: *Haloarcula, Halomicrobium, Halopiger, Halorubrum, Natrialba, Natrinema, Natronorubrum*, and *Natronococcus*. All strains proved to be polyextremophiles, with high tolerance to NaCl, UV-B radiation, and high arsenic concentration.

KEY WORDS: Archaea · Extremophiles · Halophiles · Arsenicv · UV · Andean Lakes · Puna

INTRODUCTION

Halophilic and highly halotolerant species are found in all 3 domains of life. Halophilic archaea are typically the dominant microbial group found in hypersaline or alkaline environments such as soda lakes, solar salterns, and hypersaline environments of anthropogenic origin (Ochsenreiter et al. 2002). Particulary, the *Euryarchaeota* contain diverse groups of halophilic *Archaea*, including halophilic methanogenic *Archaea* (Xu et al. 2016). Among the *Euryarchaeota*, the extremely halophilic *Archaea* belong to the class *Halobacteria* with 3 orders, *Halobacteriales*, *Haloferacales*, and *Natrialbales* (Gupta et al. 2015). Using denaturing gradient gel electrophoresis (DGGE), new halophilic archaeal phylotypes were identified in marine salterns (Antón et al. 1999), soda lakes (Grant et al. 1999, Ochsenreiter et al. 2002), Antarctic lakes (Bowman et al. 2000), coastal salt marshes (Munson et al. 1997), and in ponds from potassium mines (Ochsenreiter et al. 2002).

Halophilic *Archaea* display particular physiological traits; they can be either aerobic or anaerobic chemoorganotrophs, and they can use nitrate,

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dimethyl sulfoxide, trimethylamine-N-oxide, and fumarate as electron acceptors (Oren & Trüper 1990, Oren 1991, 2006, Müller & DasSarma 2005, Liu et al. 2012). In addition, *Halobacterium* has the capability of growing photoheterotrophically in microaerophilic conditions, using bacteriorhodopsin as a light-driven proton pump (Ochsenreiter et al. 2002).

Life under high salt concentrations demands specific metabolic adaptations. Halophilic *Archaea* store potassium intracellularly in order to maintain balance with the external salt concentration (Ochsenreiter et al. 2002, Oren 2013), whereas halophilic *Bacteria* and *Eukarya* generate solutes (betaine, ectoine) and polyols (glycerol) for the same purpose (Ochsenreiter et al. 2002, Roberts 2005). Moreover, the enzymes of halophilic *Archaea* and many halophilic *Bacteria* present an excess of acidic over basic residues, which charge them negatively as required for functioning properly on high salt concentration up to 4–5 M (Karan et al. 2012, DasSarma & DasSarma 2015).

Haloarchaea are also able to grow under toxic arsenic concentrations in natural ecosystems or under laboratory culture conditions (Wang et al. 2004, Volant et al. 2012, Li et al. 2014, Rascovan et al. 2016). Arsenic salts containing arsenate (As[V]; predominantly H_3AsO_4 at low pH) are highly toxic, as they structurally mimic phosphate and impede many physiological processes and in particular the production of ATP (Dopson et al. 2003). The toxicity of arsenite (As[III]; $As[OH]_3$) is even higher due to its strong affinity for sulfhydryl groups in proteins (Saha et al. 1999), and in addition, it depletes intracellular glutathione, resulting in cytosol oxidation and free radical production.

The hypersaline and extreme environments of the so-called high-altitude Andean lakes (HAALs) in the dry central region of the Andes are an important source for the isolation of novel *Archaea* (Fernández Zenoff et al. 2006a, Flores et al. 2009, Albarracín et al. 2015, 2016). These ecosystems are exposed to aridic to semi-aridic climates and host diverse saline and hypersaline lakes as well as saline deposits, some of them unique in the world, that form evaporitic basins or salt flats (Dorador et al. 2010). The combination of both arid climate and closed basins provides a high rate of evaporation, increasing the salt content (Davila et al. 2008). In addition to the hypersaline conditions, HAALs feature high arsenic concentrations and are exposed to high UV radiation (UVR) levels (Albarracín et al. 2015, 2016). UVR is a limiting factor for planktonic and microbial soil communities in these ecosystems, because UV-B levels at doses higher than 200 J m^{-2} cause cell damage (Visser et al. 2002, Fernández Zenoff et al. 2006a,b, Flores et al. 2009, Albarracín et al. 2015, 2016).

Based on the 'salty' nature of HAALs, a great diversity of halophiles has been documented, suggesting that these areas are reservoirs of yet unidentified microbial taxa with great biotechnological potential (Flores et al. 2009, Dorador et al. 2010, Albarracín et al. 2015, 2016). Nevertheless, most previous studies have focused on bacterial diversity, giving less attention to *Archaea*, which are expected to dominate the populations of these hypersaline environments (Dorador et al. 2010, Rascovan et al. 2016). By using culture-dependent and -independent methods, we aimed to describe the phylogenetic affiliation and ecological importance of *Archaea* thriving in the extreme, hypersaline lakes and salt flats of the HAAL area of the Argentinean and Chilean Puna. In addition, we compared our results to the archaeal diversity of other high-altitude environments in the region and other similar environments around the world.

MATERIALS AND METHODS

Sampling

Water and sediment samples were collected from 11 lakes and 3 salt flats, locally called 'laguna' (L.) and 'salar' (S.), respectively: L. Antofalla (LA), L. Brava (LB), L. Chiro (LC), L. Diamante (LD), L. Socompa (LS), Ojos de Mar Tolar Grande (OMTG), L. Vilama (LV), L. Negra (LN), L. Azul (LAZ), L. Tebenquiche (LT), L. Cejar (LCJ), S. del Hombre Muerto (SHM), S. Llullaillaco (SL), and S. Pocitos (SP) (Fig. 1, see Table 1). We chose environments located between 3350 and 4700 m above sea level (a.s.l.) in the High Andean Puna Ecoregion in Argentina and Chile. Water samples were collected from the surface in 1.5 l plastic sterile bottles. Sediment samples were taken with a shovel and directly stored in sterile plastic bags. These samples were then kept at 4°C until further processing in the laboratory (within 24 h after collection).

Media and enrichment cultures

The aerobic medium used for enrichment and isolation was WJK medium (pH 8.0) (Maldonado 2015), containing (in g l^{-1}): NaCl (252.16), $MgCl_2{\cdot}6H_2O$ (0.5), $MgSO_4{\cdot}7H_2O$ (0.5), KNO_3 (1.011), KCl (5.84),

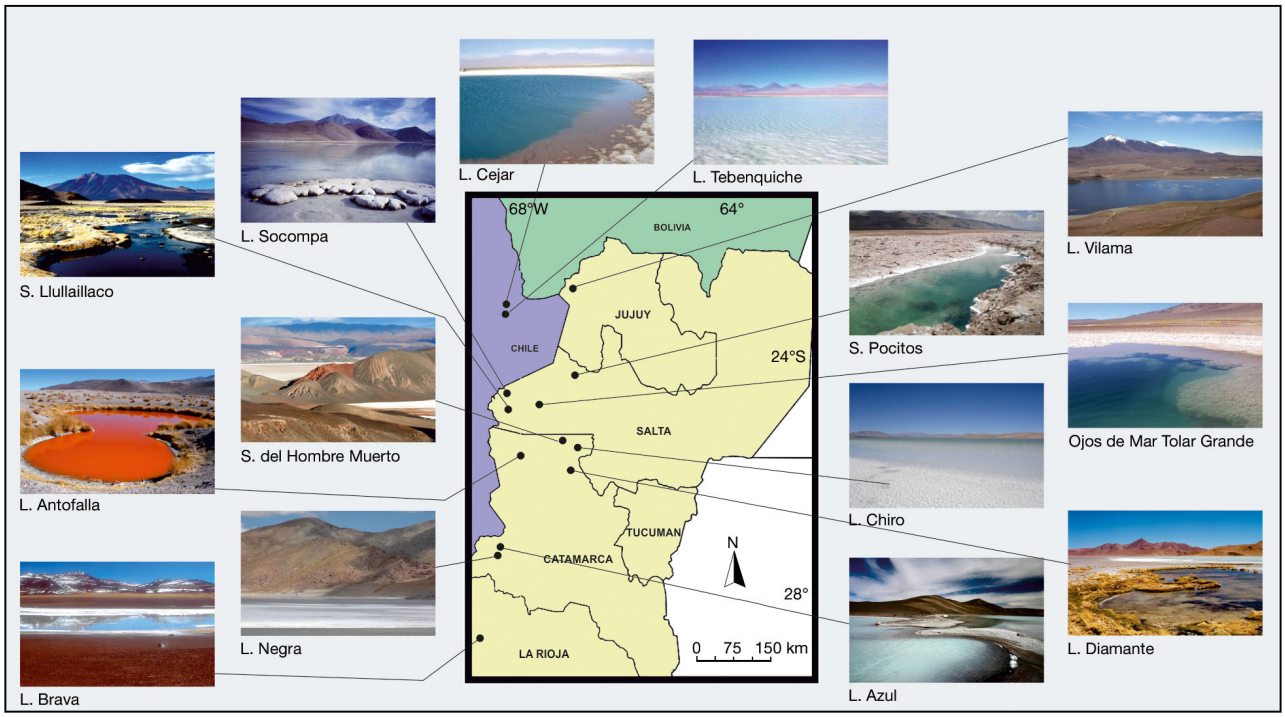

Fig. 1. Geographic location of the studied high-altitude Andean lakes (L.) and salt flats (S.)

peptone (Oxoid) (5), and yeast extract (Oxoid) (1), as well as trace element solution 1 ml (HCl 25%, 7.7 M, 10 ml; $FeCl_2$ 1.5 g; $ZnCl_2$ 70 mg; $MnCl_2 \cdot 4H_2O$ 100 mg; H_3BO_3 6 mg; $CoCl_2 \cdot 2H_2O$ 190 mg; $CuCl_2 \cdot 2H_2O$ 2 mg; $NiCl_2 \cdot 6H_2O$ 24 mg; $Na_2MoO_4 \cdot 2H_2O$ 36 mg; H_2O 1 l). For agarized media, 1.45% agar (Oxoid) was used.

For the isolation procedure, 1 ml of water samples or 5 g of sediment samples were pre-inoculated in fresh WJK medium and left for enrichment over 12 d (37°C; shaken at 160 rpm). An aliquot (100 µl) of each enriched sample was plated on agar media at the beginning and after 12 d of enrichment. The plates were incubated aerobically at 37°C for 12 d. Pure colonies were streaked onto agar plates, and representative colonies were transferred to fresh liquid medium and grown in a shaker at 37°C (160 rpm). The cultivation conditions chosen in this study did not select for methanogens (Bräuer et al. 2006).

Phenotypic characterization

Colony morphologies and pigmentation were determined on agar plates after 14 d of growth. Cell morphology and motility were examined on freshly prepared wet mounts or after Gram staining under a light microscope (XSZ-107 BN ARCANO®). Cell staining was carried out as described by Dussault (1955).

Assays were also conducted in order to determine the pH growth range, salinity and arsenic tolerance. To this end, strains were grown in WJK medium, the pH range was evaluated with WJK medium at pH 3, 5, 8 and 10, incubated at 37°C with constant shaking (160 rpm); in identical conditions, they were grown in increasing concentrations of NaCl (5, 10, 20, and 30%). Arsenic tolerance was determined by growing the isolated strains at different arsenic concentrations: 100, 150, 200, and 400 mM of As[V] in the form of sodium arsenate dibasic heptahydrate ($Na_2HAsO_4 \cdot 7H_2O$) and 2.5, 5, 7.5, and 10 mM of As[III] in the form of sodium arsenite ($NaAsO_2$). In all cases, growth was recorded by measuring OD_{600} of culture aliquots at intervals of 12 h for 5 d. Specific growth rates were calculated using the formula:

$$\mu = (\ln OD_{600}t_2 - \ln OD_{600}t_1)/t_2 - t_1;\ t_2 > t_1 \qquad (1)$$

where $\ln OD_{600}t_2$ and $\ln OD_{600}t_1$ are the natural logarithm at time 1 (t_1) and time 2 (t_2), respectively. t_1 and t_2 are the extremes of exponential phase in the corresponding growth curve.

The effect of UV-B exposure on the strains was tested by growing in WJK medium (37°C, 160 rpm) until exponential phase (OD_{600} = 0.8). Aliquots (5 µl) of serial dilutions of the preculture (10^{-1} to 10^{-7}) were inoculated in drops on agar plates. The plates were exposed to UV-B radiation for 6, 12, and 24 h using

2 lamps (09815-6 lamps, Cole Parmer Instruments, major emission line at 312 nm), and the temperature was controlled at 37°C. UV-B lamps were covered with acetate film to block out the UV-C radiation. UV-B doses were quantified using a radiometer (09811-56, Cole Parmer Instruments) at 312 nm with an intensity I = 0.7 W m^{-2}, and the doses were 16.53 kJ m^{-2} at 6 h, 33.06 kJ m^{-2} at 12 h and 66.11 kJ m^{-2} at 24 h. For a control, the same experiment was performed in the dark. The plates were incubated upside down in the dark to prevent photoreactivation for 12 d at 37°C.

DNA extraction

Environmental DNA was extracted from water and sediment samples from each site. Water samples (200 ml) were filtered on-site in a 0.22 µm pore-size filter, and DNA was extracted from the filters using the CTAB method (Fernández Zenoff et al. 2006b). For the sediment samples, 5 g were weighed in a sterile Erlenmeyer flask, and 10 ml of WJK medium and 500 µl 20 Tween (100%) were added. Samples were agitated in a shaker at 37°C (180 rpm) for 1 h and then centrifuged at 671 × g (10 min). The supernatant was filtered using polycarbonate Whatman filters of 47 mm diameter (0.22 µm pore size). After that, the DNA was extracted from the retained community in the filters (total DNA), using a modified CTAB method (Bailey 1995, Fernández-Zenoff et al. 2006a).

The CTAB method was similarly applied to obtain pure DNA from cultures of isolated strains grown on WJK medium for 12 d at 37°C and harvested by centrifugation (3000 × g for 10 min at 4°C).

PCR amplification of 16S rRNA genes and DGGE analysis

Archaeal 16S rRNA genes were amplified in an XP Thermal Cycler Block XP-A (Bioer). From cultivated organisms, DNA fragments of 591 bp from V3 to V5 variable regions of the 16S rRNA gene with *Archaea*-specific primers 344F (5′-ACG GGG YGC AGC AGG CGC GA-3′) and 915R (5′-GTG CTC CCC CGC CAA TTC CT-3′) were obtained (Yu et al. 2008). The 344F primer contained an additional 40 nucleotide GC-rich sequence (GC clamp) at its 5′-end in order to maintain stable melting behavior during DGGE (the GC sequence is 5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3′) (Stahl & Amann 1991, Top 1992, Muyzer et al. 1993, Casamayor et al. 2000). Each PCR reaction (25 µl) contained 20 ng template DNA, 0.25 µM of oligonucleotides, 200 µM dNTPs, and 1.5 U recombinant *Taq* DNA polymerase (Invitrogen). To amplify the archaeal genes, a touchdown protocol for 20 cycles with temperatures ranging from 71 to 61°C was used; the annealing temperature was reduced 1°C every 2 cycles, and then PCR was continued for 15 additional cycles at an annealing temperature of 61°C. Except for the initial denaturation step (94°C, 5 min), denaturation and annealing phase steps were 1 min in length, while most of the polymerization phase steps were 3 min; the only exception was the final cycle, which was 10 min long (Casamayor et al. 2000).

PCR products were verified by 0.8% agarose gel electrophoresis using a 100 bp DNA ladder (Genbiotech) as a molecular marker. Amplified 16S rRNA gene fragments from environmental samples were analyzed by DGGE in 5 different gels (see Fig. 2), using a Dcode™ Universal Mutation Detection System (Bio-Rad Laboratories). PCR products were separated on an 8% polyacrylamide gel, with a linear gradient of the denaturants urea and formamide increasing from 20% at the top of the gel to 70% at the bottom. PCR product (20 µl) was loaded for each sample, and the gel was run at 120 V for 4.5 h at 60°C. After the electrophoresis, the gel was incubated in the dark for 15 min in 1× TAE containing SYBR® Gold (Invitrogen) and visualized under UV in a Gel Doc™ 2000 Gel Documentation System (Bio-Rad) with the Quantity-One Analyst software (Bio-Rad).

Individual DGGE bands were excised from the gel with a sterile scalpel. Each piece was transferred into 30 µl of sterile water and incubated overnight at 4°C to allow diffusion of DNA. Eluted DNA was re-amplified using 344-F and 915-R primers with identical conditions as before.

Cluster analysis of DGGE bands

Conventional culture-dependent methods are inadequate for the analysis of microbial communities in natural environments, especially for extremophilic archaea (Ward et al. 1990). To overcome this situation, DGGE was applied to study the structure and composition of dominant members of the archaeal communities in different hypersaline environments (Muyzer et al. 1993, Yeates et al. 1998, Read et al. 2011). However, the technique has resolution limitations (Eichner et al. 1999, Nicol et al. 2003). One organism may produce several bands because of multiple heterogeneous rRNA operons (Cilia et al.

1996, Rainey et al. 1996). Moreover, partial 16S rDNA sequences do not always allow discrimination among species, as 1 band may represent more than 1 species with identical partial 16S rDNA sequences (Vallaeys et al. 1997).

The DGGE gel image analyses were performed with the Cross Checker program (version 2.91, Plant Breeding, Wageningen University and Research Center, The Netherlands) to analyze the archaeal community structure.

Each band in the DGGE profile was considered a different operational taxonomic unit (OTU), and the band intensity indicated the relative abundance of each OTU within the sample. Bands with equivalent positions in different wells corresponded to the same OTU. Consequently, the same taxonomic affiliation was assigned to the non-sequenced bands positioned at the same level as those sequenced. For this reason, in some cases the number of OTUs per sampled site was higher than the number of bands sequenced.

To determine the content of the band pattern information (similarities and differences) in terms of structural diversity of the sampled sites, a binary matrix was constructed and a cluster analysis was performed and visualized in a dendrogram for the 5 different gels. The cluster analysis was applied to the obtained images from the 5 gels with the statistical software InfoStat version 2.0, using the weighted pair group method with arithmetic mean (WPGMA) method (simple matching (sqrt 1-S), where S is a similarity coefficient), which was based on the presence or absence of bands at positions in each lane of the gel (Di Rienzo et al. 2013).

16S rRNA gene sequencing and phylogenetic analysis

Purified amplicons obtained as previously described were subjected to DNA sequencing. The dideoxy chain termination method was employed with an ABI Prism 3730XL DNA analyzer, using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reactions Kit (PE Biosystems) according to the manufacturer's protocol.

Phylogenetic trees were constructed by the neighbor-joining method and evaluated by bootstrap sampling (1000 replicates) using the MEGA 4.1 program (Kumar et al. 2008). Bands and isolates were identified using the EzTaxon-e server (www.ezbiocloud.net) and NCBI server (https://www.ncbi.nlm.nih.gov), respectively, with 16S rRNA gene sequence data. The 16S rRNA gene sequences reported in this paper were deposited in GenBank under accession numbers (see Table 2).

Sequence data were verified using Chromas 1.55 and aligned using DNAMAN Version 4.03, and 16S rDNA sequences were aligned using ClustalW (Thompson et al. 1994).

RESULTS

Physico-chemical properties of selected sites

A summary of the geographical location, altitude, and other parameters of the studied systems is shown in Table 1. Due to the low latitude and high altitude (Fig. 1), the Puna region is exposed to extreme levels of solar radiation (Luccini et al. 2006). Accordingly, we were able to detect high *in situ* UV-B levels (in the range of 280–312 nm), up to 8.94 W m^{-2}. All of the lakes are shallow except for OMTG (up to 800 cm depth), which suggests that even benthic communities are exposed to considerable UVR doses. In addition, conductivity was measured in all water samples where the values ranged from 9 to 22 S m^{-1}, except from LC. Assuming a similar saline composition to seawater, the salinity was calculated (Table 1). All lakes except LC are true brines, i.e. they displayed much higher salinity (3- to 8-fold) than that of seawater (3.5%). Most of the studied lakes had a pH in the alkaline range (8–11). These data reveal that the sampled wetlands are alkaline and typical hypersaline environments. In addition, they have a high content of dissolved arsenic (Table 1), features suggesting that microbes inhabiting these lakes are polyextremophiles.

Cluster analysis

DGGE profiles were compared based on the presence, precise position, and relative intensity of bands. A cluster analysis (WPGMA) of DGGE bands corresponding to 16S rRNA gene fragments (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/a081p171_supp.pdf) was used to compare profiles of archaeal communities from different lakes and different type of samples (water/sediments).

The cluster analysis obtained from the LV, SHM, and LS sites showed that sediment sequences clustered together while those of LB water formed an outgroup, indicating differences in archaeal biodiversity between sediment and water samples (Fig. 2a). In turn, the cluster analysis obtained from the SHM sed-

Table 1. Physico-chemical characteristics of the aquatic ecosystems under study including information on the type of samples taken and the number of isolates or DGGE bands sequenced per sample. Abbreviations are Laguna Antofalla (LA), Laguna Brava (LB), Laguna Chiro (LC), Laguna Diamante (LD), Laguna Socompa (LS), Ojos de Mar Tolar Grande (OMTG), Laguna Vilama (LV), Laguna Negra (LN), Laguna Azul (LAZ), Laguna Tebenquiche (LT), Laguna Cejar (LCJ), Salar del Hombre Muerto (SHM), Salar Llullaillaco (SL), and Salar Pocitos (SP). Sediment (S); Water (W). ND: no data

Wetland	LA	LB	LC	LD	LS	OMTG	LV	LN	LAZ	LT	LCJ	SHM	SL	SP
Global position	25°39′S 67°42′W	28°19′S 68°51′W	25°32′S 66°55′W	26°00′S 67°02′W	24°32′S 68°12′W	24°37′S 67°22′W	22°36′S 66°55′W	27°39′S 68°33′W	27°34′S 68°32′W	23°08′S 68°15′W	23°03′S 68°13′W	25°27′S 67°04′W	24°51′S 68°16′W	24°23′S 66°59′W
Depth (cm)	10	75	15	20	20–100	300–800	20	20	100	40	>100	15	20	50
Altitude (m a.s.l.)	3350	4300	4050	4580	3600	3510	4500	4400	4400	2320	2350	4000	3800	3660
Temp (°C)	18	ND	ND	14	14	14	8.5	ND	ND	25.3	35.4	ND	15	ND
pH	8.5	8.7	7.5	11	9	6.5	7.1	6.8	7.5	8.6	7.90	8.5	8.5	8
Arsenic[a]	ND	33.6	ND	117	18,5	0.88	11.8	3	0.8	4	ND	ND	<0.01	<0.01
Salinity (%)	25	20	3.55	19.4	17.0	17.5	11.7	19.5	5	22	21	27	9.6	22.6
Conductivity (S m^{-1})	22	19.8	3.3	17.4	11.5	16.9	9.5	17.6	4.64	21.27	20.38	22.1	9.17	21.4
Number of identified DGGE bands	0/9	3/11	3/16	0/26	6/21	15/22	13/22	1/1	4/9	1/3	6/12	26/111	0/7	0/9
Type of Sample (DGGE bands)	W (9)	W (11)	W (12) S (4)	W (13) S (13)	W S (21)	W (22) S	S (22)	W (1)	W (9)	W (3)	W (12)	W (80) S (31)	W (7)	W (9)
Number of isolates	18	0	0	3	4	13	0	0	0	0	0	5	5	5

[a]mg l^{-1} for water samples; mg kg^{-1} for sediment samples

iment and from SHM water (4 different points) clustered together with sequences of LC water forming an outgroup, indicating differences in community structure between the 2 lakes (Fig. 2b). The cluster analysis obtained from SHM sediment, LV sediment, LC sediment, LCJ water, LT water, LN water, LAZ water, and SHM (4 different points) water, showed no obvious clusters that could be identified based on sample origin (Fig. 2c). DGGE bands from OMTG sediments (3 different points) clustered together and LS sediment formed an outgroup (Fig. 2d). Furthermore, DGGE bands from LD sediment, LA water, LD water, SL water, and SP water clustered together. A sub-cluster from LD indicted the similarity in the profiles between the 2 samples (water and sediment) from the same lake (Fig. 2e).

Cluster analysis revealed that the archaeal community structure was diverse, and no consistent differences were observed between water and sediment samples, confirming spatial heterogeneity despite the similar and extreme physico-chemical conditions of the lakes.

Occurrence and phylogenetic diversity of uncultured archaea

DGGE analyses of samples from all lakes produced 279 different 16s rRNA gene amplified products (bands) which were re-amplified and sequenced. Only 78 bands gave significant sequence information sufficient for comparison with public databases, i.e. GenBank and the Ez-taxon server (Table 2). BLAST showed that more than 70% of the DGGE band sequences exhibited a similarity value below 97% with their closest relatives in GenBank (environmental or cultured strains), which could indicate novelty of these uncultured strains.

All DGGE band sequences were assigned to the phylum *Euryarchaeota*, with most of them belonging to the class *Halobacteria*, family *Halobacteriaceae* (81% of the bands). In general, the archaeal communities were dominated by a few genera like *Halorubrum* (61%), *Natrialba* (16%), and *Haloarcula* (9%), but only few methanogens were identified. Only 14 OTUs were assigned to methanogenic *Archaea*: these included members of the classes *Methanobacteria* (5 bands in LV and LS), *Methanococci* (7 bands in LS, LV, LB, and SHM), and *Methanomicrobia* (2 bands in LS), including the following families: *Methanocaldococcaceae* (6%), *Methanobacteriaceae* (5%), *Methanococcaceae* (4%), *Methanosarcinaceae* (3%), and *Methanothermaceae* (1%) (Fig. 3).

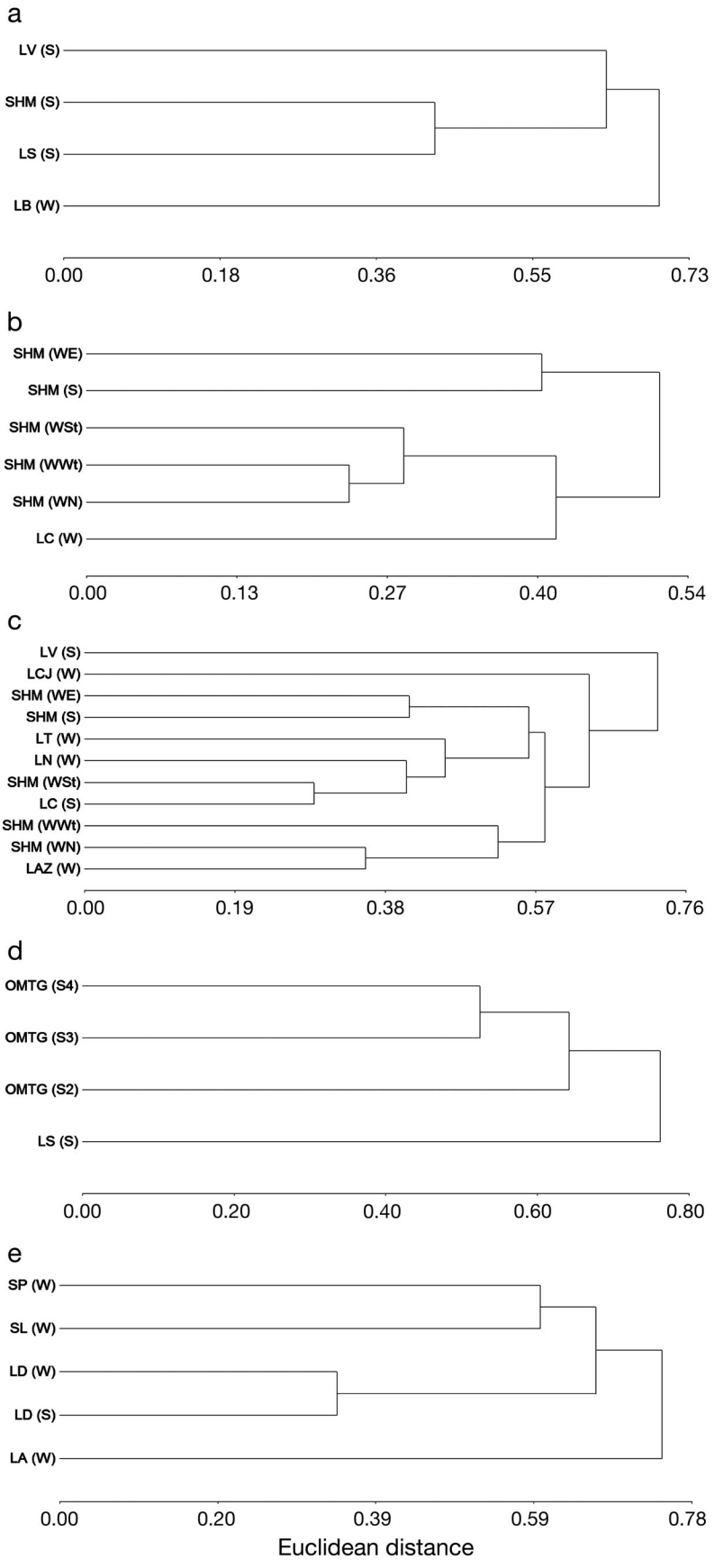

Fig. 2. Cluster analysis of the archaeal DGGE profiles from high-altitude Andean lakes based on the 16S rRNA gene. The dendrograms were calculated on the basis of the weighted pair group method with arithmetic mean (WPGMA). Abbreviations are Laguna Vilama (LV), Salar del Hombre Muerto (SHM), Laguna Socompa (LS), Laguna Brava (LB), Laguna Chiro (LC), Laguna Cejar (LCJ), Laguna Tebenquiche (LT), Laguna Negra (LN), Laguna Azul (LAZ), Ojos de Mar Tolar Grande (OMTG), Salar Pocitos (SP), Salar Llullaillaco (SL), Laguna Diamante (LD), and Laguna Antofalla (LA). S: sediment, W: water, S2: sediment 2, S3: sediment 3, S4: sediment 4, WE: water east, WSt: water south, WWt: water west, WN: water north

Interestingly, 10 different genera were detected from 2 sampled sites: LV showed representatives of *Halovivax*, *Natrinema*, *Halobellus*, *Natronorubrum*, *Halaarchaeum*, *Halorubrum*, *Methanobrevibacter*, *Methanothermobacter*, *Methanothermococcus*, and *Methanothermus*, and SHM displayed sequences of *Halorubrum*, *Haloasta*, *Halonotius*, *Halobellus*, *Haloarcula*, *Natronorubrum*, *Natrinema*, *Haloterrigena*, and *Methanococcus*. In OMTG, sequences were retrieved for 15 out of 17 bands, which were affiliated with 4 different genera: *Natrialba*, *Natrinema*, *Natronorubrum*, and *Halobiforma* (Fig. 3) A phylogenetic tree (Fig. 4) was constructed using 16s rRNA gene sequences of DGGE bands, with the closest taxonomic neighbor found in the Ez-taxon database, and using NCBI for reference strains.

Identification and characterization of cultivable *Archaea*

Sample enrichments were transferred to agar plates with WJK medium. After 12 d, single colonies of 53 pure isolates were obtained (Table 3). Despite all efforts, we were unable to obtain any *Archaea* from sediment and/or water samples collected from LB, LC, LV, LN, LAZ, LT, and LCJ. However, the culture-independent technique we used in this work indicated the presence of archaeal sequences in those lakes (Table 2).

According to their 16S rRNA gene sequences, all isolates belonged to the phylum *Euryarchaeota*, family *Halobacteriaceae* and the following genera: *Haloarcula*, *Halomi-*

Table 2. Identification number of the operational taxonomic units (OTUs) from DGGE bands retrieved from 8 high-altitude Andean lakes in Argentina and 2 in Chile, taxonomic affiliation, and closest representative sequence (Repr. Seq.) for each OTU. Site abbreviations as in Table 1

OTU ID	Repr. Seq.	Class	Taxonomic affiliation Order	Family	GenBank acc. no.	Closest relative (% identity) Cultured strains in Ez-taxon database	Uncultured/Cultured strains in NCBI
1 (LAZ)	AW38	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793345	*Halorubrum californiense* (98.56)	*Halorubrum californiense* (98.56)
2 (LAZ)	AW40	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793346	*Halorubrum californiense* (98.74)	*Halorubrum californiense* (98.74)
3 (LAZ)	AW41	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793347	*Halorubrum californiense* (98.85)	*Halorubrum californiense* (98.85)
4 (LAZ)	AW42	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793348	*Halorubrum californiense* (98.72)	*Halorubrum californiense* (98.72)
5 (LB)	BW12	*Methanococci*	*Methanococcales*	*Methanocaldococcaceae*	FN998965	*Methanocaldococcus jannaschii* (77.63)	FJ655634 SAS_3E9 (90.87)
6 (LB)	BW13	*Methanococci*	*Methanococcales*	*Methanocaldococcaceae*	FN998966	*Methanotorris formicicus* (76.80)	FJ655628 SAS_3D10 (94.75)
7 (LB)	BW10	*Methanococci*	*Methanococcales*	*Methanococcaceae*	FN998964	*Methanococcus voltae* (75.52)	EF444637 Thp_A_90 (92.37)
8 (LC)	CHW42	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN998986	*Halorubrum distributum* (81.34)	*Halorubrum distributum* (81.34)
9 (LC)	CHW61	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793354	*Natrinema salaciae* (98.20)	*Natrinema salaciae* (98.20)
10 (LC)	CHW63	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793355	*Haloterrigena hispanica* (96.15)	*Haloterrigena hispanica* (96.15)
11 (LCJ)	CJW2	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793328	*Halopelagius longus* (91.58)	CU467236 (95.18)
12 (LCJ)	CJW4	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793329	*Halopelagius longus* (89.49)	CU467236 (93.33)
13 (LCJ)	CJW 5	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793330	*Halopelagius longus* (94.32)	CU467236 (98.86)
14 (LCJ)	CJW6	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793331	*Halobellus salinus* (97.45)	*Halobellus salinus* (97.45)
15 (LCJ)	CJW7	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793332	*Halogranum salarium* (89.80)	*Halogranum salarium* (89.80)
16 (LCJ)	CJW9	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793333	*Halorubrum aidingense* (92.35)	*Halorubrum aidingense* (92.35)
17 (LN)	NW37	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793344	*Halorubrum californiense* (98.36)	*Halorubrum californiense* (98.36)
18 (LS)	SOC10	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN999920	*Haloterrigena hispanica* (97.82)	*Haloterrigena hispanica* (97.82)
19 (LS)	SS6	*Methanobacteria*	*Methanobacteriales*	*Methanobacteriaceae*	FN999915	*Methanobacterium palustre* (77.19)	EU731093 GNA02H12 (96.29)
20 (LS)	SS4	*Methanococci*	*Methanococcales*	*Methanocaldococcaceae*	FN999913	*Methanotorris formicicus* (70.56)	AY454657 EI_D10 (79.32)
21 (LS)	SS5	*Methanococci*	*Methanococcales*	*Methanococcaceae*	FN999914	*Methanococcus voltae* (77.14)	EU731138 GNA01D07 (92.71)
22 (LS)	SS3	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcinaceae*	HG793360	*Methanohalophilus mahii* (76.99)	AY454688 D_H09 (77.76)
23 (LS)	SS7	*Methanomicrobia*	*Methanosarcinales*	*Methanosarcinaceae*	FN999916	*Methanohalophilus mahii* (98.46)	*Methanohalophilus mahii* (98.46)
24 (LT)	TW1	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793327	*Halorubrum lipolyticum* (97.17)	*Halorubrum lipolyticum* (97.17)
25 (LV)	VS1	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN999936	*Halovivax ruber* (96.18)	*Halovivax ruber* (96.18)
26 (LV)	VS15	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793359	*Natrinema salaciae* (97.05)	*Natrinema salaciae* (97.05)
27 (LV)	VS2	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN999937	*Halobellus limi* (96.03)	*Halobellus limi* (96.03)
28 (LV)	VS18	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793336	*Natronorubrum aibiense* (63.95)	*Halomicroarcula pellucida* (62.36)
29 (LV)	VS19	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793337	*Halarchaeum acidiphilum* (69.14)	CU467236 (71.04)
30 (LV)	VS21	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793338	*Halorubrum kocurii* (92.04)	*Halorubrum kocurii* (92.04)
31 (LV)	VS23	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793340	*Halorubrum kocurii* (83.82)	EF106538 G34A (89.86)
32 (LV)	VS35	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793343	*Halorubrum aidingense* (79.89)	*Halorubrum aidingense* (79.89)
33 (LV)	VS16	*Methanobacteria*	*Methanobacteriales*	*Methanobacteriaceae*	HG793334	*Methanobrevibacter boviskoreani* (68.14)	AY454664 D_A12 (74.18)
34 (LV)	VS17	*Methanobacteria*	*Methanobacteriales*	*Methanobacteriaceae*	HG793335	*Methanothermobacter thermophilus* (60.96)	EU731948 GNA05H08 (60.50)
35 (LV)	VS24	*Methanobacteria*	*Methanobacteriales*	*Methanobacteriaceae*	HG793341	*Methanothermobacter wolfeii* (76.65)	DQ103669 ArcA01 (90.19)
36 (LV)	VS22	*Methanococci*	*Methanococcales*	*Methanococcaceae*	HG793339	*Methanothermococcus okinawensis* (72.58)	FJ655628 SAS_3D10 (86.40)
37 (LV)	VS32	*Methanobacteria*	*Methanobacteriales*	*Methanothermaceae*	HG793342	*Methanothermus fervidus* (61.93)	DQ837291 56S_3A_45 (64.05)
38 (OMTG)	TS4	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN999924	*Natrialba aegyptia* (98.59)	*Natrialba aegyptia* (98.59)
39 (OMTG)	TS5	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN999925	*Natrialba aegyptia* (99.20)	*Natrialba aegyptia* (99.20)
40 (OMTG)	TS6	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN999926	*Natrialba aegyptia* (98.04)	*Natrialba aegyptia* (98.04)
41 (OMTG)	TS20	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN999931	*Natrialba aegyptia* (91.07)	*Natrialba aegyptia* (91.07)
42 (OMTG)	TS8	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN999934	*Natrialba aegyptia* (98.79)	*Natrialba aegyptia* (98.79)
43 (OMTG)	TS9	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG810030	*Natrialba aegyptia* (92.44)	*Natrialba aegyptia* (92.44)
44 (OMTG)	TS7	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN999933	*Natrialba aegyptia* (96.55)	*Natrialba aegyptia* (96.55)
45 (OMTG)	TS1	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN999921	*Natrialba aegyptia* (98.99)	*Natrialba aegyptia* (98.99)
46 (OMTG)	TS14	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN999927	*Natrinema pallidum* (95.79)	*Natrinema pallidum* (95.79)

47 (OMTG)	TS15	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN999928	*Natrinema pallidum* (97.74)	*Natrinema pallidum* (97.74)
48 (OMTG)	TS16	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN999929	*Natrinema pallidum* (97.01)	*Natrinema pallidum* (97.01)
49 (OMTG)	TS3	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN999923	*Natrinema pallidum* (96.46)	*Natrinema pallidum* (96.46)
50 (OMTG)	TS2	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN999922	*Natronorubrum tibetense* (90.13)	*Natronorubrum tibetense* (90.13)
51 (OMTG)	TS21	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN999932	*Natronorubrum tibetense* (93.60)	*Natronorubrum tibetense* (93.60)
52 (OMTG)	TS19	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN999930	*Halobiforma nitratireducens* (76.67)	EF690634 TX4CA_79 (77.41)
53 (SHM)	SHM17	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN998967	*Halorubrum xinjiangense* (93.17)	FJ492047 DV427 (93.17)
54 (SHM)	SHM18	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN998968	*Halorubrum xinjiangense* (91.60)	*Halorubrum xinjiangense* (91.60)
55 (SHM)	SHM26	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN998976	*Halorubrum xinjiangense* (92.87)	*Halorubrum xinjiangense* (92.87)
56 (SHM)	SHM19	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN998969	*Halorubrum trapanicum* (92.19)	*Halorubrum trapanicum* (92.19)
57 (SHM)	SHM20	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN998970	*Halorubrum alkaliphilum* (91.11)	FJ492047 DV427 (97.43)
58 (SHM)	SHM36	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN998984	*Halorubrum aquaticum* (93.06)	*Halorubrum aquaticum* (93.06)
59 (SHM)	SHM23	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN998973	*Halorubrum chaoviator* (95.74)	*Halorubrum chaoviator* (95.74)
60 (SHM)	SHM24	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN998974	*Halorubrum chaoviator* (96.57)	*Halorubrum chaoviator* (96.57)
61 (SHM)	SHM35	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN998983	*Halorubrum californiense* (94.38)	*Halorubrum californiense* (94.38)
62 (SHM)	SHM9	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG810032	*Halorubrum aidingense* (95.12)	*Halorubrum aidingense* (95.12)
63 (SHM)	SHM16	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG810033	*Halorubrum aquaticum* (98.17)	*Halorubrum aquaticum* (98.17)
64 (SHM)	SHM22	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN998972	*Halohasta litorea* (88.76)	*Halohasta litorea* (88.76)
65 (SHM)	SHM30	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN998979	*Halonotius pteroides* (95.65)	AM947441 SFG1G041 (95.82)
66 (SHM)	SHM31	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN998980	*Halonotius pteroides* (94.83)	AM947441 SFG1G041 (95.18)
67 (SHM)	SHM32	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN998981	*Halonotius pteroides* (94.33)	AM947441 SFG1G041 (95.06)
68 (SHM)	SHM33	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN998982	*Halohasta litorea* (82.01)	*Halohasta litorea* (82.01)
69 (SHM)	SHM38	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN998985	*Halonotius pteroides* (83.69)	*Halonotius pteroides* (83.69)
70 (SHM)	SHM28	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN998977	*Halobellus salinus* (92.28)	*Halobellus salinus* (92.28)
71 (SHM)	SHM21	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN998971	*Haloarcula marismortui* (94.24)	*Haloarcula marismortui* (94.24)
72 (SHM)	SHM8	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG810031	*Natronorubrum tibetense* (95.54)	*Natronorubrum tibetense* (95.54)
73 (SHM)	SHM53	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793351	*Halorubrum californiense* (98.05)	*Halorubrum californiense* (98.05)
74 (SHM)	SHM59	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793352	*Halorubrum californiense* (95.68)	*Halorubrum californiense* (95.68)
75 (SHM)	SHM60	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793353	*Halorubrum californiense* (99.14)	*Halorubrum californiense* (99.14)
76 (SHM)	SHM64	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793356	*Halorubrum kocurii* (59.47)	*Halorubrum kocurii* (59.47)
77 (SHM)	SHM68	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG793357	*Halorubrum sodomense* (71.84)	FJ561281 B6-RDX (71.84)
78 (SHM)	SHM29	*Methanococci*	*Methanococcales*	*Methanococcaceae*	FN998978	*Methanococcus aeolicus* (73.33)	EU731323 GNA01C06 (85.90)

crobium, *Halopiger*, *Halorubrum*, *Natrialba*, *Natrinema*, *Natronorubrum*, and *Natronococcus* (Table 3). Nine strains exhibited a similarity value lower than 97% with their closest relative (type cultured strains from Ez-Taxon server: www.ezbiocloud.net). However, all other isolates exhibited 97–100% sequence similarities with respect to the type strains of the closest relatives (Table 3).

Halorubrum was the most abundant genus. Representatives of *Halorubrum* were obtained from all 7 samples: LA, LD, SHM, SL, SP, LS, and OMTG. For most samples, *Halorubrum* strains constituted more than 50% of total isolates (Fig. 5). A phylogenetic tree (Fig. 6) was constructed using 16s rRNA gene sequences of isolates, the closest taxonomic neighbor found in the Ez-taxon database for reference strains.

Phenotypic characterization was performed concerning 4 parameters: salinity and pH ranges and UV-B and As tolerance (Table S1 in the Supplement). All strains showed high tolerance to NaCl. Indeed, strains could only grow when the media salinity was 10% or higher (≤30%), with the optimal growth achieved in WJK (25% NaCl). Furthermore, only 5 of the strains (A25, AJ67, AD153, AD156, and D1.1.25) grew better on NaCl 30% than on NaCl 10%.

All isolates analyzed here were neutrophils, growing well in the pH range 5–10, with pH 8 being their optimal pH value.

All strains tolerated UV-B continuous irradiation up to 24 h (total dose: 66.11 kJ m^{-2}). There were 44 pigmented strains showing a high tolerance to UVR-B, whereas the 9 non-pigmented strains were less resistant, tolerating only 12 h of UVR-B (total dose: 33.06 kJ m^{-2}).

The majority of the isolates proved to be arsenic resistant, being tolerant to As[V] and As[III], in the range of 100–400 mM and 2.5–10 mM, respectively (Table S1).

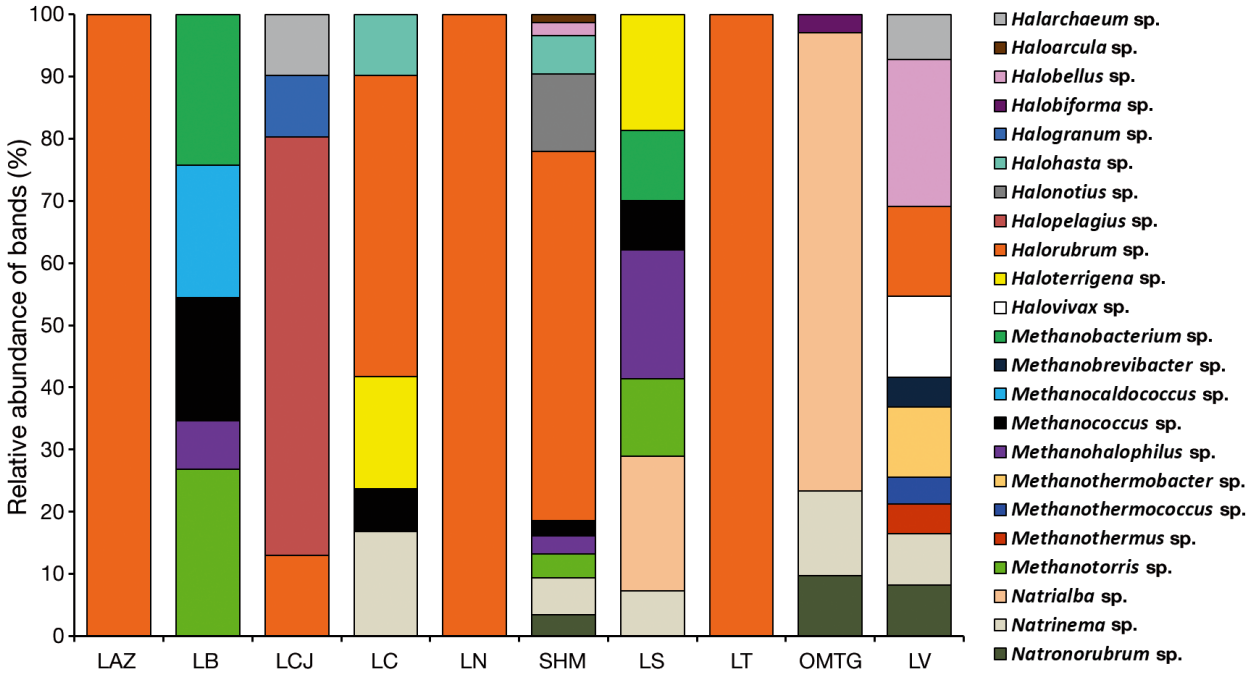

Fig. 3. Relative abundance of the genera in identified operational taxonomic units (OTUs) from DGGE bands among sampled sites (see Table 1 for abbreviations)

DISCUSSION

Microbial diversity of extreme environments in remote areas such as HAALs is poorly known due to difficult access and geographic isolation from urban and scientific centers (Fernández-Zenoff et al. 2006a,b, Souza et al. 2006, Dib et al. 2009, Flores et al. 2009, Dorador et al. 2010, 2013). Recent studies have highlighted the rich biodiversity and novel microbial associations (microbialites, stromatolites) of HAALs, indicating that they may be sources for novel cultivates of *Archaea* (Fernández Zenoff et al. 2006a,b, Dib et al. 2008, 2009, Flores et al. 2009, Dorador et al. 2010, 2013, Farías et al. 2011, 2014, Albarracín et al. 2012, Burguener et al. 2014, Rascovan et al. 2016). In this study, we used culture-dependent and -independent methods to describe archaeal biodiversity of athalassohaline systems in the Argentinean and Chilean Puna (Dorador et al. 2010).

The taxa identified include the genera *Haloarcula*, *Halomicrobium*, *Halopiger*, *Halorubrum*, *Haloterrigena*, *Methanobacterium*, *Methanobrevibacter*, *Methanocaldococcus*, *Methanococcus*, and *Methanothermobacter*. These microorganisms are comparable to the ones found in other halophile environments: Lakes Qinghai, Aibi, Aiding, Ejinor, Bagaejinnor, Shangmatala, and Ayakekum (China), Fuente de la Piedra (Spain), Sebkha Ezzemoul (Algeria), Lake Tebenquiche (Chile), evaporites (Baja California), Naxo Islands (Greece), salt flats in Tunisia, and saline lakes in Antarctica and the Dead Sea (Baliga et al. 2004, Karr et al. 2006, Kharroub et al. 2006, Castillo et al. 2007, Gutiérrez et al. 2007, Romano et al. 2007, Mancinelli et al. 2009, Trigui et al. 2011, Anderson et al. 2016).

Methods for isolation and molecular characterization are often combined to identify novel microorganisms in natural systems and to compare their community composition (Amann et al. 1995, Smit et al. 2001). In this study, analysis of the sampled communities by DGGE combined with cluster analysis (Fig. 2) indicated that at some sites, sediment communities differed from those of lake water, while in most cases, water and sediment communities clustered together. These findings suggest a substantial variation in sediment and water characteristics in some lakes (Table 1), but on the other hand, they revealed limited variability within the archaeal community. We did not detect any clear effect of the distance between sampling sites on community composition; DGGE band profiles overlapped substantially between samples from sites separated by hundreds of kilometers. It is generally assumed that the combination of environmental factors such as organic matter (Parkes et al. 2005), salinity (Edlund et al. 2006), and nutrients (Nelson et al. 2007) influence the structure of microbial communities, causing differences among

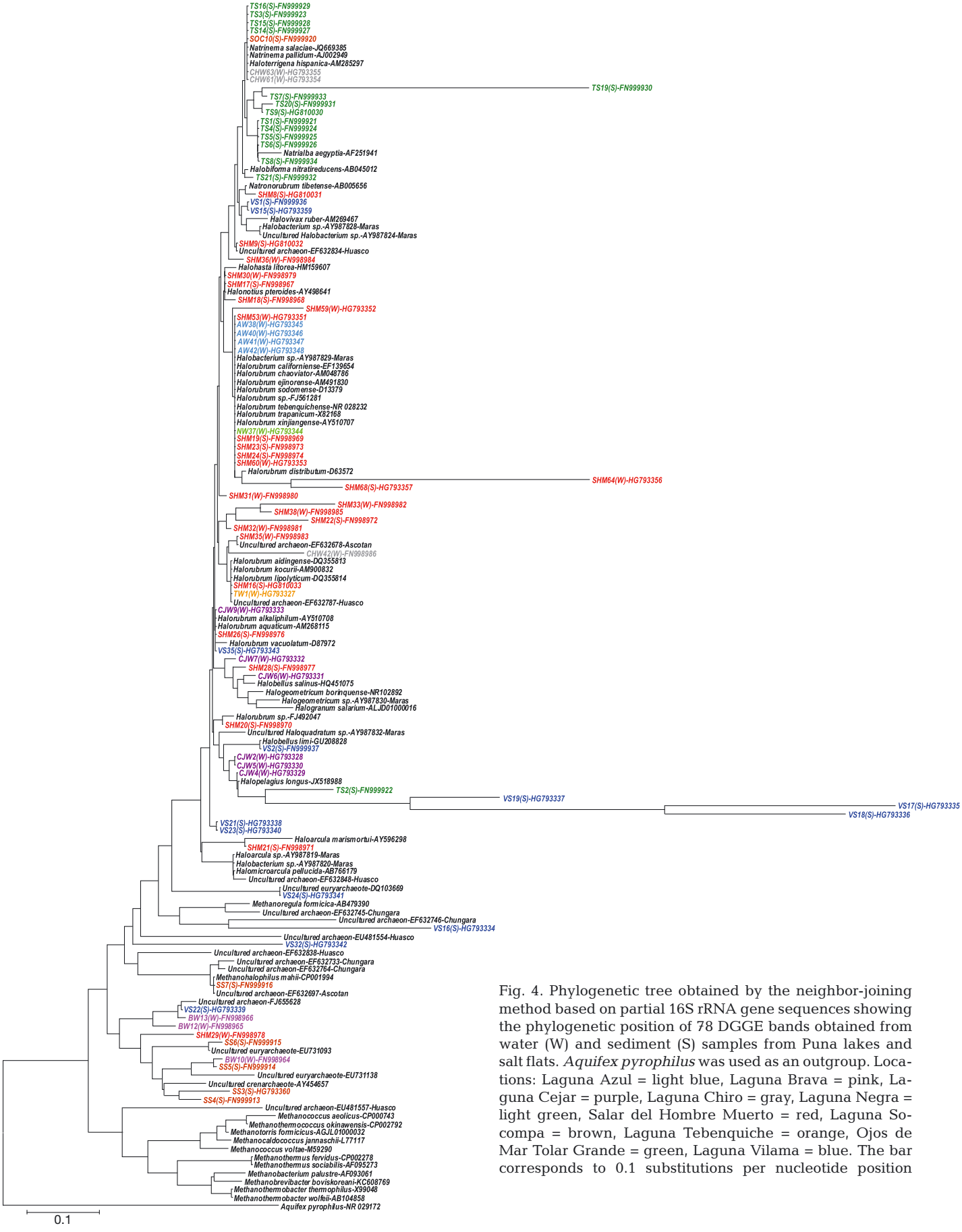

Fig. 4. Phylogenetic tree obtained by the neighbor-joining method based on partial 16S rRNA gene sequences showing the phylogenetic position of 78 DGGE bands obtained from water (W) and sediment (S) samples from Puna lakes and salt flats. *Aquifex pyrophilus* was used as an outgroup. Locations: Laguna Azul = light blue, Laguna Brava = pink, Laguna Cejar = purple, Laguna Chiro = gray, Laguna Negra = light green, Salar del Hombre Muerto = red, Laguna Socompa = brown, Laguna Tebenquiche = orange, Ojos de Mar Tolar Grande = green, Laguna Vilama = blue. The bar corresponds to 0.1 substitutions per nucleotide position

Table 3. Taxonomic affiliation of 53 *Archaea* isolated from high-altitude Andean lakes. S: sediment, W: water; site abbreviations as in Table 1

Strain	Taxonomic affiliation			GenBank acc. no.	Closest relative (% identity) in Ez-taxon database	Isolation source/ type of sample
	Class	Order	Family			
AJ57	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE801346	*Halorubrum californiense* (99.07)	LA/W
AJ58	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE801347	*Halorubrum californiense* (99.01)	LA/W
AJ62	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE801348	*Halorubrum californiense* (99.09)	LA/W
AJ63	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE801349	*Halorubrum californiense* (99.01)	LA/W
AJ67	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE801350	*Halorubrum californiense* (99.19)	LA/W
AJ68	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE801351	*Halorubrum californiense* (99.02)	LA/W
AJ69	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE801352	*Halorubrum californiense* (99.01)	LA/W
AJ81	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE801353	*Halorubrum californiense* (99.01)	LA/W
AJ83	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE801354	*Halorubrum californiense* (99.01)	LA/W
AJ93	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE801355	*Halorubrum californiense* (99.00)	LA/W
AJ100	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802580	*Halorubrum californiense* (98.98)	LA/W
AJ101	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802581	*Halorubrum californiense* (99.08)	LA/W
AJ102	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802582	*Halorubrum saccharovorum* (98.88)	LA/W
AJ115	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802587	*Halorubrum californiense* (98.63)	LA/W
AJ126	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802596	*Halorubrum californiense* (98.84)	LA/W
AJ128	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802597	*Halorubrum californiense* (99.00)	LA/W
AJ130	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802599	*Halorubrum lipolyticum* (98.17)	LA/W
AJ131	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802600	*Halorubrum lipolyticum* (98.35)	LA/W
AD156	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HF558673	*Halorubrum kocurii* (98.36)	LD/S
AD153	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HF558672	*Halorubrum aidingense* (97.64)	LD/W
D1.1.25	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HG515528	*Natronococcus jeotgali* (99.44)	LD/W
A26	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FR690813	*Natrinema salaciae* (98.68)	LS/S
A29	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FR690814	*Halorubrum lipolyticum* (99.45)	LS/S
A30	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FR690815	*Natronorubrum tibetense* (97.20)	LS/S
AJ113	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE805212	*Halorubrum californiense* (99.20)	LS/W
AT4	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN870351	*Natrialba aegyptia* (97.92)	OMTG/S
AT5	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN870352	*Natrialba aegyptia* (93.49)	OMTG/S
AT6	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN870353	*Natrialba aegyptia* (99.17)	OMTG/S
AT7	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN870354	*Natrialba aegyptia* (91.65)	OMTG/S
AT8	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN870355	*Natrialba aegyptia* (97.78)	OMTG/S
AT9	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN870356	*Natrialba aegyptia* (96.50)	OMTG/S
AT14	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN870357	*Natrialba aegyptia* (99.00)	OMTG/S
AT23	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN870358	*Halopiger aswanensis* (97.34)	OMTG/S
A25	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN870359	*Halomicrobium katesii* (83.84)	OMTG/S
A31	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FR690812	*Natronorubrum tibetense* (99.79)	OMTG/S
AJ104	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802583	*Halorubrum californiense* (99.07)	OMTG/W
AJ120	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802591	*Halorubrum californiense* (99.02)	OMTG/W
AJ121	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802592	*Halorubrum californiense* (98.52)	OMTG/W
AC1	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN870360	*Halorubrum californiense* (98.97)	SHM/W
AC2	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN870361	*Halorubrum arcis* (96.44)	SHM/W
AC3	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN870362	*Halorubrum arcis* (95.74)	SHM/W
AC10	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN870363	*Natrialba aegyptia* (97.52)	SHM/W
AC12	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	FN870364	*Natrialba aegyptia* (98.55)	SHM/W
AJ112	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802586	*Halomicrobium katesii* (96.50)	SL/W
AJ117	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802588	*Halorubrum californiense* (99.03)	SL/W
AJ118	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802589	*Halorubrum californiense* (99.03)	SL/W
AJ119	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802590	*Halorubrum terrestre* (91.14)	SL/W
AJ111	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802585	*Haloarcula argentinensis* (98.55)	SL/W
AJ123	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802593	*Haloarcula argentinensis* (98.42)	SP/W
AJ109	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802584	*Haloarcula argentinensis* (99.08)	SP/W
AJ110	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE805211	*Haloarcula argentinensis* (99.27)	SP/W
AJ124	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802594	*Halorubrum californiense* (96.37)	SP/W
AJ125	*Halobacteria*	*Halobacteriales*	*Halobacteriaceae*	HE802595	*Haloarcula argentinensis* (98.72)	SP/W

isolated sites (Oren 1999). In this study, a direct correlation between salinity or pH variability and the structure of the archaeal community in the sampled sites was not evident. Hence, the identified difference among the archaeal communities may have resulted from variation in other parameters not explored here, such as nutrient availability (Nicol et al. 2003, Nam et al. 2008).

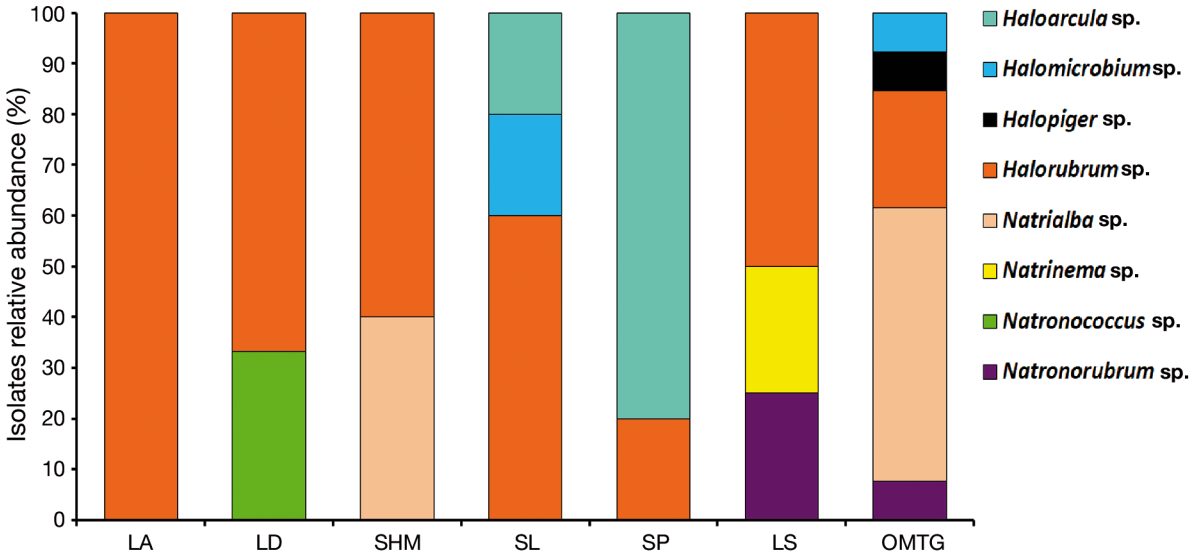

Fig. 5. Relative abundance of genera in isolates from various sampled sites. Site abbreviations as in Table 1

Eighteen percent of the DGGE sequences were related to 9 methanogenic archaeal genera, indicating the presence of methanogens in saline environments and emphasizing their ecological importance in those systems (Orphan et al. 2001, Michaelis et al. 2002, Lloyd et al. 2006). The most halotolerant methanogenic *Archaea* known, namely *Methanohalobium evestigatum* and *Methanohalophilus portucalensis*, can grow in salinities up to 25–26% (Zhilina & Zavarzin 1987, Lai & Gunsalus, 1992, Boone et al. 1993), and the moderately halophilic *Methanohalophilus mahii*, *Methanohalophilus halophilus*, *Methanohalophilus portocalensis*, and *Methanohalophilus zhilinae* can grow in salinities up to 4–12% (Oren 2013). These data correlate with the salinity values found in the HAALs (Table 1), demonstrating the presence of methanogens in the Puna sediments (Dorador et al. 2010, 2013).

Most of the DGGE band sequences showed similarity values <97% with their closest relatives in GenBank, reflecting the scarce information on microbial life in these types of extreme environments. This confirms HAALs as a promising source of unknown *Archaea* and, probably, of novel metabolic processes.

The microbial diversity observed in the studied lakes and salterns is comparable to that found in similar environments, e.g. Huasco Saltern, Ascotan saltern, Chungara Lake, Parinacota wetland, and Piacota Lake in the Chilean Puna. All of these microbes show similar Shannon diversity index values (data not shown) (Dorador et al. 2013) and share some genera with La Palma salt flats in Spain (*Halorubrum* and *Haloarcula*) (Ochsenreiter et al. 2002) and with Victoria salt flats in Australia (*Halorubrum*, *Halogranum*, and *Halobellus*) (Emerson et al. 2013). The most abundant genera (*Halorubrum*, *Natrialba*, and *Haloarcula*) were evenly distributed among the sampled sites, whereas the difference among the archaeal populations was due to the presence of less abundant genera (Boon et al. 2000).

In contrast to the findings of Dorador et al. (2010) about uncultured diversity, DGGE analysis revealed that *Halobacteria* dominated over methanogens in the sampled sites. Unlike the study by Benlloch et al. (2001) and Maturrano et al. (2006), the culture-independent methods that we used assessed much more diversity than the culture-dependent ones; in fact, DGGE revealed that the microbial communities of the HAALs comprised up to 23 genera, while we were able to isolate 53 strains corresponding to only 8 genera. The diversity of cultivable *Archaea* was low at some lakes (LB, LC, LV, LN, LAZ, LT, and LCJ). This might be due to the physiological state of the organisms and the lack of nutrients or appropriate conditions to support their growth during incubation (Barer & Harwood 1999, Laiz et al. 2003). According to their 16S rRNA gene sequences, all of the isolates belonged to the phylum *Euryarcheaota*, family *Halobacteriaceae*, a taxon that is widespread in hypersaline environments (Ochsenreiter et al. 2002, Oren 2002, Burns et al. 2004, Dorador et al. 2010, 2013).

All isolates were able to grow in a media salinity between 10 and 30% NaCl, a typical feature of the *Halobacteriaceae* (Oren 2006). The strains A25, AJ67, AD153, AD156, and D1.1.25 showed the high-

Fig. 6. Phylogenetic tree obtained by the neighbor-joining method based on partial 16S rRNA gene sequences showing the phylogenetic position of 53 isolates from water (W) and sediment (S) samples from Puna lakes and salt flats. *Aquifex pyrophilus* was used as an outgroup. Locations: Laguna Antofalla = dark red, Laguna Diamante = navy blue, Laguna Socompa = brown, Ojos de Mar Tolar Grande = green, Salar del Hombre Muerto = red, Salar Llullaillaco = light brown, Salar Pocitos = yellow. The bar corresponds to 0.1 substitutions per nucleotide position

est tolerance to NaCl (≤30%). This was due to their phylogenetic proximity to *Halomicrobium katesii*, *Halorubrum californiense*, *Halorubrum chaoviator*, *Halorubrum kocurii*, and *Natronococcus jeotgali*, respectively, and coincides with the highest NaCl concentration up to which such species can grow (Roh et al. 2007, Gutiérrez et al. 2008, Kharroub et al. 2008, Pesenti et al. 2008, Bowers & Wiegel 2011).

All strains were considered neutrophiles, with pH 8 being the optimum value for growth as proposed for other *Halobacteriaceae* strains (Oren 2012) with limits for growth at pH 5 and pH 10. Strains AT4, AT5, AT6, AT7, AT8, AT9, and AT14 grew better at pH 5 than at pH 10 and are phylogenetically related to *Natrialba aegyptia*, with a theoretical pH optimum of 7.5 and a pH range of 6–9 (Boon et al. 2000, Bowers & Wiegel 2011). Strains AD153, AD156, and D1.1.25, which are phylogenetically related to *Halorubrum chaoviator*, *Halorubrum kocurii* and *Natronococcus jeotgali*, respectively, grew better at pH 10 than at pH 5, despite the theoretical optimum of pH 7.5 and optimal ranges of 7–9, 6–9, and 7–9.5, respectively (Bowers & Wiegel 2011). These results demonstrated that the HAAL *Archaea* readily adapt to varied and extreme pH.

In accordance with their isolation origin, all strains were able to tolerate a total UV-B dose of up to 66.11 kJ m^{-2}. The pigmented strains (44) displayed a higher tolerance to UV-B than the non-pigmented strains (9), suggesting a protective mechanism of carotenoid pigments against UVR damage through the capture of triplet states of photosensitizers and reactive oxygen species (Moeller et al. 2005, Chattopadhyay 2008). Our results concur with previously reported evidence indicating *Haloarchaea* as one of the groups of microorganisms most resistant to radiation (DeVeaux et al. 2007), and suggest an adaptation of the isolates to the high-altitude environment.

Similar UV tolerance profiles were reported for HAAL bacteria such as *Actinobacteria*, *Firmicutes*, and *Gammaproteobacteria* from L. Azul (4450 m a.s.l.) and L. Vilama (4600 m a.s.l.), with a high UV-B tolerance (1.4 kJ m^{-2}) (Dib et al. 2008, Albarracín et al. 2012). *Acinetobacter johnsonii* A2 from L. Azul, *Serratia marcescens* MF42, *Pseudomonas* sp. strain MF8, and *Cytophaga* sp. strain MF7 from L. Pozuelos (3600 m a.s.l.) demonstrated a high tolerance when subjected to 3.93 kJ m^{-2} UV-B dose (Fernández Zenoff et al. 2006b). *Bacillus megaterium*, *Staphylococcus saprophyticus*, *Nocardia* sp., and *Acinetobacter johnsonii* from L. Azul were tolerant to a UV-B dose of more than 27 kJ m^{-2} (Fernández Zenoff et al. 2006a). Lastly, *Pseudomonas* sp. V1 and *Brachybacterium* sp. V5 from L. Vilama also proved to have efficient mechanisms against UV damage (Farías et al. 2009).

All archaeal isolates were tolerant of As[V] and As[III]. Previous microbiological studies showed diverse mechanisms of arsenic resistance in *Halobacterium* sp. strain NRC-1 (Wang et al. 2004). Microorganisms that oxidize and reduce arsenic were reported in acid environments such as the Iron Mountain Mine in northern California (Edwards et al. 2000) and Yellowstone National Park (Kan et al. 2011). Dib et al. (2008) showed that the majority of isolates from high-altitude lakes in the Argentinian Puna are highly tolerant of arsenic due to their genetic detoxification and metabolism systems, and their capability of using arsenic as a substrate in bioenergetics processes (Rascovan et al. 2016).

In summary, this work combined culture-dependent and -independent methods to describe the phylogenetic affiliation and ecological niche of *Euryarchaeota* thriving in 14 hypersaline wetlands in the Argentinean and Chilean Puna. More studies will be required to examine the effect of abiotic parameters such as seasonal variation in salinity, pH, and temperature on archaeal community structure, composition, and diversity. Current genomic analysis of the HAAL isolates described herein will help explain the importance of the Domain *Archaea* in these natural ecosystems.

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