ANALYSIS OF CYANOBACTERIAL DIVERSITY OF SOME HOT SPRINGS IN AFYONKARAHISAR, TURKEY

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Abstract. The cyanobacterial diversity of Ömerli, Akkuş-Gazlıgöl, and Hüdai-Sandıklı hot springs in Afyonkarahisar District in Turkey were analysed and compared using cultivation and cultivation-independent methods, including DGGE (denaturing gradient gel electrophoresis) and cloning of PCR-amplified fragments of 16S rRNA genes. Cultivation studies revealed that a total of 74 isolates had 6 different ARDRA (Amplified Ribosomal DNA Restriction Analysis) profiles and they were identified at the genus level and all cyanobacterial isolates were phylogenetically related to *Fischerella, Geitlerinema/Leptolyngbya laminosa* and uncultured *cyanobacterium* genus. A total of 97 clones from 16S rRNA gene library was analysed by ARDRA. 16S rRNA sequence analysis of these clones revealed that the cyanobacterial clones were related to 16S rRNA gene sequences retrieved from environmental samples, *Geitlerinema* and *Leptolyngbya laminosa* genus members. DGGE analysis revealed *Geitlerinema, Cyanobacterium,* and *Phormidium* genus, and 16S rRNA gene sequences retrieved from environmental samples. High-throughput 16S rRNA gene sequencing with DGGE analysis showed that the most frequent sequences in Ömerli, Akkuş-Gazlıgöl and Hüdai-Sandıklı samples were affiliated with *Geitlerinema*. This work highlights the cyanobacterial diversity of Afyonkarahisar hot springs.
Keywords: *thermophilic cyanobacteria, hot spring, Afyonkarahisar, cloning, DGGE (Denaturating Gradient Gel Electrophoresis)*

Introduction

Cyanobacteria are Gram-negative bacteria which have the ability of oxygenic photosynthesis. They are the most adaptive photosynthetic organisms and live in almost every habitat on earth. They are found in fresh water, marine water, soil in thermophilic and psychrophilic conditions. Cyanobacterial morphology varies from unicellular to multicellular. Cyanobacteria that can develop over 45°C are called thermophilic cyanobacteria. These cyanobacteria have a more limited distribution area than mesophilic cyanobacteria (Whitton and Potts, 2000). Thermal hot springs are found in different geographical areas with different physical and chemical features which severely limit the survival of photoautotrophic organisms in them. Thermal environments make the cyanobacteria living there endemic (Castenholz, 1996; Papke et al., 2003; McGregor and Rasmussen, 2008).

Thus, except a few cosmopolitan thermophilic cyanobacterial species (i.e. *Mastigocladus laminosus* Cohn) (Castenholz, 1996; Miller et al., 2007), most thermophilic cyanobacteria are new operational taxonomic units (OTUs) (Ward et al., 1998; Taton et al., 2006; McGregor and Rasmussen, 2008).

Cyanobacteria are the most commonly reported microbial groups constituting thermophilic mats and considered as the major primary producers in these habitats (Castenholtz, 1973). Other bacteria living in the same environment have also important

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roles within these microbial communities (Ward et al., 1990; Weller et al., 1992; Moyer et al., 1995).

Afyonkarahisar is a district in western Turkey (*Figure 1*) with well known hot springs. Although several hot springs in different regions of Afyonkarahisar have been in use for many years, their cyanobacterial diversity has not yet been investigated by molecular phylogenetic approaches.

Figure 1. *Sampling locations in Afyonkarahisar (shown with circles on the map). GPS coordinates of the sampling points are 38°56'09.43N-30°29'48.11E (Akkuş-Gazlıgöl); 38°25'59.26N-30°10'54.89E (Hüdai-Sandıklı); 38°50'24.45N-30°25'01.08E (Ömerli). (Satellite imagery: Google/Google Earth).*

In this study, we applied the 16S rRNA gene analysis both for isolates and environmental DNA to determine the cyanobacterial community structure of three most popular hot springs in Afyonkarahisar.

Materials and Methods

Study site and sample collection

Three hot springs Ömerli (98°C), Akkuş-Gazlıgöl (64°C), and Hüdai-Sandıklı (68°C) in Afyonkarahisar were selected as our study sites (*Figure 2*).

Water and mat samples were collected as study material. Water samples were taken from the epilimnion at a depth of 1 m using sterile glass bottles and tubes during January and October 2012 and were kept on ice baths until they were analysed. Microscopic analyses of water samples were performed with Olympos microscope BX51 equipped with digital microphoto-camera DP70. Morphological analyses were carried out at the genus level based on the identification systems proposed by Geitler, 1932 and the "form-genus" approach of Castenholz, 2001. The samples were cultivated with or without nitrogen in BG-11 medium (Rippka et al., 1981; Boutte et al., 2005; Cuzman et al., 2010) supplemented with 50 mg l^{-1} of cycloheximide to avoid eukaryotic cells. Cultures were grown at 55°C for several weeks until a green active biomass became visible and purification process was then performed. Subsequent cultures were incubated in the BG11 solid medium at 55°C.

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(a)

(b)

(c)

Figure 2. *(a) Ömerli (b) Akkuş-Gazlıgöl (c) Hüdai-Sandıklı hot springs*

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Chemical analysis of water samples

Chemical analysis of all of the water samples from the hot springs (*Table 1*) was performed with the Spectroquant® NOVA 60 photometer using Merc cell tests.

DNA extraction from pure cultures, mat and water samples

The bacterial genomic DNAs from purified cultures were prepared using the protocol described by DNeasy® Plant Mini Kit (Qiagen). 500 ml water samples were filtered on 0.2 µm-pore-size filters (Millipore) to obtain the total bacterial genomic DNA. The filters and mat samples (0.5 g) with yellow, green and dark-green layers were obtained from 3-5 mm below the mat surface using a clean razor blade and placed in 15 ml sterile tubes containing 2 ml lysis buffer (40 mM EDTA, 400 mM NaCl, 0.75 M sucrose, 50 mM Tris HCl pH 8.3). Each sample was frozen at -20°C (Giovannoni et al., 1990). For the enzymatic lysis step, a volume of 50 µl lyzozyme (50 mg/ml) was added to the filters and incubated for 20 minutes at 37ºC. After then 100 µl SDS (10%) and 43 µl proteinase K (14 mg/ml) was added and incubated 2 hours at 37ºC. 2 ml of phenol/chloroform/isoamylalcohol (Merck, Germany) (25:24:1) were added and incubated for 10 minutes at 56ºC. The nucleic acids were precipitated from the supernatant (divided in Eppendorf tubes) by adding 2 volumes of ethanol and kept for 2 hours at -20ºC. Then, the tubes were centrifuged for 20 minutes at 16000 g. After extraction, DNA was subjected to purification step using the Wizard DNA Clean-Up System (Promega). To check the quality of nucleic acids, they were run in 1% agarose (LE, FMC Products, Rockland, ME) gel and visualized under UV light after ethidium bromide staining. The purified DNA were stored at –85°C (Wilmotte et al., 2002; Boutte et al., 2005).

Cloning of 16S rDNA and ARDRA (Amplified Ribosomal DNA Restriction Analysis)

The water samples were used for cloning. PCR amplification of the cyanobacterial 16S rRNA gene plus ITS and 5'end of 23S was made in a 3 x 50 µl reaction mixtures. Set of primers used was CYA 359F (5'-ggggaattttccgcaatggg-3') and 23S30R (5'-cttcgcctctgtgtgcctaggt-3') (Taton et al., 2003).

Applied Biosystems® thermal cycler was used for the amplification reaction contained 1 X of Q5® Reaction Buffer, 1 mg ml^{-1} of BSA (bovine serum albumin), 200 µM of dNTP mix, 0.5 µM of the forward and reverse primers, 1 U/µl of Q5® High-Fidelity DNA Polymerase (New England Biolabs, Inc) with proofreading activity in a final volume of 50 µl. The PCR amplification cycle was 5 min at 94ºC; 10 cycles of 45 s at 94ºC, 45 s at 57ºC, and 2 min at 68ºC; 25 cycles of 45 s at 92ºC, 45 s at 54ºC, and 2 min at 68ºC; and a final elongation step of 7 min at 68ºC. PCR products were purified with Quantum Prep® PCR Kleen Spin Columns (Bio-Rad). Poly (A) extension was performed using Qiagen® A-Addition Kit according to manufacturer's instructions. Cloning of the PCR products was done with a TOPO® TA Cloning Kit (Invitrogen) according to manufacturer's instructions. White and light blue transformants were purified twice by streaking and then were screened by performing colony PCR with the primer pair CYA359F (5'-ggggaattttccgcaatggg-3') and CYA783R (5'-gactactggggtatctaatcccatt-3'). The amplification conditions were as follows: incubation for 10 min at 94ºC; 35 cycles of 1 min at 94ºC, 1 min at 60ºC,

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1 min 68ºC; and a final elongation step of 7 min at 68ºC (Wilmotte et al., 2002; Boutte et al., 2005).

Plasmid DNAs were extracted with a Quantum Prep Plasmid Miniprep kit (Bio-Rad) by following the manufacturer's instructions. The inserted 16S rRNA gene plus ITS was reamplified with CYA 359F (5'-ggggaattttccgcaatggg-3') and 23S30R (5'-cttcgcctctgtgtgcctaggt-3') primers as described above and subjected to ARDRA to screen the clone libraries.

Additionally, the 16S rRNA gene amplicons obtained from the genomic DNA of the culture were used for ARDRA with two different restriction endonucleases, MspI and MboI (MBI Fermentas). The preperation of the digestion reactions were as follows: add 10 µl of PCR product to 2 µl of 10 x reaction buffer (buffer R +-MboI, and Y+/Tango – MspI) and 0.5 µl of restriction enzyme and complete to final volume of 20 µl with water. Incubation was carried out for 3 h in the water bath at the optimal temperature of 37ºC (according to company's instruction). The reaction was stopped by incubating at 65ºC for 25 minutes. Electrophoresis was performed at constant 90 V/cm for 180 minutes. The gel was stained with ethidium bromide after the migration and just visualized with UV light. pBR322 DNA/Alu I Marker (MBI Fermentas) was used as a marker (Boutte et al., 2005). The ARDRA patterns were compared according to the band positions, and identical patterns were considered as the same group. Partial sequences of 16S rDNAs from representatives of each group were determined. For each ARDRA type, sequencing was made with primers 23S30R (5'-CTTCGCCTCTGTGTGCCTAGGT-3'), 1492R (5′-GTA CGG CTA CCT TGT TAC GAC-3′), and 1092R (GCG CTC GTT GCG GGA CTT) by Macrogen (Seoul, Korea) and then these sequences were assembled.

DGGE Analysis

Three filter samples and three mat samples from the hot springs were used for the DGGE. A semi-nested PCR was performed so as to increase the sensitivity and to facilitate of the DGGE analysis.

For the first PCR, the forward primer 16S359F (5′-GGG GAA TTT TCC GCA ATG GG-3′) and the reverse primer 23S30R (5′-CTT CGC CTC TGT GTG CCT AGG T-3′) were used. 0.5µl of the DNA was added to 49.5 µl of the amplification mixture, where the final concentrations of the components were 1 X of Q5® Reaction Buffer, 1 mg ml^{-1} of BSA (bovine serum albumin), 200 µM of dNTP mix, 0.5 µM of the forward and reverse primers, 1 U/µl of Q5® High-Fidelity DNA Polymerase (New England Biolabs, Inc) with proofreading activity in a final volume of 50 µl. The amplification procedure was as follows: one cycle of 5 min at 94ºC; Touch down 10 cycles of (6 cycles of 45 s at 94ºC, 1 min 60ºC, 1.5 min at 68ºC; 4 cycles of 45 s at 94ºC, 1 min 60ºC, 1.5 min at 68ºC) and the final elongation step was done for 7 min at 68ºC for the b1 reaction. For the a1 reaction, 5 min at 94ºC, 27 cycles of 45 s at 94ºC, 1 min at 54ºC, and 1.5 min at 68ºC and final elongation step was done for 7 min at 68ºC. The resulting PCR products (0.5 µl) were served as templates for the second PCR, which was performed with the forward primer 16S359F and reverse primers 16S781R (a) (5′-GAC TAC TGG GGT ATC TAA TCC CAT T-3′) and 16S781R (b) (5′-GAC TAC AGG GGT ATC TAA TCC CTT T-3′). A 38-nucleotide GC-rich sequence (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CC-3') was attached to the 5′ end of each of the reverse primers (Nübel et al., 1997; Boutte et al., 2006).

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The reaction conditions were the same as those described above except the amplification process which involved incubation for 5 min at 94°C, followed by 35 cycles (b2) /30 cycles (a2) of 1 min at 94°C, 1 min at 60°C, and 1 min at 68°C and then a final elongation step of 7 min at 68°C. Two distinct reactions were performed for each reverse primer. The negative control for the first PCR was used in the second PCR to check for contamination. DGGE was made by Ingeny system and performed as described by Nübel et al., 1997 with the following modifications. The PCR products obtained with primers 16S781R (a) and 16S781R (b) were applied separately to the polyacrylamide gel. The gel contained a linear 40 to 65% denaturant gradient, the pH of the TAE buffer was adjusted to 7.4, and the electrophoresis was applied for 22 h at 80 V and 60°C. After being stained with ethidium bromide, the gel was visualized under UV light and then photographed by Uvitec gel documentation system. The DGGE bands were excised with a surgical scalpel. Each small gel block was placed in 100 µl of sterile water for 2 h at room temperature. Each solution was used as a template for PCR amplification as described above. The PCR products were then electrophoresed to confirm the bands. 350-bp part of 16S rRNA gene was sequenced with primer 16S784R (5′-GGA CTA CWG GGG TAT CTA ATC CC-3′).

Nucleotide sequence accession numbers

Environmental 16S rRNA gene sequences from Afyonkarahisar hot springs are available at the GenBank with the accession numbers KJ461812-KJ461849, KT715745-KT715753, and KT793918-KT793927.

Analyses of sequence data

All sequences were compared to the sequences in the BLAST search program at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov). 16S rRNA sequences of the hits for our sequences were obtained through the RDP (Ribosomal Database Project) site at Michigan State University (http://rdp.cme.msu.edu/). The top five hits as well as some additional relevant sequences were used for phylogenetic analysis. Sequences of partial 16S rDNA of cultures, clones and DGGE fragments were analysed by BioEdit v7.2.5 software. All sequences were checked for chimera formation using the DECIPHER (Wright et al., 2012). CHECK-CHIMERA software developed by the Ribosomal Database Project and the phylogenetic affiliations of their 5′ and 3′ ends were compared. Phylogenetic trees were constructed using the maximum likelihood treeing algorithm and Nearest-Neighbor-Interchange (NNI) method in the MEGA 6 (Tamura et al., 2007). The Distance Matrix was calculated using the Jukes-Cantor correction. Validity of the tree topology was checked using the bootstrap method (1000 replicates).

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Results

Chemical properties of the hot spring water samples

As shown in *Table 1*, waters of three springs have little difference in their properties.

Table 1. *Some properties of the water samples (mg/l)*

	Akkuş-Gazlıgöl (64°C)	**Hüdai-Sandıklı** (68°C)	**Ömerli** (98°C)
pH	8.3	7.06	7.06
Mg^{2+}	19.3	30	20.6
NO_3^-	<0.5	<0.5	<0.5
Mn^{2+}	0.14	0.10	0.13
NH_4^+	<0.5	<0.5	<0.5
P	0.4	0.4	1.7
N	<0.5	<0.5	0.0
Cl_2	0.18 *0.15	0.07 *0.07	0.13 *0.12
CSB/COD	<10	<10	14
NO_2^-	0.020	0.044	0.048
F^-	1.85	1.68	1.83

*amount of free Cl_2

Microscopic observation and cyanobacterial cultivation analysis of the water samples of the springs

Microscopical examinations of the water samples of the hot springs showed a dominance of filamentous cyanobacteria *(Figure 3).* The cyanobacteria found were described based on their morphologies. *Oscillatoria*-like cyanobacteria were dominant and *Gleocapsa*-like unicellular cyanobacteria were encountered less frequently. 74 pure isolates having 6 different ARDRA profiles *(Table 2)* were obtained from the hot springs. BLAST analysis of partial 16S rDNA obtained from the isolates showed that Ömerli hot spring has more cyanobacterial diversity than the other two. ARDRA profiles of the isolates and their closest Genbank matches are shown in *Table 3*.

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In our ARDRA results, 73% of the isolates belong to the group S1. The groups S2, S3, S4, S5, and S6 are 4.05%, 6.75%, 2.70%, 12.16% and 1.35%, respectively. The similarities among the isolates with the closest relative sequences in Gen Bank can be seen in *Table 3.*

The sequences of the isolates CY2, CY9, and CY16 have similar with the genus *Fischerella* sp. isolated from Costa Rica (Unpublished, Acc. Number DQ786171). The CY8 isolate sequence showed identity with the *Uncultured cyanobacterium* obtained from as-rich and DIC-limited geothermal waters of El Tatio, Chile (Unpublished, Acc. Number KP794044.1) and *Fischerella* sp. (Acc. Number HM636645). The sequences of CY32, CY20, CY11, CY13 and CY31 were similar with the genus *Geitlerinema* sp./*Leptolyngbya laminosa* isolated from Euganean thermal muds, Padova, Italy (Unpublished, Acc. Number FM210758).

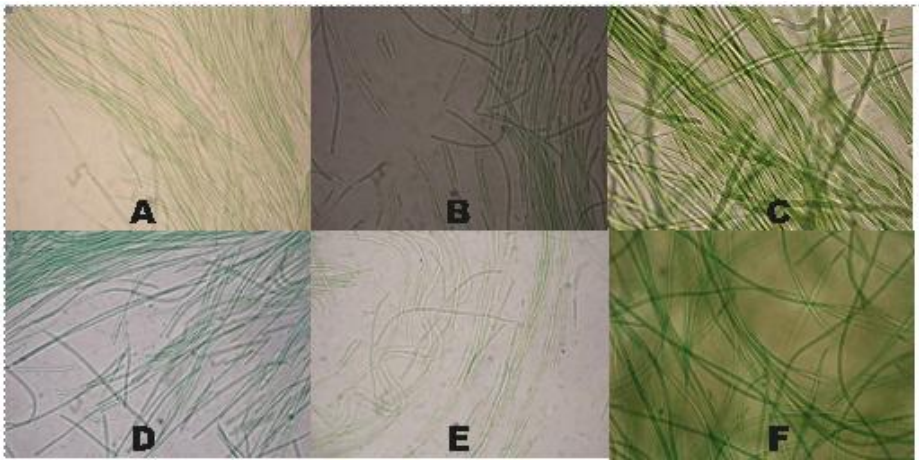

Figure 3. *Light microscopy images of some cultures (40X objective). A, B, C, D, E, F trichome morphology of Oscillatoria- like field colonies in BG-11 medium.*

Table 2. *Isolates, clones and the DGGE bands of Akkuş-Gazlıgöl, Hüdai-Sandıklı and Ömerli hot springs and their ARDRA groups*

	Isolates	**Clone libraries**	**DGGE bands**
Akkuş-Gazlıgöl	**14 isolates** Group S1 (9 isolates) Group S3 (5 isolates)	**30 clones** Group K1 (26 clones) Group K2 (2 clones) Group K6 (2 clones)	**2 DGGE (a2) band** (A.2.1, A.2.2) **7 DGGE (b2) band** (B.2.1, B.2.2, B.2.3, B.2.4, B.2.5, B.8.1, B.8.2)
Hüdai-Sandıklı	**17 isolates** Group S1 (15 isolates) Group S5 (2 isolates)	**32 clones** Group K1 (2 clones) Group K2 (22 clones) Group K3 (1 clones) Group K4 (7 clones)	**3 DGGE (a2) band** (A.5.3, A.5.4, A.5.5) **2 DGGE (b2) band** (B.6.1, B.6.3)

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Ömerli	**43 isolates**	**35 clones**	**2 DGGE (a2) band**
	Group S1 (30 isolates)	Group K1 (19 clones)	(A.1.2, A.1.3)
	Group S2 (3 isolates)	Group K2 (5 clones)	
	Group S4 (2 isolates)	Group K4 (8 clones)	**16 DGGE (b2) band**
	Group S5 (7 isolates)	Group K5 (3 clones)	(B.3.1, B.3.2, B.3.3, B.3.4, B.3.5, B.3.6, B.3.7, B.3.8, B.4.1, B.4.2, B.4.3, B.7.1, B.7.2, B.7.3, B.7.4, B.7.5)
	Group S6 (1 isolates)		

Table 3. *Cyanobacterial isolates, clones and their closest GenBank Matches.*

	ARDRA Group No	Sample Code (Accession no)	% of identity with the closest relative	Closest relative according to BLAST search/ Accession no/ Length
Isolates	S1	CY11 (KT715748)	99% 93%	*Geitlerinema* sp. (FM210758) / 1745 *Uncultured cyanobacterium* clone MSmat.3.11 (JQ612141) / 1745
	S1	CY20 (KT715750)	99% 93%	*Geitlerinema* sp. (FM210758) / 1780 *Uncultured cyanobacterium* clone MSmat.3.11 (JQ612141) / 1780
	S2	CY8 (KT715753)	99% 99%	*Uncultured cyanobacterium* (KP794044.1) /1087 *Fischerella sp.* (HM636645) / 1087
	S3	CY32 (KT715752)	99% 99%	*Geitlerinema* sp. (FM210758) /1089 *Uncultured Leptolyngbya* sp. clone Tsenher12otu4-1 (KT258783) / 1089
	S3	CY31 (KT715751)	96% 96%	*Geitlerinema* sp.(FM210758) /1526 *Uncultured Leptolyngbya* sp. clone Tsenher12otu4-1 (KT258783) / 1526
	S4	CY9 (KT715746)	99% 99%	*Fischerella* sp. (DQ786171) /1483 *Uncultured bacterium* clone B95 (AF407731) / 1483
	S4	CY2 (KT715745)	99% 99%	*Fischerella* sp. (DQ786171) /1491 *Uncultured bacterium* clone B95 (AF407731) / 1491
	S5	CY13 (KT715749)	98% 98%	*Geitlerinema* sp. (FM210758) /1531 *Uncultured Leptolyngbya* sp. clone Tsenher12otu4-1 (KT258783) / 1531
	S6	CY16 (KT715747)	97% 97%	*Fischerella* sp. (DQ786171) /1428 *Uncultured bacterium* clone B95 (AF407731) /

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				1428
Clones	K1	Clone 7 (KT793923)	97% 90%	*Uncultured bacterium* (AB757744) /1501 *Cyanothece* sp. (CP001344) / 1501
	K1	Clone 21 (KT793924)	99% 91%	*Uncultured bacterium* (AB757744) /1539 *Cyanothece* sp. (CP001344) / 1539
	K2	Clone 15 (KT793921)	99% 93%	*Geitlerinema* sp. (FM210758) /1737 *Uncultured cyanobacterium* clone MSmat.3.11 (JQ612141) /1737
	K3	Clone 12 (KT793922)	93% 93%	*Geitlerinema* sp. (FM210758) /1427 *Uncultured Leptolyngbya* sp. clone Tsenher12otu4-1 (KT258783) / 1427
	K4	Clone 62 (KT793926)	99% 91%	*Uncultured bacterium* (AB757744) /1470 *Cyanothece sp.* (CP001344) / 1470
	K5	Clone 46 (KT793925)	95% 88%	*Uncultured bacterium* (AB757744) /1623 *Oculatella atacamensis* (KF761587) / 1623
	K6	Clone 66 (KT793927)	93% 91%	*Leptolyngbya laminose* (FM210757) / 1646 *Uncultured cyanobacterium* clone MSmat.3.11 (JQ612141) /1646

Molecular Analysis

In addition to cultivation studies, we carried out culture-independent method cloning and Denaturating Gradient Gel Electrophoresis (DGGE) analysis for the determination of the biodiversity of Afyonkarahisar hot springs. To create a clone library containing 16S rRNA + ITS + 5 parts of 23S rRNA inserts, we used cyano-specific PCR primers which are CYA359F and 23S30R pairs. A total of 97 clones containing inserts of the right size were analysed. First, they were classified on the basis of their restriction profiles (*Table 2*). Akkuş-Gazlıgöl, Hüdai-Sandıklı, and Ömerli hot springs have 3, 4, and 4 different ARDRA profiles, respectively. These groups are K1, K2, and K6 for Akkuş-Gazlıgöl, K1, K2, K3 and K4 for Hüdai-Sandıklı, and K1, K2, K4 and K5 for Ömerli hot spring *(Table 2)*. The group K1 clones are the major ones in Akkuş-Gazlıgöl and Ömerli hot springs. One or two representatives from 6 different restriction groups were sequenced with the sequence primers 1092-1492-23SR. The BLAST analysis of the selected clones is shown in *Table 3*. 67% of the cyanobacterial clones were related to *Uncultured bacterium* (≥ 95-97% similarity) / *Cyanothece* sp. (90-91% similarity) or *Oculatella atacamensis* (88% similarity) as culture.

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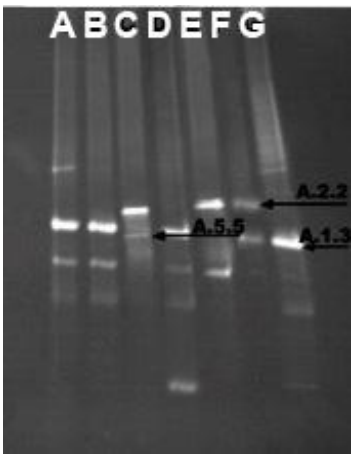
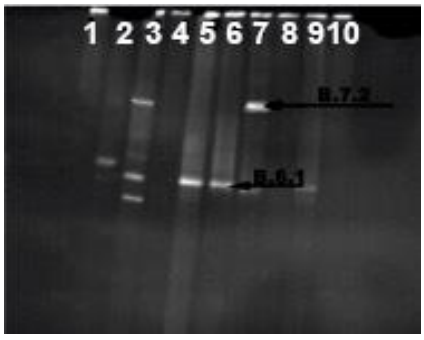
The similarities of the clone 7, 21, 62 sequences with *Uncultured bacterium* obtained from Padang Cermin Hot Spring Water (unpublished, Acc. Number AB757744) as environmental sample and with *Cyanothece* sp. (unpublished, Acc. Number CP001344) isolated from rice fields in Senegal as isolate were 90%, 91%, and 91%, respectively. Also, the similarities of clone 46 sequence with *Uncultured bacterium* (Acc. Number AB757744) and *Oculatella atacamensis* obtained from Atacama Desert (Karina et al., 2014, Acc. Number KF761587) as culture were 95% and 88%, respectively. The sequence of clone 12 and 15 were 93% and 99% identity with the genus *Geitlerinema* isolated from Euganean thermal muds, Padova, Italy (Unpublished, Acc. Number FM210758). Clone 66 sequence was 93% similarity with the *Leptolyngbya laminosa* from Euganean thermal muds, Padova, Italy (Unpublished, Acc. Number FM210757) *(Table 3).*

For the DGGE fingerprint analyses, we studied the 16S rRNA gene-defined community diversity in cyanobacterial water samples and mats from three hot springs. CYA359F and the CYA781R primer pair were used in DGGE method for the second PCR reaction. 25 and 7 positive bands were identified using DGGE (b2) and (a2), respectively. Representative samples of DGGE separations and some major bands of hot springs cyanobacterial 16S rRNA are presented in *Figure 4.*



DGGE (a2)

A) Hüdai-Sandıklı water, B) Akkuş-Gazlıgöl mat, C) Hüdai-Sandıklı mat, D)-E) Ömerli mat, F) Akkuş-Gazlıgöl water, G) Ömerli water



DGGE (b2)

B) (1)-(4) Ömerli water, (2) Akkuş-Gazlıgöl water, (3)-(7) Ömerli mat, (5)-(6) Hüdai-Sandıklı mat, C) (8) Akkuş-Gazlıgöl mat, (9) Hüdai-Sandıklı water

Figure 4. *DGGE gels (a2) and (b2) of the hot springs and some major bands*

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The DGGE band numbers from Akkuş-Gazlıgöl, Hüdai-Sandıklı, and Ömerli hot springs are shown in *Table 2*. The sequences obtained from most of the the bands (87.5%) yielded similarities to *Geitlerinema* with high percentages of similarity (99%/100%) that was isolated from thermal waters in İzmir (Unpublished, Acc. Number HQ197683.1) and one band (B.6.3) was related to the *Uncultured bacterium/Cyanobacterium* with similarity 99%/100%, respectively that was isolated from Yellowstone National Park, Fairy Springs (Boomer et al., 2009; FJ206395) and Yellowstone National Park, octopus spring cyanobacterial mat (Weller et al., 1992; Acc. Number L04709.1), two bands (A.1.2 and A.1.3) were related to the *Uncultured cyanobacterium* with similarity 99% and 94%, respectively (Unpublished, Acc. Number GQ480617), and one band (A.5.5) was related to the *Uncultured cyanobacterium/Phormidium* with similarity 99%/98% (Unpublished, Acc. Number EU728938.1 and DQ408370.1), respectively (*Table 4*).

In our DGGE results, Ömerli may have sequences belonging to the *Geitlerinema, Uncultured cyanobacterium*, Hüdai-Sandıklı may have *Geitlerinema, Uncultured bacterium/Cyanobacterium, Uncultured cyanobacterium/Phormidium* while Akkuş-Gazlıgöl may have only belonging to the *Geitlerinema* sp. sequences.

Table 4. *Obtained DGGE bands and their closest relatives in GenBank*

DGGE bands	% Similarity closest relative in BLAST search of Gen Bank
B.2.1, B.2.2, B.2.3, B.2.4, B.2.5, B.3.1, B.3.2, B.3.3, B.3.4, B.3.5, B.3.6, B.3.7, B.3.8, B.4.1, B.4.2, B.4.3, B.6.1, B.7.1, B.7.2, B.7.3, B.7.4, B.7.5, B.8.1, B.8.2, A.2.1, A.2.2, A.5.3, A.5.4	(99-100%) *Geitlerinema* sp. (HQ197683.1)
B.6.3	(100%) *Uncultured bacterium* (FJ206395) (99%) *Cyanobacterium* sp. (L04709.1)
A.1.2, A.1.3	(99%, 94%) *Uncultured cyanobacterium* (GQ480617)
A.5.5	(99%) *Uncultured cyanobacterium* (EU728938.1) (98%) *Phormidium* sp. (DQ408370.1)

Phylogenetic analysis

Sequences obtained from the isolates, clones and DGGE bands were aligned with the closest strains and retrieved from environmental samples obtained from RDP II and NCBI. Phylogenetic trees for cyanobacteria constructed based on partial 16S rRNA sequences were shown in *Figure 5*, *6*, and *7*. Aligned partial 16S rRNA gene sequences corresponding to *E. coli* sequence positions 388 to 1442 for the isolates, 551 to 1525 for the clones and 415 to 717 for the DGGE bands were used but the indels were not taken into account.

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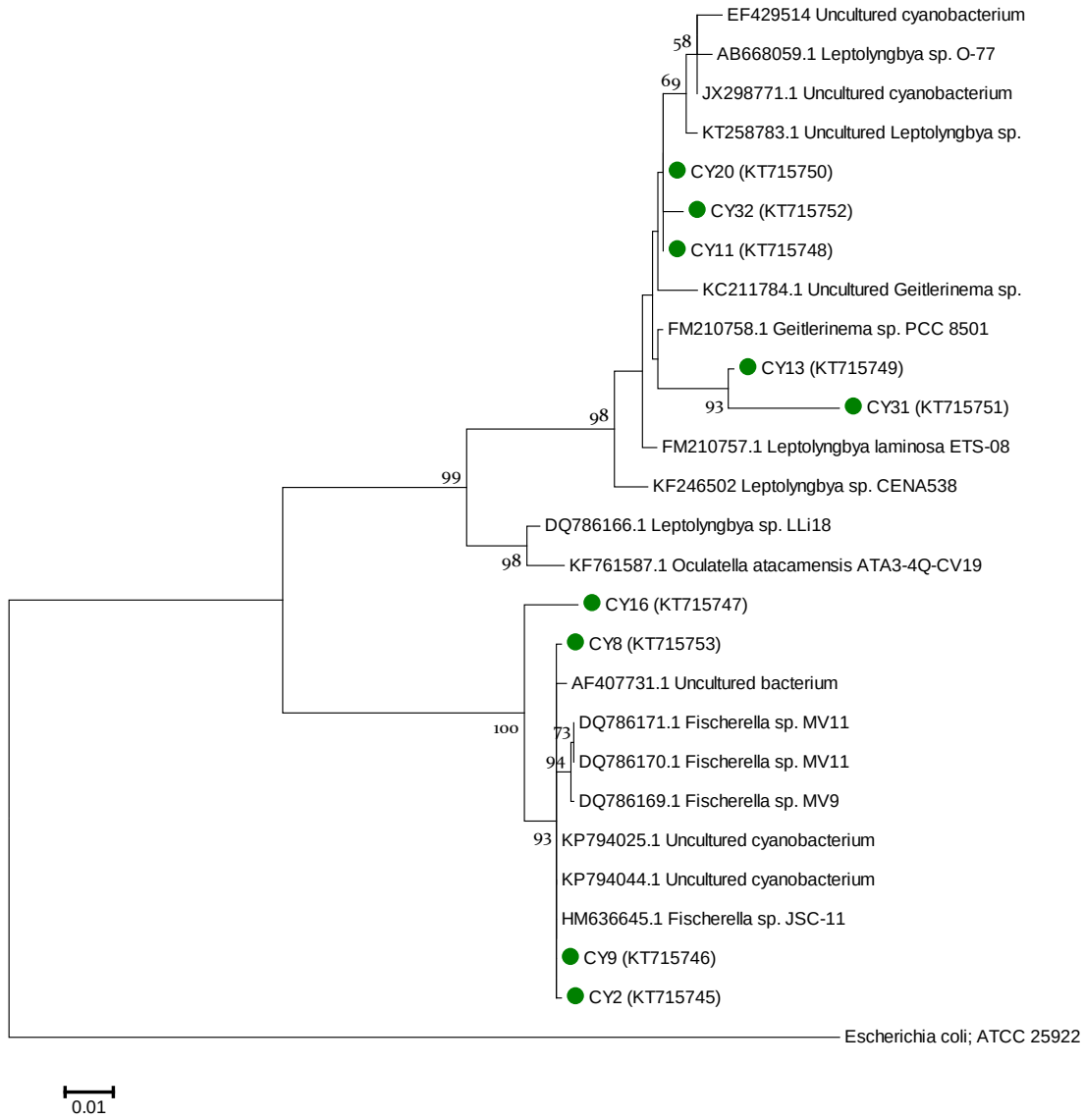

Figure 5*. Phylogenetic inferences based on 16S rRNA gene sequences from isolates (indicated by green circle). Sequence of the E.coli was used as outgroup. Scale bar represents expected number of substitutions per site. Bootstrap support values below 50% were not included in the figure.*

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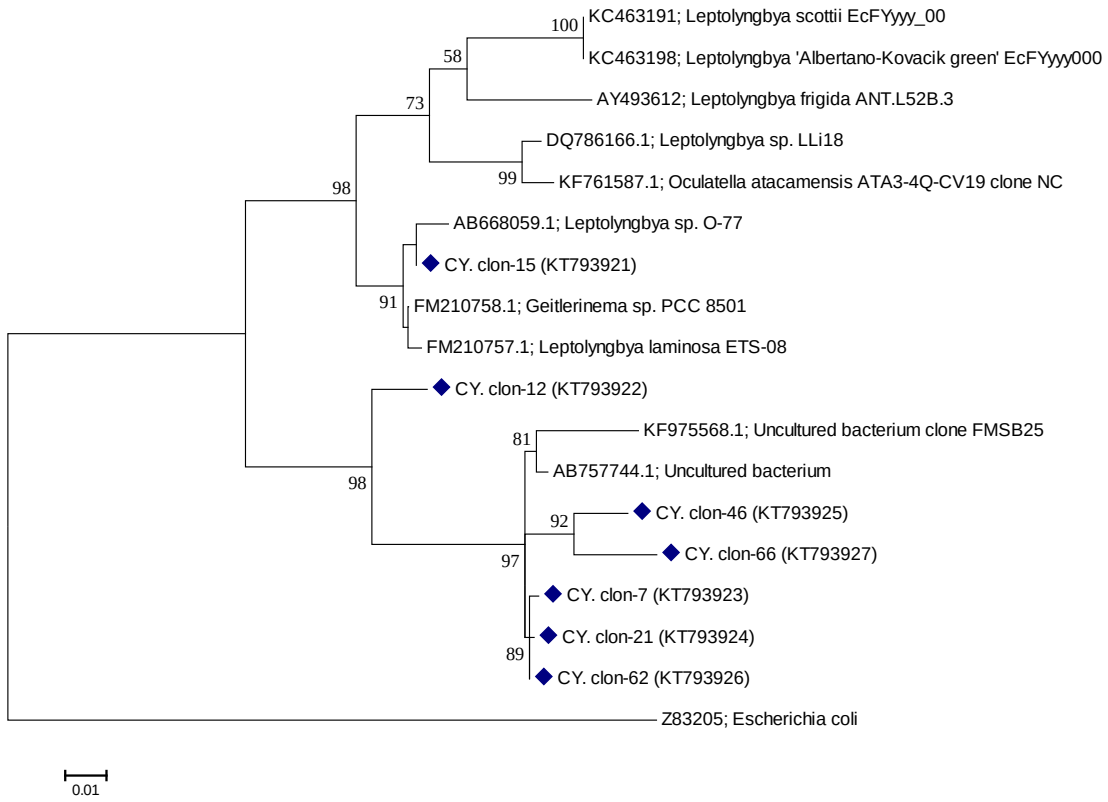

Figure 6. *Phylogenetic inferences based on 16S rRNA gene sequences from clones (indicated by blue diamond) belonging to the cyanobacteria. Bootstrap support values below 50% were not included in the figure.*

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Figure 7*. Phylogenetic inferences based on 16S rRNA gene sequences from some DGGE bands (indicated by violet trigon) belonging to the cyanobacteria. Bootstrap support values below 50% were not included in the figure.*

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Conclusions

The microbial diversity analysis has shifted in the last two decades from cultivation-dependent approaches to 16S rRNA-based cultivation-independent approaches, which led to the discovery of many novel microbial taxa. Nevertheless, this new approach also has limitations and is often restricted 16S rRNA clones through sequence similarity. Therefore, cultivation is still the method of choice to understand fully the physiology and complex ecological interactions in which microorganisms engage (Gunde-Cimmerman et al., 2005). The biodiversity of thermophilic cyanobacteria in terrestrial geothermal locations is fairly well documented, where they occur as part of a microbial mat community at temperatures up to ~65°C, and in biofilms at higher temperatures up to 75°C (Ward et al., 1998). The restriction fragment length polymorphisms (RFLPs) of particular PCR products can provide signature profiles specific to the genus, species, or even strain. Genetic characterization of cyanobacterial strains has been undertaken using RFLPs of the 16S rRNA gene (16S-ARDRA) (Lyra et al., 1997) and of the intergenic transcribed spacer region (ITS-ARDRA) (Lu et al., 1997; West and Adams, 1997).

Furthermore, amplification of the 16S–23S rRNA ITS, whose length and number was shown to be variable in cyanobacteria (West and Adams, 1997; Rocap et al., 2002; Iteman et al., 2002; Neilan, 2002, Laloui et al., 2002; Iteman et al., 2002) in cyanobacteria, can also be used as an identification tool. In this research, we used cultivation and molecular approaches, including DGGE analysis and cloning based on the 16S rRNA gene to reveal the cyanobacterial community composition of three Afyonkarahisar hot springs.

A large number of new thermophilic cyanobacterial isolates have been obtained for a long time by many researchers (Stetter, 1996; Casamatta et al., 2003). Our cyanobacterial isolates and previously cultured cyanobacteria, such as *Uncultured cyanobacterium/Fischerella* sp and *Geitlerinema* sp/ *Leptolyngbya laminosa* in Gene-Bank are very similar to each other. The sequence of our isolate CY31 had 96% similarity with the genus *Geitlerinema* sp. isolated from Euganean thermal muds, Padova, Italy (Unpublished, Acc. Number FM210758). 16S rRNA sequence similarity (96%) of CY31 indicates that this strain might be a novel species of the genus. Therefore, further studies, e.g. DNA: DNA homology should be carried out with different *Geitlerinema* species.

According to the cultivation results, the hot springs of Afyonkarahisar are inhabited by mainly thermophilic filamentous cyanobacteria including the *Fischerella* sp. and *Geitlerinema* sp., which are known to exist in thermal springs worldwide (Brock 1978; Ward and Castenholz 2000). *Spirulina labyrinthiformis* was earlier reported as the dominant organism in material collected by Aaronson from a 52 °C spring of Zerka Ma'in (Rayss 1944). However, we did not encounter *Spirulina* sp. in these hot springs. In addition, although one of the best-documented genera of thermophilic cyanobacteria is the genus *Synechococcus* (Ward et al., 1990, 1998; Miller and Castenholz, 2000; Ramsing et al., 2000; Papke et al., 2003). We did not determine any sequence related this genus in the hot springs.

The diversity and distribution of cyanobacterial groups in the springs were not high. The results obtained from Ömerli, Akkuş-Gazlıgöl and Hüdai-Sandıklı had some similarities with each other and those obtained from the other studies (Reysenbach et al., 1994; Ghosh et al., 2003; Komarek et al., 2003; Hongmei et al., 2005; Sompong et al., 2005; Jing et al., 2006; Sompong et al., 2006; Moro et al., 2007; Ionescu et al., 2009).

Cyanobacterial clones recovered from Ömerli hot spring were phylogenetically related to *Uncultured bacterium* and *Geitlerinema* sp. For Hüdai-Sandıklı hot spring, clones were phylogenetically related to *Uncultured bacterium* and *Geitlerinema* sp.

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Uncultured bacterium, *Geitlerinema* sp, and *Leptolyngbya laminose* sequences were also obtained in Akkuş-Gazlıgöl hot spring. Although Ömerli has the highest cyanobacterial diversity, Akkuş-Gazlıgöl has more cyanobacterial genus diversity. Contradictory results of same samples were obtained when analyzing the cyanobacterial community inhabiting the hot springs with the culturing. *Getilerinema* sp. was dominated in the culturing method while *Uncultured bacterium/ Cyanothece* sp. became major ones with the cloning.

16S rRNA PCR-DGGE is one of the most frequently used technique to assess the genetic diversity of microbial communities (Muyzer, 1999; Ercolini, 2004). This method allows the separation of small DNA fragments (maximum size of 1000 bp) of the same length but of different sequence according to their melting properties. Fragments with only one single base substitution can be separated with this technique (Nollau and Wagener, 1997). The analysis of these group-specific PCR fragments on a DGGE gel provides a valuable tool for monitoring the structure and dynamics of microbial populations over time or under the influence of environmental changes. Although limitations exist, such as the difficulty on band isolation and too short sequences retrieved to provide a robust phylogenetic analysis, this methodology has been revealed to be a valuable technique to monitor changes in bacterial community structure, both in relative abundance and in bacterial diversity (Lemarchand et al., 2005). We used the specific primers that have the advantage of giving a PCR product which corresponds to the variable regions V3 and V4, and contains significant information for phylogenetic assignments (Nübel et al., 1997; Boutte et al., 2006).

Casamatta et al., 2003 found various cyanobacteria like *Pleurocapsa, Phormidium, Anabaena, Synechocystis, Oscillatoria, Microcoleus* and *Pseudanabaena* with 16S rRNA gene sequences along with DGGE inhabiting Octopus hot spring. Similarly, *Geitlerinema* and *Phormidium* genus were also detected in our study. Bands that migrated to the same position in the DGGE gel and displayed no ambiguous differences in nucleotide sequences were considered to represent unique 16S rRNA sequence types. Moreover, identical sequences were obtained from different bands in the same lane. This is in agreement with the observation of Nikolausz et al., 2005 that dominant amplicons could be distributed at different positions in the same pattern. If several domains have similar melting properties, stochastic effects might cause one to denaturation before the other in a fraction of the amplicon population and could also explain the presence of different bands with the same sequence in one lane.

Consistent with cloning and DGGE, the 16S rRNA gene sequences from Ömerli showed that *Geitlerinema* sequences were recovered from both method and was dominated with DGGE. Similar to our cultivation results, *Geitlerinema* sp. was obtained with the cloning and DGGE analysis. But the *Fischerella* sp. could not be determined with cloning and DGGE sequences.

In this study, although high diversity emerged in ARDRA profile, low diversity in terms of genera was obtained from culture-dependent and culture independent assay results. Previous studies in thermal springs have shown an increasing diversity with decreasing temperature (Kullberg, 1968; Brock, 1978; Castenholz, 1978; Miller and Castenholz, 2000; Sompong et al., 2005). However, this result has some conflicts with ours. According to our results, the cyanobacterial diversity increased with the increasing temperature. Clearly, thermal stress is not a limiting factor since the hottest spring were most biodiverse. The cyanobacteria identified in this study do not follow a single pattern when compared with similar organisms from different thermal sites in the world. Some

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clones and isolates showed low similarities (93–97%) to those from other environments while others showed over 97% similarity to their counterparts.

Since thermophiles are notoriously difficult to culture, this study significantly expanded the known range of cultivated thermophilic cyanobacteria and provided a biological resource for future biotechnological exploitation. Furthermore, cultivation and characterization of axenic thermophilic cyanobacterial strains provided a further insight into the physiology of thermophiles and yielded a source of organisms for possible biotechnological exploitation.

It is intriguing that the unique environment of Afyonkarahisar hot springs has not been surveyed for cyanobacterial diversity before. This is the first study in which both culture-dependent and culture-independent techniques have been used simultaneously to target unique regions of the 16S rRNA +ITS gene in samples obtained from Afyonkarahisar hot springs.

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