

ULTRAMORPHOLOGY AND MOLECULAR STUDIES OF DIPLOSTOMUM PARACAUDUM (TREMATODA) COLLECTED IN GREATER ZAB RIVER FROM KURDISTAN REGION, IRAQ

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Abstract. The genus *Diplostomum* includes a large group of freshwater parasites with global distribution and complex life cycles. Larval stages are important pathogens that cause eye fluke disease, which contributes to significant impacts on natural fish populations and losses in aquaculture. The taxonomy of the genus *Diplostomum* is complicated and has been based mainly on the morphological features. The aim of the present study was to reveal the light on ultramorphology and molecular aspects of *Diplostomum paracaudum* (Iles, 1959) Shigin, isolated from the eyes of four species of fishes (*Capoeta trutta*=92, *Carassius auratus* =29, *Chondrostoma regium*=65 and *Cyprinion macrostomum*=7) collected from Greater Zab River from Kurdistan Region, Iraq. The study revealed that *C. regium* were most infected with a prevalence of 23%, while *C. macrostomum* was less infected with a prevalence of 14.2%. Morphological examination and measurements were conducted with optical microscopy and scanning electron microscope. Also, the genomic DNA of *D. paracaudum* was extracted and the ITS-2 regions were amplified by using PCR. The sequence of ITS1-5.8S-ITS2 of *D. paracaudum* in this study completely matched with the sequence of *D. paracaudum* that was identified in Denmark. The sequence of ITS1-5.8S-ITS2 of *D. paracaudum* provides a valuable resource for novel genetic markers, disease ecology and molecular epidemiology studies. Morphological features as well as molecular analysis showed that the collected specimens belonged to *D. paracaudum*. This is the first record in Iraq.

Keywords: phylogeny, *Diplostomum paracaudum*, morphology, eye fluke disease, ITS1-5-8s-ITS2 rDNA

Introduction

The Genus *Diplostomum* belongs to the Family Diplostomidae, Class Trematoda and Phylum Platyhelminth (Niewiadomska, 1996). *Diplostomum*, like other digenean trematodes, have attracted attention of countless studies due to their complex life cycles, The life cycle begins with adult *Diplostomum* flukes residing in the intestines of definitive hosts, which are typically piscivorous birds. The adult flukes lay eggs, which are passed through the bird's feces into freshwater environments and hatched in the water, releasing miracidia, which are ciliated larvae. The miracidia actively swim in search of their first intermediate host, freshwater snails, they penetrate its tissues and undergo development within the snail and transform into sporocysts, which then produce cercariae, (Caffara, 2019) the cercariae are the free-swimming, infective larvae. They leave the snail and swim in the water, actively searching for their second intermediate host, which is usually a fish. The cercariae penetrate the skin of the fish and then encyst within the fish's tissues. Here, they transform into metacercariae, (Locke et al., 2020) which are the next larval stage and are infectious to the definitive host. When the infected fish is consumed by a definitive host (piscivorous bird or other suitable predator), the metacercariae in the fish's tissues are ingested. Once inside the

bird's digestive system, the metacercariae excyst, and the juvenile flukes mature into adult *Diplostomum* flukes, completing the life cycle (Seppälä et al., 2006). The larval stages of *Diplostomum* needs a first intermediate host which is usually a snail. Also, most species needs a second intermediate host that can be vertebrate (Moszczyńska et al., 2009). Metacercariae of *Diplostomum* live in the eyeballs of freshwater fish (Karami et al., 2022). In the fish infected with *Diplostomum* parasite, one of the clearest signs is the formation of white spots or cataracts in the black background of the pupil, which can be seen unilaterally or bilaterally (Woo and Poynton, 1995). Blind fish lose the ability to adapt to the color of the environment and become very bright or very dark, therefore, they can be hunted by fish-eating animals and birds. also this parasite causes anemia and thinness in individuals, decrease the population of farmed aquatic animals and reduce the overall economic efficiency of production (Ashton et al., 1969).

The metacercariae of different species show a high morphological similarity and therefore species identification is difficult. Metacercariae of *Diplostomum* are important fish pathogens, but reliable data on their diversity in natural fish populations are virtually lacking (Kudlai et al., 2017). Also, the taxonomy of the genus *Diplostomum* is complicated, and has been based mainly on the molecular studies. Molecular investigation and sequencing DNA can be applied to determine species at all developmental stages, which is a major advantage for investigating parasites with complex life cycles (Locke et al., 2010).

The aim of present study was to shed the light on ultramorphology and molecular aspects of *Diplostomum paracaudum*.

Materials and Methods

Description of study area

Greater Zab River is one of the major Tigris River tributaries. It flows 392 km in length downstream of its original source from Turkey (Al-Ansari, 2013). Samples of fishes were collected inear Girda Rasha, Aski Kalak and Chamadbz City (36°37'0"N, 43°44'0"E) 40 km west of Erbil City in northern Iraq (Figure 1).

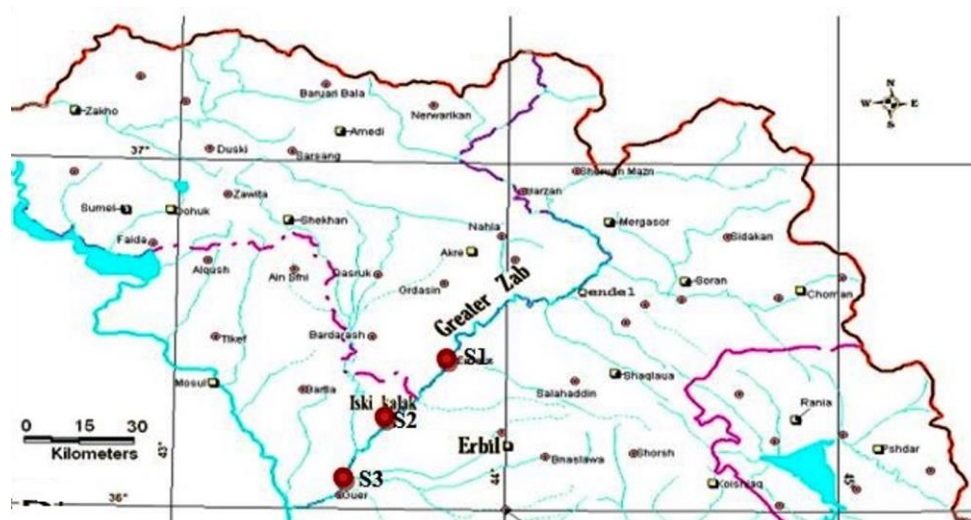


Figure 1. Map of Geater Zab River in northern Iraq

Sampling

Fish samples were collected from Greater Zab River once a month for a period from November 2020 to October 2021. Samples were collected in the morning in the middle of each month by fishermen using gill nets and transported alive in a cool box with local river water to the laboratory of College of Agricultural Engineering Sciences and examined within 24 hours after their capture. Totally, 193 fish belonged to four species including *Capoeta trutta* (n=92), *Carassius auratus* (n=29), *Chondrostoma regium* (n=65), *Cyprinion macrostomum* (n=7). The fish were identified based on their morphometric and meristic characteristics (Coad, 2010) and the scientific names for fishes were identified based on Forese and Pauly (Froese and Pauly, 2019).

Parasite collection and preservation

In the laboratory, fish were sacrificed by pithing. Their total length and weight were measured, and their gender were determined after dissection. The fish's entire eyes were removed, and the lenses were put in separate glass petri dish, then dissected out and teased apart using a stage 4 and 12 power-dissecting microscopes. The number of metacercariae in each lens was counted. The metacercariae were washed in saline solution before being fixed in 70% ethanol. Worms were stained in Mayer's acid carmine, dehydrated in ascending concentrations of ethanol, cleared in xylene and whole mounted in Canada balsam (Amlacher, 1970).

Margolis recommended using the terms infection parameters (prevalence, mean intensity, and mean abundance) to denote the percentage of infected hosts in a sample, the mean number of worms recovered from all infected fishes in a sample, and the mean number of worms recovered from all examined fishes (infected and uninfected) in a sample (Margolis et al., 1982).

Morphological identification

After opening the lens of eye, the metacercaria were washed with saline solution (0.9%), fixed in hot 4% formaldehyde solution (60°C) to relax the body, and preserved in 70% ethanol. A number of metacercaria were stored in absolute ethanol for molecular study (Amlacher, 1970). All measurements of the parasites were made with an Olympus ocular micrometre eyepiece and are given in millimeters. Photos were taken with a Sony Optical Steady Shot digital camera (model DSC-W570, 16.1 mega pixels).

Scanning electron microscopy

The metacercaria were prepared for scanning electron microscope by methods described by Erdtman (1952) and were washed three times with phosphate buffer solution (PBS). The samples later were dehydrated using series of acetone concentration percentages ranging from 50, 70, 80, 85, 90, 95% and three times in 100% acetone for 30 min each. Then, they were dried at critical point, were coated with gold in a sputter coater and were observed under the specimens to examine using a Quanta 450 scanning electron microscope at an accelerating voltage of 25 kV.

DNA extraction

Genomic DNA was isolated from each metacercaria after being preserved directly in absolute ethanol (99%). The genomic DNA was extracted using a QIAamp® DNA Mini

Kit. In brief, the parasite digested for 1 – 3 h at 56 °C with proteinase K in ATL buffer and eluted in 50 µl of AE buffer (QIAamp® DNA Mini Kit).

DNA amplification and sequencing

The polymerase chain reaction (PCR) was used to amplify the ITS1-5.8S-ITS2 regions. The specific primer SS2F/NC2R (Niewiadomska, 1996) were used to amplify the two nuclear ribosomal markers (ITS1-5.8S-ITS2).

The PCR reaction (in a volume of 30 µl) was performed in 25 mM Tris-HCl, pH 9.0 at 25°C and contained 50 mM KCl, 2 mM MgCl₂, 0.1 mg/ml gelatin, 200 µM dATP, dGTP and dTTP, 100 µM [α 32-P] dCTP (0.05 µCi/nmol), 12.5 µg of activated salmon sperm DNA, 10 pmol of each primer and 1.5 U *Taq* polymerase (Canvax Biotech, S.L.).

The PCR reactions were carried out in a thermocycler (Applied Biosystems 2720, USA) using the following cycling instructions: 94°C for 5 min (initial denaturation), 35 cycles of 94°C, 30 sec (denaturation), 55°C, 30 sec (annealing), 72°C, 30 sec (extension) and a final extension of 72°C for 7 min, followed by holding at 4°C. Two microliters of genomic DNA (20 – 40 ng) in nuclease-free deionised distilled water were added to each PCR reaction.

Five microliters of each PCR product were examined on a 1.5% w/v agarose gel, stained with DNA stain (Good View™ SBS Genetech Beijing, China) and photographed using a gel documentation system. A 1000 bp DNA ladder (Vivantis, Malaysia) was used. The resulting products were sent to Macrogen in South Korea for nucleotide sequence analysis by a dideoxy termination method using a Genetic Analyzer 3500 DNA sequencer (Applied Biosystems, USA) in both directions (forward and reverse) using the same PCR primers (Pérez-del-Olmo et al., 2014).

Phylogenetic analysis

The resulted ITS1-5.8S-ITS2 sequences (forwards) were compared with their complements (reverses) and then adjusted using online software tool (www.bioinformatics.org/sms/rev_comp.html) to obtain reverse complement. Then the resulted sequences were aligned to each other using multiple sequence alignment program using the online software tool CLUSTALW (genome.jp/tools-bin/clustalw) to get the most homologous sequences (one sequence).

The obtained sequences were then compared to previously published sequence data for identification using the Basic Local Alignment Search Tool (BLAST) from the GenBank database (<http://www.ncbi.nlm.nih.gov/>) (Altschul et al., 1990). Individual *Diplostomum paracaudum* ITS1-5.8S-ITS2 sequences from different fish host species in the current study were aligned using the online computer program CLASTALW (<https://www.genome.jp/tools-bin/clustalw>) and then manually adjusted for searching nucleotide variations in *Diplostomum paracaudum* in various fish host species.

The sequence data of ITS1-5.8S-ITS2 fragments obtained from *Diplostomum* metacercaria collected from all different fish host species were installed into the MEGAX version 10.7.1 software program for the phylogenetic study (Kumar et al., 2018). To unify the sequence lengths, the common 447, 268, and 475 bp lengths of ITS1-5.8S-ITS2 segments, were chosen and used for phylogenetic analysis to determine the most appropriate sequence evolution model for the given data, with gaps and missing data treated with the partial deletion option. CLUSTALW alignment was used to align the sequences in order to build evolutionary development trees. The trees for all

isolated species were built using the Maximum Likelihood (ML) method and the Tamura-Nei model (Tamura and Nei, 1993).

Results

Prevalence of metacercaria of Diplostomum

A total of 193 cyprinid fishes (92 *C. trutta*, 29 *C. auratus*, 65 *C. regium* and 7 *C. macrostomum*) were collected from Greater Zab River during the present study. Eye lenses of these fishes were infected with metacercariae of *D. paracaudum*. The overall prevalence of infection was 7.6%, 13.7%, 23% and 14.2, respectively. The mean intensity of infection for each species was 2, 2.75, 4.2 and 4, respectively (*Table 1*).

Table 1. Prevalence of *D. paracaudum* and mean of intensity among infected fish species

| Host | Examined | Infected | Prevalence % | No. of parasite | Mean intensity |
|-----------------------|----------|----------|--------------|-----------------|----------------|
| Capoeta trutta | 92 | 7 | 7.6 | 14 | 2 |
| Carassius auratus | 29 | 4 | 13.7 | 11 | 2.75 |
| Chondrostoma regium | 65 | 15 | 23 | 63 | 4.2 |
| Cyprinion macrostomum | 7 | 1 | 14.2 | 4 | 4 |

Morphological identification

Morphological examination and measurements were conducted with optical microscopy and identified that the metacercaria in the present study were *D. paracaudum*. The metacercaria appeared off-white, body elongate-oval and flattened (*Figure 2*). The total length of the larvae was 0.190 - 0.273 mm, with a width of 0.106 - 0.179 mm. The length of esophagus was 0.7-1.3 mm, the length of intestinal caecum was 0.20 - 0.35 mm and the length of esophageal caecum was 0.25 - 0.40 mm.

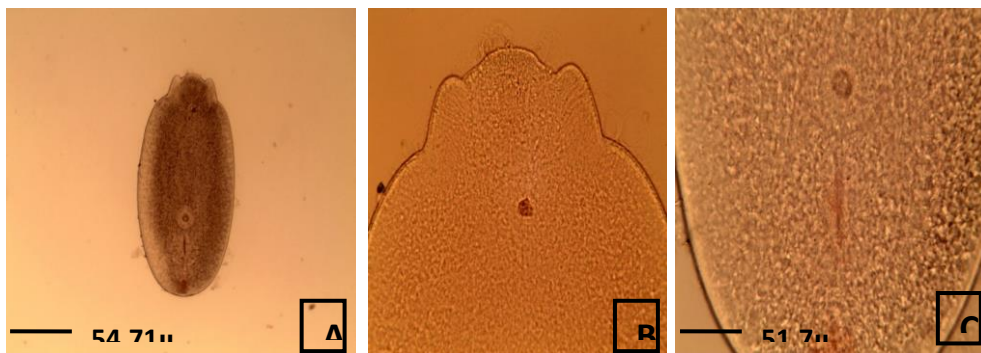


Figure 2. Metacercariae of *Diplostomum paracaudum*. Scale-bars: μm . A – Whole body of the metacercariae, B - Pseudo sucker region and oral sucker of the metacercariae, C - Ventral sucker and excretory pore of the metacercariae

Primordial hind body not distinct. The dimensions of oral sucker spherical were $29 \times 29 \mu\text{m}$ and the dimensions of ventral sucker subspherical were $37 \times 42 \mu\text{m}$. There were two small contractile lappets (pseudosuckers) on each side of oral sucker, 31 - 32 μm long and 15 - 16 μm in maximum width. There was no prepharynx and pharynx was

subspherical with dimensions of $24 \times 23 \mu\text{m}$. The oesophagus was very short, and caeca was long, narrow, reach posterior to holdfast organ. The Holdfast organ was large, transversely elongated, with dimensions of $50 \times 84 \mu\text{m}$. Reserve excretory system was covered with numerous, dispersed, relatively large excretory granules.

Scanning electron microscope (SEM)

Examination of the metacercariae by SEM technique showed the general body surface and represented the external features including the oral and ventral suckers, the pseudo suckers and the tribocytic organ (*Figure 3*).

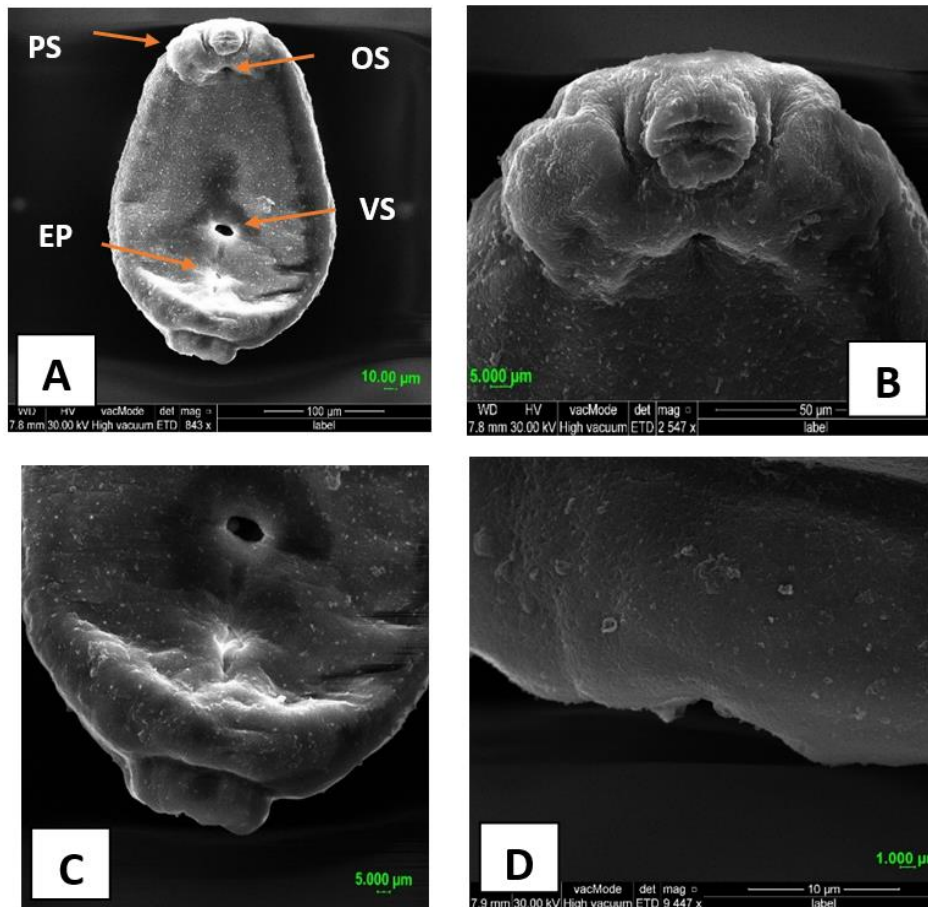


Figure 3. Scanning electron microscopy images of *Metacercariae* of *Diplostomum paracaudum*. (A) General morphology of ventral view with pseudo sucker region (PS), oral sucker (OS), ventral sucker (VS) and excretory pore (EP) of the metacercariae. (B) Anterior part of the metacercariae with pseudo sucker region and oral sucker. (C) Posterior part of the metacercariae with ventral sucker and excretory pore. (D) Tegument part of the metacercariae

The anterior portion comprises most of the worm, while the posterior body is reduced to a small, posterodorsal, conical projection, at the tip of which the excretory pore can be discerned. The ventral surface of the frontal is flat or slightly concave, and the dorsal surface is somewhat convex. Its tegument is unarmed and smooth, but somewhat irregular and porose in the region of the lappets. The mouth is subterminal ventral.

DNA amplification and sequencing

The internal transcribed spacer cluster (ITS1-5.8S-ITS2) of the rRNA gene were amplified and sequenced. Totally, 11 sequences were found and deposited in GenBank database with accession numbers of ON885465 to ON885476 (Table 2).

Table 2. Accession numbers provided by NCBI for the collected *Diplostomum metacercaria* in different fish hosts in the present study

| Sample ID | Samples Accession Number | Query Cover % | Identic Number % | Genbank Accession Number | Genbank Species Identification | Country Identification |
|-----------|--------------------------|---------------|------------------|--------------------------|--------------------------------|------------------------|
| 1 | ON885465 | 100 | 100 | OK329895.1 | Diplostomum paracaudum | Denmark |
| 2 | ON885466 | | | | | |
| 3 | ON885467 | | | | | |
| 4 | ON885468 | | | | | |
| 5 | ON885469 | 100 | 99.91 | KJ888995.1 | Diplostomum paracaudum | Denmark |
| 6 | ON885470 | | | | | |
| 7 | ON885471 | | | | | |
| 8 | ON885473 | | | | | |
| 9 | ON885474 | 93 | 99.81 | JQ665457 | Diplostomum paracaudum | Germany |
| 10 | ON885475 | | | | | |
| 11 | ON885476 | 93 | 99.89 | MW135078.1 | Diplostomum paracaudum | Denmark |

Phylogenetic analysis

A molecular phylogenetic analysis was performed based on ITS1-5.8S-ITS2 sequences from the present study together with ITS1-5.8S-ITS2 sequences of *Diplostomum* spp. and one outgroup sequences of *Braunina cordiformis* (Figueroa and Franjola, 1988), (GenBank accession No. KY951725) retrieved from GenBank. The results showed that the sequence of *D. paracaudum* in this study completely align with the sequence of *Diplostomum paracaudum* that was identified in Denmark (with Identic Number and Query Cover of 100%).

The foundation behind choosing the above 20 *Diplostomum* spp. sequences was that these have all been published in peer-reviewed literature, and the origin of parasites and the methodology used for species identification can thereby be corroborated. These sequences were chosen as representatives for certain species/isolates and it should be noted that identical GenBank sequences, which did not add further to the present analysis, were not included. Sequences were aligned with Clustal X v2.1 and the phylogenetic analysis was conducted in MEGA using the Maximum Likelihood method based on the K2+G model (suggested as the best-fit model in MEGA). A bootstrap consensus tree was inferred from 1000 replicates.

The results of phylogeny analysis showed that all of 20 ITS1-5.8S-ITS2 sequences of *Diplostomum* spp. were clustered together and the outgroup sequence was placed in a separate cluster (Figure 4). ITS1-5.8S-ITS2 sequences of *D. paracaudum* clustered based on fish species, in such a way that there were five major cluster belonged to five fish species. However, the sequences of *Carassius auratus* showed the most distance from other ITS1-5.8S-ITS2 sequences of *D. paracaudum*.

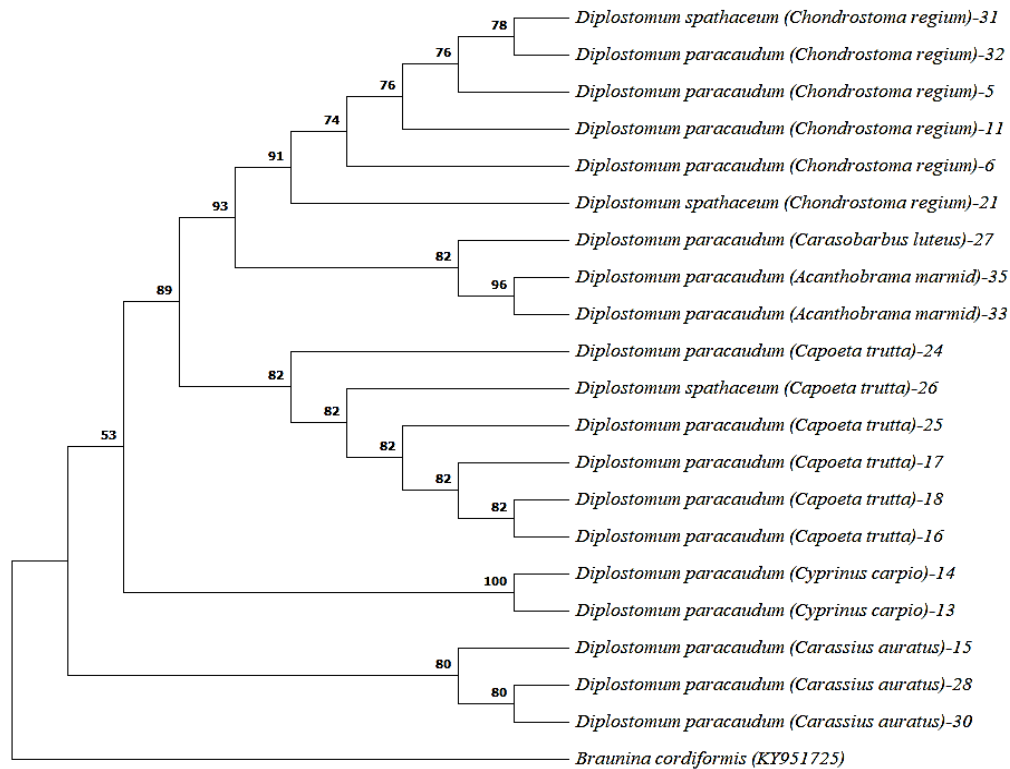


Figure 4. Phylogenetic analysis inferred using the Maximum Likelihood method based on an alignment comprising 20 ITS1-5.8S-ITS2 sequences of *Diplostomum* spp. and one outgroup sequences

Discussion

In present study, four different species of fish were distinguished and prevalence of *D. paracaudum* was examined. Morphological examination and measurements were conducted with optical microscopy and identified that the metacercariae were *D. paracaudum* examination of the metacercariae by SEM technique shows the general body surface and confirms the external features. Also, molecular techniques were applied to confirmation and distinction of this species.

The first information on the occurrence of *Diplostomum* sp. metacercariae in Iraq was by Ali (1989) who detected it from six cyprinid fish species from Greater Zab River. After that, Abdullah (1990) recorded *D. spathaceum* from *Barbus luteus*, *Cyprinus carpio* and *Cyprinion macrostomum* from Dokan Lake. According to Mhaisen (2023), ten species of *Diplostomum* were recorded from the lenses of freshwater fishes of Iraq (*D. commutatum*, *D. flexicaudum*, *D. indistinctum*, *D. montanum*, *D. paraspathaceum*, *D. phoxini*, *D. pungiti*, *D. spathaceum*, *D. volvens* and *D. yogoenum*), in addition to the record of unidentified species of this genus from lenses of 16 fish species. Since no previous report about recording of this species is available in Iraq, the present record represents the first record of *D. paracaudum* in Iraq.

Niewiadomska investigated morphological characteristics of *Diplostomum paracaudum*. The results represented a technique based on the bootstrap method for increasing the discriminant power between this species and *D. pseudospathaceum* (Niewiadomska and Niewiadomska-Bugaj, 1995). In another study, metacercariae of two species of *Diplostomum paracaudum* and *Diplostomum pseudospathaceum* were

identified using DNA polymorphism study (Laskowski, 1996). The variation of the metacercariae of diplostomid in Tanzania was investigated. The results showed doubt about the reliability of measurements in the determination of species in these trematodes (Chibwana and Nkwengulila, 2010). The morphology and genetics of *Diplostomum* species were studied in Portugal. The outcomes of their study showed existence of consistent differences between the *Diplostomum* species metacercariae (Cavaleiro et al., 2012).

Karami et al. (2022) investigated the existence of parasites in salmonid aquaculture in Denmark and recorded *D. paracaudum*. The sequence of *D. paracaudum* in their study completely match to the sequence of this parasite in present study.

In this study, *D. paracaudum* was investigated and the taxonomy of the genus *Diplostomum* is complicated and ITS1-5.8S-ITS2 sequences of *D. paracaudum* is similar to other species of *Diplostomum*. Therefore, few researches studied this species. Also, eye fluke infection status in Baltic cod was investigated and *Diplostomum paracaudum* was identified in eye lenses of fish. The sequence of this parasite is closely similar to the sequence in our study (with Identical Number of 99.91% and Query Cover of 100%) (Mehrdana et al., 2015).

Understanding the phylogeny of *D. paracaudum* is crucial for elucidating its evolutionary relationships, taxonomic placement, and potential implications for host-parasite interactions. In this scientific discussion, we will explore the current knowledge regarding the phylogenetic position of *D. paracaudum* based on available molecular and morphological data.

Utilized ITS1 and ITS2 sequences by Pérez-del-Olmo et al. (2014) to investigate the phylogenetic relationships of *Diplostomum* species, including *D. paracaudum*. Their findings revealed distinct genetic lineages for *D. paracaudum*, supporting its classification as a separate species within the *Diplostomum* genus.

Conclusion

The investigation of *D. paracaudum* in Greater Zab River based on molecular and morphological features showed the existence of this parasite in the eye lenses of fishes. The sequenced region of this parasite was matched to previous sequenced region of *D. paracaudum*. Also, the results revealed a high host-specificity in *D. paracaudum*. The sequence data can be used further for ecological studies on the distribution and host ranges of these major fish parasites in Iraq. In conclusion, that is the first record for the existence of *D. paracaudum* in Iraq. The morphological characters and DNA sequence-based analysis revealed the identity of the species.

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