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Prevalence, molecular identification and genotyping of the crayfish plague pathogen, *Aphanomyces astaci* in major narrow-clawed crayfish (*Pontastacus leptodactylus* Eschscholtz, 1823) populations from Türkiye

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Abstract

Introduction: Crayfish plague is considered the most important crayfish disease globally. It is caused by the fungus-like agent, *Aphanomyces astaci.* This study aimed to identify and determine the prevalence of *A. astaci* using PCR in narrow-clawed crayfish (*Pontastacus leptodactylus*) populations from across Türkiye. **Material and Methods:** A PCR was carried out with primers specific to the internal transcribed spacer region of the *A. astaci* pathogen on both telson and abdominal cuticle tissues from crayfish individuals from 41 different locations. **Results:** *Aphanomyces astaci* was detected in the crayfish from 34 of the locations. Molecular diagnosis showed the prevalence rates of *A. astaci* to be between 0% and 68.2%. For 7 of the 34 locations, the strain of *A. astaci* was determined. Microsatellite analysis of tissue from individuals with positive PCR results revealed the *A. astaci* genotypes in seven populations. Genotype B was found to be the predominant genotype responsible for crayfish plague in Turkish crayfish populations. The Psl genotype (genotype B) was determined in six of the populations, and the As genotype (genotype A) was detected in only one. **Conclusion:** Crayfish plague poses a significant threat to crayfish populations, necessitating the development of rapid, highly sensitive diagnostic methods. An understanding of the sensitivity of the PCR detection method and of the prevalence and genotyping of *A. astaci* in Turkish crayfish populations has been gained from this study.

Keywords: crayfish, clinical samples, *Aphanomyces astaci*, PCR.

Introduction

Freshwater crayfish are represented by two species (*Pontastacus leptodactylus* and *Austropotamobius torrentium*) in Türkiye. The narrow-clawed or Turkish crayfish, *P. leptodactylus*, is the major species and is distributed in most lakes and rivers across Türkiye. However, the stone crayfish *Austropotamobius torrentium* is distributed only in the Thrace Region (1). The Turkish crayfish is an economically valuable crayfish species indigenous to Türkiye, which was caught commercially from 1970 until 1986 in significant quantities and exported to Europe. By the year 1985, the total crayfish catch had decreased dramatically because of crayfish plague (4, 23). This plague was first reported in crayfish from Lake Çivril in the Denizli Province of Türkiye in 1984, and the sole aetiological agent was identified in 1985 by Baran and Soylu (4) as being *Aphanomyces astaci*. This pathogen is a fungus-like microorganism belonging to the Oomycota class (Schikora 1906). Timur (27) reported crayfish plague disease from Lake Çivril in Denizli province, Lake Eğirdir in the Isparta province and Lake Karataş in the Burdur province in 1985. In subsequent years, the disease has continued to spread and affect Turkish crayfish populations (13, 14, 26).

Crayfish plague is considered the most important crayfish disease globally. This lethal disease has caused the collapse of the populations of the European, noble or broad-fingered crayfish, *Astacus astacus*, and the white-clawed or Atlantic stream crayfish, *Austropotamobius pallipes* in Europe. Although not proven to be caused by crayfish plague, the first mass crayfish mortalities in Europe were reported in northern Italy in the 1860s and in France in 1879; the crayfish plague agent subsequently spread throughout Europe (7). Outbreaks were reported in Spain in the 1960s, and the UK, Türkiye, Greece and Norway in the 1980s (2). Besides the Turkish crayfish (*P. leptodactylus*), all European crayfish species (*Astacus astacus*, *Austropotamobius torrentium* and *Austropotamobius pallipes*) are also highly susceptible to crayfish plague (5). Infection causes melanisation and tissue erosion in the crayfish cuticle, and in some cases white tufts of oomycete may be visible on the body. Infected crayfish may exhibit unusual behaviour a few days before death, such as appearing during daylight hours, wandering out of the water, and walking strangely because of paralysis (3).

Diagnosis of *A. astaci* infection is performed by gross observation, wet mount microscopy, histopathology and oomycete culture. Polymerase chain reaction–based identification methods have been commonly used in recent decades because they are rapid and give accurate results. The first PCR identification method for *A. astaci* was developed by Oidtmann *et al.* (21), and following this Vrålstad *et al.* (29) developed a real-time PCR technique for diagnosis of the pathogen. This has been used by many to diagnose *A. astaci* infection of crayfish populations in different countries: in *Astacus astacus* from Finland and Czechia (11, 15), *Austropotamobius pallipes* from Italy (5) and non-indigenous signal crayfish (*Pacifastacus leniusculus*), spiny-cheek crayfish (*Orconectes limosus*), calico crayfish (*O. immunis*) and red swamp crayfish (*Procambarus clarkii*) from France (7).

There have been several studies performed by conventional (4, 23, 27) and molecular identification methods (13, 14, 26) on crayfish plague in Turkish crayfish populations. However, these studies were conducted at a limited number of locations, and there is currently no information about the prevalence of crayfish plague disease throughout Türkiye. There is also no information on the prevalence of individual *A. astaci* strains.

In this study, a PCR was used to identify the crayfish plague pathogen (*A. astaci*) in tissue samples taken from crayfish captured at 41 different locations throughout Türkiye, and the prevalence of *A. astaci* was determined. For seven of the locations, microsatellite analysis was carried out to determine the genotypes of the *A. astaci* strains.

Material and Methods

Crayfish sampling and determination of individuals with crayfish plague. Turkish crayfish specimens were captured with fyke nets between August 2015 and November 2016. Approximately 20 crayfish were obtained from each of 41 major crayfish populations throughout Türkiye (Fig. 1). The crayfish from each location were transferred alive to the laboratory for gross examination and tissue sampling. Each crayfish was assessed for the clinical signs of crayfish plague. After gross observations were made, the crayfish were euthanised with chloroform and the presence of melanisation and tissue erosion determined.

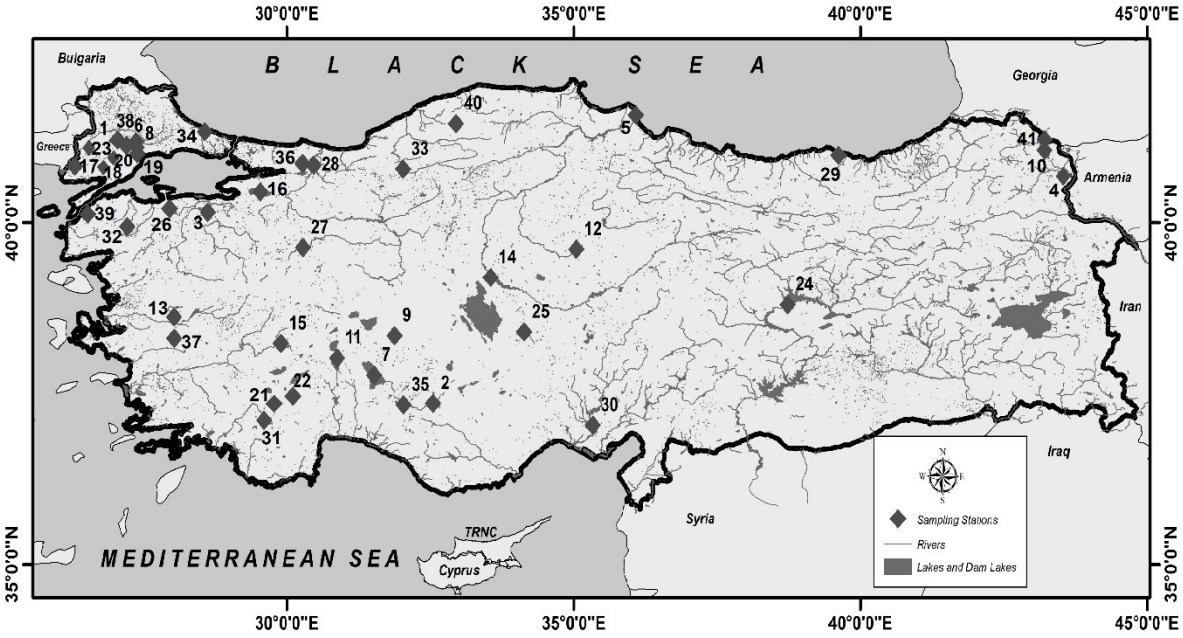

Fig. 1. Map of sampling stations where Turkish crayfish (*Pontastacus leptodactylus*) were caught to determine the prevalence of crayfish plague

Tissue sampling, DNA extraction and purification. Tissues were removed from the abdomen, melanised cuticle and telson of euthanised crayfish and fixed in ethanol for DNA isolation. Extraction and purification of DNA was performed using a commercial genomic DNA extraction kit (DNeasy Blood & Tissue kit, Qiagen, Hilden, Germany) following the manufacturer's instructions. The purified DNA's quality was checked by 2% agarose gel electrophoresis and its quantity was measured with a BioDrop μLITE micro-volume spectrophotometer (BioDrop, Cambridge, UK). The purified DNA samples were adjusted to 50 ng/μL for PCR.

PCR protocol. The identification of the *A. astaci* was made by PCR using DNA samples purified from both telson and abdominal cuticle tissues. Positive control DNA (*A. astaci* genotypes A and B supplied by Dr. J. Makkonen from the University of Eastern Finland, Kuopio) and a negative control containing no DNA were included in each PCR. After PCR optimisation, partial sequences were amplified which were approximately 264 base pairs (bp) in the internal transcribed spacer region of the nuclear ribosomal gene from the *A. astaci* present in the sampled tissues. The amplifications were conducted in a 50 μL PCR reaction volume containing 10× PCR Master Mix containing $MgCl_2$ (BioBasic, Ontario, Canada), 10 mM dNTPs (BioBasic), the primers at a final concentration of 0.5 μM for each reagent and 1 μL of template DNA (50 ng). The primers were as described by Oidtmann *et al.* (20, 21) and designated 42 with the sequence 5′-GCTTGTGCTGAG GATGTTCT-3′ and 264R with the sequence 3′-GGA CTAACCCGAAAGTGCAA-5′. The PCRs were performed in a T100 gradient thermal cycler (Bio-Rad, Hercules, CA, USA) using the following protocol: an initial denaturation step of 5 min at 95°C; 40 cycles of 30 s at 96°C, 30 s at 59°C and 40 s at 72°C, and a final extension step of 5 min at 72°C.

PCR sensitivity test and validation of amplified template DNA. The sensitivity of the PCR protocol was tested using DNA isolated from the mycelium of laboratory-cultured *A. astaci*. The isolated DNA was adjusted to 50 ng/μL using the A260nm/A280nm ratio. Ten-fold serial dilutions were prepared and diluted samples subjected to 30-cycle and 40-cycle PCR amplification using the primers described above to determine the minimum diagnostic concentration. The presence of *A. astaci* in tissues from symptomatic and asymptomatic crayfish was tested. For this, DNA was used which was isolated from tissues commonly infected by *A. astaci*, namely abdominal cuticle, telson and melanised tissue. The abdominal cuticle and telson tissues from 20 symptomatic and 20 asymptomatic crayfish and melanised cuticle tissues from 20 symptomatic crayfish were evaluated. Amplified PCR products from the samples and a positive control were subjected to nucleotide sequence analysis. Sequencing reactions were performed in a Master Cycler Pro 384 thermal cycler (Eppendorf, Westbury, NY, USA) using an ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA), following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the primers detailed above. The sequences of both positive control and clinical samples were edited and aligned by BioEdit v. 7.2.5, and National Center for Biotechnology Information basic local alignment search tool analyses were carried out in GenBank. Subsequently, for each crayfish collected (n = 800), a 40-cycle PCR was performed on abdominal cuticle and telson tissue samples.

Microsatellite analysis and genotyping of *A. astaci* strains from crayfish samples. Eight microsatellite markers (Aast-2, 4, 6, 7, 9, 10, 12 and 13) generating specific and polymorphic amplification patterns (8, 30) were used for genotyping the crayfish plague pathogen strains. The forward primer of each locus was 5′-labelled with a fluorescent 6-FAM dye (Applied Biosystems). Microsatellite analysis was carried out on crayfish samples that gave a strong positive PCR result for *A. astaci* and on reference strains (genotypes A and B) for genotyping. The amplified microsatellite fragments were checked by 2% agarose gel electrophoresis. Next, the fluorescent-labelled fragments were purified from the unincorporated terminators with the BigDye XTerminator Purification Kit (Applied Biosystems). The samples were resolved with an ABI 3730XL DNA analyser (Applied Biosystems) using the GENSCAN 500LIZ (500 bp) internal standard size marker from Macrogen (Amsterdam, the Netherlands). The sizes of resolved microsatellite alleles were scored using Peak Scanner v. 2.0 (Applied Biosystems).

Results

PCR sensitivity test. According to the results of 30-and 40-cycle PCR tests with 50 ng of *A. astaci* DNA serially diluted 10-fold, the 40-cycle PCR was found to be sensitive, with a DNA detection concentration limit of 500 fg/μL (Fig. 2).

Detection of *A. astaci* in crayfish tissue. Abdominal cuticle tissue had the highest detection rate of *A. astaci* of all test material from symptomatic individuals (Table 1, Fig. 3). As expected, lower positive detection rates were obtained from asymptomatic telson and abdominal cuticle tissue (Table 1, Fig. 4).

Validation of PCR results. All amplified fragments had between 97 and 100% similarity with the reference nucleotide sequence for *A. astaci* (Fig. 5).

Prevalence of *A. astaci* in Turkish crayfish stocks. The results showed that of the 800 crayfish specimens collected from 41 different locations, 193 specimens exhibited crayfish plague symptoms (Table 2). According to the molecular identification results, the mean prevalence of *A. astaci* in the tested whole individuals was 14.4% (Table 2).

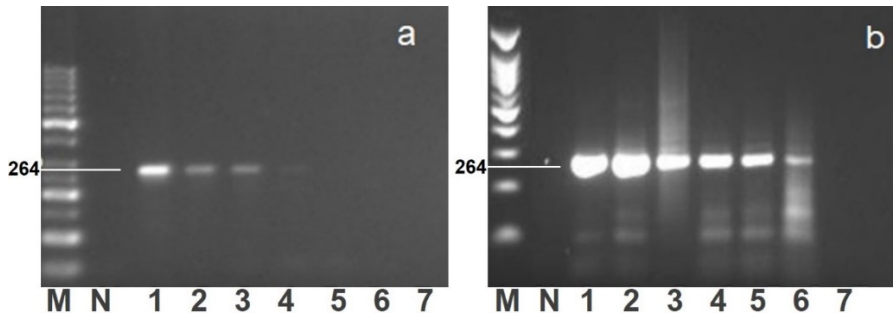

Fig. 2. PCR sensitivity test results for detection of *Aphanomyces astaci*
M – ladder; N – negative control; Lanes: 1 – 50 ng; 2 – 5 ng; 3 – 500 pg; 4 – 50 pg; 5 – 5 pg; 6 – 500 fg; 7 – 50 fg; a – 30-cycle PCR; b – 40-cycle PCR

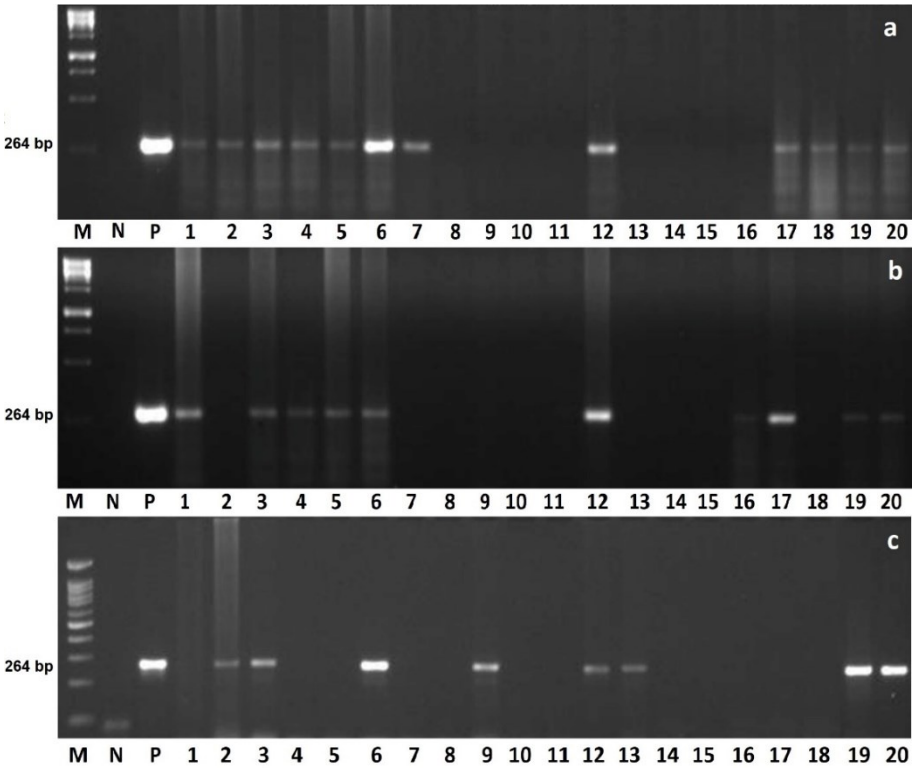

Fig. 3. PCR amplification results for *Aphanomyces astaci* from the tissue of Turkish crayfish symptomatic for crayfish plague
a – abdominal cuticle tissue (n = 20); b – telson tissue (n = 20); c – melanised tissue (n = 20)

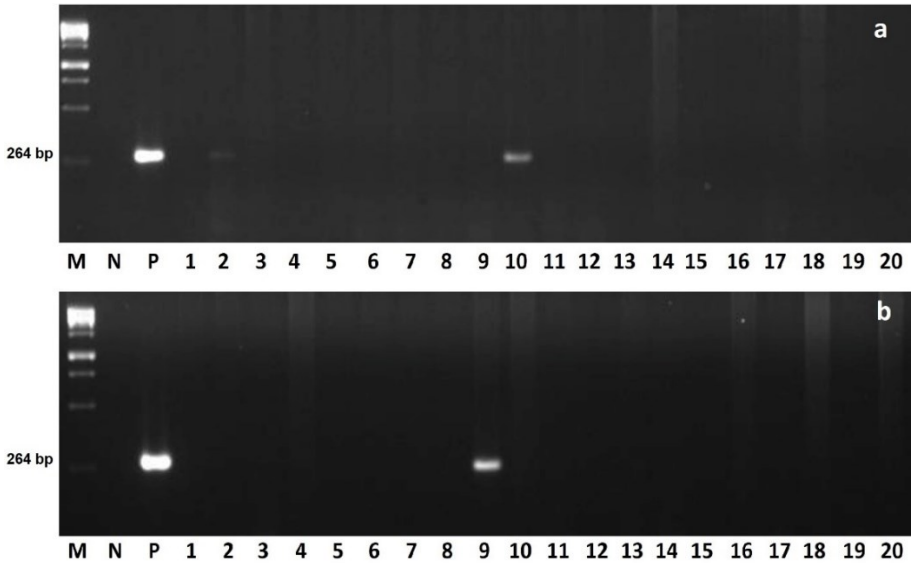

Fig. 4. PCR amplification results for *Aphanomyces astaci* from the tissue of Turkish crayfish asymptomatic for crayfish plague
a – abdominal cuticle tissue (n = 20); b – telson tissue (n = 20)

Table 1. PCR detection of *Aphanomyces astaci* from various crayfish tissue

Sample type	Tissue type	n	Number of PCR-positive samples	Detection rate (%)
Symptomatic specimens	Telson	20	10	50
	Abdominal cuticle	20	12	60
	Melanised cuticle	20	8	40
Asymptomatic specimens	Telson	20	1	5
	Abdominal cuticle	20	2	10

Table 2. The prevalence of the crayfish plague pathogen *Aphanomyces astaci* in samples of Turkish crayfish populations from 41 locations

Sampling station		Latitude (N)	Longitude (E)	Sampling date	n	Sympto-matic crayfish	PCR-positive crayfish	Symptomatic crayfish (%)	PCR-positive crayfish (%)
1	Altınyazı reservoir	41°04′45.48″	26°35′16.08″	27/09/2016	20	5	0	25	0
2	Apa reservoir	37°21′34.92″	32°32′43.44″	18/08/2016	20	7	1	35	5
3	Lake Apolyont	40°08′55.98″	28°36′53.32″	14/08/2015	20	5	2	25	10
4	Arpaçay reservoir	40°37′09.38″	43°41′37.67″	26/08/2016	20	4	3	20	15
5	Lakes Bafra and Balık	41°34′26.34″	36°04′49.91″	7/06/2016	20	5	6	25	30
6	Lake Bayramşah	41°07′31.66″	27°11′57.34″	18/04/2016	5	0	0	0	0
7	Lake Beyşehi	37°46′20.64″	31°31′16.36″	11/11/2016	20	4	8	20	40
8	Bıyıkali reservoir	41°00′26.23″	27°23′30.33″	18/04/2016	20	5	1	25	5
9	Lake Çavuşçu	38°21′02.00″	31°52′37.00″	18/08/2016	20	3	2	15	10
10	Lake Çıldır	41°03′28.77″	43°12′41.70″	26/08/2016	20	4	1	20	5
11	Lake Eğirdir	38°01′25.52″	30°52′22.30″	26/10/2015	21	8	9	38	42.9
12	Gelingüllü reservoir	39°36′33.84″	35°02′37.32″	18/12/2015	20	15	1	75	5
13	Lake Gölmarmara	38°37′17.76″	28°01′49.15″	12/01/2016	20	1	0	5	0
14	Hirfanlı reservoir	39°11′58.37″	33°33′01.47″	18/08/2016	20	8	1	40	5
15	Lake Işıklı	38°14′07.70″	29°53′35.13″	28/08/2015	20	5	12	25	55
16	Lake İznik	40°26′59.71″	29°32′02.30″	31/08/2016	23	6	8	26.1	34.8
17	Kadıköy reservoir	40°47′39.87″	26°46′28.57″	27/09/2016	20	6	4	30	20
18	Karababa reservoir	41°12′41.35″	27°02′56.03″	18/04/2016	20	3	3	15	15
19	Karacakılavuz reservoir	41°06′52.31″	27°21′39.29″	8/08/2015	20	3	0	15	0
20	Karaidemir reservoir	40°57′21.47″	27°00′38.23″	8/08/2015	20	2	1	10	5
21	Karamanlı reservoir	37°23′59.50″	29°50′12.04″	30/09/2016	20	4	1	20	5
22	Lake Karataş	37°23′11.35″	29°58′05.78″	3/09/2015	20	3	3	15	15
23	Lake Karpuzlu	40°49′11.88″	26°18′28.67″	2/09/2015	20	4	2	20	10
24	Keban reservoir	38°48′08.03″	38°43′45.74″	10/11/2015	20	10	1	50	5
25	Mamasın reservoir	38°24′06.07″	34°07′55.84″	13/07/2016	20	3	1	15	5
26	Lake Manyas	40°12′10.56″	27°56′53.99″	14/08/2015	24	1	0	4	0
27	Porsuk reservoir	39°38′07.80″	30°16′45.84″	17.06.2015	20	14	0	70	0
28	Lake Poyrazlar	40°50′18.01″	30°28′01.87″	14.08.2015	15	1	4	6.7	26.7
29	Lake Sera	40°59′10.85″	39°36′55.41″	10.11.2015	20	3	6	15	30
30	Seyhan reservoir	37°02′24.00″	35°19′55.00″	1.11.2016	20	5	1	25	5
31	Lake Gölhisar	37°06′51.19″	29°36′02.34″	16.03.2016	20	4	1	20	5
32	Yenice reservoir	39°56′21.01″	27°12′50.26″	22.04.2016	15	2	0	13.3	0
33	Lake Yeniçağa	40°46′51.95″	32°01′22.14″	10.10.2016	20	8	3	40	15
34	Durusu reservoir	41°20′00.00″	28°34′00.00″	15.09.2015	20	3	4	15	20
35	Lake Suğla	37°20′15.00″	32°01′56.00″	22.10.2015	20	9	7	45	35
36	Lake Taşkısığı	40°52′07.00″	30°24′12.00″	10.10.2016	22	3	15	13.6	68.2
37	Lake Gölcük	38°18′45.25″	28°01′43.88″	28.10.2015	20	2	7	10	35
38	Hanoğlu reservoir	41°11′24.19″	27°22′08.37″	2.11.2016	20	3	8	15	40
39	Atikhisar reservoir	40°07′26.04″	26°31′24.24″	30.10.2016	15	3	3	20	20
40	Lake Bostancılar	41°27′04.56″	32°56′59.07″	11.10.2016	20	3	3	15	15
41	Lake Aktaş	41°13′00.06″	43°12′56.87″	12.11.2016	20	6	4	30	20
					Σ = 800	*Σ = 193*	*Σ = 137*	x̄ = 23.6	x̄ = 16.5

Table 3. Crayfish samples from which *Aphanomyces astaci* was isolated and genotyped by multi-locus microsatellite analysis and compared to reference strains

Locations of examined crayfish	Allele sizes of amplified microsatellite loci								Genotypes
	Aast2	Aast4	Aast6	Aast7	Aast9	Aast10	Aast12	Aast13	
Genotype A[1] (reference strain)	160	103	157	205	179	142	226/240	194	A
Genotype B[2] (reference strain)	142	87	148	214	164/182	132/134	226/240	202	B
Eğirdir Lake	143	87	148	211	164/182	134	226/241	194	B
Karababa reservoir	159	87	158	214	164/182	132	226/241	194	B
Karaidemir reservoir	142	87	148	211	163/185	134	226/240	194	B
Mamasın reservoir	142	87	148	211	164/183	134	226/240	194	B
Lake Suğla	158	101	158	211	180	142	226/240	194	A
Hanoğlu reservoir	142	87	148	214	164/181	132	226/240	194/202	B
Atikhisar reservoir	142	87	149/158	211	164/183	134	226/240	194	B

[1]UEF-VEN5/14 As (A) genotype DNA and [2]UEF-8866-2 PsI (B) genotype DNA were supplied by the University of Eastern Finland

	Variable Base Positions					
	11	1111112222	1111 33345597778		Identical	GenBank
Sequences	1234568901	3467890235	01294772566	Organism	(%)	Acces. No
#P	TTAAATACAT	CTATAAATTG	CAGTTAGCTCC	A. astaci	100	KX555484.1
#S-A1	....	....	....	A. astaci	100	KX555484.1
#S-A2	....	....	....	A. astaci	100	KX555484.1
#S-A3	.A..CA..GC	TGG.T.....	.CTA.......	A. astaci	98	KX555484.1
#S-A4	C....A....	....	....	A. astaci	99	KX555484.1
#S-A5	....	....	....	A. astaci	100	KX555484.1
#S-A6	C....A....	....	.......TCG.	A. astaci	99	KX555484.1
#S-A7	.C..CACTGC	GGGCTG.GA.	GCTACC.....	A. astaci	97	GU320221.1
#S-A12	C.T.......	....	....	A. astaci	99	KX555484.1
#S-A17	....	.........A	....	A. astaci	99	KX555484.1
#S-A18	CA...AC.GC	.GG.......	.CT........	A. astaci	99	KX555484.1
#S-A19	....	....	....	A. astaci	100	KX555484.1
#S-A20	....	....	....	A. astaci	100	KX555484.1
#S-T1	....	....	....	A. astaci	100	KX555484.1
#S-T3	C....A....	....	....	A. astaci	99	KX555484.1
#S-T4	....	....	....	A. astaci	100	KX555484.1
#S-T5	....	....	....	A. astaci	100	KX555484.1
#S-T6	....	....	..........A	A. astaci	99	KX555484.1
#S-T12	....	....	........CG.	A. astaci	100	KX555484.1
#S-T16	C.T.CACTGC	TGGCTG.G..	.CT.CCC....	A. astaci	97	GU320221.1
#S-T17	....	....	........CG.	A. astaci	99	KX555484.1
#S-T19	....	....	....	A. astaci	100	KX555484.1
#S-T20	.A..CA..GC	TGG.T.....	.CTA.......	A. astaci	98	KX555484.1
#S-M2	....	....	....	A. astaci	100	KX555484.1
#S-M3	....	.C........	....	A. astaci	100	KX555484.1
#S-M6	....	....	....	A. astaci	100	KX555484.1
#S-M9	....	....	....	A. astaci	100	KX555484.1
#S-M12	....	.........A	....	A. astaci	100	KX555484.1
#S-M13	....	....	....	A. astaci	100	KX555484.1
#S-M19	....	....	.........G.	A. astaci	100	KX555484.1
#S-M20	C..GC.G...	....	....	A. astaci	100	KX555484.1
#AS-A2	....	....	....	A. astaci	100	KX555484.1
#AS-A10	....	....	........CG.	A. astaci	100	KX555484.1
#AS-T9	C.T...C.CC	GCGCTTG...	....	A. astaci	98	KX555484.1

Fig. 5. National Center for Biotechnology Information basic local alignment search tool results of amplified partial internal transcribed spacer region of *Aphanomyces astaci* from GenBank
P – positive control; S-A – symptomatic abdomen tissue; S-T – symptomatic telson tissue, S-M – symptomatic melanised tissue, AS-A – asymptomatic abdomen tissues, AS-T – asymptomatic telson tissues

However, the prevalence ranged over the different locations from 0.0% to 68.2%. *Aphanomyces astaci* was detected by PCR in 34 of the 41 populations. The highest prevalence was 68.2% in Lake Taşkısığı. The fungus was not detected in crayfish from the Altınyazı, Yenice, Porsuk or Karacakılavuz reservoirs or Lakes Gölmarmara, Bayramşah and Manyas. Notably, only Lake Bayramşah had no symptomatic crayfish.

A total of 137 out of 800 crayfish tested positive (Table 2). Of these, 84 tested positive in the telson, while 73 tested positive in the abdominal cuticle; 20 individuals had positive PCR results in both telson and abdominal cuticle tissue. These results validated the use of both tissue types for detection of *A. astaci*.

Microsatellite analysis and genotyping of *A. astaci* strains from crayfish samples. The eight microsatellite markers (Aast-2, 4, 6, 7, 9, 10, 12 and 13) produced specific and polymorphic amplicons which were used for genotyping crayfish plague pathogen strains (8, 30). Microsatellite analyses were performed on samples from seven of the locations (Lakes Eğirdir and Suğla and the Karababa, Karaidemir, Mamasın, Hanoğlu and Atikhisar reservoirs). The microsatellite motifs obtained are given in Table 3. When compared with the reference strains (genotypes A and B), only A was present in Lake Suğla while B was present at the six other locations (Lake Eğirdir and the Karababa, Karaidemir, Mamasın, Hanoğlu and Atikhisar reservoirs).

Discussion

Crayfish plague is undoubtedly the most significant factor jeopardising European crayfish populations. Rapid diagnostic methods for this disease with high sensitivity can help inform crayfish management and conservation efforts. In this study we investigated *A. astaci* from the tissue of infected crayfish by a PCR-based molecular detection method. The single-round PCR with 40 cycles was found to have a detection threshold for *A. astaci* genomic DNA of 500 fg. This quantity is greater than the 100 fg that Oidtmann *et al.* (20, 21) reported. Our minimum detection limit, however, is lower than the 1 pg DNA concentration that Phadee *et al*. (22) reported as the minimum detectable concentration for *A. piscicida*. This study also supports the use of both abdominal cuticle and telson tissue to detect *A. astaci,* which aligns well with the findings of Oidtmann *et al.* (20).

Although we aimed to sample 20 individuals per locality, for various reasons sample sizes differed in some locations, as can be seen in Table 2. Symptomatic crayfish were identified at all except one of the 41 locations and *A. astaci* was detected in crayfish from 34 of them, confirming the widespread distribution of the disease and the pathogen. We were not able to detect the presence of *A. astaci* in the crayfish captured from the Altınyazı (n = 20), Karacakılavuz (n = 20), Porsuk (n = 20) or Yenice reservoirs (n = 15) or Lakes Bayramşah (n = 5), Gölmarmara (n = 20) or Manyas (n = 24). However, in all locations except Lake Bayramşah (n = 5) individuals with crayfish plague symptoms were observed. This observation is consistent with that of Schrimpf *et al.* (25), who demonstrated that it is virtually impossible to prove the absence of the pathogen with such small sample sizes. In contrast to the PCR results in this study, but consistently with the physical examination of the crayfish in this study, Rahe and Soylu (23) reported crayfish plague in Lakes Gölmarmara and Manyas, and recently Kokko *et al.* (13) provided evidence of the presence of *A. astaci* in the crayfish population inhabiting Porsuk reservoir.

It is possible that the number of diseased crayfish was underrepresented in this study because of the sampling method; this method also reduced the probability of detecting *A. astaci*. Given that uncoordinated limb movements, paralysis, muscle weakness, loss of extremities and abnormal behaviour are among the symptoms of crayfish plague (4, 23, 27), and since the crayfish capture for each sample was carried out using fyke nets with bait, it is probable that the crayfish which were suffering these symptoms were not capable of competing for food or had limited mobility, and were thus less likely to be caught. In addition to this, several other factors (*i.e.* temporal variations and/or detectability) as discussed by Filipova *et al.* (7) could also affect the detection probability of the pathogen. Therefore, these results do not mean that the seven localities where 0% prevalence resulted are truly free of crayfish plague.

Of the 41 locations reported in this study on the prevalence of *A. astaci* in Turkish narrow-clawed crayfish populations, 6 were previously studied by Svoboda *et al.* (26) and Kokko *et al.* (13, 14); however, the infection status of 35 populations was reported for the first time in this study (Table 2). The test results and calculated prevalence values in PCR-positive populations are low compared to those of other studies. This may be due to the lower diagnostic capability of conventional PCRs compared to real-time PCRs and the random selection of individuals used in the study. However, Svoboda *et al.* (26) randomly selected healthy and symptomatic samples from 32 individuals in Lake Eğirdir and were able to detect pathogens in 4 of them (12.5%) with real-time and conventional PCR analysis. On the other hand, Kokko *et al.* (14) reported high prevalence rates (95% and 100%) in Lake Iznik and the Hirfanlı reservoir in symptomatic and asymptomatic individuals from two populations. Kokko *et al*. (13) reported a relatively lower prevalence of the disease in six populations examined in their study of seven populations from Türkiye. Among these populations, 100% prevalence in the Hirfanlı reservoir (n = 5), 80% in Lake Egirdir (n = 5), 60% in Lake Çıldır (n = 5), 100% in the Porsuk reservoir, (n = 5), 80% in the Sarımsaklı reservoir (n = 5) and 50% in the Yenikarpuzlu reservoir (n = 4) was calculated, while no infected individuals were identified in the Keban reservoir (n = 6). Kuzbikova *et al.* (15) analysed 28 populations of two invasive *Pacifastacus leniusculus* and *Orconectes limosus* by conventional PCR in Czechia. The pathogen was found in 17 of the 28 populations examined.

In *O. limosus* populations, prevalences of positive reactions ranging from 0% to 100% were reported, while in *Pacifasticus leniusculus*, only 1 of the 124 individuals examined was positive for the pathogen. In another study, Maguire *et al.* (17) reported similar prevalence rates in Croatia. They reported higher prevalence of infection in samples tested from three native species (*Astacus astacus*, *Austropotamobius pallipes* and *Austropotamobius torrentium*) than in *P. leptodactylus* samples, at 68%, 38% and 40%, respectively, against 27%. The same researchers reported infection prevalence in samples from two exotic species as 58% in *O. limosus* and 25% in *Pacifastacus leniusculus*, but could not detect infected individuals in a third exotic species, the redclaw crayfish (*Cherax quadricarinatus*).

Among the crayfish populations that tested positive for *A. astaci* infection, the highest prevalence percentages were in the populations inhabiting Lakes Taşkısığı (68.2%) Işıklı (60%), Eğirdir (42.9%) and Beyşehir (40%) and the Hanoğlu reservoir (40%). In Türkiye, the crayfish plague first appeared in Lake Işıklı in 1984, then in Lake Eğirdir in 1985, and in several others in the following years (4, 23). Since then, various studies have reported the crayfish plague from different locations. Earlier studies postulated that Turkish crayfish populations are recovering, based on the hypothesis that the causative agent has long been persistent in Turkish water bodies and despite the high prevalence of the disease, crayfish populations have been reproductive and have managed to co-exist with the pathogen (13, 14, 26). It has been proposed that this is because *P. leptodactylus* has developed resistance and/or the virulence of the pathogen has decreased (13, 14, 26). This study supports this notion in some of its findings. In Lake Eğirdir, for example, *A. astaci* was detected at a high level compared to other locations; from this together with the previous observations in Lake Eğirdir (4, 13 26), it can be inferred that *A. astaci* has been persistent in the lake for over 30 years. However, despite the high prevalence observed in the present study (42.9%), the recent catch amount of 891 tonnes in 2020 (28) suggests that the crayfish population is indeed recovering.

In this study, the strains of *A. astaci* present in the tissue samples were determined for seven of the populations sampled. Genotype B was present at six of the locations (Lakes Eğirdir, Karababa, Karaidemir and Mamasin and the Hanoğlu and Atikhisar reservoirs), while genotype A was present only at Lake Suğla. Previously, five different genotype groups (A, B, C, D and E) of *A. astaci* isolates from various populations of crayfish were identified using the random amplified polymorphic PCR technique (6, 9, 16). Among these groups, genotype A is known as the As genotype and was isolated from *Astacus astacus* and *P. leptodactylus* (9). Genotypes B, C, D and E were first noted in North American crayfish species. Genotype B is known as the PsI genotype and genotype C as PsII and they were detected in *Pacifastacus leniusculus* from California and Canada (9). Genotype D is known as Pc and was reported from *Procambarus clarkii* (6), and finally, genotype E is known as Or and was identified in *O. limosus* (16). Huang *et al.* (9) first classified an isolate from Türkiye as genotype A (AsI). In another study, Kokko *et al.* (13) reported both genotype A (Lake Çıldır and the Porsuk and Sarimsakli reservoirs) and genotype B (the Yenikarpuzlu and Hirfanlı reservoirs) in some crayfish populations different to the populations used in this study. Similarly, in a study conducted by Maguire *et al.* (17) on the Croatian *P. leptodactylus* population, *A. astaci* isolates were reported as both genotypes A and B. In this study, most of the *A. astaci* samples obtained from Turkish crayfish populations were genotype B. This genotype has been reported in the *Pacifastacus leniusculus* population (6, 9). Rezinciuc *et al.* (24) reported all *A. astaci* isolates as genotype B in *Austropotamobius pallipes* populations from the Iberian Peninsula of Spain. Grandjean *et al.* (8) reported genotype B in *Pacifastacus leniusculus*, *Astacus astacus* and *Austropotamobius pallipes* from France, Finland, Norway and Czechia, whereas genotype A was found in different French and Czech populations of noble crayfish.

Although crayfish plague is a highly destructive phenomenon in susceptible crayfish species, some *A. astaci* strains appear to show signs of lower virulence (11, 19). Therefore, knowing the genotype of the plague pathogen can be of great benefit for the management of crayfish stocks. Some studies on virulence variation of *A. astaci* genotypes in different outbreaks documented that genotypes and isolates varied in virulence (10, 12, 18, 19). During the spread of this agent in Europe, some genotypes have reportedly adapted to their hosts (12), and two genotypes, A (As) and B (Psl) detected in some water bodies in Türkiye in this study, are currently causing crayfish plague with variable mortality rates in Europe (12, 19). There is a lack of information on the virulence of these genotypes in controlled laboratory conditions on crayfish from *P. leptodactylus* populations from Türkiye. However, it was reported that genotype A caused very variable mortality among native European crayfish in laboratory trials (19) as well as in wild populations (11, 14). Makkonen *et al.* (18) reported that genotype B virulence was higher in Finnish *Astacus astacus* populations, whereas that of genotype As was more variable and lower in groups infected with these isolates. Furthermore, the researchers reported signs of increased resistance to some of the tested strains belonging to the As genotype of *A. astaci* in different noble crayfish populations. Therefore, it is of great importance to determine the virulence of the *A. astaci* strains causing crayfish plague in Türkiye.

Conclusion

Crayfish plague poses a significant threat to crayfish populations, necessitating the development of rapid, highly sensitive diagnostic methods. This study has yielded significant insights into the prevalence, detection sensitivity and genetic diversity of *A. astaci* in Turkish crayfish populations. The impressive sensitivity

limit of 500 fg/µL which resulted underscores the effectiveness of PCR assays, particularly the 40-cycle PCR, in detecting *A. astaci* DNA. Tissue examinations revealed a variable prevalence of crayfish plague across different locations, with the highest (68.2%) observed in Lake Taşkısığı. Additionally, microsatellite analysis unveiled the presence of distinct genetic strains (genotypes A and B) of *A. astaci* in various locations, genotype A being exclusively identified in Lake Suğla and genotype B predominating in the remaining sampled locations. These findings emphasise the need for continuous monitoring and conservation measures in Turkish crayfish populations to protect them from the detrimental effects of crayfish plague. The high sensitivity of PCR assays and the identification of genetic strains offer valuable tools for disease management and population conservation strategies. An in-depth understanding of crayfish plague dynamics is crucial for preserving the biodiversity of Turkish crayfish and their ecosystems. The needs for future research and action have become evident, as expanding the geographical scope of sampling, especially those water bodies previously unexamined, and concomitant analysis would provide a more comprehensive picture of the distribution of crayfish plague in Türkiye.

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