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DNA Sequence Based Molecular Identification of *Eustrongylides excisus* Larvae in *Sander Lucioperca* from Lake Eğirdir

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ABSTRACT

In this study, 926-954 bp partially distinct nucleotide sequences which belong to the 18S and 28SrDNA gene loci from 3 *Eustrongylides excisus* samples were identified as identical and registered in GenBank (OP480437-39). Nucleotide values in 18S and 28S rDNA gene sequences of *Eustrongylides excisus* samples were defined respectively: 26.94% 27.26 A; 26.78%-26.84% T; 28.30-28.62% G; 17.39-17.92% C. According to the sequence dataset distance matrix obtained by the Pairwise comparison method, there was a complete agreement (0%) between the *Eustrongylides excisus* isolates identified in this study (OP480437-39) and the *Eustrongylides excisus* isolate registered in the Gen-Bank (MK007967, MT415236, MK545494).The *Eustrongylides excisus* isolates were collected in the same cluster in Maximum likelihood filogram analysis. The internal transcribed spacer (ITS) region gene sequence results of the isolates confirmed the taxonomic position of *Eustrongylides excisus*, which was defined according to its anatomical and morphological features. More, 18S and 28S rDNA gene sequences were defined for the first time in this study on 3 samples of *Eustrongylides excisus* species in Lake Eğirdir, and contributions were made to the determination of genetic characteristics of the species.

Keywords: *Eustrongylides excisus*, DNA sequencing, *Sander lucioperca*

Eğirdir Gölü'nden *Sander Lucioperca'daki Eustrongylides excisus Larvalarının* DNA Dizisi Tabanlı Moleküler Tanımlaması

ÖZ

Bu çalışmada, 3 *Eustrongylides excisus* örneğinden 18S ve 28SrDNA gen lokuslarına ait 926-954 bp kısmi belirgin nükleotid dizileri özdeş olarak tanımlanmış ve GenBank'ta kayıt altına alınmıştır (OP480437-39). *Eustrongylides excisus* örneklerinin 18S ve 28SrDNA gen dizilerindeki nükleotid değerleri tanımlanmıştır: % 26,94-27,26 A; %26,78-26,84 T; % 28,30-28,62 G; % 17,39-17,92 C. Pairwise comparison metodu ile elde edilen dizi veri kümesi mesafe matrisine göre, bu çalışmada tanımlanan *Eustrongylides excisus* izolatları (OP480437-39) ile GenBank'ta kayıtlı *Eustrongylides excisus* izolatları (MK007967, MT415236, MK545494) arasında tam bir uyum görülmüştür (%0,0). Bu *Eustrongylides excisus* izolatları Maximum likelihood filogram analizinde aynı kümede toplanmıştır. İzolatların internal transkripsiyonlu ayırıcı (ITS) bölgesi gen sekansı sonuçları, *Eustrongylides excisus*'un anatomik ve morfolojik özelliklerine göre tanımlanan taksonomik konumunu doğrulamıştır. Ayrıca, Eğirdir Gölü'ndeki *Eustrongylides excisus* türüne ait 3 örnek üzerinden 18S ve 28SrDNA gen dizileri ilk defa bu çalışmada tanımlanmış ve bu türe ait genetik özelliklerin belirlenmesi çalışmalarına katkı yapılmıştır.

Anahtar Kelimeler: *Eustrongylides excisus*, DNA sekanslama, *Sander lucioperca*

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INTRODUCTION

Eustrongylides spp, which includes several parasitic species, is distributed in wide geographical areas including North and South America, Europe, East Africa and Asia. Three valid species are accepted in the genus *Eustrongylides*, *E.tubifex*, *E.ignotus* and *E.excisus* (Moravec et al., 2003).

Currently, molecular analyses using sequencing of the Internal transcribed spacer (ITS rDNA) and mitochondrial cytochrome c oxidase subunit I (mtCOI DNA) regions have proven useful for accurate identification of *Eustrongylides* spp at the larval and adult level (Abe, 2011; Xiong et al., 2013; Guardone et al., 2021). In this context, Mazzone et al. (2019) identified both adults and L4 larval stages of *Eustrongylides excisus* with ITS array-based molecular methods along with anatomical and morphological descriptions. Similarly, Pekmezci and Bolukbas (2021) reported the results of binary genetic distance analysis based on ITS regions of *Eustrongylides excisus* specimens at the L4 stage larvae by Mazzone et al. (2019) with the adult *Eustrongylides excisus* specimens and concluded that they belong to the same taxon.

In this study; The taxonomic positions of the samples of *Eustrongylides excisus* at the Larval-4 stage recorded in *Sander lucioperca* from Lake Eğirdir were defined using sequence data of 18S and 28SrDNA loci. The sequence data of *Eustrongylides excisus* samples, which were the research material, were analyzed for compatibility with *Eustrongylides excisus* isolates in the NCBI database. The obtained results will contribute to, it will contribute to revealing the genetic characteristics of the *Eustrongylides excisus*species on a local and global scale.

MATERIAL AND METHOD

Material

Lake Eğirdir is located within the Mediterranean Region, between 37°50'-38°16' north latitudes and 30°57'-30°44' east longitudes. The Lake, located within the borders of Isparta province in the south of Türkiye, is Türkiye's fourth-largest lake and also the second-largest freshwater lake with an area of 457 km². The height of the lake is 918 m above sea level and its average depth is 8 meters. Egirdir Lake, located in the Lakes Region, has a length of 50 km in the north-south direction, a coastline of 150 km, 16 km at its widest area (Kaptan and Özan, 2014).

Eustrongylides excisus specimens, were collected from 15 *Sander lucioperca* caught from Lake Eğirdir. These nematodes were removed from the fish's abdominal peritoneum and identified by stereo microscopy using cephalic papilla and similar anatomical and morphological features according to the (Moravec, 1994). Parasitic worms were fixed in 70% ethanol for DNA extraction.

Method

Samples in 70% ethanol were used for the extraction of *Eustrongylides excisus* DNA. For this, EurX Gene MATRIX Tissue and Bacterial DNA isolation kit (Poland) were used. DNA isolation was carried out according to the methodology established by Koehler et al. (2009).

Total DNA was suspended in nuclease-free ultrapure water. DNA concentration and purity for each sample were measured by spectrophotometry using NanoDrop ND 2000 (ThermoScientific, Wilmington, USA) at absorbance wavelength ratios of 260/280 nm and 260/230 nm, respectively. DNA integrity was evaluated by agarose gel electrophoresis and 1.5% ethidium bromide staining. DNA isolates were stored at -20 °C (Koehler et al., 2009).

One-step PCR was performed to amplify the approximately 700 base regions of 18S and 28S rDNA. The PCR reaction was carried out with Solis Biodyne (Estonia) FIREPol® DNA Polymerase Taq polymerase enzyme. 18SF and 28SR primers were used for the targeted 18SrDNA and 28SrDNA regions in the PCR study (Table 1).

It was confirmed that the band size of the PCR products belonged to the desired gene regions by comparison with the DNA standard criterion. During the purification phase of the PCR product, the MAGBIO "High Prep™ PZR Clean-up System" (AC-60005) purification kit was used for the single band samples obtained and purified by following the kit procedures. DNA bands were cut from the gel and purified with the help of EurXGeneMATRIX Agarose Out DNA purification kit (Poland). Next, bidirectional sequencing was performed using the same primers as the ABI PRISM 3130xl automated sequencer (Applied Biosystems), Macrogen (Amsterdam, The Netherlands)'s BigDye Terminator v3.1 Loop Sequencing kit. The obtained sequences were registered in the GenBank database with accession numbers OP480437, OP480438 and OP480439.

Table 1. Primers used for amplification and sequencing of 18S and 28S rDNA gene locus of *Eustrongylides excisus* samples

Target gen	Primers	Sequences (5'-3')	Reference
18S, 28S rDNA	18SF	TTGGATGATTCGGTGAGGT	Xiong et al., 2013
	28SR	AACCGCTTAGTAATATGCT	

The resulting raw sequence was evaluated for quality score (Q ≥ 20) before combining data (Kearse et al., 2012). The sequences were then compared with reference *Eustrongylides excisus* sequences in the NCBI BLAST database environment (Altschul et al., 1990). Confirmed ITS sequences were aligned using Clustal-X. Alignments were visually inspected in Seaview. Pairwise estimates of variations between isolate sequences were calculated using the Kimura two-parameter (K2P) model on the MEGA X base (Kumar et al., 2018). 18S and 28SrDNA sequences were used in phylogenetic analysis (Table 2).

The maximum Likelihood method (ML) was used for phylogenetic analysis (Tamura et al., 2004). *Pellioditis marina* was used as the outgroup. Bootstrap analysis was performed with 1,000 replicates to evaluate the support of each branch in the tree. Values of 70% and above were taken as significant.

RESULTS AND DISCUSSION

Results

Eustrongylides excisus samples were found in 3 of the 15 *Sander lucioperca* examined within the scope of the research. The body weight values of the host fish samples were between 20.1-39.2 g and the length were between 13.0-17.7 cm. Specimens of the larval *Eustrongylides excisus* were recorded in the peritoneum of the host fishes. Redness and necrosis findings were observed in the peritoneal tissue where the parasites were found.

Table 2. Data of *Eustrongylides* sp. isolates used in phyllogram analysis

Species	Locality	Gen locus	NCBI Numbers	References
Eustrongylides excisus	Eğirdir Lake, Türkiye	18S, 5.8S, 28S rDNA	OP480437 OP480438 OP480439	This study
Eustrongylides excisus	Derbent Dam Lake, Samsun, Türkiye	18S, 5.8S, 28S rDNA	MK007967	Pekmezci and Bolukbas, 2021
Eustrongylides sp.	Freidoonkenar, Mazandaran, Iran	18S, 5.8S, 28S rDNA	KU963206	Youssefi et al., 2020
Eustrongylides sp.	Yangtze River, Hubei, China	18S, 5.8S, 28S rDNA	GQ215501	Xiong et al., 2013
Eustrongylides excisus	Trasimeno Lake, Italy	18S, 5.8S, 28S rDNA	MK545493	Mazzone et al., 2019
Eustrongylides excisus	Massaciuccoli Lake, Lucca, Italy	18S, 5.8S, 28S rDNA	MT415236	Guardone et al., 2021
Eustrongylides ignotus	Norman, Oklahoma, USA	18S rDNA	EU394732	Koehler et al., 2009
Pellioditis marina *-Out group*	Sudbury, Ontario, Canada	mtCOI	EU394733	Guardone et al., 2021

Eustrongylides excisus specimens in the larval stage have a thick filamentous shape. It is covered by a thick cuticle with transverse stripes on the body surface. There are 12 cephalic papillae in two rows at the anterior end. All of the *Eustrongylides excisus* specimens recorded in this study were female. The body measured 23.6-32.3 (27.4) mm long and 9.6-1.3 (1.2) mm wide. The esophagus were 3.10-5.59 (4.23) mm long. The papillae were arranged throughout the body, lateral over the cuticle. The posterior tip was cup-shaped. The genitals were located on the posterior side of the body. The vulva opened out from the edge of the anus at the posterior terminal.

During the study, 18S and 28S rDNA gene loci of 4 *Eustrogylides excisus* samples collected from *Sander lucioperca* fish in Lake Eğirdir were identified. A single profile and reliable bands of the locus were recorded. Band size was determined as 926, 954 and 950 bp. The nucleotides of its locus were directly sequenced from each sample and registered in GenBank (OP480437-39). While the lowest A+T value in the 18S and 28S rDNA gene locus of the 3 isolates that were not recognized in this study was observed in the isolate Eig2 (OP480438), the highest A+T ratio was found in Eig3 (OP480439). The lowest G+C event is in the Eig3 (OP480439) sample. On the other hand, the highest G+C values were recorded in the samples Eig2 (OP480438) and Eig1 (OP480437) (Table 3).

The nucleotide sequences at the 18S and 28S rDNA loci of the three *Eustrongylides excisus* samples showed a perfect match among themselves in terms of diversity and no variation was found. The sequences of these 3 isolates also showed complete similarity with the closest isolate (MK007967) on NCBI. So, it has been determined that the existing isolates belong to the same taxon (Table 4). In addition, no haplotype variation was found among the nucleotide sequences in the 18S and 28S rDNA location of the present *Eustrogylides excisus* isolates. And thus, the haplotype (gene) diversity (Hd) value was found to be "0" (Table 4).

Table 3. Percentage values (%) of A, T, G, C nucleotides at 18S and 28S rDNAlocus of *Eustrongylides excisus* samples

References	GenBank No	A (%)	T (%)	G (%)	C (%)
This study, Eig1	OP480437	27,21	26,78	28,62	17,39
This study, Eig2	OP480438	26,94	26,83	28,30	17,92
This study, Eig3	OP480439	27,26	26,84	28,32	17,58
Xiong et al. 2013	GQ215501	27,77	27,27	27,87	17,08
Mazzone et al. 2019	MK545495	27,73	26,60	28,11	17,57
Youssefi et al. 2020	KU963206	27,87	26,91	27,87	17,34
Guardone et al. 2021	MT415237	27,67	26,54	28,18	17,61
Pekmezci andBolukbas 2021	MK007967	27,23	26,81	28,27	17,70

Table 4. Nucleotide variation and percentage values (%) at the 18S and 28S rDNA locus of *Eustrongylides excisus* samples (based on MK007967 reference isolate)

Sample No	GenBank No	Number of nucleotide variations and (%)	Positions and names of Variated Nucleotides
Eig1	OP480437	0	-
Eig2	OP480438	0	-
Eig3	OP480439	0	-

In this study, *Eustrongylides excisus* isolates (OP480437-39) and *Eustrongylides* spp isolates in the GenBank database were compared in pairwise analysis (%) over 18S and 28S rDNA nucleotide sequences. First of all, the 3 isolates of this study (OP480437-39) showed perfect agreement among themselves. Similarly, a complete agreement was determined between our 3 isolates (OP480437-39) and MT415236 and MK545494 *Eustrongylides excisus* isolates. However, among our isolates numbered OP480437-39, the taxa that are located at long distances, provided that they are within the intraspecific distance limit values, are GQ215501 and KU963206 *Eustrongylides* sp isolates, respectively. Significantly, a different species-level distance was defined between *Eustrongylides excisus* isolates and *Eustrogylides ignotus* (EU394732). *Dioctophyme renale* (EU394733) was used as the outgroup in this analysis (Table 5).

Phylogram analysis was performed using the Maximum Likelihood (ML) method using the 18S and 28S rDNA gene sequences of the *Eustrongylides excisus* isolates obtained in this study (OP480437-39) and Eustrongylides spp in the GenBank database. As a result of this analysis, *Eustrongylides excisus* isolates and *Eustrongylides ignotus* as a different species strongly supported the formation of pyelograms with a separation value of 99%. In the phylogram, OP480437-39 and MK007967 isolates from *Eustrongylides excisus* isolates obtained in the current

study showed the closest positioning among themselves. This positioning was followed by the other isolates (KU963206, MK545493, MT415236). On the other hand, *Eustrongylides* sp. isolates GQ215501, GQ215539 and GQ215514 were located further to the present isolates. Moreover, while isolates GQ215544, GQ215547, and GQ215567 formed a group among themselves, isolate MK650418 was located in a single branch. As AM398823 *Pellioditis marina* is an outgroup, it formed a different branch in the tree (Figure 1).

Table 5. Pairwise analysis data of *Eustrongylides* spp. samples according to 18S and 28S rDNA locus sequences.

	OP480437.1 *E. excisus*	OP480438.1 *E. excisus*	MK007967.1 *E. excisus*	OP480439.1 *E. excisus*	GQ215501.1 *Eustrongylides* sp.	KU963206.1 *Eustrongylides* sp.	MT415236.1 *E. excisus*	MK545494.1 *E. excisus*	EU394732.1 *E. ignotus*	EU394733.1 *Dioctophyme renale* -Dış grup
OP480437.1 *Eustrongylides excisus*										
OP480438.1 *Eustrongylides excisus*	0,000									
MK007967.1 *Eustrongylides excisus*	0,000	0,001								
OP480439.1 *Eustrongylides excisus*	0,000	0,003	0,003							
GQ215501.1 *Eustrongylides* sp.	0,026	0,030	0,027	0,027						
KU963206.1 *Eustrongylides* sp.	0,004	0,004	0,005	0,005	0,033					
MT415236.1 *Eustrongylides excisus*	0,000	0,000	0,000	0,000	0,027	0,000				
MK545494.1 *Eustrongylides excisus*	0,000	0,000	0,000	0,000	0,024	0,000	0,000			
EU394732.1 *Eustrongylides ignotus*	1,354	1,362	1,344	1,359	1,413	1,390	1,409	1,408		
AM398823.1 *Pellioditis marina* -*Out group*	1,363	1,363	1,363	1,363	1,429	1,363	1,363	1,363	1,812	

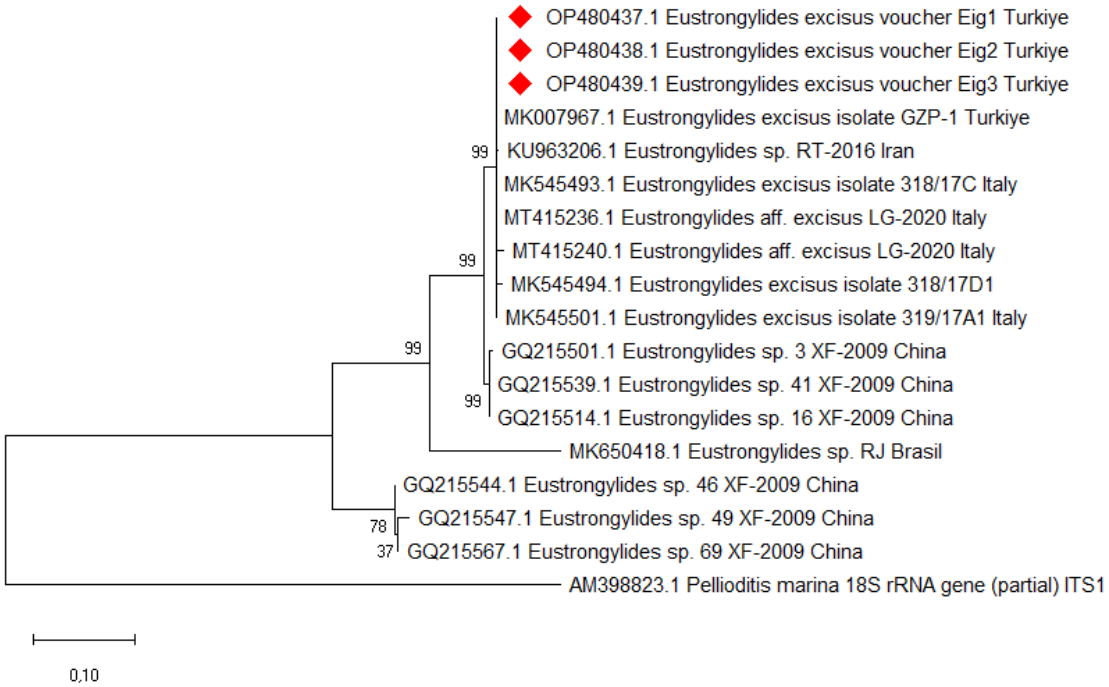

Figure 1. Maximum likelihood (ML) tree based on 18S and 28S rDNA locus sequence data of *Eustrongylides* spp samples from the current study and the GenBank database

Discussion

Various fish species are paratenic hostsfor *Eustrongylides excisus* in the taxon Dioctophymatidae (Spalding et al., 1993). *Eustrongylides excisus* larvae cause various pathogenic phenomena in fish species which serve as intermediate hosts. Findings such as large scars, lesions, and kidney damage were observed in the tissues where these nematode larvae were found (Spalding et al., 1993; Bjelić-Čabrilo et al., 2013). In addition, it has been reported that this parasite causes hyperemia, oedema, mild bleeding, inflammatory reactions, hyperemia in mesenteric vessels, external nodules, and necrosis in muscles (Innal et al., 2019; Youssefi et al., 2020). Tissue thickening, nodules, and redness were observed in the peritoneum of infected host fish in this study.

Sander lucioperca is an important commercial fish species in Türkiye and is widely used in traditional cuisine (Balık et al., 2006; Çelik et al., 2005; Çağlak and Karslı, 2013; İlhan and Sarı, 2013). Similarly, it has commercial importance in countries such as Croatia, Greece, Spain, and Italy (Maci and Basset, 2010). Recently, the preference for raw or undercooked fish as food is a rising trend. Therefore, parasitological monitoring of *E. excisus* is important for public health (Eiras et al., 2018). Cases such as gastritis and intestinal tract infection and outgrowth of parasites have been described in humans eating raw or undercooked fish containing *Eustrongylides* nematodes (Ibiwoye et al., 2005). In support of these data, *Eustrongylides excisus* can be defined as a potentially dangerous parasite for public health in Türkiye due to its wide distribution in various freshwater fish and the increase in the preference for eating undercooked or raw fish in recent years.

Anatomical and morphological structures of the current research samples were examined in terms of their diagnostic features (caudal morphology, caudal and anterior papilla features, nerve ring and esophagus, and vulva). The anatomical and morphological features of *Eustrongylides excisus* specimens examined in this study showed complete similarity with the data of other researchers who studied this species (Measures, 1988; Mazzone et al., 2019; Pekmezci and Bolukbas, 2021).

In Türkiye, *Eustrongylides excisus* has been recorded in various fish species according to their anatomical and morphological features: *Gobius fluviatilis* (Öztürk et al., 2001), *Perca fluviatilis* (Soylu, 2013), *Atherina boyeri* (Çolak, 2013), *Sander lucioperca* (Metin et al., 2014; Özmen et al., 2021; Tanrıkul et al., 2019), *Aphanius transgrediens* (İnnal et al., 2019).

The first molecular study in Türkiye to describe *Eustrongylides excisus* from *Sander lucioperca* on ITS, SSU rRNA, and COI sequences was carried out by Pekmezci and Bolukbas, 2021). This is the second study in Türkiye to identify the *E. excisus* species recorded from *Sander lucioperca* in Lake Eğirdir using a molecular tool based on the 18S and 28S rDNA locus.

The base value ratios in the sequences at the 18S and 28S rDNA locus of the *Eustrongylides excisus* samples examined in the present study were defined. Among these samples, the lowest A+T value was observed in isolate Eig2 (OP480438), while the highest A+T ratio was found in Eig3 (OP480439). While the lowest G+C phenomenon was Eig3 (OP480439), the highest G+C value was recorded in Eig2 (OP480438) and Eig1 (OP480437) samples.

In addition, a great similarity was found between the A, T, G, C, % values at the 18S and 28S rDNA locus of *Eustrongylides excisus* isolates identified by former researchers (Xiong et al., 2013; Mazzone et al., 2019; Youssefiet al., 2020; Guardone et al., 2021; Pekmezci and Bolukbas, 2021) and the distribution ratios of the bases in the 18S and 28S rDNA sequence of the isolates examined in this study. The great similarity between the base distribution ratios can be considered strong evidence showing the common ancestral homology of these isolates.

No variation was found between the 18S and 28S rDNA locus nucleotides of the three isolates (OP480437-OP480439) identified during the research process. Thus, it was determined that these isolates belong to the same taxon according to the sequence data of this locus. More, according to pairwise genetic distance analysis, 100% identity was defined between the 18S and 28S rDNA sequences (OP480437-39) of *Eustrongylides excisus* larvae identified in this study and the sequences of *Eustrongylides excisus* isolates MT415236 and MK545494 from Italy (Mazzone et al., 2019; Guardone et al., 2021). A difference of 0.1% was found with *Eustrongylides* spp isolate (MK007967) from Türkiye (Pekmezci and Bolukbas, 2021). 0.4% variation was also seen with 18S rDNA sequences (KU963206) of the same species identified from Iran (Youssefi et al., 2020). However, genetic variation among 18S rDNA sequences of *Eustrongylides excisus* isolate GQ215501 identified by Xiong et al. (2013) (China) is greater, 0.26%. Variation values between isolates showed that these samples represent the same taxon. In addition, it was determined that the sequence variation among *Eustrongylides excisus* isolates increased as the geographic isolation distance increased.

As a result of the maximum likelihood analysis, it was observed that *Eustrogylides excisus* isolates (OP480437-39) and other *Eustrogylides excisus* isolates (MK007967, KU963206, MK545493, MT415236, MT415240) obtained in the present study clustered on a monophyletic branch with a bootstrap value of 99%. The results showed that the 18S and 28S rDNA locus can be successfully used to identify the relevant nematode species in phylogenetic studies. The 18S and 28S rDNA gene sequence of *Eustrongylides excisus* isolates (OP480437-39) was not different from the MK007967 *Eustrongylides excisus* isolate and was located in the nearest branch. *Eustrogylides excisus* specimens at the larval 4 stages examined in this study showed exact matches with the 18S and 28S rDNA sequence of the *Eustrogylides excisus* isolate (MK007967, MK545493, MT415236). This result confirmed that our research samples belong to the genus *Eustrogylides excisus* genetically according to the 18S and 28S rDNA locus. Pekmezci and Bolukbas (2021) stated that the exact and correct identity of larval nematodes can be identified through genetic sequences if they show the same or very high similarity rates with well-defined adult nematode sequences. The present study results also supported this view.

CONCLUSION

In conclusion, with this study, the 18S and 28S rDNA gene sequence of the parasite *Eustrongylides excisus* in *Sander lucioperca* collected from Lake Eğirdir in Türkiye was identified for the first time. The 18S and 28S rDNA sequence of 3 *Eustrongylides excisus* specimens confirmed its taxonomic position by identifying it according to morphological structure and molecular data. *Eustrongylides excisus* isolates examined in the present study showed complete identity among themselves in terms of the gene sequence. These valid genetic data contributed to the establishment of the phylogenetic relationships of *Eustrongylides* species, the creation of the DNA barcode library of *Eustrongylides excisus*, and its taxonomic origin. In addition, further studies using the same genetic markers are needed to examine genetic variability and population genetic structure in larvae and adults of *Eustrongylides* species in Türkiye.

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