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RESEARCH ARTICLE

ASSESSING THE DIVERSITY OF PROKARYOTIC COMMUNITIES AND NANOHALOARCHAEAL LINEAGES IN VARIOUS AQUATIC HYPERSALINE HABITATS (TURKEY) USING HIGH-THROUGHPUT SEQUENCING AND CLONING

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ABSTRACT

In this study, distribution of the prokaryotic groups in Tuz Lake, Ayvalık and Tuzlagözü solar salterns was investigated using 16S rRNA gene targeted approaches. The relative abundance of prokaryotic taxa in the samples was detected by using high-throughput sequencing. Operational taxonomic units (OTUs) associated with *Haloquadratum* were the most abundant in MiSeq reads. *Nanohaloarchaeota*-related OTUs were rare (<1%) in Ayvalık and Tuzlagözü solar salterns, and around 5% in Tuz Lake. Other OTUs frequently found and shared in the samples were associated with *Halorubrum*, *Halonotius* and *Salinibacter* genera.

Nanohaloarchaeota lineages, whose phylotypes have been frequently detected in diverse hypersaline environments, were examined in more detail by 16S rRNA gene cloning using group-specific primer. Some of the highly represented nanohaloarchaeal phylotypes in the clone libraries showed low similarity to any sequence in the database, generating two distinct clades. One of the novel lineages was found to be prominent in the clone library constructed from Ayvalık sample. Phylotypes showing 95-97% sequence similarity to *Ca.* Nanopetramus were also highly represented in Ayvalık. Phylotypes frequent in the clone libraries of Tuz Lake and Tuzlagözü samples were associated with a novel lineage, as well as *Ca.* Nanosalina and its relatives.

Keywords: *Nanohaloarchaeota*, High-Throughput Sequencing, Cloning, Halophiles

1. INTRODUCTION

Microbial diversity in the hypersaline lakes and solar salterns in different parts of the world has been extensively investigated, and the majority of microbial groups developing in these environments have been isolated, identified and characterized. Isolation and cultivation of the microorganisms are essential to understand the whole biology. However, most microorganisms cannot be cultured in the laboratory conditions due to technical difficulties. In recent years, knowledge about uncultured microbial lineages has expanded with advances in technology and the development of new approaches and tools.

Haloquadratum and *Salinibacter* have been reported to be abundant archaeal and bacterial taxa in the hypersaline environments, particularly in thalassohaline habitats (reflecting the ionic composition of seawater) [1-5]. In addition to such well-defined taxa, the presence of novel lineages such as *Nanohaloarchaeota*, which is globally distributed in these ecosystems and does not yet have a pure culture, has been revealed using methods based on high-throughput sequencing. Grant et al. (1999) obtained phylotypes belonging to *Nanohaloarchaeota* from the clone library constructed from the sample of Lake Magadi (Kenya), an extremely alkaline salt lake, and reported that they belong to a novel lineage in the archaea. This novel lineage could not be classified due to the lack of closely related sequences to be compared. Narasingarao et al. (2012) performed a metagenomic analysis of the size-fractionated hypersaline water samples from Tyrrell Lake (Australia) and obtained two genomic sequences of the novel taxon that they proposed as the class *Nanohaloarchaea* (currently *Nanohaloarchaeota* phylum).

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Nanohaloarchaeota exhibits a distant phylogenetic relationship with *Halobacteria*, and the cells of this group are rather small (<0.8 μm). Based on microscopic observations and genome analysis, it was initially suggested that *Nanohaloarchaeota* cells are free-living. In recent studies, nanohaloarchaeal and haloarchaeal cells have been co-cultured, and it has been reported that they required haloarchaeal cells for growth [7-9]. Nanohaloarchaeal phylotypes and genomic sequences have been recovered from clone libraries, metagenomic and single-cell genomic analyzes from thalassohaline and athalassohaline (non-marine origin) environments including solar salterns, neutral to alkaline or slightly acidic or perennially cold hypersaline lakes, magnesium-rich deep-sea hypersaline basins, high altitude salt flats rich in boron and lithium [4-7, 10-18].

In this study, we aimed to examine the structure of prokaryotic communities in a thalassohaline lake, a coastal solar saltern and an inland solar saltern fed with hypersaline spring water, using small-subunit (SSU) rRNA gene-targeted amplicon sequencing which allowed us to detect taxa that are difficult to culture and / or rare as well as abundant ones. The relative abundance of the prokaryotic taxa as well as *Nanohaloarchaeota* in the samples has been revealed. Despite their widespread distribution, so far very little is known about the occurence of nanohaloarchaeal lineages in distinct hypersaline environments in Turkey. We also conducted a preliminary study on the distribution of nanohaloarchaeal lineages in these environments and carried out molecular cloning for the phylogenetic analysis of this group.

2. MATERIALS AND METHODS

2.1. Sampling

Surface water samples were taken from different hypersaline environments. Tuz Lake is located within the borders of Ankara, Konya and Aksaray provinces of Central Anatolia and sampling was carried out near the district of Şereflikoçhisar (39°07″N, 33°41″E). Another sampling area was the crystallization pond of Ayvalık solar saltern on the Aegean Sea coast (Balıkesir province; 39°26″N, 26°71″E). Sampling was also done from the crystallization pond of an inland solar saltern, which was fed by a hypersaline spring water in Tuzlagözü village (Sivas city; 39°71″N, 37°67″E) (Figure 1). Total salt concentrations and pH of the samples were measured by using hand refractometer (Eclipse) and pH meter (Mettler Toledo).

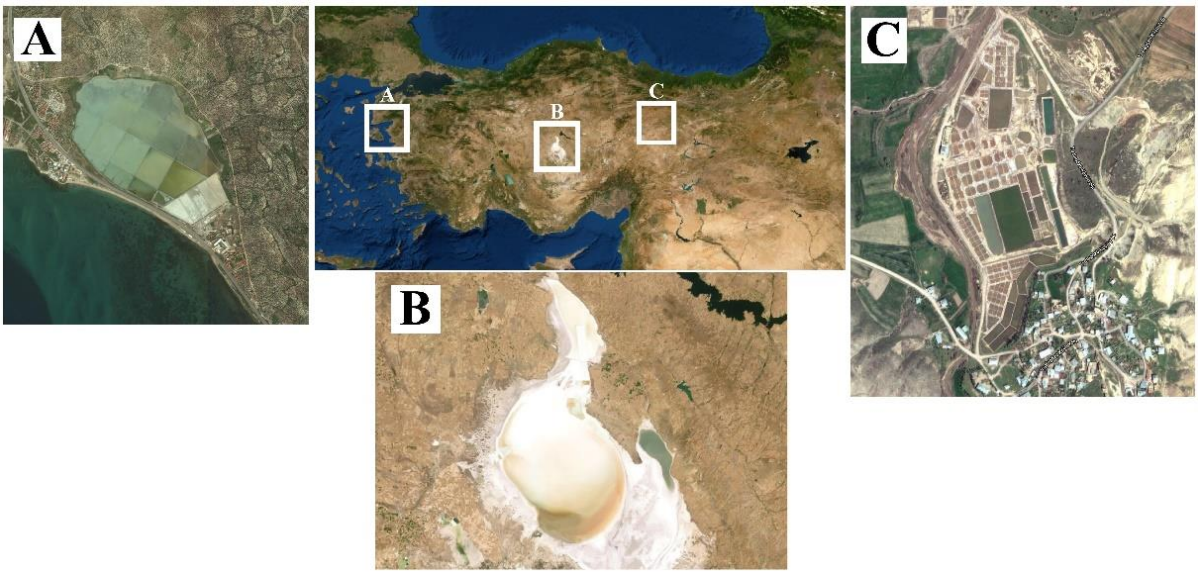

Figure 1. Sampling locations. Ayvalık Saltern (A), Tuz Lake (B), and Tuzlagözü Saltern (C).

2.2. DNA Extraction

100 mL of water sample was passed through 0.22 µm pore diameter filters (Millipore, Isopore GTTP) and total nucleic acid extraction was performed from these filters as described by Mutlu et al. (2008). After the filters were cut into tiny pieces, they were placed in tubes (2 ml), and extraction buffer (100 mM Tris-HCl and 100 mM EDTA, pH 8.0) and lysozyme (3 mg/ml) were added. The tubes were incubated in a shaker at 37°C, then proteinase K (150 mg/ml) and 10% sodium dodecyl sulfate were added, and the incubation process was repeated. After the addition of NaCl (5 M) and CTAB (10% CTAB, 0.7 M NaCl) solutions, the tubes were put in a 65°C water bath. The tubes were chilled in liquid nitrogen. The heat shock treatment was repeated three times. Phenol-chloroform-isoamyl alcohol (25:24:1) was used for the purification of nucleic acids, and the precipitation step was carried out using ethanol. The DNA pellets were resuspended in Milli-Q water.

2.3. High-Throughput Sequencing

Modified 515f/806rB primer pair targeting V4 region of 16S rDNA genes was used in the amplification step (https://earthmicrobiome.org/protocols-and-standards/16s/). Polymerase chain reaction (PCR) components were as follows, for a total volume of 25 µl: water 13.0 µl, PCR master mix (2x) 10.0 µl, forward primer (10 µM) 0.5 µl, reverse primer (10 µM) 0.5 µl, and template DNA 1.0 µl. PCR reactions were prepared in triplicate. PCR was performed under the following conditions: 94°C 3 min (1 cycle); 94°C 45 s, 50°C 60 s, 72°C 90 s (35 cycles); 72°C 10 min (1 cycle). Triplicate PCR reactions were pooled. PCR amplicons were analyzed on the gel to check if visible and expected size bands were obtained. Amplicons were purified using MoBio UltraClean PCR Clean-Up Kit. Paired-end sequencing was performed by using Illumina MiSeq platform (Argonne National Laboratory, Chicago, USA). QIIME (Quantitative Insights into Microbial Ecology) software was used to analyze 16S rRNA amplicon sequences [19-23]. PyNAST [24] and Uclust [25] were used for alignment and OTU picking, respectively. Since SILVA database is up-to-date, we preferred the use of the results from this database (v132) [26]. Similarity threshold for OTU clustering was chosen as 97%. The taxa names from SILVA database have been replaced with the corresponding ones in Genome Taxonomy Database (GTDB), which references bacterial and archaeal genomes and contains validated taxa names [27].

2.4. PCR Amplification and Cloning of Nanohaloarchaeal 16S rRNA Genes

Nanohaloarchaeota-specific LT1215R (5’ ggccgcgtgtatcccagagc) [7] and archaea-specific Arc21F (5’ ttccggttgatccygcccga) [28] primers were used for amplification of 16S rRNA genes. PCR conditions for the amplification are as follows: 94ºC 3 min (1 cycle); 94ºC 30 sec, 59ºC 1 min, 72ºC 2 min (30 cycles) 72ºC 10 min (1 cycle). PCR reactions were carried out in parallel and then collected in a tube. PCR products of the expected size (~1200 bp) were purified from the gel using the purification kit (Wizard SV Gel and PCR Cleaning System; Promega). Gene cloning was performed according to the instructions of TOPO TA cloning kit (Invitrogen). pCR2.1-TOPO vector in the ligation step and *Escherichia coli* TOP10 cells in the transformation step were used. Colony PCR was performed with M13F (5'-gtaaaacgacggccag -3') and M13R (5'- caggaaacagctatgac -3') primers from white colonies. Appropriate sized PCR products were used in amplified ribosomal DNA restriction analysis (ARDRA). The restriction reaction contains 5 µl of PCR product, 1 µl of buffer (NEB), enzyme 0.5 µl (*Hinf*I, NEB), MQ-water 3.5 µl. The tubes were incubated for 16 h at 37°C and the reactions were loaded on a 2% agarose gel prepared with 1X TBE buffer (60 V, 3 h). After comparing ARDRA patterns, representatives from each profile were selected and sequenced. VecScreen program was used to detect vector contamination and the sequences were compared against NCBI database (https://www.ncbi.nlm.nih.gov/). The sequences were aligned with Muscle program [29]. Phylogenetic tree was created with RaxML [30] and visualized with Figtree (https://github.com/rambaut/figtree/).

3. RESULTS

Total salinity and pH of the samples, the number of the valid reads and OTUs obtained from MiSeq sequencing were given in Table 1. The average length of the reads was 253 bases.

Table 1. Properties of the samples, sampling dates and number of the OTUs obtained from MiSeq data

Sampling Sites	Salinity (%)	pH	Altitude	Collection Date	Valid reads	Observed OTUs
Ayvalık	32.0	7.50	0	Sept 2015	21140	522
Tuz Lake	29.0	7.52	905 m	June 2016	20668	454
Tuzlagözü	26.2	7.45	1390 m	August 2014	49824	613

The reads associated with *Halobacteriales* were found to be predominant in the samples. The relative abundance of reads on *Haloquadratum* was highest in all samples, and it was found that it constituted 55%, 52% and 29% of the reads of Tuz Lake, Tuzlagözü and Ayvalık samples, respectively. *Bacteroidota*-related reads were detected in the samples with low abundance (Figure 2). The most abundant bacterial group in all samples was *Salinibacter*. Majority of the sequences were assigned to known genera (86% of the total reads), and the sequences that could not be assigned to any known taxonomic rank was very low (0.2% of the total reads) (Figure 2).

Other abundant OTUs detected in Ayvalık saltern were associated with *Halonotius* (19%), *Haloferacaceae*_g1 (19%), *Halorubrum* (11%) and *Natronomonas* (7%). *Halobellus* (3.6%), *Haloplanus* (3.5%), *Salinibacter* (2.2%) and *Haloarcula* (1.9%) genera related OTUs were also notable (Figure 2).

Haloparvum-associated OTUs found only in Tuz Lake were quite abundant (17.5%). *Halonotius* (6.5%), *Salinibacter* (4.1%), *Nanosalinaceae*_g1 (3.9%), *Halobacteriales*_g2 (2.4%), *Ca.* Nanosalinarum (1.2%), *Halapricum* (1.2%) and *Halorubrum* (1.1%) were other low-abundant taxa (Figure 2).

Other remarkable taxa in Tuzlagözü sample were *Halorubrum* (7.7%), *Salinibacter* (5.8%), *Halonotius* (4.8%), *Haloarcula* (4.2%), *Natronomonas* (3.9%), *Bradymonadaceae*_g (3.6%), *Salicola* (3.1%), *Haloferacaceae*_g1 (2.9%), *Haloplanus* (2.2%), *Halovenus* (1.4%) and *Halobellus* (1.1%). *Salicola*-related OTUs obtained only from Tuzlagözü sample.

Among the rare taxa (<1%), OTUs associated with *Halomicroarcula*, *Halomicrobiaceae*_g2, *Nanosalinaceae*_g2 and *Chitinophagales*_g were shared in all three samples (Figure 2).

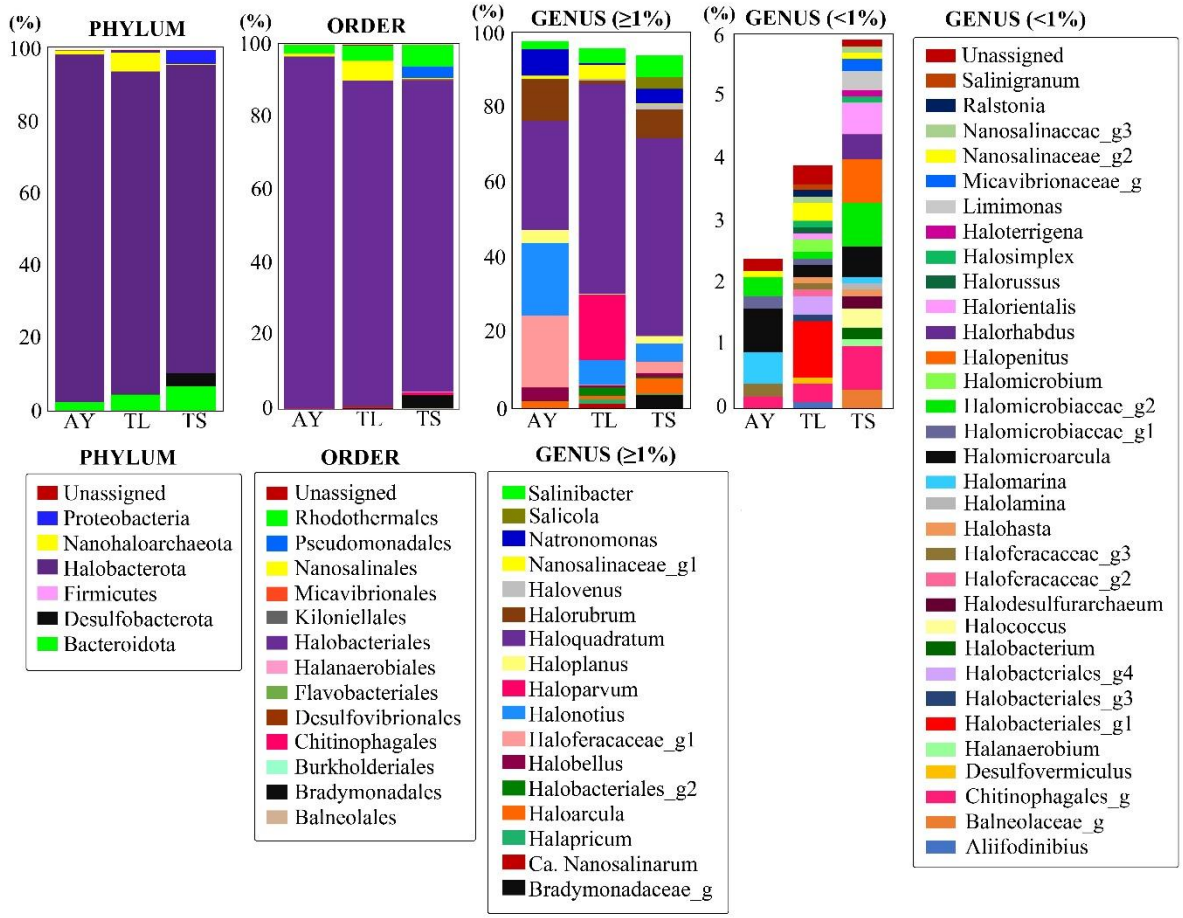

Figure 2. The relative abundance of the prokaryotic taxa in the communities were given at the phylum, order and genus level. AY: Ayvalık Saltern; TL: Tuz Lake; TS: Tuzlagözü Saltern.

16S rRNA gene cloning was performed to obtain phylotypes belonging to *Nanohaloarchaeota* in hypersaline samples. A total of 132 clones were analyzed by ARDRA, which yielded a total of 11 patterns for *Nanohaloarchaeota* and 2 patterns for *Halobacterota* (Figure 3). Profile 3 was highly represented in Ayvalık sample. Profile 1 and 8 were relatively high in Tuz Lake and Tuzlagözü samples (Figure 4). At least one representative was selected from each profile and 29 of them were sequenced.

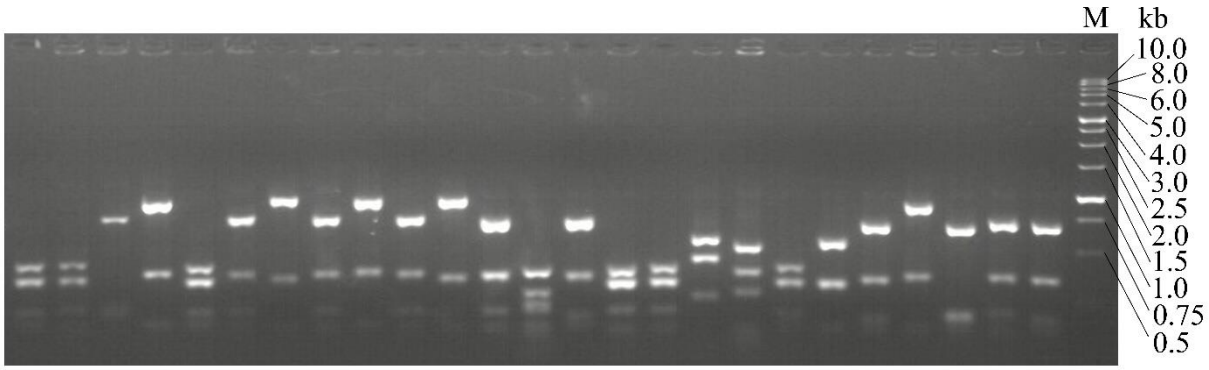

Figure 3. A representative agarose gel image showing ARDRA patterns of 16S rRNA genes obtained through cloning. M, DNA ladder.

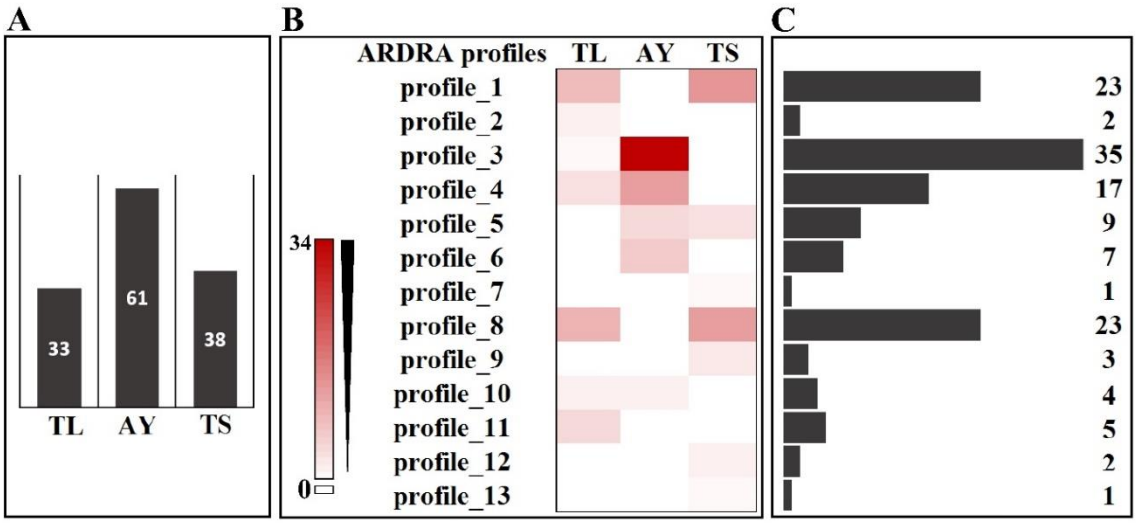

Figure 4. The number of clones screened (A). Heatmap displaying the number of clones for each profile (B). Total number of clones corresponding to each profile (C). AY: Ayvalık Saltern; TL: Tuz Lake; TS: Tuzlagözü Saltern.

The phylogenetic position of *Nanohaloarchaeota* phylotypes was given in Figure 5. In addition to nanohaloarchaeal phylotypes, *Halobellus* and *Haloplanus* related phylotypes were also obtained from the Tuzlagözü sample. For the primers used in our study, the annealing temperature can be used between 59-63ºC, and we preferred 59ºC and obtained a few haloarchaeal sequences. Apparently, the annealing temperature can be increased by a few degrees to prevent non-specific products.

4. DISCUSSION

There are detailed studies to reveal the microbial diversity in Tuz Lake. Mutlu et al. (2008) investigated prokaryotic diversity in Tuz Lake by 16S rRNA gene-targeted denaturing gradient gel electrophoresis and cloning. They reported that the most frequently represented phylotypes were related to *Haloquadratum* and *Salinibacter*. In addition to these groups, *Halorubrum*, *Halobellus*, *Halonotius*, *Halanaerobacter* and *Acinetobacter* phylotypes were recovered. Strains of *Salinibacter* and *Haloarcula* were also isolated. Illumina MiSeq sequencing of 16S/18S rRNA gene amplicons was performed by Ünal & Küçükyıldırım-Çelik (2020) to reveal microbial groups in Tuz Lake. It has been reported that the most abundant OTUs were associated with the genus *Haloquadratum*. Novel OTUs clustered in *Halobacteriaceae* were also highly represented. *Salinibacter*, the most commonly represented bacterial genus, *Halorubrum*, *Halonotius* and *Halapricum*-associated OTUs were also detected in a remarkable abundance. OTUs of the genera *Halobonum*, *Halobellus*, *Haloplanus* and the phylum *Nanohaloarchaeota* were detected, albeit at a low rate. In this study, dominant OTUs in Tuz Lake were found to be related to *Haloquadratum* and this result overlaps with the results obtained from other studies. Other abundant OTUs were associated with *Haloparvum*, which currently has two species isolated from a commercial salt (seawater origin, Philippines) and salt mine (China) [32, 33]. Affiliated OTUs were not detected in the samples belonging to Ayvalık and Tuzlagözü salterns. In the work of Ünal & Küçükyıldırım-Çelik (2020), some of the novel OTUs that clustered in *Halobacteriaceae* may correspond to the genus *Haloparvum*. Other notable OTUs detected in this study were associated with *Halonotius*, *Salinibacter*, *Halapricum*, *Halorubrum*, *Halobacteriales*, *Ca.* Nanosalinarum and *Nanosalinaceae*, and it coincides with the previous work [31]. The abundance of *Halorubrum* OTUs obtained in this study is slightly lower than those obtained from the previous study, and *Nanohaloarchaeota* OTUs were slightly higher. Differences in the relative abundance of OTUs are possible because sampling from Tuz Lake in this study was conducted in June, and in the previous study in March [31].

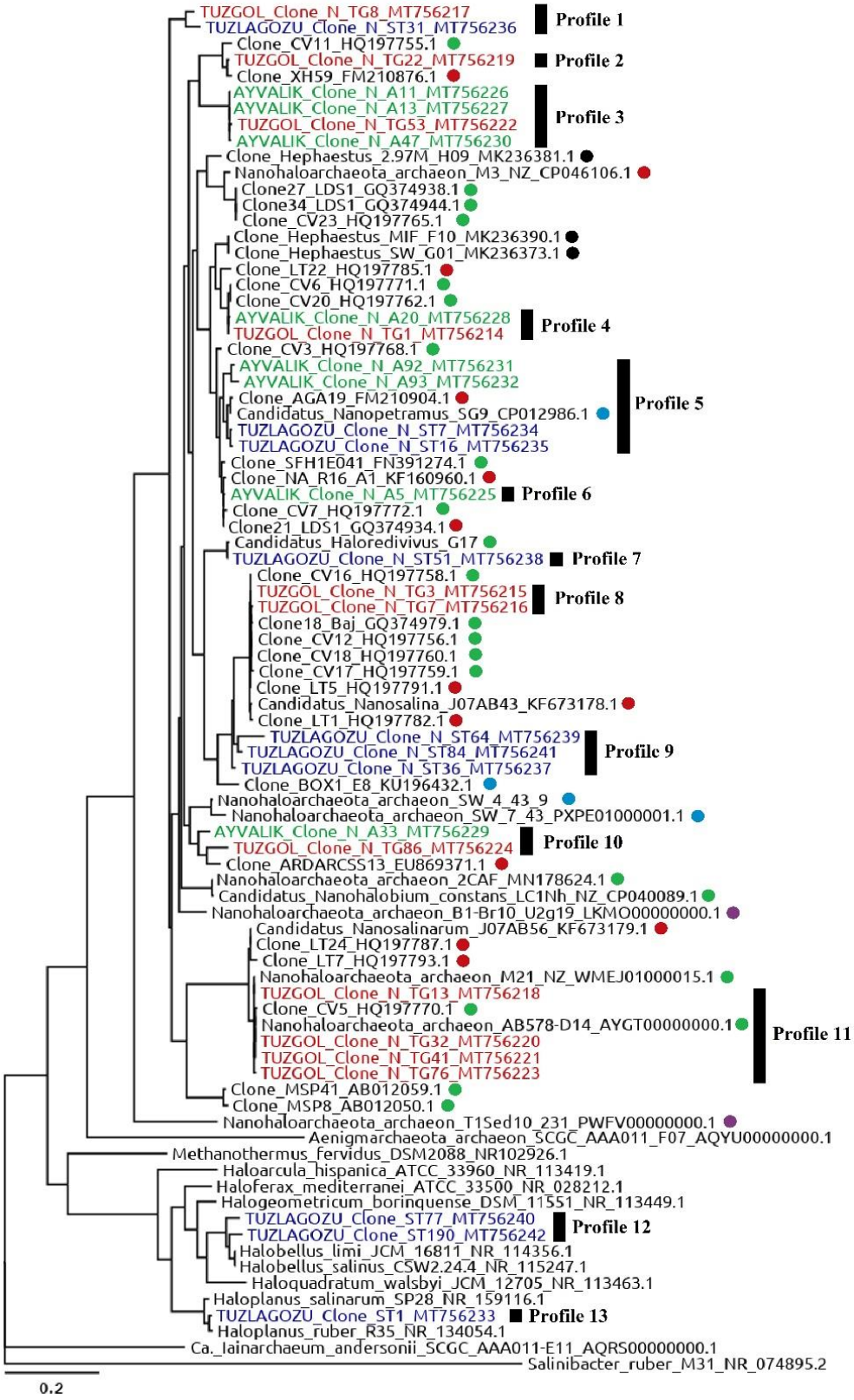

Figure 5. Maximum likelihood phylogenetic tree constructed from 16S rRNA gene sequences obtained from *Nanohaloarchaeota* clone libraries (100 bootstrap replicates). The circles indicate the sources from which the nanohaloarchaeal sequences were derived; solar salterns (green), salt (red) and soda (purple) lakes, salt flats (blue) and deep-sea basin (black).

Elevi et al. (2004) previously conducted a study on the isolation and characterization of halophilic archaeal strains from Ayvalık saltern. Apart from this study, there is not much information about microbial groups developing in the saltern. In this study, it was found that besides *Haloquadratum* OTUs, *Halonotius*, *Halorubrum* and the novel OTUs clustered within the family *Haloferacaceae* were also highly represented in the Ayvalık sample. OTUs related to *Natronomonas*, *Halobellus*, *Haloplanus*, *Salinibacter* and *Haloarcula* genera were also noteworthy.

In our previous study, pyrosequencing from 16S rRNA gene amplicons was performed to detect the archaeal and bacterial groups in Tuzlagözü crystallization pond [35]. Since the library was constructed separately for the archaea and bacteria in pyrosequencing, the ratio of these groups was evaluated by fluorescence in situ hybridization (FISH) analysis, and the archaeal cells were found to be more abundant. The reads from MiSeq sequencing (~250bp) were shorter than those obtained from pyrosequencing (~440bp), but the number of the reads from MiSeq analysis for Tuzlagözü sample was about 5 times higher than those previously obtained from pyrosequencing. Although there were similarities in the results obtained from pyrosequencing and MiSeq analysis, there was no complete overlap. Samplings were carried out in June (20% salinity) in the previous study and in August (~26% salinity) in this study, and the total salinity of the samples also differed. It can be expected that the composition of community in small inland salterns fed with spring water may be more variable over time than that of natural hypersaline environments. Among the OTUs, which were abundant in both pyrosequencing and MiSeq analyzes, *Haloquadratum*, *Halorubrum*, *Haloarcula*, *Salinibacter* and *Haloplanus* have been shared, but with varying relative abundances. In this study, *Haloquadratum* related OTUs were found to be dominant. Following *Salinibacter*, bacterial OTUs of *Salicola* and *Bradymonadaceae* were also remarkably represented. Bacterial OTUs were represented more in Tuzlagözü sample than in Tuz Lake and Ayvalık samples.

Narasingarao et al. (2012) designed a lineage-specific probe and a primer for the *Nanohaloarchaeota* group and reported that the nanohaloarchaeal cells accounted for ~14% and 8-11% of the total cells in Lake Tyrrell and Chula-Vista solar saltern (California, USA), respectively. They further investigated nanohaloarchaeal lineages in those environments through cloning and metagenomic analysis and estimated that their members could make up 10-25% of the total archaeal communities. In our study, the majority of nanohaloarchaeal phylotypes, particularly those recovered from Tuz Lake, showed high sequence similarity (97-99%) with those from Tyrrell Lake and Chula-Vista crystallizer. The relative abundance of the nanohaloarchaeal OTUs in Tuz Lake sample was found to be 5.4%. Relevant OTUs were detected in very low abundance in the samples belonging to Ayvalık (0.9%) and Tuzlagözü salterns (0.4%). The distribution of metagenomic 16S reads from crystallizer ponds in Santa Pola coastal saltern (Spain) was examined, and the reads captured from the pond with 33% salinity were reported to be mostly related to *Haloquadratum* and *Halorubrum*, followed by *Natronomonas*, *Salinibacter* and *Haloplanus* [36]. These were also among the most frequently represented taxa in Tuzlagözü sample. While *Haloquadratum* was the dominant taxon in the pond (Santa Pola) with 37% salinity, reads belonging to *Ca.* Nanosalina (4%) and Nanosalinarum (1.7%) were also represented in low abundance [36]. It has been reported that most of the reads obtained from metagenomic studies from salt flats and pools in the Atacama Desert (Chile) were associated with *Halobacterota* followed by *Bacteroidetes*, and sequences related to *Nanohaloarchaeota* were detected in low abundance (~2%) [14, 16]. Samples from Salar de Uyuni (Bolivia), another salt-flat, were analyzed by 16S rRNA gene-targeted cloning and amplicon sequencing. It has been stated that members of the phylum *Halobacterota* are dominant, the most abundant haloarchaeal OTUs in all samples are associated with the genus *Halonotius*, and OTUs related to *Nanohaloarchaeota* have been detected in low abundance (<1%) [18, 37].

A few phylotypes (profile 8) from Tuz Lake showed 99% sequence similarity to that of *Ca.* Nanosalina sp. J07AB43, whose genome sequence was captured from the metagenomic data of Tyrrell Lake [7]. Similar ARDRA patterns were obtained from Tuzlagözü sample, but the representative selected for sequencing was excluded because the sequencing data was of poor quality. A sister clade to Nanosalina was formed by the phylotypes (profile 9) obtained only from Tuzlagözü.

Several phylotypes obtained from Tuz Lake (profile 11) formed a distinct clade and showed 98-99% similarity to those of *Ca.* Nanosalinarum sp. J07AB56, *Nanohaloarchaeota* archaeon strain AB578 and M21. Genome sequence of J07AB56 was derived from the metagenomic dataset of Tyrrell Lake, and genomes of AB578 and M21 were obtained by single cell isolation from a solar saltern (Alicante, Spain) [7, 12, 38]. The sequences assigned to *Ca.* Nanosalinarum were obtained only from Tuz Lake.

Some of the phylotypes (profiles 4, 5 and 6) retrieved from Tuzlagözü (98%), Ayvalık (95-97%) and Tuz Lake (95%) showed sequence similarity to that of *Ca.* Nanopetramus sp. SG9, whose sequence originated from a metagenome data (Salar Grande, Atacama Desert) [16].

A phylotype (profile 7) obtained only from Tuzlagözü was 99% similar to the sequence of *Ca.* Haloredivivus sp. G17, which was obtained by single cell isolation from the pond of a solar saltern (Alicante, Spain) [5].

The phylotypes (profile 10) obtained from Tuz Lake and Ayvalık showed low sequence similarity to a clone sequence (95%) obtained from Sidi Ameur Salt Lake (Algerian Sahara) and the metagenome originated sequences (92-95%) from a salt crust (Atacama Desert) [13, 14]. Another phylotype (profile 2) obtained only from Tuz Lake showed 98% similarity with the clone sequences retrieved from a salt lake (Inner Mongolia, China) [15].

Phylotypes clustered in profiles 1 and 3 displayed low similarity (≤92%) to any known nanohaloarchaeal sequences in NCBI database and formed separate branches in the phylogenetic tree. 16s rRNA genes of *Ca.* Nanohalobium constans LC1Nh from a solar saltern (Sicily, Italy), B1-Br10_U2g19 from a soda lake (Kulunda steppe, Russia) and M3_22 from Dead Sea (Israel) showed low sequence similarity (≤93%) with the nanohaloarchaeal phylotypes obtained in this study [8, 38, 39].

A few phylotypes obtained only from Tuzlagözü sample belonged to *Halobacteria*. Two of these phylotypes (profile 12) were 97% similar to that of *Halobellus salinus* strain CSW2.24.4 isolated from a solar saltern (Victoria, Australia), and another one (profile 13) was 99% similar to that of *Haloplanus ruber* strain R35 isolated from an aquaculture farm (China) [40, 41].

In conclusion, the high abundance of OTUs associated with the genus *Haloquadratum* in the samples was quite characteristic, in agreement with previous studies. The vast majority of the OTUs belonged to well-characterized taxa. Abundance of nanohaloarchaeal OTUs were found to be notable in Tuz Lake. Novel nanohaloarchaeal lineages were revealed by cloning, and one of them appeared to be prevalent in the clone library of Ayvalık sample. When the phylogenetic clusters of *Nanohaloarchaeota* were examined considering the geographical region from which they were retrieved, or the features of the environment, no characteristic grouping was observed.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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