

Morphological and Molecular Characterization of *Eustrongylides excisus* in *Neogobius fluviatilis* from Lake Uluabat with Global Phylogenetic Analyses

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Abstract

DNA-based species identification methods enable the reliable determination of species and a more accurate elucidation of evolutionary relationships. Internal transcribed spacer (ITS) rDNA regions are used as a reliable molecular marker in species discrimination. This study investigated the zoonotic nematode *Eustrongylides excisus* in *Neogobius fluviatilis* population of Lake Uluabat, Türkiye, contributing to the understanding of its genetic diversity, taxonomic status, and zoonotic risk. Sequencing of the ITS region identified a single, distinct haplotype from the Lake Uluabat isolates. Comparative genetic analysis revealed low-to-moderate intraspecific variation (pairwise distances: 0.000–0.0404) among global isolates. Phylogenetic analysis confirmed the monophyly of *E. excisus* and revealed geographically correlated clustering. Turkish isolates formed a distinct, well-supported clade most closely related to a homogeneous Italian clade. Chinese and Australian isolates showed greater internal diversity and grouped separately. The genetic data provide new insights into the structure of *E. excisus*, demonstrating both widespread, conserved lineages and region-specific diversification. The close phylogenetic relationship between Turkish and Italian isolates points to historical connectivity within the Mediterranean basin. These findings are crucial for monitoring the parasite's spread, understanding its evolutionary history, and developing targeted risk assessments for human infection.

Introduction

Nematodes of the genus *Eustrongylides* are cosmopolitan parasites that utilize a complex life cycle involving aquatic oligochaetes as first intermediate hosts, various fish species as second intermediate or paratenic hosts, and piscivorous birds as definitive hosts (Novakov et al., 2013). The larval stages, particularly *Eustrongylides excisus*, are of significant ecological and sanitary concern due to their pathogenicity in fish and

their zoonotic potential, causing human eustrongylidosis through the consumption of raw or undercooked infected fish (Measures, 1988).

Accurate species identification and an understanding of their population genetics are crucial for assessing their distribution, host specificity, and evolutionary history. While morphological characterization remains a fundamental tool for identification, molecular markers, such as the internal transcribed spacer (ITS) region of ribosomal DNA, have

proven indispensable for resolving taxonomic ambiguities and revealing cryptic diversity within this genus (Nadler and De León, 2011).

The aim of this study is to investigate the presence and genetic structure of *Eustrongylides excisus* in *Neogobius fluviatilis* host fish caught from Lake Uluabat in Türkiye. Morphological observations were combined with molecular and phylogenetic analyses of the ITS rDNA region to characterize present parasite isolates. The aims were to confirm species identification, evaluate genetic diversity and determine the phylogenetic relationships of Turkish isolates and other geographical regions. These results provide current insights into the speciation dynamics of *Eustrongylides* populations in different geographical regions, based on ITS gene region characteristics, and contribute to a broader understanding of the parasite's genetic diversity, taxonomic status.

Material and Methods

Study Area

Lake Uluabat is situated approximately 30 km west of Bursa and east of the Mustafakemalpaşa district, at coordinates 40°12' N and 28°40' E. The lake encompasses a surface area of approximately 116 km² and is characterized as a shallow freshwater body, with a mean depth of roughly 2.5 meters. It is situated at an elevation of about 9 meters above sea level. The lake's primary hydrological outflow drains into the Sea of Marmara via the Kocasu Stream (İleri, 2010).

Sampling Study

Fish sampling was carried out in the coastal zone of the Eskikaraağaç region of Lake Uluabat in September 2024. Specimens were collected using a seine net fitted with anchovy-style mesh. The net measured 5 m in arm length, had a 5 m mouth opening, a height of 1.5 m, and a mesh size of 5 mm. Using this gear, a total of 128 *Neogobius fluviatilis* samples were captured.

The *Neogobius fluviatilis* samples were examined macroscopically for *E. excisus*. The parasites were carefully removed, rinsed with distilled water, and preserved in cryogenic tubes containing 70% ethanol. Subsequently, the samples were transferred to the Fish Health Laboratory of the Eğirdir Fisheries Research Institute for further analysis. Nematode specimens were identified as *E. excisus* according to Moravec (1994), taking into account specific species characteristics.

DNA Isolation and PCR

Genomic DNA was extracted from three *Eustrongylides excisus* specimens using the High Pure PCR Template Preparation Kit (Roche Applied Science), following the manufacturer's protocol. Polymerase Chain Reaction (PCR) amplification was subsequently

performed with the 2× PCR Master Mix kit (Thermo Scientific, Carlsbad, California, USA).

Specific primer sequences used for amplification of the nuclear ribosomal Internal Transcribed Spacer (ITS) region are provided in Table 1. Polymerase Chain Reaction (PCR) mixtures were prepared in a total volume of 25 µl. Each reaction contained 1 µM of each primer, 12.5 µl of 2× PCR Master Mix, 1–2 µl of genomic DNA, and nuclease-free distilled water (Thermo Scientific).

PCR amplification was conducted under the following thermal cycling conditions: an initial denaturation at 95°C for 5 min, followed by 35 cycles consisting of denaturation at 95°C for 30 s, primer annealing at 53°C for 45 s, and extension at 72°C for 90 s. A final extension step was performed at 72°C for 10 min. Amplified products were resolved on a 1.5% agarose gel prepared with TBE buffer (0.045 M Tris-borate, 0.001 M EDTA, pH 8.0) through electrophoresis to confirm successful amplification of the target region. Bidirectional sequencing of the PCR products was subsequently carried out by BM Lab (Ankara, Turkey) using the same primer set and the Sanger sequencing method.

Genetics and Phylogenetic Analysis

On a global scale, sequences obtained from different geographical localities were aligned using BioEdit software and formatted in FASTA files. The number of haplotypes and mutations among haplotypes was determined with DnaSP v6 (Rozas et al., 2017). Haplotype network data were organized in DnaSP v6 and exported in NEXUS format (Maddison et al., 1997). A haplotype network was subsequently constructed in PopART software using the median-joining algorithm (Leigh and Bryant, 2015).

The obtained sequence data were edited using BioEdit v7.0 and submitted to the NCBI GenBank database under the accession number PQ433131. Genomic sequences of *Eustrongylides* species from diverse geographical regions, including Turkey, Italy, China, France, Japan, India, Iran, and Australia, were retrieved via BLAST search (Altschul et al., 1990) (Table 2).

Sequences were aligned using the ClustalW algorithm implemented in MEGA X. The optimal substitution model for phylogenetic analysis was identified with the model selection function in MEGA X, based on the Bayesian Information Criterion (BIC). According to this analysis, the Tamura 3-parameter model was determined as the most appropriate for the dataset (Tamura, 1992; Kumar et al., 2018). Phylogenetic reconstruction was performed using the Maximum Likelihood (ML) method, and tree topology was evaluated with 1000 bootstrap replicates to assess the robustness of the inferred relationships. Bootstrap values were used to estimate the statistical support of the nodes. *Soboliphyme baturini* (AY277895) was

included as the outgroup species. Genetic diversity analyses were conducted in DnaSP v6.0, where parameters such as the number of haplotypes, haplotype diversity, and nucleotide diversity were calculated (Julio et al., 2017).

Results

Occurrence of *Eustrongylides excisus*

The occurrence of *E. excisus* was investigated in 128 *Neogobius fluviatilis* specimens collected from Lake Uluabat, and 63 of the host fish were infected with *E. excisus*, representing 49.2%. Parasite samples were collected from the cloacal opening of the host fish. *Eustrongylides excisus* specimens identification was carried out based on specific characteristics. Such as the segmented cuticle structure, morphology of the mouth and lips, structural features of the oral and caudal

papillae, the position of the nerve ring on the esophagus, connection shape between the esophagus and intestine, and the location of the vulva. The characteristic morphological and anatomical structures were presented in Figure 1.

Molecular and Phylogenetic Analyses

Nucleotide Polymorphism

The ITS rDNA region of three *Eustrongylides excisus* specimens collected from *Neogobius fluviatilis* was successfully amplified and sequenced. PCR amplification produced a fragment of approximately 950 bp in all samples. The nucleotide sequences obtained from the three specimens were identical, showing no intraspecific variation. Consequently, the ITS sequence from one representative isolate was submitted to the GenBank database under accession number PQ433131.

Table 1. Primer sequences targeting the ITS gene region (18S–28S)

Primer	Sequence	Reference
18SF	(5' TTGGATGATTCGGTGAGGT 3')	Xiong et al. 2013
28SR	(5' AACCGTTAGTAATATGCT 3')	

Table 2. Data of *Eustrongylides* spp isolates used in phylogram analysis

Species	Host	Locality	NCBI Numbers	References
<i>Eustrongylides excisus</i>	<i>Neogobius fluviatilis</i>	Uluabat Lake, Turkiye	PQ433131	This study
<i>Eustrongylides excisus</i>	<i>Gambusia holbrooki</i>	Egirdir Lake, Turkiye	PP333225	Eren et al., 2025
<i>Eustrongylides excisus</i>	<i>Sander lucioperca</i>	Egirdir Lake, Turkiye	OP480437 OP480438 OP480439	Ozturk and Ozturk, 2023
<i>Eustrongylides excisus</i>	<i>Sander lucioperca</i>	Derbent Dam Lake, Turkiye	MK007967	Pekmezci and Bolukbas, 2021
<i>Eustrongylides excisus</i>	<i>Phalacrocorax carbo</i>	Trasimeno Lake, Italy	MK545509 MK545510 MK545517 MK545530 MK545539 MK545540 MK545541	Mazzone et al., 2019
<i>Eustrongylides excisus</i>	<i>Perca fluviatilis</i>	Trasimeno Lake, Italy	MK545493	Mazzone et al., 2019
<i>Eustrongylides excisus</i>	<i>Perca fluviatilis</i>	Trasimeno Lake, Italy	OK380960 OK380961	Franceschini et al. 2022
<i>Eustrongylides excisus</i>	<i>Atherina boyeri</i>	Massaciucoli Lake, Italy	MT415236	Guardone et al., 2021
<i>Eustrongylides excisus</i>	<i>Perca fluviatilis</i>	Padula reservoir, Corsica, France	PP888044 PP888045 PP888046	Esposito et al. 2024
<i>Eustrongylides</i> sp.	<i>Rhinogobius</i> sp.	Tokyo, Japan	LC708132 LC708133	Anonymous 1
<i>Eustrongylides</i> sp.	<i>Esox lucius</i>	Iran	KU963206	Youssefi et al. 2020
<i>Eustrongylides</i> sp.	<i>Monopterus albus</i>	China	GQ215502 GQ215513 GQ215514 GQ215515 GQ215516 GQ215540 GQ215543 GQ215549 GQ215550 GQ215552 GQ215561 GQ215572 GQ215551 GQ215562	Xiong et al. 2013
<i>Eustrongylides excisus</i>	<i>Maccullochella peelii</i>	Australia	OP830361 OP830362 OP830363	Shamsi et al. 2023
<i>Eustrongylides</i> sp.	<i>Channa punctatus</i>	India	KJ458967	Kundu and Mandal, 2022
<i>Eustrongylides</i> sp.	<i>Alligator sinensis</i>	Anhui Province, China	PP256050	Anonymous 2
<i>Soboliphyme baturini</i> - Out group	<i>Mustela zibellina</i>	Kolyma Province, Siberia	AY277895	Rusin et al., 2003

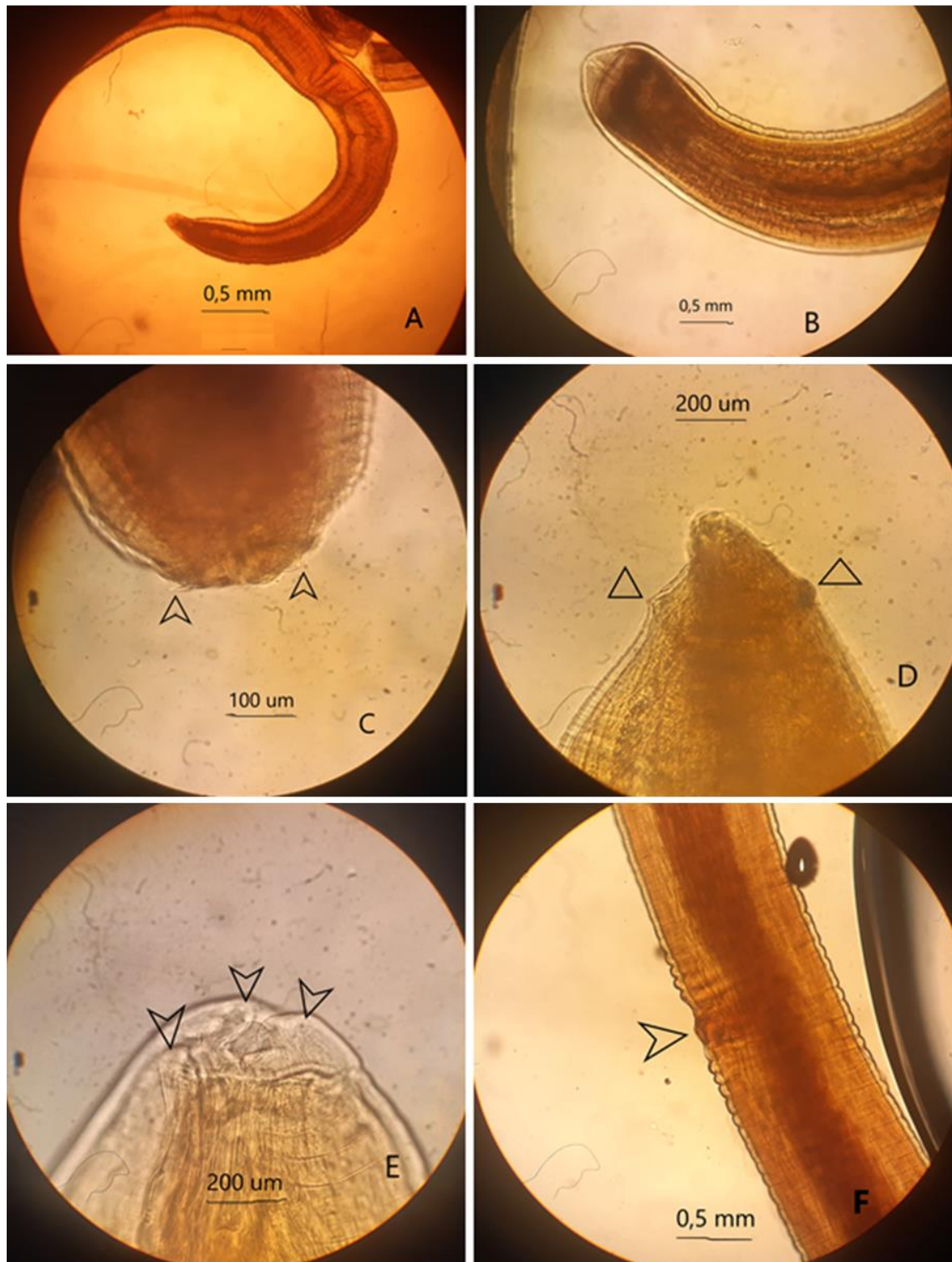


Figure 1. Visual representations of the anatomical and morphological structures of *Eustrongylides excisus* specimens. A: Anterior end, B: Posterior end, C: Minor papillae, D: Major papillae, E: Lip structures at the anterior terminal, F: Vulva (Original).

BLAST analysis against sequences available at the National Center for Biotechnology Information confirmed complete sequence identity with the closest available isolate (OP480437), indicating that the obtained specimen belongs to the same taxon as previously reported *E. excisus* isolates.

Comparative analysis of the ITS region revealed several nucleotide polymorphisms among *E. excisus*

isolates from different geographic regions. In the isolate obtained in the present study, positions 945 and 946 were identified as adenine (A) and cytosine (C), respectively. This nucleotide pattern was identical to that reported for an isolate from Iran but differed from sequences obtained from China and from an isolate collected in Lake Eğirdir, Türkiye (PP333225), which possessed thymine (T) and adenine (A) at the

corresponding positions. Notably, this region was absent in European sequences of *Eustrongylides excisus*, preventing direct comparison. Additional sequence variation among Turkish isolates was observed beyond position 850. For example, isolate MK007967.1 contained guanine (G) at position 893 and thymine (T) at position 909, whereas these sites were absent (indels) in isolates OP480437.1, OP480438.1, and OP480439.1. At position 955, MK007967.1 contained adenine (A), while the Uluabat isolate (PQ433131.1) and the other Turkish isolates possessed thymine (T). Furthermore, position 941 was guanine (G) in isolate OP480439.1 but adenine (A) in the Uluabat isolate and the remaining sequences (Figure 2).

Pairwise Genetic Distance

Pairwise genetic distance analysis based on ITS rRNA sequences revealed values ranging from 0.000 to 0.0404 (Table 3), indicating low to moderate levels of intraspecific variation. The Turkish isolates PP333225.1 (Sarem_Egirdir-1) and MK007967.1 (Turkey_GZP-1) exhibited identical ITS rRNA sequences, both between themselves and with isolates from geographically distant regions, including China (MK545493.1), Italy (MK545530.1), Japan (LC708133.1), and France (PP888046.1). Minor genetic differences (0.0013–0.0065) were observed among several Chinese isolates (GQ215543.1, GQ215549.1, GQ215552.1, and

GQ215572.1). In contrast, moderate genetic distances were detected between some Chinese isolates and those from Australia, India, and Japan. The highest genetic divergence (0.0404) occurred between the Chinese isolates GQ215502.1 and GQ215552.1.

To further evaluate genetic variation independent of geographic origin, isolates were grouped into haplotypes and analyzed separately. A total of 10 haplotypes were identified among the 42 *E. excisus* isolates included in the analysis. Pairwise genetic distance values among haplotypes ranged from 0.00131 to 0.04052. The lowest genetic distances (0.00131) were observed between several haplotype pairs, including Hap_1–Hap_7, Hap_1–Hap_10, Hap_5–Hap_6, Hap_7–Hap_9, and Hap_2–Hap_8, indicating a high degree of sequence similarity. In contrast, the greatest genetic divergence was detected between Hap_4 and Hap_9 (0.04052), followed by Hap_4–Hap_7 and Hap_4–Hap_10 (0.03922) (Table 4).

Genetic Diversity and Neutrality Test

Genetic diversity indices calculated from ITS rRNA sequence data revealed moderate haplotype diversity ($H_d = 0.630 \pm 0.080$) but low nucleotide diversity ($\pi = 0.00994$). The mean number of pairwise nucleotide differences was 7.60, indicating that although multiple haplotypes were present, sequence divergence among them remained limited. Mutation-based diversity

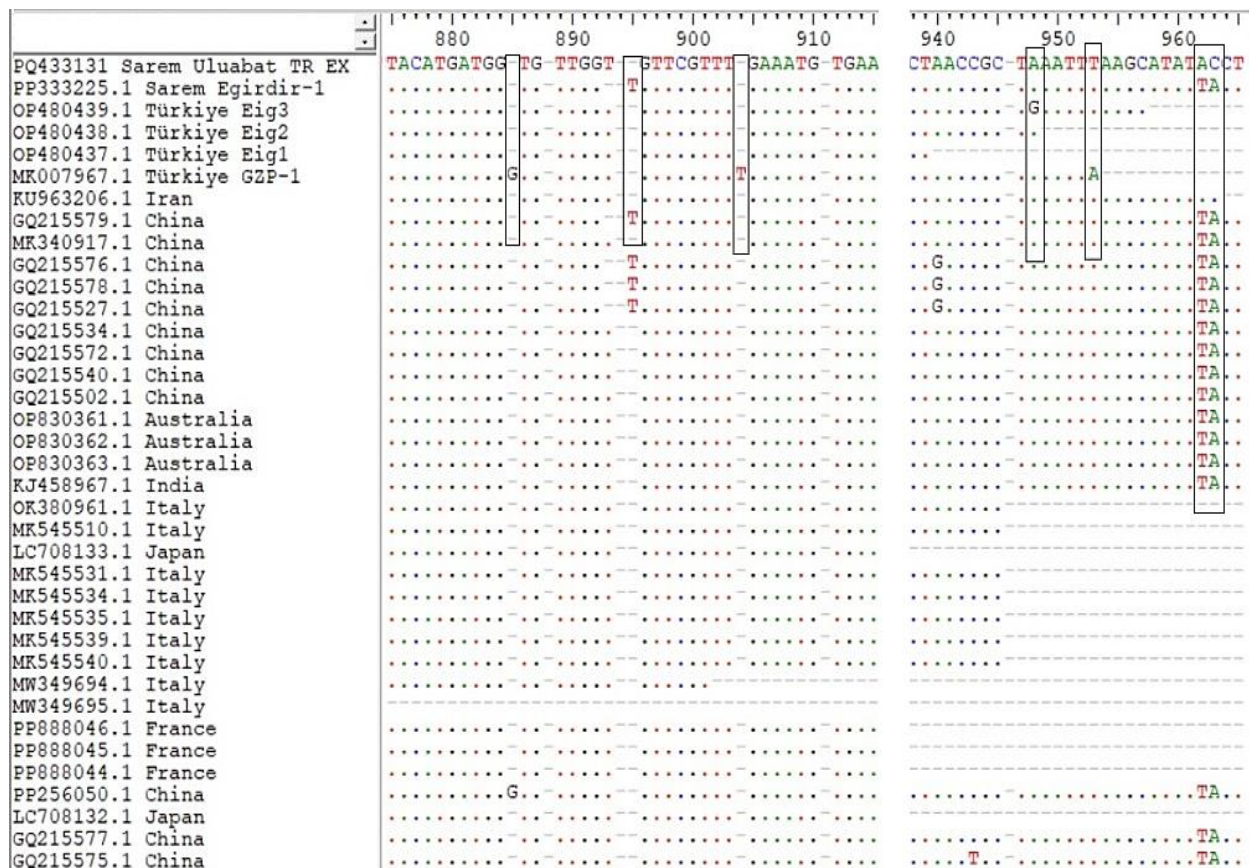


Figure 2. Sequence variation in *Eustrongylides excisus* isolates from diverse geographical regions.

estimates yielded comparable values, with theta per site ($\theta_s = 0.011$) and theta per gene ($\theta_g = 9.063$) (Table 5).

Neutrality tests produced predominantly negative values. Tajima's D was negative but not statistically significant ($D = -0.56196$; $P > 0.05$), suggesting a slight excess of low-frequency polymorphisms without strong evidence for deviation from neutral evolution. In contrast, Fu and Li's D was significantly negative (-2.55808 ; $P < 0.05$), indicating an excess of recent mutations that may reflect recent population expansion or directional selection. Fu and Li's F also yielded a negative value (-2.21131), although this result was not statistically significant ($P > 0.05$). Overall, these results indicate low to moderate genetic variation within *E. excisus*, with limited but detectable signals consistent with recent demographic or selective processes.

The haplotype network constructed from ITS rRNA sequence data displayed a predominantly star-like topology, characterized by a central haplotype connected to several peripheral haplotypes by a small number of mutational steps. This structure indicates low overall genetic differentiation among haplotypes and is consistent with a recent demographic expansion or rapid dispersal of *E. excisus*. The short mutational distances and low connectivity among nodes further suggest that the ITS region is relatively conserved within the species, in agreement with the low to moderate genetic divergence observed in pairwise distance analyses (Figure 3).

The central haplotype included isolates originating from diverse geographic regions, including Türkiye, Italy, China, Iran, Japan, France, Australia, and Indonesia,

Table 3. Pairwise genetic distance matrix of *Eustrongylides excisus* isolates from different geographic regions based on the ITS rRNA gene

PQ433131_Sarem_Uluabat	PP333225.1	MK007967.1	KU963206.1	GQ215540.1	GQ215543.1	GQ215549.1	GQ215552.1	GQ215502.1	GQ215572.1	MK545493.1	OP830361.1	OP830362.1	KJ458967.1	MK545530.1	LC708133.1	PP888046.1
PP333225.1_Sarem_Egirdir-1	0,0000000000															
MK007967.1_Turkiye_GZP-1	0,0000000000	0,0000000000														
KU963206.1_Iran	0,0000000000	0,0000000000	0,0000000000													
GQ215540.1_China	0,0234375000	0,0234680574	0,0234375000	0,0234375000												
GQ215543.1_China	0,0013037810	0,0013054830	0,0013037810	0,0013020833	0,0247395833											
GQ215549.1_China	0,0026075619	0,0026109661	0,0026075619	0,0026041667	0,0260416667	0,0039062500										
GQ215552.1_China	0,0221354167	0,0221642764	0,0221354167	0,0221354167	0,0038709677	0,0234375000	0,0247395833									
GQ215502.1_China	0,0378096480	0,0378590078	0,0378096480	0,0378096480	0,0258397933	0,0391134289	0,0404172099	0,0378096480								
GQ215572.1_China	0,0208333333	0,0208604954	0,0208333333	0,0208333333	0,0025806452	0,0221354167	0,0234375000	0,0208333333	0,0232558140							
MK545493.1_China	0,0000000000	0,0000000000	0,0000000000	0,0000000000	0,0234375000	0,0013020833	0,0026041667	0,0000000000	0,0378096480	0,0208333333						
OP830361.1_Australia	0,0182291667	0,0182529335	0,0182291667	0,0182291667	0,0077419355	0,0195312500	0,0208333333	0,0182291667	0,0232558140	0,0051612903	0,0182291667					
OP830362.1_Australia	0,0169270833	0,0169491525	0,0169270833	0,0169270833	0,0064516129	0,0182291667	0,0195312500	0,0169270833	0,0219638243	0,0038709677	0,0169270833	0,0012903226				
KJ458967.1_India	0,0208333333	0,0208604954	0,0208333333	0,0208333333	0,0025806452	0,0221354167	0,0234375000	0,0208333333	0,0232558140	0,0000000000	0,0208333333	0,0051612903	0,0038709677			
MK545530.1_Italy	0,0000000000	0,0000000000	0,0000000000	0,0000000000	0,0234375000	0,0013037810	0,0026075619	0,0000000000	0,0378096480	0,0208333333	0,0000000000	0,0182291667	0,0169270833	0,0208333333		
LC708133.1_Japan	0,0000000000	0,0000000000	0,0000000000	0,0000000000	0,0234375000	0,0013020833	0,0026041667	0,0000000000	0,0378096480	0,0208333333	0,0000000000	0,0182291667	0,0169270833	0,0208333333	0,0000000000	
PP888046.1_France	0,0000000000	0,0000000000	0,0000000000	0,0000000000	0,0234375000	0,0013020833	0,0026041667	0,0000000000	0,0378096480	0,0208333333	0,0000000000	0,0182291667	0,0169270833	0,0208333333	0,0000000000	0,0000000000

Table 4. Pairwise genetic distance values of ITS rRNA gene of 10 haplotypes of *Eustrongylides excisus*.

	A	B	C	D	E	F	G	H	I	J
1 Hap_1										
2 Hap_2	0,02092									
3 Hap_3	0,02353	0,00261								
4 Hap_4	0,03791	0,02353	0,02614							
5 Hap_5	0,01830	0,00523	0,00784	0,02353						
6 Hap_6	0,01699	0,00392	0,00654	0,02222	0,00131					
7 Hap_7	0,00131	0,02222	0,02484	0,03922	0,01961	0,01830				
8 Hap_8	0,02222	0,00131	0,00392	0,02484	0,00654	0,00523	0,02353			
9 Hap_9	0,00261	0,02353	0,02614	0,04052	0,02092	0,01961	0,00131	0,02484		
10 Hap_10	0,00131	0,02222	0,02484	0,03922	0,01961	0,01830	0,00261	0,02353	0,00392	

Table 5. Summary of Genetic Diversity Indices and Neutrality Tests in *Eustrongylides excisus* samples based on ITS rRNA Sequence Data

N	H	hd±SD	π (k)	θ_s	θ_g	Tajima's D	p value	FLD	p value	FLF	p value
42	10	0.630±0.080	0.00994 (7.60163)	0.011	9.063	-0.56196	P>0.05	-2.55808	P<0.05	-2.21131	0.05<P

Abbreviations: n: number of sequences, h: number of haplotypes, hd: haplotype diverse, SD standard deviation, π : nucleotide diversity, k: mean number of pairwise nucleotide differences, θ_s : theta per site, θ_g : theta per gene FLD: Fu and Li's D test statistic; FLF: Fu and Li's F test statistic (Fu and Li 1993, Tajima 1989).

indicating a widely distributed and genetically homogeneous lineage. In contrast, peripheral haplotypes were represented by fewer isolates and often showed more restricted geographic distributions, suggesting the accumulation of recent localized mutations. The occurrence of identical or closely related haplotypes across distant regions, together with the distribution of isolates from the same geographic origin among multiple haplotypes, indicates limited phylogeographic structuring and suggests ongoing or historical gene flow among populations (Figure 4).

Phylogenetic Analysis

Phylogenetic analysis further supported these findings. The phylogenetic tree showed that Turkish isolates, including those generated in the present study, formed a distinct and well-supported clade. Italian isolates constituted a separate and homogeneous cluster with minimal internal variation and were the closest phylogenetic lineage to the Turkish clade, suggesting a relatively close evolutionary relationship between these Mediterranean populations. In contrast,

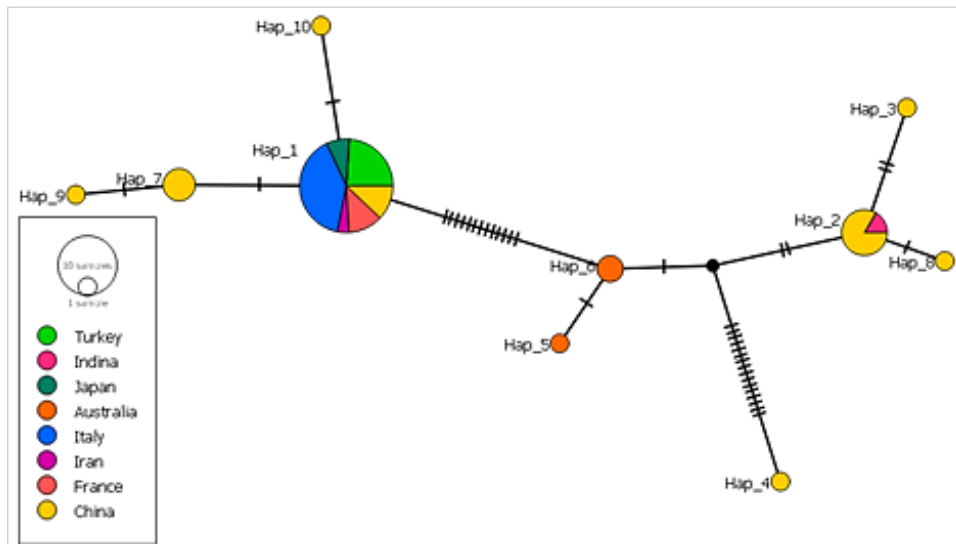


Figure 3. Worldwide haplotype network of *Eustrongylides excisus* isolates constructed using the ITS rRNA gene region.



Figure 4. Location of the haplotype network of *Eustrongylides excisus* isolates based on the ITS rRNA gene regions on a world map.

Chinese isolates exhibited considerable intraspecific diversity and formed several subclades. Australian isolates grouped into a distinct subclade positioned adjacent to the Chinese clusters, indicating a closer evolutionary relationship between these regional groups than with other lineages included in the analysis. The phylogenetic tree was rooted using *Soboliphyme baturini* as the outgroup taxon (Figure 5).

Discussion

Eustrongylides excisus has a complex, multi-host life cycle involving oligochaetes, fish, and aquatic birds. It is the causative agent of eustrongylidosis, a parasitic disease primarily affecting piscivorous birds such as herons, cormorants, moorhens, and ducks, often leading to host mortality (Moravec, 1994; Branciarini et al., 2016). Research at Lake Bracciano, Italy, identified cormorants (*Phalacrocorax carbo*) as definitive hosts and found a positive correlation between the local cormorant population size and the prevalence of *E.*

excisus (Rusconi et al., 2022). This dynamic is also relevant to Lake Uluabat, an important habitat for migratory waterbirds. A 2024 census recorded 67,918 individuals from 38 species at this lake (Kosk, 2024). The substantial presence of these potential definitive hosts suggests that Lake Uluabat's ecosystem supports the completion and maintenance of the *E. Excisus* life cycle.

Pathological impact of *Eustrongylides excisus* larvae is typically more severe in larval-stage or small-bodied fish species than in larger ones, resulting in proportionally higher mortality rates (Morey et al., 2022). This heightened susceptibility was demonstrated by Fusco et al. (2023), who reported 100% mortality in zebrafish experimentally infected with *E. excisus*. In wild fish, infections also impair vital host functions. For instance, Kaur et al. (2013) documented encapsulated *Eustrongylides* sp. larvae within the ovaries of *Glossogobius giuris*, an infection associated with reduced reproductive capacity. The present study examined another small-bodied species, *Neogobius fluviatilis*, and observed associated pathologies

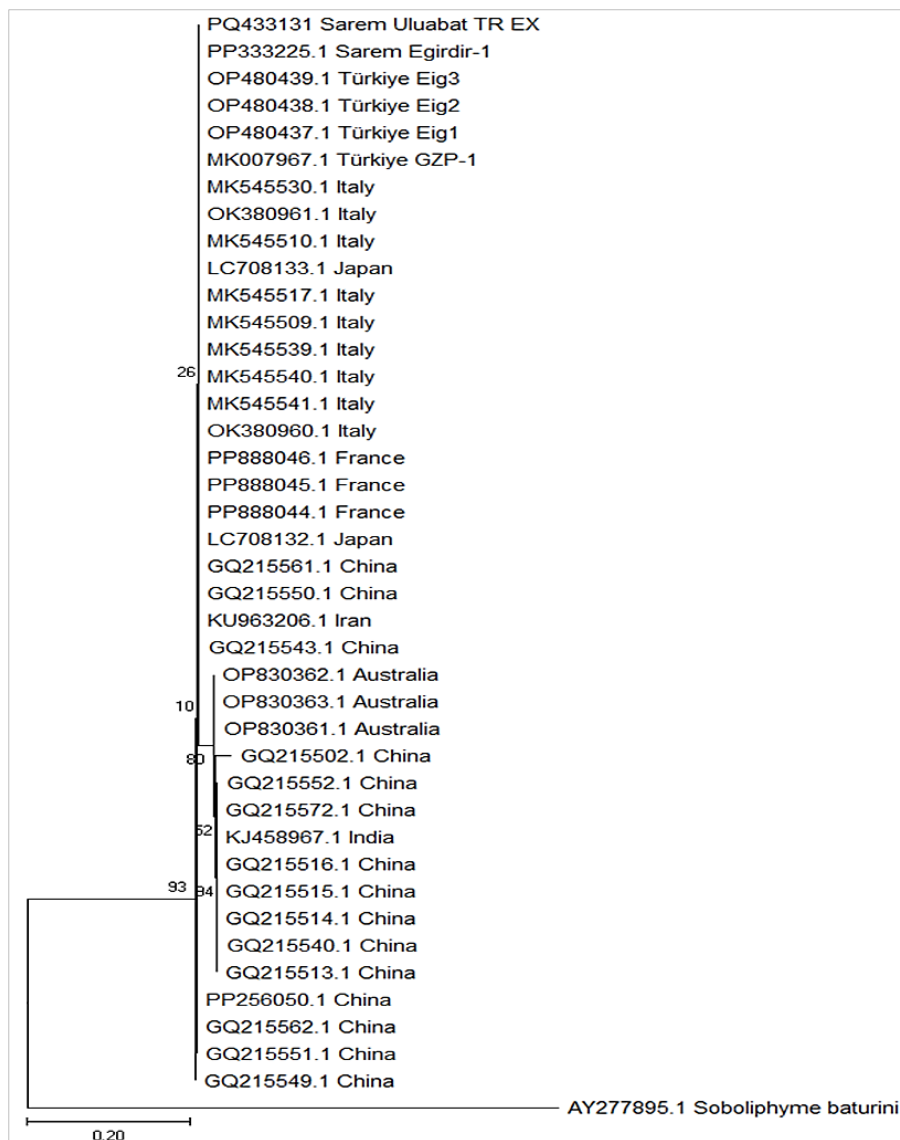


Figure 5. Phylogenetic tree based on ITS rDNA sequence data of *E. excisus* isolates.

including tissue degeneration, tearing, and hemorrhage in infected specimens. During this study, redness, necrosis, and thinning of the peritoneum were observed in the cloacal region of the host fish samples infected with *E. excisus*.

Eustrongylides excisus is a zoonotic nematode of significant public health concern due to its transmission via the consumption of raw or undercooked fish (Branciaro et al., 2016; Honcharov et al., 2022). Human infection, which can lead to severe gastritis and intestinal perforation, is difficult to treat as larvae migrate and encyst in extra-intestinal tissues, rendering antihelminthics ineffective (Guardone et al., 2021). The growing trend of consuming raw fish elevates this risk, underscoring the need for enhanced, practical mitigation strategies. While complete exclusion of infected fish from the food chain is ideal, the culling of wild fish populations is neither ecologically nor economically feasible (Fusco et al., 2023). Therefore, mitigation must focus on post-harvest interventions and public guidance. Given that larvae can be found in edible muscle tissue, robust protocols are essential, including immediate evisceration, freezing at -20°C for ≥ 7 days, or cooking to a core temperature of $\geq 63^{\circ}\text{C}$ (Shamsi and Pearce, 2025). Fish species from Lake Uluabat are a preferred food source for the local population, occurrence a risk of contracting fish-borne parasitic diseases.

The ITS rDNA sequence analysis of *Eustrongylides excisus* obtained in this study, the absence of intraspecific variation among the three specimens is consistent with previous reports that ITS regions often display limited polymorphism within populations, making them reliable markers for species-level identification (Blouin, 2002). However, the comparative analysis revealed distinct nucleotide polymorphisms among isolates from different geographic regions, underscoring the potential role of microevolutionary processes and geographic isolation in shaping genetic diversity. Similar geographic structuring has been documented in other nematodes, where ITS variation reflects both host associations and biogeographic history (Gendron et al. 2024).

The analysis of genetic variation among *Eustrongylides excisus* isolates revealed low levels of nucleotide divergence across geographic regions, with p-distance values ranging from 0.000 to 0.040. Such limited intraspecific variation is consistent with patterns observed in other parasites associated with highly mobile hosts, where dispersal of infected hosts facilitates gene flow among distant populations (Geller et al., 2013). In addition, the relatively low sequence variability observed here may also reflect the evolutionary conservation of the ITS rRNA gene, which is subject to strong functional constraints and typically evolves at a low substitution rate (Hillis & Dixon, 1991).

More detailed genetic distance analysis among *E. excisus* haplotypes showed p-distance values ranging from 0.00131 to 0.04052, confirming an overall low level

of genetic differentiation within the dataset (Nadler & Pérez-Ponce de León, 2011). The lowest genetic distances likely reflect recent common ancestry or shared geographic origins among closely related haplotypes (Avice, 2000), whereas the higher values may indicate partial geographic isolation or the persistence of relatively divergent lineages within the species (Grant & Bowen, 1998). Notably, Hap_4 exhibited comparatively higher pairwise distances (0.023–0.040) relative to other haplotypes, suggesting that it may represent a more divergent lineage, a pattern frequently associated with ancestral haplotypes or geographically structured populations (Posada & Crandall, 2001). Nevertheless, the maximum observed divergence remains within the reported range of intraspecific variation for nematode ribosomal genes, confirming that Hap_4 still lies within the species boundaries of *E. excisus* (Nadler et al., 2006).

The moderate haplotype diversity ($H_d = 0.630 \pm 0.080$) combined with low nucleotide diversity (π) further suggests a recent population expansion following a founder event or demographic bottleneck (Grant & Bowen, 1998). This interpretation is supported by the discrepancy between nucleotide diversity (π) and Watterson's theta (θ), indicating deviation from the neutral model of evolution (Tajima, 1989), as well as by the significantly negative F_u and L_i 's D value, which reflects an excess of low-frequency polymorphisms typical of expanding populations (Ramos-Onsins & Rozas, 2002).

Consistent with these results, the median-joining haplotype network revealed a dominant central haplotype shared across multiple geographic regions, with several low-frequency haplotypes connected by one or a few mutational steps, forming a characteristic star-like topology. Such a structure is widely interpreted as evidence of recent demographic expansion from a common ancestral population (Posada & Crandall, 2001) and suggests a high level of population connectivity, potentially reflecting panmixia or very recent divergence among populations (Templeton, 2004).

The phylogenetic reconstruction, supported by bootstrap analysis (Hillis & Bull, 1993), also indicated geographic clustering among *E. excisus* isolates. Sequences from proximate regions, such as Turkey, Italy, and France, formed closely related clades characterized by short internal branches, suggesting recent common ancestry and limited mutation accumulation (Nei & Kumar, 2000). This phylogeographic concordance supports the interpretation of shared evolutionary history or restricted dispersal within this regional cluster (Hickerson et al., 2010). Interestingly, the inclusion of Japanese isolates within this clade may reflect convergent evolutionary patterns or similar selective pressures acting in comparable ecological environments (Losos, 2011). In contrast, isolates from more distant regions, including China and Australia, formed separate and more deeply divergent clades, consistent with long-

term independent evolutionary trajectories, potentially shaped by biogeographic barriers limiting gene flow. The pronounced substructuring observed among Chinese isolates further suggests higher intra-regional genetic diversity, possibly reflecting a large effective population size or the persistence of multiple refugial populations within the region (Excoffier et al., 2009).

Conclusion

This study confirms the presence of the zoonotic nematode *Eustrongylides excisus* in the *Neogobius fluviatilis* population of Lake Uluabat and provides the first molecular characterization of this parasite in this host and region using the ITS marker. Genetic analyses revealed moderate haplotype diversity with low nucleotide diversity and a star-like haplotype network, suggesting a recent demographic expansion from a common ancestral population, likely facilitated by highly mobile avian definitive hosts. Despite this expansion signal, the phylogeographic structuring and presence of divergent lineages indicate underlying population complexity at a broader geographic scale. These findings highlight Lake Uluabat as an ecological hotspot for the *E. excisus* life cycle and emphasize the importance of integrating molecular surveillance into parasitological monitoring programs to better understand the parasite's distribution and evolutionary dynamics.

Ethical Statement

This study is not subject to the permission of HADYEK in accordance with the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees" 8 (k). The data, information and documents presented in this article were obtained within the framework of academic and ethical rules.

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Author Contribution

First Author: Conceptualization, Writing -review and editing; Second Author: Data Curation, Formal Analysis, Investigation, Methodology, Visualization and Writing -original draft; Third Author: review and editing; Fourth Author: Investigation, Methodology; Fifth author; Data Curation, Writing and Sixth author: Supervision, Writing - review and editing.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or

personal conflicts that could have appeared to influence the work reported in this paper.

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