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Diversity of extremely halophilic cultivable prokaryotes in Mediterranean, Atlantic and Pacific solar salterns: evidence that unexplored sites constitute sources of cultivable novelty

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Abstract

The culturable fraction of aerobic, heterotrophic and extremely halophilic microbiota retrieved from sediment and brine samples of eight sampling sites in the Mediterranean, Canary Islands and Chile was studied by means of a tandem approach combining large-scale cultivation, MALDI-TOF MS targeting whole cell biomass, and phylogenetic reconstruction based on 16S rRNA gene analysis. The approach allowed the identification of more than 4,200 strains and a comparison between different sampling sites. The results indicated that the method constituted an excellent tool for the discovery of taxonomic novelty. Four new genera and nine new species could be identified within the archaeal family *Halobacteriaceae*, as well as one new bacterial species, and a representative of *Salinibacter ruber* phylotype II, a group that had been refractory to isolation for the last fifteen years. Altogether, the results indicated that in order to provide better yields for the retrieval of novel taxa from the environment, performance of non-redundant environment sampling is recommended together with the screening of large sets of strains.

Introduction

Culture-dependent microbiology suffers from being empirical and time and effort intensive, but it is essential to basic science and biotechnology [7]. In addition, obtaining pure cultures of the vast majority of microorganisms in the environment is difficult due to slow growth, metabolic needs or the incapacity to find appropriate media [17], as well as additional microbial interactions that could be related to the modification of their connections with the environment, other prokaryotes or viruses [23]. Therefore, there is a need to develop strategies to culture organisms in the laboratory, and this is a prerequisite for biodiscovery [23]. The search for novelty by means of culture techniques can be approached using different methodologies, such as large-scale cultivation, innovative culturing strategies or enrichment by micromanipulation [17]. One of the important advantages of large-scale cultivation is that the extent of any novelty may be related to the extent of the screening itself.

The exhaustive studies on 16S rRNA gene sequences as a measure of the microbial diversity thriving on the Earth have led to a compilation of a vast database, which currently contains more than 3.5 million environmental sequences [52]. The current measurements of the extent of diversity indicate that 0.5 to 2 million species may exist in the biosphere and that this is an achievable amount for classification purposes [52]. On the other hand, it seems that there is a redundancy in the environments studied, and that perhaps the search for novelty might be more successful in unexplored systems [52]. This may also hold true for the cultivable fraction, and perhaps unexplored environments should be studied in order to retrieve novel strains. Additionally, large-scale cultivation may also be successful in retrieving members of the rare biosphere [38].

The screening of large sets of organisms may require extensive (and to some extent expensive) work by means of genetic studies, such as partial sequencing of 16S rRNA genes [54], molecular fingerprints [16], phenotypic analyses, fatty acid [13] or polar lipid profiles [24, 44], and infrared mass spectroscopy [51]. Of special relevance, given its relatively low cost and reliable screening of a large number of cultures, is MALDI-TOF mass spectrometry using whole cell biomass [50]. This approach has been shown to be very effective in sorting almost 290,000 clinical isolates in a relatively short period of time, as well as in the identification of rare bacterial species that may be implicated in pathogenesis [46]. Moreover, this technique was successfully applied for the identification of clusters of isolates in a given environmental sample as single but non-clonal species [34].

The different disciplines that can benefit from large culture screenings range from very applied sciences, such as biotechnology, to taxonomy which is one of the most fundamental disciplines. Actually, taxonomic practices changed drastically at the beginning of this century when species descriptions based on a single isolate overtook those with two or more strains [48]. In the International Journal of Systematic and Evolutionary Microbiology, between June 2013 and June 2014, 82% of the published species descriptions included one strain, 8.3% had two strains, 5.3% had three strains and 3.6% had four or more strains. The tolerance for classifying taxa with a single isolate has greatly increased the speed of describing cultured diversity. However, the description of a given taxon based on just one representative has been criticized as inaccurate scientific practice [10,14] because these descriptions may not reflect the actual diversity of the taxon. However, others have justified this practice since the whole biological diversity must be described with reasonable speed [12]. In order to overcome the difficulties in isolating several organisms of the same taxon, the screening of large sets of cultures may be of help.

Hypersaline environments, such as crystallizer ponds of solar salterns, are extreme environments characterized by a reduction of microbial diversity with increasing salt concentrations [32]. The dominant organisms inhabiting these environments belong to the archaeal domain, whereas members of the bacterial domain are generally less abundant [5,19,20,32]. Molecular microbial ecology studies have revealed the archaeal taxa *Haloquadratum walsbyi* (the so-called “square archaeon”) and the recently described *Nanohaloarchaea* [19] as highly abundant. On the other hand, *Halorubrum*, *Haloferax*, *Halobacterium* and *Haloarcula* were the dominant genera recovered by cultivation techniques [49]. The most abundant bacterial genera thriving in such environments, as revealed by both culture-dependent and –independent methods, were *Salinibacter* and *Salicola* [5,33]. In general, diversity studies have been performed mostly in brines [5,15,20,34], with very few in corresponding sediments [29].

Most of the current studies on the diversity of halophilic microorganisms in hypersaline systems have been performed by means of culture-independent molecular techniques, such as, for example, on either 16S rRNA gene diversity [20] or by metagenomic approaches [15].

Despite the fact that molecular studies describe to a great extent the taxonomic and genetic diversity of the key players in their environments, they have failed to culture living organisms that can be potentially important sources of information for biotechnological, pharmaceutical and even taxonomic purposes. Culturing techniques may satisfy the needs of many microbiologists, as exemplified very well by the statement of Steve Giovannoni that “Nothing beats actually having the organism in culture” [8].

In the current study, the isolation and identification of over 4,200 extremely halophilic strains from eight different locations in the world are presented by means of a tandem approach using Matrix-Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry (MALDI-TOF/MS) and 16S rRNA gene sequencing. The study confirmed that the approach was very suitable for understanding the diversity of the culturable fraction, as well as for isolating rare representatives of known taxa. Moreover, the results pointed to the fact that extending the studies to scarcely explored (e.g. hypersaline sediments in comparison to brines) or as yet unexplored sites (e.g. South American salterns) enhanced the success of retrieving representatives of novel taxa.

Materials and methods

Samples and processing

Sediment and brine samples for this study were obtained from eight different solar salterns: S’Avall (AV) and Campos (CA), both from the island of Mallorca, and Formentera (FM), all three located in the Balearic Islands; Janubio (LZ) and Fuerteventura (FV) both located in the Canary Islands; La Trinitat (ST) in Tarragona, and Santa Pola (SP) in Alicante, both on the east coast of the Spanish peninsula; and Lo Valdivia (LV) located on the coast of Curicó in Chile (Table 1). At each location the samples were taken from two different crystallizers. Brines were collected in 1 L sterile flasks from three different sampling points in the ponds. Triplicates of the sediment samples were taken with methacrylate cores, as previously reported [28]. Samples were transported to the laboratory within 24-48 h after collection and processed immediately. Brines were directly diluted and plated. The three sediment cores were initially sliced, the first 0.5 cm and the overlaying salt crust were removed, and the following 30 cm were homogenized and further diluted for cultivation purposes.

Growth media, plating and isolation

In all cases, a surface-spread plating method was used to isolate aerobic heterotrophic extreme halophiles. One milliliter of homogenized sediment or 1 mL of brines were used to prepare the serial dilutions (to 10^{-5}) in seawater medium (SW) at a salt concentration of 25% [43]. All samples and their respective dilutions were plated in duplicate on SW at two different salt concentrations: 20% and 30%. In both cases, Yeast Extract (YE, Cultimed Panreac Química S.A.) was added at a final concentration of 0.05% as a carbon and energy source. Plates were

incubated at room temperature (22 °C) for at least one month until growth was observed. Approximately 100 colonies from each sample (i.e. each of the duplicate samples of brines or sediments, and at the two respective growth conditions) were selected taking into account different size, morphology and color in order to obtain the largest diversity possible. Selected colonies were brought to pure culture by re-streaking them on solid media ensuring the recovery of a single morphology for each. For storage purposes, individual isolates were grown in liquid medium (SW 20% and 30% with 0.05% YE), and the resultant suspensions were mixed with 40% (v/v) glycerol and stored at -80 °C. Subculturing of the glycerolated strains reactivated approximately 95% of the collection checked.

MALDI-TOF analyses

The initial screening of the isolated strains was carried out with MALDI-TOF MS using whole cell biomass, as previously published [34]. All isolates were refreshed by replicating them onto agar plates with their respective isolation media (i.e. 20% or 30% SW with 0.05% YE). Cells were grown until the colony size was approximately 1 mm in diameter. A small amount of biomass (1-2 mg) was picked from the agar plates with a 1-µL sterile plastic loop, and deposited onto a ground steel 384-target plate (Bruker Daltonik Leipzig, Germany). Samples were overlaid with 2 µL of matrix solution (saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and air dried at room temperature. Measurements were performed with an Autoflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Leipzig, Germany) equipped with a 200 Hz Smartbeam laser. Spectra were recorded in the linear, positive mode at a laser frequency of 200 Hz within a mass range from 2000 to 20,000 Da. The IS1 voltage was 20 kV, the IS2 voltage was maintained at 18.7 kV, the lens voltage was 6.50 kV, and the extraction delay time was 120 ns. For each spectrum, approximately 500 shots at different positions of the target spot were collected and analyzed. The spectra were externally calibrated using the Bruker Bacterial Test Standard (*Escherichia coli* extract including the additional proteins RNAse A and myoglobin). Calibration masses were as follows: RL29 3637.8 Da; RS32, 5096.8 Da; RS34, 5381.4 Da; RL33meth, 6255.4 Da; L29, 7274.5 Da; RS19, 10,300.1 Da; RNase A, 13,683.2 Da; myoglobin, 16,952.3 Da. Spectra analyses were carried out with BioTyper software 3.0 (Bruker Daltonics) and were used to construct similarity dendrograms. Each single similarity cluster in the dendrograms was regarded as an operational taxonomic unit (OTU), and this was the minimal unit used for further identification by means of 16S rRNA gene sequence analysis.

PCR amplification and sequencing of 16S rRNA genes

16S rRNA gene PCR amplification of the selected isolates was performed by taking a small amount of biomass with a sterile toothpick and directly suspending it in the PCR mix. The reaction mix (50 µL final volume) contained 5 µL of 10x Ex Taq™ buffer (20 mM $MgCl_2$), 1 µL of

each forward and reverse primers (10 µM each), 4 µL of dNTP Mix 10x (25 µM each) and 0.25 µL Taq polymerase TaKaRa Ex Taq™ (Takara Bio Inc, Japan; 5 units/µL). Amplification for the *Bacteria* domain was conducted using the universal [24] primers GM3 (5'-AGAGTTTGATCATGGCTCAG-3') and S (5'-GGTTACCTTGTTACGACTT-3'). For the archaeal domain the primers used were 21F (5'-TTCCGGTTGATCCTGCCGGA-3' [11] and 1492R (5'-TACGGYTACCTTGTTACG-3' [25]. The amplification reaction was performed in a Mastercycler® gradient (Eppendorf, Germany) using the following steps: one denaturing cycle at 94 °C (5 min) and 35 cycles of: 94 °C (1 min), 55 °C (30 s), 72 °C (2 min); and a final extension step at 72 °C (10 min). Electrophoresis was performed in a 1% agarose gel, and visualization was carried out after staining with ethidium bromide. PCR products were purified with MSB® Spin PCRapace (INVITEK GmbH, Berlin), following the manufacturer's indications, and then sent for sequencing to Secugen S.L. (Spain). The sequences have been deposited in the public repositories with the entries LN649797 to LN650054.

Tree reconstructions

Sequences were reviewed, corrected and assembled using Sequencher v4.9 software (Gene Codes Corp., USA). Alignments and tree reconstructions were performed using the ARB software package version 5.5 [30]. The new sequences were added to the reference datasets SILVA REF111 and LTP115 [42, 53], respectively, and aligned using the SINA tool (SILVA Incremental Aligner, [41]) implemented in the ARB software package. Final alignments were manually improved following the reference alignment in ARB-editor. Complete sequences were used to reconstruct *de novo* trees using the neighbor-joining algorithm, while the partial sequences were added into a pre-existing tree using the ARB-Parsimony tool, both implemented in the ARB software package. Sequences were grouped in operational phylogenetic units (OPUs) as an alternative to using strict cut-off values of identity thresholds in order to identify isolated clades derived from the phylogenetic tree topology that produce biologically meaningful units [16,29]. An OPU was considered as the smallest clade containing one or more amplified sequences affiliating together with reference sequences available in the public repositories. When possible, the OPUs should include a type strain sequence present in the LTP database [53], and for identity values >98.7% with type strain sequences the amplicons were considered to belong to the same species using this conservative threshold, as previously recommended [47]. On the other hand, for the identity values <98.7% and >94.5% with the closest relative type strain 16S rRNA gene sequence of the same OPU, the amplicons were considered to be the same genus (according to Yarza et al. [52]) but from a different unclassified species.

Statistical analyses

The presence or absence of isolates detected for each OTU was coded as a binary matrix and imported into the statistical program. Data ordination was undertaken considering location and

type of sample (sediment or brines). Non-metric multi-dimensional scaling (nMDS) was performed using PRIMER 5 software version 5.2.8 (PRIMER-E Ltd., UK) and the previous matrix distance was elaborated using the Euclidean distance. Rarefaction curves were calculated using PAST software version 1.82b [22]. Good's coverage values were also calculated in order to estimate the diversity coverage of the strain collection [21].

Results

Isolates and MALDI-TOF MS analyses

A total of 32 different samples (sediments and brines of two crystallizer ponds in each of the eight sampled salterns) were screened for the cultivable fraction of heterotrophic aerobic extreme halophilic microbiota. In all cases, the salinities in the crystallizer ponds were higher than 27%, ranging between 27% in ST2 and 37.6% in LV2 (Table 1). Cultivation yields from the different samples and media were very variable, ranging between $3.2 \cdot x\ 10^4$ colony forming units per milliliter (CFU/mL) in FM brines (on 30% salinity medium) and $2.05 \cdot x\ 10^6$ CFU in FV brines (with the 20% salinity medium) (Supplementary Table S1). Unexpectedly, no growth was obtained at 30% SW for the FV sample. It was intended to cover the widest diversity range possible by selecting all colonies with distinguishable morphologies, sizes and colors from the incubated agar plates at SW salt concentrations of 20% and 30%, from their respective brines or sediments, with a minimum of 77 strains for each sample and condition. A total of 5,076 isolates were recovered, with a minimum of 378 isolates from FV and a maximum of 792 from CA. More than 720 isolates were isolated from five samples (SP, AV, CA, LZ and LV).

All isolates were analyzed by whole-cell MALDI-TOF/MS within the 4 weeks following their isolation to pure cultures. Spectrometric profiles were manually inspected and only those with a stable baseline and good signals were considered for further analysis. After sieving the profiles, the discarded fraction ranged between 3.5% and 22% (SP and AV, respectively) of the initial dataset. Poor baselines could have been due to the salt present in the culture medium, but for pragmatic reasons bad profiles were discarded. The number of valid spectra was approximately 86% of the total measured (Supplementary Table S1). In order to generate a global dendrogram (Supplementary Figure S1) and select representative strains, dendrograms for each location were constructed (Supplementary Figures S2 to S9). Independent clusters of profiles were recognized as different operational taxonomic units (OTUs) following similar criteria in previous studies [34]. In general, two different major clusters (with the exception of LV and AV) at each location could be determined that, upon phylogenetic inference, could be distinguished as *Bacteria* or *Archaea* (Supplementary Figures S2 to S9), respectively. For further analysis, members of both domains were treated independently. The global archaeal dendrogram (Supplementary Figure S1A) was constructed with 1,017 representative profiles with a total of 73 OTUs: 46 OTUs were formed from isolates originating in only one location; 18 OTUs were from two to three locations; and 9 OTUs from four or more locations. In this regard, OTU 23 consisted of isolates from the eight solar salterns analyzed. Furthermore, 24 OTUs

were represented only in sediment isolates, and 4 OTUs only from brine isolates. On the other hand, the global bacterial dendrogram (Supplementary Figure S1b) was constructed with 1,226 profiles and exhibited a much simpler composition where only 6 OTUs could be distinguished. It was remarkable that the LV and AV samples did not render any bacterial isolate. OTU 74 harbored the majority of the profiles (1,161 strains isolated from all samples except LV and AV). Five OTUs embraced isolates from both sediment and brine samples, and the other one was composed of strains originating only from sediment samples.

Affiliation of the OTUs corresponding to the archaeal fraction

Since it was intended to construct a spectra database of extreme halophilic microorganisms, a large set of representative strains from the samples studied initially (LV, CA, AV and SP) was selected for 16S rRNA gene sequencing. For this purpose, an attempt was made to cover the maximum diversity in each dendrogram. One strain within each OTU was selected for sequencing of its almost complete 16S rRNA gene, and two or more additional strains only for partial sequencing. For the latter studied samples (ST, FM, LZ, and FV), the sequencing effort was reduced significantly as most of the OTUs detected could be readily identified (Table 1). The representatives of each OTU were used to reconstruct a domain phylogeny and recognize the different OPUs present in the samples.

From the archaeal phylogenetic reconstruction (Figure 1), 35 OPUs could be identified that affiliated with 15 distinct putative genera and 25 species within the family *Halobacteriaceae*, using the conservative thresholds of 94.5% [52] for the genus category, and 98.7% for species [47]. Among them, four putative novel genera and 17 additional novel species were recognized (11 with identity values below 98.1% with their closest relative sequence of an existing type strain; Figure 1, Table 2). The OPUs affiliated with the genera *Halorubrum* (*Hrr.*; 2,251 isolates), *Haloarcula* (*Har.*; 126 isolates), *Haloterrigena* (*Htg.*; 121 isolates), *Halolamina (Hlm.*; 94 isolates*)*, *Haloplanus* (*Hpn.*; 94 isolates), *Haloferax* (*Hfx.*; 83 isolates), *Halonotius* (*Hns.*; 61 isolates), *Natronomonas* (*Nmn.*; 60 isolates), *Halovivax* (*Hvx.*; 53 isolates), *Halomicrobium* (*Hmc.*; 51 isolates), *Halogeometricum* (*Hgm.*; 38 isolates), *Halobellus* (*Hbs.*; 17 isolates), *Halorientalis* (*Hos.*; 13 isolates), *Natronoarchaeum* (*Nac.*; 12 isolates) and *Halobacterium* (*Hbt.*; 3 isolates) (Figure 1). Since colony selection was not random (as the highest diversity possible was sought by identifying different colony shapes) no diversity indices could be deduced. However, when analyzing the rarefaction curves (Supplementary Figure S10), they were already saturated when the collection size was ~300 colonies. In all samples, the number of colonies in the study largely exceeded this number and in most of them it was double. Moreover, the *minimum sample size* (i.e. the smallest number of colonies to be selected from each sample to obtain enough representativeness of the total cultivable) recommended for each sample collection [31] (Supplementary Table S2) was exceeded between two to four fold. Altogether, the results agreed with the calculated Good's indices that, in all cases, were greater than 95.8% of the total expected culturable diversity. Therefore, we could be confident that a

considerable large fraction of the culturable diversity was covered under the conditions of this study.

The branch comprising the *Halorubrum* genus was the most represented and accounted for 2,251 strains representing 52% of the total, and 71% of the archaeal isolates. Moreover, with this genus 14 out of the 35 OPUs of this domain could be affiliated. Among the 14 OPUs of this lineage, one putative new genus (OPU 14) and eight putative new species of *Halorubrum* (OPUs 2, 3, 6, 7, 10, 11, 12 and 13) could be identified. OPU 14, with 27 isolates, appeared as an isolated branch, and the closest relative was *Hrr. tibetense* with a 92.3% 16S rRNA sequence identity. The remaining OPUs detected affiliated with classified *Halorubrum* species with identity values above 98.7%. Most of the OPUs were present in two or more locations, and OPU 8 was the only one detected in one sample (LV). The clade comprising *Hrr. californiense* (OPUs 1, 2 and 3), with 955 isolates, was the largest (23.1% of the total and 42.4% of the genus) and was present in high numbers at all locations except LV. Contrarily, LV showed higher representation of OPUs 7, 8, and 9 that were closely related to *Hrr. coriense* (112 isolates; OPU 7) and *Hrr. litoreum* (211 isolates; OPUs 8 and 9). Almost all OPUs affiliating with *Halorubrum* were isolated from both brines and sediments. Interestingly, OPU 14 was isolated only from sediment samples in CA, SP and LZ.

The branch comprising the genus *Haloarcula* was the second most diverse and accounted for 126 strains that represented 3.1% of the total, and 4.3% of the archaeal isolates. The lineage harbored seven OPUs, four of which (OPUs 24, 25, 26 and 28) were putative new species, and one was different enough to be considered as a putative new genus (OPU 27 with 93.8% identity to the closest type strain *Har. salaria*). The presence of *Har. hispanica* (13 isolates in OPU 22), *Har. salaria* (66 isolates in OPUs 23, 24, 25, 26 and 27) and *Har. marismortui* (47 isolates in OPU 28) species could also be identified. However, this genus was unevenly represented as only LV, FV and FM samples contained these isolates. LV exhibited the highest OPU diversity, and OPUs 22, 25, 26 and 28 were exclusively found in this location. Similarly, OPUs 23 and 27 were exclusive to FV (Table 3[CJR1]). All other archaeal branches detected were represented by only one OPU, and the representatives of the genera *Haloferax*, *Halolamina* and *Haloplanus* were isolated in four or more locations.

All samples rendered between 11 to 15 OPUs, except for LV that showed the highest richness with 22 OPUs (Table 2). In general, brines showed smaller numbers of OPUs than sediments. The former presented a minimum of 9 OPUs at CA and a maximum of 18 OPUs at LV, whereas sediments presented a minimum of 11 at CA and LZ, and a maximum of 21 OPUs at LV. Only ST exhibited the same number of OPUs in both brines and sediments. In this regard, 26 of the 35 archaeal OPUs were isolated from both sediment and brine. *Hbt. noricense* (OPU 31) was a unique group recovered only from brines, and was only present in LV. Contrarily, the putative new genus OPU 14, as well as OPU 17 (*Hgm. rufum*), OPU 21 (*Natronoarchaeum* sp.), OPU 23 (*Haloarcula* sp.), OPU 25 (*Haloarcula* sp.) and OPU 26 (*Haloarcula* sp.), were only isolated from sediment samples.

In some cases, different OTUs (i.e. clusters based on MALDI-TOF MS profiles) affiliated with the same OPU (i.e. unique phylogenetic clades affiliating the new isolates with reference sequences; Supplementary Figure S1). For example, OPU 1 embraced OTUs 5, 22 and 28. However, the reconstruction based on the 16S rRNA gene showed that each OTU represented slightly distinct lineages within the OPU, indicating that they could represent different populations of the same species. Contrarily, there were few cases (OTUs 29, 32 and 65) where the isolates of the same cluster affiliated with two different OPUs (e.g. OTU 29 affiliated with OPUs 10 and 11 that corresponded to *Hrr. arcis* with 96.2% and *Hrr. aidingense* with 97.9% sequence identities, respectively). However, in all such cases, a detailed observation of the MALDI-TOF MS clustering topology (Supplementary Figure S11) showed two slightly different subpopulations that clustered below the threshold settings.

Affiliation of the OTUs corresponding to the bacterial fraction

The bacterial set of isolates was much less diverse (Figure 2). All isolates affiliated with five genera, with *Salinibacter* (1,163 isolates) being the most commonly retrieved organism, followed by the very low occurrence of *Salicola* (21 isolates), *Halovibrio* (5 isolates), *Rhodovibrio* (31 isolates), and *Pontibacillus* (10 isolates). The percentages of bacterial isolates varied between the different locations and ranged between 26.7% (FM) and 64.6% (FV) (Table 1). Surprisingly, no bacterium could be isolated from more than 1,188 strains at the LV and AV locations. *Salinibacter ruber* was the most retrieved species among the bacterial isolates with nearly 95% of the total (corresponding to OPUs 36 and 37). Interestingly, one isolate of OPU 37 affiliated with the sequence of the hitherto uncultured phylotype II (EHB-2) of *S. ruber* species [5]. Sequences from genus *Rhodovibrio* (OPU 40) were retrieved in FM and CA, *Salicola* (OPU 38) in ST and SP, *Halovibrio* (OPU 39) in ST and *Pontibacillus* (OPU 41) in SP. OPU 38, affiliating with *S. marasensis* (DQ019934), possibly represented a novel species of the genus *Salicola* with 97.7% 16S rRNA sequence identity with the closest relative.

Detection of putative novel taxa

A total of 22 unique groups were detected among the 41 OPUs identified in the *Archaea* and *Bacteria* domains (Figures 1 and 2), and they had 16S rRNA gene identities below conservative thresholds with their closest relatives for species and genus (98.7% and 94.9% identity levels, respectively). These comprised 53% of the total, and could represent 18 new species (labeled with a white circle, Figures 1 and 2), and four new genera (labeled with a black circle, Figure 1). Only one putative new species occurred in the bacterial domain. The majority of putative new taxa were simultaneously isolated from different locations, such as OPUs 2, 3, 10, 11, 12, 14, 15, 19 and 20 that were common to at least three different locations (Table 2). The single southern hemisphere sample (LV) provided the highest number of new taxa, where 14 of the 22 potential new taxa were isolated, nine of which were shared by other samples (OPUs 2, 3, 7,

11, 12, 13, 15, 24 and 32; Figure 1). The remaining five species were exclusive to this sample (OPUs 21, 25, 26, 28, and 34; Figure 1).

Analyses of the Euclidean distances between the different diversity measurements, plotted as nMDS (Figure 3), showed that sediment diversity was coincident with that of the overlaying brines. The diversity measurements of the LV, SP and ST samples exhibited larger differences compared to those observed in the island samples (AV, CM, FM, LZ, and FV). Among the samples studied, those from Chile (LV) exhibited the highest diversity and heterogeneity.

Discussion

In this study, a comprehensive analysis is presented for the species retrieved from eight solar salterns distributed among different locations in the Spanish Mediterranean, Canary Islands' Atlantic and Chilean Pacific coasts by means of standard culture methods. A collection of 5,085 isolates was compiled and their MALDI-TOF/MS profiles were obtained. For pragmatic reasons, approximately 16.5% inadequate profiles were discarded and a final set of 4,243 strains was processed. This study may be regarded as one with the largest set of identified cultures obtained from environmental samples. Although this culture set may seem small compared to the one of 284,899 clinical isolates [46], it is comparable to the 3,626 isolates from bottled natural mineral water identified by random amplified polymorphic DNA (RAPD) fingerprinting and 16S rRNA gene analyses [16]. MALDI-TOF/MS profiling has been shown to be very advantageous for analyzing the microbial diversity of the cultured fraction of environmental samples [34]. This technique has also been applied to the study of isolates from sewage sludge [45], PCB-contaminated sediments [25], intra-specific diversity of *S. ruber* [4], and identification of 845 yeast strains isolated from grape musts [1].

The values obtained for the different indices used (i.e. rarefaction curves and Good's coverage) gave us the confidence that most of the cultivable diversity was sampled using the culture media and conditions established for this work. The tandem study combining MALDI-TOF/MS and 16S rRNA gene sequencing rendered a total of 41 different OPUs, of which 22 could be regarded as putative new species according to their genealogic affiliation and identity with the closest related type strain sequences (Figures 1 and 2). This observation was reinforced by previous reports indicating that single clusters in the MALDI-TOF/MS dendrogram (OTUs) can be regarded as individual species [34]. The diversity observed was in accordance with haloarchaea shown to be the principal prokaryotic component of hypersaline habitats [2], and the fact that bacteria (despite having been underestimated for decades) could constitute up to 20% of their total diversity [5]. Our isolates were distributed among 35 distinct archaeal and 6 bacterial OPUs or species.

The most frequently retrieved bacterial species was *S. ruber*, which has been reported to be the most relevant member of this domain thriving in brines [5] and is widely distributed in

many hypersaline systems worldwide [3]. One of the most remarkable results from this survey was the unexpected successful isolation of representatives of phylotype II (OPU 37) (EHB-2; [5]). This phylotype was reported to co-occur with *S. ruber* (EHB-1) in lower amounts, but has been refractory to pure culture for more than a decade [5]. The large number of isolates belonging to this taxon (over 1,100) permitted the recognition of two members of the second phylotype (Figure 2), and was an example of the benefits of large-scale cultivation approaches. It was remarkable that neither the Mallorcan AV nor the Chilean LV samples rendered a single bacterial isolate. These results were very surprising because *Salinibacter* had been isolated in previous studies from AV [35], and sequences of this bacterium and others had been retrieved by a culture-independent pyrosequencing approach (unpublished data). This phenomenon cannot be easily explained but could be related to either the culture media used (although this is improbable given the previous isolation successes), or that the organisms in the samples were in a "viable but not cultivable" state [36]. Other bacterial isolates were representatives of known halophiles but to a much lesser extent, and some of them, such as *Salicola* and "*Pseudomonas halophila*", are of high relevance in hypersaline environments, with the latter actually being a member of *Halovibrio denitrificans* [33].

The archaeal fraction was more diverse than the bacterial component, and all cultures were members of the *Halobacteriaceae* [37]. Members of the genus *Halorubrum* were by far the most frequently recovered in all samples. Actually, this genus accounts for the largest number of species with validly published names within the *Halobacteriaceae* family [37], has been exhaustively studied by means of multilocus sequence analysis (MLSA) and genome analyses, and is a prominent example for understanding the genetic properties of the archaeal species [18]. In fact, the members of this group have also been reported to be the most recovered culture types in similar environments [6,35]. In all cases, most of the retrieved species of this genus were related to *Hrr. californiense*, which was originally described from a crystallizer pond at the Cargill Solar Salt Plant in California [40]. This species was especially relevant in numbers in the Mediterranean and Atlantic sites, although it was present in all samples (Figure 1, Table 3[CJR2]). On the other hand, relatives of *Hrr. coriense* and *Hrr. litoreum* had a major relevance in the Chilean samples. The second most recovered genus was *Haloarcula*, which is also known for being a readily culturable haloarchaeon [6,35]. The remaining 13 cultured genera were less abundant.

Almost all OPUs affiliated with known genera but, surprisingly, 22 of the 41 OPUs could constitute new species considering the minimal conservative threshold of 98.7% (Table 2) 16S rRNA gene identity [47]. However, even if this threshold was considered too conservative, 14 of these OPUs shared 16S rRNA gene identities <98.1% with their closest relative type strains. Moreover, among the putative new species, four of them exhibited identity values <94.5% with the closest relative type strains, which is a threshold that can be considered to discriminate between different genera [53]. The observation that approximately 50% of the detected OPUs could be regarded as new unclassified taxa makes the approach of large-scale screening a good source of taxonomic novelty.

It is remarkable that all taxa detected in brine samples were also retrieved from their corresponding sediment fraction. Contrarily, not all taxa retrieved from sediments could be isolated from their corresponding brines. In this case, sediments appeared to be a source of higher diversity yields of aerobic heterotrophic extreme halophilic taxa compared to brines. The sediments studied here were most probably anaerobic given their moody structure [CJR3](fine-grained sediments exhibit a very low oxygen penetration which occurs only in the first mm, [9]), their blackish color (because of the formation of FeS due to sulfate respiration), and that the first 0.5 cm (out of a 30 cm deep core) had been discarded. Actually, oxygen may already be a limiting factor for aerobiosis in brines given its low solubility [2]. Hypersaline sediments are much more diverse than the overlaying brines, containing larger amounts of bacterial representatives and lower amounts of the archaeal domain [28]. However, among the archaeal representatives, a significant proportion of the taxonomic diversity may correspond to *Halobacteriales* that coexist with other methanogenic extreme halophilic archaea [28]. Not much is known about the role of *Halobacteria* in anaerobic sediments, or whether they only occur as inactive cells that have been sedimented from the overlaying brines. However, some *Halobacteria* have been demonstrated to grow anaerobically by either fermentation or anaerobic respiration using alternative electron acceptors, such as nitrate, dimethyl-sulfoxide or fumarate, among others [2]. The fact that a larger diversity was retrieved in this study from the sediments compared to the overlaying brines at each site might be related to either the higher abundances of cells in the former or to the higher diversity in ecological niches given the distinct availability of substrates and electron acceptors.

Finally, it was also remarkable that the largest source of diversity occurred in the Chilean samples, from where most of the novel taxa could be retrieved, some of which were exclusive to this site (i.e. OPUs 21, 25, 26, 28, and 34). As already hypothesized, studying unexplored sites avoiding environmental sampling redundancy may constitute a source of discovery for microbial novelty [52]. The Chilean salterns of Lo Valdivia were the most remote in this study, and both the water origin and the artisanal operation for the salt production and harvest may be responsible for the larger and novel diversity observed. In this regard, the Chilean saltern operation differs significantly from the other salterns studied. Chilean salterns are constructed with small ponds (approximately 50 m^3) and water is manually transferred between ponds of different salinities. The other salterns contain much larger brine bodies (greater than 1,500 m^3) and water is transferred through inlets with nearly continuous brine feeding.

Altogether, the results of this study indicated that the strategy of screening large sets of isolates constituted a proportional source of novelty. In addition, success in finding new taxa may be enhanced by sampling as yet unexplored sites (such as LV here), or poorly studied sources (such as hypersaline sediments here). The tandem approach combining MALDI-TOF/MS and 16S rRNA gene sequencing allowed cultivable diversity to be studied at a relatively low cost. Moreover, the large-scale screening of cultures provided an excellent approach for gathering more than single strains representing new species from distinct samples

and sampling sites. This approach may help to avoid the important problems of understanding intraspecific diversity promoted by the current practice of classifying taxa based on only a single isolate [48].

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Tables and Figures:

Table 1. Solar salterns, location and salinity of the sampled ponds, percentage of the isolates corresponding to the archaeal and bacterial domains, number of partial and complete sequences of the 16S rRNA gene, number of OTUs and OPUs detected in sediment and brine samples and at each location.

Solar Saltern	Location and coordinates	Sampling date	% Salinity		Nr. OTUs	Archaea (%)	Bacteria (%)	Partial sequences	Complete sequences	Nr. OPUs		
			Cr. 1	Cr. 2						S	B	TOTAL
Trinitat (ST)	Tarragona 40º34'22''N 0º39'13''E	June - 2010	29	27	18	65.3	34.7	23	0	14	14	14
Santa Pola* (SP)	Alicante 38°11'5''N 2º37'46''W	June - 2010	32.8	34.4	23	54.5	45.5	27	11	14	10	15
S'Avall* (AV)	Sant Jordi, Mallorca (IB) 39°19'26"N 2°59'22"E	October - 2010	28	31.5	13	100	0	35	8	13	10	13
Campos* (CA)	Campos, Mallorca (IB) 39º20'46"N 2º59'57''E	October - 2010	33	31	14	66.4	33.6	12	11	10	9	11
Formentera (FM)	Formentera (IB) 38º43'34"N 1º24'14"E	July - 2012	36	34	11	73.3	26.7	5	1	12	11	13
Janubio (LZ)	Yaiza, Lanzarote (CI) 28º55'47"N 13º49'51"W	July - 2012	33.8	35	15	59.7	40.3	7	1	11	7	11
Carmen (FV)	El Carmen, Fuerteventura (CI) 28°27'30"N 13°56'30"W	July - 2012	28	29.5	10	35.4	64.6	1	2	14	5	14
Lo Valdivia* (LV)	Boyeruca, Chile 34º42'16''S 72º1'4''W	December - 2011	36.8	37.6	25	100	0	54	24	21	18	22
					16.1^{B}	69.3^{B}	40.9^{B}	164^{A}	56^{A}	13.6^{B}	10.5^{B}	14.1^{B}

S: sediments; B: brines. ^{A}Total, ^{B}Mean, *Initial set of solar salterns analyzed, Cr: crystallizer pond, IB: Balearic Islands, CI: Canary Islands

Table 2. Distribution of isolates according to their origin and 16S rRNA gene sequence identity with the closest relative type strains.

	Nº OPU	% Similarity	Number of isolates from solar salterns															
			Spanish Peninsula				Balearic Islands						Canary Islands				Chilean Coast	
			ST		SP		AV		CA		FM		LZ		FV		LV	
			S	B	S	B	S	B	S	B	S	B	S	B	S	B	S	B
<94.9%	14	92.3			10				4				13					
	15	93.2					3	27					16				10	5
	27	93.8													6	4		
	16	94.3	6	1			7	3							15	13		
95%-98.1%	26	95															10	
	28	95.1															23	24
	35	95.2			6	7												
	10	96.2	4	20	21				12				36		6			
	25	96.3															20	
	34	96.6															21	3
	20	97.2	4	8	15	1	10		10	5			24	12	5			
	21	97.2															12	
	38[B]	97.7			9	12												
	11	97.9			15				14	2	13	4			11		5	9
	24	98.1									10						1	10
98.2%-98.7%	3	98.2	17	7	19	36	51	23	32	31	7	18	17	25	7		11	15
	6	98.2			25	16							15	19	2			
	19	98.3	5	8	4	1			25	5			18	11			14	3
	12	98.4	7	9	56		30	91	15	13		15			9	5	5	4
	7	98.5													1		38	73
	32	98.7					27										23	3
	2	98.7	15	9	20	12	54	47	23	20	8	15	20	15	14	6	5	5
	13	98.7					7				14	27			2	4	9	3
98.8%-100%	9	98.8	12	3			2	5			5	10					25	63
	23	98.9													5			
	29	98.9	6	1			6				12	26						
	1	98.9	21	11	37	17	32	21	45	57	10	20	31	15	12	14	10	8
	37[B]	99	1		1													
	5	99	3	10	17						7	15						
	30	99.1	5	13	13	3					26							
	8	99.1															26	60
	33	99.2			5	18	35	3			14	22						
	22	99.3															1	12
	39[B]	99.4	5															
	18	99.6	9	6				4	13	9			27				10	5
	17	99.6													13		32	
	40[B]	99.6							22	4	5							
	41[B]	99.6			3	7												
	4	99.7	7	18	10	7	6	75	20	56	9	6	20	21	4			
	31	99.7																3
	36[B]	99.8	55	75	69	229			62	119	108	2	87	153	63	143		
TOTAL number of isolates			182	199	355	366	270	299	297	321	467	180	324	271	153	176	321	308
Total number of new species per solar saltern			7	7	10	6	8	5	8	6	5	5	8	5	10	4	15	12
			7BS		6BS + 4S		5BS + 3S		6BS + 2S		5BS		5BS + 3S		4BS + 6S		12BS + 3S	
Number of new species per solar saltern <98.2%			3	3	5	2	3	2	4	2	2	1	4	1	4	1	8	5
			3BS		2BS + 3S		2BS + 1S		2BS + 2S		1BS + 1S		1BS + 3S		1BS + 3S		5BS + 3S	

[B]Bacteria OPUs[CJR10]

679

680 Figure legends:

681 FIGURE 1. Phylogenetic reconstruction based on 16S rRNA genes of the haloarchaeal isolates
682 and their closest representative type strains. The percentage sequence identity of each OPU
683 with the closest relative is indicated in brackets, and the type strain sequence used to calculate
684 the identities is framed in grey. In addition, sequences <94.9% were considered as putative new
685 genera (black star), and <98.7% as putative new species (white star). Novel taxa occurring in
686 the Chilean sample are indicated with a white circle when co-occurring in other sampling sites,
687 and a black circle when exclusive to this location. The numbering of the OTUs for each OPU is
688 given in the second column, and the third column indicates the location where the OPU was
689 present, and the number of isolates recovered in sediment (S) and brine (B) samples is in
690 brackets. [CJR11]

691

692 FIGURE 2. Phylogenetic reconstruction based on 16S rRNA genes of the bacterial isolates and
693 their closest representative type strains. The percentage sequence identity of each OPU with
694 the closest relative is indicated in brackets, and the type strain sequence used to calculate the
695 identities is framed in grey. In addition, sequences <94.9% were considered as putative new
696 genera (black star)[CJR12], and <98.7% as putative new species (white star). The numbering of
697 the OTUs for each OPU is given in the second column, and the third column indicates the
698 location where the OPU was present, and the number of isolates recovered in sediment (S) and
699 brine (B) samples is in brackets. [CJR13]

700

701 FIGURE 3. nMDS (non-metric multi-dimensional scaling) analysis based on Euclidean distances
702 considering the presence or absence of isolates for each OTU by location and type of sample
703 (sediment or brine). Squares indicate insular and triangles mainland samples. The abbreviations
704 of the symbols are: Trinitat (ST), Santa Pola (SP), Avall (AV), Campos (CA), Formentera (FM),
705 Janubio (LZ), Carment (FV) and Lo Valdivia (LV). The suffix –S indicates sediments and –B
706 indicates brines.

707

708

709

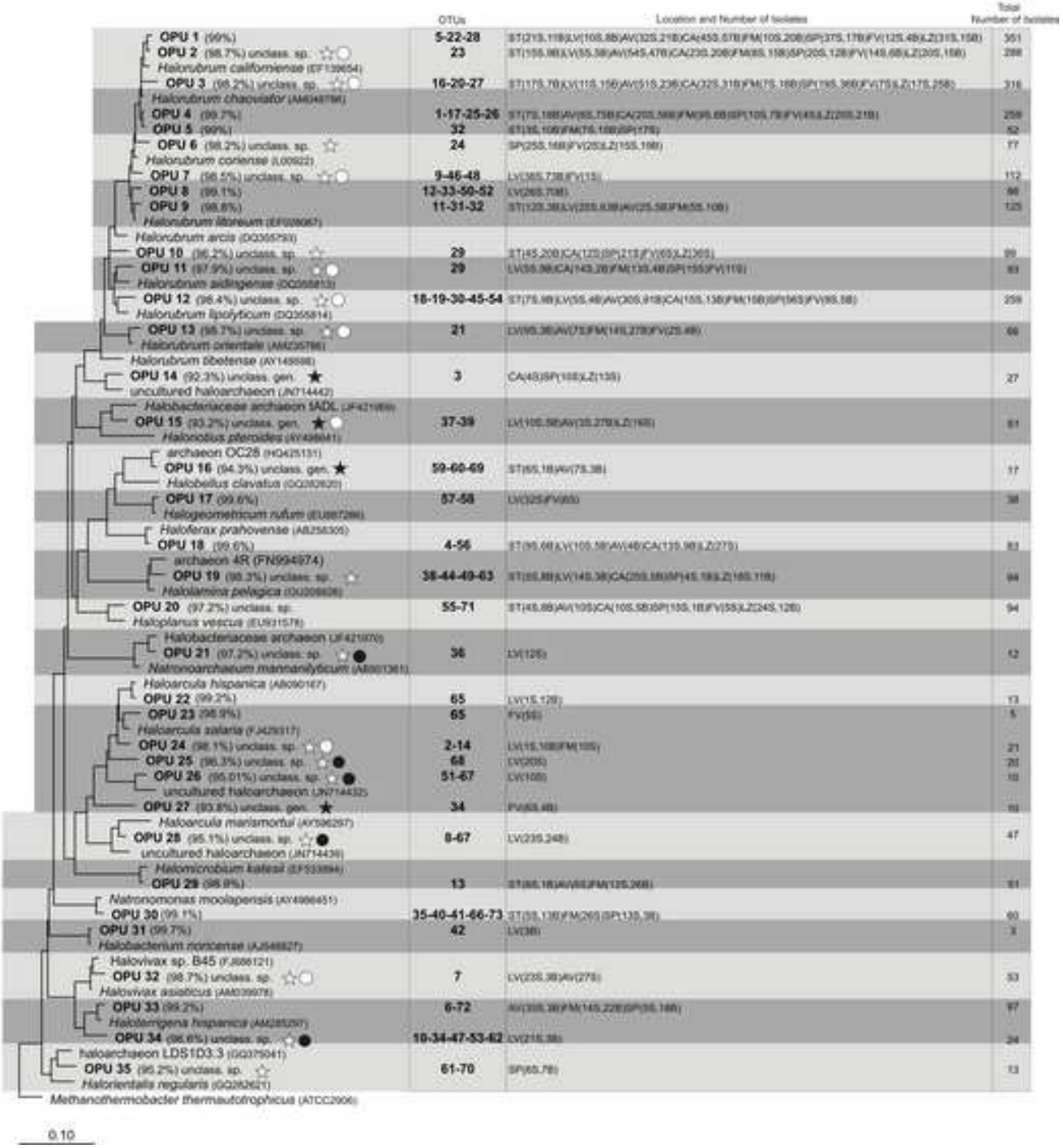


Figure_2

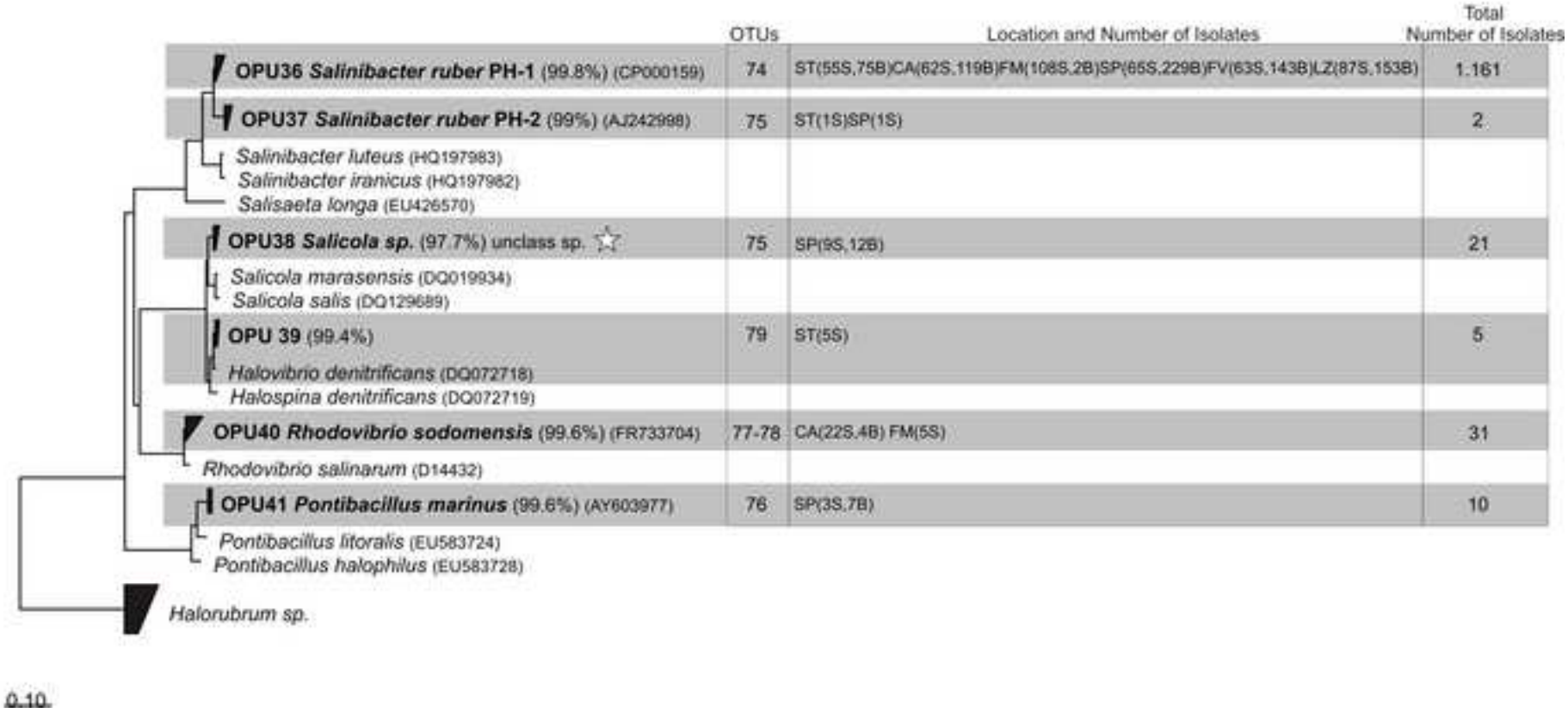
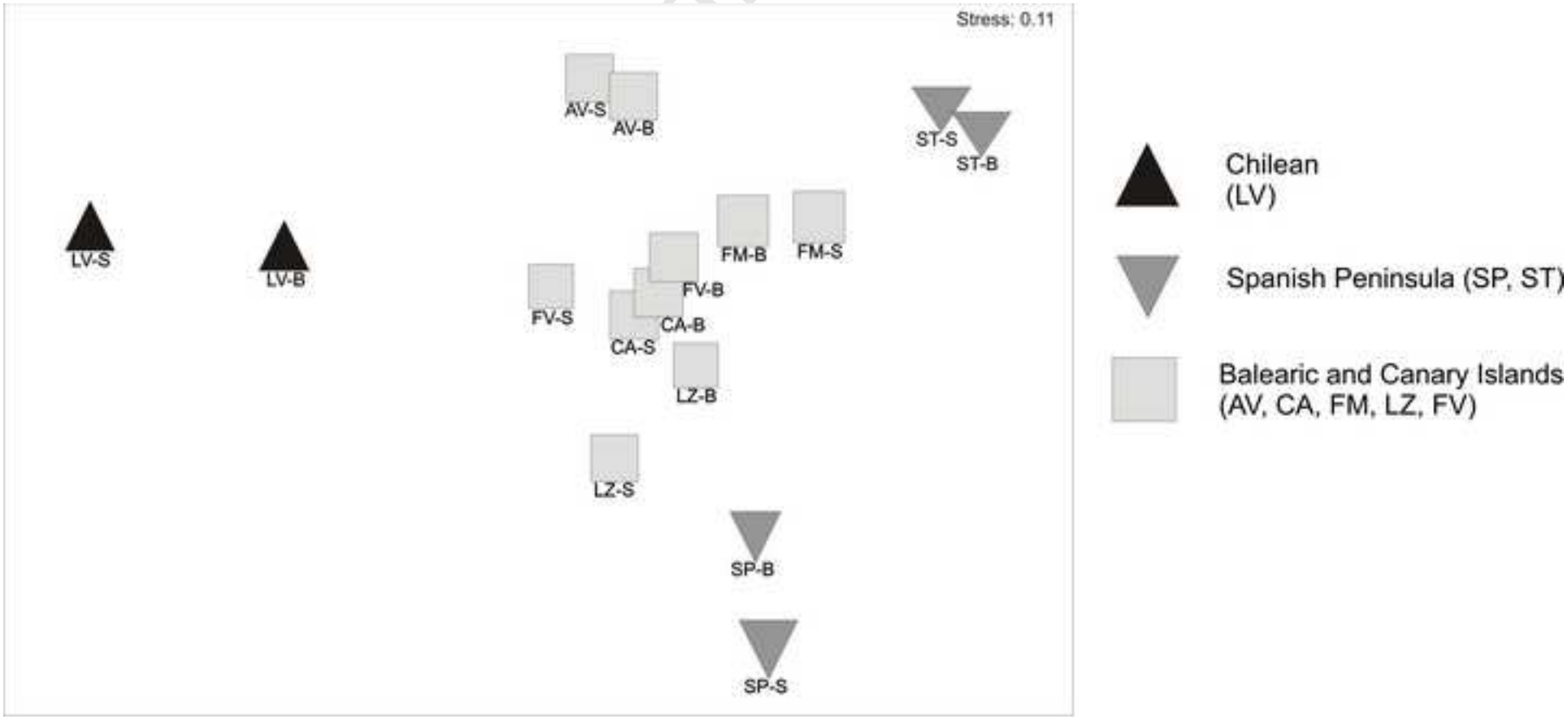

OTUs
Location and Number of Isolates
Total Number of Isolates
OPU36 Salinibacter ruber PH-1 (99.8%) (CP000159)
74
ST(55S,75B)CA(62S,119B)FM(108S,2B)SP(65S,229B)FV(63S,143B)LZ(87S,153B)
1,161
OPU37 Salinibacter ruber PH-2 (99%) (AJ242998)
75
ST(1S)SP(1S)
2
Salinibacter luteus (HQ197983)
Salinibacter iranicus (HQ197982)
Salisaeta longa (EU426570)
OPU38 Salicola sp. (97.7%) unclass sp.
75
SP(9S,12B)
21
Salicola marasensis (DQ019934)
Salicola salis (DQ129689)
OPU 39 (99.4%)
79
ST(5S)
5
Halovibrio denitrificans (DQ072718)
Halospina denitrificans (DQ072719)
OPU40 Rhodovibrio sodomensis (99.6%) (FR733704)
77-78
CA(22S,4B) FM(5S)
31
Rhodovibrio salinarum (D14432)
OPU41 Pontibacillus marinus (99.6%) (AY603977)
76
SP(3S,7B)
10
Pontibacillus litoralis (EU583724)
Pontibacillus halophilus (EU583728)
Halorubrum sp.
0.10

Figure_3


Stress: 0.11
AV-S
AV-B
ST-S
ST-B
LV-S
LV-B
FM-B
FM-S
FV-B
FV-S
CA-B
CA-S
LZ-B
LZ-S
SP-B
SP-S
Chilean
(LV)
Spanish Peninsula (SP, ST)
Balearic and Canary Islands
(AV, CA, FM, LZ, FV)