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Haloalkalitolerant and Haloalkaliphilic Fungal Diversity of Acıgöl/Turkey

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Abstract: Microfungi are the most common microorganisms found in range from environment. They are well known as producer of some product in industrial and food fields, decomposer of organic matter, of important mycotoxins, reason major economic and health effects on plant, animal and human life. Because of these reasons, studies on the determination of biodiversity of microfungi are vitally important. Aim of this study is investigation of biodiversity of microfungi in Acıgöl Lake that is the second largest alkaline lake in the world.
For this purpose, the water sample was compositely taken from a saltern of Acıgöl Lake in November 2019. The samples have been analysed in terms of pH, and salinity. To isolate and enumerate the fungal species from water, filtration method and different DRBC medium types were used. Salt tolerance range of isolates were determinated.
A total of 260 CFUs/L and 65 CFUs/L were counted from DRBC and DRBC28 media, respectively. After purification steps, totally 52 isolates were obtained and identified by using conventional methods and multi locus genes sequencing.
The results indicated that the Acıgöl Lake Region has rich for *Aspergillus* (25%) and *Penicillium* (27%) genera, respectively. Although other members of the genus were determined in the region, other members were found to be 48% in total. In addition, *Cladosporium acalypha* and *Penicillium sizovae* was determined as a new recorded for Turkey. The fact that the microfungus biodiversity determined by this study has the ability to produce toxins (such as *Aspergillus flavus*), contains pathogenic (such as plant pathogen *Fusarium* genus members) and saprophyte species, has been identified as an issue to be considered for public health.

Key words: Acıgöl, Lake, Microfungus, Biodiversity

Acıgöl/Türkiye'deki Haloalkalitolerant ve Haloalkalifilik Fungus Çeşitliliği

Öz: Mikrofunguslar, çevrede en yayın olarak bulunan mikroorganizmalardır. Bu organizmalar, organik maddelerin ayrıştırıcısı, endüstriyel ve gıda alanlarında bazı ürünlerin üreticisi, önemli mikotoksinlerin, bitki, hayvan ve insan yaşamı üzerindeki önemli ekonomik ve sağlık etkilerinin nedeni olarak bilinirler. Bu nedenlerden dolayı mikrofungusların biyolojik çeşitliliğinin belirlenmesine yönelik çalışmalar hayati önem taşımaktadır. Bu çalışmanın amacı, dünyanın ikinci büyük alkali gölü olan Acıgöl Gölü'ndeki mikrofungusların biyolojik çeşitliliğinin araştırılmasıdır.

Bu amaçla, su örneği Kasım 2019'da Acıgöl Gölü'ndeki tuzladan kompozit olarak alınmıştır. Su örneği pH ve tuzluluk açısından analiz edilmiştir. Mantar türlerinin sudan izole edilmesi ve sayılması için filtrasyon yöntemi ve farklı DRCB besi ortamları kullanılmıştır. İzolatların tuz tolerans aralığı belirlenmiştir.

DRBC ve DRBC28 ortamlarından sırasıyla toplam 260 KOB/L ve 65 KOB/L sayılmıştır. Saflaştırma adımlarından sonra, toplam 52 izolat elde edilmiştir ve geleneksel yöntemler ve çoklu lokus genleri dizilimi kullanılarak izolatlar tanımlanmıştır.

Sonuçlar, Acıgöl Gölü bölgesinin sırasıyla *Aspergillus* (% 25) ve *Penicillium* (% 27) cinsleri bakımından zengin olduğunu göstermiştir. Bölgede cinsin diğer üyeleri belirlenmekle birlikte toplam diğer üyeler % 48 oranında bulunmuştur. Ayrıca *Cladosporium acalypha* ve *Penicillium sizovae* Türkiye için yeni kayıt olarak belirlenmiştir. Bu çalışma ile belirlenen mikrofungus biyoçeşitliliğinin toksin üretme kabiliyetine sahip olması, patojenik ve saprofit türleri içermesi, halk sağlığı için dikkate alınması gereken bir konu olarak belirlenmiştir.

Anahtar kelimeler: Acıgöl, Göl, Mikrofungus, Biyoçeşitlilik

Introduction

Microfungi are important eukaryotic microorganisms for plants, animals and humans with their beneficial and harmful activities (Amaeze et al., 2010). Microfungi are commonly found in soil, air, in various food products, from fresh waters to seas and in extreme environments (Kayış et al., 2018). Some microfungi have been noted in many extreme environments such as salt water, rock surface, ocean bottoms, hot ecological regions. Many species developed, especially *Acremonium, Alternaria, Aspergillus, Cladosporium, Fusarium* and *Penicillium* genera members have been observed in different level saline environments (Orwa et al., 2020).

Saline lakes are alkaline with pH values often ranging between 9 and 12. This condition is characterized by particularly high concentrations of carbonate salts. The presence of sodium chloride and other dissolved salts at high concentration creates salty waters (Salano et al., 2017). Fungi in saline environments have been found to produce extremoliths and extremozymes to cope with osmotic stress. They can also prevent water loss by accumulating K^+ ions (Orwa et al., 2020).

Many abilities have been discovered in haloalkaliphilic microfungi such as absorbing salt ions, producing organic acids, providing macromolecules beneficial for health (Wei and Hong-Zhang, 2019). As a result of many studies, haloalkaliphilic microfungi are rare, while haloalkalitelorans are more intense (Eliades et al., 2011). Microfungi growing in extreme environments are of great importance for humans, animals and plants as microbial biodiversity. These microfungi are important pathogens of humans, animals and plants, and they have many properties, such as producing certain enzymes and compounds, producing low-molecular-weight low metabolites, mycotoxins for this reason, it is very important to reveal the biotechnological features (Schuster and Kahmann, 2019).

Haloalkalitolerant microfungi are important for some enzymes in biotechnological field as well as molecular characterization in adaptation (Eliades et al., 2011). It supports both ecological roles and industrial applications with microfungi isolated from extreme environments (Sharma et al., 2016). Some halotolerant fungi are able to produce different hydrolytic enzymes such as cellulase (CMCase), amylase, protease, lipase, and laccase with tolerance to salt (Li et al., 2018).

More studies are needed to discover more features of these microorganisms. For these reason, the main idea of this study investigation of biodiversity of microfungi in

Acıgöl Lake that is the second largest alkaline lake in the world and located between Afyonkarahisar-Denizli-Isparta city boundaries, in the southwest Anatolia, Turkey.

Material and Metod

Research Area, Features of Acıgöl Lake and Sampling

The research area is Acıgöl Lake, which is one of most important sodium sulphate production fields in Turkey, located between Afyonkarahisar-Denizli-Isparta provinces boundaries (Map 1). It is a tectonic lake and has center coordinates 37º 49' N 29º 48' E, and is 836 m altitude above the sea level. The average depth of the lake is 150 and 210 cm with an average area of 41.34 km^2. Acıgöl Lake, which is fed by precipitation, ground-water and the springs that occur along tectonic faults, has no water output other than evaporation and industrial activities. The modern Acıgöl Lake is the second largest alkaline lake in the world, with active precipitation of sodium, calcium and magnesium salts and its surface varies greatly due to seasonal drought (Kuşçu et al., 2017).

Sampling, Isolation and Enumeration of Microfungi

The water sample was compositely taken from a saltern of North site (37°51′14.8″N 29°52′32.2″E) of Acıgöl Lake in November 2019 (Map 1). Variables such as sample pH and salinity (%) are analyzed in Eskişehir Osmangazi University Biology Department Laboratories.

Map 1. Acıgöl Lake and sampling point

To isolate and enumerate the fungal species from water, 20 ml of water sample has been filtered through the sterile Cellulose Nitrate Membrane Filters (pore size 0.45µm, Ø 47 mm Sartorius) and placed onto the Petri plates containing DRBC and DRBC with water sample (DRBC+28% salty) media with chloramphenicol (100 mg/L). For DRBC28 medium, untreated saltern water from the Acıgöl Lake has been used. The plates have been incubated for 5 weeks at 25°C. Fungal colony forming units (CFUs) were counted on 3rd, 5th, 7th, 14th and 30th days of incubation, and subcultures were made of all of the morphologically distinct colonies from each Petri dish on Malt Extract Agar (Merck) slants and kept at 4°C. Individual pure strains have been deposited in the culture collection of the Department of Biology, Eskisehir Osmangazi University (Turkey).

Water samples of ten aliquots have been filtered in parallel and the average number of colonies has been calculated as CFUs/1000 ml. Water activities of the media have been determined using the water activity meter (Aqualab, Decagon Devices, USA).

Halotolerans and Haloalkalitolerant Tests

In the first step, the isolated fungi were inoculated with three point technique in Petri plates containing PDA supplemented with 0%, 5%, 10%, 15% and 20% NaCl. After 7 days incubation at 27 ºC, colony diameters were measured in mm.

In the second step, the isolates with halotolerant properties were inoculated in plates containing PDA adjusted to high pH values (8 and 10) in addition to salt. After 7 days incubation at 27 ºC, colony diameters were measured in mm. The buffer solutions used in the pH settings of the media were used from Grum et al (2016).

Morphological and Multi Locus Gene

Taxonomic identification of fungal isolates was based on their cultural characteristics and morphological structures. Briefly, for identification, isolates were streaked onto potato dextrose agar (PDA) (MERCK) and incubated at 25°C for 7 days. Subsequently, colony diameters were measured and fungal cultures were examined under stereomicroscope (Prior James Swift, England) and a high-resolution light microscope (OLYMPUS CH20BIMF200, OLYMPUS Optical Co, Ltd. Japan) to determine colonial and morphological features. For morphological examinations fungal material was mounted in a modified mounting medium, Lacto-Cotton Blue, as proposed by Sime et al. (2002). Microfungi obtained from Acıgöl Lake were identified to genus level according to Barnett and Hunter (1999).

The isolates which are accepted as *Aspergillus* and *Penicillium* genus members of haloalkalitolerant and haloalkaliphilic microfungi were initially detected at the

genus level according to their microscopic and colonial characteristics.

For traditional identification of *Penicillium* species, Czapek Yeast Extract Agar (CYA; incubation at 25°C and 37°C), Malt Extract Agar (MEA; at 25°C), Yeast Extract Agar (YES; at 25°C) and Creatine Sucrose Agar (CREA; at 25°C) were used. For traditional identification of *Aspergillus* species, CYA (at 25°C and 37°C), MEA (at 25°C) and CREA (at 25°C) were used. The isolates were incubated for 7 days. At the end of incubation, the isolates were distinguished at the species level according to their morphological and microscopic characteristics (Klich, 2002; Samson et al. 2010; 2011).

All isolates were grown on Potato Dextrose Agar (PDA) at 25 °C 7 days for DNA extraction. Fungal genomic DNA was extracted by using ''Mobio Ultraclean Microbial DNA Isolation Kit'' according to the manufacturer's instructions. Obtained DNA used as template for PCR amplification of internal transcribed spacer (ITS) regions of the rDNA genes, part of the β-tubulin (BenA) and calmodulin (CaM), genes. The ITS regions and BenA genes were amplified using described methods (Visagie et al. 2014). For CaM, the CL1 and CL2 primer sets were used (Serra et al. 2006). PCRs were performed by using a Veriti® 96-Well Thermal Cycler (Applied Biosystems®) using described methods (Visagie et al. 2014).PCR products were confirmed by agarose gel electrophoresis (1% w/v in 1xTAE) and visualized by GelRed staining and examined via the Gel Documentation System (Uvitec M02 4611). Sequencing reactions were performed with the Applied Biosystems (3130 XL Genetic Analyser) by the RefGen Biotechnology (http://www.refgen.com).

Data Analysis

The data obtained as a result of the sequence were compared with the NCBI GenBank Database type (Altschul et al., 1990; https://blast.ncbi.nlm.nih.gov/Blast.cgi). The closest Blast result to each taxon was reported.

Alignment of all data obtained as a result of comparisons was carried out using Muscle in the MEGA X software program and together with other sequences of type species obtained from NCBI GenBank (Kumar et al., 2018). Aligned datasets were analyzed in MEGA X 1000 repeats (1000 bootstrap) using the Maximum Likelihood (ML) analysis based on the Tamura-Nei model (Tamura and Nei, 1993). All positions with less than 50% site coverage, containing gaps, or missing data were eliminated.

The author and current species names of the isolates have been standardized according to the Index Fungorum website (http://www.indexfungorum.org/names/names.asp)

Results and Discussions

Halotolerans and Haloalkalitolerant Tests

Microfungi are common microorganisms found in range habitats from soil, air, water and extreme environments (Chavez et al., 2015). As a matter of fact, we were faced with a remarkable fungal biomass values in our study of isolation from Acıgöl that is an important in sodium sulphate production area in Turkey. A total of 52 and 13 isolates were obtained from water sample by using DRBC and DRBC28 media, respectively. Similarly isolates numbers, the highest colony count were recorded from DRBC as 260 CFUs/L. A total of 65 CFUs/L colonies were counted from DRBC28 medium. It is seen that the number of isolates and colonies obtained from 20% (percentage of colonies) and 19,12% (percentage of isolates) are from DRBC28 medium containing 28% salt. A total of 58 isolates from obtained isolates were detailed in this study.

Many studies have demonstrated that fungal biodiversity is high in hypersaline and salty soil environments (Plemenitaš et al., 2014).

Fungi living in hipersaline environments need a minimum salt concentration to adapt to different salt concentrations. Concentrations of Na^+ ions are much greater than K^+ ions. Therefore, the mechanisms that maintain a stable and high intracellular K^+ / Na^+ ratio are crucial to survival in such environments (Plemenitaš et al., 2014). Studies investigating the effects of different salt concentrations on fungal growth; They determined that at low salt concentrations fungal colonies had large diameters and that the colony diameters decreased with the increase in salt concentration, and that alkaline pH values limited the growth of fungal colonies. Another remarkable record is that the pH values of the culture media increase to the alkaline level; It is also the decrease in the level of sport creation (Samson et al., 2010; Kayış et al., 2018).

Isolates were found to have a certain amount of salt tolerance. When the growth performance of the isolates at different salt concentrations was evaluated, it was determined that colonies with the largest diameter were formed in the medium containing 5% salt concentration compared to the medium with 0% salt concentration (Figure 1). While isolates were exhibited moderately

smaller colonies than 0% on 10% and 15% salinity media, micro colonies or not colony formation were on 20% salinity medium (Figure 2).

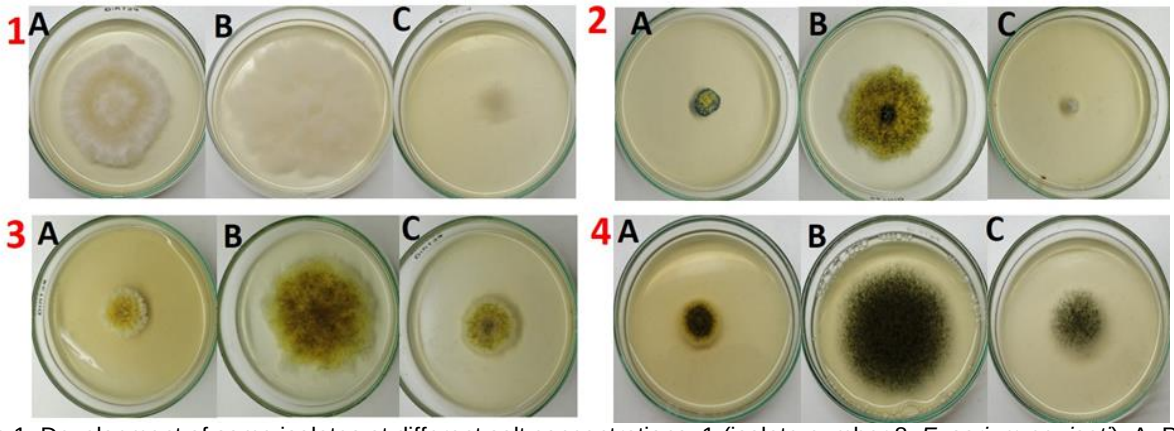

Figure 1. Development of some isolates at different salt concentrations. 1 (isolate number 8, *Fusarium equiseti*); A: PDA 0% salt, B: PDA 5% salt, C: PDA 10% salt; **2** (isolate number 48, *Aspergillus cristatus*) A: PDA 0% salt, B: PDA 5% salt, C: PDA 20% salt; **3** (isolate number 50, *Aspergillus pseudoglaucus*) A: PDA 0% salt, B: PDA 5% salt, C: PDA 10% salt; **4**; (isolate number 54, *Aspergillus amstelodami*) A: PDA 0% salt, B: PDA 5% salt, C: PDA 15% salt.

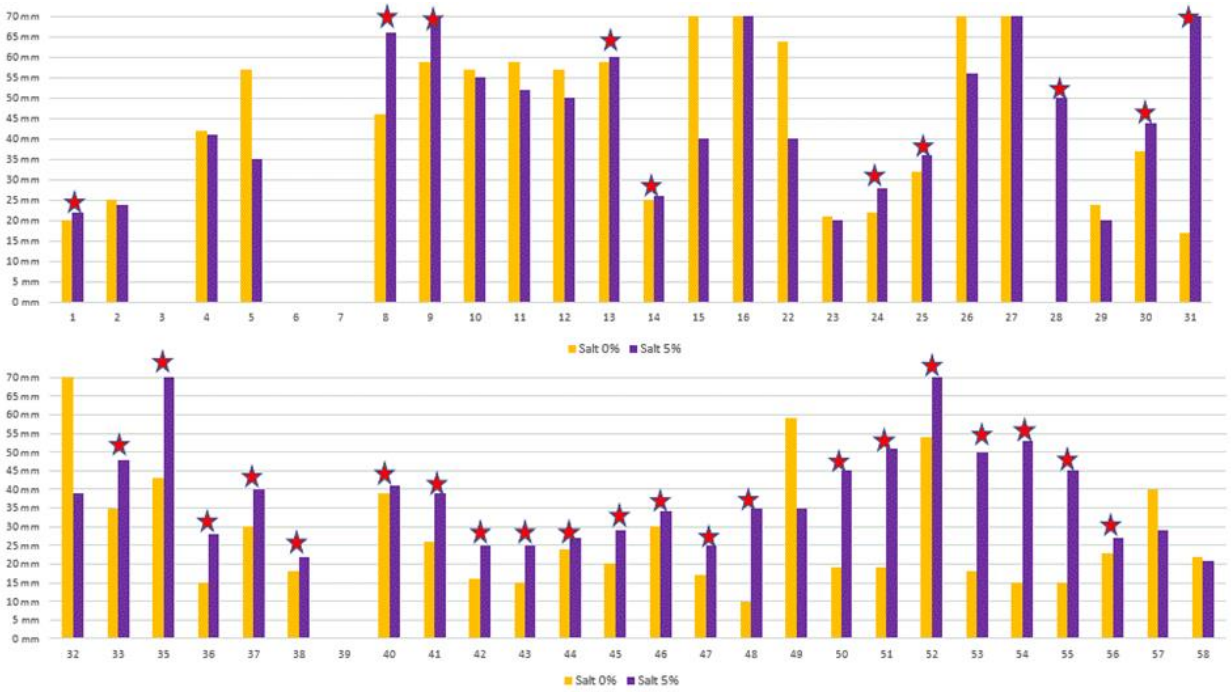

Figure 2. The development of organisms at different salt concentrations (Labeled isolates formed larger colonies on 5% salt medium than of 0%.

Morphological and Multi Locus Gene Identification

The dominant fungal group in the hypersaline and alkaline environments known as *Aspergillus* sp. and *Penicillium* sp. genera in addition to *Alternaria* sp., *Fusarium* sp.and melanized fungi (Plemenitaš et al., 2014; Orwa et al., 2020). According to morphologic and multi-locus genes sequencing results, the isolates were found to be members of *Acremonium* (2%), *Alternaria* (13 %), *Aspergillus* (25%), *Cladosporium* (6%), *Fusarium* (19%), *Penicillium* (27%), *Rhizopus* (2%), and *Trichoderma* (2%). The *Penicillium* genus was recorded as a common genus in Acıgöl Lake with 27% (Figure 3). When we focused on biodiversity and distribution of members of the *Penicillium*, *P. brevicompactum* Dierckx 1901 was determined as the most common with 36%.

This is followed by *P. dipodomyicola* (Frisvad, Filt. & Wicklow) Frisvad 2000 (21.5%), *P. sizovae* Baghd. 1968 (21.5%) and *P. bilaiae* Chalab. 1950, *P. chrysogenum* Thom 1910, *P. solitum* Westling 1911 (each 7%). Up to now, many species of *Penicillium* have been reported from saline habitats such as saline soil, hypersaline regions, hypersaline lakes (Chung et al., 2019). In addition to the widespread determination of *P. brevicompactum* especially in the salterns, *P. chrysogenum, P. citrinum, P. digitatum* has also been recorded with a high frequency (Yadav et all, 2018).

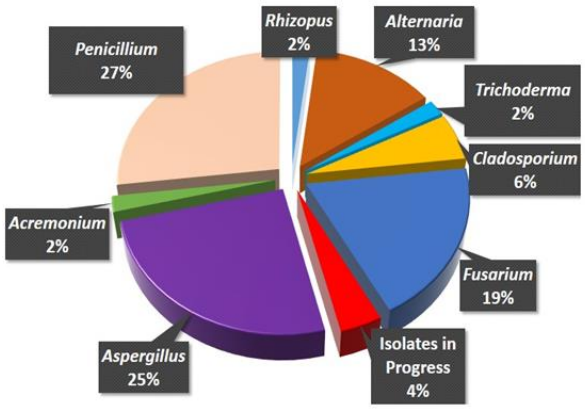

Figure 3. Distribution of isolates in level of genus

About distribution of *Aspergillus* genus from investigated area, although this genus is less in number than *Penicillium* in the research area, it is remarkable that it is higher in terms of diversity. Another remarkable issue is the prevalence of members with strong body structures such as sexual reproduction and sclerotium. *Aspergillus amstelodami* (L. Mangin) Thom & Church 1926, *A. intermedius* Blaser 1976, *A. ochraceus G.* Wilh. 1877 and *A. terreus* Thom 1918 species were recorded as common species (each 15%). This is followed by A. *alliaceus* Thom & Church 1945, *A. cristatus* Raper & Fennell 1965, *A. flavus* Link, Mag. Gesell. naturf. Freunde 1809, *A. pseudoglaucus* Blochwitz 1929 and *A. tubingensis* Mosseray 1934 (each 8%). Already, Eurotiales order within Ascomycota is most important group of both xerotolerant and halotolerant species (Gunde-Cimerman et al., 2009).

Fusarium genus follows *Aspergillus* and *Penicillium* genera at a rate of 19%. *F. solani* (*Neocosmospora solani) (Mart.) L. Lombard & Crous 2015* 40%, *F. keratoplasticum* (*Neocosmospora keratoplasticum*) (Geiser, O'Donnell, D.P.G. Short & Ning Zhang) Sand.-Den. & Crous 2017 20% were obtained. *F. equiseti* (Corda) Sacc. 1886, *F. fujikuroi* Nirenberg 1976, and *Fusarium sp*. species were obtained at a rate of 10%.

All *Alternaria* genus were identified as *Alternaria alternata* (Fr.) Keissl. 1912. *Cladosporium* breed members were obtained in 6% (*C. acalyphae* Bensch, H.D. Shin, Crous & U. Braun 2010, *C. cladosporioides* (Fresen.) G.A. de Vries 1952 and *C. pseudocladosporioides* Bensch, Crous & U. Braun 2010 33.3%). In addition to their ability to survive in low water activity, these black fungi can protect themselves and survive against ultraviolet rays that occur directly due to their pigments and indirectly due to habitat components. Therefore, the frequency of being encountered in hypersalin environments worldwide is high (Chung et al., 2019). Other species obtained; *Acremonium sclerotigenum* (Moreau & R. Moreau ex Valenta) W. Gams 1971, *Rhizopus oryzae* A. Fisch. 1892 and *Trichoderma harzianum* Rifai 1969 (Table 1).

Saline waters are chemically rich by Cl^+ and Na^+ ions. It also contains ions such as Mg^{2+}, SO_4^{2-}. Chemical structure of water together with other environmental factors such as temperature, humidity, wind, sun can affect microbial diversity (Chung et al., 2019). Intense water evaporation and leaching of surrounding rocks with Ca^{2+} and Mg^{2+} deficiency cause the formation of water with high pH values (Bondarenko et al., 2018). In our study, it has been determined that this kind of environmental characteristics may be suitable for the development of certain breed members.

Microfungi that develop only in saline environments are called halophilic (Wei and Hong-Zhang, 2018). The species identified as a result of the study are also seen in different environments such as air, soil, food, and we can say that these microfungi are halotolerant because they have also developed in different salt ratios. Some of the *Acremonium, Aspergillus, Fusarium* and *Penicillium* species are known to be secondary metabolite producers. Secondary metabolites cause anticancer, antioxidant, antiviral and antibacterial effects (Orwa et al., 2020). In this study, it is seen that the microfungi isolated from Acıgöl Lake are rich in these species. Since lake water is rich in potassium, sodium and sulfate, it is used by some businesses (denizli.gov.tr). Therefore, determination of the microfungal diversity of the lake and the characteristics of the species obtained are of great importance. Identified species are known to cause beneficial and harmful effects in economic, biotechnological, food industry, agricultural activities and many other areas.

According to the checklists (Asan (2004), Asan et al. (2016) and Asan (2017)); *Cladosporium acalypha* (23) and *Penicillium sizovae* (37, 46, 56) was determined as a new recorded for Turkey. Contribution to future studies can be made by determining the fungal variety of Acıgöl Lake in terms of Halotolerant and Haloalkalitolerant and the development of these species in different concentrations of salt.

Table 1. Distribution of species isolated from Acıgöl Lake

Species Name	GenBank accession numbers	Number of the isolate (Percentage)
Acremonium sclerotigenum	MT472511	58 (1.92)
Alternaria alternata	MT472471, MT472472, MT472473, MT472474, MT472475, MT472476, MT472482	10, 11, 12, 13, 14, 15, 26 (13.46)
Aspergillus flavus	MT472489	35 (1.92)
Aspergillus alliaceus	MT472488	33 (1.92)
Aspergillus intermedius	MT472504, MT472506	**51, 53** (3.85)
Aspergillus amstelodami	MT472507, MT472508	**54, 55** (3.85)
Aspergillus cristatus	MT472502	**48** (1.92)
Aspergillus ochraceus	MT472486, MT472505	**31, 52** (3.85)
Aspergillus pseudoglaucus	MT472503	50 (1.92)
Aspergillus terreus	MT472484, MT472485	**29**, **30**(3.85)
Aspergillus tubingensis	MT472487	**32** (1.92)
Cladosporium acalyphae	MT472479	**23** (1.92)
Cladosporium cladosporioides	MT472481	**25** (1.92)
Cladosporium pseudocladosporioides	MT472480	**24** (1.92)
Fusarium equiseti	MT472469, MT472470	**8, 9** (3.85)
Fusarium fujikuroi Nirenberg 1976	MT472465	**4** (1.92)
Neocosmospora keratoplastica	MT472463, MT472478	**2, 22** (3.85)
Neocosmospora solani	MT472462, MT472464, MT472466, MT472467	**1, 3, 5, 6** (7.69)
Fusarium sp.	MT472468	**7** (1.92)
Penicillium bilaiae	MT472498	**44** (1.92)
Penicillium brevicompactum	MT472490, MT472493, MT472496, MT472497, MT472501	**36, 39, 42, 43, 47** (9.62)
Penicillium chrysogenum	MT472510	**57** (1.92)
Penicillium solitum	MT472494	**40** (1.92)
Penicillium dipodomyicola	MT472492, MT472495, MT472499	**38, 41, 45** (5.77)
Penicillium sizovae	MT472491, MT472500, MT472509	**37, 46, 56** (5.77)
Rhizopus arrhizus	MT472477	**16** (1.92)
Trichoderma harzianum	MT472483	**28** (1.92)
Isolates in Progress	-	**27, 49** (3.85)

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