PROKARYOTIC DIVERSITY OF HOT SPRINGS IN BALIKESİR AND KÜTAHYA, TURKEY

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(Received 2nd Jul 2016; accepted 12th Sep 2016)

Abstract. The prokaryotic diversity of the hot springs Sındırgı, Güre, and Havran in the Balıkesir Province and Eynal in the Kütahya Province, both located in the western part of central Anatolia, was analyzed and compared using cultivation and cultivation-independent methods, including Fluorescence In Situ Hybridization (FISH) and cloning of PCR-amplified fragments of 16S rRNA genes. A total of 66 isolates were obtained from the cultures. They were identified to the species or genus level as *Geobacillus, Bacillus, Brevibacillus, Aneurinibacillus, Anoxybacillus*, and *Aeribacillus*. As a result of the FISH study, only bacteria domain signals were received, but arkea domain signals were not. A total of 265 clones from the 16S rRNA gene library were analyzed with Amplified Ribosomal DNA Restriction Analysis. These bacterial clones were identified as the genera *Anoxybacillus*, *Meiothermus*, Uncultured bacterium, *Aneurinibacillus, Brevibacillus, Bacillus,* and *Geobacillus*. This research highlights the prokaryotic diversity of hot springs in the Balıkesir and Kütahya Provinces.
Keywords: *thermophilic bacteria, 16S rRNA, cloning, FISH, ARDRA*

Introduction

Waters that are located near the magma layer, formed by the heating of groundwater, and disturbed on the fault line are called hot springs. Turkey is one of seven countries in the world that are rich in hot springs, with almost 1300 thermal springs throughout Anatolia (Akkaya and Kıvanç, 2002). Nowadays, thermophilic bacteria have significance due to their characteristic enzymes that resist high temperatures and biogas production by decomposition (Kardos et al., 2011). Hyperthermophile enzymes such as amylase, xylenase, protease enzymes, and DNA polymerase from thermophiles are extremely important in industrial areas and genetic engineering research. Therefore, demonstrating the microbial properties of thermal springs is important for industry and the economy. For this reason, studies on the discovery of microorganisms and new enzymes have become more common (Rifaat et al., 2005; Demirjian et al., 2001).

The Balıkesir and Kütahya Provinces are located in western Turkey, which are both well known for their hot springs. Several springs in different regions of these provinces have been known to geologists for many years. However, their prokaryotic diversity has not been explored with molecular phylogenetic approaches.

In the present study, we have used the 16S rRNA methodology to determine the prokaryotic community structure of some of the most popular hot springs of Balıkesir and Kütahya: Sındırgı (93°C), Güre (65°C), Havran (62°C), and Eynal (70°C).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/1404_429440

Materials and Methods

Isolation and morphological determination of cultures

Four hot springs were analyzed *(Figure 1,2).* Water samples were collected with sterile glass bottles during February and August 2014. Samples were brought to the laboratory on the same day. For inoculation, 1 liter of each sample was filtered with 0.2-µm pore size sterilized filters, which were placed in a 0.2% Nutrient broth (Sigma Aldrich) and Low Phosphate Basal Medium (LPBM). It contained the following, in grams per liter: NH_4C1, 1.0; KH_2P0_4, 1.0; Na_2HP0_4. 7 H_20, 0.1; $MgS0_4$. $7H_20$, 0.2; $CaC1_2$. $2H_20$, 0.2; and yeast extract 1.0). It was then left at 55°C and 70°C for several weeks until active biomass became visible. After this, it was purified. Gram staining was applied to isolates and morphological properties were determined with an OLYMPOS BX50 light microscope.

Figure 1. *The hot spring Sındırgı in Balıkesir (A, B) and hot springs Güre (C) and Eynal (D) in Kütahya.*

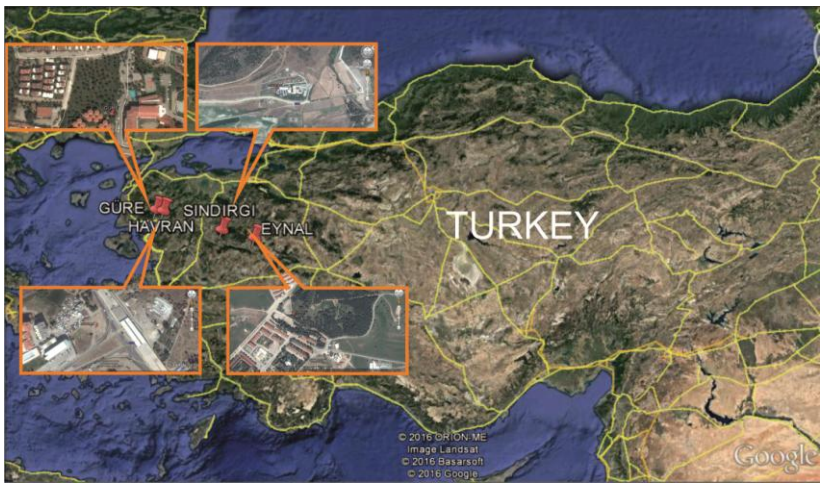

Figure 2. *Sampling locations were marked on the map. GPS coordinates of the sampling points are 39°15'50.11"N 28°18'21.59"E (Sındırgı); 39°15'18.5"N 26°52'58.9"E (Güre); 39°34'12.1"N 27°02'38.6"E (Havran); 39°07'40.4"N 28°59'45.3"E (Eynal) (Satellite imagery: Google/Google Earth).*

Chemical analysis of thermal water samples

Chemical analysis of water samples was performed with an ICP (Perkin Elmer Optical Emission Spectrofotometer Optima 4300 DV). Na, K, Mg, Ca, Mn, and Fe were detected.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Fluorescence In Situ Hybridization (FISH) analysis of thermal water samples

For fixation, 1.2 ml formaldehyde (Sigma Aldrich) (37%) was added to 5-ml water samples and incubated at 4°C overnight. Then samples were filtered with a 0.2-μm pore size GTTP filter (Milipore). For hybridization, we used 16S rRNA targeted oligonucleotide probes with a Cy3-tagged Eub 338 and an Arc 344 universal probe. Hybridization was carried out at 46°C for 2.5 h. Next, filters were washed with washing buffer and stained DNA-specific fluorescent DAPI dye, rinsed ethyl alcohol, distilled water, and finally analyzed with a Leica DMIRE2 epifluorescence microscope (Anton et al., 1999).

DNA extraction from pure cultures and water samples

The bacterial genomic DNA from purified cultures were prepared using the protocol described by the ZR Fungal/Bacterial DNA MicroPrep™ Kit. To obtain total bacterial genomic DNA, water samples (2 liters) were filtered with 0.2-μm pore size filters (Millipore). DNA extraction from filters was performed according to Boutte et al. (2005). Extracted DNA was stored at –85°C until used.

Cloning of 16S rDNA

The water samples were used for cloning. PCR amplification of the 16S rRNA gene was done in a 3 × 50 μl mixture with a set of universal primers: 27F (AGAGTTTGATCMTGGCTCAG), 21F (TCCGGTTGATCCYGCCGG), and 1492R (TACGGYTACCTTGTTACGACTT) (Nakagawa et al., 2002; Antoniou et al., 2015). PCR reactions were carried out with an Applied Biosystems® thermal cycler under the following conditions: 25 μl One*Taq*® 2X Master Mix with Standard Buffer (New England Biolabs, Inc), 0.2 μM of the forward and reverse primers, and 2 μl DNA in a final volume of 50 μl. Amplification was carried out as follows: one cycle of 3 min at 94°C; 30 cycles of 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C; and a final elongation step of 10 min at 72°C. PCR products were purified with Wizard® SV Gel and a PCR Clean-Up System. Poly (A) extension was performed using a Qiagen® A-Addition kit according to the manufacturer's instructions. Cloning of the PCR products was done with a Qiagen PCR Cloning plus kit following the manufacturer's instructions. White and light blue transformants were purified twice by streaking and then were screened by performing colony PCR with the primer pairs M13F (5'-GTAAAACGACGGCCAGT-3') and M13R (5'-GTTTTCCCAGTCACGAC-3'). The amplification conditions were those described in by Piterina et al. (2010). Primers were subjected to Amplified Ribosomal DNA Restriction Analysis (ARDRA) to screen the clone libraries.

ARDRA (Amplified Ribosomal DNA Restriction Analysis)

The 16S rRNA gene amplicons obtained from the genomic DNA of the isolates and clones were used for ARDRA with the restriction endonuclease *Mbo*I (MBI Fermentas) (Table 1). The digestion reaction was prepared according to the manufacturer's instructions. ARDRA patterns were compared and identical patterns were considered to be from the same group. Partial sequences of 16S rDNAs from representatives of each group were determined. For each ARDRA type, sequencing was carried out with primers 1492R (5′-GTACGGCTACCTTGTTACGAC-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) (Lane et al., 1985) by Macrogen (Seoul, Korea) and then these sequences were assembled. Environmental 16S rRNA gene sequences

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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from the hot springs were deposited in the GenBank with these accession numbers: KR864865, KR864866, KR864867, KR864868, KR864869, KR864870, KR864871, KR864872, KR864873, KR864874, KR864875, KR864876, KR864877, KR864878, KR864879, KR864880, KR864881, KR864882, KR864883, KT893400, KT893401.

Analyses of sequence data

All sequences were compared with the BLAST search program on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). The top five hits as well as some additional relevant sequences were used for phylogenetic analysis. 16S rRNA sequences from hits of our sequences were obtained through the RDP (Ribosomal Database Project) site at Michigan State University (http://rdp.cme.msu.edu/). Sequences of partial 16S rDNA of cultures and clones were analyzed with the MEGA 6.0 program (Tamura et al., 2007). All sequences were checked for chimera formation using DECIPHER (ES Wright et al., 2012). The CHECK-CHIMERA program, developed by the Ribosomal Database Project, and the phylogenetic affiliations of their 5′ and 3′ ends were compared.

Phylogenetic analysis

Sequences obtained from the isolates and clones were aligned with the closest strains and retrieved from environmental samples obtained from RDP II and NCBI. Phylogenetic trees were constructed using the MEGA 6.0 program. The Distance Matrix was calculated using the Jukes-Cantor algorithm and the trees were constructed using the Nearest-Neighbor-Interchange (NNI) method. Validity of tree topology was evaluated with the bootstrap method (1000 replicates).

Results and Discussion

Chemical properties of the water samples

Thermal water samples of the springs had different chemical properties, but pH was about 7.13–8.04 in all springs. Chemical analysis of the thermal waters shows that they are neutral and slightly alkaline. Na (1054 ppm), Mg (12.23 ppm), Ca (42.34 ppm), Fe (1.7 ppm), and K (66.38 ppm) ions were highest in the Eynal spring whereas Mn ions (0.03 ppm) were highest in the Güre spring. Each water sample had different chemical properties because of the temperature differences of the sources and geological structure of the schist.

Culture identifications

In total, we obtained 66 pure isolates with different morphologies on agar plates. Like the Gram reaction, 66 isolates were Gram positive bacilli. Their colony colors were white, cream, and yellow and some of them had a mucoid structure. These isolates had 20 different ARDRA profiles with the *Mbo*I restriction enzyme. ARDRA profiles of the isolates and their closest Genbank matches are shown in *Table 1*.

The Eynal hot spring is more diverse, with 10 different ARDRA profiles and 8 that were not in other springs. According to the cultivation result, *Bacillus* sp. and *Geobacillus* sp. were most abundant in all springs. The *Aeribacillus* genus was obtained from only the Eynal hot spring. Group 13 has most isolates whereas groups 3, 6, 8, 10, 11, 14, 15, and 20 were represented by only 1 isolate each.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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Table 1. *Thermophilic isolates and their closest GenBank matches.*

ARDRA Group No	Isolate Code * (GenBank No)	Closest GenBank Match		
		% of identity with the closest relative	Closest relative according to BLAST search/Length	Accession no./Source
1	S2 (KR864865)	99%	*Geobacillus thermoleovorans*/1434	NR074931/ United Kingdom hot spring
2	S5 (KR864881)	100%	*Geobacillus thermoparaffinivorans*/1291	KT266806/ China Fujian hot spring
3	E4 (KR864882)	99%	*Geobacillus thermoleovorans*/1457	AJ564612/ United Kingdom hot spring
4	E6 (KR864883)	99%	*Geobacillus kaustophilus*/1211	FJ823105/ Lakeshore duff
5	G1 (KR864876)	100%	*Bacillus licheniformis*/1353	KF879248 / Morocco hot spring
6	G2 (KT893401)	99%	*Brevibacillus thermoruber*/1304	KJ842630/ Indonesia hot spring
7	G9 (KR864879)	100%	*Bacillus licheniformis*/1353	KF879248 / Morocco hot spring
8	G5 (KR864877)	100%	*Aneurinibacillus thermoaerophilus*/1341	EF032876/ Selangor
9	H4 (KR864869)	99%	*Anoxybacillus flavithermus*/1379	KJ722464/ China Fujian hot spring
10	H6 (KR864870)	99%	*Bacillus* sp./1174	LN681603/ Tapovan, India

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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11	H7 (KR864871)	99%	*Aneurinibacillus* sp./1123	FJ268961/ Pendula leaf
12	EY (KR864873)	97%	*Geobacillus* sp./1329	HQ703944/ Manikaran hot spring
13	EY5 (KR864866)	100%	*Bacillus sonorensis*/1131	KP282741/ Crude oil samples
14	EY13 (KR864874)	100%	*Geobacillus thermoparaffinivorans*/1369	KC252981/ Benguet hot spring
15	EY18 (KR864867)	100%	*Aeribacillus pallidus*/1128	KR611619/ Reservoir fromatiion water
16	EY21 (KR864875)	100%	*Bacillus licheniformis*/1284	KJ572278 / Morocco hot spring
17	SY5 (KR864880)	100%	*Bacillus licheniformis*/1192	GU945232 / Morocco hot spring
18	GY4 (KR864868)	99%	*Anoxybacillus* sp./1393	GQ184213/ Sungai Klah hot spring
19	H9 (KR864872)	99%	*Brevibacillus thermoruber*/1357	KJ722521/ Schoenebeck geothermal station
20	EY12 (KT893400)	100%	*Bacillus licheniformis*/1191	KF879248 / Morocco hot spring

*S (Sındırgı), E (Eynal), G (Güre), H (Havran)

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Uncultural molecular analysis based on the 16S rRNA gene

To determine the biodiversity of the thermal sources, we first cultivated water samples and identified those with 16S rRNA gene sequences. In addition, we used culture-independent methods, FISH and cloning. A total of 265 clones containing inserts of the right size were analyzed. First, they were grouped on the basis of their restriction profiles and then representatives from 15 different restriction groups were sequenced with the sequence primer 1492R. The best match to databases was obtained from BLAST analyses of the selected clones (*Table 2*). Most clones were from Group 1, related to *Anoxybacillus flavithermus* (99% similarity). Groups 7 and 8 were also related to *Anoxybacillus flavithermus*, with 99% and 100% similarities.

Table 2. *Clones and their closest GenBank matches.*

ARDRA Group No	**Clone Code**	**Closest GenBank Match**		
		% of identity with the closest relative	**Closest relative according to BLAST search/Length**	**Accession no./Source**
1	Clone G1 (KT893391)	99%	*Anoxybacillus flavithermus* /792	KJ722464.1/ Schoenebeck geothermal station
2	Clone G7 (KT893392)	99%	*Meiothermus silvanus* /906	NR074273 / Portugal hot spring
3	Clone G12 (KT893393)	95%	*Uncultured bacterium* /670	HQ639470.1/ China
4	Clone H4 (KT893394)	93%	*Uncultured bacterium* /889	EF648061.1 / Activated sludge-China
5	Clone H12 (KT893395)	99%	*Aneurinibacillus thermoaerophilus* /559	KJ190161.1 / Activated sludge-China
6	Clone H13 (KT893396)	99%	*Brevibacillus thermoruber* /826	KJ842631.1 / China
7	Clone H15 (KT893397)	99%	*Anoxybacillus flavithermus* /859	KJ842638.1/ Tanjung Sakti Hot spring
8	Clone S12 (KT893399)	100%	*Anoxybacillus flavithermus* /809	KJ722464.1 / Schoenebeck geothermal station
9	Clone E21 (KT893383)	97%	*Bacillus licheniformis* /735	KP216563.1 / High salt wastewater
10	Clone E25 (KT893384)	91%	*Uncultured bacterium* /940	HM184957 / China
11	Clone E27 (KT893385)	98%	*Geobacillus* sp. /484	CP001638.1 / Hot wood compost, Middleton
12	Clone E33 (KT893386)	91%	*Meiothermus* sp. /565	AY845055.1 / Hot spring
13	Clone E36 (KT893387)	99%	*Bacillus amyloliquefaciens* /542	KR109267.1 / Hot spring
14	Clone E41 (KT893389)	99%	*Uncultured organism* /837	HQ767379.1 / Gastrointestinal specimens
15	Clone E42 (KT893390)	99%	*Geobacillus* sp. /883	CP001638.1 / Hot wood compost, Middleton

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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The Eynal hot spring had the most diversity. Groups 9–15 were only found at Eynal. Groups 1 and 2 were present in all hot springs whereas Groups 3 and 8 were found only in the Güre and Sındırgı hot springs, respectively.

The sequences of clone G12, H4, and E25 were related to *Uncultured bacterium* (Acc. Number HQ639470; EF648061; HM184957) and Clone G7 and E33 were affiliated with *Meiothermus silvanus* (Acc. Number NR074273) and *Meiothermus* sp. (Acc. Number AY845055). Clone H12, H13, and E21 were related to *Aneurinibacillus thermoaerophilus* (Acc. Number KJ190161), *Brevibacillus thermoruber* (Acc. Number KJ842631), and *Bacillus licheniformis* (Acc. Number KP216563), respectively. Clone E27 and E42 were affiliated with *Geobacillus* sp. (Acc. Number CP001638). Clone E36 and 41 were related to *Bacillus amyloliquefaciens* (Acc. Number KR109267.1) and *Uncultured organism* (Acc. Number HQ767379), respectively.

Phylogenetic trees

Phylogenetic analysis was performed using the maximum likelihood treeing algorithm in the MEGA 6.0 program. Phylogenetic trees for bacteria constructed based on partial 16S rRNA sequences are shown *Figures 3* and *4*.

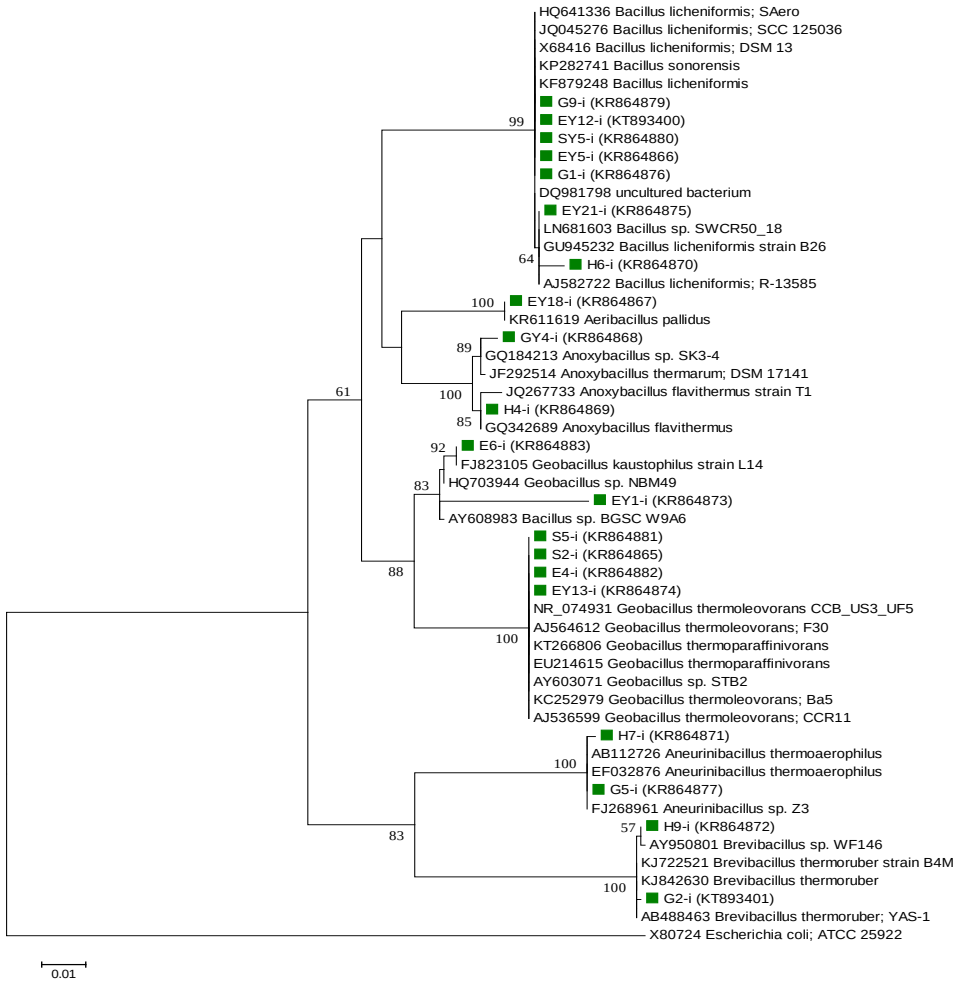

Figure 3. *Phylogenetic inferences based on 16S rRNA gene sequences from isolates (indicated by green squares). The scale bar represents the expected number of substitutions per site. Bootstrap support values below 50% were not included in the figure.*

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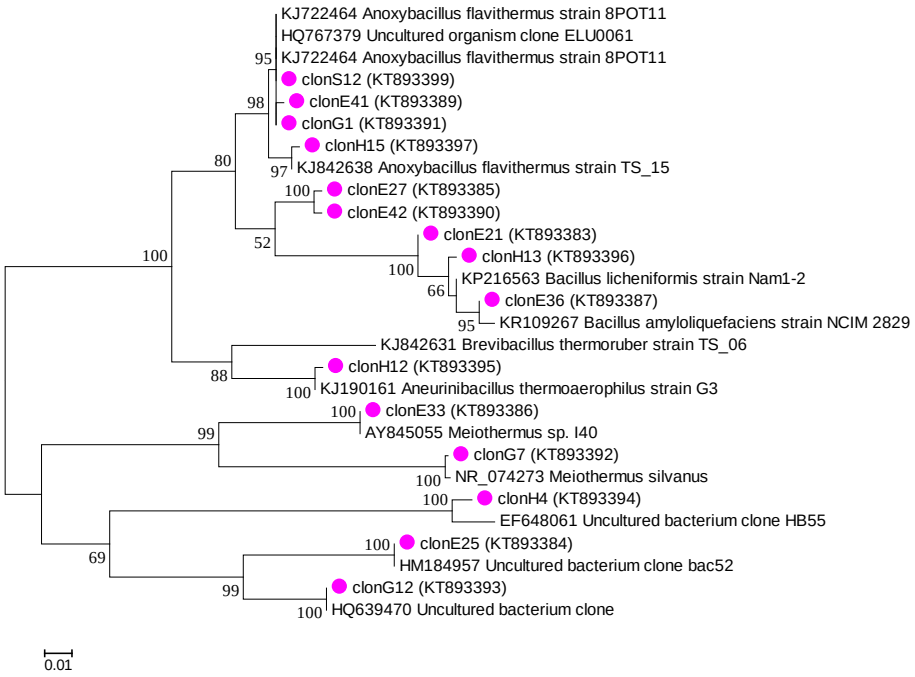

Figure 4. *Phylogenetic inferences based on 16S rRNA gene sequences from clones (indicated by pink circles) belonging to the bacteria. The scale bar represents the expected number of substitutions per site. Bootstrap support values below 50% were not included in the figure.*

FISH Analysis

FISH analysis of the hot springs' prokaryotic communities showed that bacterial species were dominant. The signals of the bacteria specific Eub 338 probe showed that Eynal hot spring had the most species. Long and short bacilli were dominant in all springs (*Figure 5*). Also, there were cells with long chain morphology and spontaneous radiation. These cells were most probably a member of the Cyanobacteria.

In agreement with the culture and cloning results, the FISH results showed that bacilli morphology was abundant in all hot springs.

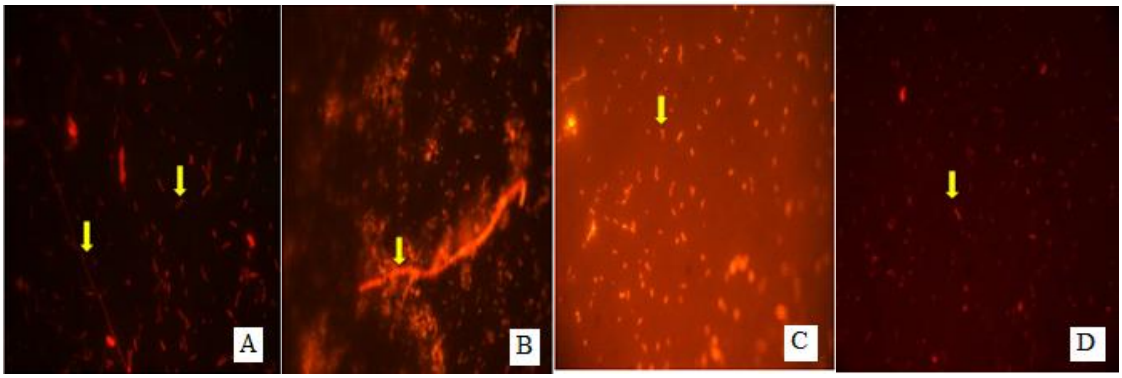

Figure 5. A. *Eub 338 positive cells with thin bacilli morphology from the Sındırgı hot spring.* ***B.*** *Autoflourescent chain cell from the Eynal hot spring.* ***C.*** *Eub 338 positive cells with thin, twisted bacilli morphology from the Güre hot spring.* ***D.*** *Eub 338 positive cells with thin, twisted bacilli morphology from the Havran hot spring.*

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Conclusions

Since 16S rRNA is the best conserved part of the 30S rRNA operon, it has been proposed as an "evolutionary clock," which has led to the reconstruction of the tree of life (Woese, 1987). The 16S sequence analysis is used to identify and classify isolated pure cultures and estimate bacterial diversity in environmental samples without culturing through metagenomic approaches. The cloning and sequencing of 16S genes amplified directly from extreme environments through metagenomic approaches has demonstrated that microbial diversity is far more extensive than we ever imagined from culture-based studies alone. Culture methods continue to improve to reveal the exact diversity (Janssen et al., 2002; Lopez-Garcia and Moreira, 2008). The ARDRA is a technique based on the cutting with restriction enzymes of 16S rRNA gene region after amplification with appropriate primers. Restriction profiles obtained by cutting with restriction enzymes are chosen as representative of different species (Sklarz et al., 2009).

Our thermophilic isolates have high similarities to previously cultured thermophilic bacteria such as *Aneurinibacillus, Aeribacillus, Anoxybacillus, Bacillus, Brevibacillus,* and *Geobacillus*, which were obtained from the Gene-Bank (Canganella and Trovatelli, 1995; Nazina et al., 2004; Yasawong et al., 2011; Özdemir et al., 2012; İnan et al., 2012).

The species *Meiothermus silvanus, Aneurinibacillus thermoaerophilus,* and *Bacillus amiloliquefaciens* were only detected using cloning and the species *Geobacillus thermoleovorans, Geobacillus thermoleovorans, Geobacillus thermoparaffinivorans, Bacillus sonorensis, Aeribacillus pallidus,* and *Geobacillus kaustophilus* were obtained only from the culture studies.

Aneurinibacillus, Aeribacillus, Anoxybacillus, Bacillus, Brevibacillus, Geobacillus, *Thermus, Pseudoxanthomonas, Acinetobacter, Paenibacillus,* and *Thermoactinomycetes* were found in earlier studies from different hot springs (Palmisano *et al.*, 2001; Adıgüzel et al., 2009; Ghati et al., 2013). Although *Geobacillus* and *Bacillus* species were dominant in our samples, *Thermus, Pseudoxanthomonas, Acinetobacter, Paenibacillus,* and *Thermoactinomycetes* species were not encountered in our culture and cloning studies.

This is the first study to reveal the diversity of the Güre, Havran, Sındırgı, and Eynal hot springs. Culture-dependent and culture-independent techniques were used simultaneously to target unique regions of the 16S rRNA gene. The isolates obtained in this study have potentially important biotechnological applications because their industrial enzymes are resistant to harsh conditions. These abilities of our isolates will be investigated in future projects.

Acknowledgments. The authors would like to thank Anadolu University Research Project Department, Project 1403F100.

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DOI: http://dx.doi.org/10.15666/aeer/1404_429440

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