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Diversity of halophilic archaea in the crystallizers of an Adriatic solar saltern

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Abstract

Haloarchaeal diversity in the crystallizers of Adriatic Sečovlje salterns was investigated using gene fragments encoding 16S rRNA and bacteriorhodopsin as molecular markers. Screening of 180 clones from five gene libraries constructed for each gene targeted revealed 15 different 16S rRNA and 10 different bacteriorhodopsin phylotypes, indicating higher haloarchaeal diversity than previously reported in such hypersaline environments. Furthermore, results of rarefaction analysis indicated that analysis of an increasing number of clones would have revealed additional diversity. Finally, most sequences from the crystallizers grouped within the *Halorubrum* branch, whereas square-shaped '*Haloquadratum*' relatives, repeatedly reported to dominate crystallizer communities, were rare. Presence of such special and diverse haloarchaeal community could be attributed to the Sečovlje salterns rare continuous short-cycling salt production mechanism.

Keywords: 16S rRNA; Bacteriorhodopsin; *Haloarchaea*; Saltern; Hypersaline; Halophiles

1. Introduction

Hypersaline waters of a saltern crystallizer at salinities at or near saturation are one of the most extreme environments with respect to the sodium chloride concentration. The dominant microorganisms in such systems are members of the halophilic archaeal family *Halobacteriaceae* [1–3], co-existing with a small percentage of halophilic bacteria, such as the recently described *Salinibacter ruber* [4], halophilic fungi and protists.

The microbial diversity of this type of hypersaline environments has been extensively studied, focusing on the use of both molecular ecological [1,5–8] and cultivation-based methods [9,10]. All these efforts have been concentrated on crystallizers of solar salterns located in areas with Mediterranean or arid climate, allowing sufficient solar energy for year-round solar salt production with one to two salt harvests per year.

In areas with no dry season, solar salt production is enabled by modifications to an almost universal solar salt production technology. To date, the effect of such changes on microbial assemblages remains unknown. Thus, our objective was to investigate the abundance and diversity of halophilic archaea in the crystallizers of this type of solar salterns and make comparisons with studies of geographically different saltern crystallizers. To this aim, we have chosen the most northerly located

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solar salt production system on the Adriatic Sea coast. Slovenian Sečovlje solar salterns exhibit several interesting features. Dating from the 12th century, the salterns are located in southeast part of Piran Bay along the estuary of the Dragonja River. The salterns receive enough solar energy for salt production from May to October. During the salt harvesting season the evaporation of seawater is strongly enhanced by offshore winds, which cool the evaporating surface so that the brine temperature rarely exceeds 32 °C [11]. The crystallizer sedimentation surface is stabilized with a firm stromatolitic mat, allowing only manual salt collection. Here, the brine is led from the concentrators to crystallizers once or twice a day. Unlike in other salterns studied, the salt is harvested almost continuously as soon as it starts to crystallize, as opposed to one or two yearly harvests. Such continuous short-cycling technology is enabled by keeping the crystallizer brine levels extremely shallow, measuring less than 10 cm in depth. The crystallizers do not have the typical red brine coloration.

Sequencing of PCR-amplified 16S rRNA genes from DNAs extracted from environmental samples has proven to be a powerful and very reliable means of describing microbial community structure. Recently, the studies of genes encoding relevant ecological activities came in focus gaining insight into ecological functions of groups under investigation [12,13]. To further assess haloarchaeal diversity, we have chosen to investigate – in parallel – the diversity of bacteriorhodopsins, a wide and diverse group of proteins occurring almost ubiquitously within haloarchaea [14]. Containing retinal as a chromophore, bacteriorhodopsins function as proton pumps within the membrane, generate an electrochemical gradient and are assumed to have a role in a bioenergetics of the cell, providing a light-driven energy source.

2. Materials and methods

2.1. Saltern and biomass collection

Water samples were collected from the crystallizer pond at the Sečovlje solar saltern located in Sečovlje (near Portorož), Slovenia (E 13°36′, N 45°28′), in August 2003. The water was kept in 0.5-l sterile flasks for 2–3 hours until further processing in the laboratory. Samples (5 μl) were analyzed using a Zetopan Binolux microscope (Reichert, Germany). For cell counts, cells were stained with acridine orange (Sigma–Aldrich Chemie, GmbH, Steinheim, Germany) and 20–30 microscopic fields were analyzed.

The following physicochemical parameters were determined: pH (ISO 10523: 1994E, electrometric method), temperature and water activity (a_w) of the sample (CX-1 system, Campbell Scientific Ltd.). Concentrations of nitrate (NO_3^-), ammonium (NH_4^+) and phosphate (PO_4^{3-}) were analyzed using standard colorimetric procedures [15]. Extraction of total microbial community DNA was performed as described earlier [16]. Samples were stored in Tris–EDTA buffer, pH 8 at 4 °C.

2.2. Molecular techniques

16S rRNA PCR amplifications were performed using *Taq* DNA polymerase (Fermentas), primers D30 (5′-ATT CCG GTT GAT CCT GC) and D56 (5′-GYT ACC TTG TTA CGA CTT) from Arahal et al. [17] and the following program: 94 °C (2 min), followed by 30 cycles of 94 °C (45 s), 50 °C (45 s) and 72 °C (90 s), with an additional 5 s added for each cycle and a final 10 min extension step of 72 °C.

The second set of 16S rRNA PCR amplifications was performed using primers Arch21F (5′-TTC CGG TTG ATC CYG CCG GA) and Arch958R (5′-YCC GGC GTT GAM TCC AAT T), following the protocol described by DeLong [18].

Rhodopsins were PCR-amplified using primers bop1 (5′-GAC TGG YTG TTC ACS ACR CC) and bop2 (5′-ASG TCR AKS ACC ATG AA) as described earlier [19]. PCR products were then cloned using the pGEM-T Easy cloning kit (Promega) according to the manufacturer's directions and five clone libraries were constructed for each gene targeted. Clones positive for inserts were then sequenced using Perkin Elmer 377 DNA sequencer and edited using Sequencher (Gene Codes, Ann Arbor, USA). For bidirectional sequencing of 16S rRNA fragments, the additional primer B99 (5′-GTG TTA CCG CGG CTG CTG) was used [17]. Clone sequences have been deposited into GenBank under Accession numbers DQ071586–DQ071610.

2.3. Sequence comparisons and rarefaction analysis

Relevant sequences were obtained from GenBank (www.ncbi.nlm.nih.gov) using BLASTN and BLASTP. Alignments of the 16S rRNA region corresponding to nucleotides 74–710 (*Escherichia coli* numbering, [20]) and the bacteriorhodopsin region corresponding to Helix C to Helix G [14] were created using ClustalX [21]. The stability of the alignments has been assessed through comparison of ClustalW alignments produced under different gap opening/extension penalties using SOAP [22]. This conservative approach assumes that confidence in homology assessment increases with stability to variation in alignment parameters, and only unaffected positions across the spectrum of settings are considered to be unambiguously aligned and are kept for phylogenetic analysis. In the 16S rRNA analysis, gap penalties were incrementally adjusted from 7 to 17

by steps of 2 and extension penalties were adjusted from 0.01 to 0.09 by steps of 0.02. For bacteriorhodopsin analysis, gap penalties were incrementally adjusted from 2 to 17 by steps of 3 and extension penalties were adjusted from 0.01 to 0.09 by steps of 0.02. Finally, identical bacteriorhodopsin sequences were removed.

Putative chimeric sequences were recognized and omitted from further studies: (i) by using the CHECK-CHIMERA program available from Ribosomal Database Project II [23]; (ii) by looking for taxa that changed positions in trees based on sequences of 250 nucleotides from both 5′ and 3′ end and (iii) by looking into sequences with unrealistically long branches or unique branching sites.

We used ∫-LIBSHUFF [24] to compare our 16S rRNA and bacteriorhodopsin libraries, as well as our bacteriorhodopsin library to published diversity results of Santa Pola solar salterns [19]. For diversity indices calculations, defining operational taxonomic units and rarefaction analysis computer software DOTUR [25] was used.

2.4. Phylogenetic reconstruction

For 16S rRNA analysis, alternative evolutionary models were tested employing PAUP* [26] and Modeltest 3.6 [27]. The optimal model of nucleotide substitution was the Tamura–Nei substitution model, with gamma-distributed rate heterogeneity and a proportion of invariable sites (TrN + Γ + *I*).

The most likely relationship between bacteriorhodopsin sequences was identified using maximum likelihood reconstruction methods as implemented in PROML version 3.6a2.1 of the PHYLIP package [28], with a JTT substitution matrix combined to a Γ8 + INV model and parameters optimized by TREE-PUZZLE 5.0 [29]. Global rearrangement was enforced and sequences were jumbled two times.

Topology supports for best topologies were assessed using Bayesian inference [30] and non-parametric bootstrapping. Bayesian posterior probabilities were computed under the same ML model with MrBayes 3.0b4 [31] with a Metropolis-coupled Markov chain Monte Carlo algorithm [32], by running four chains for 10^6 generations, taking samples every 100 generations. The initial 6% of trees was discarded as 'burn-in' to ensure that the chains have reached stationarity. From the resulting 9400 trees, posterior probabilities were assessed for individual clades based on their observed frequencies.

MP bootstrap support values were assessed by 1000 bootstrap replication and ML bootstrap support values by 100 bootstrap replications. The heuristic branch-swapping algorithm TBR with 10× addition sequences randomized was applied under this optimality criterion.

3. Results

3.1. Sample characteristics

The water activity (a_w) of the sample originating from the crystallizer pond of Sečovlje solar salterns was 0.759. At the time of sampling, the temperature of the hypersaline brine was 30 °C. The sample was visibly turbid, with a pale-yellow color, and viscous with slightly alkaline pH 8.0. Light microscopy (phase contrast; 1000× magnification) revealed rods and polymorphic forms, typical of haloarchaea. Cell counts were estimated at 3×10^6 cells ml^{-1}. The concentrations of phosphate (PO_4^{3-}), nitrate (NO_3^-) and ammonia (NH_4^+) were 2.04 μM (0.194 mg/l), 28.33 μM (1.757 mg/l), and 133.33 μM (2.400 mg/l), respectively. These values are in consistence with the values obtained in 1997, when psysico-chemical parameters were measured throughout the salt production season [11].

3.2. Sequence analysis of 16S rRNA gene fragments

A sample of 120 clones from five 16S rRNA libraries was randomly selected and sequenced. A PHYLIP generated distance matrix was used to assign sequences to OTUs using furthest neighbor algorithm at a distance of 3% as implemented in DOTUR. Within this sample, 15 OTUs were detected, with 6 OTUs represented by at least three clones. Next, the sequences were used to construct phylogenetic trees including other 16S rRNA sequences from the database. Representative clones from all phylotypes detected in phylogenetic analysis were sequenced on both strands and are presented in Fig. 1. The 16S rRNA tree was divided into nine groups, based on the least inclusive monophily composed by Sečovlje sequences and next nearest relatives from GenBank.

Represented by clone Sec16SA5, 43 sequences of group I formed a distinct branch with high affinity to *Halorubrum lacusprofundi*, a species originating from Antarctic monomictic Deep Lake, with salinities up to 32%. *H. lacusprofundi* is not a psychrophilic haloarchaeon, as its temperature optimum and maximum are 31–36 and ~42 °C, respectively [33]. Relatives of *H. lacusprofundi* represented a dominant fraction of halophilic archaea in Deep Lake [6], but were, to our knowledge, not encountered in other hypersaline systems.

Thirty-six sequences clustered in group II as a second *Halorubrum* group. These sequences, represented by clone Sec16SD8, were placed next to haloarchaeon CSW5.28.5, originating from an Australian solar saltern crystallizer to whom Sec16SD8 shared 99% sequence similarity. Altogether, 66% of the 16S rRNA sequences recovered (79 clone sequences) were affiliated with members of the genus *Halorubrum*. In addition, sequences belonging to group III (10% of total sequences recovered)

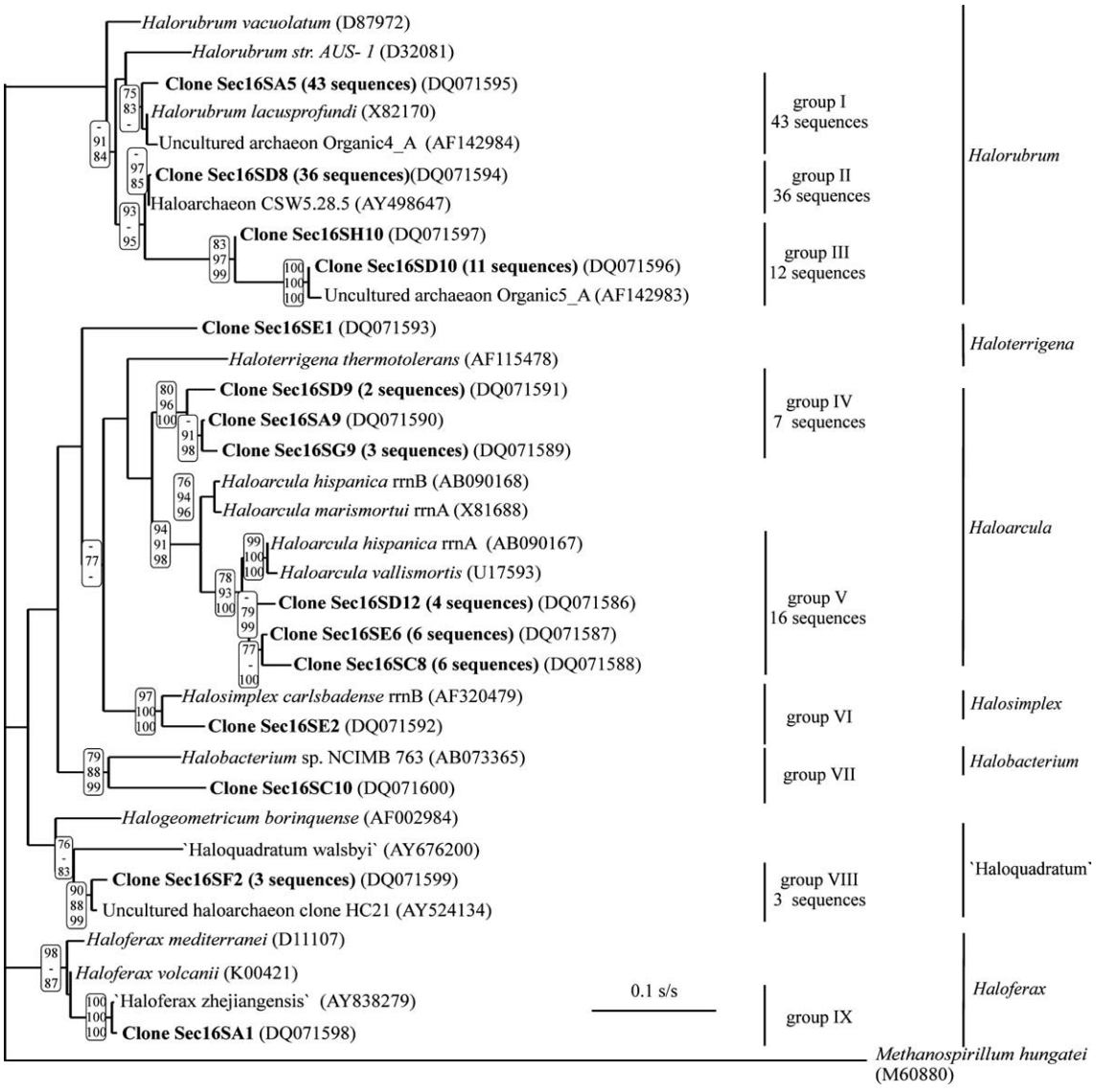

Fig. 1. Phylogenetic reconstruction for 16S rRNA sequences recovered from a crystallizer pond. Most likely topology shown here was obtained under a TRN model of sequence evolution [30] + Γ distribution of rate heterogeneity among sites + fraction (INV) of invariant sites. Scale represents expected number of substitutions per site. Numbers represent bootstrap values for node of special interest. From top to bottom: maximum likelihood, maximum parsimony and posterior probability (expressed as percentages).

were found to be related to *Halorubrum*-placed environmental 16S rRNA sequences, recovered in previous studies on hypersaline Antarctic lakes [6]. Related sequences were also recovered from Australian Cheetham salterns, where they represented 16% of total sequences recovered [10].

The second most recovered 16S rRNA sequences, termed group V, represented 13.3% of total sequences recovered. These sequences formed a clade with moderate affinity for *Haloarcula* clade. These groups were followed in abundance by group IV (5.8% of total sequences recovered), which formed a new monophyletic clade within *Halobacteriaceae*, with low-to-moderate affinities to genus *Haloarcula*. Sequences forming group VIII were affiliated with '*Haloquadratum walsbyi*', to which they shared 91% sequence similarity. The representative of group VIII, clone Sec16SF2, grouped with saltern-originating clone HC21 (Bidle et al., 2004, unpublished). Clone Sec16SE2 was most similar to *Halobaculum gommorense*, to whom it shared 92% sequence similarity.

The remaining three groups, represented by one sequence, each showed affinities to *Halosimplex carlsbadense* (group VI), '*Haloferax zhejiangensis*' (group VII) and *Halobacterium* sp. NCIMB 763 (group IX).

3.3. Sequence analysis of bacteriorhodopsin gene fragments

A sample of 60 clones from each of the five bacteriorhodopsin libraries constructed was randomly selected

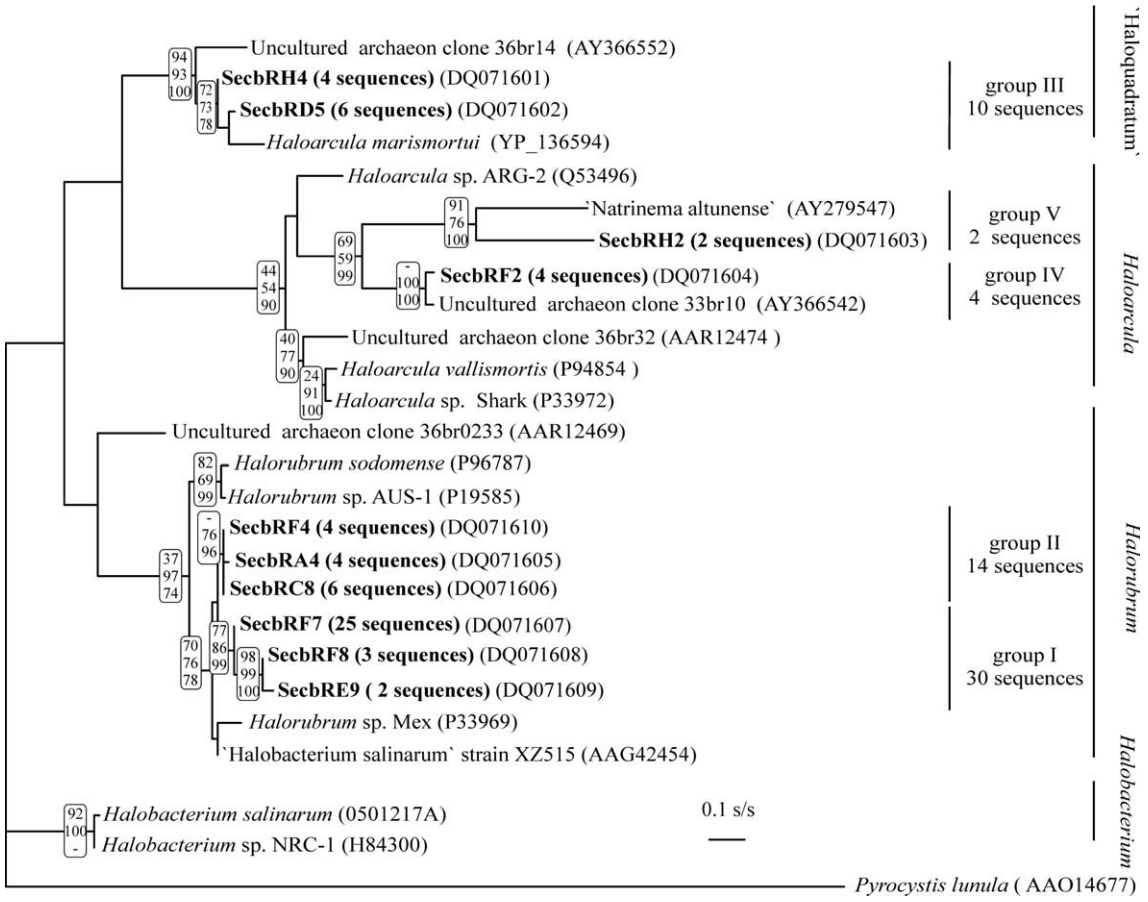

Fig. 2. Phylogenetic relationships among translated bacteriorhodopsin sequences. Most likely topology show here was obtained under a JTT model of sequence evolution + Γ distribution of rate heterogeneity among sites. Ln $L = -4153.82069$. Scale represents expected number of substitutions per site. Numbers represent bootstrap values for node of special interest. From top to bottom: maximum likelihood, maximum parsimony and posterior probability (expressed as percentages).

and sequenced. All of the sequences recovered shared similarities with haloarchaeal bacteriorhodopsins of the proton pump class.

The bacteriorhodopsin tree could be divided into five groups (Fig. 2). Concurrent with 16S rRNA environmental sequences analysis, the vast majority of the recovered sequences (73%) showed affinity for the bacteriorhodopsin *Halorubrum* clade. These sequences formed groups I and II, represented by 30 and 14 protein sequences, respectively. Within *Halorubrum*, these individual clades showed affinities for AR1/AR3 and XZ515/Mex clades. Two different protein sequences formed group III (altogether, 120 sequences form this clade, but 8 were deemed identical for phylogenetic purposes, see Section 2). This well-defined clade was found to be related to '*H. walsbyi*' clade, environmental bacteriorhodopsin sequences found to dominate the haloarchaeal community in the crystallizers of Spanish Santa Pola solar salterns [19]. Two more groups (IV and V) showed affinities to environmental bacteriorhodopsin sequences, not related to any previously known sequences and related to the '*Natrinema altunensis*' bacteriorhodopsin sequence, respectively. Further protein alignment analysis (data not shown) suggested that the reported PCR products are likely to function as proton pumps in the environment, showing characteristics unique to and consistent with bacteriorhodopsins.

3.4. Sequence comparisons, diversity indices and rarefaction analysis

The 16S rRNA and bacteriorhodopsin libraries constructed during this study were compared to themselves using the integral from of statistic in ∫-LIBSHUFF with 10,000 randomizations. In this comparison, we found that for 16S rRNA gene libraries, P values ranged from 0.069 to 0.8136 with margin of error for the P value's 95% confidence interval of 0.001, whereas bacteriorhodopsin libraries P values ranged from 0.061 to 0.9975 with margin of error for the P value's 95% confidence interval of 0.008. Thus, we were unable to identify a significant difference with an experimental error rate of 0.05.

Although the biodiversity of hypersaline systems has been extensivelly studied, we were unable to compare

Table 1
Diversity indices for Sečovlje solar salterns compared with Santa Pola solar salterns and Antartic Deep Lake

Diversity index	Sečovlje 16S rRNA library	Santa Pola 16S rRNA library	Deep Lake 16S rRNA library	Sečovlje bacteriorhod opsin library	Santa Pola bacteriorhod opsin library
Diversity (H')	2.235	1.673	0.94	1.485	1.427
Dominance (SI')	0.14	n.d.	0.14	0.26	0.25
Species richness (Chao)	27	n.d.	15	9	9
Species richness (ACE)	20.32	n.d.	n.d.	6.52	9.19

Data from Bowman et al. [6], Papke et al. [19] and this study; n.d., not determined.

our 16S rRNA data to published diversity of other hypersaline systems due to the lack of data available in databases – almost in all cases a consequence of RFLP library screening and sequencing representatives of detected phylotypes. Therefore, we had to limit our comparisons to the bacteriorhodopsin gene library, obtained in Santa Pola solar salterns [19]. The obtained P values were less than 0.001, suggesting that there is a high probability that the bacteriorhodopsin libraries constructed in Sečovlje and Santa Pola solar salterns contain different taxonomic lineages.

Indices indicating diversity (Shannon–Weaver index), dominance (Simpson index) and species richness (Chao 1 and ACE index) are shown in Table 1. The indices showed that there was a shift towards lower diversity and species richness when Sečovlje solar salterns were compared to previously published indices, available only from studies on Santa Pola solar salterns and Antarctic Deep Lake.

Rarefaction analysis was applied to evaluate whether screening on 120 16S rRNA clones and 60 bacteriorhodopsin clones was sufficient to estimate diversity within clone libraries (Fig. 3.). The rarefaction curves did not reach a clear plateau, indicating that analysis of an increasing number of clones would have revealed further diversity.

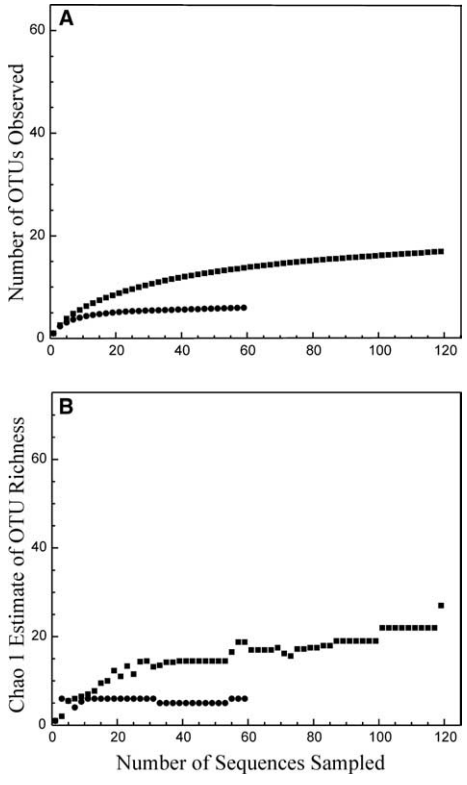

Fig. 3. Rarefaction (A) and Chao 1 richness estimator collector's curves (B) using 16S rRNA (■) and bacteriorhodopsin (●) gene sequences from Sečovlje salterns.

4. Discussion

4.1. Saltern crystallizers worldwide

The microbiota of a crystallizer, as one of the most extreme environments with respect to sodium chloride concentration, received much attention over the years, with large body of evidence pointing both towards long-term survival of archaea [34] and presence of extraterrestrial salt deposits [35]. Extensive microbial ecology data from several in situ PCR studies on saltern crystallizers established low overall diversity with prevalence of halophilic members of *Archaea*. Moreover, the dominant fraction of microbial community in the crystallizers of Spanish Santa Pola was represented by relatives of only recently cultivated '*H. walsbyi*' [9]. Over the years, this enigmatic square-shaped microbe was reported to dominate microbial populations in the crystallizers of Israeli Eilat salterns, Australian Cheetham salterns and solar salterns in Peru [36,10,37]. Given the almost universal solar salt production technology, the crystallizers worldwide have been assumed to support similar microbial communities. The impact of local geographical differences was not emphasized until differences in whole community lipid and pigment patterns of two geographically distant solar salterns were described [38]. During a five-year period, whole lipid and pigment patterns were found to be more complex in Californian Newark saltern compared to Israeli Eilat saltern. This difference in complexity (and presumably, microbial populations) was attributed to the Newark salterns milder weather conditions (cooler and more rainfall) as well as the nutrient-enriched water source. These observations pointed out the need to further investigate different salt productions systems worldwide.

4.2. Bacteriorhodopsins versus 16S rRNA as molecular markers

In the study presented here, we have focused on haloarchaeal diversity in the crystallizers of a solar saltern in the Adriatic region, where prevailing weather conditions act to shorten the microbial community development time by limiting the solar salt production to the arid part of the year. For this in situ PCR study, we took into account the limitations of 16S rRNA gene as molecular marker: its inability to provide useful genetic information at or below the species level or to provide useful ecological/physiological information regarding the organisms under investigation. When the taxonomic diversity, as in case of a saltern crystallizer, is well established at 16S rRNA level, the study of genes that code for relevant ecological activities can be considered a practical alternative. For these reasons, bacteriorhodopsins were introduced as a molecular marker. Besides confirming useful mechanism of generating maintenance energy for cells at low nutrient regimes, these proteins were proven to be present in significant amounts in solar salterns by spectroscopic and other analysis [38,39]. Moreover, recently analyzed environmental bacteriorhodopsin sequences showed considerable amount of diversity below the species level [19].

Diversity indices calculated in this study indicated that the overall diversity revealed using 16S rRNA as molecular marker is higher in Adriatic Sečovlje salterns than in Santa Pola salterns, while bacteriorhodopsin libraries diversity indices were comparable. Furthermore, results of libraries comparisons indicated that Santa Pola and Sečovlje libraries probably contain different taxonomic lineages. Using 16S rRNA as molecular marker, we found that the haloarchaeal community in the crystallizers was strongly dominated by two groups of *Halorubrum* related environmental phylotypes. In addition, members of four other haloarchaeal genera and two groups of environmental phylotypes encountered in other hypersaline environments with one group of sequences forming a separate clade, were found in the sample studied. Community structure found using bacteriorhodopsin as molecular marker parallels that found by in situ 16S rRNA analysis, yet confirming two novel groups undetected by 16S rRNA analysis. Some of the diversity encountered in the Adriatic region parallels the diversity in other salterns studied, such as abundance of two *Halorubrum* groups encountered in Australian Cheetham salterns, and the presence of novel branch of bacteriorhodopsins, initially described in Spanish Santa Pola salterns. However, the square-shaped morphotype was not observed in our study and only a minor fraction of the recovered sequences was found to be related to the '*Haloquadratum*' clade (91% sequence similarity). Therefore, we must conclude that the haloarchaeal community structure in the Adriatic region is different than in other salterns studied.

4.3. Influence of the production technique on the archael community

With prevailing weather conditions limiting the salt production to the summer period, Sečovlje salterns represent a yet unstudied type of solar salterns employing continuous short-cycling solar salt production technology. Here, the eutrophic concentrator brine is led to the crystallizers once or twice a day. To ensure sufficient evaporation, the crystallizer brine is extremely low in depth (<10 cm) compared to 30–50 cm in other salterns studied. The solar energy collection is enhanced by dark colored microbial mat at ponds bottom, while strong offshore winds act to aid evaporation and cool the brine temperature. To prevent possible damage from rainfall, the salt is harvested almost continuously, sometimes as soon as it starts to crystallize at 27% of total salts concentration. The time for archaeal community development is thus extremely shortened, allowing haloarchaeal population density at only 10^6 cells ml^{-1}.

In conclusion, the dynamic continuous short-cycling production system appears to act to drastically reduce the number of slow-growing species (e.g. '*H. walsbyi*' compared to members of *Halorubrum*, *Haloferax* and *Haloarcula* [9,10,40]). Combined with nutrient-enriched source water and mild weather conditions, this solar salt production technology can give rise to a very diverse archaeal community, dominated by faster growing members of halophilic archaea.

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