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PROKARYOTIC DIVERSTY OF ACID MINE DRAINAGE PONDS IN ORE ENRICHMENT PLANT

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ABSTRACT

The biodiversity of acidophilic prokaryotes was determined in three AMD ponds (pH 2.7-6.5) in Turkey (İzmir-Halıköy antimony ore enrichment plant) using 16S rRNA cloning and denaturing gradient gel electrophoresis methods. Water samples were taken two times in March 2014 and June 2015. The microbial diversity identified includes species such as *Acidiphilium angustum, Acidocella sp., Ferroplasma acidiphilum, Acidithiobacillus ferriphilus, Acidithiobacillus ferrivorans, Acidiphilium rubrum, Thiomonas* sp., *Acidiphilium multivorum, Acidiphilium cryptum, Ferrovum myxofaciens, Acidocella aluminiidurans* with the used techniques. In addition to, it has been determined that biodiversity is variable in the operating mine pools. *Aciditihobacillus ferriphilus*, *Acidiphilium angustum,* and *Acidiphilium rubrum* are new records for Turkey.

Keywords: acidic mine drainage, acidophiles, prokaryotic diversity, Turkey

INTRODUCTION

Acid mine drainage (AMD) is the largest environmental problem caused by normally associated with mining activities **(Garcia-Moyano *et al.*, 2015)**. The mining wastewater is defined by properties such as low pH, high metal ions (e.g., iron, nickel, copper) and mineral concentrations. Acidophilic microorganisms living in this habitat are very interesting because of their adaptability to extreme pH values, their metabolic diversity, and their ability to be used in biomining applications. Especially, due to their availability in biomining and bioremediation applications, it is important to identify the acidophiles living in AMD. As determined in previous studies, the AMD microbial community changes over time **(McGinnes and Johnson, 1993; Edwards *et al.*, 1999; Volant *et al.*, 2014)**. The variety of microbial community is attached to seasonal changes and environmental conditions in AMD **(Auld *et al.*, 2017)**.
Classical microbial ecology methods remain limited in determining microbial diversity. Therefore, culture-independent methods such as 16s rRNA gene cloning, fluorescent in situ hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE) are often used to investigate the diversity of microbial community that adapts to this unique environments **(Gonzalez-Toril et al., 2003; Nicomrat *et al.*, 2006; Garcia-Moyano *et al.*, 2015)**. Acidophilic chemolithotrophs such as *Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans, Leptospirillium ferrooxidans* have been identified in AMD which extremely low pH and high concentrations of iron, sulfates and other heavy metals **(Edwards *et al.*, 1999; Kuang *et al.*, 2012)**. At the same time, the investigation of microbial community by molecular methods is difficult because of the inhibition of PCR by metals such as Fe and Cu **(Nicomrat *et al.*, 2006)**. For the reason, DGGE and 16s rRNA gene cloning methods are used together to support each other in determining the microbial diversity of AMD.
The aim of this research was to determine the acidophilic prokaryotic community of acid mine drainage in ore enrichment plant Halıköy, İzmir (Turkey). Our study area in Halıköy is within the Menderes Massif in western Turkey. The antimony mine was discovered in 1870 and continued to operate until 1918 in Halıköy area. After a long-standing period, production began again in 1974 **(Akcay *et al.*, 2006)**. Our results are the first knowledge about the prokaryotic community of the selected AMD area.

MATERIALS AND METHODS

Site description and sample collection

The water samples were collected from the operating antimony mine, ore enrichment plant Halıköy site (38°5'28.09"N, 28°10'09.6"E) in İzmir, Turkey (Fig 1), in two different time (March 2014 and June 2015). The samples were taken three different points from mine area as drainage water (sample #1 and sample #4), iron oxide pool water (sample #2 and sample #5), and stationary water before iron oxide pool (sample #3 and sample #6) (Fig 2). Water samples were taken in sterile Duran bottles and were filtered from 0.2 um GTTP filter. In situ measurements for pH were made using a WTW Multi350i/SET (WTW, Germany). Metal concentrations of water samples were measured by inductively coupled plasma atomic emission spectrometry (ICP-AES).

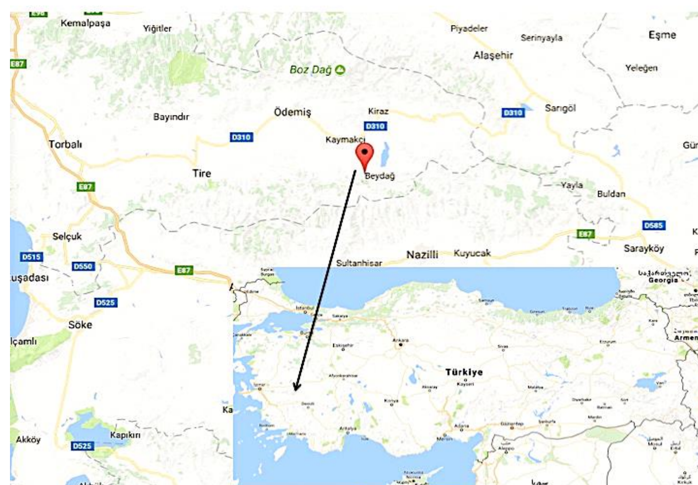

Figure 1 Map of Halıköy, İzmir (Turkey), where AMD ponds are located in antimony mine site

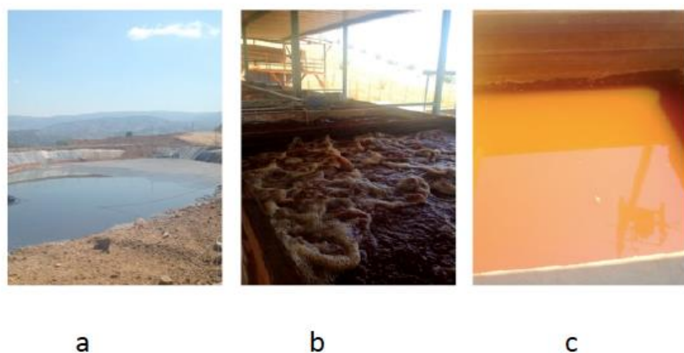

Figure 2 Points of water samples (a: #1 and #4; b: #2 and #5; c: #3 and #6)

DNA isolation and 16S rRNA gene amplification

DNA isolations were performed following the procedure explained by Cifuentes *et al*. (2000) and Nogales *et al*. (1999) as modified. We used the primer set 27F (AGAGTTTGATCMTGGCTCAG)-1387R (GGGCGG(AT)GTGTACAAGGC) and 20F (AGAGTTTGATC(AC)TGGCTCAG)-915R (GTGCTCCCCCGCCAATTCCT) for bacteria and archaea, respectively.
PCR cycles were as follows: one cycle at 95 °C for five minutes, 30 cycles at 95 °C for 30 seconds, one minute at the corresponding annealing temperature 55 °C and 62 °C (for bacteria and archaea, respectively) and 72 °C for 1.5 minutes, and a final extension step at 72 °C for ten minutes.

16S rRNA gene cloning

The PCR products were cloned using the pGEM-T easy vector system II and colony PCRs were set up with 27F and 1387R; 20F and 907R primer sets of selected colonies, for bacteria and archaea, respectively. Similar profiles were determined by amplified ribosomal DNA restriction analysis (ARDRA) in the 16S rRNA gene libraries. Clones were divided into categories (3 h, 37°C) based on pattern generated by the restriction enzymes MspI and HaeIII (5 units each).

DGGE

Amplification of the 16S rRNA gene was carried out with specific primers for DGGE analysis. Primer set including 344F-GC (CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGACGGGGC GCAGCAGGCGCGA) and 907R (CCGTCAATTCCTTTGAGTTT) was used for the archaeal gene amplification while for the bacterial gene, the forward primer, 341F-GC (CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCTACGG GAGGCAGCAG) in combination with 907R were used (**Muyzer *et al*., 1993**).
DGGE PCR condition for bacteria: one cycle at 94 °C for five minutes, one minute at 65 °C and three minutes at 72 °C and nine cycles at 94 °C for one minute, one minute at the annealing temperature was decrease 64- 55 °C and 72 °C for three minutes, and one cycle at 94 °C for five minutes, one minute at 55 °C and three minutes at 72 °C and a final step 94 °C for five minutes, one minute at 55 °C and ten minutes at 72 °C. DGGE PCR condition for archaea: one cycle at 94 °C for five minutes, 29 cycles at 94 °C for three seconds, 56 °C for 45 seconds, 72 °C for two minutes, and one cycle at 94 °C for 30 seconds, 56 °C for 45 seconds, 72 °C for seven minutes. The PCR products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Italy).
Denaturing gradient gel electrophoresis (DGGE) was performed with the DCode Universal Mutation Detection System (Bio-Rad Laboratories, Inc.). PCR product was loaded on 1 mm thick 8% (w/v) polyacrylamide (37.5: 1 acrylamide: bisacrylamide) gels containing a 45-60% linear denaturing gradient. Gels were run in 1X TAE buffer at 60°C and 90 V for 18 h. Gels were stained in 1X TAE buffer containing ethidium bromide solution (1 µg/ ml) and photographed under UV transillumination.
Each band in different positions was cut with a sterile lancet from the polyacrylamide gel and was stored at 37 °C in solvent buffer (ammonium acetate 5M, magnesium acetate 10 mM, EDTA (pH 8.0) 1mM, SDS 0.1%) during overnight and DNA fragments were isolated. The DNA fragments were used re-amplification with same primer pairs without GC clamps and were sequenced.

Accession numbers and construction phylogenetic tree of nucleotide sequences

Multiple gene alignments were applied using MUSCLE software. Phylogenetic trees were made using MEGA version 7 and the neighbor-joining method (**Saitou and Nei, 1987**). The 16S rRNA gene sequences and results of DGGE analyses were uploaded into the GenBank Database.

RESULTS

Characteristics of site and samples

The sampling site is antimony mine site being operated. The AMD samples were characterized by acidic pH values ranging from 2.7 to 6.5 and high concentrations of dissolved metals (Table 1). The sample points show the typical orange and red colors of dissolved ferric iron as shown in Figure 1. It was determined that the samples had high iron, zinc, lead and manganese ratios. Treatment and neutralization studies of outlet water caused increase the pH value and decrease iron concentration between two sampling times (sample #5). This pH change also affected the prokaryotic diversity at the sample site (Table 1).

Table 1 pH values and dissolved metal concentrations of water samples

Samples code- pH	#1 3.0	#2 2.9	#3 2.7	#4 2.8	#5 6.5	#6 3.6
Elements	**Concentrations (mg L^{-1})**					
Fe	205.3	237.8	*	198.4	40.959	200.7
Zn	1.693	7.547	28.98	2.006	5.328	15.952
Mn	4.701	*	*	4.351	8.937	*
Cr	0.185	0.302	0.784	0.145	0.267	0.935
Co	0.066	1.794	6.643	0.045	1.539	5.628
Cu	0.077	0.820	1.637	0.058	0.754	1.756
Ni	0.241	1.624	6.481	0.321	0.954	4.522
Pb	0.029	0.065	0.063	0.019	0.017	0.052

*Not determined because it has a high concentration.

Cloning

Clone library technique offers the opportunity to do very sensitive taxonomic studies. By this method, uncultured and unspecified microorganisms are also possible to define (**Sanz and Kochling, 2007**). The clones from sample #1 and #3 YT_K1, YT_2, YT_K12, YT_K14, YT_K16 matched with an uncultured bacterium (%99), an uncultured archaeon (%97), *Acidithiobacillus ferrivorans* (%99), *Acidithiobacillus ferriphilus* (%99), and *Acidiphilium rubrum*, respectively. According to ARDRA of the plasmid insert, it has been determined that there are 4 and 14 different profiles from sample #2 and #5 (same points, different periods) coded samples taken in March 2014 and June 2015, respectively (Fig. 3). The sequence of clones (from water samples #2) YT_K3, YT_K4, YT_K7, and YT_K8 showed 99% similarities with *Acidiphilium* sp., *Acidithiobacillus ferriphilus*, *Acidocella sp.*, *Acidiphilium angustrum,* respectively. Clones from water sample #3, YT_K12 and YT_K13 matched with *Acidithiobacillus ferrivorans* (similarity 99%), YT_K14 and YT_K16 matched with *Acidithiobacillus ferriphilus, Acidiphilium rubrum* (similarity 99%), respectively. The archaeal profile was only determined on AMD samples #1 and #3. According to the results of sequence analysis the clones YT_K11, and YT_K20 matched with *Ferroplasma acidiphilium* (similarity 99%), and clone YT_K9 showed 99% similarities with Thermoplasmatales archaeon. Other clones from water samples #4 and #6 showed 99% similarities with *Acidiphilium* sp. *and Thiomonas* sp. The sequencing results of the clones were given in Table 2.

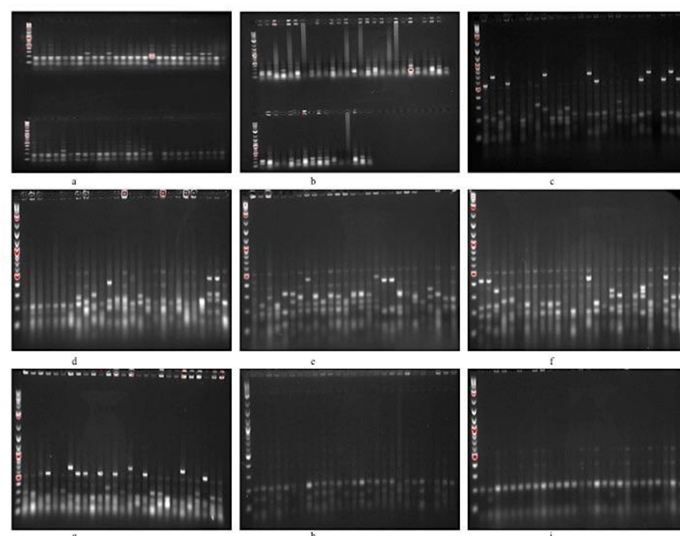

Figure 3 ARDRA profiles of water samples (for bacteria: a, b, c, d, e, f, g: #1, #2 #3, #4, #5, #6; for archaea: h, i: #1, #3 respectively).

Table 2 Prokaryotes16S rRNA gene clones of environmental water samples and their closest matches in GenBank

Clone no	GenBank accession no	Organism	Water sample no	16S rRNA Gene Sequence Similarity (%)	Number of sequencing base
YT_K1	MH057124	Uncultured bacterium partial 16S rRNA gene, clone BioPlate3_A12 HE587229.1	1	99%	754
YT_K2	MH057125	Uncultured archaeon gene for 16S rRNA, partial sequence, clone: HO28S9A63 AB600346.1	1	97%	650
YT_K3	MH057126	*Acidiphilium* sp. DBS4-1 16S ribosomal RNA gene, partial sequence EU003879.1	2	99%	1004
YT_K4	MH057127	*Acidithiobacillus ferriphilus* strain DSM 100412 tyrosyl-tRNA synthetase gene, complete cds; KY002491.1	2	99%	850
YT_K7	MH057128	*Acidocella* sp. M21 16S ribosomal RNA gene, partial sequence AY765998.1	2	99%	854
YT_K8	MH057129	*Acidiphilium angustum* strain Colony6 16S ribosomal RNA gene, partial sequence KC924944.1	2	99%	873
YT_K9	MH057130	Uncultured Thermoplasmatales archaeon clone B_DKE 16S ribosomal RNA gene, partial sequence KY825129.1	3	99%	546
YT_K10	MH057131	Uncultured archaeon clone AMD-archD26 16S ribosomal RNA gene sequence KC537536.1	3	98%	941
YT_K11	MH057132	*Ferroplasma acidiphilum* strain Y, complete genome CP015363.1	3	99%	809
YT_K12	MH057133	*Acidithiobacillus ferrivorans* strain NO-37 16S ribosomal RNA gene, partial sequence NR_114620.1	3	99%	1024
YT_K13	MH057134	Acidithiobacillus ferrivorans SS3, complete genome CP002985.1	3	99%	704
YT_K14	MH057135	*Acidithiobacillus ferriphilus* strain DSM 100412 tyrosyl-tRNA synthetase gene, complete cds; KY002491.1	3	99%	1010
YT_K16	MH057136	*Acidiphilium rubrum* strain Colony11 16S ribosomal RNA gene, partial sequence KC924949.1	3	99%	1016
YT_K17	MH057137	Uncultured *Thiomonas* sp. clone dw10 16S ribosomal RNA gene, partial sequence KF287769.1	4	99%	865
YT_K18	MH057138	Uncultured bacterium clone RT11-ant10-e08-W 16S ribosomal RNA gene, partial sequence JF737920.1	4	100%	862
YT_K20	MH057139	*Ferroplasma acidiphilum* strain DX-m 16S ribosomal RNA gene, partial sequence KX694511.1	3	99%	747
YT_K21	MH057140	Uncultured bacterium clone NSC0m-bac_d12 16S ribosomal RNA gene, partial sequence KC619550.1	4	98%	671
YT_K23	MH057141	Uncultured bacterium partial 16S rRNA gene, clone BioPlate2_H11 HE587131.1	4	95%	894
YT_K25	MH057142	*Acidiphilium multivorum* strain AIU301 16S ribosomal RNA gene, complete sequence NR_074327.1	4	99%	877
YT_K27	MH057143	Uncultured bacterium clone LRE22B44 16S ribosomal RNA gene, partial sequence HQ420129.1	4	99%	963
YT_K28	MH057144	*Acidiphilium cryptum* JF-5, complete genome CP000697.1	4	99%	824
YT_K29	MH057145	Uncultured bacterium clone A13 16S ribosomal RNA gene, partial sequence KF031176.1	5	98%	805
YT_K30	MH057146	Uncultured alpha proteobacterium clone AKYG835 16S ribosomal RNA gene, partial sequence AY922070.1	5	99%	809
YT_K31	MH057147	Uncultured bacterium clone BE326_BF2_otu4 16S ribosomal RNA gene, partial sequence JX298447.1	5	96%	848
YT_K32	MH057148	Uncultured bacterium clone BDP28WS24 16S ribosomal RNA gene, partial sequence KF841202.1	5	98%	668
YT_K33	MH057149	Uncultured bacterium clone T7-82 16S ribosomal RNA gene, partial sequence GQ487952.1	5	99%	857
YT_K34	MH057150	Uncultured beta proteobacterium clone 220T36 16S ribosomal RNA gene, partial sequence DQ110071.1	5	98%	816
YT_K35	MH057151	Uncultured bacterium partial 16S rRNA gene, clone Iron-rich microbial mat clone Hoffnungsstollen_#5-1A_E11 LN870830.1	5	96%	726
YT_K36	MH057152	Uncultured bacterium clone SX2-12 16S ribosomal RNA gene, partial sequence DQ469219.1	5	99%	858
YT_K37	MH057153	Uncultured bacterium clone EPS09_OK_001A_57 16S ribosomal RNA gene, partial sequence JX521231.1	5	99%	838
YT_K38	MH057154	Uncultured bacterium clone F-19 16S ribosomal RNA gene, partial sequence HQ132424.1	5	97%	702
YT_K40	MH057155	Uncultured bacterium clone SH201209-31 16S ribosomal RNA gene, partial sequence KX508591.1	5	98%	646
YT_K41	MH057156	Uncultured bacterium clone SX2-10 16S ribosomal RNA gene, partial sequence DQ469201.1	5	99%	752
YT_K42	MH057157	Uncultured bacterium clone AMD1-Plate1-B06 16S ribosomal RNA gene, partial sequence JN127499.1	5	99%	455
YT_K43	MH057158	Uncultured bacterium clone BCWCWP1A42 16S ribosomal RNA gene, partial sequence FJ598380.1	5	99%	925
YT_K44	MH057159	*Acidiphilium* sp. BGR 75a 16S ribosomal RNA gene, partial sequence GU167999.1	6	99%	843
YT_K45	MH057160	Uncultured *Thiomonas* sp. clone S-K6-C18 16S ribosomal RNA gene, partial sequence EF612428.1	6	99%	878
YT_K46	MH057161	Uncultured bacterium partial 16S rRNA gene, clone BioPlate2_G10 HE587210.1	6	99%	798
YT_K47	MH057162	Uncultured bacterium partial 16S rRNA gene, clone BioPlate2_A10 HE587139.1	6	99%	827

DGGE

DGGE analyses were performed with each different sample to determine the level of microbial diversity in the mine area. (Fig 4). The samples collected from the same sample points determined to have different profiles. Especially, due to pH change, it was found that bacterial diversity quite different sample #5 and sample #2. Blast analyses of DGGE bands sequences are given in Table 3. Archaeal diversity was determined only in sample #1 and sample #3. The sequence of bands YT_D1, YT_D2, YT_D3, YT_D4, and YT_D5 showed similarity with uncultured archaeon clones, as a show that in Table 3. Differences were observed in the DGGE profiles of taken water samples at different times from the same sampling points. It was determined that the sample #4 have more bacterial diversity from sample #1. According to sequence analysis results, there are bands matched with *Ferrovum myxofaciens, Acidithiobacillus ferrooxidans, Acidithiobacillus ferrivorans, Acidithiobacillus ferriphilus* and uncultured *Acidithiobacillus* sp. in sample #1. In the case of water sample #4, it was determined that the majority of the species are *Acidocella* (Table 3). Although samples #2 and #5 were taken from the same spot, it was thought that the change in pH at sample #5 caused the formation of different profiles. Bands at sample #5 were showed similarity with *Acidocella aluminiidurans,* uncultured *Acidocella* sp. and uncultured *Acidiphilium* sp. In sample #3, bacterial diversity is less than in sample #6. The band of sample #6, it was determined to match with *Thiomonas* sp. (Table 3).

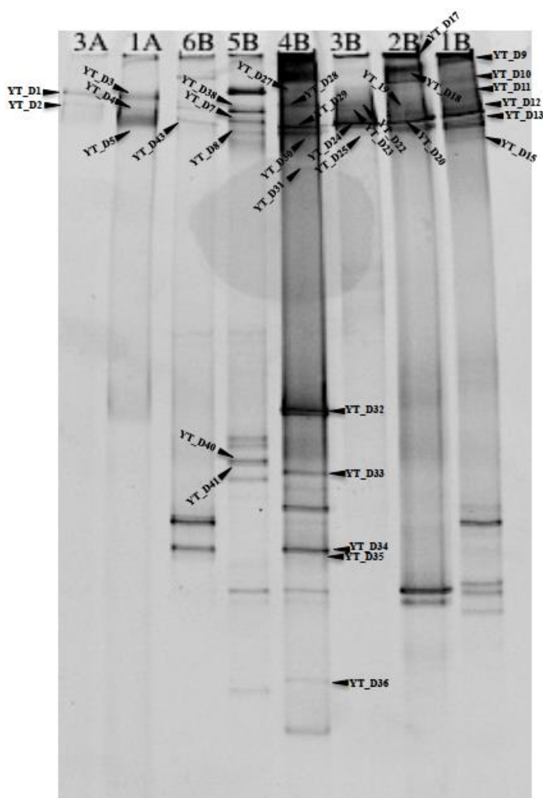

Figure 4 DGGE profiles of the AMD water samples.

Accession numbers and construction phylogenetic tree of nucleotide sequences

16S rRNA gene sequences were deposited in GenBank under accession numbers MH057089-H057162. In order to determine the phylogenetic group, the phylogenetic tree was constructed with sequences obtained by 16 rRNA clone library and DGGE analyses (Fig 5, 6).

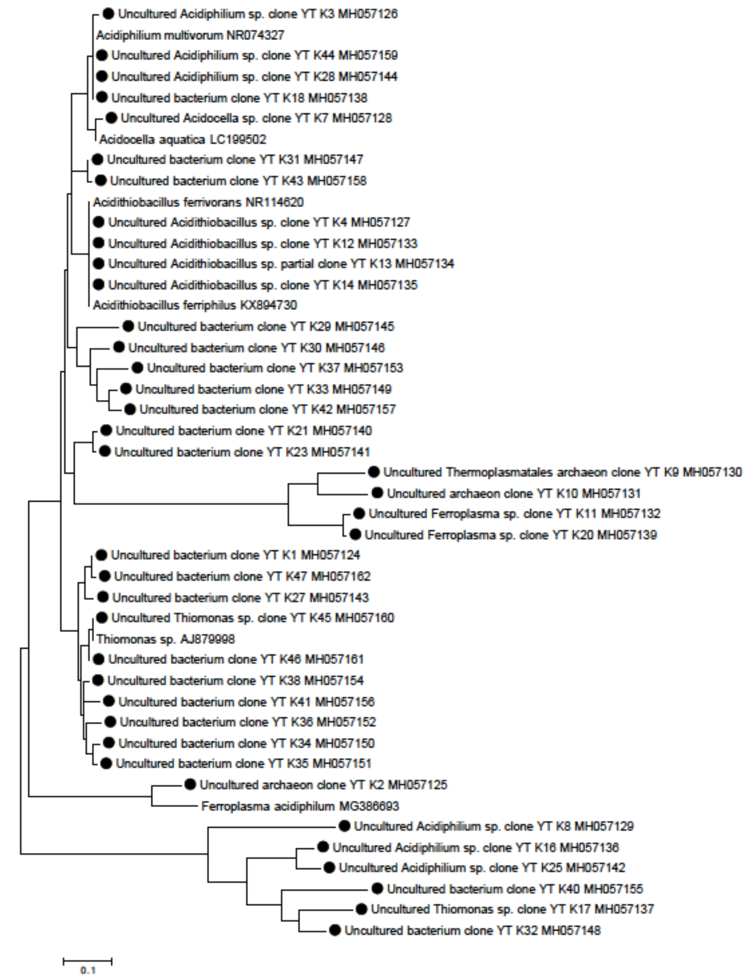

Figure 5 Phylogenetic tree based on 16S rRNA gene sequences of 16S rRNA clone libraries.

Table 3 Prokaryotes of environmental water samples closest matches of DGGE bands in GenBank

Clone no	GenBank accession no	Organism	Water sample no	16S rRNA Gene Sequence Similarity (%)	Number of sequencing base
YT_D1	MH057089	Uncultured archaeon clone AMD-archI04 16S ribosomal RNA gene sequence KC537677.1	3	99%	520
YT_D2	MH057090	Uncultured Thermoplasmatales archaeon clone B_DKE 16S ribosomal RNA gene, partial sequence KY825129.1	3	100%	437
YT_D3	MH057091	Uncultured euryarchaeote clone RT10A_3A_4 16S ribosomal RNA gene, partial sequence EF441876.1	1	99%	457
YT_D4	MH057092	Uncultured archaeon clone AMD-archF17 16S ribosomal RNA gene sequence KC537589.1	1	99%	449
YT_D5	MH057093	Uncultured archaeon clone AMD-archE05 16S ribosomal RNA gene sequence KC537545.1	1	99%	458
YT_D7	MH057094	Uncultured *Acidiphilium* sp. partial 16S rRNA gene, clone A4_89 AM940514.1	5	93%	542
YT_D8	MH057095	Uncultured *Acidocella* sp. clone M3O29 16S ribosomal RNA gene, partial sequence JX568779.1	5	94%	519
YT_D9	MH057096	*Ferrovum myxofaciens* strain P3G 16S ribosomal RNA gene, partial sequence NR_117782.1	1	97%	477
YT_D10	MH057097	*Acidithiobacillus ferrooxidans* strain S1 16S ribosomal RNA gene, partial sequence FJ913262.1	1	95%	465
YT_D11	MH057098	*Acidithiobacillus ferriphilus* strain M20 16S ribosomal RNA, partial sequence NR_147744.1	1	98%	308
YT_D12	MH057099	*Acidithiobacillus ferrivorans* strain NO-37 16S ribosomal RNA gene, partial sequence NR_114620.1	1	99%	297
YT_D13	MH057100	*Acidithiobacillus* sp. YB18 16S ribosomal RNA gene, partial sequence KM369916.1	1	99%	443
YT_D15	MH057101	*Ferrovum myxofaciens* strain EHS8 16S ribosomal RNA gene, partial sequence KC155322.1	1	99%	522
YT_D17	MH057102	*Acidocella* sp. CFR23 16S ribosomal RNA gene, partial sequence KC662252.1	2	98%	529
YT_D18	MH057103	Uncultured *Acidocella* sp. clone M3O29 16S ribosomal RNA gene, partial sequence JX568779.1	2	95%	515
YT_D19	MH057104	*Acidiphilium* sp. strain MPLK-302 16S ribosomal RNA gene, partial sequence KX689773.1	2	93%	542
YT_D20	MH057105	Uncultured *Acidocella* sp. clone M3O29 16S ribosomal RNA gene, partial sequence JX568779.1	2	93%	511
YT_D22	MH057106	*Acidocella aquatica* gene for 16S ribosomal RNA, partial sequence LC199502.1	3	93%	562
YT_D23	MH057107	Acidocella facilis strain PW2 16S ribosomal RNA gene, partial sequence NR_025852.1	3	95%	520
YT_D24	MH057108	Acidiphilium sp. strain MPLK-302 16S ribosomal RNA gene, partial sequence KX689773.1	3	91%	532
YT_D25	MH057109	Acidocella facilis strain PW2 16S ribosomal RNA gene, partial sequence NR_025852.1	3	86%	523
YT_D27	MH057110	Uncultured Acidiphilium sp. partial 16S rRNA gene, clone A4_89 AM940514.1	4	93%	544
YT_D28	MH057111	*Acidocella aluminiidurans* strain AL46 16S ribosomal RNA gene, partial sequence NR_112716.1	4	97%	546
YT_D29	MH057112	Acidiphilium angustum strain KLB 16S ribosomal RNA gene, partial sequence NR_025850.1	4	91%	523
YT_D30	MH057113	Uncultured Acidocella sp. clone G16O27 16S ribosomal RNA gene, partial sequence JX568562.1	4	95%	533
YT_D31	MH057114	Uncultured Acidocella sp. clone M2C26 16S ribosomal RNA gene, partial sequence JX568698.1	4	97%	519
YT_D32	MH057115	Uncultured Acidocella sp. clone M3O29 16S ribosomal RNA gene, partial sequence JX568779.1	4	93%	525
YT_D33	MH057116	*Acidiphilium multivorum* strain AIU301 16S ribosomal RNA, complete sequence NR_074327.1	4	94%	513
YT_D34	MH057117	Acidocella aminolytica strain 101 16S ribosomal RNA gene, partial sequence NR_025849.1	4	91%	481
YT_D35	MH057118	Uncultured alpha proteobacterium clone LKC_Acid_38p 16S ribosomal RNA gene, partial sequence EU038054.1	4	87%	534
YT_D36	MH057119	Acidocella facilis strain PW2 16S ribosomal RNA gene, partial sequence NR_025852.1	4	93%	505
YT_D38	MH057120	Acidocella aluminiidurans strain NBRC 104303 16S ribosomal RNA gene, partial sequence NR_114266.1	5	95%	514
YT_D40	MH057121	Acidocella sp. strain MPLK-72 16S ribosomal RNA gene, partial sequence KX689753.1	5	93%	517
YT_D41	MH057122	Uncultured alpha proteobacterium gene for 16S ribosomal RNA, partial sequence, clone: AN037 AB809968.1	5	97%	519
YT_D43	MH057123	*Thiomonas* sp. Dg-E17 partial 16S rRNA gene, isolate Dg-E17 LN864672.1	6	96%	521

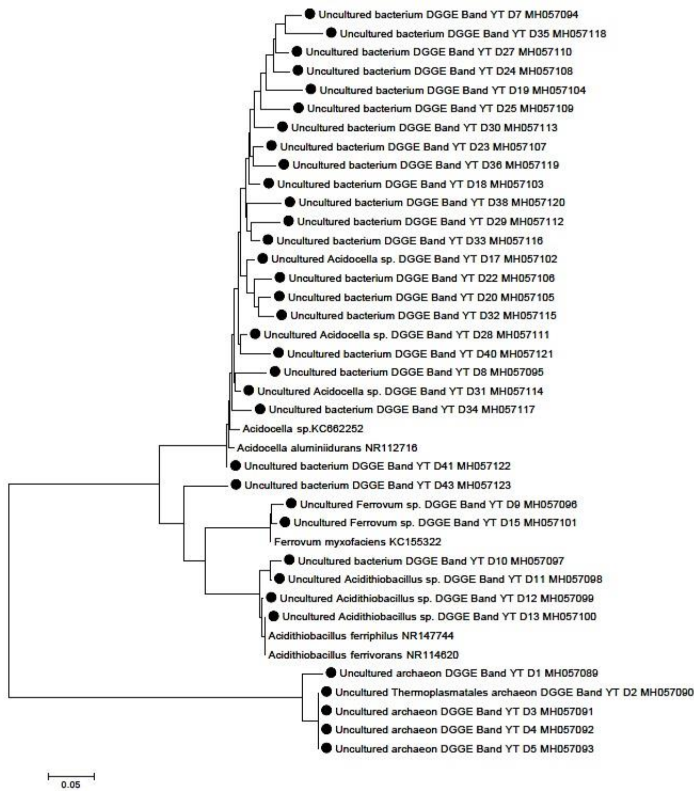

Figure 6 Phylogenetic tree based on 16S rRNA gene sequences of DGGE bands.

DISCUSSION

Determining of communities in AMD provides important clues in terms of the diversity and functions of these organisms with the development of molecular approaches. Furthermore, the development of sequencing technologies is rare in acidic environments allowing the identification of numerous taxa found **(Kuang *et al*., 2012; Aliaga Goltsman *et al*., 2014)**.

The pH value and metal concentrations of the AMD ponds seem to be suitable for existence in the determined species. Especially, high iron concentration is determinant for the life of species such as *Acidithiobacillus ferrivorans, Aciditihobacillus ferriphilus.* Mendez and coworkers have determined *Acidithiobacillus ferrivorans* with similar properties samples (pH 2.7, Fe: 38.100 mg kg $^{-1}$) **(Mendez *et al*., 2008)**. One of the sequences identified in the study of microbial diversity of Xiang Mountain sulfide mine was matched with *Ferrovum myxofaciens,* which was recently isolated from an abandoned copper mine (pH 3.0, Fe: 100.6 mg L^{-1}) **(Hao *et al*., 2010)**. *Acidiphilium* sp. was determined by community composition analysis in acid mine drainage from Fankou Pb/Zn mine, China (pH 1.9, Fe 1240 mg L^{-1}) **(Chen *et al*., 2014,a)**.

Autotrophic and heterotrophic groups were identified from selected sample points in this study. It is noteworthy that archaea domain members cannot be determined from samples #4 and #6 in June 2015, while the archaea were determined in March 2014 (samples #1 and #3). It is thought that this may be due to the continuing effects of the mining activity. Changes in the environment created by anthropogenic effects are rapidly affecting microbial diversity. *Ferroplasma* spp., which was identified in this study, has been determined as dominant after the period of the acidification processes of mine wastes **(Chen *et al*., 2013; Chen *et al*., 2014,b)**.

As seen in Figure 7, the variety of prokaryotic diversity was observed by used molecular techniques in AMD ponds. As one of the reasons for this, some of the technical difficulties of the methods used can be shown. While sequencing over a short region of DNA by the DGGE method, the cloning longer base chain can be evaluated. With all of these drawbacks, both methods complement each other's deficiencies so that can determine the prokaryotic diversity of AMD ponds to a significant extent. For example, *Ferroplasma* sp., *Thiomonas* sp. species could be identified only by 16S cloning, while *Ferrovum* sp. could be identified only by DGGE method. Other less frequently detected bacterial taxa the heterotrophic growers *Thiomonas* spp. **(Chen *et al*., 2016)** that has been isolated was not detected by molecular techniques, probably reflecting its low abundance **(Bruneel *et al*., 2005)**.

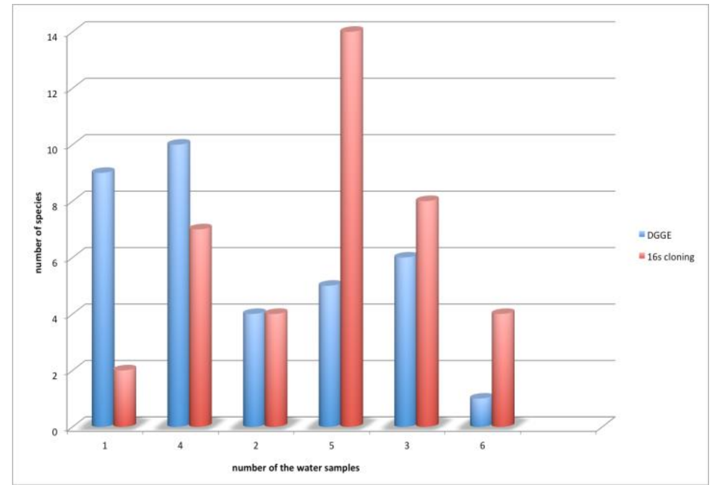

Figure 7 The diversity profile of AMD ponds obtained by 16S rRNA clone libraries and DGGE methods.

Gonzalez-Toril and collaborates studied by DGGE using 16S rRNA and, by 16S rRNA gene amplification for research molecular ecology an extreme acidic Tinto River (Spain). Comparative sequence analysis of DGGE bands determined the entity of the respective microorganisms like *Leptospirillum* spp., *Acidithiobacillus ferrooxidans, Acidiphilium* spp., *Ferrimicrobium acidiphilum, Ferroplasma acidiphilum*, and *Thermoplasma acidophilum* **(Gonzalez-Toril *et al*., 2003)**. Aytar and coworkers have identified prokaryotic diversity in two different AMD sites (Balya and Çan) in Turkey **(Aytar *et al*., 2014)**. Some species identified in this study were as *Acidithiobacillus* sp., *Leptosipirillum* sp., *Ferooplasma* sp. Saglam and colleagues determined bacterial diversity in the Acisu effluent with cloning 16s rRNA sequences. The bacterial population was identified to occur *Acidithiobacillus ferrivorans, Ferrovum myxofaciens, Leptospirillum ferrooxidans*, *Acidithiobacillus ferrooxidans, Acidocella facilis, Acidocella aluminiidurans, Acidiphilium cryptum, Acidiphilium multivorum, Acidithiobacillus ferrivorans, Acidithiobacillus ferrooxidans, Acidithiobacillus thiooxidans*, *Acidiphilium cryptum* **(Saglam *et al*., 2016)**.

Alphaproteobacteria *(Acidiphilium, Acidocella)* **(Liu *et al*., 2011; Falagan *et al*., 2013)**, Betaproteobacteria *(Thiomonas, Ferrovum),* **(Mendez *et al*., 2008; Johnson *et al*., 2013)**, Acidithiobacillia *(Acidithiobacillus)* **(Williams and Kelly, 2013)** are seen in other studies. *Betaproteobacteria*, mostly belonging to the '*Ferrovum*' genus, was clearly predominant in the community below middle pH conditions, whereas Alphaproteobacteria, Euryarchaeota, Gammaproteobacteria, and *Nitrospira* exposed a powerful adaptation to more acidic conditions **(Kuang *et al*., 2012)**.

As a result of matches, it has been determined that some sequences are similar to those of *Aciditihobacillus ferriphilus* **(Nunez *et al*., 2017)**, *Acidiphilium angustum,* and *Acidiphilium rubrum* **(Auld *et al*., 2013)**. These species are new records for Turkey. The iron-oxidizing acidithiobacilli *Acidithiobacillus ferriphilus* was also isolated from different global locations such as metal-rich waters sample deep within the mine **(Kay *et al*., 2014)**. The type strain $M20^T$ was isolated from a pond in a geothermal area of Montserrat (pH 1.5-3.0) (West Indies) which lived optimally pH 2.0 and 30 °C of temperature **(Atkinson *et al*., 2000)**. It was determined later that this strain separated from other acidithiobacilli **(Falagan and Johnson, 2016)**. The clones and DGGE bands sequences showed to match the most *Acidiphilium* genus. The mesophilic and obligately acidophilic bacteria *Acidiphilium angustum* grow in the pH range of 2.0-5.9. The clone YT_K8 matched with *Acidiphilium angustum* (99% similarity) was obtained from water sample #2 (pH 2.9). Auld and coworkers have isolated *Acidiphilium rubrum* from an AMD site in Copper Cliff, Ontario **(Auld *et al*., 2013)**. The isolate was isolated from AMD water at pH 2.5, similar to the water sample (pH 2.7) in which identified YT_K16.

CONCLUSION

Future studies will focus on the roles of these species on the biogeochemical cycles of the region where microbial diversity is determined. The AMD is also likely to contain new species. To better understand community dynamics in acid formation, more studies are needed to identify predominant species in AMD environments.

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