

Survey and first report of *Acanthamoeba* T4 genotype in natural spring water resources in the Black Sea, Turkey

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ABSTRACT

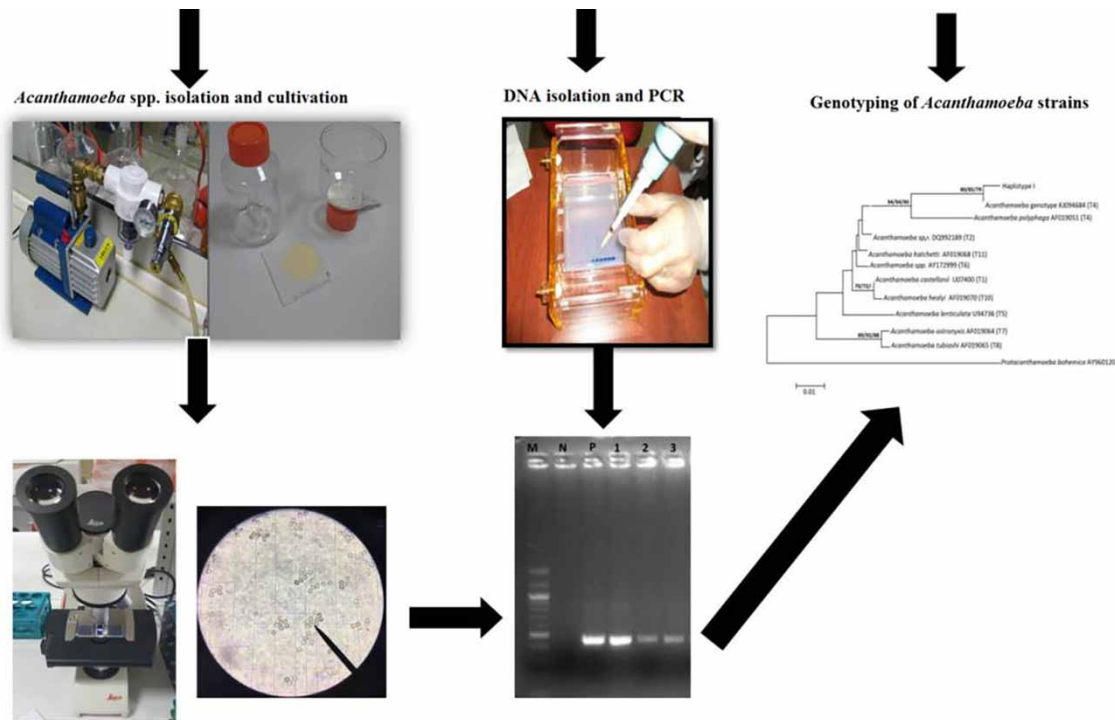
Infection with *Acanthamoeba* spp. may result in granulomatous amoebic encephalitis and *Acanthamoeba* keratitis. Water is an important habitat where *Acanthamoeba* species thrive. Therefore, studying the occurrence of this free-living amoeba in water sources will help understand the infection dynamics. The aim of the study was to survey and report on the presence of *Acanthamoeba* spp. in water resources from the Ordu and Giresun provinces in Black Sea. *Acanthamoeba* spp. was found in 1/17 natural spring water samples from Ordu and in 2/18 from Giresun. *Acanthamoeba* species were not detected in any of the investigated tap water samples. Sequencing of the (SSU) rDNA gene resulted in the identification of haplotype I (*Acanthamoeba* genotype: KJ094684). T4 (8.6%) was the only isolated genotype in both Ordu and Giresun provinces. This is the first report of *Acanthamoeba* T4 genotype in natural spring water resources in the Black Sea. The occurrence of *Acanthamoeba* species in natural spring water sources should be considered as a potential risk for human infection, especially to high-risk populations.

Key words: *Acanthamoeba* spp., Black Sea, genotype, natural spring water, PCR, T4 genotype

HIGHLIGHTS

- Pathogenic *Acanthamoeba* detection in natural spring water in Black Sea, Turkey.
- Identification of pathogenic *Acanthamoeba* genotypes in Black Sea, Turkey.
- First report of *Acanthamoeba* T4 genotype in natural spring water in Black Sea, Turkey.
- *Acanthamoeba* T4 genotype in natural spring water is a potential health risk for high-risk humans.

GRAPHICAL ABSTRACT



INTRODUCTION

Habitats of *Acanthamoeba* species that have an extensive distribution in the environment are air, soil and various water types (Brown *et al.* 1982; John & Howard 1995; Lorenzo-Morales *et al.* 2005; Todd *et al.* 2015; Mahmoudi *et al.* 2021). They have even been found from air-cleaning units (Astorga *et al.* 2011; Chan *et al.* 2011). In addition, they have been found in dialysis units and in the human upper respiratory tract (Marciano-Cabral & Cabral 2003).

Acanthamoeba infection can lead to granulomatous amoebic encephalitis and amoebic keratitis (AK) (Marciano-Cabral & Cabral 2003; Khan 2006; Todd *et al.* 2015). AK has been reported in persons after swimming (Todd *et al.* 2015), while wearing contact lenses, or from individuals who insufficiently clean their contact lens (Parija *et al.* 2001; Marciano-Cabral & Cabral 2003; Schuster & Visvesvara 2004).

Since *Acanthamoeba* species have been isolated from various sources (tap water, seawater, chlorinated swimming pools), and surprisingly from contact lens fluids and containers, the risk for infection is relatively high, especially for the immunocompromised or individuals from high-risk groups. Rapid identification of *Acanthamoeba* spp. from various sources (environmental and clinical) has been reported to be essential for the diagnosis and control of AK (Koyun *et al.* 2020).

Gast *et al.* (1996) and Stothard *et al.* (1998) have reported the detection of *Acanthamoeba* based on the (SSU) rDNA gene. Twelve genotypes (T1–T12) have been shown in three morphological groups by the examination of more than 50 strains (Stothard *et al.* 1998). Genotypes T13–T16 have been identified by Horn *et al.* (1999), Hewett *et al.* (2003), and Corsaro & Venditti (2010). Fuerst *et al.* (2015) has also reported on genotypes T1–T20. There have been 21 diverse genotypes of *Acanthamoeba* (T1–T21) identified by (SSU) rDNA gene sequencing that have been investigated by Corsaro *et al.* (2017).

The most predominant genotype has been isolated from corneal, skin and nasal samples (Booton *et al.* 2005; Niyiyati *et al.* 2009), as well as samples collected from hot springs, swimming pools and beaches (Basher *et al.* 2018), recreational and domestic water sources (Todd *et al.* 2015) that revealed the T4 genotype. These results are supported by other studies from hot spring (Badirzadeh *et al.* 2011; Solgi *et al.* 2012) and soil samples (Reyes-Batlle *et al.* 2014).

Various studies worldwide have reported the presence of *Acanthamoeba* in different water types: isolation of *Acanthamoeba* from tap, river and seawater samples in Jamaica (Lorenzo-Morales *et al.* 2005); tap water in the UK (Shoff *et al.* 2008) and municipal sources in France (Delafont *et al.* 2013) have been documented. Other reports on *Acanthamoeba*

detection come from two water basins in Southern Taiwan (Kao *et al.* 2012) and various water sources in the Khyber Pakhtunkhwa region of Pakistan (Tanveer *et al.* 2013). Several studies have been done on the distribution of *Acanthamoeba* genotype from mineral water in the south of Brazil (Maschio *et al.* 2015), from recreational and domestic water sources in Jamaica (Todd *et al.* 2015) and from recreational hot springs in Malaysia (Mohd Hussain *et al.* 2019).

Limited reports are available about the pathogenic potential of amoebae from water samples in Turkey. Saygi & Akin (2000) have reported on *Acanthamoeba* and *Naegleria* in soil and thermal water samples in Sivas; *Acanthamoeba* spp. have been documented from environmental samples in Ankara (Kilic *et al.* 2004); and amoebae have been found in well water from Kayseri (Kuk *et al.* 2013). Recently, other reports have drawn attention to potentially pathogenic free-living *Amoebae* (FLA) isolated from water supplies from different provinces (Kayseri, Bingöl, Erzurum, Elazığ, Kocaeli, Diyarbakır and İzmir) in Turkey (Yazar *et al.* 2016), and *Acanthamoeba* spp. have been found in river water samples from the Black Sea region provided (Koyun *et al.* 2020).

In this paper, we aimed to report on *Acanthamoeba* genotypes found in the natural spring water samples taken from Ordu and Giresun provinces in the Black Sea region of Turkey based of the (SSU) rDNA target DNA of *Acanthamoeba* spp. Understanding the distribution and pathogenic potential of *Acanthamoeba* in the natural spring waters from these areas will provide perceptions into potential risks for infection and may help with the mitigation of *Acanthamoeba*-related morbidity and mortality.

MATERIALS AND METHODS

Geography

Ordu is a province in the Central and Eastern Black Sea regions. There is an annual average rainfall of 968 mm in Ordu with groundwater potential of 59 million m³. While the centre of Ordu municipality collects a large part of its drinking water supply from the treatment of the Melet River and underground spring waters, the districts surrounding Ordu mostly use underground spring waters. Groundwater reserves in Ordu are located around the Mesudiye, Fatsa and Unye districts.

These resources are used for drinking and potable water supply, industrial supply and irrigation. Generally, there are drinking water treatment plants in Ordu centre and its districts. However, due to the increasing population, their drinking water cannot meet the water quality demands in several villages and communities.

The average number of rainy days in Giresun province in the Eastern Black Sea Region is 184 days. There is an annual average rainfall of 1,585 mm in Giresun. Groundwater aquifers in Giresun province are filled by alluvial deposits come from Pazarsuyu, Aksu and Yağlıdere rivers.

The most efficient basin in terms of groundwater is the Harsit valley. Groundwater resources are mainly used for irrigation, drinking water and industrial purposes throughout the province. In addition, streams and well water resources are used for drinking and utility water in Giresun.

A collection of tap water and natural spring water samples from Black Sea sites in this study was performed in the spring of 2018. Rainfall was observed to be high for several days each month during this season. A total of 35 natural spring water and 60 tap water samples were collected from Ordu and Giresun provinces (Tables 1 and 2).

Acanthamoeba species isolation and cultivation

For the isolation of *Acanthamoeba* species, 500 ml of the natural spring water and tap water samples were filtrated with a cellulose nitrate membrane with a pore size of 0.45 µm, as previously as described by Mahmoudi *et al.* (2012) and Koyun *et al.* (2020). The filters were placed in Ringer agar plates seeded with *Escherichia coli*.

Table 1 | Occurrence of *Acanthamoeba* spp. by the PCR in various water samples collected from Ordu and Giresun provinces

Water sort	Examined sites	Investigated water samples	PCR-positives samples
Tap water	All sites	60	0
Natural spring water	Ordu Province	17	1
	Giresun Province	18	2
Total positive (%)	–	95	3 (3.2%)

Table 2 | Sequencing for *Acanthamoeba* spp. in natural spring water samples collected from Ordu and Giresun provinces

Investigated site	Number of examined sample	Sequence analysis
Ordu provinces	17	Haplotype I <i>Acanthamoeba</i> genotype (KJ094684) T4
O1	-	-
O2	-	-
O3	-	-
O4	-	-
O5	-	-
O6	-	-
O7	-	-
O8	1	1
O9	-	-
O10	-	-
O11	-	-
O12	-	-
O13	-	-
O14	-	-
O15	-	-
O16	-	-
O17	-	-
Giresun provinces	18	2
G1	-	-
G2	-	-
G3	-	-
G4	1	1
G5	-	-
G6	-	-
G7	-	-
G8	-	-
G9	-	-
G10	-	-
G11	-	-
G12	-	-
G13	-	-
G14	-	-
G15	-	-
G16	-	-
G17	1	1
G18	-	-
Total positive (%)	35	3 (8.6%)

Plates were incubated at 26 °C for 3 days and were microscopically examined for *Acanthamoeba* trophozoites and monitored for 14 days for the presence of cysts. The identification of the genus *Acanthamoeba* was performed using morphologically distinct characteristics of trophozoites and cysts under light microscopy (Figure 1).

The pellets were resuspended with PBS after centrifugation. Amoeba cells were counted in 1 ml volume of suspension using a haemocytometer (Thoma cell counting chamber, Figure 2). The samples were stored at 4 °C for DNA isolation.

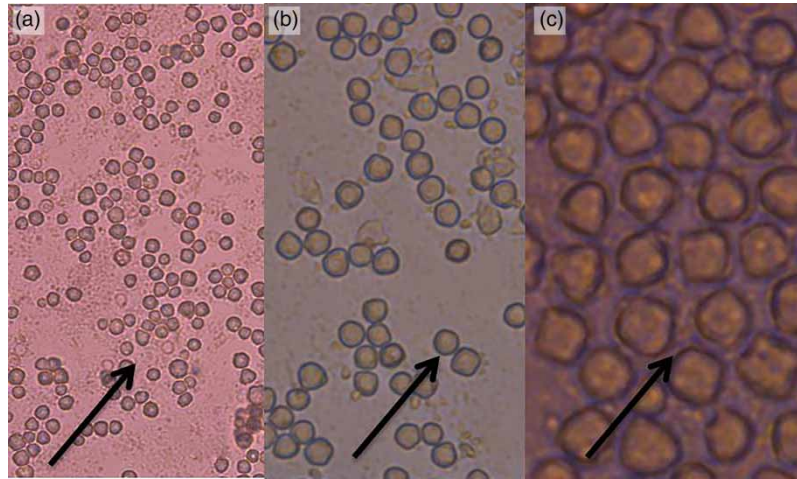


Figure 1 | *Acanthamoeba* cysts cultivated from natural spring water samples in inverted microscopy: (a) $\times 10$, (b) $\times 20$, and (c) $\times 40$; scale bar 10 μm .

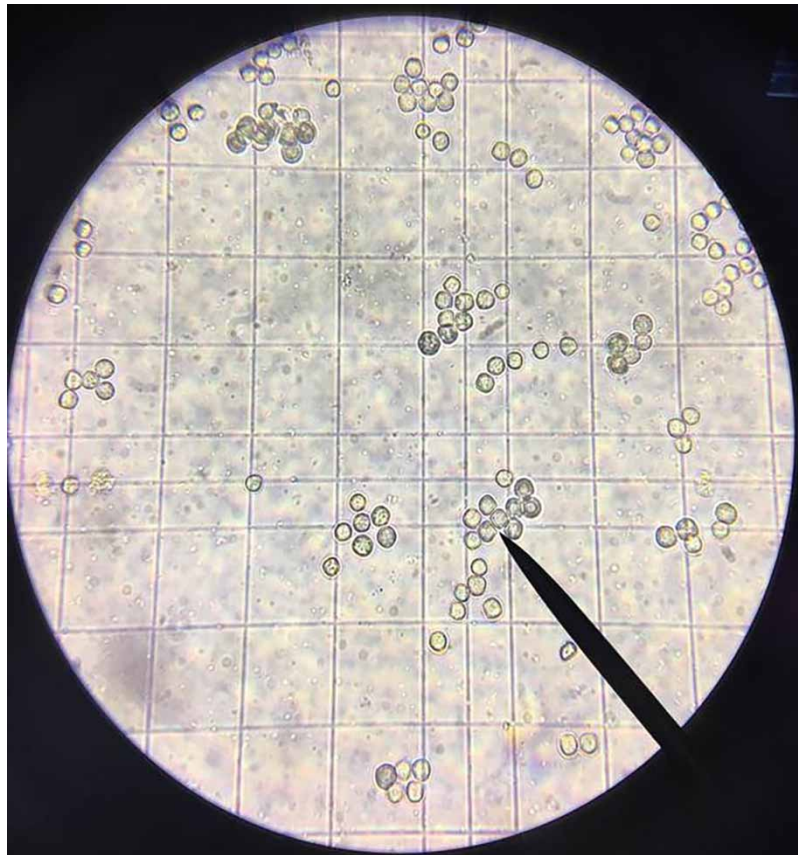


Figure 2 | *Acanthamoeba* cysts counted with haemocytometer (40 \times).

DNA isolation and PCR

Lysozyme (100 mg/ml) was used to extract DNA from the pellets and were subjected to 15 freezing (liquid nitrogen) and thawing (100 °C) cycles as previously described by Koloren *et al.* (2011) and Koyun *et al.* (2020). The samples were further treated with proteinase K (20 mg/ml) before DNA extraction using the QIAamp DNA Mini Kit (Qiagen) as performed by Plutzer *et al.* (2008), Koloren *et al.* (2011) and Koyun *et al.* (2020). The primers JDP1 and JDP2 (Schroeder *et al.* 2001) were used for the amplification of 500-bp fragment of SSU rRNA by the PCR. The final volume of 25 µl PCR mixture was prepared with 10× PCR buffer, 25 mM MgCl₂ and HotstarTaq DNA polymerase 5 U/µl and 20 pmol of both primers, 5×Q solution. Thermal cycling condition was set at 35 cycles (94 °C for 60 s, 50 °C for 45 s, 72 °C for 60 s), followed by a final extension at 72 °C for 10 min. PCR products were observed on a 1.5% agarose gel electrophoresis and stained with ethidium bromide solution under UV light.

Sequencing and genotyping of *Acanthamoeba* strains

PCR products were purified with Millipore Multiscreen[®] HTS PCR Plates (Filter Plates) according to the manufacturer's instructions, and the purified products were sequenced in both directions by Genetic Analyzer with the Big Dye Terminator V.3.1 cycle sequencing kit (Applied Biosystems) and an ABI PRISM3730×L Analyzer (96 capillary types). BioEdit software was used for the bi-directional sequences with both primers. The common base sequences from the water samples and the reference base sequences from the GenBank were aligned by Clustal W. Accession numbers of (SSU) rRNA reference sequences for *Acanthamoeba* were U07400 (T1), DQ992189 (T2), KJ094684 (T4), AF019051 (T4), U94736 (T5), AY172999 (T6), AF019064 (T7), AF019065 (T8), AF019070 (T10) and AF019068 (T11).

Phylogenetic analyses were carried out using MEGA version 5.05 for the Neighbour-Joining (NJ), Maximum-Parsimony (MP) and Maximum-Likelihood (ML) for the alignment visualization of evolutionary background that was calculated with the Kimura two-parameter version with 10,000 pseudo replication bootstrap tests. The percent of the nucleotide likeness and pairwise background among haplotypes were evaluated using BioEdit and MEGA 5.05, respectively.

RESULTS

Identification of *Acanthamoeba* cysts

Acanthamoeba cysts were identified and defined by observing double-walled (ectocyst and endocyst) cyst structures in an inverted microscope (Figure 1) and their numbers counted with haemocytometer (Figure 2) as described by Koyun *et al.* (2020).

PCR assay for the positive water samples

The PCR results for the presence of *Acanthamoeba* spp. in positive natural spring water samples collected from the Black Sea are demonstrated in Table 1. *Acanthamoeba* spp. were not identified in any of the 60 tap water samples, while 3 out of 35 (8.6%) natural spring water samples were positive for *Acanthamoeba*. The positive *Acanthamoeba* natural spring water samples taken from investigated sites were identified by the PCR (Figure 3) are represented in Table 1.

The amplified PCR product from Ordu is shown in lane 1 and the other two positive PCR products are shown in lanes 2 and 3 in Figure 3.

In Table 1, one of 17 natural spring water samples from Ordu province was found positive for *Acanthamoeba* spp. Two of 18 natural spring water samples from Giresun province were found positive for *Acanthamoeba* spp. Three of 35 samples from Ordu and Giresun provinces yielded a positive result for the detection of *Acanthamoeba* spp. (8.6%) and were identified as haplotype I (*Acanthamoeba* genotype, KJ094684, T4).

Genotyping and divergence of pairwise evolutionary

PCR-positive natural spring water samples in Ordu and Giresun provinces were analysed, and phylogenetic trees were constructed with NJ, MP and ML algorithms for *Acanthamoeba* spp. (SSU) rDNA target sequences. The phylogenetic tree is presented in Figure 4. NJ phylogenetic tree for *Acanthamoeba*-positive natural spring water samples (haplotype I) and (SSU) rDNA gene region of all *Acanthamoeba* genotypes received from GenBank (T1, T2, T4, T5, T6, T7, T8, T10, T11) is shown in Figure 4. According to the NJ phylogenetic tree, haplotype I and *Acanthamoeba* genotype (KJ094684) are represented by 80, 85 and 79% homology with sequences of *Acanthamoeba* T4 given with bootstrap values in NJ, ML and MP trees, respectively (Figure 4).

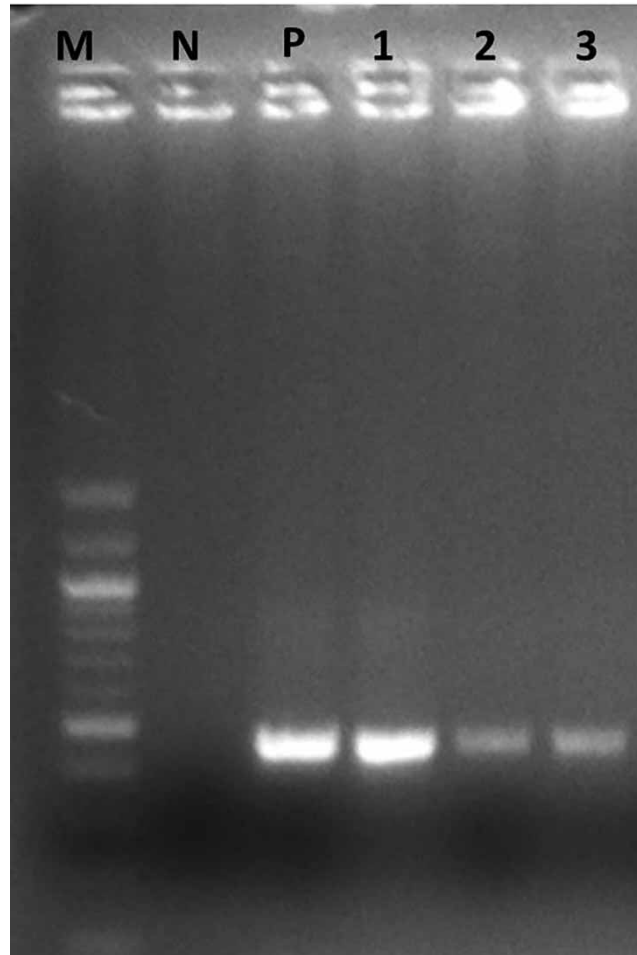


Figure 3 | The amplified (SSU) rDNA gene by the PCR from natural spring water samples taken from Ordu and Giresun provinces. M: 100 bp ladder; N: distilled water (negative); P: *Acanthamoeba castellanii* (ATCC30010); 1–3: positive water samples.

Table 2 demonstrates the results of the sequence analysis and generated haplotypes. One haplotype was found among three sequenced natural spring water samples. Three (8.6%) positive water samples of the 35 examined samples were identified as haplotype I.

The pairwise distance and nucleotide sequence percentage similarities between (SSU) rDNA sequence haplotypes in Ordu and Giresun provinces and target sequences for *Acanthamoeba* spp. taken from GenBank are shown in Table 3.

DISCUSSION

Acanthamoeba species and genotypes were found in natural spring water samples in the Ordu and Giresun provinces of the Black Sea in Turkey. The highest number of *Acanthamoeba* spp. was found in the Giresun province. This is the first study to demonstrate the presence and molecular characterization of *Acanthamoeba* in natural spring waters in the investigated areas.

Acanthamoeba spp. were found in 5.9% (1/17) and 11.1% (2/18) of the natural spring water samples in Giresun and Ordu provinces, respectively. *Acanthamoeba* species were not detected in tap water samples. Three samples have been found positive for *Acanthamoeba* spp. and they were successfully sequenced. All three *Acanthamoeba* isolates from the present study belonged to the T4 genotype. These results are in accordance with a previous study reported in Turkey by Kilic *et al.* (2004), Kuk *et al.* (2013), and Koyun *et al.* (2020). The finding supported outcomes from other studies which have shown that the most frequently *Acanthamoeba* genotype in the world is T4 (Walochnik *et al.* 2000; Schroeder *et al.* 2001; De Jonckheere 2002; Ledee *et al.* 2003; Booton *et al.* 2005; Badirzadeh *et al.* 2011; Rahdar *et al.* 2012; Solgi *et al.* 2012; Qvarnstrom *et al.* 2013; Reyes-Batlle *et al.* 2014). In the present study, all three *Acanthamoeba* isolates were sequenced and belonged to the T4 genotype. This was consistent with other studies that demonstrated that the most frequently detected *Acanthamoeba*

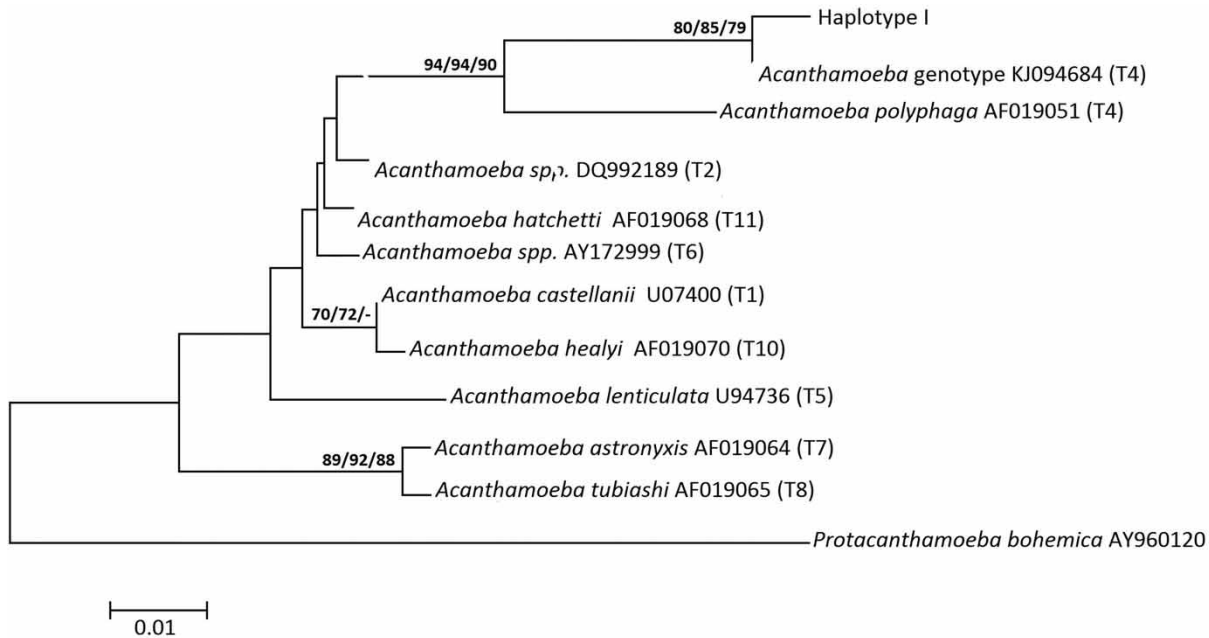


Figure 4 | Phylogenetic tree of *Acanthamoeba* (SSU) rDNA target sequences in natural spring water samples collected from Ordu and Giresun by using NJ, ML and MP analyses. *Protacanthamoeba bohemica* was used as an outgroup. Bootstrap values (higher than 50%) from NJ, ML and MP analyses, respectively, are represented in this tree.

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In addition, the *Acanthamoeba* T4 genotype corresponding to *A. castellanii* is the main genotype connected with more than 90% of AK cases as reported in previous studies (Mohd Hussain *et al.* 2019).

AK has previously been reported in Turkey by Akyol *et al.* (1996), Akisu *et al.* (1999), Demirci *et al.* (2006), and Ertabaklar *et al.* (2007). Additionally, one study conducted in Sivas reported the isolation, characterization, and pathogenicity testing of FLA from soil and freshwater samples (Akin 2000). The first report to identify *Acanthamoeba* in well water sediments was documented in Kayseri (Kuk *et al.* 2013), and another study demonstrated FLA in soil and water samples in Kayseri, Bingöl, Erzurum, Elazığ, Kocaeli, Diyarbakır and İzmir provinces (Yazar *et al.* 2016).

Acanthamoeba from hot springs has been documented in Nicaragua, Taiwan, Switzerland and Iran (Leiva *et al.* 2008; Hsu *et al.* 2009; Gianinazzi *et al.* 2010; Solgi *et al.* 2012). Delafont *et al.* (2013) reported *Acanthamoeba* from municipal water sources in France. Furthermore, the isolation of *Acanthamoeba* was also documented in the Gilan region in Iran's surface water (lagoons, lakes and rivers) and freshwater resources, in natural water samples from 11 provinces in Northeast Thailand (Thammaratana *et al.* 2016), in recreational hot springs from Malaysia (Mohd Hussain *et al.* 2019) and two major water reservoirs in the Philippines (Milanez *et al.* 2020a), including a nationwide survey of *Acanthamoeba* species and genotypes in various freshwater systems from the Philippines (Milanez *et al.* 2020b).

The current study has demonstrated the nucleotide sequence identity and evolutionary distance connection of the (SSU) rDNA gene region of *Acanthamoeba* genotypes that was confirmed by the GenBank from the examined natural spring water samples (Table 3).

The minimum genetic distance between haplotype I and *Acanthamoeba* genotypes (KJ094684) was computed to be 0.0057. The maximum genetic distance between haplotype I and *A. tubiashi* was determined to be 0.0938. The nucleotide sequence similarity between the haplotype I *Acanthamoeba* genotypes (KJ094684) was 99.5% (Table 3).

There was a similarity in NJ, ML and MP phylogenetic trees with 80, 85 and 79% bootstrap between haplotype I and *Acanthamoeba* genotypes (KJ094684) (Figure 4). T4 genotypes were isolated from natural spring water samples in Ordu and Giresun provinces. This finding was in concordance with reports from Walochnik *et al.* (2000), Schroeder *et al.* (2001), Ledee *et al.* (2003), Kilic *et al.* (2004), Booton *et al.* (2005), Kuk *et al.* (2013) and Koyun *et al.* (2020).

Table 3 | Estimates of evolutionary divergence between sequences

	Haplotype I	<i>Acanthamoeba</i> genotype	<i>Acanthamoeba castellanii</i>	<i>Acanthamoeba polyphaga</i>	<i>Acanthamoeba hatchetti</i>	<i>Protacanthamoeba bohemica</i>	<i>Acanthamoeba</i> sp.	<i>Acanthamoeba lenticulata</i>	<i>Acanthamoeba</i> sp. (T6)	<i>Acanthamoeba astronyxis</i>	<i>Acanthamoeba tubiashi</i>	<i>Acanthamoeba healyi</i>
Haplotype I	ID	0.995	0.86	0.898	0.565	0.53	0.553	0.495	0.545	0.376	0.42	0.55
<i>Acanthamoeba</i> genotype KJ094684 (T4)	0.0057	ID	0.865	0.903	0.568	0.533	0.556	0.498	0.548	0.379	0.423	0.553
<i>Acanthamoeba castellanii</i> U07400 (T1)	0.0593	0.0532	ID	0.854	0.616	0.56	0.6	0.537	0.591	0.403	0.45	0.618
<i>Acanthamoeba polyphaga</i> AF019051 (T4)	0.0531	0.0471	0.0531	ID	0.557	0.537	0.56	0.502	0.552	0.378	0.415	0.548
<i>Acanthamoeba hatchetti</i> AF019068 (T11)	0.0470	0.0410	0.0114	0.0471	ID	0.63	0.877	0.746	0.858	0.557	0.613	0.79
<i>Protacanthamoeba bohemica</i> AY960120	0.1682	0.1613	0.1254	0.1542	0.1186	ID	0.617	0.583	0.607	0.458	0.508	0.588
<i>Acanthamoeba</i> sp. DQ992189 (T2)	0.0563	0.0502	0.0143	0.0379	0.0086	0.1186	ID	0.746	0.926	0.547	0.603	0.793
<i>Acanthamoeba lenticulata</i> U94736 (T5)	0.0751	0.0689	0.0290	0.0656	0.0260	0.1216	0.0260	ID	0.741	0.549	0.592	0.706
<i>Acanthamoeba</i> sp. AY172999 (T6)	0.0561	0.0500	0.0143	0.0440	0.0085	0.1121	0.0085	0.0289	ID	0.543	0.598	0.781
<i>Acanthamoeba astronyxis</i> AF019064 (T7)	0.0906	0.0843	0.0438	0.0811	0.0438	0.1257	0.0499	0.0560	0.0438	ID	0.825	0.55
<i>Acanthamoeba tubiashi</i> AF019065 (T8)	0.0938	0.0875	0.0378	0.0811	0.0468	0.1257	0.0499	0.0621	0.0438	0.0057	ID	0.616
<i>Acanthamoeba healyi</i> AF019070 (T10)	0.0623	0.0563	0.0028	0.0562	0.0143	0.1286	0.0172	0.0319	0.0172	0.0468	0.0408	ID

The nucleotide sequence percentage similarities and pairwise evolutionary differences between (SSU) rDNA sequences were found in natural spring water samples from Ordu and Giresun provinces and reference sequences of *Acanthamoeba* spp. from GenBank.

In some rural areas in Ordu province, where there is no sewerage network, wastewater is discharged to the septic tanks of the houses. Especially in the summer months, the population living in the village increases due to hazelnut agriculture. Therefore, septic tank complaints are intense in these months. Efforts are needed to recycle and reuse wastewater in the province of Ordu. Studies can also be carried out on the use of treatment sludge in the soil (Osiki 2015–2019).

It is necessary to establish social and public awareness to protect water basins in the area and to make more efficient use of water resources. Groundwater use must be regulated and effectively controlled. National policy must be followed to protect groundwater reserves and prevent their depletion. Because rivers vary seasonally in terms of flow rate and quality, they are also affected by pollution and the local governments must formulate plans for the storage and treatment of surface water for the coming years. In addition, aquifers and groundwater sources need to be protected from pollution, so they can be used when necessary (Gültekin 2019).

Turkey is characterized by temperate, semi-arid climate zone and temperature extremes. The average annual precipitation in Turkey is approximately 643 mm (Kirtorun & Karaer 2018).

With Turkey's increasing population and growing cities, the country is rapidly progressing towards water demand and supply. Unlike surface water pollution, groundwater pollution is difficult to control. Most of the natural spring waters in Ordu and Giresun provinces are used for drinking. The detection of *Acanthamoeba* T4 genotype from these water sources poses a public health risk, especially to the immunocompromised population. The local governments must formulate policies to monitor and maintain the quality of spring water sources for a sustainable source of clean water for human use and consumption.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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