Morphological and molecular identification of Euclinostomum heterostomum in the spotted snakehead (Channa punctata) in Narayanganj, Bangladesh

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Abstract

Parasites of the Clinostomidae family are widely distributed worldwide. Freshwater fish of Bangladesh frequently serve as hosts for various Clinostomidae species. The metacercariae stage of *Euclinostomum heterostomum*, a prevalent digenetic trematode, exhibits a distinct affinity for infecting Channidae species and becomes encysted within the liver, kidneys, and muscles of these species. This study focused on identifying *E. heterostomum* in *Channa punctata* using a combination of morphological and molecular approaches. The morphological characteristics of the species were examined using both light and scanning electron microscope. Encysted flukes, inflammatory infiltrates of leukocytes, along with degeneration and loosening of hepatic tissue were observed in the histopathological examination of the infected liver tissue. Molecular analysis of the partial nuclear ribosomal internal transcribed spacer (ITS rDNA) sequence confirmed the presence of *Euclinostomum heterostomum*. A comparison of the collected metacercariae sequence (Accession no. OR591452) with the NCBI GeneBank databases found similarity to other species within the same genus in India and Thailand. The phylogenetic reconstruction provided compelling evidence of genetic similarity among different strains of the genus *Euclinostomum*, indicating their shared genetic heritage.

Introduction

The spotted murrel, scientifically known as *Channa punctata* (Bloch, 1793), is a species of snakehead fish belonging to the Channidae family. This warm-water teleost, commonly referred to as the spotted murrel, is native to Southeast Asia and is widely distributed in freshwater ecosystems such as lakes, ponds, and rivers, as well as in confined water (Jayaram, 2010). However, freshwater murres serve as a common host and habitat for a diverse array of parasites, fostering a complex ecological relationship within their aquatic environment (Gautam et al., 2018; P. A. A. Shareef & Abidi, 2015; Sharma & Sharma, 1980; Verma et al., 2018). They harbor a significant quantity of helminth parasites and infections induced by *Euclinostomum heterostomum* metacercariae (Donges, 1974; Gupta & Agarwal, 1983). Metacercariae from *Euclinostomum* species exhibit encystment in the kidneys, liver, and muscles across various fish species, with adults commonly found residing in the mouth, pharynx, larynx, trachea, and upper esophagus (Caffara et al., 2016). Parasite-infected fish lose their capacity to absorb nutrients from their diet as their energy is redirected to combat the infection, resulting in malnutrition and hindered growth. Internal parasites harm vital organs, such as the liver, kidney, stomach, and intestines, causing functional disruption, while prolonged infections weaken the fish's immune system, increasing susceptibility to secondary infections (Feist & Longshaw, 2008; Okon et al., 2023).

Histopathological changes induced by parasites of the Clinostomidae family in *Channa* spp. involved a decrease in glomerular size, severe degeneration, and necrosis of hemopoietic tissue. In *C. marulius*, the infected kidney displays melano-macrophage centers, hypertrophied nuclei of tubule cells, and detached epithelial cells (Kaur et al., 2016). Heavy infiltration of immune cells at cyst attachment sites was observed, leading to tissue damage (P. A. Shareef & Abidi, 2012; Suanyuk et al., 2013). Tubule cells exhibited hypertrophied nuclei, and these renal alterations were associated with liver melanosis and visceral fibrosis. Parasitic cysts replacing liver and kidney tissues, along with dilated renal tubules with leucocytic infiltrations, hepatic tissue degeneration, compression, and loosening, accompanied by enucleated and reshaped hepatocytes (Kaur et al., 2012; P. A. A. Shareef & Abidi, 2015; Younis et al., 2022).

The trematode species *E. heterostomum* (Rudolphi, 1809) of the Clinostomidae family is notable for its wide-ranging presence across the globe. Records of its occurrence extend from southern Europe through Russia to Africa and across Asia (Gokcen, 2008). *E. heterostomum* adults are commonly located within the oral cavity of herons, primarily at the base of the tongue, and the metacercarial stage employs fish (*Channa* spp.) as their intermediate hosts (Caffara et al., 2016; Donges, 1974; Jhansilakshmibai & Madhavi, 1997). Although eight *Euclinostomum* species have been documented, their taxonomic validity remains controversial. Traditionally, *Euclinostomum* species have been identified based on their morphological features. Combining molecular techniques with morphological studies helps to clarify their distinct taxonomic positions within this fluke group (Caffara et al., 2016).

Narayanganj is a densely populated district in Bangladesh with a high industrial area (Noman et al., 2016). Every year, a huge amount of industrial wastage is released into the surrounding rivers. These chemical agents hamper natural and cultured fish species, especially bottom dwelling fishes (Kumar Maurya et al., 2019). Fish parasites have emerged as effective markers of environmental well-being, showcasing variations in their population in direct correlation with shifts in water parameters and the influx of contaminants (Jeronimo et al., 2022). Furthermore, there is a dearth of data on Euclinostomid flukes in Bangladesh. The objective of this study was to conduct a comprehensive investigation encompassing morphological, histopathological, and molecular identification of *Euclinostomum heterostomum* in spotted snakehead (*C. punctata*) from the Shitalakshya River of Narayanganj district, Bangladesh.

Materials and Methods

Study Area

From January to March 2023, 160 live *Channa punctatus* specimens were collected from aquaculture farms situated in eight different locations along the Shitalakshya River in Narayanganj district in Bangladesh. The geographical location of the study area was presented using the GIS software QGIS 3.34 version (Moyroud & Portet, 2018). Following collection, live fish were transported to the Fish Disease Laboratory, Department of Aquatic Animal Health Management, Sher-e-Bangla Agricultural University, Bangladesh, in aerated polyethylene containers.

Specimen Collection

Fish specimens were anesthetized, followed by the assessment of weight, measurements, and thorough inspection. A standardized procedure was carefully followed to assess the fish for parasites, involving necropsy and visual examination of the exposed body cavity (gastrointestinal tract, muscle tissue, and other internal organs) for any parasitic infestations (Meyers, 2004). Parasites were obtained by dissecting fish specimens kept in a physiological saline solution (0.75% NaCl), specifically targeting encysted trematodes (metacercarial stage) within the liver, kidney, and peritoneal membrane. Collected trematode parasites were compressed between two slides or placed under the slight pressure of a slide and coverslip. Alternate specimens were stored in vials containing 100% ethanol for subsequent molecular analysis. The prevalence and mean intensity were also determined (Bush et al., 1997).

Morphological study

After initial flattening and fixation in alcohol, formalin, and acetic acid in 85:10:5, the specimens were dehydrated in an ascending ethanol series, cleared in xylene, stained with borax carmine, and then mounted on glass slides using DPX (Gokcen, 2008). Using the stereo microscope (Motic SMZ160T, Hong Kong), photomicrographs of stained specimens were studied, then sketched, and identified according to the standard literature (Yamaguti, 1971). The internal organs of mounted parasites were measured using a light microscope (Euromex D. 1355 F050, Netherlands).

Scanning electron microscopy

The collected metacercaria were subjected to ultra-morphological examination using a scanning electron microscope (SEM). Two specimens intended for scanning electron microscopy (SEM) were dehydrated in a graded series of ethyl alcohol and then dried at 89 degrees centigrade for 24 hr. in a microwave oven. To obtain fine focus, the sample was mounted on metal stubs using silver paste, coated with gold, and examined with an FE-SEM Model Sigma 300 VP, Carl Ziess, Germany) (Briosio-Aguilar et al., 2019).

Histopathological study

The liver tissues afflicted with infection underwent thorough washing with water before preservation in buffered 10% formalin for approximately 24 h, preparing them for subsequent histological examinations. Subsequently, the specimens underwent a dehydration process involving a series of ethanol concentrations (50%, 70%, 90%, and 100%) and 2-propanol, followed by clearing in xylene (Viktorov & Proshin, 2003). Following dehydration in a graded ethanol series, the samples were embedded in

paraffin wax. Paraffin blocks were fashioned from molten wax and maintained at 65°C for 5–6 h. Histopathological evaluations and analysis of cellular infiltration at the attachment site were conducted by cutting serial sections of 5 µm thickness using a rotatory microtome (MicroTec, Germany). The sliced samples were placed in a hot water bath at 45^0C for a while, transferred to an oven at 70°C for 30 min, and cleared again in xylene and 2-propanol. Subsequently, staining with Heidenhain's hematoxylin and eosin was performed, followed by another round of dehydration using a succession of ethanol concentrations (70%, 90%, and 100%), clearing with xylene, and finally mounting with DPX. The DPX-mounted sections were examined, and photographs were captured using a light microscope (Euromex D. 1355 F050, Netherlands).

Molecular identification

The collected metacercarial specimens underwent saline rinsing before preservation in (96–100%) ethanol until DNA extraction. Genomic DNA was extracted from the samples using the TIANamp Marine Animals DNA Kit, following the manufacturer's instructions. For the amplification of the ITS region, a primer pair set (forward 5'- GTCGTAACAAGGTTTCCGTA – 3' and reverse 5'- TATGCTTAAATTCAGCGGGT – 3') with a product size of 1021 base pairs was designed and used (Kaur et al., 2020). The polymerase chain reaction (PCR) mix included Green Buffer 2.5 L, dNTP mix 0.5 L, ITS (F-1) 2 L, ITS (R-1) 2 L, Nuclease-free water 16.5 L, Template DNA 3 L, and Taq DNA polymerase 0.5 L, totaling a reaction volume of 27 L. The thermocycler program comprised an initial denaturation at 94.0°C for 5 min, followed by 35 cycles of 94.0°C for 1 min, 52°C for 1 min, 72°C for 1 min, and a final elongation at 72°C for 10 min. Subsequently, the PCR products were confirmed via gel electrophoresis in 0.5 g agarose gels, 50 L 10 TE buffer, and staining with 2 g ethidium bromide. The purified PCR products were sequenced in Wahan, China. The acquired partial ITS rDNA sequence (forward) was edited using BioEdit version 7.2 software, and the resulting 830-bp sequence was deposited in NCBI GenBank (Accession no. OR591452). This sequence was compared for identity with available sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST). The retrieved similar sequences from the BLAST result multiple sequence alignment was conducted using the ClustalW system with default parameters (K.-B. Li, 2003). The phylogenetic tree was constructed using MEGA 11 software using the neighbor-joining method with a bootstrap method test of phylogeny involving 1,000 bootstrap replications (Tamura et al., 2021).

Results

Collection and prevalence of E. heterostomum in C. punctata

This study conducted a thorough examination of *E. heterostomum* prevalence in diverse locations within Narayanganj, Bangladesh. Among the 160 fish under scrutiny, 41 were identified as infected, yielding an overall prevalence rate of 25.63%. Geographical coordinates for each area are as follows: Narayanganj Sadar (23.6000° N, 90.5000° E), Fatulla (23.6423° N, 90.4817° E), Araihazar (23.79267° N, 90.65315° E), Bandar (23.61698° N, 90.51280° E), Sonargaon (23.64216° N, 90.60234° E), Nabiganj (23.62006° N, 90.50147° E), Bhulta (23.78368° N, 90.56734° E), and Gopaldi (23.80209° N, 90.71099° E) (Table 1) (Fig. 1).

Table 1
Prevalence of *E. heterostomum* in different locations in Narayanganj, Bangladesh.

Areas	No. of fish infected/ No. of fish examined	Prevalence (%)	Geographical coordinates
Narayanganj Sadar	2/20	10	23.6000° N, 90.5000° E
Fatulla	5/20	25	23.6423° N, 90.4817° E
Araihazar	5/20	25	23.79267°N 90.65315°E
Bandar	9/20	45	23.61698°N 90.51280°E
Sonargaon	6/20	30	23.64216°N 90.60234°E
Nabiganj	7/20	35	23.62006°N 90.50147°E
Bhulta	3/20	15	23.78368°N 90.56734°E
Gopaldi	4/20	20	23.80209°N 90.71099°E
Total	41/160	25.63 (avg.)	

The collected *C. punctata* exhibited infestations with diverse types of endoparasites. Encysted metacercariae of *E. heterostomum* were found embedded in the liver, kidney, and peritoneal membrane of the infected fish (Fig. 2).

Morphometric identification of the collected metacercaria

This study provides morphometric characterization for the identification of *E. heterostomum* in *C. punctata*. In this study, various morphometric observations, including organ sizes, their positions, and the ratio of body length to body width, were examined to validate the identity of the parasite, in line with the existing literature. The identification of metacercaria in this study as *E. heterostomum* (Rudolphi, 1809, Travassos, 1928) was accomplished by observing essential morphological characteristics, including body shape, oral and ventral suckers, and reproductive structures, and comparing them with established descriptions in the literature (Yamaguti, 1971). Examining *E. heterostomum* under a microscope revealed distinct features in its body structure, marked by two suckers, varying in size, branched intestinal ceca and reproductive organs (Fig. 3).

The entire *E. heterostomum* specimen displayed a body length ranging from 8.2 to 10.5 mm and a width of 2.3 to 3.6 mm. The oral sucker, small, rounded, and subterminal, had a diameter of 0.112 × 0.33 mm, whereas the well-developed ventral sucker, larger than the oral sucker, measured 1.5 × 1.7 mm and was positioned at the first third of the body. Within the reproductive system, the testes are in pairs, intercaecal, in the body's middle and posterior thirds. The anterior testis appeared bean or U-shaped, with a length ranging from 0.32 to 0.54 mm and a width of 0.41–0.72 mm, whereas the posterior testis exhibited a triangle shape with dimensions of 0.41–0.54 mm in length and 0.59–0.68 mm in width. The ovary was situated between the two testes, measuring 0.19–0.28 mm in length and 0.16–0.22 mm in width (Table 2). The intestinal ceca exhibit branching, with enlargement in the pre-acetabular region, extending laterally to the ventral sucker at the posterior end of the body. The major ceca located posterior to the ventral sucker generate secondary blind diverticula, with varying numbers (ranging from 9 to 13 on both the right and left sides). The genital pore opens on the margin of the anterior testis, and a tegument surface devoid of spines is evident.

Table 2
Morphometric value of four *Euclinostomum heterostomum* evaluated in the present study.

Measurements	This study (n-4) *E. heterostomum* (mm)	Yonis et al. (2022) *E. heterostomum* (mm) (Younis et al., 2022)	Purivirojkul and Sumontha (2013) *E. heterostomum* (mm)
Host species	*Channa punctata*	*Oreochromis niloticus*	*Channa punctata*
Microhabitat	Body cavity	Body cavity	Body cavity
Metacercaria form	Liver, Peritoneal cavity	Liver, Kidney	Liver, Kidney
Body length	8.2–10.5	9.3–10.0	5.78–7.77
Body width	2.3–3.6	3.0–3.5	2.25–2.73
Oral sucker diameter	0.11 × 0.33	0.13–0.39 × 0.75–0.95	0.19 × 0.26
Ventral sucker diameter	1.5 × 1.7	1.8–2.1 × 1.5–1.95	0.97 × 0.93
Distance between suckers	1.1–1.6	1.5–1.8	
Anterior testis			
Shape of anterior testis	Bean or Lobed	Lobed	Lobed
Length	0.32–0.54	0.25–0.3	0.13
Width	0.41–0.72	0.63–0.7	0.179
Posterior testis			
Shape of posterior testis	Y-shaped	Y-shaped	Y-shaped
Length	0.41–0.54	0.38–0.43	0.530
Width	0.59–0.68	0.5–0.58	0.680
Ovary			
Shape of ovary	Oval	Oval or bean	Oval or bean
Length	0.19–0.28	0.18–0.23	0.179
Width	0.16–0.22		0.149
No. of caeca diverticula	9–13	8–10	10–12

Scanning electron microscopy analysis

The SEM examination exposes a fascinating view of this parasitic organism, which is characterized by a flattened, leaf-shaped body adorned with surface structures. SEM imaging revealed a clearly defined elongated and broad-bodied structure, rounded at both ends, featuring a strategically positioned subterminal oral sucker in the anterior region of the organism. The microphotographs from SEM also revealed the existence of a collar-like ventral sucker, contributing to the parasite's ability to adhere firmly to the host tissues. The domed ventral sucker was identified in the anterior one-third of the body. The body surface, as observed through SEM, displays a textured pattern, possibly associated with sensory or locomotory adaptations. Minute details such as sensory papillae or tegumental spines may become apparent, providing insights into the parasite's interaction with its environment and host (Fig. 4).

Histopathological analysis of the liver

Histological analysis of *C. punctata* liver caused by trematode infection demonstrates unique alterations that reflect the effects on the organ. Microscopic analysis demonstrated the presence of inflammatory infiltrates surrounding the hepatic tissues. The infiltration of basophils around the main blood vessel and central artery indicates an immune response to the parasite infection. The parasites were deeply embedded throughout the hepatic tissue, forming cysts that constrained the liver parenchyma and creating lumps between the cysts (Fig. 5).

Molecular Identification

For molecular analysis, the ITS rRNA region was partially sequenced to discern and differentiate the causative agents from closely related species (Fig. 6).

Raw sequences were obtained and subjected to a BLAST search against the NCBI sequence database to assess sequence similarity with closely related species. In comparison, the sequence displayed 100% identity with the *E. heterostomum* isolate recorded in India (GenBank No. MT785768) and Thailand (GenBank No. KC894799), whereas 99.16% identity with the *E. heterostomum* isolate from Israel (GenBank No. KP721430) (Table 3).

Table 3
Information on the sequences obtained from NCBI GeneBank and used in the phylogenetic analysis.

GeneBank accession number	Species	Host	Geographic origin	Gene	Length (bp)	Query Cover (%)	Percent Identity (%)	References
KP721424	*E. heterostomum*	Cichlids	Lake Kinneret, Israel	ITS1, 5.8 rRNA, ITS2	996	99	99.16	(Caffara et al., 2016)
MT78578	*E. heterostomum*	*Channa striata*	India	ITS1, 5.8 rRNA, ITS2	1005	100	100	(Kaur et al., 2020)
KC894798	*Euclinostomum sp.*	*Trichopsis vittata*	Mueang, Thailand	ITS1, 5.8 rRNA, ITS2	1082	100	99.88	(Senapin et al., 2014)
OR591452	*E. heterostomum*	*Channa Punctata*	Narayangan, Bangladesh	ITS1, 5.8 rRNA, ITS2	830	Sequence generated in this study	Sequence generated in this study	Sequence generated in this study
KC894799	*Euclinostomum sp.*	*Trichopsis schalleri*	Mueang, Thailand	18 rRNA, ITS1, ITS2, 5.8 rRNA, 28 rRNA	1082	100	100	(Senapin et al., 2014)
KC894800	*Euclinostomum sp.*	*Trichopsis vittata*	Rattanawapi, Thailand	18 rRNA, ITS1, ITS2, 5.8 rRNA, 28 rRNA.	1082	100	99.88	(Senapin et al., 2014)
KP721427	*E. heterostomum*	Cichlids	Israel: Lake Kinneret	ITS1, 5.8 rRNA, ITS2	977	97	99.26	(Caffara et al., 2016)
KP721438	*E. heterostomum*	Cichilids	Israel: Lake Kinneret	ITS1, 5.8 rRNA, ITS2	977	97	99.38	(Caffara et al., 2016)
KF577720	*Clinostomidae gen. sp.*		Mpumalang, South Africa	ITS1, 5.8 rRNA, ITS2	699	83	94.01	
MH282566	*Clinostomum album*	*Planorbella trivolvis*	Mississippi, USA	ITS1, 5.8 rRNA, ITS2	1011	78	90.69	(Rosser et al., 2018)

GeneBank accession number	Species	Host	Geographic origin	Gene	Length (bp)	Query Cover (%)	Percent Identity (%)	References
MH282564	*Clinostomum album*	*Planorbella trivolvis*	Mississippi, USA	ITS1, 5.8 rRNA, ITS2	1016	78	90.69	(Rosser et al., 2018)

The obtained results confirmed the identity of the specimens as *E. heterostomum*, providing a robust molecular basis for morphological identification. To explore the molecular variability within *E. heterostomum*, multiple sequence alignments were performed. The alignment highlighted conserved regions and allowed the identification of potential genetic markers for future studies on population genetics and evolutionary relationships. In phylogenetic analysis was the studies *E. heterostomum* (GenBank No. OR591452) formed a monophyletic clade alongside a sister clade containing *E. heterostomum* (GenBank No. MT785768) within the family Clinostomidae (Fig. 7**)**.

Discussion

Channa punctata is an important snakehead in the Indian subcontinent, which is cultivated and has a good preference in the Indian fish diet. The present study provides additional insights into the integration of morphological, histopathological, and molecular data for the identification of *E. heterostomum*. As aquaculture continues to grow, challenges have surfaced, with parasitic infestation emerging as a primary daily hurdle for fish farms (Radwan, 2022). Parasite-infected fish lose their capacity to absorb nutrients from their diet as their energy is redirected to combat the infection, resulting in malnutrition and hindered growth (Barber, 2012). Several factors contribute to the variability in prevalence, such as differences in habitat, food supply, the availability of suitable hosts, including gastropods (aquatic snails) and fishes as intermediate hosts, and the influence of aquatic piscivorous birds (act as final hosts), which are crucial in the completion of life cycles for many digenetic trematodes (Chung et al., n. d.; Mutengu & Mhlanga, 2018). Knowledge of the life cycle is crucial for identifying the various stages at which transmission occurs. This information is essential for implementing targeted control measures to break the transmission cycle and reduce the prevalence of infections. (Donges, 1974) and (Jhansilakshmibai & Madhavi, 1997) published comprehensive documentations of the life cycle of *E. heterostomum*.

Several combined morphometric and molecular approaches were used to identify metacercariae of *E. heterostomum* in cichlids (Caffara et al., 2016). The observed sucker ratios align with various studies that report the oral sucker to ventral sucker size ratio ranging from one-third to one-half (Choudhary et al., 2022). *Clinostomum* can be distinguished on the basis of size, tegumental spines, and various aspects of the genital complex from named and unnamed species in the same region (Caffara et al., 2020). The shape and position of the cirrus sac relative to the testes and ovary have emerged as consistent and trustworthy features for differentiating species within the *Clinostomum* genus (Sereno-Uribe et al., 2018). Moreover, the genital complex may be a more reliable diagnostic feature in identifying species of *Clinostomum* (B. F. Li et al., 2018). Moreover, scanning electron microscopy (SEM) of *E. heterostomum* confirmed the species and documented the structural details (Kaur et al., 2020).

The use of histopathological changes as a vital biomarker in environmental monitoring facilitates the scrutiny of specific target organs (Salamat & Zarie, 2016). Distinguishing between normal and abnormal cells, comparative histology plays a pivotal role in diagnosing various diseases (Wolf et al., 2015). Various histological alterations have been documented in the liver of fish in various investigations because of parasite cysts. Previous studies have discovered comparable patterns in the liver of infected fish, where histological changes such as vacuolar degeneration, necrosis, and substantial tissue destruction near the portal vein have been noted (Parbin & Mech, 2019; Pinky Kaur, 2012). The liver of *Clarias gariepinus* in the Anambra River basin, infected with *Euclinostomum clarias* metacercariae, showed significant pathological abnormalities. These included degeneration of the liver, leakage of bilirubin, tissue death, formation of scar tissue, inflammatory reactions, and growth of bile ducts around the liver's central blood vessels (Onucha, 2010).

The present study represents the first molecular report of *E. heterostomum* in the country. This finding is consistent with previous research using molecular approaches for identifying *E. heterostomum* in *Channa striata* (Kaur et al., 2020)d *heterostomum* and *E. ardeolae* in Tilapia (Younis et al., 2022). The distinctive morphological variances were validated through a comprehensive analysis that compared the sequences of internal transcribed spacers (ITS) within ribosomal DNA and the mitochondrial gene cytochrome c oxidase I (CO1) across 39 individual specimens (Caffara et al., 2011). It is noteworthy that various conserved genes were employed to identify Clinostomid metacercariae from snakeheads. The ITS2 and CO1 genes played a crucial role in identifying it as *C. philippinense* (Zimik et al., 2019). Moreover, several morpho-molecular identifications were conducted using different genes to distinguish parasites within the Clinostomidae family, including *Clinostomum phalacrocoracis, Clinostomum complanatum, C. marginatum*, and *Clinostomum tilapiae* (A. Salem et al., 2021; Caffara et al., 2011).

Overall, the integration of morphological and molecular approaches collectively confirms the presence of *Euclinostomum heterostomum* in *Channa punctata*, to address the heightened abiotic and biotic stresses caused by the constantly shifting global climate, recent efforts aim to prevent and manage pathogenic diseases, thereby mitigating microbial infections and fostering sustainable fish production.

Declarations

Author Contributions

Conceptualization and Methodology, Sayed Mashequl Bari and Kazi Ahsan Habib; Software, Sayed Mashequl Bari, Aktia Amina and Zubyda Mushtari Nadia; Investigation: Sayed Mashequl Bari and Aktia Amina; Data Curation: Aktia Amina and Zubyda Mushtari Nadia; Writing-Original Draft preparation: Sayed Mashequl Bari and Zubaida Mushtari Nadia; Writing – Review & Editing: Kazi Ahsan Habib, Raf Ana Rabbi Shawon and Md. Matiur Rahman. All authors have read and agreed to the published version of the manuscript.

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Conflicts of interest

Financial interests

The authors declare they have no financial interests.

Non-financial interests

none.

Ethics approval

This is an observational study. The Sher-e-Bangla Agricultural University research ethics committee has confirmed that no ethical approval is required.

References

1. A. Salem, M., Z. Abdel- Maogood, S., Abdelsalam, M., & A. Mahdy, O. (2021). Comparative morpho-molecular identification of *Clinostomum phalacrocoracis* and *Clinostomum complanatum* metacercaria coinfecting Nile tilapia in Egypt. *Egyptian Journal of Aquatic Biology and Fisheries*, *25*(1), 461–476. https://doi.org/10.21608/ejabf.2021.145698
2. Briosio-Aguilar, R., Garcia-Varela, M., Hernandez-Mena, D. I., Rubio-Godoy, M., & Perez-Ponce de Leon, G. (2019). Morphological and molecular characterization of an enigmatic clinostomid trematode (Digenea: Clinostomidae) parasitic

as metacercariae in the body cavity of freshwater fishes (Cichlidae) across Middle America. *Journal of Helminthology*, *93*(4), 461–474. https://doi.org/10.1017/S0022149X18000445

3. Bush, A. O., Lafferty, K. D., Lotz, J. M., & Shostak, A. W. (1997). Parasitology Meets Ecology on Its Own Terms: Margolis et al. Revisited. *The Journal of Parasitology*, *83*(4), 575–583. https://doi.org/10.2307/3284227
4. Caffara, M., Locke, S. A., Cristanini, C., Davidovich, N., Markovich, M. P., & Fioravanti, M. L. (2016). A combined morphometric and molecular approach to identifying metacercariae of *Euclinostomum heterostomum* (Digenea: Clinostomidae). *Journal of Parasitology*, *102*(2), 239–248. https://doi.org/10.1645/15-823
5. Caffara, M., Locke, S. A., Echi, P. C., Halajian, A., Luus-Powell, W. J., Benini, D., Tedesco, P., & Fioravanti, M. L. (2020). A new species of *Clinostomum Leidy*, 1856 based on molecular and morphological analysis of metacercariae from African siluriform fishes. *Parasitology Research*, *119*(3), 885–892. https://doi.org/10.1007/s00436-019-06586-2
6. Caffara, M., Locke, S. A., Gustinelli, A., Marcogliese, D. J., David J. Marcogliese, David J. Marcogliese, Marcogliese, D. J., Fioravanti, M. L., Fioravanti, M. L., & Fioravanti, M. (2011). Morphological and molecular differentiation of *Clinostomum complanatum* and *Clinostomum marginatum* (Digenea: Clinostomidae) metacercariae and adults. *Journal of Parasitology*, *97*(5), 884–891. https://doi.org/10.1645/ge-2781.1
7. Choudhary, K., Ray, S., Shamsi, S., & Agrawal, N. (2022). Characterization of *Clinostomum* (Digenea: Clinostomidae) spp. In India. *Parasitology Research*, *121*(11), 3083–3089. https://doi.org/10.1007/s00436-022-07644-y
8. Chung, D. I., Kong, H. H., & Moon, C. H. (1995). Demonstration of the second intermediate hosts of *Clinostomum complanatum* in Korea. *The Korean Journal of Parasitology*, *33*(4), 305–312. http://dx.doi.org/10.3347/kjp.1995.33.4.305
9. Donges, J. (1974). The life cycle of *Euclinostomum heterostomum* (Rudolphi, 1809) (Trematoda: Clinostomatidae). *International Journal for Parasitology*, *4*(1), 79–90. https://doi.org/10.1016/0020-7519(74)90012-5
10. Feist, S. W., & Longshaw, M. (2008). Histopathology of fish parasite infections – importance for populations. *Journal of Fish Biology*, *73*(9), 2143–2160. https://doi.org/10.1111/j.1095-8649.2008.02060.x
11. Gautam, N. K., Misra, P. K., & Saxena, A. M. (2018). Seasonal variation in helminth parasites of snakeheads *Channa punctatus* and *Channa striatus* (Perciformes: Channidae) in Uttar Pradesh, India. *Helminthologia*, *55*(3), 230–239. https://doi.org/10.2478/helm-2018-0020
12. Gokcen, A. (2008). Fixation, staining and preparation of permanent mounts of helminths. *Turkiye parazitolojii dergisi*, *32*(2), 177–181.
13. Gupta, A. K., & Agarwal, S. M. (1983). Host Parasite Relationships in *Channa Punctatus* and *Euclinostomum heterostomum*. *Current Science*, *52*(10), 474–476.
14. Jayaram, K. C. (2010). *The freshwater fishes of the Indian region* (2nd ed). Narendra Pub. House. https://cir.nii.ac.jp/crid/1130282272794762880
15. Jeronimo, G. T., da Cruz, M. G., Bertaglia, E. de A., Furtado, W. E., & Martins, M. L. (2022). Fish parasites can reflect environmental quality in fish farms. *Reviews in Aquaculture*, *14*(3), 1558–1571. https://doi.org/10.1111/raq.12662
16. Jhansilakshmibai, K., & Madhavi, R. (1997). *Euclinostomum heterostomum* (Rudolphi, 1809) (Trematoda): Life-cycle, growth and development of the metacercaria and adult. *Systematic Parasitology*, *38*(1), 51–64. https://doi.org/10.1023/A:1005829625739
17. Kaur, P., Minare, A., & Mourya, B. (2020). Molecular and scanning electron microscopy (sem) associated energy dispersive X-Ray analysis (EDXA) characterization of *Euclinostomum heterostomum* (Rudolphi, 1809) (Trematoda) from Channa striata (Bloch, 1793). *International Journal of Fisheries and Aquatic Studies*, *8*(5), 107–111. https://doi.org/10.22271/fish.2020.v8.i5b.2311
18. Kaur, P., Qureshi, T. A., & Shrivastav, R. (2012). Histo-architectural alterations induced by *Euclinostomum heterostomum* (metacercariae) infected liver of *Channa punctata* (Bloch). *Journal of Parasitic Diseases*, *36*(2), 197–199. https://doi.org/10.1007/s12639-012-0102-7

19. Kaur, P., Shrivastav, R., & Qureshi, T. A. (2016). Pathological effect on naturally infected kidneys of freshwater murrels by *Euclinostomum heterostomum* metacercariae. *Journal of Parasitic Diseases : Official Organ of the Indian Society for Parasitology*, *40*(1), 157–160. https://doi.org/10.1007/s12639-014-0468-9
20. Kumar Maurya, P., Malik, D. S., Kumar Yadav, K., Gupta, N., & Kumar, S. (2019). Haematological and histological changes in fish *Heteropneustes fossilis* exposed to pesticides from industrial waste water. *Human and Ecological Risk Assessment: An International Journal*, *25*(5), 1251–1278. https://doi.org/10.1080/10807039.2018.1482736
21. Li, B. F., Liu, X. H., Ge, H.-L., Xie, C. Y., Cai, R. Y., Hu, Z. C., Zhang, Y. G., & Wang, Z. J. (2018). The discovery of *Clinostomum complanatum* metacercariae in farmed Chinese sucker, *Myxocyprinus asiaticus*. *Aquaculture*, *495*, 273–280. https://doi.org/10.1016/j.aquaculture.2018.05.052
22. Li, K.-B. (2003). ClustalW-MPI: ClustalW analysis using distributed and parallel computing. *Bioinformatics*, *19*(12), 1585–1586. https://doi.org/10.1093/bioinformatics/btg192
23. Meyers, T. R. (2004). *Standard Necropsy Procedures for Finfish* (pp. 1–10).
24. Moyroud, N., & Portet, F. (2018). Introduction to QGIS. In *QGIS and Generic Tools* (pp. 1–17). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781119457091.ch1
25. Mutengu, C., & Mhlanga, W. (2018). Occurrence of *Clinostomum* Metacercariae in *Oreochromis mossambicus* from Mashoko Dam, Masvingo Province, Zimbabwe. *Scientifica*, *2018*, 1–6. https://doi.org/10.1155/2018/9565049
26. Noman, A. H. Md., Mia, M. A., Banna, H., Rana, Md. S., Alam, A. S. A. F., Gee, C. S., Isa, C. R., & Er, A. C. (2016). City profile: Narayanganj, Bangladesh. *Cities*, *59*, 8–19. https://doi.org/10.1016/j.cities.2016.05.020
27. Okon, E. M., Okocha, R. C., Taiwo, A. B., Michael, F. B., & Bolanle, A. M. (2023). Dynamics of co-infection in fish: A review of pathogen-host interaction and clinical outcome. *Fish and Shellfish Immunology Reports*, *4*, 100096. https://doi.org/10.1016/j.fsirep.2023.100096
28. Onucha, E. (2010). Liver pathology of the African catfish, *Clarias gariepinus* infected with *Euclinostomum clarias* metacercariae in Anambra River Basin, Nigeria. *Animal Research International*, *7*(1), 1151–1155.
29. Parbin, H., & Mech, B. (2019). Histopathological study of parasite infested *Channa punctata*. *International Journal of Life Sciences Research*, *7*(3), 44–52.
30. Pinky Kaur. (2012). Histopathological and haematological investigations on *Nandus nandus* (Ham.) parasitized by metacercariae of *Clinostomum complanatum* (Rudolphi, 1819). *International Journal of Environmental Sciences*, *2*(3), 1324–1330. https://doi.org/10.6088/ijes.00202030019
31. Radwan, M. (2022). Vital economic threat of predatory birds and parasites to cultivated fishes in Egypt. *Aquaculture*, *548*, 737666. https://doi.org/10.1016/j.aquaculture.2021.737666
32. Rosser, T. G., Baumgartner, W. A., Alberson, N. R., Noto, T. W., Woodyard, E. T., Tommy King, D., Wise, D. J., & Griffin, M. J. (2018). *Clinostomum poteae* n. sp. (Digenea: Clinostomidae), in the trachea of a double-crested cormorant Phalacrocorax auritus Lesson, 1831 and molecular data linking the life-cycle stages of *Clinostomum album* Rosser, Alberson, Woodyard, Cunningham, Pote & Griffin, 2017 in Mississippi, USA. *Systematic Parasitology*, *95*(6), 543–566. https://doi.org/10.1007/s11230-018-9801-5
33. Salamat, N., & Zarie, M. (2016). Fish histopathology as a tool for use in marine environment monitoring: A review. *Comparative Clinical Pathology*, *25*(6), 1273–1278. https://doi.org/10.1007/s00580-014-2037-0
34. Senapin, S., Phiwsaiya, K., Laosinchai, P., Kowasupat, C., Ruenwongsa, P., & Panijpan, B. (2014). Phylogenetic analysis of parasitic trematodes of the genus *Euclinostomum* found in Trichopsis and Betta fish. *Journal of Parasitology*, *100*(3), 368–371. https://doi.org/10.1645/13-285.1
35. Sereno-Uribe, A. L., Garcia-Varela, M., Pinacho-Pinacho, C. D., & Perez-Ponce de Leon, G. (2018). Three new species of *Clinostomum Leidy*, 1856 (Trematoda) from Middle American fish-eating birds. *Parasitology Research*, *117*(7), 2171–2185. https://doi.org/10.1007/s00436-018-5905-8
36. Shareef, P. A. A., & Abidi, S. M. A. (2015). Studies on the epidemiology and histopathology of *Euclinostomum heterostomum* (Trematoda; Digenea) infection in Channa punctata from North India. *Archives of Polish Fisheries*, *23*(3),

133–140. https://doi.org/10.1515/aopf-2015-0015

37. Shareef, P. A., & Abidi, S. (2012). Incidence and histopathology of encysted progenetic metacercaria of *Clinostomum complanatum* (Digenea: Clinostomidae) in *Channa punctatus* and its development in experimental host. *Asian Pacific Journal of Tropical Biomedicine*, *2*(6), 421–426. https://doi.org/10.1016/S2221-1691(12)60068-9
38. Sharma, R. K., & Sharma, P. K. (1980). A new nematode *Neocamallanus bareilliensis* from a piscine host *Channa punctatus* Bloch. *Journal of Helminthology*, *54*(1), 21–23. https://doi.org/10.1017/S0022149X00006283
39. Suanyuk, N., Mankhakhet, S., Soliman, H., Saleh, M., & El-Matbouli, M. (2013). *Euclinostomum heterostomum* infection in guppies *Poecilia reticulata* cultured in Southern Thailand. *Diseases of Aquatic Organisms*, *104*(2), 121–127. https://doi.org/10.3354/dao02595
40. Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution*, *38*(7), 3022–3027. https://doi.org/10.1093/molbev/msab120
41. Verma, S. K., Yadav, S., & Saxena, A. (2018). An ecological analysis of acanthocephalan parasites of *Channa punctatus* of river Gomti, Lucknow, Uttar Pradesh, India. *International Journal of Zoology Studies*, *3*(2), 1–4.
42. Viktorov, I. V., & Proshin, S. S. (2003). Use of isopropyl alcohol in histological assays: Dehydration of tissue, enbessing into paraffin, and processing of paraffin sections. *Bulletin of Experimental Biology and Medicine*, *136*(1), 105–106. https://doi.org/10.1023/A:1026017719668
43. Wolf, J. C., Baumgartner, W. A., Blazer, V. S., Camus, A. C., Engelhardt, J. A., Fournie, J. W., Frasca, S., Groman, D. B., Kent, M. L., Khoo, L. H., Law, J. M., Lombardini, E. D., Ruehl-Fehlert, C., Segner, H. E., Smith, S. A., Spitsbergen, J. M., Weber, K., & Wolfe, M. J. (2015). Nonlesions, misdiagnoses, missed diagnoses, and other interpretive challenges in fish histopathology studies: A guide for investigators, authors, reviewers, and readers. *Toxicologic Pathology*, *43*(3), 297–325. https://doi.org/10.1177/0192623314540229
44. Yamaguti, S. (1971). Synopsis of digenetic trematodes of vertebrates. Vols I and II. *Synopsis of Digenetic Trematodes of Vertebrates. Vols I and II.* https://www.cabdirect.org/cabdirect/abstract/19730809427
45. Younis, A. E., Hamouda, A. H., & Moustafa, E. M. (2022). *Euclinostomum heterostomum* and *E. ardeolae* in tilapia species of Aswan Governorate, Egypt: Morphological, molecular, and histopathological characterization. *Aquaculture International*, *30*(2), 825–844. https://doi.org/10.1007/s10499-022-00839-1
46. Zimik, P., Sharma, S., & Roy, B. (2019). Characterization of *Clinostomum metacercariae* using microscopic and molecular approaches. *Annals of Parasitology*, *65*, 87–97.

Figures

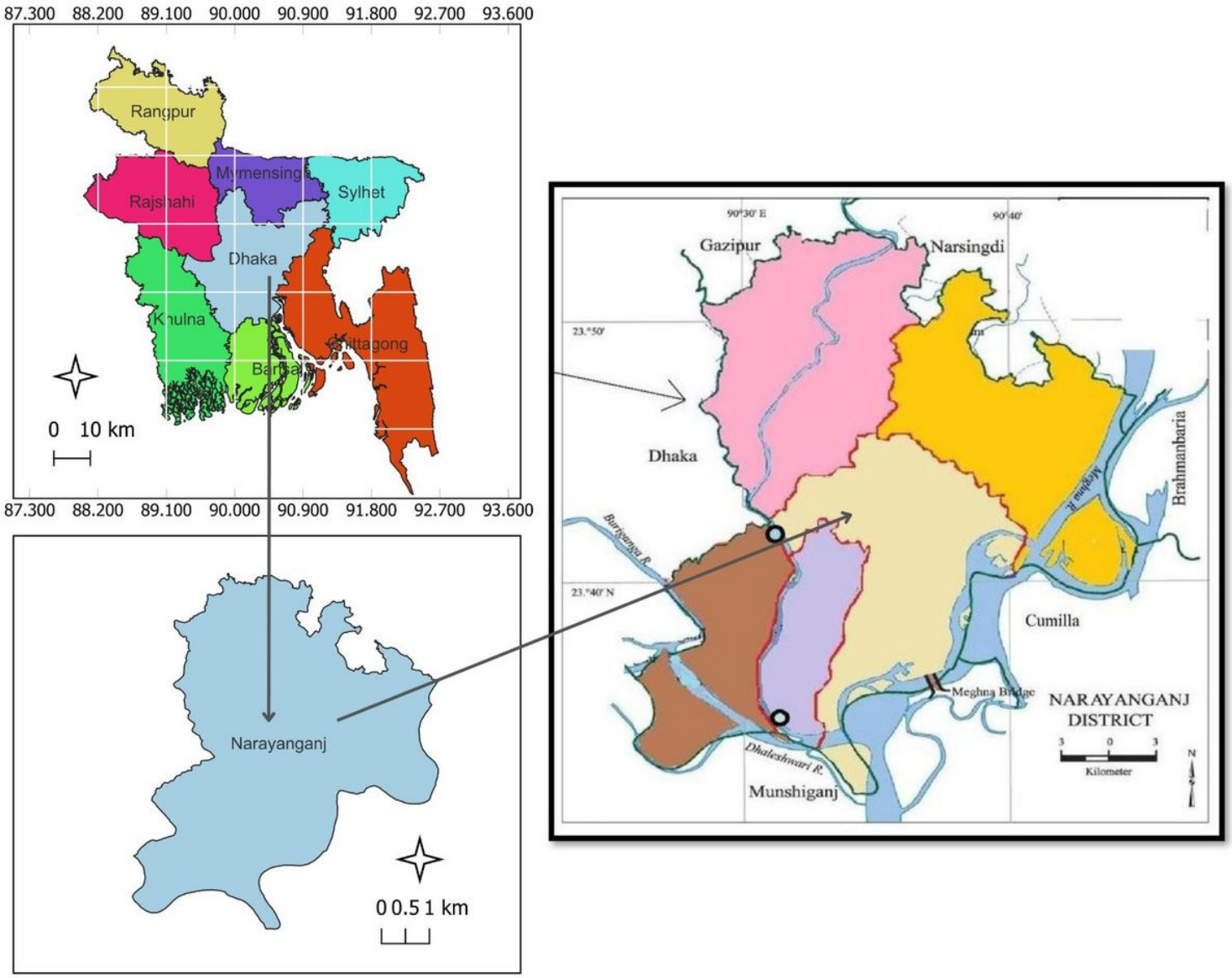

Figure 1

Location map of Narayanganj district, Bangladesh. Red points given in the map are sampling sites from where the samples were collected.

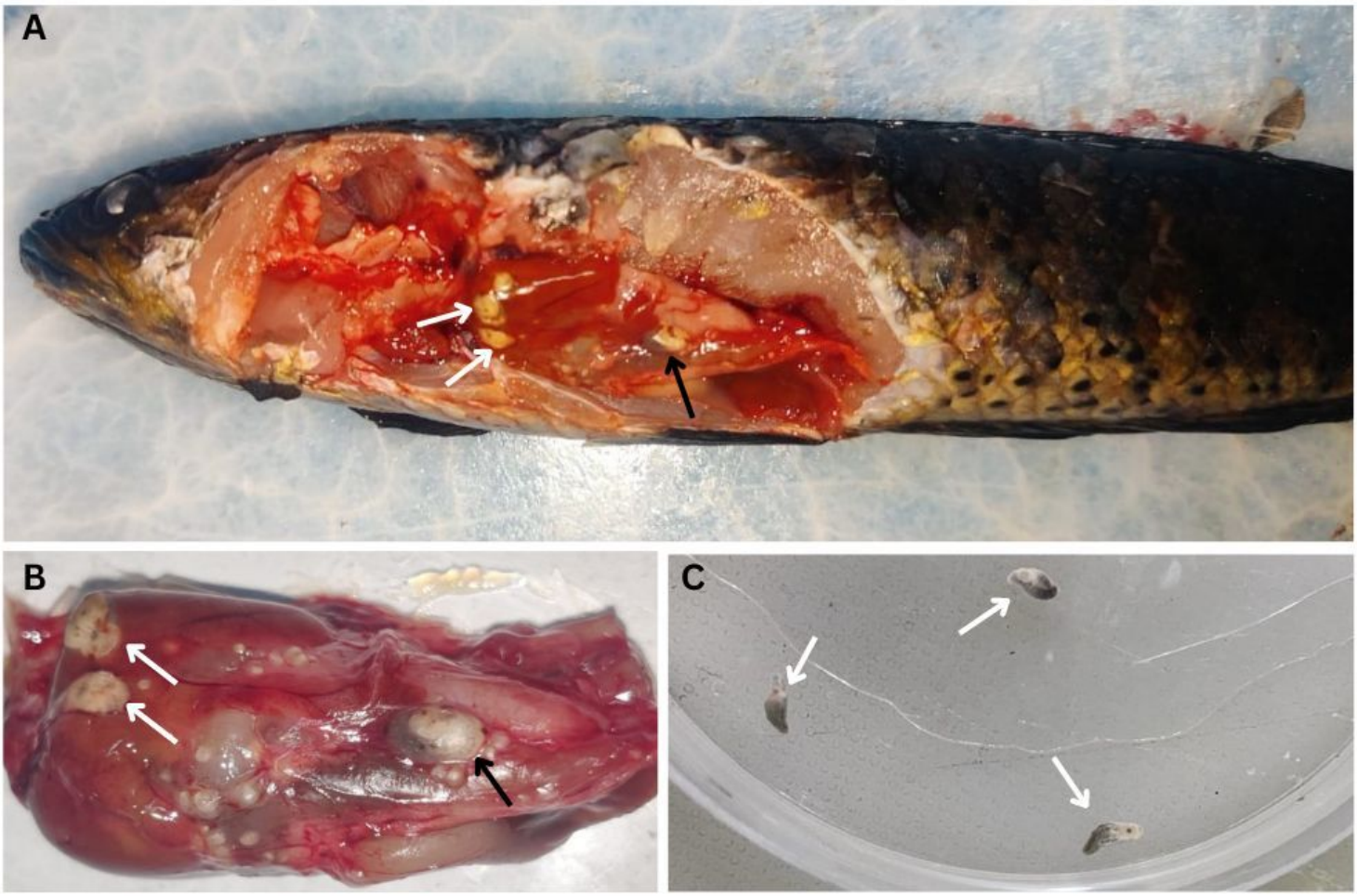

Figure 2

(a) Dissected *C. punctata* showing the encysted progenetic metacercariae of *E. heterostomum* in liver (white arrows) and peritoneal membrane (black arrow). (b) Liver surface (white arrow) and peritoneal membrane (black arrow) encysted metacercaria of *E. heterostomum* separated gut content. (c) progenetic metacercariae of *E. heterostomum* (white arrow) opened from cysts.

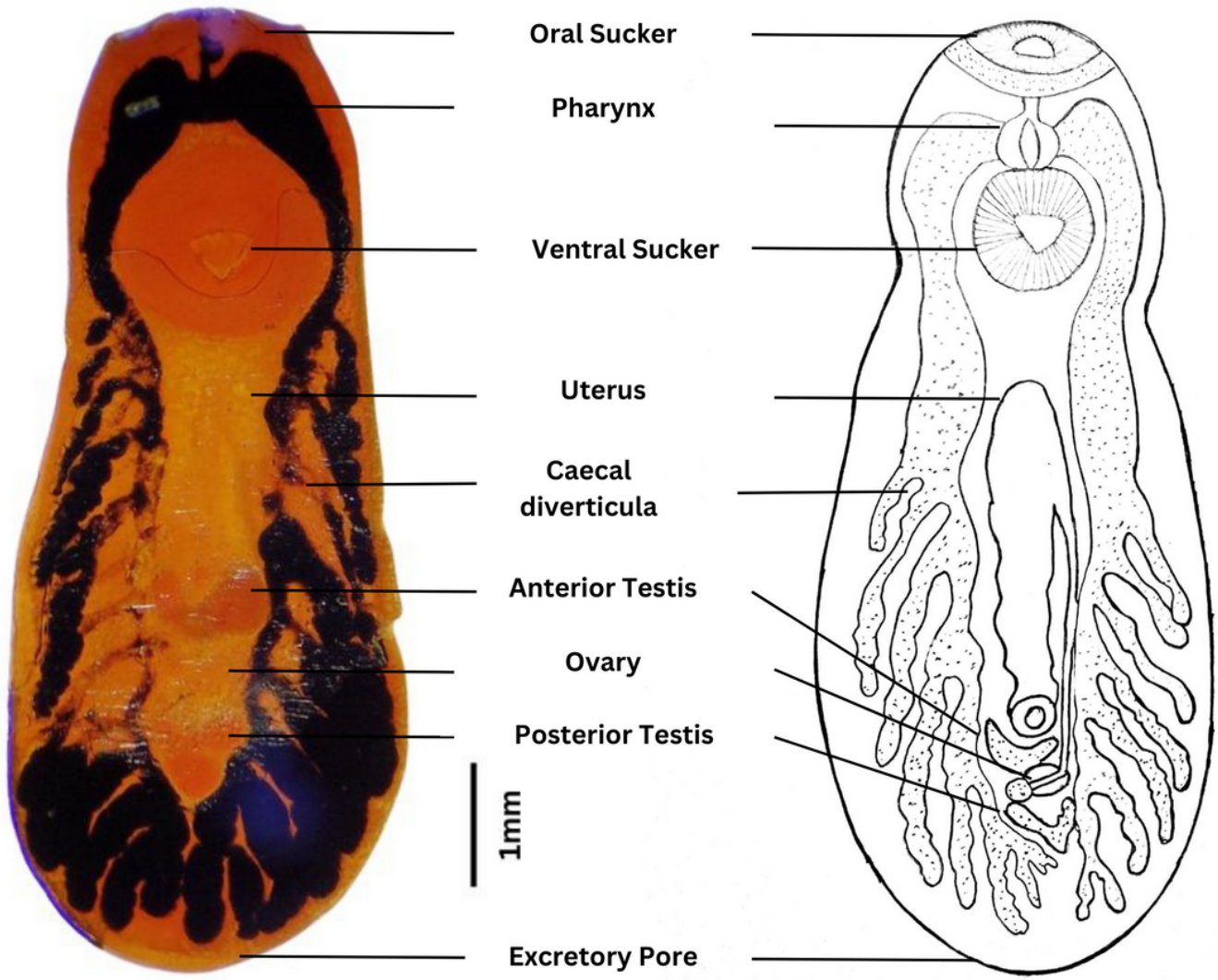

Figure 3

Stereo microscopic view of *E. heterostomum* collected in fish from Narayanganj, Bangladesh (left); Sketch of *E. heterostomum* (Right). The scale bar is 1 mm.

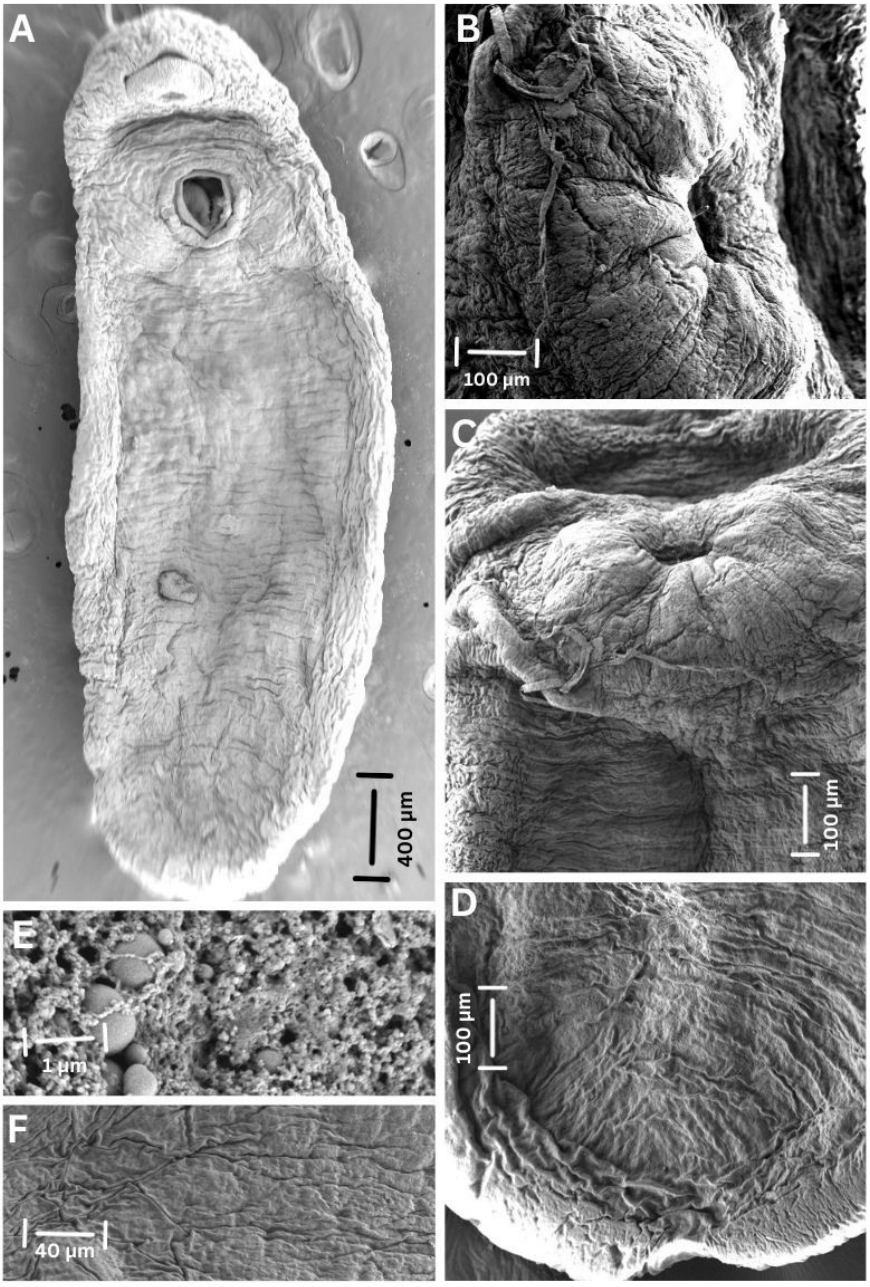

Figure 4

Scanning electron micrographs of *E. heterostomum* collected in a snakehead, Channa punctata from Narayanganj, Bangladesh. A- Ventral view of whole specimen (Scale bar = 400 µm), B-oral sucker (Scale bar = 100 µm), C-ventral sucker (Scale bar = 100 µm), D-1/4 dorso-posterior portion of the body, E- Tegument od mid body region (Scale bar = 40 µm), F-Tegumental surface (Scale bar = µm).

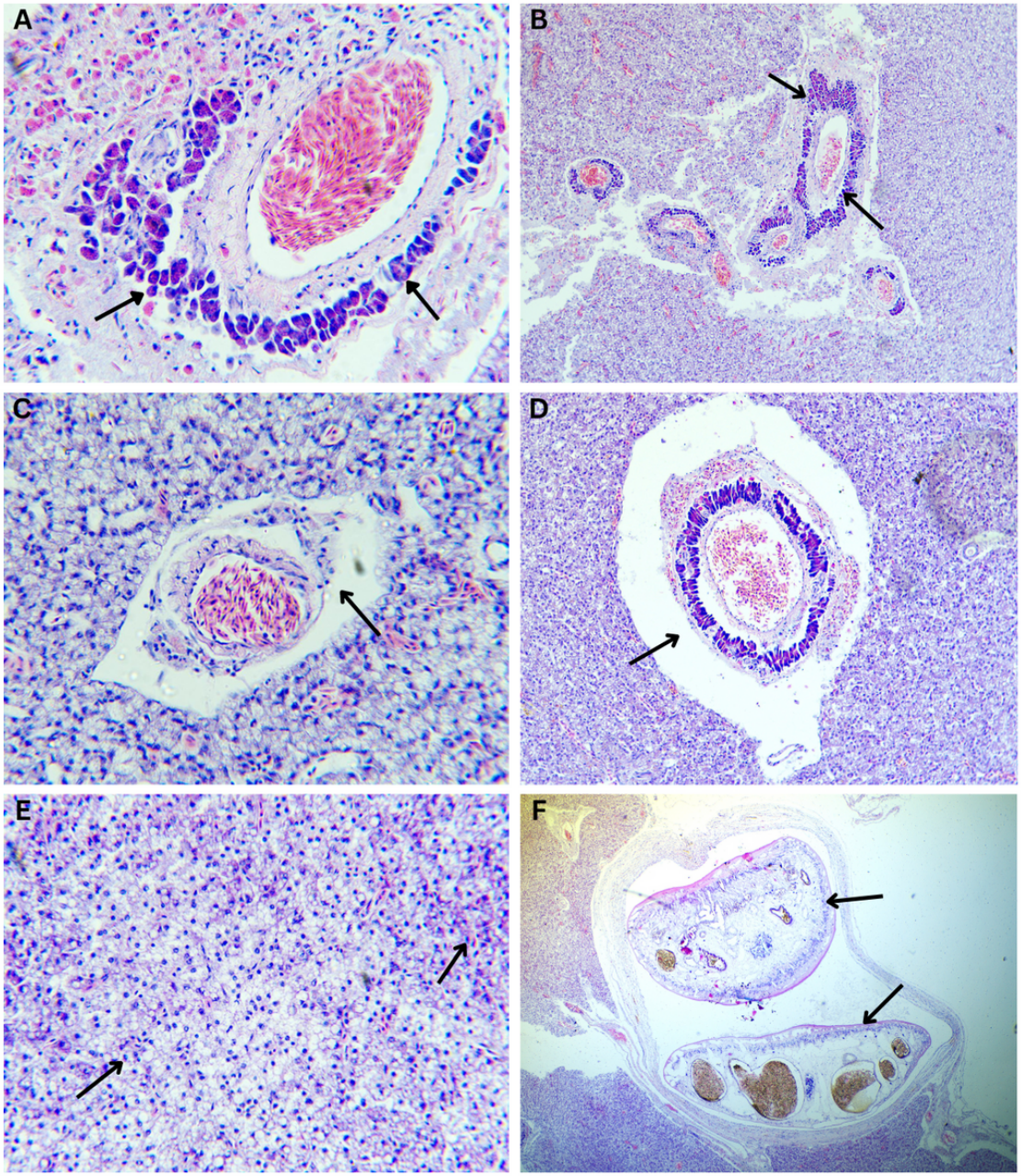

Figure 5

Histological structure of liver of Channa punctata showing histopathological alternations. A & B. Infiltration of basophil around large vessel (arrow); C. Infiltration of infiltrating cells around central vein which is desquamationated (arrowhead); D. Infiltration of basophil around central vein, E. Infiltration of mononuclear inflammatory cell (arrow) (10 × 40x resolution); F. Section of liver showing two encysted metacercaria (arrows) (10 × 10x resolution).

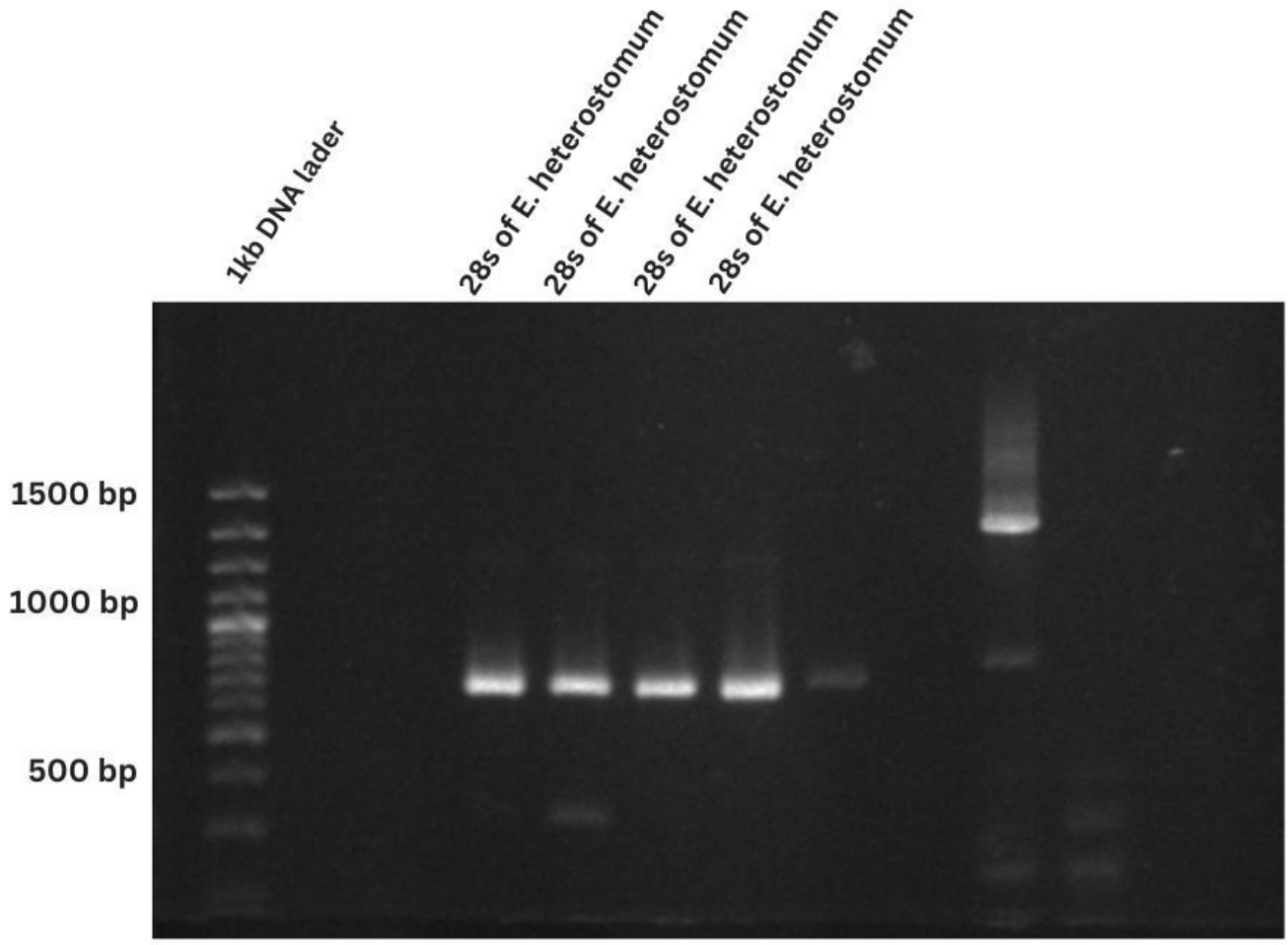

Figure 6

Sequence analysis. a. Gel analysis of PCR products from the two detected *E. heterostomum* under UV light stained with ethidium bromide. The ampilicons for 28S gene marker were ~ 1300 bp. (M.W. = 688 bp).

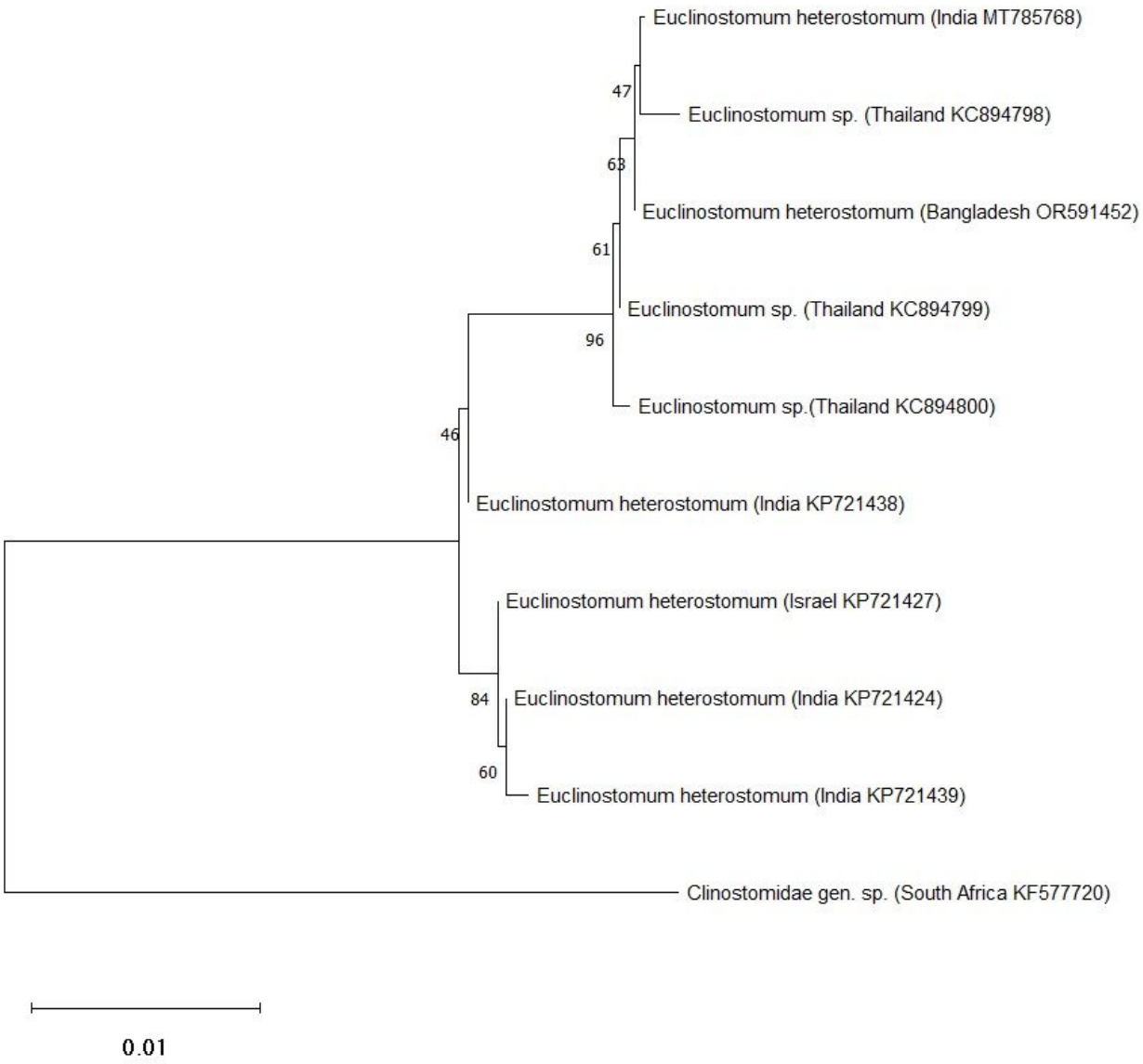

Figure 7

Phylogenetic tree constructed in MEGA11 by neighbor-joining method resulting from 830bp 28S rDNA sequence of the detected *E. heterostomum*. The bootstrap original tree inferred from 1000 replicates represents the evolutionary history of analyzed taxa. The matched sequences are marked by accession numbers, names, and locations.