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Morphological and molecular characterization of *Posthodiplostomum* sp. (Digenea: Diplostomidae) metacercaria in the muscles of snakeheads (*Channa punctata*) from Manipur, India

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Summary

The spotted snakehead, *Channa punctata* Bloch, 1793, is a locally important fish species commonly consumed by the natives in the state of Manipur, Northeast India. The fish host *C. punctata* from Lamphel area revealed a diplostomid metacercarial infection. Morphologically, the recovered metacercaria was identified as a species of *Posthodiplostomum* Dubois, 1936. Molecular characterization using the ribosomal RNA genes (rDNA 18S, ITS2 and 28S regions) and the mitochondrial CO1 region supplements the identification. Molecular analysis revealed the metacercaria to be closely related to *Posthodiplostomum* sp. Japan isolate, with sequence similarity variation from 97.5 – 99.7 % while considering for the three rDNA markers. The secondary structure of the ITS2 region further corroborated these results; the typical four-helix model, when compared to the taxon from Japan, showed differences only in twelve bases (with seven transitions and five transversions). In phylogenetic analysis also, the metacercaria claded with the genus *Posthodiplostomum,* coming closer to the Japanese isolate, thus supplementing the morphological identification of the metacercaria.

Keywords: fish; *Channa punctata;* metacercaria; *Posthodiplostomum* sp.; morphology; morphometry; rDNA; mtCO1

Introduction

Species of *Posthodiplostomum* Dubois, 1936*,* a genus of digenean flukes in the family Diplostomidae has been frequently reported in Cypriniformes (Ishii, 1951; Nagasawa *et al*., 1989; Zrnčić *et al*., 2009), Channiformes (Nguyen et al., 2012) and Perciformes (Karimian *et al*., 2013). There are about 30 species of *Posthodiplostomum* reported from bird hosts (Yamaguti, 1971; Sonin, 1986), which utilize a wide range of cypriniform fishes as their second intermediate host; the family Cyprinidae in particular constitutes the largest group of hosts being affected (Lucky, 1970; Chubb, 1977; Jalali, 1998; Rolbiecki, 2004; Ondračková *et al*., 2004a; Vytautas & Èeslovas, 2009; Karimian *et al*., 2013). Of these species, the metacercariae of *P. cuticola* are responsible for causing 'Black spot' disease in the fish host (Dönges, 1964; Schäpperclaus, 1990; Ondračková *et al*., 2004b; Karimian *et al*., 2013) and those of *P. brevicaudatum* encysting in the eye lens cause ocular parasitosis (Dönges, 1969; Stanevičiūtė *et al*., 1998). However, another species, *P. minimum*, is responsible for causing 'white grub' in fishes (Colley & Olson, 1963; Lewis & Nickum, 1964; Avault & Smitherman, 1965; Lane & Morris, 2000). There is only one report of *Posthodiplostomum* metacercarial infection occurring in the channid fish (Nguyen *et al*., 2012). In fish intermediate hosts, the parasite occurs encysted in the skin, fins, cornea and superficial muscles as the etiological agent of black-spot disease (Kurochkin & Biserova, 1996; Shukhgalter & Chukalova, 2002; Ondračková *et al*., 2002, 2004b). Piscivorous birds, mainly herons (Ardeidae), are definitive host for the parasite in Eurasia (Skrjabin, 1964; Yamaguti, 1971).

Molecular characterization of larval and adult forms of various diplostomid taxa utilizing data from rDNA markers has proven to be useful in supplementing their morphology-based identification (Galazzo *et al*., 2002; Niewiadomska & Laskowski, 2002; Criscione *et al*., 2005; Nguyen *et al*., 2012; Zhao *et al*., 2012). The mitochondrial cytochrome oxidase 1 (mtCO1) gene has also been largely utilized as a tool in molecular discrimination of digenean species (Nolan & Cribb, 2005; Olson & Tkach, 2005).

In a pilot study carried out to evaluate the status of metacercarial infections in freshwater fishes in Manipur, the snakehead fish, *Channa punctata* Bloch, 1793, was frequently found harbouring a diplostomid (*Posthodiplostomum* sp.) infection in the skin (Athokpam & Tandon, 2013). As a sequel to the same, the present study was taken

up to morphologically and molecularly characterize this metacercaria to determine its systematic position. To achieve this goal, rDNA ITS2, 18S and 28S and mtCO1 gene regions were used as molecular markers.

Material and methods

Study area and morphology study

Live *C. punctata* were collected from the focal area of *Posthodiplostomum* infection, i.e., Lamphel (Imphal West District, Manipur). The host muscle, skin, fins, gills, oral cavity and eyes were examined under a stereomicroscope to detect any infection with metacercariae.

For morphological identification, the metacercarial cysts were excysted artificially by providing a gentle mechanical pressure and flattened between a glass slide and a cover glass. They were then fixed in 70 % ethyl alcohol, processed for whole mount preparation and stained with acetocarmine stain following the standard protocol. Observations were made using Leitz Ortholux-2 research microscope and line drawing sketched using camera lucida. Morphometric measurements (in millimetre) of the cyst and excysted metacercaria were taken with the help of ocular micrometer.

Molecular study

DNA isolation, amplification and sequencing

DNA was extracted from the excysted unflattened metacercariae separated from a single fish host, fixed in 70 % ethyl alcohol using QIAamp DNA Mini Kit (50) according to the manufacturer's instructions.

For PCR-amplification we used three rDNA marker gene regions: 18S, 28S and internal transcribed spacer 2 (ITS2) and the mitochondrial cytochrome oxidase subunit 1 (CO1) region. Primers used for the respective gene region are detailed as follows:

ITS2: 3S (Forward)/ A28 (Reverse) (Bowles *et al*., 1995)
18S: EukA (Forward)/ EukB (Reverse) (Diez *et al*., 2001)
28S: dig12 (Forward)/ 1500R (Reverse) (Tkach *et al*., 2000)
mtCO1: JB3 (Forward)/ JB4.5 (Reverse) (Bowles *et al*., 1993).

The thermal gradient of these marker regions started with an initial denaturation at 94 ºC (5min), annealing – for 18S at 58 ºC (1.10 min), 28S at 57 ºC (1 min), ITS2 at 57 ºC (1.10 min) and CO1 at 56 ºC (1.10 min), and final extension at 72 ºC (10 min). The amplified PCR products were separated by electrophoresis through 1.6 % (w/v) agarose gels in TAE buffer, stained with ethidium bromide, transilluminated under ultraviolet light, and then photographed.

PCR products were purified using Genei Quick PCR purification kit for DNA sequencing, and sequenced in both directions using PCR primer sets utilizing Macrogen sequencing service, Korea.

Sequence analysis

Similarity search was carried out using Basic Local Alignment Search Tool (BLAST) available at http://www.ncbi.nlm.nih.go v/blast. Since the full length of the 18S and 28S rRNA could not be retrieved from one direction sequencing, the contigs were created by assembling the forward and reversed sequences of the genes using DNA Baser v3.5.3 (http://www.dnabaser.com/). Multiple sequence alignments were done for each of the amplified markers from the studied metacercaria, with related sequences from superfamily Diplostomoidea retrieved from GenBank (Table 2), using ClustalW of Bioedit software (http://www. ebi.ac.uk/clustalw). For sequence identities Bioedit software version 7.0.9.0 (Hall, 1999) was used.

Phylogenetic analysis

The rDNA ITS2 sequences were used for phylogenetic studies, using various bioinformatic tools. The ITS2 rDNA plus the flanking 5.8S and 28S sequences were first annotated using the hidden Markov models (HMM)-based annotation to retrieve the exact sequence of the region (Keller *et al*., 2009) available at http://its2.bioapps.biozentrum.uni-wuerzburg.de/. The output fasta format files from Bioedit were then entered into MEGA5 for phylogenetic tree construction using the distance-based Neighbour Joining (NJ) method and character-based Maximum Parsimony (MP). Bayesian Inference (BI) analysis was also done by aligning the sequences using Clustal X 2.0.7; the NEXUS file format was generated and alignments were imported to Mr.Bayes v3.1.2 programme (Huelsenbeck & Ronquist, 2001), using GTR+I+G model. Markov Chain Monte Carlo (MCMC) chains were generated following Shylla *et al*. (2011) with some modifications: ngen = 250000 (ITS2); 100000 (18S); 200000 (28S); 'sump burnin' = 6250 (ITS2); 2500 (18S); 5000 (28S) and 'sumt burnin' = 6250 (ITS2); 2500 (18S); 5000 (28S). In addition to the sequence data set used in the analysis, *Clinostomum* sequence was included as the outgroup taxon. Phylogenetic accuracy was done by bootstrapping the constructed trees.

Predicted ITS2 RNA secondary structures

The ITS2 secondary structure was used as a supplementary tool to phylogenetic analysis. Secondary structure of the annotated ITS2 sequence was reconstructed with MFOLD software version 3.2, using free energy folding algorithms (Zuker, 2003) and the structure with lowest free energy was taken for analysis. The consensus secondary structure was generated with *Posthodiplostomum* sp. Japan isolate (accession number: AB693170.1) using 4Sale (Seibel *et al*., 2006).

Results

Morphology and morphometry

Based on its morphological attributes, the metacercaria recovered from the infected host's muscle was identified as belonging to the genus *Posthodiplostomum* Dubois, 1936 (Family: Diplostomidae Poirier, 1886) and is describe as follows.

Description (based on 10 specimens). Metacercarial cyst oval or round in shape, surrounded by a single layered cyst

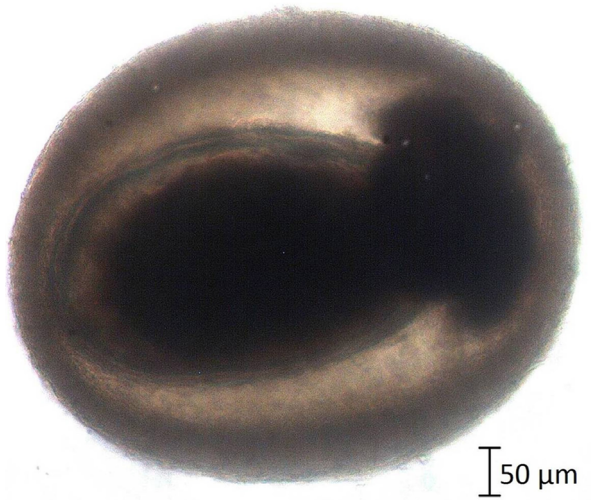

Fig. 1. Encysted metacercaria recovered from the muscle tissue of *C. punctata*

wall, 0.64 – 0.67 (0.65 ± 0.01) mm by 0.53 – 0.55 (0.51 ± 0.06) mm in size (Fig. 1). Excysted metacercaria with distinctly bipartite body, typically 'Neascus' type. Forebody: somewhat foliaceous or lanceolate; pseudosuckers absent; oral and ventral suckers feebly developed, small in size; pharynx small, conspicuous; intestinal caeca not prominent; trybocytic organ disc-like or almond-shaped, with cavity opening via median slit, holdfast gland present; reserve bladder composed of 3 longitudinal canals (one median, two lateral) ramifying and forming net in forebody, excretory bodies free in canal ramifications. Hindbody: somewhat oval, stump-like, lodging primordia of gonads that occupy greater space. Testes two, one behind other in median field; anterior testis smaller, oval, laterally or submedially placed; posterior testis larger in size, transversely elongate. Ovary small, rounded, submedian or lateral or diagonal to anterior testis. Copulatory bursa evertible, with terminal opening (Fig. 2).

Morphometric measurements of the particular body parts of the mounted metacercaria are given in Table 1.

Molecular study

Molecular analysis and characterization

The select marker regions as mentioned above were amplified successfully; the obtained sequences were deposited in GenBank and their accession numbers obtained [KF738447 (ITS2), KF738450 (28S), KF738455 (18S) and KF738453 (CO1)]. The amplicon size of ITS2, 28S, 18S and CO1 was 441 bp, 1117 bp, 1877 bp and 366 bp in length, respectively. The sequences generated from the present study were aligned with those of strigeidae and

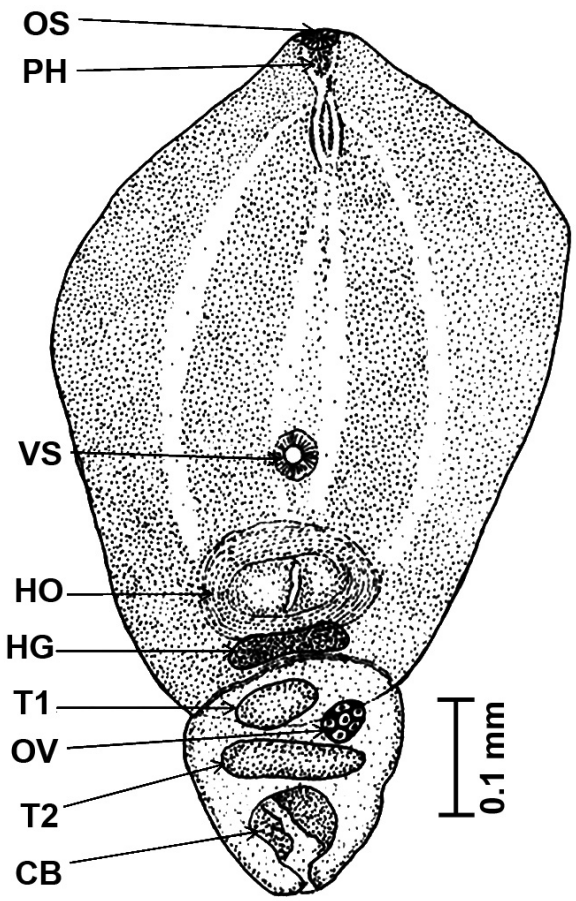

Fig. 2. Excysted metacercaria–line drawing (camera lucida), CB – copulatory bursa, HG – holdfast gland, HO – holdfast organ, OS – oral sucker, OV – ovary, PH – pharynx, T1 – anterior testis, T2 – posterior testis, VS – ventral sucker

Table 1. Morphometric measurements (in mm) of the excysted metacercaria of *Posthodiplostomum* sp.

Characters	**Range (mm)**	**Mean**	**±SD**
Body length	0.504 – 0.675	0.635	±0.069
Forebody			
Length	0.396 – 0.495	0.445	±0.074
Width	0.315 – 0.567	0.444	±0.085
Hindbody			
Length	0.099 – 0.18	0.150	±0.029
Width	0.135 – 0.198	0.158	±0.035
Oral Sucker			
Length	0.014 – 0.030	0.023	±0.007
Width	0.020 – 0.032	0.027	±0.005
Ventral sucker			
Length	0.048 – 0.05	0.049	±0.001
Width	0.048 – 0.05	0.049	±0.001
Pharynx			
Length	0.030 – 0.036	0.033	±0.004
Width	0.016	0.016	0
Holdfast gland			
Length	0.012 – 0.018	0.016	±0.003
Width	0.060 – 0.075	0.069	±0.007
Holdfast:			
Length	0.060 – 0.075	0.064	±0.007
Width	0.090 – 0.105	0.099	±0.008
Anterior Testis			
Length	0.021 – 0.210	0.087	±0.107
Width	0.042 – 0.054	0.047	±0.005
Posterior Testis			
Length	0.018 – 0.030	0.022	±0.007
Width	0.078 – 0.090	0.084	±0.006
Ovary			
Length	0.018 – 0.021	0.019	±0.001
Width	0.027 – 0.030	0.028	±0.001
Bursa			
Length	0.060 – 0.660	0.063	±0.003
Width	0.045 – 0.057	0.052	±0.005

diplostomid taxa retrieved from the GenBank (Table 2). The metacercaria under the present study stands very close to *Posthodiplostomum* sp. Japan isolate with sequence identities of 97.5 % (ITS2), 99.7 % (18S) and 97.5 % (28S), showing very low variation (0.3 – 2.5 %) between the two (Tables 3 – 5).

Phylogenetic study

The phylogenetic trees constructed for the three rDNA genes using MP, NJ and BI methods showed almost the same topology of taxa; therefore, to avoid any repetition only the BI tree is shown herein (Fig. 3a-c). All trees revealed that the present metacercaria clades with *Posthodiplostomum* sp. Japan isolate.

Table 2. Diplostomidae and Strigeidae species sequences of rDNA used in the analysis with their respective GenBank accession numbers

Sr. No.	Name of species	Family	Host Species	Locality	Marker Region	Accession no.
1.	*Alaria alata*	Diplostomidae	-	France	ITS2	JF340223.1
2.	*A. alata*		*Nyctereutes procyonoides*	Ukraine	18S rDNA	AY222091.1
		"			28S rDNA	AF184263.1
			-			
3.	*A. alata*	"	*Sus scrofa*	Italy	CO1	HM022223.1
4.	*A. taxideae*	"	-	USA	ITS2	JF820609.1
			Lithobates sylvaticus		28S rDNA	JF820607.1
5.	*Bolbophorus* sp.	"	-	USA	ITS2	AF470611.1
					18S rDNA	AF490575.1
					CO1	AF470615.1
6.	*B. confuses*	"	-	Israel	ITS2	AY242851.1
					18S rDNA	AY242851.1
7.	*B. damnificus*	"	Pelican	USA	18S rDNA	AF490574.1
			-		CO1	AF470609.1
8.	*B. levantinus*	"	Night heron	USA	18S rDNA	AF490576.1
9.	*Diplostomum* sp.	"	*Lithobates sylvaticus*	Canada	ITS2	GQ292510.1
10.	*Diplostomum* sp.	"	*Lymnaea stagnalis*	USA	28S rDNA	JX262945.1
11.	*D. compactum*	"	-	USA	18S rDNA	AY245764.1
12.	*D. phoxini*	"	*Phoxinus phoxinus*	UK	18S rDNA	AY222090.1
					28S rDNA	AY222173.1
13.	*D. pseudospathaceum*	"	*Larus cachinnans*	Czech Republic	ITS2	JX986854.1
14.	*D. spathaceum indistinctum*	"	-	USA	18S rDNA	AY245761.1
15.	*Neodiplostomum seoulense*	"	-	Korea	CO1	AF096233.2
16.	*Ornithodiplostomum* sp.	"	*Percina caprodes*	Canada	ITS2	HM064934.1
17.	*Posthodiplostomum* sp.	"	*Channa argus*	Japan	ITS2	AB693170.1
					18S rDNA	AB693170.1
					28S rDNA	AB693170.1
18.	*Posthodiplostomum* sp.	"	*Lepomis gibbosus*	Canada	ITS2	HM064957.1
19.	*P. minimum*	"	-	USA	18S rDNA	AY245767.1
20.	*Tylodelphys scheuringi*	"	-	Canada	ITS2	FJ469596.1
21.	*T. mashonensis*	"	-	Czech Republic	ITS2	KC685362.1
22.	*Apharyngostrigea pipientis*	Strigeidae	*Nycticorax nycticorax*	USA	28S rDNA	JF820597.1
23.	*Cardiocephaloides longicollis*	"	*Larus ridibundus*	Ukraine	28S rDNA	AY222171.1
24.	Strigeidae gen.	"	-	Canada	ITS2	HM064970.1
25.	Strigeidae sp.		-	Israel	ITS2	AY245711.1
26.	Strigeidae sp.		*Cominella glandiformis*	New Zealand	CO1	FJ765509.1
27.	Strigeidida sp.		-	USA	18S rDNA	EU371593.1

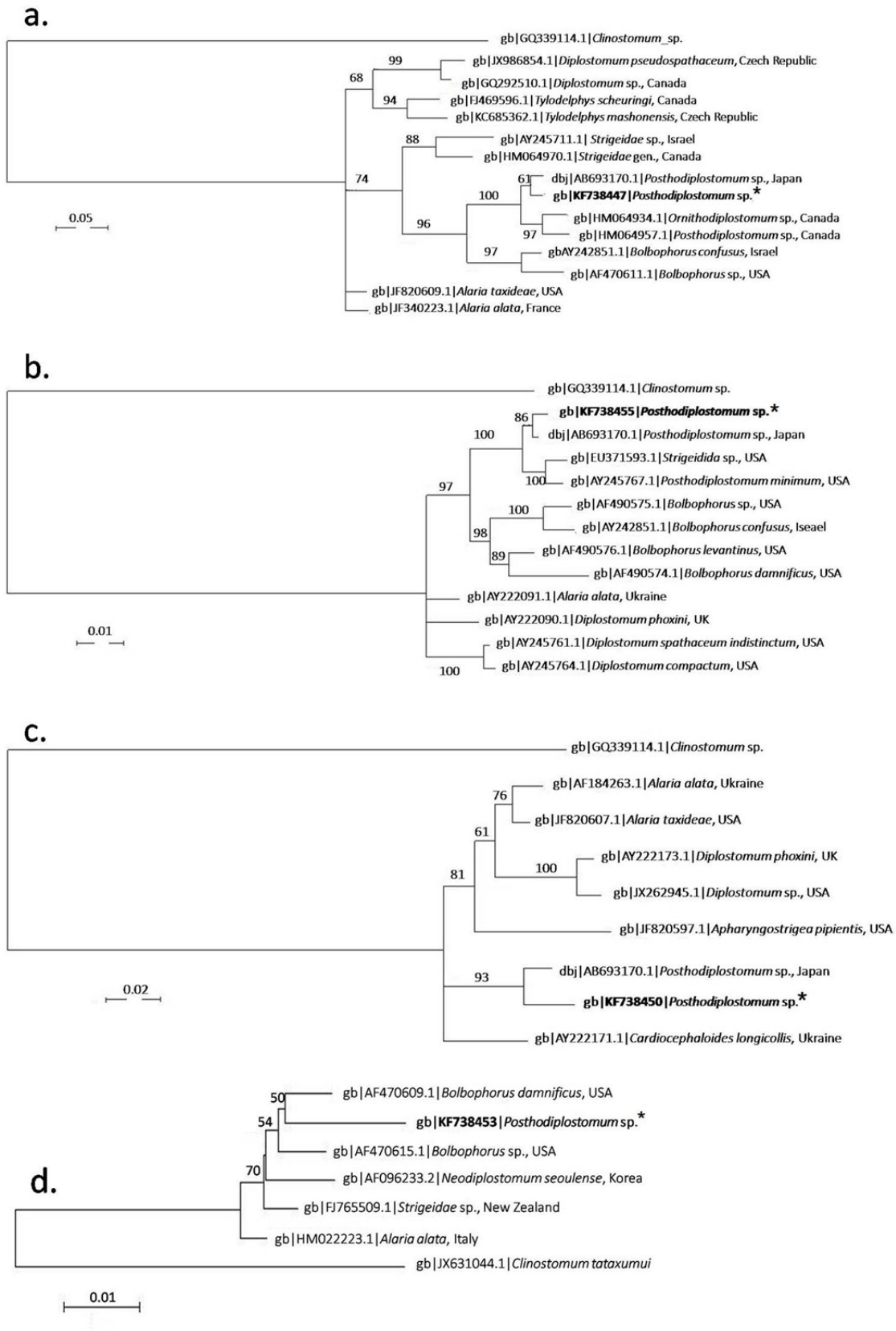

Fig. 3. Phylogenetic trees depicting relationships of the diplostomid and strigeid taxa inferred from rDNA and mtCO1 markers data. **a – c.** Bayesian Inference (BI) tree: (a) ITS2, (b) 18S, (c) 28S. **d.** NJ tree: mtCO1.
('*'- query sequence generated in the study)

Table 3. Sequence identity matrix (%) among ITS2 sequences of the various streigid /diplostomid taxa as retrieve from GenBank. (ID – Identical)

	Sequences	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1.	*Alaria alata*, France	ID													
2.	*Alaria taxideae*, USA	94.7	ID												
3.	*Bolbophorus confuses*, Israel	82.4	81.4	ID											
4.	*Bolbophorus* sp., USA	83.8	83.5	92.9	ID										
5.	*Diplostomum* sp., Canada	87.3	88.7	82.3	83.4	ID									
6.	*Diplostomum pseudospathaceum*, Czech Republic	85.9	88.0	80.3	82.1	96.4	ID								
7.	*Ornithodiplostomum* sp., Canada	84.1	83.8	87.2	87.2	83.0	81.6	ID							
8.	*Posthodiplostomum* sp., India	85.2	85.9	87.5	87.2	85.1	83.0	94.6	ID						
9.	*Posthodiplostomum* sp., Japan	85.9	86.2	88.2	89.0	84.4	83.0	95.3	**97.5**	ID					
10.	*Posthodiplostomum* sp., Canada	80.2	80.2	85.0	80.8	80.1	77.8	90.3	90.3	89.6	ID				
11.	*Tylodelphys scheuringi*, Canada	87.3	88.0	80.3	81.0	88.7	88.0	80.9	83.4	82.7	77.8	ID			
12.	*Tylodelphys mashonensis*, Czech Republic	88.7	89.7	80.0	81.0	88.0	88.0	82.0	83.8	83.8	78.1	91.1	ID		
13.	Strigeidae sp., Israel	84.5	84.2	82.7	83.4	82.1	81.7	86.9	85.8	86.5	79.8	80.3	82.8	ID	
14	Strigeidae gen., Canada	85.9	86.3	83.8	84.1	82.8	81.7	85.5	86.2	86.2	80.9	82.1	82.4	91.1	ID

Table 4. Sequence identity matrix (%) among 18S sequences of the various streigid /diplostomid taxa as retrieve from GenBank. (ID – Identical)

	Sequences	1	2	3	4	5	6	7	8	9	10	11	12
1.	*Alaria alata*, Ukraine	ID											
2.	*Bolbophorus confusus*, Iseael	97.3	ID										
3.	*Bolbophorus damnificus*, USA	96.8	97.1	ID									
4.	*Bolbophorus levantinus*, USA	97.7	98.1	98.2	ID								
5.	*Bolbophorus* sp., USA	97.3	99.0	97.0	97.9	ID							
6.	*Diplostomum compactum*, USA	98.1	96.8	96.5	97.2	96.8	ID						
7.	*Diplostomum phoxini*, UK	97.9	97.1	97.3	97.8	97.1	97.9	ID					
8.	*Diplostomum spathaceum indistinctum*, USA	98.2	96.8	96.5	97.2	96.8	99.7	98.0	ID				
9.	*Posthodiplostomum* sp., India	97.6	97.3	97.5	98.0	97.5	97.3	97.5	97.3	ID			
10.	*Posthodiplostomum* sp., Japan	97.7	97.4	97.7	98.2	97.7	97.4	97.5	97.4	**99.7**	ID		
11.	*Posthodiplostomum minimum*, USA	97.5	97.2	97.3	97.8	97.3	97.3	97.3	97.4	99.0	99.2	ID	
12.	Strigeidida sp., USA	97.8	97.3	97.2	97.8	97.4	97.4	97.5	97.6	99.0	99.2	99.3	ID

Table 5. Sequence identity matrix (%) among 28S sequences of the various streigid /diplostomid taxa as retrieve from GenBank. (ID – Identical)

	Sequences	1	2	3	4	5	6	7	8
1.	*Alaria alata*, Ukraine	ID							
2.	*Alaria taxideae*, USA	98.5	ID						
3.	*Diplostomum phoxini*, UK	96.0	96.0	ID					
4.	*Diplostomum* sp., USA	95.7	95.7	98.7	ID				
5.	*Posthodiplostomum* sp., India	93.0	93.2	92.1	91.9	ID			
6.	*Posthodiplostomum* sp., Japan	93.6	94.1	92.7	92.5	**97.5**	ID		
7.	*Apharyngostrigea pipientis*, USA	94.2	94.7	93.5	93.1	92.5	92.5	ID	
8.	*Cardiocephaloides longicollis*, Ukraine	94.7	95.2	94.5	94.0	93.0	93.2	93.0	ID

For mtCO1 region, only the NJ tree could be constructed using the sequence data pertaining to Diplostomidae and Strigeidae taxa, available in the public domain (Table 2, Fig. 3d). The CO1 tree showed that the query sequence claded with *Bolbophorus damnificus* (family Diplostomidae), though with a low bootstrap value. However, at the family level a higher bootstrap value (70 %) with *Neodiplostomum seoulense* was revealed. In all trees *Clinostomum* sp., the outgroup, formed a distinct clade.

ITS2 RNA secondary structures analysis

The secondary structure of the annotated ITS2 sequence having a length of 292 bp was analyzed. It displayed the typical four-helix model: helix I and IV being very short, helix II having three U-U mismatches, and helix III being the longest with two UGGG motifs (Fig. 4a).

The consensus secondary structure constructed using ITS2 sequences of *Posthodiplostomum* sp. under study and the Japan isolate (Fig. 4b) also revealed a typical four-helix

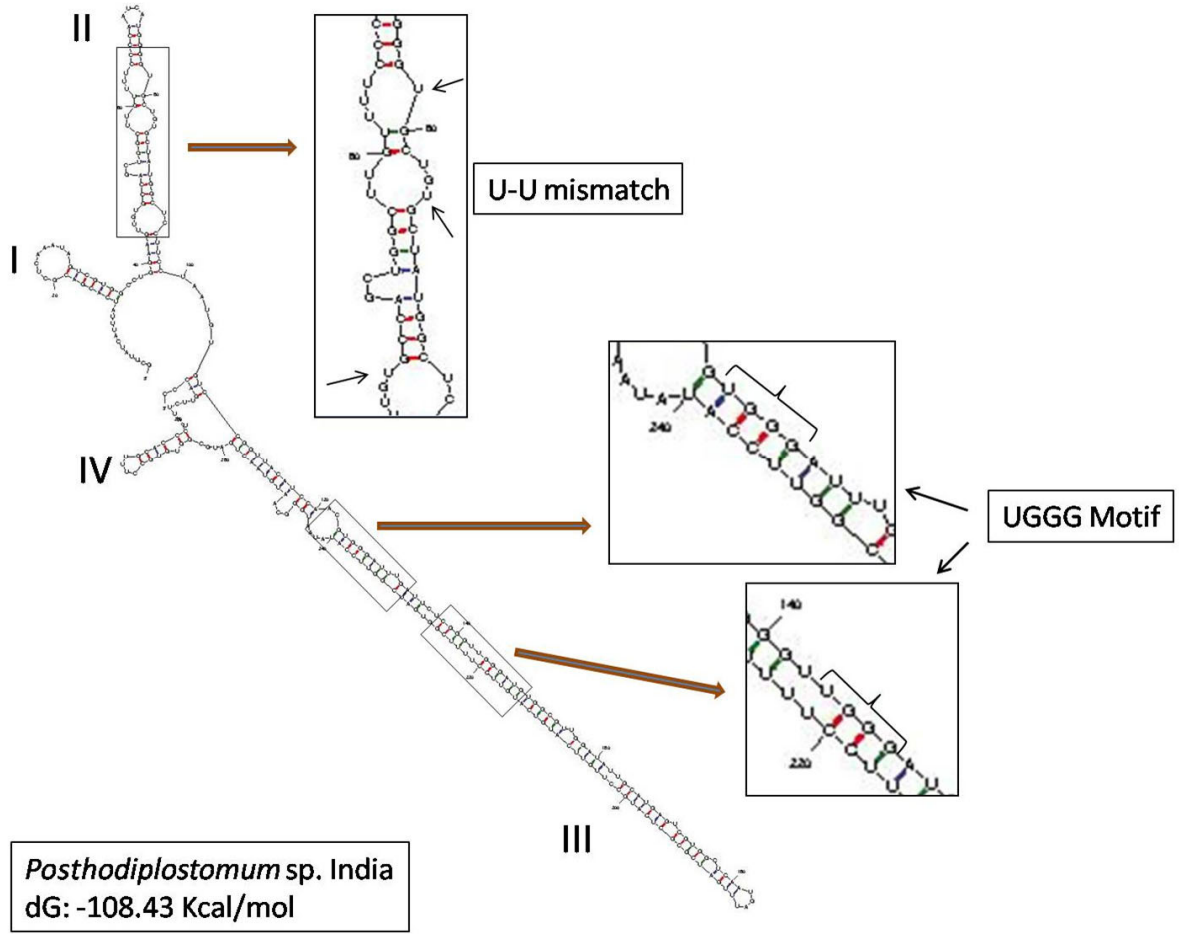

Fig. 4a. Inferred secondary structure of the ITS2 region; the motifs: U-U mismatch in helix II and UGGG in helix IV are shown

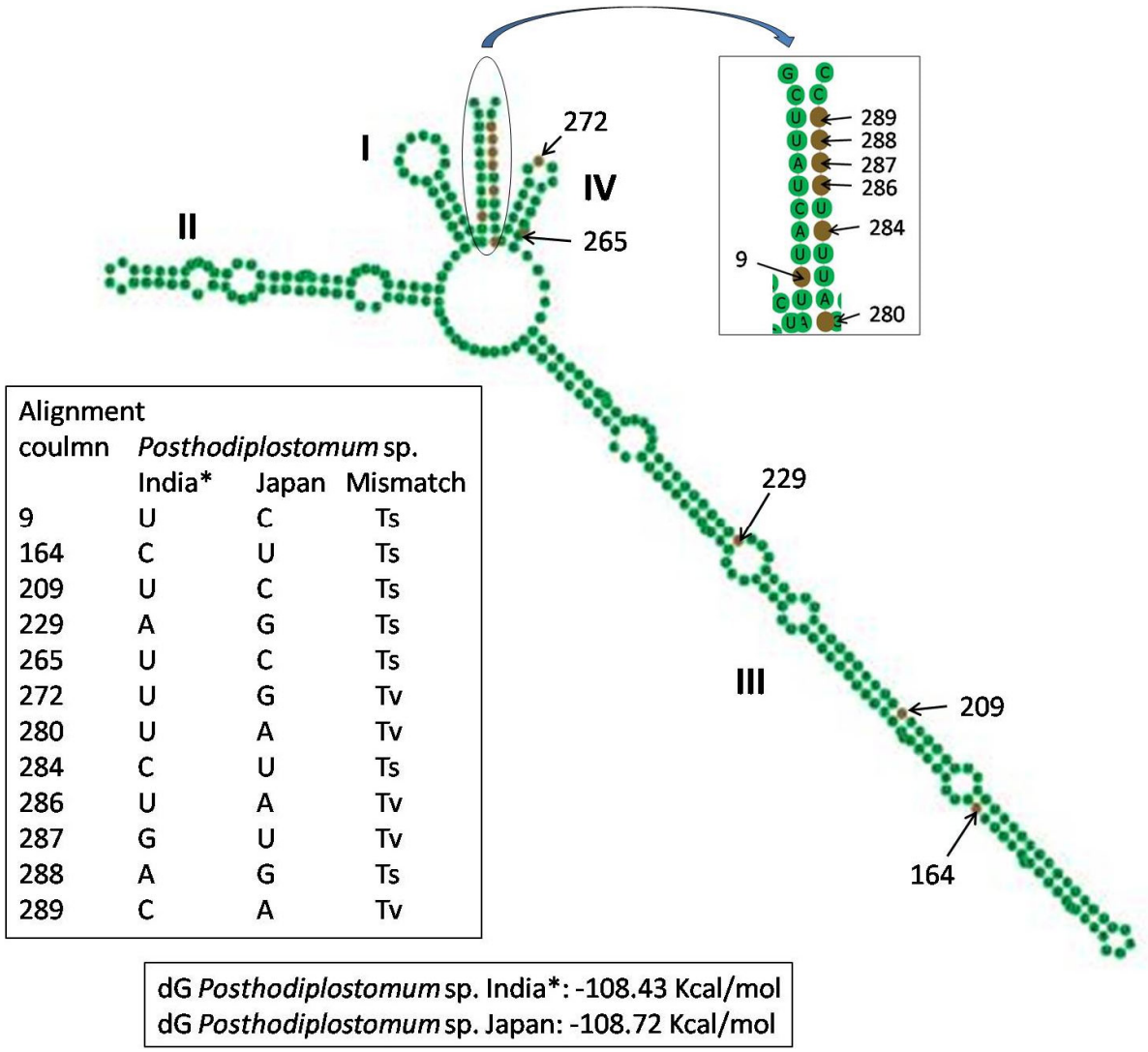

Fig. 4b. Consensus secondary structure of the ITS2 marker in the presently studied metacercaria and *Posthodiplostomum* sp. Japan isolate. U – Uracil, C – Cytosine, G – Guanine, Ts – Transition, Tv - Transversion. ('*' - query sequence)

model, showing twelve mismatches with seven transitions and five transversions at various positions in helix III, helix IV, and 3' and 5' open ends; helix I and II show complete conservedness in their sequences with no mismatches between them.

Discussion

In recent years DNA-based PCR amplification techniques have especially proven useful in quick and accurate identification of morphologically similar but genetically distinct species, and thus helped in resolving taxonomic issues involving various animal taxa including platyhelminth (McManus & Bowles, 1996; Thompson *et al*., 2004; Olson & Tkach, 2005). Nguyen *et al*. (2012) utilized both morphometric and molecular techniques to characterize a diplostomid metacercarial stage found infecting the muscle of *Channa argus*. In the present study, we tried to identify the metacercarial parasite of *C. punctata* by utilizing both morphological and molecular approaches.

The present form was found to belong to the family Diplostomidae Poirier, 1886 based on morphological criteria (Yamaguti, 1971; Niewiadomska, 2001). The present metacercaria possesses all morphological features typical of *Posthodiplostomum* genus (e.g. distinctly bipartite body, trybocytic organ disc-shaped with a median slit) and shows well-developed gonads and anlagen of some genital organs, but no melanin. From India, only five species of *Posthodiplostomum* have been reported till date; these are: *P. cuticola* (v Nordman, 1832) Dubois, 1936; *P. botauri* Vidyarthi, 1938; *P. grayii* (Verma, 1936) Dubois, 1938; *P. mehtai* Gupta and Mishra, 1974 and *P. milvi* Fotedar and Raina, 1965 from the avian (final) hosts (Yamaguti, 1971). The degree of development of the reproductive anlagen may be related to the host age and the time length of infection in the fish host (Graczyk, 1991; Niewiadomska & Szymański, 1991). While metacercariae of *P. podicipitis* and other *Posthodiplostomum* species (occurring in the fish

host *Oryzias latipes*) possess well-developed genital organs (Toyooka & Okada, 1954), those of *Posthodiplostomum* sp. reported from muscle of *Channa argus* in Japan and *P. cuticola* from skin, fins and muscle of *Phoxinus laevis* have primordial form of genital organs (Dönges, 1964; Nguyen *et al*., 2012).

In phylogenetic analysis based on ribosomal or mitochondrial gene markers, our query sequences claded close to *Posthodiplostomum* sp. and members of the family Diplostomidae. Our results thus corroborate the morphology-based classification (Niewiadomska, 2001).

The ITS2 secondary structure is typically a four-helix model, which is common to almost all eukaryotic taxa (Coleman, 2003; Schultz *et al*., 2005) and is true for digenean trematodes as well (Morgan & Blair, 1998). The conserved nature of ITS2 secondary structural core allows utility in inferring phylogenies at higher taxonomic levels, in addition to its role for low-level phylogenetic analyses on the species and genus levels (Schultz *et al*., 2006; Selig *et al*., 2008). The identical nature of the ITS2 secondary structure can be attributed to similarity in rRNA biogenesis among eukaryotes; its folding pattern plays an important role in processing of mature rRNA (Joseph *et al*., 1999) and structures are maintained during evolution through transitions/transversions, although mutations occur frequently (Coleman, 2007; Keller *et al*., 2010). The utility of ITS2 secondary structure for characterizing parasites has also been demonstrated (Shylla *et al*., 2011; Ghatani *et al*., 2012). A high degree of similarity in the ITS2 secondary structures of *Posthodiplostomum* sp. isolates corroborates the results of the primary sequence analysis in the present study.

On the basis of morphological study, supplemented with molecular characterization, the diplostomid metacercarial parasite studied herein is identified as belonging to the genus *Posthodiplostomum* Dubois, 1936.

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