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Diversity of microfungi in acid mine drainages

Bükay YENICE GÜRSU [*1], Pınar AYTAR[2], Semra İLHAN[3], Yaşar Erçin KOCABIYIK[4], Serap GEDIKLI[4], Ahmet ÇABUK[3]

[1] Eskisehir Osmangazi University; Central Research Laboratory Application and Research Center (ARUM) 26480, Odunpazari, Eskisehir-Turkey
[2]Department of Biotechnology and Biosafety, Graduate School of Natural and Applied Sciences, Eskişehir Osmangazi University, 26480, Eskişehir, Turkey
[3]Department of Biology, Faculty of Arts and Science, Eskişehir Osmangazi University, 26480 Eskişehir, Turkey
[4]Graduate School of Natural and Applied Sciences, Eskişehir Osmangazi University, 26480 Eskişehir, Turkey

Abstract

Acid mine drainage (AMD) is an anthropogenic system having lower acidic pH value and high specific conductivity. In this study, from two different acidic environments, the AMDs of Çan (Çanakkale) (pH 2.85) and of Balya (Balıkesir) (pH 2.75) the microfungi was isolated and identification. Morphological and molecular techniques were used to identify microfungi. Then fifteen species belonging to nine genera were identified. They are listed as follows: *Aspergillus awamori, A. repens, Cladosporium herbarum, C. oxysporum, Penicillium citrinum, P. montanense, P. ochrochloron, P. spinulosum, P. verrucosum, Penidiella sp., Phialophora sp., Talaromyces aculeatus, T. helicus, Trichoderma harzianum,* ve *Umbelopsis autotrophica.* Most of these species have been commonly found in acidic and/or heavy metal-rich environments. Consequently, such isolates from AMDs, which represents an extreme environment, are important because of revealing the fungal diversity and their potential biotechnological applications.

Key words: molecular ecology, community structure, acidic environment, fungal diversity

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Asit maden drenajlarında mikrofungus çeşitliliği

Özet

Asit maden drenajı (AMD), daha düşük asidik pH değerine ve yüksek spesifik iletkenliğe sahip olan bir antropojenik sistemdir. Bu çalışmada, Çanakkale (pH 2.85) ve Balya (Balıkesir) (pH 2.75) AMD'leri olmak üzere iki farklı asidik ortamdan mikrofunguslar izole edildi ve tanılandı. Asidik maden drenaj sularından izole edilen mikrofungusları belirlemek için morfolojik ve moleküler teknikler kullanılmış ve listelendiği gibi dokuz cinse ait on beş tür tespit edilmiştir; *Aspergillus awamori, A. repens, Cladosporium herbarum, C. oxysporum, Penicillium citrinum, P. montanense, P. ochrochloron, P. spinulosum, P. verrucosum, Penidiella sp., Phialophora sp., Talaromyces aculeatus, T. helicus, Trichoderma harzianum,* ve *Umbelopsis autotrophica.* Bu türlerin çoğu asidik ve/veya ağır metal açısından zengin ortamlarda bulunur. Sonuç olarak, ekstrem çevreyi temsil eden AMD'lerinden elde edilen bu izolatlar bu ortamlardaki fungus çeşitliliğinin ortaya çıkarılması ve potansiyel biyoteknolojik uygulamaları nedeniyle önemlidir.

Anahtar kelimeler: moleküler ekoloji, komünite yapısı, asidik ortam, mantar çeşitliliği

1. Introduction

Mines contain chemolithotrophic microbial communities that are sustained by energy derived from pyrite (FeS_2) oxidation. AMD contains limited organic material, these organisms serve as a source of energy and carbon for

* *Corresponding author* / Haberleşmeden sorumlu yazar: Tel.: +902222393750/6415; Fax.: +902222393578; E-mail: bukayyenice@gmail.com
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organotrophic organisms, such as bacteria and fungi. In addition, mining increases the surface area of sulfide ores exposed to air and water, thus, increases rate of acid generation. Consequently, acid mine drainage (AMD), which has low acidic pH values and high specific conductivity, is produced when sulfide-bearing material is exposed to oxygen and water.

Despite the harsh conditions, a diverse range of microorganisms populates in AMD environments. According to the literature, some acidophilic organisms have been isolated from AMDs. Earlier studies of the microbiology of AMD systems have focused almost exclusively on the prokaryotic populations for biotechnological reasons, such as bioleaching, metal bioaccumulation and biodesulphurization (Das et al.,2009; Rawlings and Johnson, 2007). By contrast, although fungi and protista are almost omnipresent in AMD, relatively few studies have reported on their isolation, identification, systematic and roles (Brake et al., 2002; Gould et al., 1974; Gross and Robbins, 2000).

Fungi are omnipresent in every ecological niche and are necessary for the breakdown and recycling of organic materials.Fungal diversity is typically the highest in tropical regions, primarily in tropical forests (Hawksworth, 2001; Mueller and Schmit, 2007). In Turkey, fungal studies have concentrated on agricultural and polluted soils, as well as indoor /outdoor environments (Asan, 2004). However, knowledge about the fungal diversity of extreme environments in Turkey is limited.

The isolation of microorganisms from extreme environments, such as acid mine drainage, is important for understanding both biodiversity and alternative metabolic pathways for biotechnological application. As is known, many fungi can adapt to extreme environments. The presence of microfungi in AMD has been reported previously (Cooke, 1976). Two extremely acidophilic (pH 1.0) and metal-tolerant mitosporic fungi, *Acontium velatum* and *Scytalidium acidophilum* were isolated (Starkey and Waksman, 1943). Besides, Raper and Thom reported that the isolates of *P. ochrochloron* had been isolated from copper sulphate or sulphuric acid solutions from textile industry (Raper and Thom, 1949). They concluded that although such environments apparently were selective for the species it was probably soil organisms. Also, Fukami et al., (1983) reported that a large portion of Cu^{2+} taken up by *P. ochrochloron* was accumulated in the cells walls. Bååth et al., (1984) studied the effect of experimental acidification on the soil microfungal community. The result is that the abundance of *P. spinulosum* increased with increasing rates of acid application. *P. montanense* was isolated from saline and extremely acidic soils (Hujslová et al., 2010). In other a study, *Phialophora* sp. was isolated from acidic tin mine wastewater (Zhang et al., 2011).

Today, AMD is a very important and common environmental problem for all organisms especialy plants (Prasad and Jeeva, 2009). Microbe-mineral interactions have great importance, because microorganisms are biological systems to the clean-up of organic and inorganic pollution. The microorganisms isolated from AMD samples can be used in bioremediation processes, such as biosorption and biodesulfurization and as a source of novel biomolecules for industrial processes (Aytar et al., 2013; Çabuk et al., 2013). Such studies have tremendous significance as knowledge of the science and technology of bioremediation, in terms of the roles of microorganisms, including fungi.

The present study describes the isolation of culturable filamentous microfungi from two AMDs with different properties: the Pb-Zn-Ag deposits in Balya and the coal mine in Çan-Turkey. To identify microfungi isolated from these AMDs, morphological and molecular techniques were used. Fungal micro and macro morphology were used to narrow down the possible taxonomic assignment. The final decision was then based on molecular systematics.

2. Materials and methods

2.1. Site description

The Balya Pb-Zn-Ag deposit and associated mine tailings are found in Northwest Anatolia, in Balya province of Balıkesir, Turkey (Figure 1-2). It is the broadest Pb-Zn mine in the area. The Balya Pb-Zn deposit was exploited from the early 1880s to the late 1940s. Pb-Zn mine activities in Balya (Balıkesir province) have since been continued for two years. The ore contains pyrite, marcasite, sphalerite, galena, chalcopyrite, and arsenopyrite as the major ore minerals (Akyol, 2012).

The Çan Basin is located in the Biga Peninsula (northwest Turkey) and it is one of the most significant natural parks in Turkey (Figure 1-2), yet mining activities for coal, gold, and industrial minerals have been operating in this area for many years. The coal has a high sulfur content of between 3 and 8 % (Baba et al., 2008). The sulphur-rich Can coals and the morphology of the basin (half-closed basin) are important parameters for the basin ecosystem. Because of both surface discharge and underground leakage into abandoned open pit coal mines, artificial lakes have occurred. Especially, the weathering product of sulfide oxidation is the construction of iron hydroxide, thereby a red/orange-colored precipitate of streams affected by AMD (Yucel et al., 2014). At that time, the coal basin was nationalized and is now operated by Turkish Coal Enterprises. The coordinates of the sample collection sites and some physicochemical parameters are given in Table 1.

2.2. Physicochemical analysis

Physicochemical conditions were determined using portable instrumentation during sampling. Briefly, the pH and temperature of the AMD pond water were measured in situ using a combined pH and temperature meter (WTW, Germany) and the dissolved oxygen concentrations were determined using a dissolved oxygen meter (WTW, Germany). To investigate microbial and chemical properties, water samples were collected in one-liter sterile bottles. All of the samples were stored at + 4°C until processing. Metal analyses of water samples from the AMD ponds were performed using a Perkin-Elmer 3100 inductively coupled plasma-atomic emission spectrometer (ICP-AES). The elemental composition of the given samples was quantified relative to a reference standard (Watson, 1994).

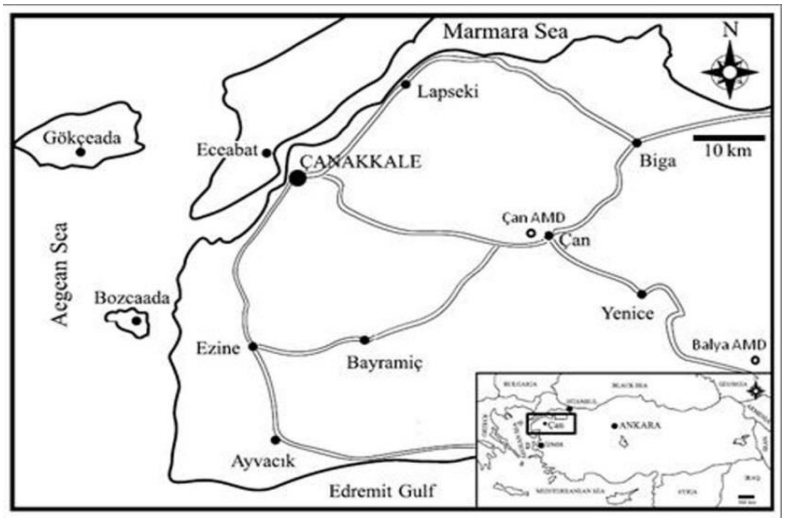

Figure 1. Detailed map showing Balikesir and Çanakkale Turkey where Balya and Çan AMDs ponds are located, respectively

a **b**

Figure 2**.** (a) A view from Balya AMD pond and (b). Çan AMD pond

2.*3. Isolation and characterization of fungi*

Water samples were collected in January 2010 and used for fungal isolation. The 20 ml aliquots of each water sample were filtered through a cellulose nitrate membrane filter (0.45 µm, Sartorius). Filters were placed onto six

different types of culture medium. Three of these media were general purpose; Dichloran Rose Bengal Chloramphenicol (DRBC) agar (Peptone, 5 g; D-Glucose, 10 g; KH_2PO_4, 1 g; $MgSO_4.H_2O$, 0.5 g; Dichloran, 0.002 g; Rose Bengal, 0.025 g; Agar, 15 g; pH 5.5–5.8, A_w > 0.95; Water, 1000 ml), Dichloran 18% Glycerol agar (DG-18) (Glucose, 10 g; Peptone, 5 g; KH_2PO_4, 1 g; $MgSO_4.7H_2O$, 0.5 g; Glycerol, 220 g; Agar, 15 g; Dichloran, 0.002 g; Chloramphenicol, 0.1 g; Water, 1000 ml; pH 5.5–5.8; A_w 0.955), and Malt Extract Agar (MEA) (Malt extract, 20 g; Glucose, 20 g; Mycological peptone, 1 g; Agar, 20 g pH 5.5). The other three medium was selective: MEACW (MEA prepared with Çan Water) and MEABW (MEA prepared with Balya Water) was prepared using water from the same sample. MEA2.5 and MEA4.0 were prepared setting pH 2.5 and 4.0 of MEA, respectively. Plates were incubated 30 days at 25-28ºC. During incubation, emerged colonies were counted every 3, 5, 7, 14, and 30 days of incubation. For every medium three aliquots were filtered in parallel and the numbers of colony forming units (cfu) were calculated. The samples were processed under sterile conditions. The isolates were chosen based on dissimilarities in the colony characteristics and then purified and numbered according to the station and the isolation medium. Purified isolates were maintained on MEA slants.

Table 1. Coordinates and physicochemical parameters of locations selected

Sampling locations	**N (North) E (East)**	**pH**	**Temperature (°C)**	**Dissolved oxygen (mg l^{-1})**
Balya AMD (1th station)	N 39° 45. 814' E 27° 35. 564'	3.08	14.8	5.30
Balya AMD (2nd station)	N 39° 45. 833' E 27° 35. 605'	3.05	14.2	5.15
Çan AMD (1th station)	N 39° 56. 824' E 26° 52. 348'	2.81	12.8	4.94
Çan- AMD (2nd station)	N 39° 57. 785' E 26° 51. 573'	2.96	14.1	4.75

Isolated fungi were identified based on macro and micro morphological characteristics such as colony diameter, mycelia and revers color, exudate, soluble pigment, sclerotia, determinative features of sexual and asexual spores for example shape, size and surface texture, using standard taxonomic references (Barnett and Hunter, 1999; Domsch et al., 1993; Ellis, 1971; Klich, 2002; Pitt and Hocking, 2009; Samson et al., 2004). The isolated pure cultures were maintained as stock in the Department of Biology, ESOGU Eskişehir-Turkey.

2.4. DNA extraction and phylogenetic analysis

Fungal mycelia were grown in MEA broth medium and the genomic DNA of selected pure cultures was extracted using the CTAB protocol (Graham et al., 1994). The ITS1–5.8S ITS2 region was amplified using primers ITS1 and ITS4. Each PCR reaction of 50 µl was performed using the following final concentrations or total amounts; 1µl of template DNA, 1X PCR buffer, 1.5 mM MgCl2, 200 µM dNTP mixture, 0.2 µM of each primer and 1.25 units Taq Polymerase (NEB). The PCR thermal cycling program used for the primer pair ITS1 (5′TCC GTA GGT GAA CCT GCG G 3′) and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3′)was as follows: initial denaturation at 95°C for 4 min, followed by 35 cycles of 95°C for 30 sec, annealing at 57°C for 55 sec, extension at 68°C for 40 sec, and final extension at 68°C for 10 min (Applied Biosystems Veriti Thermal Cycler).

Sequencing in both directions was performed on an automated sequencer (IONTEK, Istanbul, Turkey) using primers ITS1 and ITS4. Sequence similarities were obtained using the BLAST tool from NCBI (Altschul et al., 1990). Blasted sequences listed with their closest Type material GenBank sequences to render the type speices similarities.

3. Results

3.1. Physicochemical parameters

The prevailing physicochemical parameters in four different locations within the Balya and Çan AMD ponds were determined. Table 1 listed the temperatures, pH values, and dissolved oxygen levels at the sample collection sites in the Balya and Çan AMD ponds.

Table 2 lists the concentrations of several ions present in the sample solutions from the Balya and Çan AMD ponds. The Çan AMD was slightly more acidic than the Balya AMD. In the samples of from both sites, the pH (2.81-3.08), temperature (ranging from 12.8 to 14.8°C) and dissolved oxygen (4.75-5.30 mg/l) values were similar, while the concentrations of some elements were different. In the Çan AMD, the concentrations of Al (152.2 mg/l), Cr (71.00 mg/l), and Cu (206.50 mg/l) were higher than those in the Balya AMD, however, in the Balya AMD, the concentrations of total Fe (296.50 mg/l), Mg (124.30 mg/l), Mn (60.50 mg/l) and Zn (421.60 mg/l) were higher. The concentrations of other metals were also similar in both AMD ponds.

Table 2**.** Chemical analysis of water samples

Metal	**Balya AMD**	**Çan AMD**
Sulphate (gl^{-1})	3.25	3.48
Total Fe (mgl^{-1})	296.50	95.38
Aluminum (mgl^{-1})	92.70	152.20
Zinc (mgl^{-1})	421.60	4.52
Manganese (mgl^{-1})	60.50	27.61
Copper (mgl^{-1})	15.04	206.50
Nickel (mgl^{-1})	0.35	0.52
Chromium (mgl^{-1})	0.14	71.00
Cobalt (mgl^{-1})	0.42	1.78
Magnesium (mgl^{-1})	124.30	75.11
Cadmium (μgl^{-1})	6.17	<0.005
Lead (μgl^{-1})	0.015	<9.4
Vanadium (μgl^{-1})	<17.0	<17.0
Molybdenum (μgl^{-1})	<10.7	<10.7

3.2. Isolation of microfungi from AMD pond samples

The total number of isolates was counted by adding the number of appeared colonies to emerge on each membrane filter. A total of 62 colonies were calculated. A total of 15 taxa, 2 genera level and 13 species belonging to 9 genera were identified. The stations and media from which these species isolated are given in Table 3. The most common species detected in the acidic water samples were *C. herbarum*, *P. verrucosum* and *T. aculeatus*, which accounted for 15%, 13% and 13% of the total number of isolates, respectively. Additionally, *C. herbarum*, *P. citrinum*, *T. aculeatus* and *T. helicus* were isolated from both the Balya and Çan AMD samples. Several species were isolated from only one of the two AMD water samples; for example, *A. awamori*, *P. montanense*, *P. ochrochloron* and *T. harzianum* were only isolated from Balya, while *A. repens*, *C. oxysporum*, *P. spinulosum*, *Penidiella sp.*, *Phialophora sp*, and *U. autotrophica* were isolated from Çan. Only two species belonging to *Aspergillus* genus, *A. awamori* and *A. repens*, was isolated while several species of *Penicillium* were identified, including *P. citrinum*, *P. montanense*, *P. ochrocloron*, *P. spinulosum*, and *P. verrucosum*. Exept *P. citrinum*, these species were isolated from only one of AMDs.

Among six different types of medium, the highest number of isolates and the highest species diversity were obtained on the DRBC medium (Figure 3). Regarding isolate number and species diversity, this medium was followed by DG-18, MEA2.5 and MEABW/CW.

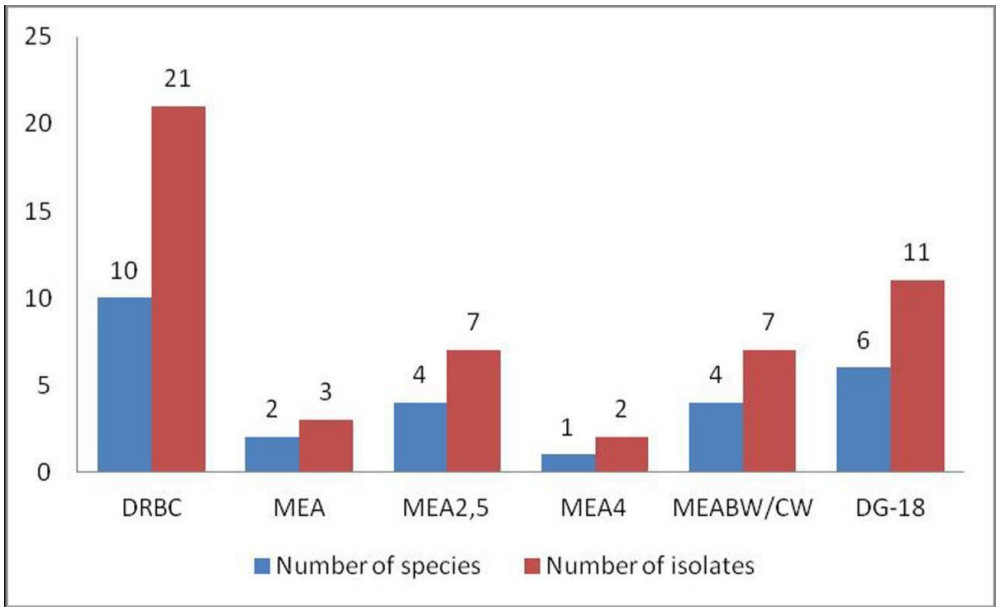

Figure 3. Distribution of the number of fungal isolate and species according to the typesof medium

Table 3. The species obtained from AMD samples and the sampling area and media from where these species were isolated

Species	**Number of isolate (cfu/total volume)**	**Media isolated**
Aspergillus awamori	AMDB(1)	DRBC
A. repens	AMDC(1)	DRBC
Cladosporium herbarum	AMDB(1), AMDC(8)	DG-18/MEABW/DRBC
C. oxysporum	AMDC(1)	DG-18
Penicillium citrinum	AMDB(1), AMDC(2)	DRBC/DG-18
P. montanense	AMDB(2)	MEA(2.5)/MEABW/MEA
P. ochrochloron	AMDB(4)	MEA(2.5)/DG-18
P. spinulosum	AMDC(3)	MEACW
P. verrucosum	AMDC(8)	DRBC/DG-18/MEA(2.5)
Penidiella sp.	AMDC(2)	DRBC
Phialophora sp.	AMDC(1)	MEACW
Talaromyces aculeatus	AMDB (1), AMDC (7)	MEA(2.5)/MEA/DRBC/MEACW
T. helicus	AMDB(6), AMDC(1)	MEA(2.5)/MEA/MEA(4.0)/DRBC
Trichoderma harzianum	AMDB(1)	DRBC
Umbelopsis autotrophica	AMDC(1)	DG-18
Unidentified filamentous fungi	Total (10)	All media

AMDB, Acid Mine Drainage Balya; AMDC: Acid Mine Drainage Çan, BW: Balya Water, CW: Çan Water DRBC, Dichloran Rose Bengal Chloramphenicol agar; DG-18, Dichloran 18% Glycerol agar; MEA, Malt Extract Agar; MEA(2.5), Malt Extract Agar adjusted pH 2.5; MEA(4.0), Malt Extract Agar adjusted pH 4.0; MEABW, Malt Extract Agar with Balya water; MEACW, Malt Extract Agar with Can water;

3.3. Phylogenetic relationships

A representative isolate was selected from the each of the isolate groups assigned according to the morphological characteristics. DNA sequence analysis was performed on the selected isolates. All of the isolates identified with the classical identification methods were confirmed using nucleotide sequences. As a result of this; *A. awamori* (AMDB-18), *C. herbarum* (AMDB-13), *C. oxysporum* (AMDC-12), *A. repens* (AMDC-6), *T. aculeatus* (AMDC-14), *P. citrinum* (AMDB-15), *P. montanense* (AMDB-8), *P. ochrochloron* (AMDB-12), *P. spinulosum* (AMDC-17), *P. verrucosum* (AMDC-9), *T. helicus* (AMDB-27), *T. harzianum* (AMDB-20) and *U. autotrophica*

(AMDC-18) were identified. *Penidiella sp.*(AMDC-8) and *Phialophora sp.*(AMDC-32) were identified to the genus level. The generated ITS sequences blasted on NCBI database to ensure the type speices similarities (Table 4).

With the exception of one isolate, all of the isolates belong to the division *Ascomycota. Umbelopsis* is a zygomyceteous fungus including the order *Mucorales.* These species were isolated from acid mine drainage and were related phylogenetically to species isolated from different environments.

Table 4**.** The generated ITS sequences with their closest type material sequences (according to GenBank data)

Species	Collection	Accession Number	Blast hit with type species (% identity/%coverage)	Type Species Accession Number
Aspergillus awamori	AMDB-18	KF588639	*Aspergillus awamori* ATCC 16877T (99/100)	NR_077143
A. repens	AMDC-6	KF588642	*A. pseudoglaucus* NRRL 40 (99/96)	NR_135336
Cladosporium herbarum	AMDB-13	KF588637	*Cladosporium herbarum* CBS:121621 (99/91)	NR_119656
C. oxysporum	AMDC-12	KF588645	*C. oxysporum* CBS 125991(99/96)	HM148118
Penicillium citrinum	AMDB-15	KF588638	*Penicillium citrinum* NRRL 1841(91/79)	NR_121224
P. montanense	AMDB-8	KF588635	*P. montanense* NRRL 3407(99/91)	NR_138270
P. ochrochloron	AMDB12	KF588636	*P. ochrochloron* CBS 357.48(99/93)	NR_111509
P. spinulosum	AMDC-17	KF588647	*P. spinulosum* FRR 1750(99/97)	NR_077158
P. verrucosum	AMDC-9	KF588644	*P. verrucosum* FRR 965(99/99)	NR_119495
Penidiella sp.	AMDC-8	KF588643	*Penidiella aggregata* CBS 128772(86/97)	NR_137772
Talaromyces helicus	AMDB-27	KF588641	*Talaromyces helicus* NRRL 2106(99/100)	AF033396
Talaromyces aculeatus	AMDC-14	KF588646	*Talaromyces aculeatus* CBS 289.48(98/100)	NR_103679
Trichoderma harzianum	AMDB-20	KF588640	*Trichoderma harzianum* CBS 226.95(98/100)	AY605713
Phialophora sp.	AMDC-32	KF588648	*Coniochaeta canina* UTHSC 11-2460 (92/97)	NR_120211
Umbelopsis autotrophica	AMDC-18	KF588649	*Umbelopsis autotrophica* CBS 310.93(99/91)	NR_111558

4. Conclusions and discussion

AMD communities that are low-complexity natural systems are characterized by a very limited number of distinct species that have probably the small number of metabolically beneficial reactions (Baker and Banfield, 2003). Although temperature, ionic composition, total organic carbon and dissolved oxygen are considered to significantly influence their microbial life, low-pH conditions also limits the diversity of microbial populations in these environments (Méndez-García et al., 2015). Procaryotic organisms are known to directly affect acid generation rates via the oxidation of iron and sulfur compounds. Most of studies on the microbiology of AMD systems have centered on these organisms. However, much less studied are the eukaryotic members of AMD communities. Especially fungi may impact the community structure and function by the consumption of organic waste products and the production of organic polymers, possibly including antibiotics but very few studies have focused on the overall fungal biodiversity of such environments and their potential ecological roles in these area remain poorly understood (Baker, et al. 2004). Despite the extreme acidity, variable temperature, and high concentrations of sulfate and toxic metals, fungal microorganisms can also populate AMD environments. Our findings are also consistent with previous results and variety fungal species have been isolated from these AMD areas (Baker and Banfield, 2003).

AMD microbial communities have classically been characterized by laboratory culturing-based techniques and more recently by direct sequencing of marker gene sequences. Traditionally, microbial diversity was also assayed through cultivation and species identification through morphology, selective media requirements, and physiological and biochemical traits l(Auld et al.,, 2013). However currently, culture independent methods are more commonly used than culture dependant methods. In this study, both traditional culturing methods and DNA sequencing techniques were used to characterize the fungal species isolated from an AMD sites in Çan and Balya, Turkey. According to our data, all of the isolates were inferred to be species previously associated with AMD by the traditional culture based methods. Six of the 15 species identified, including *C. herbarum, P. ochrochloron, P. verrucosum, Phialophora sp., T. aculeatus,* and *T. harzianum* have been identified previously in other acidic waters (Cooke, 1976; Gross and Robbins, 2000; Starkey and Waksman, 1943; Stokes and Lindsay, 1979). Identified *Penidiella* sp has also been isolated from acidic wastewater from uranium mine (He et al., 2011). *P. ochrochloron* was isolated from a plating solution containing 25% copper

sulphate and 7% sulfuric acid (Stokes and Lindsay, 1979). It has been reported that *P. ochrochloron* appears to be consistently associated with substrates containing high copper concentrations of up to 5000 ppm and that they grow at pH 2.0-8.0.

The pH values of the water samples from the Balya and Çan AMD ponds were between 2.81 and 3.08, while the copper and sulfate concentrations were between 15.04 and 206.50 g/l and 3.25 and 3.48 g/l, respectively (Table 1-2). These data also similar the results of the previous studies (ref). *C. herbarum* (9 cfu/ total volume), *P. verrucosum* (8 cfu/total volume), and *T. aculeatus* (8 cfu/total volume) accounted for 48% of the total number of species identified in the Balya and Çan AMDs. These species have been isolated in similar studies and were found to be highly abundant in those environments. Therefore, these results indicate that they are natural inhabitants of acidic mining environments, including AMDs. Every mine is unique in terms of its AMD composition; thus, the fungal biodiversity of AMDs can vary. In the present study, 15 species, 9 genera and several unidentified isolates were obtained (Figure 3). Eight species were isolated from the Balya AMD, while 11 species were isolated from the Çan AMD. Four fungal species (*C. herbarum, P. citrinum, T. aculeatus,* and *T. helicus*) were found in both the Balya and Çan sampling sites, while each of the other species were only isolated from one of the two sites. Most of the detected species belonged to *Cladosporium* and *Talaromyces* genera.

In our study, to understand the composition and structure of microbial communities in two different AMD environments, molecular diversities of 18S rRNA genes were also examined using a PCR-based cloning approach. This analysis identified 15 isolates in dominant genera: *Cladosporium, Talaromyces, Penicillium, Aspergillus, Trichoderma, Phialophora, Penidiella, Umbelopsis.* With the exception of one isolate, all of the isolates belong to the division *Ascomycota*. While *Aspergillus, Cladosporium, Penicillium* and *Trichoderma* are anamorphic, *Eurotium* and *Talaromyces* represent the teleomorphic state of ascomycetous fungi. Samson et al. (2011), by following the single name nomenclature to provide holomorphic generic diagnosis for the *Talaromyces*, transferred all accepted species of *Penicillium* subgenus *Biverticillium* to *Talaromyces* (Samson et al. 2011). Our resulted BLAST analysis showed that *Aspergillus repens* had 99% similarity to *A. repens* var. *pseudoglaucus*, synonym A. *pseudoglaucus* NRRL 40. *Penicillium citrinum* and type strain similarity was 91% and supported by the results of classic identification. *Penidiella* and *Phialophora* spp can be determined at the level of genus, so the similarity rates with type species wa 92% or less. Both two methods produced very similar results and we are able to culture the majority of the abundant species identified by direct sequencing.

In this study, six different types of medium were used for fungal isolation from AMD water samples (Table 3). The highest number of isolates and the highest diversity of species were obtained on the DRBC medium (Figure 3). Regarding isolate number and species diversity, this medium was followed by DG-18, MEA2.5 and MEABW/CW. These three media have also been used for the isolation of microfungi from various materials and environments, particularly extreme environments (Petrovic et al., 2000; Sonjak et al., 2006). It is remarkable that *T. aculeatus, P. spinulosum* and *Phialophora* sp. could also be isolated from MEA plates prepared with the water samples.

In the big picture the above mentioned that species may inhabit at acid mine drainage environments supporting by literature. The isolated other species represent fungi that are common in other non-acidic environments and they are probably originated from soils, waters, foods, and air. Identified species also exhibited growth on neutral media. This aspect needs to be further addressed such as acid tolerance test. However it seems that most of the species are acid-tolerant because of growing at very low pH.

This is the first study that documents the presence of fungal biodiversity in AMD (Balya and Çan) in Turkey). The number of total species that inhabit in acidic environments is unknown, and it is possible that the number varies depending on the properties of such habitats. Future work will concentrate on revealing other fungal inhabitants that are able to survive in similar AMD environments, using culture-independent modern molecular techniques.

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