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Morphological and Molecular Evidence of a Trematode Parasite Infected the Liver of Garfish *Xenentodon cancila* (Beloniformes: Belonidae) in India

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Research Article

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Abstract

Morphological traits to describe trematode parasites are sometimes difficult to identify and validate a species, especially in cases where many species are described from a single host. The current work uses molecular data to describe diplostomoid metacercariae supplemented with morphology found in freshwater garfish, *Xenentodon cancila* (Hamilton) collected from the River Ganga in district Bijnour, Meerut (Uttar Pradesh), India. The metacercariae were identified as *Posthodiplostomum* species, collected from the liver showed a high mass of cysts embedded in and surrounded by partially damaged liver tissue. Partial DNA sequences of the 18S, internal transcribed spacers (ITS1–5.8S–ITS2), and 28S of nuclear ribosomal DNA were generated and compared with available sequences of other congeners on the Genbank database. The phylogenetic analysis of 18S, the ITS cluster (ITS1–5.8S–ITS2), and 28S rDNA of *Posthodiplostomum* sp. from India fell within the superfamily Diplostomoidea, along with other members of *Posthodiplostomum*, which confirms its distinct status and places it close to other Indian species. In the Indian region, with morphology alone, many species are described as *Neascus*-type metacercariae that are awaiting their validation to be supplemented with molecular data. Furthermore, the validity of a few species of the genus *Posthodiplostomum* is also discussed in the present study.

Keywords: Trematode; *Posthodiplostomum*; 18S; ITS Cluster; 28S; Meerut; India

Introduction

Transmission of infectious parasites by the increasing trade of fish poses a risk to human health [1]. One of the important trades of species introduction globally is aquaculture, which causes the transmission of infectious diseases like bacterial and parasitic diseases to wild and aquaculture-raised fish [2-4]. Piscivorous migratory birds as definitive hosts are also a major factor related to parasite transmission between the continents [5]. *Posthodiplostomum* Dubois, 1936, is a trematode genus rich in diversity and comprises parasites that are widespread worldwide [6-12]. Initially, for the identification of strigeid larva types, they were categorized into different types, i.e., *Tetracotyle* Filippi, 1854; *Codonocephalus* Diesing, 1850; *Neascus* Hughes, 1927; and *Diplostomulum* Brandes, 1892. Hughes (1927) proposed a group of larval trematodes, i.e., *Neascus,* to contain metacercariae, and Dubois (1936) described the genus *Posthodiplostomum,* which is in the family Diplostomidae. Parasites of this genus have a three-host life cycle, with snails as first intermediate hosts, fish acting as second intermediate hosts, and the fish-eating birds as

definitive hosts [11,13]. According to research, during the initial phase of infection, metacercaria penetration and migration cause mechanical damage, hemorrhage, and other secondary bacterial infections in fishes [15]. Infection caused by these trematodes is called black-spot disease and may have its origins in fish muscles due to the deposition of melanocytes around metacercariae [14]. A few studies have mentioned *Posthodiplostomum cuticola* (Nordmann, 1832) as the causative agent of black-spot disease, and it is one of the common diseases that affect many fish species [15-20]. Fish infected with black-spot diseases have had reduced growth along with other deformities and mortalities [12,19, 21,22]. Sometimes, the presence of metacercaria in the liver of infected fish can be associated with changes in hepatic tissues like parenchymal atrophy, necrosis, and fibrosis that lead to dysfunction in digestion and malnutrition [23]. Additionally, *Posthodiplostomum* species infection in fishes may cause weight loss, slow developmental processes, and mortality, making them less able to avoid predation by definitive hosts, i.e., fish-eating birds [15,24-26].

Many species of *Posthodiplostomum* have been described worldwide, while more than twenty-five species are reported from India, described as *Neascus*-type metacercariae and as *Posthodiplostomum* species [27]. Their life cycle is complex, and it is very difficult to study or link different stages in the laboratory. In India, most of the *Posthodiplostomum* species are characterised based on morphology alone, which cannot be considered reliable due to a lack of descriptions or deposited specimens. Difficulties in identification can be overcome by the generation of molecular data to link different developmental stages, as suggested in various studies [28-30]. Molecular identification would eventually improve our understanding of the correct diversity of *Posthodiplostomum* species that are scarce and limited, especially in India and help in linking the adult and metacercariae stages.

As part of an ongoing survey of diplostomid metacercaria (*Neascus* type) from freshwater fishes in India, *Posthodiplostomum* species was collected in the liver of the garfish *Xenentodon cancila* with severe liver infection. A combined morphological, histological, and molecular analysis using the 18S, ITS cluster (ITS1–5.8S–ITS2), and 28S rDNA gene sequences was provided to evaluate the phylogenetic position of our specimens in relation to other closely related species.

Materials and Methods

Parasite Collection and Morphology

During a helminthological survey in Bijnor (29° 23′ N, 79° 11′ E), Uttar Pradesh, India, between November 2020 and May 2021, a total of 57 garfish, *Xenentodon cancila* (Hamilton, 1822) (Beloniformes: Belonidae), were collected. After that, fish internal organs were examined with the aid of a stereomicroscope (Motic SMZ-168 series, Xiamen, People's Republic of China) to detect the infection of parasites. Metacercariae were found to infect the liver, removed from the cysts with the help of a fine needle, and placed in a 0.9% saline solution for approximately 10–15 minutes. The morphology of metacercariae was investigated using both live and preserved specimens. For a live study of morphology, metacercariae were placed on a slide using a pipette in physiological saline (0.9%), coverslipped, and photographed by a Nikon Eclipse Ts2 microscope. Some of them were fixed in 10% formalin for permanent slide preparation, and some were immediately fixed in 95% ethanol for molecular analysis. For permanent slide preparation, they were stained with aceto-alum carmine, dehydrated in ascending grades of ethanol, cleared in xylene, and mounted in Canada balsam. Measurements were taken with the help of the digital images and NIS Elements 5.10 image analysis software. All measurements are given in millimeters. Abbreviations of the measurements are provided in the Table 2. Voucher specimens were deposited at the museum collection of the Department of Zoology, Chaudhary Charan Singh University, Meerut (U.P.), India, and at the Museum d'Histoire Naturelle, Geneva, Switzerland.

The infected fish liver sections were preserved immediately in 4% formalin and processed for histopathology. The samples were washed in 80% ethanol numerous times, implanted in paraffin wax, cut into 5- to 8-m-thick sections, and stained with hematoxylin and eosin. The histological sections were photographed by a Nikon Eclipse T*s*2 microscope equipped with NIS Elements 5.10 image analysis software.

DNA Isolation and Amplification

DNA extraction from ethanol-fixed metacercariae (*n*= 02) was performed using the QIAGEN DNeasy™ tissue kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. Sequences of the 18S, ITS cluster (ITS1–5.8S–ITS2), and 28S rDNA were obtained by PCR amplification according the primers suggested by Shinad et al. [31]. For all primers, PCR conditions were also performed according to Shinad et al. [31]. PCR products were then purified using Purelink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen, Löhne, Germany) according to the manufacturer's instructions. Sequencing reactions were performed in both directions using the ABI Big Dye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, California, USA) from the above mentioned primers and using the primers BD1 and BD2 [32] for the ITS cluster (ITS1-5.8S-ITS2) to acquire a long stretch of sequences.

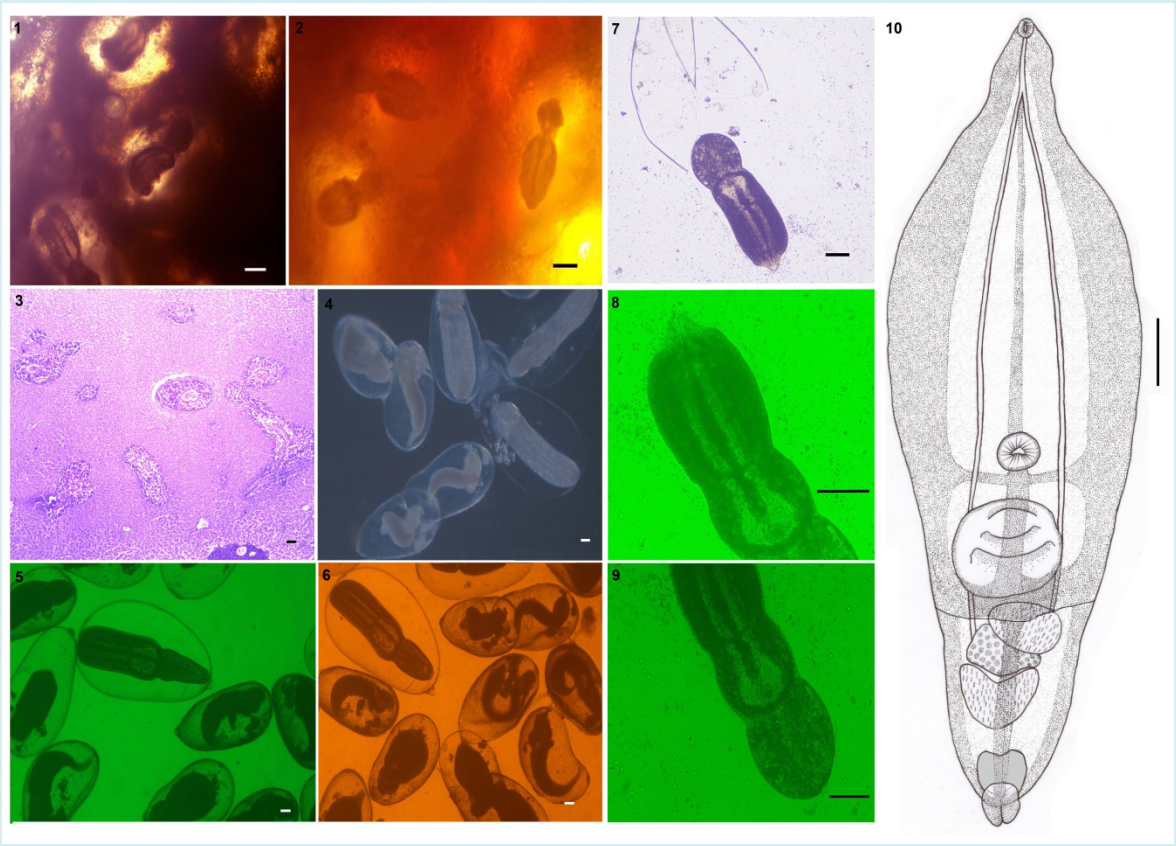
Phylogenetic Analysis

The generated sequences were visualized, assembled, and edited using BioEdit software [33]. Sequences of other diplostomids available at GenBank were retrieved after a BLASTn search. The sequences used in the phylogenetic analyses are presented in Table 1. The obtained sequences were aligned separately using ClustalW, which was implemented in MEGA vr. 11 [48]. Phylogenetic analyses were conducted using maximum likelihood (ML) and Bayesian inference (BI) algorithms. The best nucleotide substitution models for 18S, ITS cluster (ITS1-5.8S-ITS2), and 28S rDNA data sets were invariant sites and gamma-distributed among site variation (GTR + I + G). Pairwise comparisons for each region were estimated using MEGA v. 11. The ML trees were computed in MEGA vr. 11 and nodal support values were estimated from 1000 bootstrap pseudo-replicates. The BI analyses were carried out in TOPALi [49] using Markov chain Monte Carlo (MCMC) searches with two concurrent runs of four chains for 1,000,000 generations, with every 100th tree saved. The first 25% of the sampled trees were discarded as "burn-in." *Tylodelphys* species was selected as an out group for the 18S, ITS1–5.8S–ITS2 region, and 28S rDNA gene analyses.

Results

During the study, a total of 57 garfish specimens were collected, of which 54 were found infected with metacercariae. Metacercariae were found in the liver tissue of the infected fish in the form of numerous small cysts; the cyst wall was thin and delicate (Figure 1). Each fish was parasitized by 50–190 metacercariae. The excysted specimens of the present study clearly showed the morphological features of the genus *Posthodiplosomum* Dubois, 1936 and of species *Posthodiplostomum pandei* (=*Neascus pandei*) Rai and Pande, 1964 [50].

Morphological Description of Posthodiplostomum pandei (=*Neascus pandei*) Rai and Pande, 1964 (Figures 1-10)



Figures 1-10: *Posthodiplostomum pandei* (1,2) Encysted metacercariae embedded in liver tissue; (3) Histology of the liver shows encysted metacercaria within the liver tissue layer (arrow head); (4-6) Encysted metacercaria collected from *X. cancila's* liver tissue; (7) Metacercaria exit from the cyst; (8, 9) Excysted metacercaria (10) Line drawing. Scale bars (1, 2) 100 µm; (3-6) 50 µm; (7-10) 100 µm.

Metacercariae (n= 14). Encysted metacercariae with a thin, single layer of cystic wall. Spatulate, divided into two parts, or bipartite body of excysted metacercaria. Leaf-shaped forebody, larger than the hindbody. Hindbody are typically bulb-shaped. Pseudosuckers absent. At the anterior end, sub-terminal, there is a spherical-shaped oral sucker. The ventral sucker is spherical, larger than the oral sucker, and situated in the body's center, anterior to the holdfast organ. The holdfast organ is located near the forebody's posterior margin. The distinction between genital primordials is poor. The testes are divided into two parts: the anterior and posterior testes. Ovary intra-testicular. The excretory bladder is oval and terminates with a genital pore.

All the measurements of *Posthodiplostomum* species collected in the present study were compared in their morphology with those of other congeneric species in Table 2. Based on the morphology, the present species is recognised and differentiated as *Neascus pandei* Rai and Pande, 1964, and molecular biology confirms it as a *Posthodiplostomum* species. In histological sections, the cysts of *P. pandei* (=*N. pandei*) Rai and Pande, 1964 were found inside the liver and had a heavy infection (Figure 3).

- **Host:** Garfish, *Xenentodon cancila* Hamilton, 1822 (Beloniformes: Belonidae).
- **Locality:** Bijnor (29° 23′ N, 79° 11′ E), Uttar Pradesh, India.
- **Site of infection:** Liver (cysts embedded in the liver tissue).
- Prevalence in intermediate hosts: 95% (54 infected out of 57 hosts).
- **Specimens deposited:** Voucher specimens were submitted to the collection of the Museum, Department of Zoology, Chaudhary Charan Singh University, Meerut (U.P.), India (HS-TR/2022/01) and to the Museum d'Histoire Naturelle, Geneva, Switzerland (MHNG-PLAT-0144332).
- **Representative DNA sequence:** The newly generated sequences were deposited in GenBank under the following accession numbers: 18S rDNA gene: OP324624 (1068 bp), OP324628 (1079 bp); ITS1-5.8S-ITS2 region: OP324627 (1190 bp), OP324629 (1202 bp); 28S rDNA gene: OP324625 (1019 bp), OP324626 (1001 bp).

Remarks

Based on the shape of body parts and morphometric measurements, we identified our specimens as *Posthodiplostomum pandei* (=*Neascus pandei*) Rai and Pande, 1964. Many descriptions of *Posthodiplostomum* or *Neascus*-like metacercaria from *X. cancila* liver show inadequate data that results in taxonomic confusion in the identification of species, which leads to the addition of new species. Many previous studies from India were based solely on morphology and described as *Neascus*-type metacercaria species. In India, about twenty-six species of *Neascus*–type metacercariae and five of *Posthodiplostomum* were described (Table 3). Eleven of these have been reported solely from *X. cancila* as mentioned in Table 2, although they show minor variations in their morphometry. *N. mesentriformis*, *N. baughi*, *N. nanaksagrensis, N. simhai, N. moghei, N. vedi, and N. khurramnagarensis* have larger body size and width compared to our specimens, which clearly differentiate them from *P. pandei* (Table 2). There are no substantial morphometrical differences found in the body parts and morphology of *N. xenentodoni*, *N. hepatica,* and *N. srivastavi* with *P. pandei,* showing they are most closely related in nearly all metrics, which predicts they might be similar/closest species to *P. pandei* (Table 2). Although *N. xenentodoni* is close in morphology to *P. pandei,* as the molecular data of *N. xenentodoni* becomes available, it will be better to discuss it further. As we are not able to find out the deposited type specimens of *N. hepatica* and *N. srivastavi* for further clarification, it is too early to suggest them as synonyms with *P. pandei*. Thus, we consider *P. pandei* to be a valid species, and anyhow, these species (*N. hepatica* and *N. srivastavi*) should be taken into account in future studies in order to uncover their validity or synonymies with *P. pandei*.

Phylogenetic Analysis

The amplification and sequencing of the 18S gene of rDNA were successfully performed from two isolates of *Posthodiplostomum pandei* (=*N. pandei)* in the present study. The generated sequences were aligned with other diplostomids available in GenBank, as shown in the table (Table 1). The 18S sequences generated in this study show no intraspecific variations (Figure 11). The 18S sequence of *P. pandei* differed from other *Posthodiplostomum* species from different geographical regions by 1.5–3.9%. The phylogenetic tree obtained by ML and BI analyses of 18S sequences showed similar topologies. For comparison of the 18S gene with Indian species, only one sequence is available to date from *C. punctata* (KF738455), which is far away from our specimens. The analysis of 18s gene shows our specimens formed a well-supported clade found close to the Mexican isolate of *P. minimum* (PMU88074) and showing interspecific divergence of 1.5% (Figure 11).

Species	Host	Location	GenBank accession no.			References
			18S	ITS region	28S	
Ornithodiplostomum scardinii	*Scardinius erythrophthalmus*	Czech Republic	KX931443	-	KX931427	Stoyanov, et al. [11]
O. scardinii	*Ampullaceana balthica*	Denmark	-	MW001049, MW001051	-	Duan, et al. [8]
Ornithodiplostomum sp.	HNA	Canada	-	KY951727	-	Blasco-costa, et al. [29]
Mesoophorodiplostomum pricei	*Morone americana*	Canada	HM064959	HM064960	-	Locke, et al. [34]
Posthodiplostomum pricei	*L. delawarensis*	USA	-	-	MZ710972	Achatz, et al. [35]
Posthodiplostomum centrarchi	*L. gibbosus*	Slovakia	KX931442	-	-	Stoyanov, et al. [11]
P. centrarchi	*L. gibbosus*	Bulgaria	KX931441	-	-	Stoyanov, et al. [11]
Posthodiplostomum centrarchi	*L. gibbosus*	USA	-	-	OM638425	Koxlien, et al. 2022
P. centrarchi	*L. gibbosus*	Hungary	-	MN080277, MN080281	-	Cech, et al. [7]
P. centrarchi	*Ardea herodias*	Canada	-	MH521251	-	Locke, et al. [36]
Posthodiplostomum sp.	*L. gibbosus*	Canada	HM064958	HM064955	-	Locke, et al. [34]
Posthodiplostomum sp.	*Micropterus salmoides*	Canada	HM064962	-	-	Locke, et al. [34]
Posthodiplostomum sp.	*L. gibbosus*	Slovakia	-	KX931442	-	Stoyanov, et al. [11]
Posthodiplostomum brevicaudatum	*Gasterosteus aculeatus*	Bulgaria	KX931431, KX931432	-	-	Stoyanov, et al. [11]
P. brevicaudatum	*Perca fluviatilis*	Czech Republic	-	-	KX931426	Stoyanov et al. 2017 [11]
Posthodiplostomum minimum	HNA	Mexico	U88074	-	-	Campos, et al. [37]
Posthodiplostomum pandei†	*Xenentodon cancila*	India	OP324624, OP324628	OP324627, OP324629	OP324625, OP324626	Present study
Posthodiplostomum sp.	*L. gibbosus*	Canada	FJ469590	-	-	Moszczynska, et al. [38]
Posthodiplostomum cuticola	*Ardea cinerea*	Czech Republic	MK089352	-	-	Heneberg, et al. [39]
P. cuticola	*Nycticorax nycticorax*	Ukraine	-	-	MZ710955	Achatz, et al. [35]
P. cuticola	*Anisus vortex*	Denmark	-	MW001124, MW001121	-	Duan, et al. [8]

P. cuticola	*Abramis brama*	Hungary	-	MN080266, MN080287	-	Cech, et al. [7]
Posthodiplostomum sp.	*L. gibbosus*	Canada	-	HM064949	-	Locke, et al. [34]
Posthodiplostomum sp.	*Tilapia sparrmanii*	South Africa	-	MK604881	-	Hoogendoorn, et al. [19]
Posthodiplostomum sp.	*Channa argus*	Japan	AB693170	AB693170	-	Nguyen et al. 2012 [40]
Posthodiplostomum sp.	*Channa punctata*	India	KF738455	KF738447	KF738450	Athokpam, et al. [41]
P. minimum	*Lepomis macrochirus*	USA	KY809062	-	-	Lovy, et al. [42]
P. minimum	*N. nycticorax*	USA	-	-	MZ710962	Achatz, et al. [35]
P. centrarchi	*A. herodias*	Canada	MH521251	-	-	Locke, et al. [36]
P. minimum	HNA	USA	AY245767	-	-	Flowers, et al. 2003
Posthodiplostomum sp.	*Nannopterum brasilianus*	Mexico	MF398354	-	MF398331	Hernandez-mena, et al. [43]
Posthodiplostomum macrocotyle	*Busarellus nigricollis*	Brazil	-	-	MZ710958	Achatz, et al. [35]
Posthodiplostomum microsicya	*Tigrisoma lineatum*	Brazil	-	-	MZ710960	Achatz, et al. [35]
Posthodiplostomum erickgreenei	*Pandion haliaetus*	USA	-	-	MZ710956	Achatz, et al. [35]
Posthodiplostomum eurypygae	*Eurypyga helias*	Brazil	-	-	MZ710957	Achatz et al. 2021 [35]
Posthodiplostomum nanum	*Ardea alba*	USA	-	-	MZ710963	Achatz, et al. [35]
P. nanum	*Gundlachia ticaga*	Brazil	-	MH358392	-	Lopez-Hernandez, et al. [23]
P. nanum	*Poecilia reticulata*	Brazil	-	MH358393	-	Lopez-Hernandez, 2018 [23]
Posthodiplostomum sp.	*A. herodias*	USA	-	-	MZ710994	Achatz, et al. [35]
Posthodiplostomum xenentodoni (=Neascus xenentodoni)	*X. cancila*	India	-	KY234201	KY234203	Choudhary, et al. [44]
Posthodiplostomum sp.	*Channa striata*	Vietnam	-	-	MT394045	Sokolov and Gordeev, 2020 [45]

Posthodiplostomum sp.	*Trichopodus trichopterus*	Vietnam	-	-	MT394051	Sokolov, et al. [45]
Posthodiplostomum hanumanthai (=Neascus hanumanthai)	*C. punctata*	India	-	KY234199	KY042122	Choudhary, et al. [44]
Posthodiplostomum gussevi (=Neascus gussevi)	*Colisa fasciata*	India	-	KY234200	KY234202	Choudhary, et al. [44]
Posthodiplostomum pacificus	*Larus californicus*	USA	-	-	MZ710967	Achatz, et al. [35]
Posthodiplostomum cf. anterovarium	*L. gibbosus*	USA	-	-	OM688205	Koxlien, et al. 2022
P. cf. anterovarium	*L. gibbosus*	USA	-	-	MZ710942	Achatz, et al. [35]
Posthodiplostomum cf. podicipitis	*Lophodytes cucullatus*	USA	-	-	MZ710969	Achatz, et al. [35]
Posthodiplostomum sp.	*Physa* sp.	USA	-	-	MZ710982	Achatz, et al. [35]
Posthodiplostomum recurvirostrae	*Recurvirostra americana*	USA	-	-	MZ710975	Achatz, et al. [35]
Posthodiplostomum ptychocheilus	*Mergus merganser*	USA	-	-	MZ710974	Achatz, et al. [35]
Posthodiplostomum sp.	*Trichopodus pectoralis*	Thailand	ON614094	-	-	Nguyen, 2022
Posthodiplostomum sp.	*Skiffia lermae*	Mexico	-	OK315760, OK315761	-	Perez-Ponce de Leon, et al. [46]
Posthodiplostomum sp.	*Allotoca dugesii*	Mexico	-	OK315771	-	Perez-Ponce de Leon, et al. [46]
Posthodiplostomum sp.	*Gambusia* sp.	Mexico	-	OK315772	-	Perez-Ponce de Leon, et al. [46]
Posthodiplostomum sp.	*Pimephales promelas*	Mexico	-	OK315775	-	Perez-Ponce de Leon, et al. [46]
Outgroups						
Tylodelphys immer	*Gavia immer*	Canada	MH521252	-	-	Locke, et al. [36]
Tylodelphys aztecae	*Podilymbus podiceps*	Mexico	-	-	MF398337	Hernandez-mena, et al. [43]
Tylodelphys sp.	*Aechmophorus occidentalis*	Mexico	-	MK177831	-	Sereno-Uribe, et al. [47]

Table 1: Trematode species are included in the phylogenetic analysis with information on the host, locality and GenBank accession number. HNA= host name not available. †Shows species sequenced during the present study. *Sequences available on Genbank mentioned as unpublished.

Body parts	*P. pandei (=N. pandei)*	*P. pandei (=N. pandei)*	*N. mesentri formis*	*N. hepatica*	*P. xenen todoni (=N. xenen todoni)*	*N. baughi*	*N. nanaks agrensis*	*N. simhai*	*N. moghei*	*N. vedi*	*N. sriva stavi*	*N. khur ram naga rensis*
Refe rence	Present study	Rai and Pande, 1964 [50]	Rai and Pande, 1964 [50]	Chakrabarti, 1970 [51]	Pandey, 1971 [52]	Baugh and Chakrabarti, 1977 [53]	Baugh and Chakra barti, 1977 [53]	Agrawal and Khan, 1982 [54]	Agrawal and Khan, 1982 [24]	Pandey and Tiwari, 1986 [55]	Pandey and Pandey, 2000 [56]	Gupta, et al. [57]
Infected Organ	Cysts in Liver	Liver	Mesentry	Cysts in Liver	Liver and cranium	Body cavity	Cysts in liver and gonads	Body muscles	Liver	Body muscles	Liver	Liver
CSTL	0.61 (0.49-0.76)	0.51-0.7	1.4-1.8	0.72-0.87	0.52-0.67	1.32-1.50	1.05-1.03	1.17-1.19	0.99-1.00	1.13-1.33	0.58-0.69	1.13 - 1.33
CSTW	0.37 (0.28-0.46)	0.31-0.6	0.8-0.9	0.44-0.52	-	0.65-0.76	0.50-0.68	0.76-0.78	0.62-0.64	0.90-1.20	0.41-0.43-	0.90 - 1.20
WBL	0.89 (0.51-1.2)	0.78-0.85	0.64-0.72	-	-	-	-	-	-	-	-	-
WBW	0.30 (0.11z-0.42)	0.25-0.34	-	-	-	-	-	-	-	-	-	-
FBL	0.62 (0.38-0.86)	-	-	0.46-0.75	0.67-1.05	1.28-1.65	0.78-1.05	1.07-1.20	0.97-0.98	0.47-1.03	0.54-0.64	0.47 - 1.03
FBW	0.30 (0.11-0.42)	-	-	0.23-0.39	0.24-0.33	0.90-1.08	0.61-0.76	0.54-0.60	0.37-0.38	0.19-0.21	0.28-0.44	0.19 - 0.21
HBL	0.23 (0.12-0.33)	-	-	0.18-0.26	0.18-0.30	0.62-0.82	0.41-0.65	0.37-0.39	0.26-0.27	0.19-0.22	0.20-0.25	0.19 - 0.22
HBW	0.16 (0.07-0.20)	-	-	0.15-0.24	0.14-0.27	0.56-0.71	0.53-0.70	0.27-0.22	0.28-0.29	0.13-0.19	0.18-0.27	0.13 - 0.19
OSL	0.02 (0.01-0.03)	0.024-0.04	0.04-0.05	0.04-0.07	-	0.05-0.08	0.04-0.06	0.05-0.06	0.07-0.75	0.03-0.04	0.02-0.04	0.03 - 0.04
OSW	0.02 (0.01-0.02)	0.024-0.04	0.04-0.05	0.03-0.05	-	0.03-0.05	-	0.03-0.04	-	0.02-0.03	0.02-0.03	0.02 - 0.03
VSL	0.04 (0.01-0.05)	0.04-0.06	0.04-0.05	0.05-0.09	0.04-0.07	0.06-0.09	0.06-0.09	0.09-0.10	-	0.03-0.04	0.04-0.06	0.03 - 0.04
VSW	0.04 (0.02-0.05)		0.04-0.05	0.05-0.09	-	0.06-0.09	-	0.07-0.08	-	0.02-0.03	0.05-0.08	0.02 -0.03
HOL	0.12 (0.06-0.18)	0.12-0.14	0.14-0.16	-	-		-	-	0.14-0.16	-	-	0.12 - 0.17
HOW	0.14(0.06-0.19)	0.12-0.14	-	-	-		-	-	-	-	-	0.10 - 0.16
ATL	0.05 (0.02-0.07)	0.06-0.07	-	0.10-0.15	-	0.20-0.26	0.17-0.20	0.10-0.12	0.13-0.14	0.02-0.07	0.03-0.05	0.02-0.07
ATW	0.07 (0.03-0.10)	0.02-0.03	-	0.06-0.10	-	0.25-0.32	0.16-0.23	0.13-0.14	0.04-0.045	0.10-0.16	0.06-0.09	0.06 - 0.12
PTL	0.06 (0.03-0.07)	0.08-0.1	-	0.16-0.22	-	0.41-0.54	0.35-0.40	0.23-0.25	0.13-0.14	0.01-0.03	0.03-0.05	0.01 - 0.03

PTW	0.11 (0.04-0.15)	0.03-0.05	-	0.02-0.07	-	0.07-0.13	0.07-0.10	0.10-0.13	0.04-0.045	0.05 -0.09	0.06-0.09	0.05 - 0.09
OL	0.06 (0.02-0.09)	-	-	0.08-0.12	-	-	0.05-0.07	0.13-0.14	0.11-0.12	0.01-0.03	0.04-0.05	-
OW	0.08 (0.01-0.11)	-	-	0.0-0.08	-	-	0.06-0.09	0.07-0.08	0.01-0.015	0.05-0.09	0.05-0.06	-
EBL	0.07 (0.03-0.09)	-	-	-	-	-	-	-	-	-	-	-
EBW	0.05 (0.02-0.07)	-	-	-	-	-	-	-	-	-	-	-
OSVS	0.42 (0.24-0.63)	-	-	-	-	-	-	-	-	-	-	-
VSHO	0.04 (0.02-0.07)	-	-	-	-	-	-	-	-	-	-	-

CSTL= Cyst length, CSTW= Cyst width, WBL= Whole body length, WBW= Whole body width, FBL= Fore body length, FBW= Fore body width, HBL= Hind body length, HBW= Hind body width, OSL= Oral sucker length, OSW= Oral sucker width, VSL= Ventral sucker length, VSW= Ventral sucker width, HOL= Holdfast organ length, HOW= Holdfast organ width, ATL= Anterior testis length, ATW= Anterior testis width, PTL= Posterior testis length, PTW= Posterior testis width, OL= Ovary length, OW= Ovary width, EBL= Excretory bladder length, EBW= Excretory bladder width, OSVS= Distance between oral sucker and ventral sucker, VSHO= Distance between ventral sucker and holdfast organ.

Table 2: Comparative measurements shown as mean (range) of *Neascus*-type and *Posthodiplostomum* species, all infected the same host (*Xenentodon cancila*) from India.

Species	Host	Site of infection	Species status/ developmental stage	Identification evidence	References
P. botauri	NA	Intestine	Valid/A	Morphology	Vidyarthi [58]
N. vetastai	*Schizothorax esocinus, S. micropogon, S. niger*	Cyst on skin and viscera	Incertae sedis/M	Morphology	Kaw [59]
N. chelai	*Chela clupeoides*	Encysted in the integument and muscles	Incertae sedis/M	Morphology	Khera [60]
P. pandei	*Xenentodon cancila*	Liver	Valid/M	Morphology/ Molecular*	Rai, et al. [50]
N. mesentriformis	*X. cancila*	Mesentery	Incertae sedis/M	Morphology	Rai, et al. [50]
P. milvi	NA	Intestine	Valid/A	Morphology	Fotedar, et al. [61]
N. indicus	*Nuria dursica, Catla catla*	Cyst in muscles below scales	Incertae sedis/M	Morphology	Thapar [62]
N. cirrhinus	*Cirrhinus mrigala*	Cyst in muscles	Incertae sedis/M	Morphology	Thapar [62]
N. muscularis	*Channa punctata*	Muscles	Incertae sedis/M	Morphology	Rai , et al. [50]

N. elongatus	*Colisa fasciata*	Mesenteries inside body cavity	Incertae sedis/M	Morphology	Singh [63]; Pandey 64]
N. hepatica	*X. cancila*	Cyst in liver	Incertae sedis/M	Morphology	Chakrabarti [51]; Vankara, et al. [65]
P. mehtai	*Milvus migrans*	Intestine	Valid/A	Morphology	Gupta, et al. 1974 [66]
N. channi	*C. punctata*	Cranium	Incertae sedis/M	Morphology	Pandey [52]
P. xenentodoni	*X. cancila*	Liver and cranium	Valid/M	Morphology/ Molecular	Pandey [52]; Choudhary, et al. [44]
N. komiyai	*Glossgobius giuris*	Cyst attached to stomach	Incertae sedis/M	Morphology	Pandey [67]
N. hoffmani	*Nandus nandus*	Stomach	Incertae sedis/M	Morphology	Pandey [67]
N. gussevi	*C. punctata*	Cyst attached to visceral organs	Valid/M	Morphology/ Molecular	Chakrabarti [68]; Choudhary, et al. [44]
N. baughi	*X. cancila*	Body cavity	Incertae sedis/M	Morphology	Baugh, et al [53]
N. nanaksagrensis	*X. cancila*	Cysts attached to liver, gonads and in liver	Incertae sedis/M	Morphology	Baugh, et al. [53]
N. chauhani	*Heteropneustus fossilis*	Free in body muscles	Incertae sedis/M	Morphology	Agrawal and Khan, 1982 [54]
N. hanumanthai	*C. punctata*	Cyst in muscles	Valid/M	Morphology/ Molecular	Agrawal, et al. [54]; Choudhary, et al. [44]
N. simhai	*X. cancila*	Cyst in body muscles	Incertae sedis/M	Morphology	Agrawal, et al. [54]
N. moghei	*X. cancila*	Liver	Incertae sedis/M	Morphology	Agrawal, et al. [54]
N. shahjahanpurensis	*Clarius batrachus*	Muscles	Incertae sedis/M	Morphology	Pandey, et al. [55]
N. ramalingami	*Labeo rohita*	Body muscles	Incertae sedis/M	Morphology	Pandey and Tiwari, 1986 [55]
N. vedi	*X. cancila*	Body muscles	Incertae sedis/M	Morphology	Pandey, et al. [55]
N. punctatusi	*C. punctata*	Cranial cavity	Incertae sedis/M	Morphology	Dhanukumari [69]
P. grayii	*Apocheilus panchax*	Liver	Valid/A	Morphology	Verma, [70]; Madhavi, et al. [71]
N. srivastavi	*X. cancila*	Liver	Incertae sedis/M	Morphology	Pandey, et al. [56]
Posthodiplostomum sp.	*C. punctata*	Body muscles	Valid/M	Morphology/ Molecular	Athokpam, et al. [41]
N. khurramnagarensis	*X. cancila*	Liver	Incertae sedis/M	Morphology	Gupta, et al. [57]

Table 3: Species of *Neascus-type* or *Posthodiplostomum* described from India on the basis of metacercariae specimens, infected hosts, infection site, species status, identification information for the species and references related to morphological or molecular evidence. Asterisk shows molecular data generated in the present study.

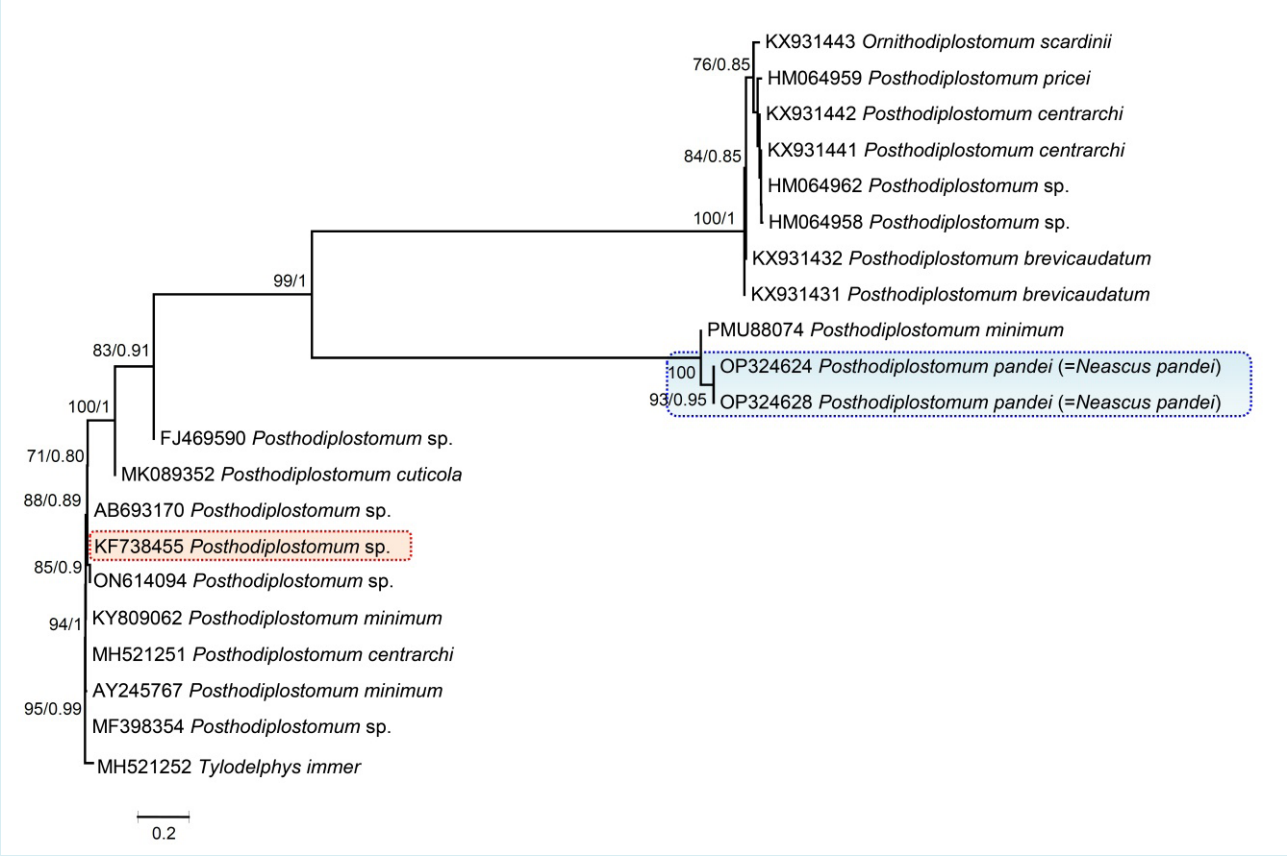


Figure 11: Phylogenetic interrelationships among *Posthodiplostomum* species based on Maximum Likelihood (ML) and Bayesian Inference (BI) analyses of partial 18S rDNA gene sequences. The scale-bar indicates the number of substitutions per site. Species names are provided after GenBank accession numbers. The numbers represent bootstrap values greater than 70%. The present studied species, *Posthodiplostomum pandei*, is indicated by the blue line box, while the red line box shows the other Indian species.

In this study, the ITS (ITS1–5.8S–ITS2) sequences of two individuals of *P. pandei* were generated and aligned with other sequences of the diplostomids (Figure 12). The genetic divergence between the different species of *Posthodiplostomum* ranged from 0.92 to 4.1%. Moreover, ITS (ITS1–5.8S–ITS2) sequences were available for the following Indian species: *N. hanumanthai* (KY042122), *N. gussevi* (KY234202), *N. xenentodoni* (KY234203), and *Posthodiplostomum* sp. (KF738447), whose genetic divergence ranged from 0.92 to 2.6%. Maximum likelihood (ML) and Bayesian inference (BI) analyses produced a similar tree in topology that shows *N. xenentodoni* to be the closest to our specimens; both belong to the same host (Figure 12).

The 28S sequences in the current phylogenetic analysis clearly demonstrated the status of *P. pandei* within *Posthodiplostomum* (Figure 13). The Indian species *N. hanumanthai* (KY042122), *N. gussevi* (KY234202), *N. xenentodoni* (KY234203), and an unknown *Posthodiplostomum* sp. (KF738450) were positioned with *Posthodiplostomum* members, validated their status, and formed a well-supported clade. Choudhary, et al. [44] were not mentioned the status of *N. xenentodoni* regarding this is a species of *Posthodiplostomum* as the molecular data predicts, the present study analysis confirmed that it is a species of *Posthodiplostomum* with good bootstrap values (90% ML and 0.92 BI) and sister to the present specimens (Figure 13). In comparison, the genetic divergence among *Posthodiplostomum* and *P. pandei* ranged from 1.2 to 4.5%. *P. pandei* and other Indian *Posthodiplostomum* species had 1.2–2.2% genetic variation. Analysis shows all the 28S sequences of Indian species and the data generated in this study are nested within a clade with strong bootstrap support and Bayesian posterior probability values of 83 and 0.9 (Figure 13).

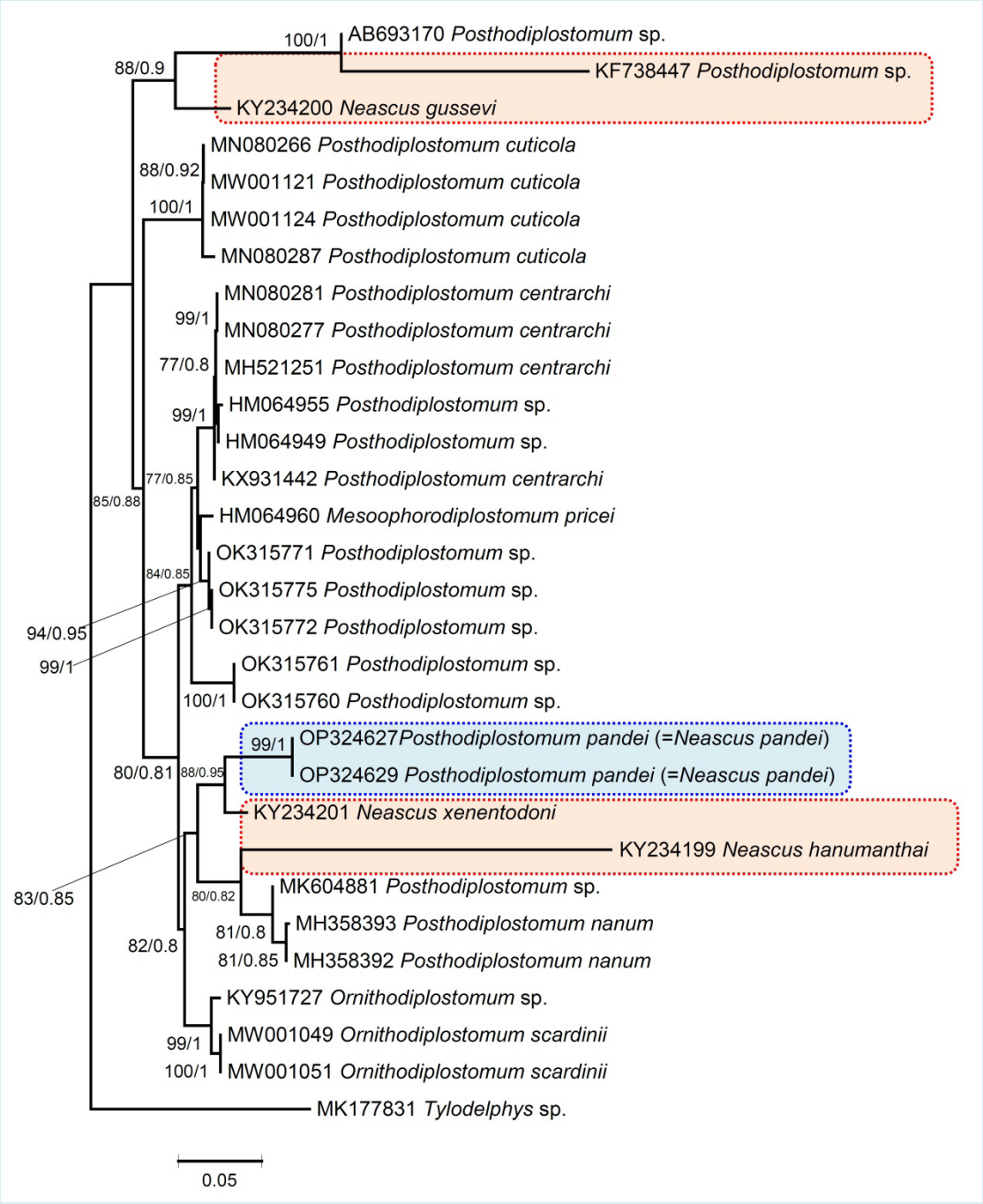


Figure 12: Phylogenetic reconstruction using ITS region (ITS1-5.8S-ITS2) sequences of *Posthodiplostomum* species (shown in blue box). The numbers represent bootstrap values greater than 70%. Numbers above branches indicate nodal support as maximum likelihood (ML) and posterior probabilities from BI. The scale bar indicates the expected number of substitutions per site. Species names are provided after Gen Bank accession numbers. The blue line box represents the currently studied species P. *pandei,* while the red line box represents the other Indian species.

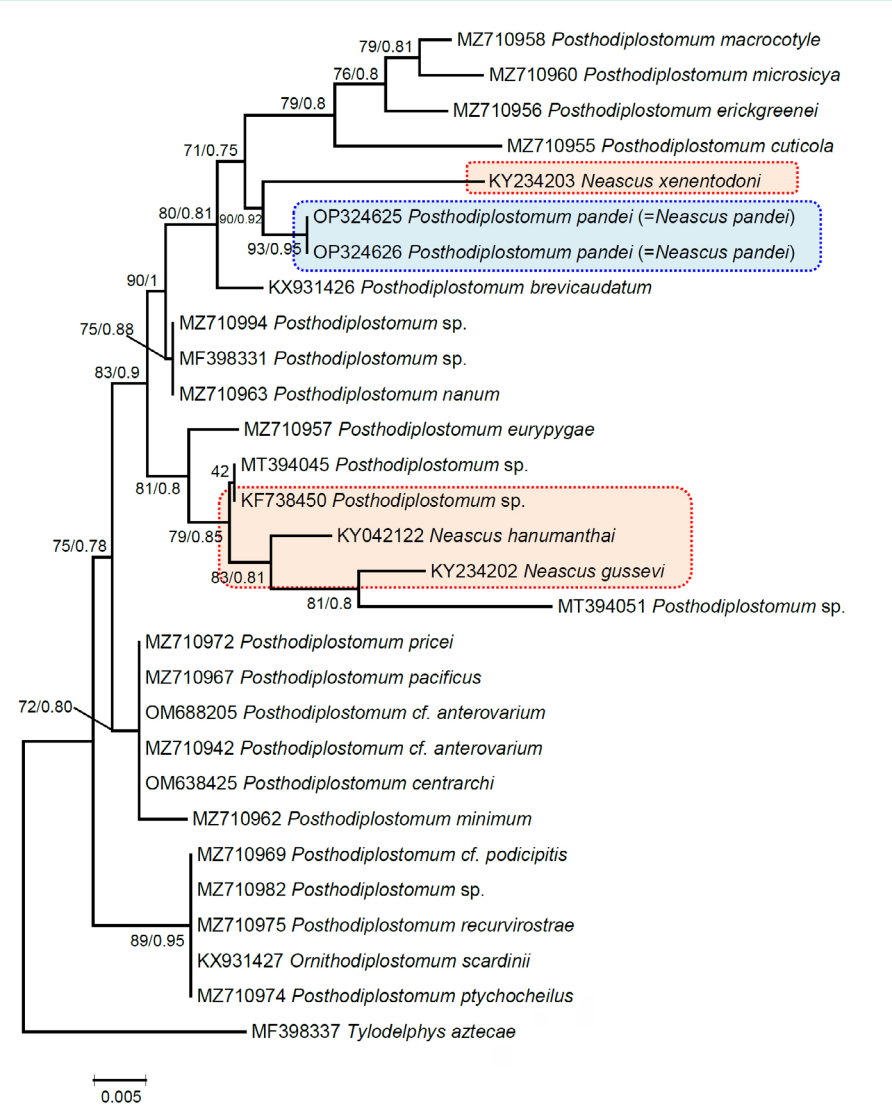


Figure 13: Phylogenetic relationship between *Posthodiplostomum* species inferred from sequences of the 28S rDNA gene based on maximum likelihood (ML) and Bayesian inference (BI) analyses. Nodal support is indicated as ML/BI. The numbers represent bootstrap values greater than 70%. The scale bar indicates the expected number of substitutions per site. Species names are provided after GenBank accession numbers. *Posthodiplostomum pandei* is indicated by the blue line box, while the red line box shows the other Indian species.

We also want to mention that in a molecular phylogenetic study by Achatz, et al. [35], the synonymy of *Posthodiplostomum, Ornithodiplostomum* Dubois, 1936, and *Mesoophorodiplostomum* Dubois, 1936, was suggested. To the best of our knowledge, the Genbank database only contains molecular data for the following *Neascus*-type species from the Indian region to date: *N. hanumanthai, N. gussevi, N. xenentodoni*, and one unknown *Posthodiplostomum* sp. The comparison of the genetic data of our specimens with the above-mentioned species molecular level and in variations of measurements of the body parts also easily differentiates them with *P. pandei*.

Discussion

Previous studies show that it is difficult to evaluate the host specificity with regard to larval helminthes because of their poor morphological identification [72,73]. But molecular methods are the best tools to deal with this problem and achieve accurate identifications of larval trematodes in fish hosts [72,74,75]. Larval trematodes often harbour multiple sites in their hosts, but most of their infection site preferences or specificities are likely mistaken [76-79]. In a study by Locke, et al. [34], different *Posthodiplostomum* species were recorded from viscera and muscles. Therefore, thorough

examinations of infection sites supplemented with molecular data are presently required to decide if infection sites are the same or differ between *Posthodiplostomum* species. In the present study, *P. pandei* was molecularly characterised for the first time. The placement of our specimens in the phylogenetic trees produced in this study supports their settlement within Diplostomidae and separates them from other representatives of different geographical regions and India too. Our species appeared as a sister taxon to the *Posthodiplostomum* species isolate that belongs to Mexico in the 18S rRNA tree, though it was far from the Indian species (KF738455), which is the only sequence available for the *Channa punctata*. However, in the ITS analysis, *P. pandei* was grouped of *N. xenentodoni*, both species infected the same host, *X. cancila;* and were fairly distinct from each other. In the distribution of species by 28S rRNA tree, it was congruent with the 18S and ITS rRNA trees in many respects, with *Posthodiplostomum* species of *C. punctata* (KF738450) appearing along with two other species, *N. hanumanthai* and *N. gussevi* from India. *N. xenentodoni* from the same host appeared as a sister taxon to *P. pandei* in the 28S rRNA tree. The phylogenetic trees generated with all molecular markers in the present study revealed that *P. pandei* belongs to a clade containing *Posthodiplostomum* species Further work is a prerequisite to establishing and understanding the Indian *Posthodiplostomum* species infections in fish and avian hosts that need to be molecularly characterised to determine their diversity, which might be different from what we know today.

About 26 species of *Neascus*-type metacercariae have been described in India, mostly based on morphology (Table 3). For many species, at this point, it is hard to establish how many species are under the genus *Posthodiplostomum,* which shows their unstable taxonomic history in India. The reason behind the unknown status of diplostomid parasites in India is that they have not been well studied, leaving a big gap with regard to their true diversity. However, in recent years, a few studies have pointed it out, contributed to the understanding of diplostomid parasites, and tried to fill the gap [80-85]. Based on morphological variation between specimens, previous authors [27] recommended the description of eleven *Neascus*-type metacercariae species from *X. cancila* (Tables 1 & 3). In our study, nearly similar morphological measurements were found in the overall body measurements for *P. pandei*, *N. hepatica*, and *N. srivastavi*. In all eleven species reported from the host *X. cancila*, only *P. xenentodoni* (= *N. xenentodoni*) has molecular sequences. With the exception of *P. xenentodoni*, no genetic sequences for the other Indian species were available for comparison from *X. cancila*. Another reason that also hampers the taxonomic studies and diversity of this group of parasites in India is the restrictions of ethical guidelines that are related to definitive hosts (birds), which make it challenging to study the relationship of adults in birds along with metacercariae and the completion of their life cycles.

In the case of diplostomid parasites, García-Varela, et al. [86] recently proposed that all South American species described on the basis of metacercaria morphology be considered *incertae sedis*. We are agreeing with that because studies based only on the morphology of metacercariae will make it difficult to reveal the relationship with their adult forms in future studies. Therefore, we also propose that, from India, those metacercariae for which molecular data is available have been considered valid species, as their phylogenetic relationships can be easily evaluated with their adults in future studies (Table 3). Under the *incertae sedis* status, we believe that those *Posthodiplostomum/Neascus*-type metacercariae species for which only morphological data is available should be taken into account, leaving the possibility for future studies to perform morphological and molecular comparisons that help to uncover their life cycle and clarify their status.

Conclusion

Overall, our identification of *P. pandei* is supported by its morphology as well as at the molecular level. Furthermore, molecular data generated here will be useful in future studies to determine whether *Neascus*-type metacercariae species belong to *Posthodiplostomum* or another genus. Though we robustly acclaim that no new species can be further described without molecular data under the genus *Posthodiplostomum,* We also proposes that some previously published *Neasus*-type species that had been misidentified should be reassigned on the basis of morphological and molecular data in future studies.

Conflicts of Interest

The authors declare no competing interests.

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