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Acanthamoeba spp. in river water samples from the Black Sea region, Turkey

İlknur Koyun, Zeynep Kolören, Ülkü Karaman, Amalia Tsiami and Panagiotis Karanis

ABSTRACT

The present study aims to investigate the occurrence of free living amoeba (FLA) in water resources (rivers and tap water) in Samsun in the Black Sea. The presence of *Acanthamoeba* spp. was confirmed in 98 of 192 water samples collected from 32 sites of Samsun province (Samsun centre, Terme, Carsamba, Tekkekoy, Bafra) by PCR. *Acanthamoeba* spp. were found in 15/36 river samples from Samsun, in 58/90 from Terme, in 12/30 from Carsamba, in 7/18 from Tekkekoy and in 6/18 from Bafra. No *Acanthamoeba* species were detected in tap water samples. The highest rate in river waters contaminated with *Acanthamoeba* species was in Terme followed by Samsun centre (41.7%), Carsamba (40%), Tekkekoy (38.9%) and Bafra districts (33.3%), respectively. The result of the subsequent sequence analysis showed Haplotype I (*A. triangularis*) in 5%, Haplotype II (*A. polyphaga*) in 29.6%, Haplotype III (*Acanthamoeba* spp.) in 62% and Haplotype IV (*A. lenticulata*) in 3%. The most common genotype was *Acanthamoeba* T4 (*Acanthamoeba* spp., *A. polyphaga*, *A. triangularis*) and T5 genotype was also found in 3%. The T4 genotype is the most common genotype associated with *Acanthamoeba* keratitis (AK) worldwide; therefore, humans and animals living in the area are at risk after contact with such waters.

Key words | *Acanthamoeba* spp., Black Sea, genotyping, Turkey, water

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INTRODUCTION

Acanthamoeba spp. are commonly found in damp and wet soil, freshwater accumulations, sewage, swimming pools, lakes, dam lakes, tap water and air (Khan 2006; Lass *et al.* 2014) and, in some cases, contact lens holders. More than 80% of people have antibodies against *Acanthamoeba* (Chappell *et al.* 2001). This suggests that *Acanthamoeba* is an organism that is frequently in contact with humans. *Acanthamoeba* species do not need a host to live; they settle into the tissues causing serious diseases (Lorenzo-Morales *et al.* 2005; Juárez *et al.* 2018). The identification of *Acanthamoeba* species at the genus level is made by distinguishing features of trophozoites and cysts, especially the shape of the double-walled cysts. *Acanthamoeba* species were initially classified as three different morphological groups (I, II and III) (Pussard & Pons 1977; Page 1991). However, the morphological classification is inadequate to discriminate at the species level of *Acanthamoeba* (Stothard *et al.* 1998; Alves *et al.* 2000). The disadvantage of diagnosis based on morphological characteristics of the cyst is that the morphology differs according to the media used; therefore, it is necessary to use cultures and specialist researchers in this field (Scheid & Balczun 2017).

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Acanthamoeba cyst environmental reservoirs have the potential to be transmitted to humans as well as other mammals (Shin & Im 2004; Edagawa *et al.* 2009; Lass *et al.* 2014). Since *Acanthamoeba* species can be found in surface waters, thermal waters, sea water, soil, air, food, drinking water, packaged spring waters, distilled water, chlorinated swimming pool waters and contact lens storage containers they therefore pose the risk of rapid spread. Rapid and reliable identification of *Acanthamoeba* spp. from both environmental sources and clinical specimens is extremely important for diagnosis and treatment of *Acanthamoeba* keratitis (AK).

Free-living amoebae (FLA) can serve as tools or hosts for phylogenetically various microorganisms, while some of them amplify intracellularly (Scheid 2014; Scheid *et al.* 2014; Balczun & Scheid 2017). Taxonomy and classification of these parasites has begun to be revised after the successful application of molecular techniques (Khan *et al.* 2001; Booton *et al.* 2002; Kong *et al.* 2002; Lorenzo-Morales *et al.* 2005). Classification based on the (SSU) rDNA gene for the detection of *Acanthamoeba* was established by Gast *et al.* (1996) and Stothard *et al.* (1998). The strains in which the differences detected in the (SSU) rDNA gene region were below 5% of all were collected under a single genotype. Analyses of more than 50 strains from three morphological groups were presented as 12 genotypes from T1 to T12 (Stothard *et al.* 1998). Other genotypes have been identified, T13 to T16, by other researchers (Horn *et al.* 1999; Hewett *et al.* 2003; Corsaro & Venditti 2010). Fuerst *et al.* (2015) showed genotyping T1 to T20 as a result of phylogenetic analysis of *Acanthamoeba* (SSU) rRNA. The variant among the isolates for the T17 genotype is heterogeneous, while the genotype T18 has only recently been reported as the new nominal *A. byersi* (Fuerst *et al.* 2015).

It was determined that 13 *Acanthamoeba* species found belong to T2, T4 and T6 genotypes. Kao *et al.* (2012) identified *Acanthamoeba* genotypes in 211 water samples collected from two water basins in southern Taiwan. *Acanthamoeba* genotypes reported were T4 ($n = 19$), T5 ($n = 8$), T15 ($n = 3$). Genotype T6, T7/T8, T11 and T12 were detected only once. Genotypes T4, T5, T6, T11 and T15 have been found to be responsible for AK and may cause potential health problems for people in contact with environmental waters (Bouheraoua *et al.* 2014; Lorenzo-Morales *et al.* 2015); it has also been recognized as a cause of keratitis in non-CLs wearers too (Juárez *et al.* 2018). Other pathogenic (T2–T10, T4, T5, T15) or non-pathogenic (T7, T16 and T17) genotypes have been identified for the first time as a result of the molecular characterization of *Acanthamoeba* genotypes from different waters in the Khyber Pakhtunkhwa region of Pakistan (Tanveer *et al.* 2013). Maschio *et al.* (2015) tested marketed mineral water bottles collected from Porto Alegre in the south of Brazil for the determination of *Acanthamoeba* genotypes. Six of the eight *Acanthamoeba* genotypes were found to belong to the T5 genotype, one to the T4 genotype and the other to the T11 genotype.

In the present study, we aimed to molecularly identify *Acanthamoeba* genotypes in river and water samples that were collected from Samsun province within the region of the Black Sea in Turkey based on the (SSU) rDNA target DNA of *Acanthamoeba* spp.

MATERIALS AND METHODS

Sampling and geography

Samsun is one of the most populated provinces and a major port of the western Black Sea in Turkey. Investigated areas in this study include Terme, Carsamba, Tekkekoy and Bafra districts of Samsun. The most important rivers are the Kizilirmak, Yesilirmak, Terme, Mert, Kurtun, Milic and Akcay. We chose to investigate areas near important rivers, such as part of the Kizilirmak in Bafra (6 samples), part of the Yesilirmak and Irmaksirti (12 samples) in Carsamba, the Terme, Milic, Akcay and Kocaman rivers (58 samples) in Terme, and the Mert and Kurtun rivers (15 samples) in Samsun centre.

Rainfall was seen to be high in a number of days each month of spring and autumn, therefore samples were taken at those periods. Water samples from rivers (as shown in Figure 1 and Tables 1 and 2) were collected from 32 stations in Samsun province during autumn 2016 and spring 2017. A total of 192 samples of river water (i.e., 96 samples twice per year) and 30 tap water samples were collected from five centres of districts, Samsun, Terme,

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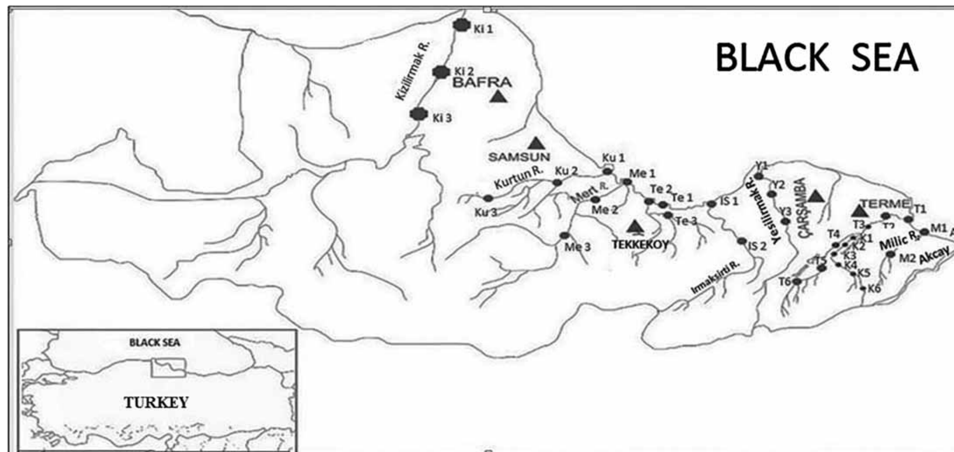

Figure 1 | The map of sampling sites from investigated areas in the Black Sea.

Table 1 | The presence of *Acanthamoeba* spp. by PCR in water samples collected from Samsun province

Water type	Investigated sites	Examined water samples	PCR-positive samples
Tap water	All sites	30	0
River water	Samsun centre	36	15
	Terme	90	58
	Carsamba	30	12
	Tekkekoy	18	7
	Bafra	18	6
Total positive (%)		222	(44%)

Carsamba, Tekkekoy and Bafra (six tap water samples from each distict) as shown in Figure 1.

Cultivation and isolation of *Acanthamoeba* species *in vitro*

For the isolation of *Acanthamoeba* species, 500 mL of the water samples were used; they were filtered through a cellulose nitrate membrane with pore size 0.45 μm, according to Mahmoudi *et al.* (2012). Filters were transferred on Ringer agar plates seeded with Gram-negative bacteria (*Escherichia coli*) as a food source. Plates were incubated at 26 °C and 3 days later, they were microscopically examined for the presence of *Acanthamoeba* trophozoites. After 3 days' incubation and in the absence of amoebae, plates were monitored at the same conditions for up to 14 days. *Acanthamoeba* were identified at the genus level, based on morphological characteristics of trophozoites and cysts using microscopy (Figure 2). Amoeba cells were scraped and harvested from culture plates, then they were washed three times with phosphate-buffered saline (PBS 7.2) and the resulting solution was concentrated by centrifugation at 3,000 × rpm for 5 min. PBS was used for resuspension of the pellets and the mixture was counted for the presence of *Acanthamoeba* in 1 mL by a hemocytometer (Thoma cell counting chamber, Figure 3). The samples were then kept at 4 °C for DNA extraction.

DNA extraction and PCR

The pellets resuspended in phosphate buffered saline (PBS) were lysed by treatment with lysozyme (100 mg/mL) and they were frozen and thawed 15 times using liquid nitrogen and heating to 100 °C, as described by Koloren *et al.* (2011). The samples were then treated with proteinase K (20 mg/mL), and DNA extraction was performed by QIAamp DNA Mini Kit (Qiagen) as described, with minor modifications adapted from Koloren *et al.* (2011). The presence of *Acanthamoeba*-specific ASA.S1 region SSU rRNA gene was amplified with primers JDP1 and JDP2 (Schroeder *et al.* 2001) by standard PCR. A 500-bp fragment of SSU rRNA was amplified by PCR. The PCRs were performed in 25 μL final volumes that included 20 pmol of both primers,

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Table 2 | Results of the sequencing for *Acanthamoeba* spp. in river water samples collected from Samsun and its districts

		Sequence analysis			
Investigated site Samsun centre	**PCR-positive samples 15**	**Haplotype I *A. triangularis* (AF316547) T4**	**Haplotype II *A. polyphaga* (AF019051) T4**	**Haplotype III *Acanthamoeba* spp. (EU168069) T4**	**Haplotype IV *A. lenticulata* (U94736) T5**
Mert River					
Me1	2	–	–	2	–
Me2	3	–	1	2	–
Me3	4	1	1	2	–
Kurtun River					
Ku1	2	–	–	2	–
Ku2	2	–	–	2	–
Ku3	2	–	1	1	–
Terme	**58**				
Akcay	–	–	–	–	–
Milic					–
Mi1	2	–	1	1	–
Mi2	1	–	–	1	–
Terme çayı					
T1	4	1	1	2	–
T2	6	–	2	3	1
T3	5	–	1	3	–
T4	5	–	2	3	–
T5	4	–	1	2	1
T6	4	1	–	3	–
Kocaman River					
K1	6	–	2	3	1
K2	5	1	3	2	–
K3	5	–	2	3	–
K4	4	–	2	2	–
K5	4	1	1	2	–
K6	3	–	–	3	–
Çarsamba	**12**				
Yesilırmak River					
Y1	1	–	–	1	–
Y2	2	–	1	1	–
Y3	3	–	1	2	–
Irmaksırtı					
I1	2	–	1	1	–
I2	4	–	2	2	–
Tekkekoy	**7**				
Gelemen (Te1)	3	–	1	2	–
Selyeri (Te2)	3	–	2	1	—

(*continued*)

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Table 2 | continued

		Sequence analysis			
Investigated site Samsun centre	**PCR-positive samples 15**	**Haplotype I *A. triangularis* (AF316547) T4**	**Haplotype II *A. polyphaga* (AF019051) T4**	**Haplotype III *Acanthamoeba* spp. (EU168069) T4**	**Haplotype IV *A. lenticulata* (U94736) T5**
Kirazlık (Te3)	1	–	–	1	–
Bafra	**6**				
Kızılırmak					
Kı1	–	–	–	–	–
Kı2	3	–	–	3	–
Kı3	3	–	1	2	–
Total positive (%)	**98**	**5 (%5)**	**29 (% 29.6)**	**61 (% 62)**	**3 (%3)**

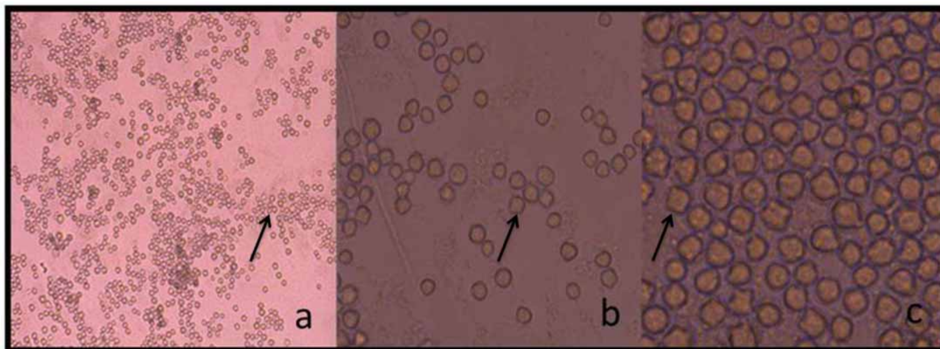

Figure 2 | *Acanthamoeba* cysts cultivated from water samples in inverted microscopy: (a) ×10, (b) ×20, (c) ×40; scale bar 10 μm.

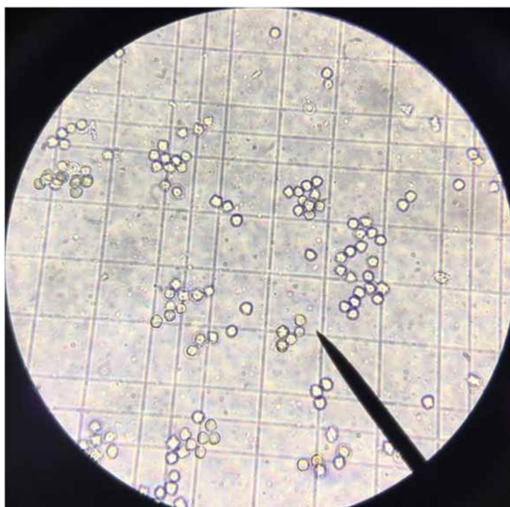

Figure 3 | Counting *Acanthamoeba* cysts collected from plates with hemocytometer (×40).

5 × Q solution, 25 mM dNTP, 10 × PCR buffer, 25 mM $MgCl_2$ and HotstarTaq DNA polymerase 5 U/μL. A Veriti thermal cycler was used for amplification that included 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 visualized on a 1.5% agarose gel electrophoresis stained with a solution of ethidium bromide under UV light.

Specificity analysis by PCR

The specificity of the *Acanthamoeba* for PCR has been demonstrated by comparing *Acanthamoeba castellanii* (ATCC30010) as a reference strain and the following DNAs were used: *Cryptosporidium parvum* (Iowa) DNA,

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Toxoplasma gondii DNA (ATCC50839), *Giardia intestinalis* (H3) DNA, *Babesia bovis* (ATCC75575), *Blastocystis hominis* (ATCC50608D).

Sequence and phylogenetic analysis

Millipore Multiscreen (R) HTSPCR Plates (filter plates) were used for the purification of PCR products according to the manufacturer's specifications. Purified PCR products were sequenced by an ABI PRISM3730 × L Analyzer (96 capillary type) and Genetic Analyzer with Big Dye Terminator V.3.1 cycle sequencing kit (Applied Biosystems). The bi-directional sequences with both primers was arranged by BioEdit software. Clustal W was used to align the reference base sequences from the GenBank and common base sequences from the water samples. Accession numbers of (SSU) rRNA reference sequences for *Acanthamoeba* were U07400 (T1), DQ992189 (T2), S81337 (T3), AF316547 (T4), U94736 (T5), AY172999 (T6), AF019064 (T7), AF019065 (T8), AF019070 (T10) and AF019068 (T11). Phylogenetic trees were drawn for *Acanthamoeba* with reference genotypes from GenBank. The neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) were performed using MEGA version 5.05, based on evolutionary distances calculated with the Kimura two-parameter model with 10,000 pseudo replications bootstrap tests. The percentage of the nucleotide similarity and pairwise distances among haplotypes were measured using BioEdit and MEGA (version 5.05), respectively.

RESULTS

Specificity analysis

A. castellanii DNA for target and other parasites such as *Cryptosporidium parvum, T. gondii*, *G. intestinalis, B. bovis* and *B. hominis* DNAs were used for the specificity of PCR. *A. castellanii* DNA was amplified by PCR, whereas DNAs from the other organisms were not amplified by PCR (Figure 4).

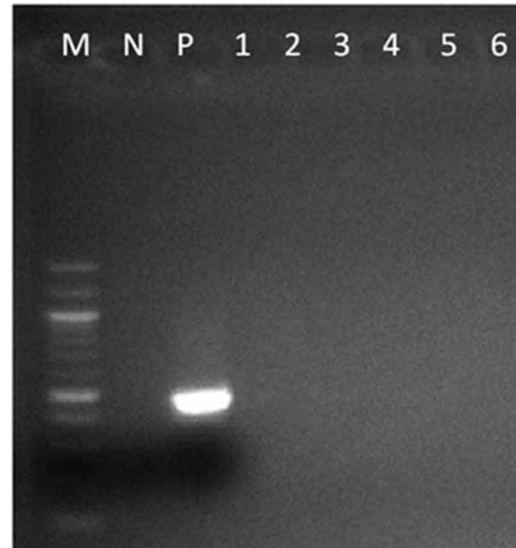

Figure 4 | Agarose gel electrophoresis of PCR specificity of *Acanthamoeba* (SSU) rDNA gene region. M: 100 bp ladder, N: distilled water (negative), P: *Acanthamoeba castellanii* (ATCC30010), DNA 1: *Cryptosporidium parvum* DNA, 2: *Toxoplasma gondii* DNA, 3: *Giardia intestinalis* DNA, 4: *Babesia bovis*, 5: *Blastocystis hominis*.

Positive water samples by PCR assay and collected from the investigated sites

The PCR results for the occurence of *Acanthamoeba* spp. in positive water samples taken from the Black Sea are shown in Table 1. *Acanthamoeba* spp. were not detected in any of the 30 drinking water samples while 98 out of 222 (44%) river water samples were positive for *Acanthamoeba*. The positive *Acanthamoeba* water samples collected from sampling sites and detected by PCR (Figure 5), are shown in Table 1. Fifteen of 36 river water samples from Samsun centre were found positive for *Acanthamoeba* spp. Fifty-eight of 90 river water samples in Terme district, 12/30 in Carsamba district, 7/18 in Tekkekoy district, 6/18 in Bafra district were found to be positive for the presence of *Acanthamoeba* spp. Ninety-eight of 192 samples from Kizilirmak, Yesilirmak, Terme, Mert, Kurtun, Milic and Akcay rivers yielded a positive result for the detection of *Acanthamoeba* spp. Five per cent were identified as Haplotype I (*A. triangularis*, T4), 29.6% as Haplotype II (*A. polyphaga*, T4), 62% as Haplotype III (*Acanthamoeba* spp., T4) and 3% as Haplotype IV (*A. lenticulata,* T5) (Table 2). Haplotype I (*A. triangularis*) for one water

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Figure 5 | The agarose gel electrophoresis of the (SSU) rDNA gene amplified by the PCR from water samples collected from Samsun province. M: 100 bp ladder, N: distilled water (negative), P: *Acanthamoeba castellanii* (ATCC30010), 1–22: water samples collected from the investigated areas.

sample, Haplotype II (*A. polyphaga*) for three samples, Haplotype III (*Acanthamoeba* spp.) for 11 samples were detected in the 15 water samples positive in the centre of Samsun, but Haplotype IV (*A. lenticulata*) was not found in the same area. Of the 58 positive water samples taken from the Terme district, four were *A. triangularis*, 18 were *A. polyphaga*, 33 were *Acanthamoeba* spp. and three were identified as *A. lenticulata*. *A. triangularis* and *A. lenticulata* were not observed in the 12 positive water samples taken from Carsamba district. It was determined that five water samples were *A. polyphaga* and seven samples were *Acanthamoeba* spp. in Carsamba district. Three of the seven positive water samples in Tekkekoy district were identified as Haplotype II (*A. polyphaga*) and four as Haplotype III (*Acanthamoeba* spp.) while Haplotype I and Haplotype IV were not found. One sample was Haplotype II (*A. polyphaga*) and five were Haplotype III (*Acanthamoeba* spp.) in six positive water samples collected from Bafra district. Haplotype I and Haplotype IV were not observed (Table 2).

Genotyping and pairwise evolutionary divergence

PCR positive river water samples in Samsun and its districts were clearly arranged and phylogenetic trees with NJ, MP, ML algorithms for *Acanthamoeba* spp. (SSU) rDNA target sequences were constructed. The tree is represented in Figure 6.

The NJ phylogeny tree for *Acanthamoeba*-positive water samples (Haplotype I–IV) and (SSU) rDNA gene region of all *Acanthamoeba* genotypes taken from GenBank (T1, T2, T3, T4, T5, T6, T7, T8, T10, T11) are given in Figure 6. According to the NJ phylogeny tree, Haplotype I and *A. triangularis* showed 68%, 70% and 78% homology with sequences of *Acanthamoeba* T4 which are supported with bootstrap values in NJ, MP, and ML trees, respectively, as shown in Figure 6. Haplotype II and *A. polyphaga* are represented as sister to *Acanthamoeba* T4 and supported with 72%, 71% and 74% bootstrap values in the NJ, MP, ML trees, respectively. Haplotype III and *Acanthamoeba* spp. showed 76%, 78% and 92% homology with sequences of *Acanthamoeba* T4 which are supported with bootstrap values in NJ, MP and ML trees, respectively. Table 2 shows the sequence analysis results and generated haplotypes. Four haplotypes were found among 98 sequenced river water samples.

Of the 98 positive water samples, five (5%) were identified as Haplotype I, 29 (29.6%) as Haplotype II, 61 (62%) as Haplotype III and three (3%) as Haplotype IV. The nucleotide sequence percentage similarities and pairwise distance between (SSU) rDNA sequences' haplotypes in Samsun and reference sequences for *Acanthamoeba* spp. from GenBank are illustrated in Table 3.

DISCUSSION

In the present study, *Acanthamoeba* spp. and *Acanthamoeba* genotypes in Samsun province of the Black Sea in Turkey were detected. It was observed that the highest number of *Acanthamoeba* spp. was in the district of Terme. The rest of the districts (Carsamba, Tekkekoy and Bafra) were less contaminated than Terme orginating from *Acanthamoeba* spp., respectively. Sequences were

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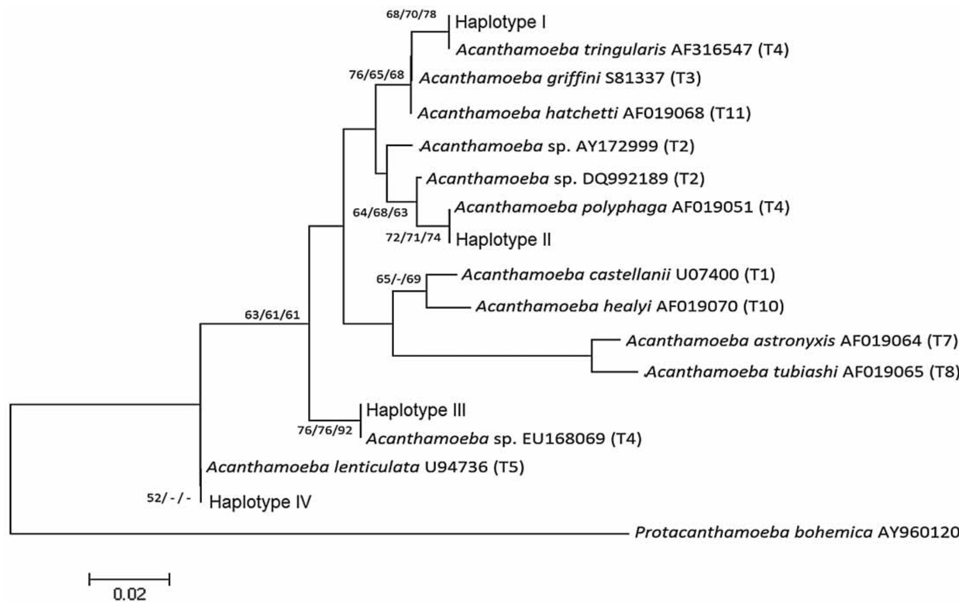

Figure 6 | Phylogenetic relationships of *Acanthamoeba* (SSU) rDNA target sequences in river water samples taken from Samsun and its districts by using NJ, ML, MP analysis. *Protacanthamoeba bohemica* was selected as an outgroup. Bootstrap values (higher than 50%) from NJ, ML and MP analysis, respectively, are shown in this tree.

successfully taken from 98 samples found positive with *Acanthamoeba* spp.

The most common genotypes of *Acanthamoeba* were T4 (96.94%), followed by T5 (3.1%). Also, many cases of *Acanthamoeba* keratitis (AK) linked to *Acanthamoeba* infections have been reported in Turkey. The first case of AK in Turkey was reported by Akyol *et al.* (1996) and the second case by Akisu *et al.* (1999), but genotyping was not included. Demirci *et al.* (2006) showed the presence of *Acanthamoeba* spp. in the eye swabs received from a five-year-old male without genotyping. Another case was reported as *A. castellanii* (T4) and detected by genotyping from a case with no contact lens (Ertabaklar *et al.* 2007). Ozkoc *et al.* (2008) reported *A. castellanii* T4 genotype in a case without contact lens but with a small trauma history. Ertabaklar *et al.* (2009) reported *A. castellani* (T4 genotype) in a 23-year-old woman who was a contact lens wearer, and had red eyes, blurred vision, stinging and burning. Yunlu *et al.* (2015) investigated the existence of free living amoebas in the case of eyelid swabs taken from 500 people. One example was *Acanthamoeba* spp. (0.2%) and the other one was *Hartmannella* spp. (0.2%). Kilic *et al.* (2004) reported the presence of *Acanthamoeba* species with genotypes T2, T3, T4, T7 in samples taken from environmental sources in Ankara greater area. All the studies presented in Turkey were contaminations reported from various sources (water, air conditioning, eye swabs, etc.) implicating *Acanthamoeba* species and in different genotypes. Researchers emphasize the importance of genotyping for the epidemiology of *Acanthamoeba* species, species separation, determination of pathogenicity and distribution of pathogenic species (Ertabaklar *et al.* 2009; Yunlu *et al.* 2015). The present study is the first report of genotyping in the Black Sea region showing the distribution of *Acanthamoeba* species in environmental waters and it fills a gap in this research area in Turkey.

The high numbers of *Acanthamoeba* spp. in water, soil and other environmental samples constitutes an important hygiene risk for people with immune deficiency and wearing contact lenses (Lorenzo-Morales *et al.* 2015). Several studies indicated that the most common *Acanthamoeba* genotype in the world was T4 (Walochnik *et al.* 2000a; Schroeder *et al.* 2001; Ledee *et al.* 2003; Booton *et al.* 2005; De Jonckheere 2007). T1, T10, T12 genotypes (Booton *et al.* 2005), T5 (Barete *et al.* 2007), T2 (Walochnik *et al.* 2008), respectively, followed the most dominant T4 genotypes, in

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Table 3 | Pairwise evolutionary difference and nucleotide sequence percentage similarities between (SSU) rDNA sequences in water samples from Samsun province and reference sequences of *Acanthamoeba* spp. from GenBank

	Haplotype I	*Acanthamoeba triangularis*	Haplotype II	*Acanthamoeba polyphaga*	Haplotype III	*Acanthamoeba* sp.	*Acanthamoeba castellanii*	*Acanthamoeba* sp.	*Acanthamoeba griffini*	*Acanthamoeba lenticulata*	Haplotype IV	*Acanthamoeba* sp.	*Acanthamoeba astronyxis*	*Acanthamoeba tubiashi*	*Acanthamoeba healyi*	*Acanthamoeba hatchetti*	*Protacanthamoeba bohemica*
Haplotype I	**ID**	0.997	0.486	0.486	0.133	0.133	0.354	0.351	0.390	0.333	0.332	0.337	0.291	0.293	0.307	0.387	0.227
Acanthamoeba triangularis **(AF316547)**	0.002	**ID**	0.487	0.487	0.133	0.133	0.354	0.351	0.390	0.333	0.332	0.337	0.290	0.293	0.307	0.387	0.227
Haplotype II	0.036036036036036	0.036	**ID**	0.999	0.215	0.215	0.393	0.422	0.410	0.394	0.394	0.427	0.389	0.369	0.379	0.407	0.271
Acanthamoeba polyphaga **(AF019051)**	0.036	0.036	0.001	**ID**	0.215	0.215	0.393	0.422	0.410	0.394	0.394	0.427	0.389	0.369	0.379	0.407	0.271
Haplotype III	0.045	0.045	0.045	0.045	**ID**	0.998	0.523	0.512	0.525	0.469	0.469	0.509	0.352	0.385	0.504	0.532	0.482
Acanthamoeba **sp. (EU168069)**	0.045	0.045	0.045	0.045	0.003	**ID**	0.523	0.512	0.525	0.469	0.469	0.509	0.352	0.385	0.504	0.532	0.482
Acanthamoeba castellanii **(U07400)**	0.054	0.054	0.054	0.054	0.054	0.054	**ID**	0.844	0.871	0.729	0.729	0.822	0.554	0.621	0.827	0.882	0.628
Acanthamoeba **sp. (DQ992189)**	0.027	0.027	0.009	0.009	0.036	0.036	0.045	**ID**	0.883	0.753	0.753	0.926	0.552	0.609	0.792	0.865	0.626
Acanthamoeba griffini **(S81337)**	0.009	0.009	0.027	0.027	0.036	0.036	0.045	0.018	**ID**	0.745	0.743	0.858	0.561	0.620	0.795	0.944	0.635
Acanthamoeba lenticulata **(U94736)**	0.054	0.054	0.063	0.063	0.063	0.063	0.045	0.054	0.045	**ID**	0.998	0.755	0.546	0.590	0.699	0.748	0.604
Haplotype IV	0.054	0.054	0.063	0.063	0.063	0.063	0.045	0.054	0.045	0.002	**ID**	0.755	0.546	0.590	0.699	0.748	0.602
Acanthamoeba **sp. (AY172999)**	0.027	0.027	0.018	0.018	0.036	0.036	0.045	0.018	0.018	0.054	0.054	**ID**	0.548	0.604	0.781	0.845	0.609
Acanthamoeba astronyxis **(AF019064)**	0.090	0.090	0.090	0.090	0.099	0.099	0.072	0.090	0.081	0.063	0.063	0.072	**ID**	0.825	0.554	0.566	0.458
Acanthamoeba tubiashi **(AF019065)**	0.099	0.099	0.090	0.090	0.099	0.099	0.054	0.090	0.090	0.081	0.081	0.072	0.018	**ID**	0.622	0.625	0.506
Acanthamoeba healyi **(AF019070)**	0.054	0.054	0.054	0.054	0.063	0.063	0.018	0.045	0.045	0.063	0.063	0.045	0.090	0.072	ID	0.793	0.587
Acanthamoeba hatchetti **(AF019068)**	0.009	0.009	0.027	0.027	0.036	0.036	0.045	0.018	0.000	0.045	0.045	0.018	0.081	0.090	0.045	**ID**	0.631
Protacanthamoeba bohemica **(AY960120)**	0.216216216216216	0.216	0.225	0.225	0.180	0.180	0.216	0.216	0.207	0.171	0.171	0.216	0.234	0.252	0.216	0.207	**ID**

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other infections without granulomatous amoebic encephalitis (GAE) and *Acanthamoeba* keratitis (AK). T4 genotype is also the most common genotype in AK (Gast *et al.* 1996; Walochnik *et al.* 2008). However, T3 (Ledee *et al.* 1996; Stothard *et al.* 1998), T5 (Spanakos *et al.* 2006; Ledee *et al.* 2009), T6 (Walochnik *et al.* 2000b), T11 (Khan *et al.* 2002), T15 (Di Cave *et al.* 2009) have also been reported to cause a number of known eye infections (Nagyova *et al.* 2010).

Regarding environmental samples, 15.9% of the natural water samples from 11 provinces in northeast Thailand were positive for *Acanthamoeba* and seven samples were T4, one sample was similar to T3, and the other two samples were similar to T5 (Thammaratana *et al.* 2016). In Iran, a total of 27 surface water samples were investigated, including natural (rivers, lakes, lagoons) and freshwater resources in the Gilan region, a large area of Iran (Mahmoudi *et al.* (2015). A total of 14 out of 19 (73.7%) water samples were confirmed by the PCR method using primers JDP1 and JDP2. Free living amoebas (*Acanthamoeba*, *Hartmannella* and *Saccamoeba limax*) were identified in 49 surface water samples collected from four provinces in Iran, Guilan, Mazandaran (north of Iran), Alborz and Tehran (capital), as reported by Mahmoudi *et al.* (2015). *Acanthamoeba* species were found to be in 18 out of 49 water samples by using primers JDP1 and JDP2 by PCR. Sequence analysis revealed that 16 samples belonged to the T4 genotype and two samples to the T5 genotype. Niyyati *et al.* (2009) and Rahdar *et al.* (2013) previously reported T2, T4 and T6 genotypes in environmental samples from rivers, stagnant water and soil in Iran. *Acanthamoeba* keratitis has been identified in the world as the T4 genotype according to (SSU) rDNA sequencing results (Visvesvara *et al.* 2007; Booton *et al.* 2009; Grun *et al.* 2014).

The nucleotide sequence similarity and evolutionary distance relationship of the (SSU) rDNA gene region of *Acanthamoeba* genotypes from the GenBank and investigated water samples is shown in Table 3. Briefly, the minimum genetic distance between Haplotype I and *A. triangularis* was calculated to be 0.002. The highest genetic distance between Haplotype I and *A. tubiashi* was found to be 0.0991. The nucleotide sequence similarity between Haplotype I and *A. triangularis* was 99.7%. The nucleotide sequence similarity and genetic divergence between Haplotype II and *A. polyphaga* was 99.9% and 0.001, respectively (Table 3).

Haplotype III is represented as a sister group to *Acanthamoeba* spp. with 99.8% nucleotide sequence similarity and the pairwise genetic divergence was 0.003. The minimum genetic distance between Haplotype IV and *A. lenticulata* was calculated to be 0.002. The highest genetic distance between Haplotype IV and *A. tubiashi* was found to be 0.081. The nucleotide sequence similarity between Haplotype IV and *A. lenticulata* was 99.8%.

There was a similarity in just NJ phylogeny trees with 52% bootstrap between Haplotype IV and *A. lenticulata* (Figure 6). At this point, our research showed similarity to studies (Walochnik *et al.* 2000a; Schroeder *et al.* 2001; Ledee *et al.* 2003; Booton *et al.* 2005) which reported that the most common *Acanthamoeba* genotype in the world was T4. At the same time, the identification of the *Acanthamoeba* T4 and T5 genotypes in our study resembled the study previously reported by Mahmoudi *et al.* (2015). In addition, there were seven out of nine *Acanthamoeba* positive samples for T4 and two samples for T15 genotype in 20 public pools in Hungary (Kiss *et al.* 2014); in Pakistan, T4 and T15 genotypes in water and soil samples (Tanveer *et al.* 2015) and in Taiwan, T4 and T2 genotypes were found to be dominant genotypes (Kao *et al.* 2015). In the following years, also reported were: T4 in seven, T3 in one, T5 in two out of samples taken from natural water resources in North East Thailand (Thammaratana *et al.* 2016); T1, T2, T4, T5, T6 and T11 in the samples collected from environmental and tap water in Uganda (Sente *et al.* 2016); T4, T3 in the samples taken from environmental waters in Egypt (Tawfeek *et al.* 2016); T4, T10 and T11 in water taken from a hospital (surgical services, intensive care unit, operating room and water storage tanks) in Tunisia (Trabelsi *et al.* 2016); and T4, T5 and T16 in tap water samples and soil samples collected from China's Yanji area (Xuan *et al.* 2017).

Worldwide, *Acanthamoeba* keratitis is the most common pathology caused by the T4 genotype. The present study suggests the possibility of AK as a risk for people and animals living in the region due to human and animal contact with such waters and due to the most common occurrence of the T4 genotype in the investigated waters, as found in our study.

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Genotyping studies are answering questions such as the difference in the specificity of *Acanthamoeba* spp., which causes infection in different organs such as the brain and eye, and whether there are differences in the pathogenicity of the species. Genotyping of *Acanthamoeba* species isolated from environmental sources may lead to an important pathway for AK diagnosis in the investigated region as both the T4 and T5 are genotypes are responsible for infections in the eye. The presence of both genotypes in the region suggests that individuals with a compromised immune system or others, such as contact lens users, should be very careful using these waters as they are a risk to public health. The spread of *Acanthamoeba* disease may increase as contact lenses are sold by non-experts which may place emphasis on the risk to users. Health authorities and employees need to raise awareness of the public, especially young people, who use lenses for cosmetic purposes, highlighting the pathogenesis of *Acanthamoeba* and the ocular risk source from this parasite.

The present study emphasizes the importance of the *Acanthamoeba* species in the investigated area and that these waters are also found to be used in agriculture and animal husbandry, increasing the risk of contamination. Since such surface, recreational waters and spring waters are used by many people, the public should be alerted to the risk of potential waterborne pathogens and water quality should be monitored periodically in these areas. The results suggest that *Acanthamoeba* strains belonging to T4 genotype, which are related to AK cases, are present in surface water samples examined in Samsun province. The diversity of *Acanthamoeba* strains, their prevalence, genotyping and pathogenic potential studies should be performed for larger-scale water samples and in different sites that have not been studied previously in the Black Sea region. *Acanthamoeba* diagnosis is under-reported, and only available in a few hospitals of large cities but not in rural areas where knowledge about personal hygiene and periodic checks of the water quality are rather limited. There is an urgent need to alert the public health authorities and draw the attention of *Acanthamoeba* to clinicians and public healthcare providers in rural and urbanized areas in Turkey.

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