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RESEARCH PAPER

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Molecular Diversity of *Diplostomum spathaceum* (Digenea: Diplostomidae) on the *Capoeta umbla* and *Cyprinus carpio* (Cypriniformes) Using Mitochondrial DNA Barcode

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Abstract

Diplostomid trematodes (Digenea) include a wide and diverse group of common digeneans. Diplostomid larval stages are significant pathogens that may exert grave effects on both natural fish and aquaculture populations. *Diplostomum* species, which use fish as a second intermediate host, is one of the most common trematode species, which affects the fish welfare negatively with the formation of cataracts by settling in the eye. This study is determined the molecular characterization of *Diplostomum* parasite in *Cyprinus carpio* and *Capoeta umbla* for the first time from Turkey, based on the mitochondrial COX1 sequence data. *Diplostomum* samples were determined as *Diplostomum spathaceum* according to the phylogenetic trees created in the light of the morphological and COX1 sequence data. Sequence results matched ~98-100% with *D. spathaceum* as a result of blast analysis. It was defined that this parasite was represented by three different haplotypes in Turkey. As a result of haplotype analysis performed on a total of 162 isolates in GenBank and obtained in this study, 40 polymorphic regions and 58 haplotypes were determined. This haplotype network had arranged within a star-like configuration with a main central haplotype. This shows that the variation within *D. spathaceum* species is quite high. The mean abundance, mean intensity, and prevalence of the parasite in *C. carpio* were 8.29 (5.80-11.24), 10.91±9.37 (1-39), and 76%, respectively. In the *C umbla*, mean abundance, mean intensity, and prevalence were calculated as 11.54 (10.16-12.92), 14.07±16.59 (1-67), and 82%, respectively.

Introduction

The majority of the trematodes (~24,000 species) are members of the Subclass Digenea and as adults; these are all obligate parasites of vertebrates (Poulin & Morand, 2004). The species of adult digenean use vertebrates as hosts. Larval stages of these parasites typically require a mollusk, usually a snail, as the first intermediate host. Most species also require a second intermediate host, which may be an invertebrate or a vertebrate, depending on the species (Moszczynska et al., 2009).

Most Diplostomid species include a wide and diverse group of common digeneans that parasitize, as adults, a wide range of piscivorous birds and sometimes mammals. They use a three-host life-cycle with freshwater snails acting as first intermediate hosts and freshwater fishes (sometimes amphibians) as second

intermediate hosts. Although the systematics of this superfamily are based largely on the morphology and to a lesser extent host associations of adults, diplostomoids are most frequently encountered and studied in second intermediate hosts, particularly freshwater fish (Blasco-Costa & Locke, 2017; Chibwana et al., 2013). The problematic identification of these larval stages is a major obstacle in the estimation of their exact role in wild fish populations and developing knowledge of the distribution ranges and evolutionary aspects of host–parasite associations of *Diplostomum* spp. (Blasco-Costa et al., 2014). Diplostomum species, which is one of the digenean parasites, were generally found in the eye lenses of freshwater fish (Desilets et al., 2013; Locke et al., 2015; McKeown & Irwin, 1997). Diplostomid larval stages are significant pathogens that may exert grave effects on both natural and aquacultured fish populations. The transmigration of large numbers of infective post-cercarial stages towards the specific sites of infection may reason fish mortalities, especially in young individuals due to hemorrhaging of capillaries and obstructed blood vessels principally in the head and brain (Chibwana et al., 2013; Lebedeva et al., 2021). All but their significance in fish health, eye-dwelling diplostomids are frequent topics of evolutionary and ecological studies. However, diplostomid metacercariae are hard to identify morphologically and molecular methods are frequently used to distinguish species (Locke et al., 2015). In contrast to morphology, DNA sequences can be used to determine species at all developmental stages, a great advantage for studying parasites with complex life cycles (Locke et al., 2010; Rellstab et al., 2011). DNA-based approaches can also perform a quick acceleration in studies of host-parasite associations and life-cycles. Therefore, for trematodes which utilise complex life-cycles, sequences of reliably identified adult stages may ensure direct and efficient means of identifying larval stages and so inferring complete life-cycles (Georgieva et al., 2013). However, recent studies have indicated that these regions do not ensure adequate resolution for species discrimination within Diplostomum and have provided a demonstration that the barcode region of the mitochondrial cytochrome c oxidase subunit 1 (COX1) gene may serve as a more efficient marker in lighting life-cycles and recognition of cryptic species variety within Diplostomum (Brabec et al., 2015).

This study aims to analyze a mtDNA-based approach to diplostomid diversity in the Eastern Anatolian Region of Turkey, which has approximately 10% freshwater potential and is the third largest fish farming of country, using the mitochondrial cytochrome c oxidase subunit 1 (COX1) gene region. The creation of this research provides an essential starting point for the development of molecular identification studies linking larval and adult parasite stages, which will ultimately improve our understanding of *Diplostomum* diversity and impact in both natural freshwater fish populations and aquaculture in Turkey.

Diplostomum species cause cataracts in fish in natural environments. This situation has a significant negative impact on fish welfare. It has been reported that due to cataracts caused by Diplostomum species, fish cannot get enough nutrients, and growth decreases (Bjerkas et al., 1996; Crowden & Broom, 1980; Ersdal et al., 2001; Savino et al., 1993), and mortality increases (Lester, 1977; Menzies et al., 2002). It has been determined that fish with cataracts have weaker escape reactions and are easier to catch than fish with healthy eyes (Brassard et al., 1982; Seppala et al., 2011). It has been determined that economic losses in the fishing sector have increased due to cataracts (Menzies et al., 2002).

Due to the significant negative effects of *Diplostomum* species on fish, it is planned to investigate this parasite molecularly in *C. carpio* and *C. umbla*, which live in large water resources in the Eastern Anatolia Region and are loved and consumed by the humans. This study was aimed for molecular identication of the parasites species inhabiting eye lens of the freshwater fish based on the COX1 gene region analysis, to determine the haplotypes and molecular phylogeny of the *Diplostomum* species and to analyzes with same identified parasite species in the world.

Materials and Methods

Study Area, Fish Sampling and Parasite Collection

This study was conducted in three daml lake (Karakaya Dam Lake, Keban Dam Lake, Uzunçayır Dam Lake), one natural lake (Hazar Lake), and one river (Dicle River) located the Eastern Anatolia of Turkey between May 2017 and August 2019. The total surface area covered by these three dam lakes and one natural lake in the region is 106.843 ha. The Dicle River, begins as the exit of the Hazar Lake in the Sivrice district of Elazig, opens to the sea in the Persian Gulf by crossing countries such as Turkey, Syria, Iraq, and Iran. The length of this river is 1900 km and the basin area includes 258,000 km².

The study was carried out on a total of 175 natural freshwater fish (*Capoeta umbla* and *Cyprinus carpio*) species. The eyes of the fish were incised with forceps and a scalpel, the lens was removed and placed in physiological saline (0.9%). Then, Diplostomum metacercerias were collected from the eye lenses of fish under a pthe stereomicroscope and processed according to Kennedy (1979). Parasites were rinsed in distilled water and gradually fixed into 1 ml eppendorf tubes using 70% and then 96% ethanol. Some metacercariae were stained with hematoxylin and placed in Canadian balsam for morphological examination, while others were used for molecular research. A total of 30 the metacercariae of *D. spataceum* collected from the eyes of each two fish were used for morphological examination. Morphologically were evaluated the body shape of the

parasite, the characteristics of the oral and ventral suckers, the location and dimensions of the pharynx, the esophagus, intestinal structure, lateral suckers, and Brandes organ. The morphological identification of parasites metacercariae was made according to Bychovskaya-Povlovskaya et al. (1964), Hoffman (1999), Kennedy (1974), Williams and Jones (1994).

Mean Abundance (A), Mean intencity (MI) and Prevalence (P) as the ecological analysis of *Diplostomum spathaceum* determined in fish species, were calculated as the following formulas according to Bush et al. (1997).

$$Mean\ Abundance\ (MA) = \frac{The\ total\ number\ of\ parasites}{The\ number\ of\ fish\ examined}$$

$$Mean\ Intensity\ (MI) = \frac{The\ total\ number\ of\ parasites}{The\ number\ of\ hosts\ infected}$$

$$Prevalence\ (P) = \frac{The\ number\ of\ hosts\ infected}{The\ number\ of\ total\ fish\ examined}\ x\ 100$$

DNA Extraction, Amplification and Sequensing

DNA extraction was isolated with DNeasy Blood and Tissue Kit (Qiagen) according to the requirements. After extraction, the products (gDNA) were electrophoresed in a 1% agarose gel with ethidium bromide added. After electrophoresis, the COX1 mtDNA gene band of around 500 bp fragment was obtained under UV light. The gel band of each specimen was cut separately and then purified.

Amount and purity analyzes of the obtained total DNA were performed in a spectrophotometer (NanoDrop 2000, Thermoscientific) from the purification of agarose gel after electrophoresis. 1µl DNA sample was placed in the device with the help of a pipette, DNA concentration was calculated considering that 1 OD at 260 nm is the absorption value of 50 ng/ml double-strand DNA. In the adjustment of DNA concentration, samples whose concentrations were determined by the spectrophotometric analysis were diluted to a DNA concentration of 200 ng / µl in order to avoid contamination of the main stocks and to standardize the DNA concentrations of all samples.

Partial fragments of the mitochondrial gene (mtDNA) of cytochrome c oxidase subunit 1 (COX1) were amplified using the diplostomides-specific designed PCR primers Plat-diploCOX1F (5'-CGT TTR AAT TAT ACG GAT CC-3') and Plat-diploCOX1R (5'-AGC ATA GTA ATM GCA GCA GC-3') which yielding approximately 500 bp size product (Moszczynska et al., 2009). The lyophilized primer used in the PCR process was diluted with distilled water up to 10 times the nmol values given by the manufacturer, and 100 pM / µl stock primer was obtained. PCR reaction was performed with a total volume of 25 µl. In this volume, 1X PCR buffer, 2.5 mM $MgCl_2$, 1.25 pmol forward and 1.25 pmol reverse primer, 50 µM dNTPs, 0.6 U Taq polymerase, 50 ng purified DNA sample and 15 µl ddH_2O were used.

The PCR conditions were set for 35 cycles at 94°C for 30 seconds, at 50°C for 30 seconds, and at 72°C for 60 seconds, followed by pre-denaturation at 94°C for 2 minutes with the Thermal cycler, followed by 72°C. 10 minutes last elongation step was applied at.

Purification and sequence analysis of single-band gene products obtained in agarose gel electrophoresis for PCR products were performed. Sequence analysis of the samples was carried out bidirectionally (forward and backward) using specific primers used in the PCR process.

DNA sequencing was performed in the Macrogen (Netherlands) laboratory using the ABI 3730XL Sanger sequencer (Applied Biosystems, Foster City, CA) and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and as a result, the raw DNA sequences were obtained.

Aligment and Phylogenetic Analysis

The chromatogram quality was checked with the use of FinchTV 1.4.0 (Geospiza Inc., Seattle Washington, USA) (http://www.geospiza.com). The sequence ends were trimmed to a final length of ~500 bp by comparing the published sequences using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) search. Clustal-Omega and ClustalW (https://www.ebi.ac.uk/Tools/msa/clustalo/) and Bioedit (Hall, 1999) were used for sequence alignment. The alignment was applied using published reference sequences retrieved from NCBI Pubmed were used as out-groups. The sequences were then uploaded to MEGAX and evolutionary analyses were conducted (Kumar et al., 2018).

Phylogenetic trees were prepared using two different methods, namely, Maximum Likelihood (ML) and Neighbor-Joining (NJ) from the COX1 mtDNA sequences obtained from Diplostomum samples, using the pipeline sequence MEGAX. There were a total of 441 positions for Neighbor-Joining and 402 positions for Maximum Likelihood in the final dataset. The evolutionary distances and history were computed using the Kimura 2-parameter model for NJ and ML trees (Kimura, 1980) and are in the units of the number of base substitutions per site. Statistical support for specific clades was obtained via 1,000 bootstrap replicates. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3951)). The rate variation model for ML allowed some sites to be evolutionarily invariable ([+I], 32.09% sites). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing

data, and ambiguous bases were allowed at any position (partial deletion option). In the creation of phylogenetic trees of the parasite, a total of 72 sequences, including the results of the research and the ones selected from the GenBank, were used. The sequences used in the creation of the phylogenetic trees, their accession numbers and their countries are listed in Table 1.

Data Analysis and Haplotype Network Analysis

DnaSPv6 was used to calculate population diversity indices (haplotype number (h), haplotype diversity (Hd), nucleotide diversity (π)), neutrality indices (Tajima's D and Fu's statistics), Fu and Li's D, and F tests (Rozas et al., 2017). DnaSPv6 was also used to generate output formats, including the NEXUS file format. Networks were created using the TCS criteria method (Clement *et al*., 2000), which provides an agglomerative approach in which clusters are gradually joined by one or more connecting edges by means of PopART-1.7 software (http://popart.otago.ac.nz) (Leigh & Bryant, 2015). While the haplotype networks were prepared at the scale of Turkey using the DNA sequences (total 33 sequences) obtained in this study, the world-scale (total 162 sequences) haplotype network was prepared by taking the data from Genbank and combining them with the data obtained from our study (Supplementary Data Table S1).

Results

Morphology

The anterior part of their body is round and the posterior part is protruding. The posterior part of the body is shorter than the anterior. A ventral sucker is twice as large as an oral sucker. The attachment organs are also larger than the ventral sucker. The pharynx is small, the esophagus is short. Esophageal branches extend into two intestinal tubes, then join together to form the letter "V". The lateral suction cup is prominent. Brandes organ is round and elongated in a transversal position.

Infection Status of *Diplostomum spathaceum*

A total of 1613 *D. spathaceum* were detected in the eye lenses of 136 out of a total of 175 fish from two different cyprinid species (*C. umbla, C. carpio*) in five important wetlands in the Eastern Anatolia Region of Turkey. While 1036 of this parasite amount was determined in *C. carpio*, 577 of them were found in *C. umbla*. It was determined that *D. spathaceum* varied between 5.80 and 11.24 of the mean abundance in *C. carpio* species and its average was 8.29. Mean Intensity was determined between 7.63±7.83 (1-22) and 14.05±11.03 (1-39) in the same species according to water resourses, it was observed that the mean was 10,91±9.37 (1-39). The prevalence of *D. spathaceum* trematode metacercariae in *C. carpio* varied between 72-80%, with an average of 76%. Mean abundance, mean intensity, and prevalence of metacercariae in the eye lens of *C. umbla* were calculated as 11.54, 14.07±16.59 (1-67), and 82%, respectively. While the three different parasitic infection indices calculated in our study were higher in *C. umbla* than in *C. carpio*, they were highest observed in the Dicle River. The mean abundance, mean intensity and prevalence of the *D. spathaceum* in these two fish species were determined as 9.22 (5.80-12.92), 11.86±12.04 (7.63 – 15.38) and 77.71% (72-84), respectively. The mean prevalence of the parasite of these two fish species in the wetlands was quite high (77.71% (72-84)), mean abundance (9.22 (5.80-12.92)), and mean intensity (11.86±12.04 (7.63 – 15.38)) was lower (Table 2).

Sequence Analyses and Phylogenetic Tree

A total of 175 *Cyprinus carpio* and *Capoeta umbla* fish from Keban Dam Lake, Karakaya Dam Lake, Hazar Lake, Uzunçayır Dam Lake, and the Dicle River were examined. As a result of the morphological analysis of the parasites, it was determined that it was *Diplostomum spathaceum*. The COI gene was amplified for definitive diagnosis. Sequence results matched ~98-100% with *D. spathaceum* as a result of blast analysis. After sequence analysis, both ends of the raw data were trimmed and all samples equalized to the 441 bp. In the current study, all nucleotide sequences were submitted in the Genbank database under the following accession numbers MW504357-MW504389 (Seq1-Seq33). It was determined that the similarity between these sequences obtained from *C. carpio* and *C. umbla* species was 99.8-100% (Supplementary Data Table S2 and Table S3).

As a result of the alignment analysis, the sequences of *Diplostomum spathaceum* are 100% similar except for the MW504364, MW504365, MW504372, MW504377, MW504385, and MW504387 sequences. Unlike these, the mutation was detected in the 136nd nucleotide (A → G) of the MW504364 and MW504377 sequence, the 190th nucleotide (T → C) of the sequence MW504365, MW504372, MW504385, and MW504387 the sequence.

It was observed that *D. spathaceum* formed unique blocky branches in trees obtained according to Maximum Likelihood (Figure 1) and Neighbor-Joining (Figure 2) methods with 33 mtDNA sequences obtained in this study and mtDNA sequences obtained from various countries before. In the light of these data, it was determined that *D. spathaceum* was clearly distinguished from other Diplostomum species. It was determined that phylogenetic trees performed according to ML and NJ methods on *D. spathaceum* mtDNA sequences showed similar branching. In the comparison of *Diplostomum spatheceum* with eight different species of *Diplostomum* (*D. lunaschiae, D. ardeae, D. mergi. D. pseudospathaceum, D. huronense,*

Table 1. Locality information and GenBank accession numbers used in phylogenetic analyses.

Taxon	Host	Specimen Cod	Location	Coordinates	Elevation (meters)	Accession No (COX1)	References
Diplostomum spathaceum	*Cyprinus carpio*	A9, B1, B3, B5, B7	Dicle River, Turkey	38.345650 N 39.692912 E	878	MW504361 MW504362 MW504363 MW504364 MW504365	Present study
	Capoeta umbla	F5, F7, G, G3, G5				MW504380 MW504381 MW504382 MW504383 MW504384	
Diplostomum spathaceum	*Cyprinus carpio*	C1, C3, C5, C7, D1	Keban Dam Lake, Turkey	38.624067 N 39.468401 E	821	MW504366 MW504367 MW504368 MW504369 MW504370	
Diplostomum spathaceum	*Cyprinus carpio*	D3, D5, D7, E1, E3	Karakaya Dam Lake, Turkey	38.418317 N 38.702853 E	676	MW504371 MW504372 MW504373 MW504374 MW504375	
Diplostomum spathaceum	*Cyprinus carpio*	E5, E7, F1, F3	Hazar Lake, Turkey	38.480846 N 39.410591 E	1240	MW504376 MW504377 MW504378 MW504379	
Diplostomum spathaceum	*Cyprinus carpio*	A1, A3, A5, A7, A9	Uzuncayir Dam Lake, Turkey	39.023187 N 39.507903 E	864	MW504357 MW504358 MW504359 MW504360	
	Capoeta umbla	G7, H1, H3, H5, H7				MW504385 MW504386 MW504387 MW504388 MW504389	
Diplostomum spathaceum	*Abramis brama*	GenBank	China			KR271430	(Locke et al., 2015)
Diplostomum spathaceum	*Abramis brama*	GenBank	China			KR271434	
Diplostomum spathaceum	*Cyprinus carpio*	GenBank	Croatia			KR271437	
Diplostomum spathaceum	*Larus cachinnans*	GenBank	Czech Republic			JX986887	(Georgieva et al., 2013)
Diplostomum spathaceum	*Larus cachinnans*	GenBank	Czech Republic			JX986895	
Diplostomum spathaceum	*Radix auricularia*	GenBank	Germany			KR149549	(Selbach et al., 2015)
Diplostomum spathaceum	*Radix auricularia*	GenBank	Germany			KR149551	
Diplostomum spathaceum	*Aspius aspius*	GenBank	Hungary			KY653976	(Kudlai et al., 2017)
Diplostomum spathaceum	*Aspius aspius*	GenBank	Hungary			KY653983	
Diplostomum spathaceum	*Salvelinus alpinus*	GenBank	Iceland			KJ726438	(Blasco-Costa et al., 2014)
Diplostomum spathaceum	*Gasterosteus aculeatus*	GenBank	Iceland			KJ726439	
Diplostomum spathaceum	*Cyprinion macrostomum*	GenBank	Iraq			KR271426	(Locke et al., 2015)
Diplostomum spathaceum	*Carasobarbus luteus*	GenBank	Iraq			KR271463	
Diplostomum spathaceum	*Perca fluviatilis*	GenBank	Italy			KR271457	
Diplostomum spathaceum	*Perca fluviatilis*	GenBank	Italy			KR271468	
Diplostomum spathaceum	*Larus argentatus*	GenBank	Poland			JX986892	(Georgieva et al., 2013)
Diplostomum spathaceum	*Rutilus rutilus*	GenBank	Romania			KR271445	(Locke et al., 2015)
Diplostomum spathaceum	*Silurus glanis*	GenBank	Romania			KR271462	
Diplostomum spathaceum	*Silurus glanis*	GenBank	Slovakia			KY653984	(Kudlai et al., 2017)
Diplostomum spathaceum	*Vimba vimba*	GenBank	Slovakia			KY653986	
Diplostomum spathaceum	*Larus argentatus michahellis*	GenBank	Spain			KP025772	(Perez-del-Olmo et al., 2014)
Diplostomum spathaceum	*Larus ridibundus*	GenBank	Spain			KP025777	
Diplostomum lunaschiae	*Tigrisoma lineatum*	GenBank	Brazil			MT324624	(Locke et al., 2020)
Diplostomum lunaschiae	*Tigrisoma lineatum*	GenBank	Brazil			MT324626	
Diplostomum ardeae	*Ardea herodias*	GenBank	Puerto Rico			MT324592	
Diplostomum ardeae	*Ardea herodias*	GenBank	Puerto Rico			MT324593	
Diplostomum mergi	*Abramis brama*	GenBank	China			KR271082	(Locke et al., 2015)
Diplostomum mergi		GenBank	China			KY271543	GenBank by Dang (2016)
Diplostomum pseudospathaceum	*Siluris glanis*	GenBank	Romania			KR271085	(Locke et al., 2015)
Diplostomum pseudospathaceum	*Lymnaea stagnalis*	GenBank	Germany			KR271095	
Diplostomum huronense	*Notemigonus crysoleucas*	GenBank	Canada			KR271072	
Diplostomum huronense	*Notemigonus crysoleucas*	GenBank	Canada			KR271075	
Diplostomum indistinctum	*Catostomidae*	GenBank	Canada			FJ477196	(Moszczynska et al., 2009)
Diplostomum indistinctum	*Notemigonus crysoleucas*	GenBank	Canada			KR271076	(Locke et al., 2015)
Diplostomum baeri	*Perca flavescens*	GenBank	USA			MF142224	(Ubels et al., 2018)
Diplostomum baeri	*Perca flavescens*	GenBank	USA			MF142227	
Diplostomum parviventosum	*Radix auricularia*	GenBank	Germany			KR149511	(Selbach et al., 2015)
Diplostomum parviventosum	*Radix auricularia*	GenBank	Germany			KR149512	
(Outgroup)							
Posthodiplostomum cuticola	*Squalius cephalus*	GenBank	Turkey			MN701652	(Simsek et al., 2020)

Table 2. Infection indices (Mean Abundance, Mean Intensity and Prevalence) of *Diplostomum spathaceum* in some cyprinids in Eastern Turkey

Host	Location	Examined Fish Number (n)	Infected Fish Number	Total number of parasites	Mean Abundance (MA)	Mean Intensity (MI)	Prevalence (P) (%)
Cyprinus carpio	Dicle River	25	18	209	8,36	11,61±10.08 (1-31)	72,00
	Karakaya Dam Lake	25	19	227	9,08	11,95±8.62 (1-27)	76,00
	Keban Dam Lake	25	20	281	11,24	14,05±11.03 (1-39)	80,00
	Hazar Lake	25	19	145	5,80	7,63±7.83 (1-22)	76,00
	Uzuncayir Dam Lake	25	19	174	6,96	9,16±8.44 (1-24)	76,00
	Total	**125**	**95**	**1036**	**8,29**	**10,91±9.37 (1-39)**	**76,00**
Capoeta umbla	Dicle River	25	21	323	12,92	15,38±19.87 (1-67)	84,00
	Uzuncayir Dam Lake	25	20	254	10,16	12,70±12.66 (1-43)	80,00
	Total	**50**	**41**	**577**	**11,54**	**14,07±16.59 (1-67)**	**82,00**
TOTAL		**175**	**136**	**1613**	**9,22**	**11,86±12.04 (1-67)**	**77,71**

D. indistinctum, D. baeri, D. parviventosum) from different countries, it is seen that the difference between them ~11-17%. Separate blocks in the phylogenetic trees created according to results of the ML and NJ evolutionary analysis have emerged due to this different too.

In the MEGAX program, the best base change models were Maximum likelihood and Neighbor-joining Phylogenetic trees. *D. spathaceum*, which we obtained in our study, was found to be similar despite collecting from five different wetlands. In phylogenetic trees created with GenBank data belonging to *D. spataceum* from different countries, it was observed that 99.3-100% similar despite they were taken from different localities. In this study, *Posthodiplostomum cuticola* species was used as the outer group. When we compare it with the outer group, a distance of ~27% has been obtained. As it can be understood from here, this rate increases as you move away from the same species.

D. spateceum has been detected in the eye lens of fish in countries such as Germany, Iraq, China, Slovakia, Spain, Iceland, Poland, Czech Republic, Romania, Italy, Hungary, and Croatia in previous molecular studies. In this study, the aforementioned parasite was determined in the eyes of *C. umbla* and *C. carpio* species living in freshwaters in the Eastern Anatolia Region of Turkey.

The mtDNA sequences of 33 samples of *Diplostomum spathaceum* obtained in this study and of the 129 sequences listed by GenBank were combined, and the results of diversity, neutrality indices and summary statistics relative to these two data sets were given in Table 3. There were detected three haplotypes and two polymorphic sites for COX1 mtDNA sequences of *D. spathaceum* of Turkey that encompassed 20% (33/162) of the total isolates in the GenBank. The Nucleotide Diversity (per site) was Pi: 0.00076±0,00024, sampling variation of Pi: 0,0000001. The haplotype (gene) diversity Hd: 0.322± 0.097 and variance of haplotype diversity 0.00941. The overall neutrality indices of Tajima's D (-0.62913), Fu and Li's F (FLF) (0.41876), Fu and Li's D (FLD) (0.79401) and Fu's Fs (-0.630) (P>0.10) were not found significant (Table 3).

When we evaluate the results depending on the distance between the geographical regions; Haplotype-1 covers all study areas including Karakaya Dam Lake, Keban Dam Lake, Hazar Lake, Uzuncayir Dam Lake, and the Dicle River. Haplotype-2 in Dicle River and Hazar Lake and Haplotype-3 in the Dicle River, Karakaya Dam Lake, and Uzuncayir Dam Lake was found. Compared to other study regions, the Dicle River offers a wider variety as it contains three haplotypes (Figure 3). When examined on the map in which region has the highest haplotype diversity according to the study regions, it is seen that the Dicle River covers all three haplotypes, while Karakaya Dam Lake, Uzuncayir Dam Lake, and Hazar Lake cover two haplotypes. However, it was determined that Keban Dam Lake contains only one haplotype (Figure 4).

As a result of haplotype analysis performed on a total of 162 isolates in GenBank together with the addition of 3 haplotypes determined for *D. spathaceum* in two different fish species from Turkey, 40 polymorphic regions and 58 haplotypes were determined. This haplotype network had arranged within a star-like configuration with a main central haplotype (Figure 5). The haplotype distribution of the world according to data of our study and Genbank was given in Figure 6. In the light of these data, it is seen that focuses *D. spathaceum* on snails, fish, and waterfowl in the Palearctic Region, excluding Africa continent. The diversity, neutrality indices and summary statistics obtained by using nucleotide data belonging *D. spathaceum* are given in Table 3.

Discussion

The metacercariae of *Diplostomum spathaceum* have been detected in previous studies lens of species such as *Abramis brama, Acanthobrama marmid, Alburnus caeruleus, Aspius aspius, Barbus luteus, Cyprinion macrostomum, Cyprinus carpio, Gasterosteus aculeatus, Larus argentatus, Larus argentatus michahellis, Larus cachinnans, Larus ridibundus*, *Misgurnus anguillicaudatus, Perca fluviatilis, Pseudochondrostoma willkommii, Radix auricularia*, *Radix cf. peregra, Rutilus rutilus, Salvelinus alpinus, Silurus glanis, Vimba vimba.* The metacercariae of this detected parasite are seen in countries such as China,

Figure 1. The evolutionary history of Diplostomum spathaceum according to the Maximum Likelihood method. The tree with the highest log likelihood (-2065.95) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3951)). The rate variation model allowed some sites to be evolutionarily invariable ([+I], 32.09% sites).

Figure 2. The evolutionary history of *Diplostomum spathaceum* according to Neighbor-Joining analyses. The optimal tree with the sum of branch length = 0.82760987 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

Table 3: Diversity, neutrality indices and summary statistics obtained by using nucleotide data of *Diplostomum spathaceum* with DnaSP-v6. SD: standard deviation (*Neutrality tests are considered statistically significant if P<0.05)

Diversity indices	Results of Eastern Turkey	Results of Word
Number of sequences (n)	33	162
Selected region	1-441	1-441
Number of polymorphic (segregating) sites (S)	2	40
Total number of mutations, Eta:	2	48
Total number of singleton mutations, Eta(s):	-	25
The number of haplotypes (h)	3	58
Haplotype diversity (Hd)	0.322±0.097	0.874±0.023
Variance of Haplotype diversity	0.00941	0.00052
Nucleotide diversity (π)	0.00076±0,00024	0.00699
Average number of nucleotide differences (k)	0,337	2.334
The neutrality tests		
Tajima's D	-0.62913, P>0.10	-2.18856, P<0.01*
Fu and Li's F (FLF)	0.41876, P>0.10	-4.12920, P<0.02*
Fu and Li's D (FLD)	0.79401, P>0.10	-4.37021, P<0.02*
Fu's (*Fs*)	-0.630, P>0.10	-33.964, <0.02*

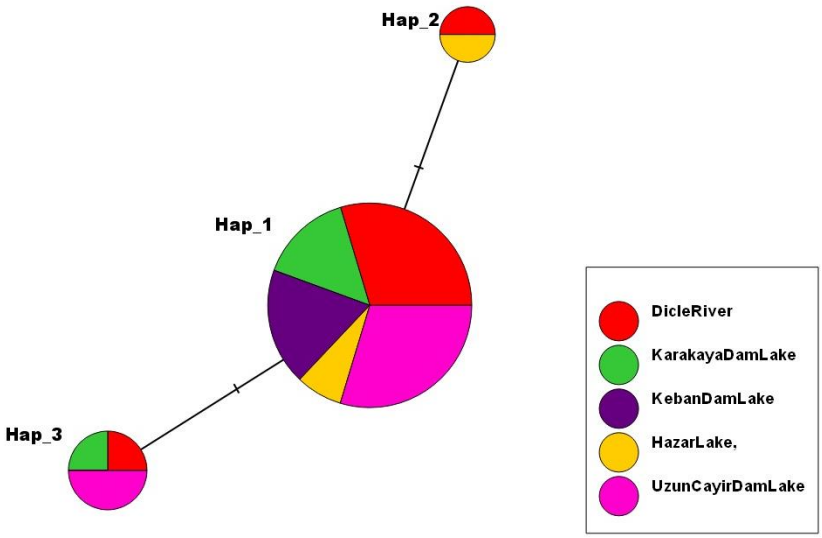

Figure 3. Haplotype network of *Diplostomum spathaceum* isolated using the COX1 (441 bp) gene from the different five freshwaters in the eastern Anatolian region of Turkey. Size of circles is proportional to the frequency of each haplotype.

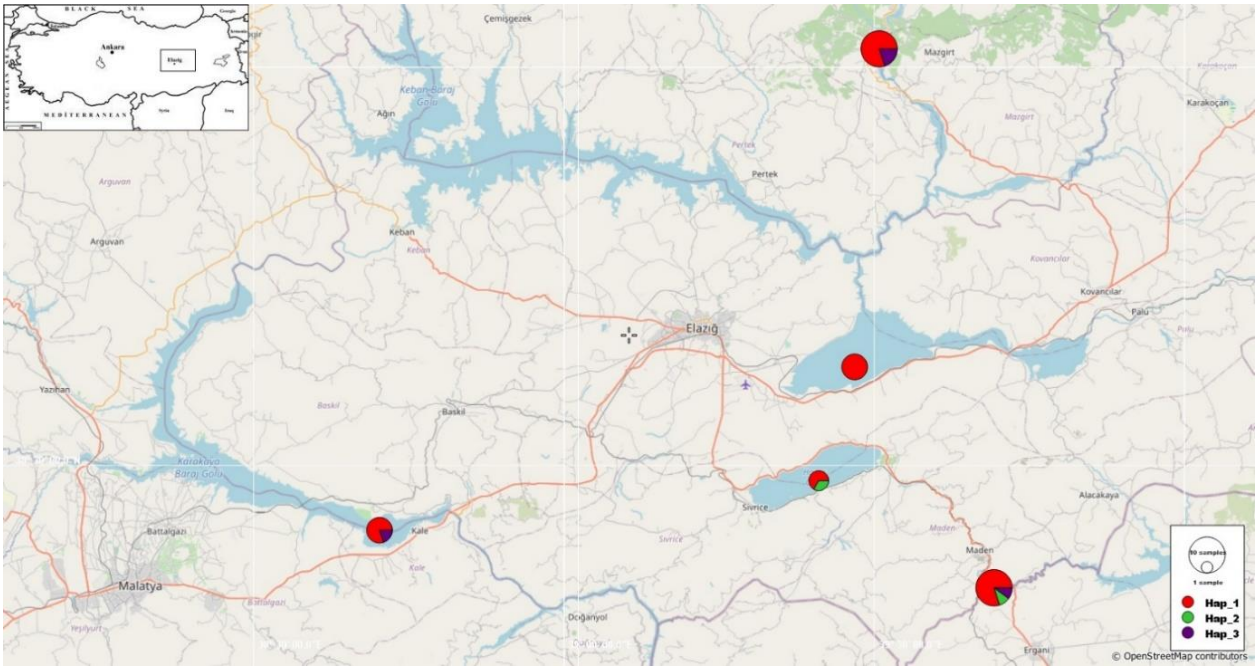

Figure 4. Distribution of haplotypes of *Diplostomum spathaceum* in wetlands in eastern Turkey. It was prepared considering the COX1 sequences produced from metacercariae of *D. spathaceum* sampled from *C. carpio* and *C. umbla* in Turkey.

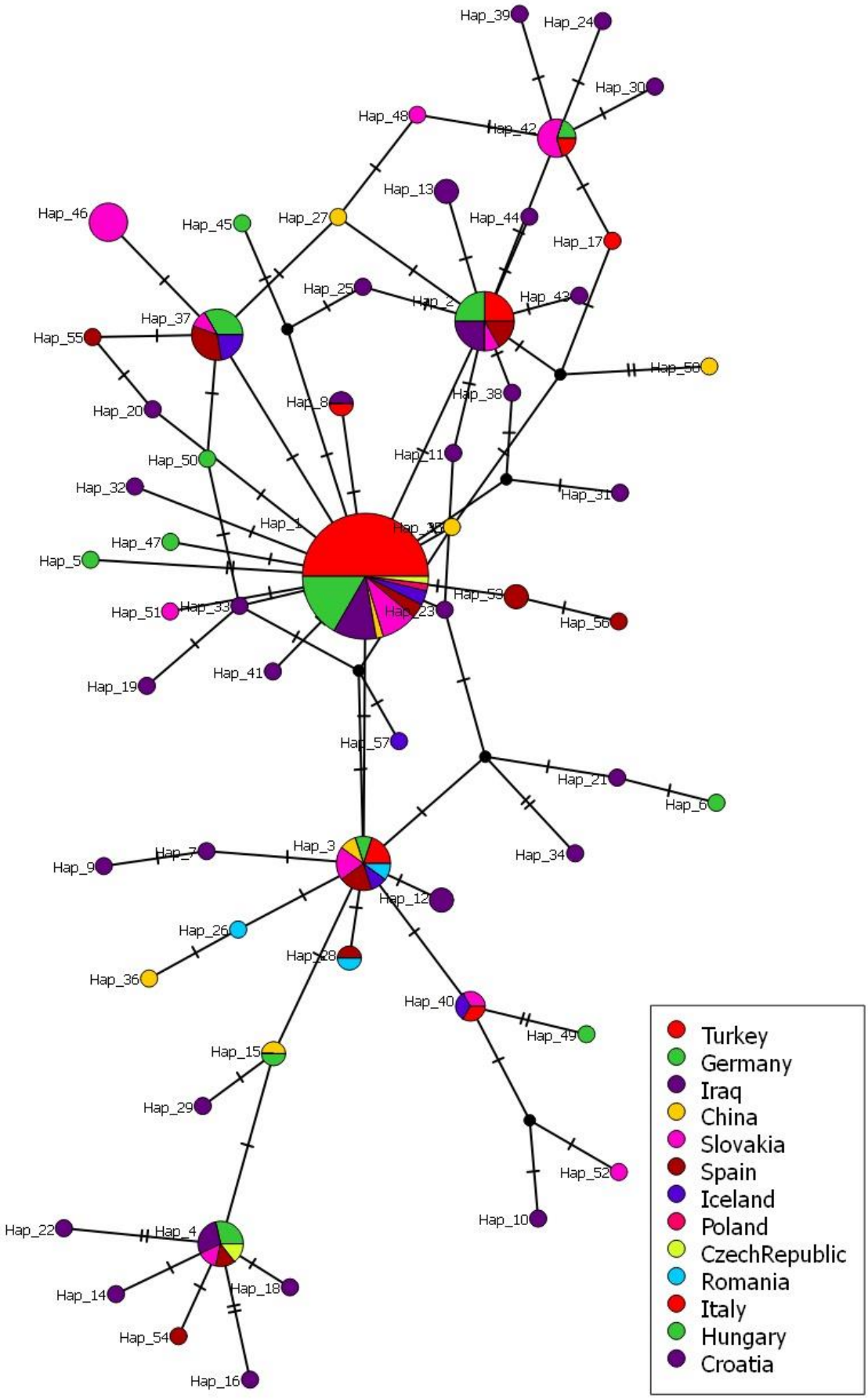

Figure 5. Haplotype network of Diplostomum spathaceum isolated from various geographical regions of world using the COX1 mtDNA gene. Size of circles is proportional to the frequency of each haplotype. Number of mutations distinguishing the haplotypes is shown by hatch marks.

Croatia, Czech Republic, Germany, Hungary, Iceland, Iraq, Italy, Poland, Romania, Slovakia, Spain located in the Eurasian continent (especially in Europe and the Middle East) (Blasco-Costa et al., 2014; Georgieva et al., 2013; Kudlai et al., 2017; Locke et al., 2015; Perez-del-Olmo et al., 2014; Selbach et al., 2015). In this study, the aforementioned parasite was determined in the eyes of *C. umbla* and *C. carpio* species living in freshwaters in the Eastern Anatolia Region of Turkey.

The morphological structure of metacercariae of Diplostomum is very close to each other in the species. Molecular studies constitute an important step especially in the diagnosis of metacercariae. The species of *Diplostomum* detected in the eyes of the fish could not be identified exactly morphological in the studies on *Acanthobrama marmid* from Keban Dam Lake (Elazig) (Dorucu et al., 2002), *Alburnus alburnus* from the Mustafakemalpasa Stream (Aydogdu & Selver, 2006), *Vimba vimba* from Golbası (Bursa) Dam Lake (A. Aydogdu et al., 2008), and in some cyprinids from Murat river (Bingol) (Gul et al., 2014). However, with this study, a definite molecular diagnosis of *Diplostomum* species, which is found in two cyprinid species (*C. carpio* and *C. umbla*) found in wetlands in Eastern Turkey according to the COX1 mtDNA gene region. According to this molecular diagnosis, it was determined that the species was exactly *D. spathaceum*. This article is the first study on *D. spathaceum* at the molecular level in Turkey.

Although morphological data create important problems in the diagnosis of Diplostomum metacercariae, the morphological characters obtained in our study showed similarities with earlier studies (Cavaleiro et al., 2012; Dörücü & Ispir, 2005).

It was determined that strong results were obtained in phylogenetic studies using the COI gene region in the light of morphological data in the identification of *Diplostomum* species. By using the sequences obtained in the study with the sequences previously given to GenBank, phylogenetic trees were formed and the proximity-distance relationships at Diplostomum species level were discussed (Blasco-Costa et al., 2014; Brabec et al., 2015). In this study, phylogenetic analysis was performed by taking the results obtained using the COI gene region and the sequence results previously given to GenBank. When the results were evaluated, it was determined that these studies were parallel and supporting each other.

The study results of Kudlai et al. (2017) have been shown similarities with our study, which although the mean intensity of the infection was low (7.63±7.83 - 15.38±19.87), the infection intensity was determined higher in some fish (67 parasites per fish). The prevalence (75%) on *D. spathaceum* of Kudlai et al. (2017), was almost equal to the prevalence of *C. carpio* investigated in our study. However, it was determined higher than the prevalence of *C. umbla*. The high prevalence of *D. spathaceum* in fish was the common result of both studies too. The prevalence of *D. spathaceum* in *Scardinius erythrophthalmus* fish of Kocadere Stream was found to be ~81-93% (Ali Aydogdu et al., 2008; Selver & Aydogdu, 2006), which is similar to the prevalence results in our study.

Locke et al. (2010) stated that the COX1 sequences they used in their study to differentiate Diplostomum at the species level were superior in determining pathogens that are taxonomically difficult to determine.

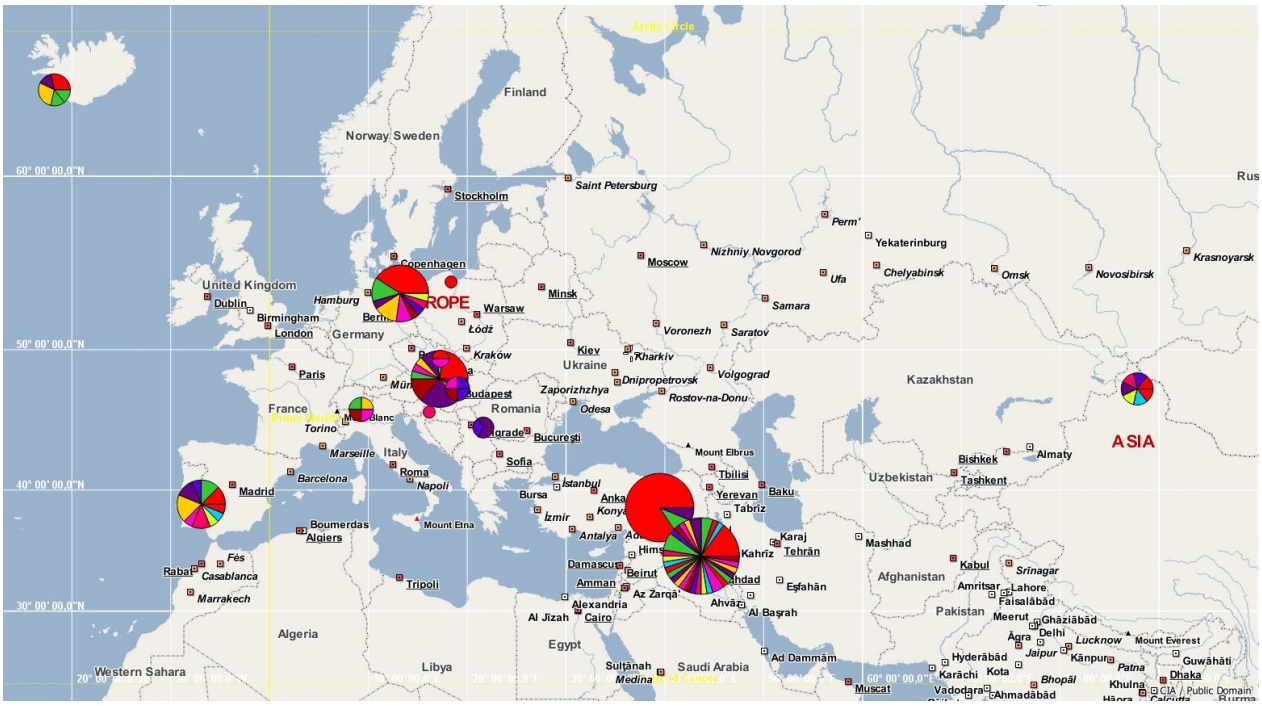

Figure 6. Distribution of haplotypes of *Diplostomum spathaceum* in world. It was prepared by considering all the COX1 sequences published so far from metacercariae of *D. spathaceum* sampled from fish in Europe and Asia.

The Plat-diploCOX1F/R primers, which were designed to amplify members of the family Diplostomidae by Moszczynska et al. (2009) have made important and successful contributions. In our study, it was seen that this primer pair, which was used for the amplification of the COX1 mtDNA gene region of D. spathaceum, gave very successful results. It was determined that the ML and NJ trees obtained by constructing the phylogenetic analysis using the Kimura K2 parameters of the COX1 mtDNA gene region sequences used in our study gave similar results to Georgieva et al. (2013) and Locke et al. (2015) (see Figure 1-2).

It is seen that mostly metacercariae in the eyes lens of fish have been studied when we look at the previous studies on *D. spathaceum* and in a small number, it is seen that the focus is on cercaria in snails and adult forms in waterfowl (Blasco-Costa et al., 2014; Georgieva et al., 2013; Kudlai et al., 2017; Locke et al., 2015; Perez-del-Olmo et al., 2014; Selbach et al., 2015). Similarly, in this study, metacercariae of *D. spathaceum* were detected in the eye lens of fish. Previous sequences obtained from both the cercaria and adult forms of the parasite and the metacercariae sequences determined in this study were found to be 98.4-100% similar. As a result of the distance matrix made on 162 sequence sets created by expanding the mtDNA sequences obtained in this study with sequences uploaded to GenBank from previous studies, it was determined that there was a maximum difference of 2.5% between *D. spathaceum* sequences. It is thought that this difference varies depending on the fish species and the geographical wetland where the samples were collected.

In a study in Europe on Diplostomum species, 10 haplotypes of *D. spathaceum* have been defined (Georgieva et al., 2013). While a total of 16 haplotypes were determined in 9 fish species in the Danube River, a total of 55 haplotypes from Asia and Europe were determined in snails and fish in the light of the COX1 sequence data of *D. spathaceum* obtained from GenBank. (Kudlai et al., 2017). It was revealed that *D. spathaceum* is grouped into 58 haplotypes in the world, with the data set created by considering the total three haplotypes of *D. spathaceum* on 33 sequences in Turkey determined as well as the sequences of all COX1 gene regions registered in Genbank.These data reveal that *D. spathaceum* can show very variable variation geographically and even within the same fish species.

The difference between the 33 COX1 mtDNA sequences (MW504357-MW504389) obtained from *C. carpio* and *C. umbla* fish living in the eastern Anatolia region of Turkey was determined to be a maximum of 0.2%. However, a maximum difference of 1.6% was detected in the distance analysis between the *D. spathaceum* sequences in our study and a total of 129 sequences from Genbank. When the expanded COX1 mtDNA sequence data were evaluated as a total, the difference between *D. spathaceum* was calculated to be approximately 2.5% maximum.

Although a total of three haplotypes were determined in the fish of the Dicle River according to our study (Figure 3-4), more haplotypes (total 30) were defined in the wetland which is the continuation of this river and continues in Iraq (Dicle-Tigris River) (Locke et al., 2015) (Figure 5-6). 14 haplotypes of *D. spathaceum* were determined in studies conducted in the Danube River (Kudlai et al. 2017). A total of three *D. spathaceum* haplotypes revealed in our study was grouped into similar haplotypes with sequences of *D. spathaceum* obtained from fish in both the Danube River of Europe (Kudlai et al., 2017) and the Dicle (Tigris) River in Iraq (Locke et al., 2015).

When we look at the parasitic distribution of *D. spathaceum* throughout the world, it is possible to say that it is distributed especially in fish in Europe, Asian, Middle East countries, and Turkey. It is seen that Haplotype-1 of this parasite species constitutes a very common group. The total number of haplotype groups containing five or more *D. spathaceum* sequences is six (Hap1, Hap2, Hap3, Hap4, Hap37, Hap42 and Hap46). The majority of the remaining haplotype groups are represented by a sequence of *D. spathaceum*.

Ethical Statement

Approval was obtained from the local ethics committee of Firat University for this study (Ethical approval number and date: 212 and 2016/131). The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

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Author Contribution

First Author: Lab and area research, Molecular analysis and Planning of the study, Data Curation, Formal Analysis, and writing -review; Second Author: Conceptualization, Funding Acquisition, Project Administration and editing; Third Author: Data Curation, Formal Analysis, Investigation, Methodology, Visualization, Molecular analysis; Resources, Writing-review; Fourth Author: Supervision, Molecular analysis, Writing - review and editing; Fifth Author: Lab and area research and writing -review.

Conflict of Interest

The author(s) 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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