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Original papers

Characterization of *Clinostomum* metacercariae using microscopic and molecular approaches

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ABSTRACT. One of the fundamental aspects in understanding the biology, diversity and epidemiology of a parasite lies in its proper identification. In the present study, morphological and molecular characterization of *Clinostomum* metacercariae recovered from an ornamental fish, *Trichogaster fasciata,* was carried out in order to ascertain its identity. To serve the purpose, scanning electron micrographs and gene sequences for two commonly used molecular markers, i.e., nuclear ribosomal internal transcribed spacer 2 (rDNA-ITS2) and mitochondrial cytochrome c oxidase subunit 1 (mtCO1) were obtained. The sequences were further used for generating similarity index matrix as well as inferring phylogenies. Light and electron microscopic observations on metacercariae of the parasite revealed that it belongs to the genus *Clinostomum*. Identification of the same up to the level of species was made possible through sequence and phylogenetic analyses. The ITS2 sequence analyses of our species (KX758630) showed similarity to unidentified *Clinostomum sp.* reported from Nigeria (KY865625) and China (KP110579), and *C. tilapiae* recorded from South Africa (KX034048) and Nigeria (KY649353). However, the CO1 gene analyses suggested it to be highly identical to *C. philippinense* and the same was also corroborated in the phylogenetic analysis. Thus, morphological and molecular characterization revealed that the recovered metacercariae belong to the species *C. philippinense.* Additionally, a brief description of secondary structures of ITS2 of various species of *Clinostomum* has also been discussed.

Key words: *Clinostomum*, *Trichogaster fasciata,* ITS2, mtCO1, metacercaria, molecular characterization

Introduction

Clinostomum species are known to have a complex life cycle involving gastropods as the first intermediate host, fishes or amphibians as the second while birds harboring the adult stages [1]. The adult parasite resides in the oral cavity and esophagus of fish eating birds; few in reptiles and occasionally in human. However, in Asian countries where eating raw and undercooked fishes is a common practice, occurrence of *Clinostomum* infection in human is recorded to be very frequent. For instance, 19 such cases have been reported only from Japan [2], pharyngitis and lacramalitis due to *Clinostomum* infection has been reported from Thailand and Korea [3,4]. In addition to human, freshwater fishes (intermediate host) incur severe damage following heavy infection with metacercariae of *Clinostomum* species (popularly known as "yellow grub") [5].

Identification of *Clinostomum* species based solely on morphology is liable to suffer from misidentification due to existence of the phenotypic variability within the same species [6–8]. Even among species, lack of reliable morphological characters has led to instability in the taxonomic status of *Clinostomum* species which lead to frequent revision in its taxonomic position [9]. Alternatively, molecular approaches such as DNA based PCR methods have proven to be useful in identification of parasites up to species level as well as differentiation of closely related helminth parasites, including digenetic trematodes [10–12]. Based on the analyses of molecular data from rDNA and mitochondrial gene so far 14 species have been considered to valid within the genus *Clinostomum,* [13]. Additionally, the taxonomic validity of *Clinostomum complanatum* and its differentiation from *C. marginatum* has also been resolved using molecular approaches [14–16].

Table 1. ITS2 sequences of *Clinostomum* species used for sequence analysis and phylogenetic inference

Sl. no.	Species	Accession No.	Locality	Host
1	*Clinostomum* sp.	KX758630*	India	*Trigogaster fasciata*
2	*Clinostomum* sp. morphotype 1	KY865625	Nigeria	*Synodontis batensoda*
3	*C. tilapiae*	KX034048	South Africa	–
4	*C. tilapiae*	KY649353	Nigeria	*Synodontis batensoda*
5	*Clinostomum* sp. 8	KP110579	China	*Ctenopharyngodon idella*
6	*C. phalacrocoracis*	FJ609423	Kenya	*Ardea cinerea*
7	*C. cutaneum*	GQ339114	Kenya	*Ardea cinerea*
8	*C. complanatum*	JF718624	Italy	*Lepomis gibbosus*
9	*C. complanatum*	MF171131	Turkey	*Squalius cephalus*
10	*C. complanatum*	KF811010	India	*Heteropneustes fossilis*
11	*C. marginatum*	KU708007	USA	*Ardea alba*
12	*C. tataxumui*	KU156742	Middle America	*Tigrisoma mexicanum*
13	*Euclinostomum heterostomum*	KP721439	Isreal	Cichlids

*Sequence generated for the study

In this context, the nuclear ribosomal internal transcribe spacer 2 (rDNA-ITS2) and mitochondrial gene cytochrome c oxidase subunit 1 (mtCO1) have been extensively used to resolve taxonomic issues and to differentiate closely related parasitic species [16,17]. Because these genes display rapid rate of evolution, they have emerged as the locus of choice in answering questions related to taxonomy, population genetics, species identification and phylogenetic relationships of various helminth parasite species, including trematode, nematode and cestode [18–22]. An additional advantage of using ITS2 is the possibility of predicting its secondary structure from the primary sequence data and is known to provide further information that can be useful in delineating closely related species [23,24]. This approach has been successfully used in discriminating closely related species among plants, fungi and parasitic groups, including cestodes and trematodes [20,25–27].

In the present study, *Clinostomum* metacercariae, recovered from the fish *Trichogaster fasciata* (an ornamental and edible fish in Manipur, India) was characterized in order to ascertain its specific identity using morphological as well as molecular approaches. Additionally, secondary structure of the ITS2 sequence of various species of *Clinostomum* is also briefly discussed.

Materials and Methods

The metacercariae (n=19) were recovered from the body cavity of the fish, *Trichogaster fasciata* that were brought alive from different local fish markets of Manipur, India. They were thoroughly washed in phosphate buffered saline and further processed for morphological and molecular studies.

For morphological studies, light and scanning electron microscopic (SEM) observations were carried out. For light microscopic studies, whole mount preparation was done for which the samples were flattened, stained in borax carmine, dehydrated in a graded series of alcohol from 30% to 100% for 10 minutes each; cleared in methyl benzoate and mounted in canada balsam. The prepared slides were viewed under Leica Microscope (DM1000). For SEM, the recovered specimens were initially fixed in 10% neutral buffered formalin followed by dehydration and drying following standard procedure [28] and viewed under JSM35CF (Jeol) operated at 20kv.

DNA isolation, amplification and sequencing. The whole worm was used for genomic DNA extraction using phenol chloroform-isoamyl method following standard protocol [29]. Complete rDNA-ITS2 region and partial CO1 were amplified by PCR using primers set 3S/A28 and Dice 1F/Dice 14R respectively [30,31]. The PCR product was purified using Genei Quick PCR purification kit

Table 2. mtCO1 sequences of *Clinostomum* species used for sequence analysis and phylogenetic inference

Sl. no.	Species	Accession No.	Locality	Host
1	*C. phillipinense*	MF947448*	India	*Trigogaster fasciata*
2	*C. philippinense*	KP110523	Thailand	*Trichogaster microlepis*
3	*C. tilapiae*	KY649364	Nigeria	*Synodontis batensoda*
4	*C. phalacrocoracis*	KY906238	South Africa	*Clarias gariepinus*
5	*C. attenuatum*	KP150306	USA	*Lithobates pipiens*
6	*C. detruncatum*	KP110519	Brazil	*Synbranchus marmoratus*
7	*C. marginatum*	JX630997	Mexico	*Ardea alba*
8	*C. complanatum*	KM923964	China	*Carassius auratus*
9	*C. tataxumui*	KJ504211	Middle America	*Tigrisom amexicanum*
10	*Euclinostomum heterostomum*	KP721421	Isreal	Cichlids

*Sequence generated for the study

followed by sequencing in both directions on an automated sequencer (Macrogen sequencing services, South Korea). The generated sequence was submitted to NCBI-GenBank and the accession number acquired.

Sequence and phylogenetic analysis. The generated sequences along with sequences of the other *Clinostomum* species retrieved from GenBank were used for analyses (Tables 1 and 2). They were firstly aligned using the ClustalW program in MEGA6 [32]. The aligned sections were then imported to BioEdit [33] for generation of sequence identity matrix. Phylogeny was inferred using Bayesian Inference (BI) in MrBayes [34] taking *Euclinostomum heterostomum* as an outgroup species. Branch support for MrBayes was given using Bayesian posterior probabilities (Bpp) that was computed using the Metropolis-Coupled Markov Chain (MCMC) method. The analysis was run for 500000 generations and sampled every 1000 generations, with the first 25% of the trees being discarded as the 'burn-in' phase. For prediction of secondary structure, ITS2 sequence was initially annotated using online tool ITS2 Database [35] and then folded using the minimum folding energy module in RNAfold [36].

Results

Light microscopic and scanning electron microscopic studies

Body medium, linguiform, dorsally convex, oral

Table 3. ITS2 sequence similarity index matrix for the various species of *Clinostomum*

	1	2	3	4	5	6	7	8	9	10	11	12
1. *Clinostomum* sp. India*	**ID**											
2. *Clinostomum* sp. Nigeria	95.9	**ID**										
3. *C. tilapiae* Nigeria	95.9	98.2	**ID**									
4. *C. tilapiae* South Africa	95.9	98.2	100.0	**ID**								
5. *Clinostomum* sp. China	95.9	98.2	98.5	98.5	**ID**							
6. *C. phalacrocoracis* Kenya	95.3	97.6	98.8	98.8	97.6	**ID**						
7. *C. cutaneum* Kenya	95.0	97.3	98.5	98.5	97.3	98.5	**ID**					
8. *C. complanatum* Italy	94.2	96.5	96.8	96.8	98.2	95.9	95.6	**ID**				
9. *C. complanatum* Turkey	94.2	96.5	96.8	96.8	98.2	95.9	95.6	100.0	**ID**			
10. *C. complanatum* India	85.8	88.1	88.4	88.4	98.2	87.5	87.2	87.2	87.2	**ID**		
11. *C. marginatum* USA	91.6	93.6	94.2	94.2	98.2	93.6	93.3	93.0	93.0	86.4	**ID**	
12. *C. tataxumui* Mexico	92.4	94.2	94.7	94.7	98.2	93.6	93.3	93.3	93.3	87.2	94.7	**ID**

Table 4. CO1 sequence similarity index matrix for the various species of *Clinostomum*

	1	2	3	4	5	6	7	8	9
1. *C. philippinense* India*	**ID**								
2. *C. philippinense* Thailand	99.5	**ID**							
3. *C. tilapiae* Nigeria	87.6	88.1	**ID**						
4. *C. phalacrocoracis* S. Africa	86.2	86.6	88.6	**ID**					
5. *C. attenuatum* USA	85.2	84.7	82.7	82.7	**ID**				
6. *C. detruncatum* Brazil	83.7	84.2	90.6	85.7	86.2	**ID**			
7. *C. marginatum* Mexico	84.7	85.2	83.2	80.7	90.1	86.2	**ID**		
8. *C. complanatum* China	85.2	85.7	87.1	89.1	83.7	85.7	85.7	**ID**	
9. *C. tataxumui* Middle America	85.2	84.7	82.2	79.8	84.7	85.7	83.7	80.7	**ID**

Fig. 1. Light microscopic image of *Clinostomum philippinense* metacercaria

sucker is smaller than the ventral sucker and surrounded by a collar like structure (Figs. 1 and 2a). It measures 2.35–5.54 mm long and 1.60–2.19 mm wide; oral suckers measures 0.25–0.35 mm in length and 0.37–0.40 in width, ventral sucker measures 0.83–1.15 mm long and 0.81–1.14 mm wide. Anterior and posterior end rounded, pharynx bifurcates posterior to oral sucker into two intestinal caeca and continues till the terminal end of the body. Ovary is intertesticular, submedian and measures 0.091–0.20 mm in length and 0.091–0.19 mm in width. The anterior testis range from 0.30–0.54 mm in length and 0.32–0.51 mm in width. The posterior testis range from 0.19–0.49 mm in length and 0.31–0.70 mm in width.

Scanning electron micrographs showed the collar-like rim of the oral sucker is thick with barely perceptible protrusions; without any papillae and the internal surface of the oral sucker has an uneven appearance (Fig. 2b). The ventral sucker shows the presence of thin thread like structure internally (Fig. 2c). Antero-lateral tegument is spinated while postero-lateral tegument has a cobblestone like structure (Figs. 2d,e). The ventral part of the body has a papillary tegument and genital pore is also present (Fig. 2f). The posterior part of the fluke ends in excretory pore surrounded by spines (Figs. 2g,h).

Molecular characterization

The complete fragment of ITS2 and partial CO1 region were successfully amplified using the aforementioned primer sets. The sequences were submitted to GenBank under the accession numbers KX758630 and MF947448 for ITS2 and CO1, respectively. Further analyses (Similarity index matrix) of the sequences supported supported the light and scanning electron microscopic finding and the recovered metacercaria showed maximum sequence identity with *Clinostomum* sp. for ITS2. The sequence similarity index matrix generated for the ITS2 gene revealed maximum similarity (95.9%) of our species with unidentified Nigerian (KY865625) and Chinese (KP110579) (Table 3) isolate of *Clinostomum* sp. and also with *C. tilapiae* of Nigeria (KY649353) and South Africa (KX034048) (Table 3). The CO1 sequence similarity analysis, on the other hand, showed that the sequence of our specimen is highly similar (99.5%) with *C. philippinense* (Table 4).

In order to corroborate the sequence analysis results, phylogenetic trees were constructed for the various species of *Clinostomum* for both the genes. Both the phylogenetic trees were well resolved and

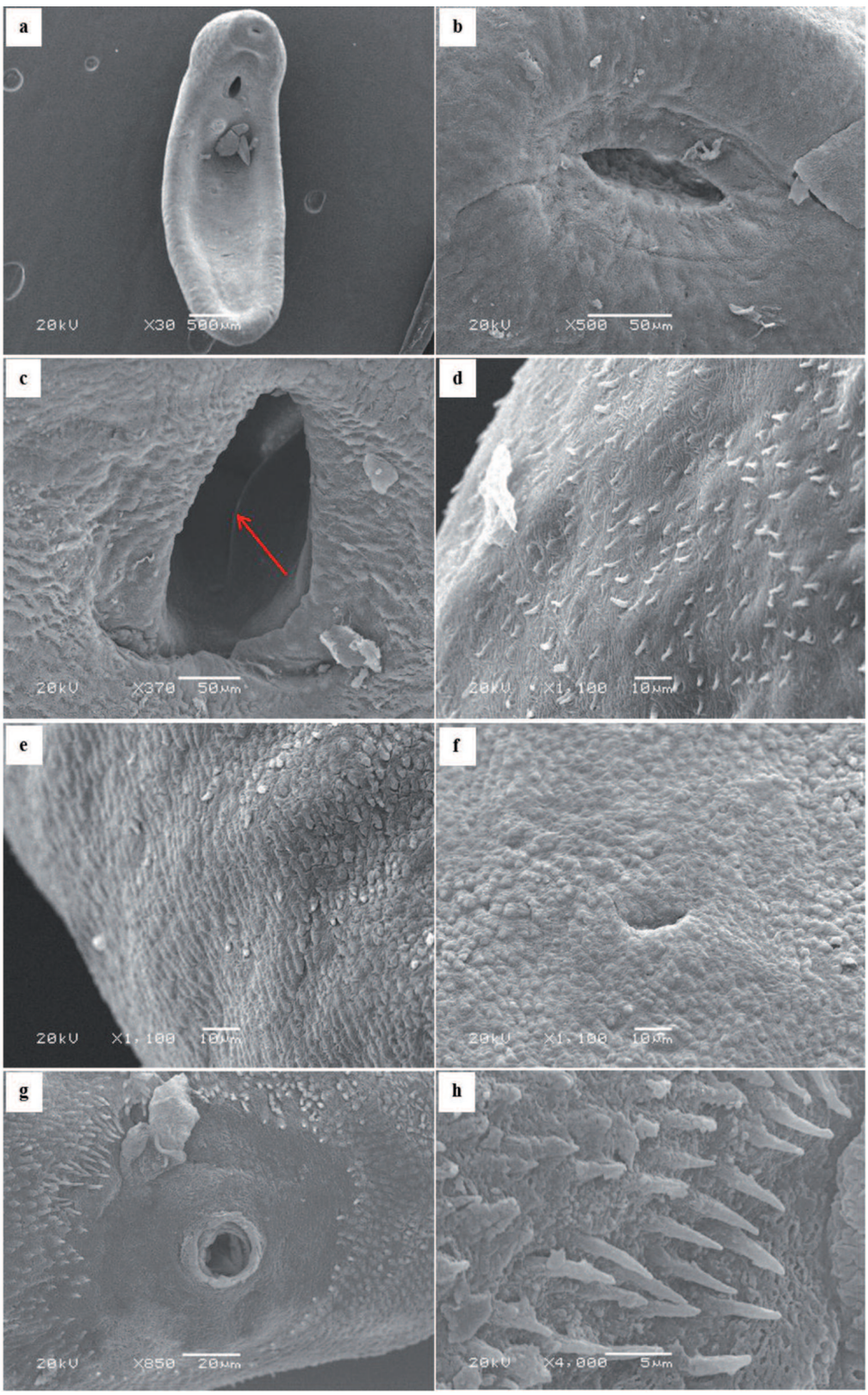


Fig. 2. (a-h). Scanning electron micrographs of *Clinostomum philippinense*. a. General view of the whole fluke, ventral view; b. Magnified view of oral sucker; c. Close–up view of ventral sucker showing presence of thin thread like structure internally; d. Spinated antero-lateral tegument; e. Postero-lateral tegument showing cobblestone like structure; f. Ventral median part of body showing papillary surface topography and presence of genital pore; g. Excretory pore in posterior end of the body; h. Enlarged view of spines surrounding the excretory pore.

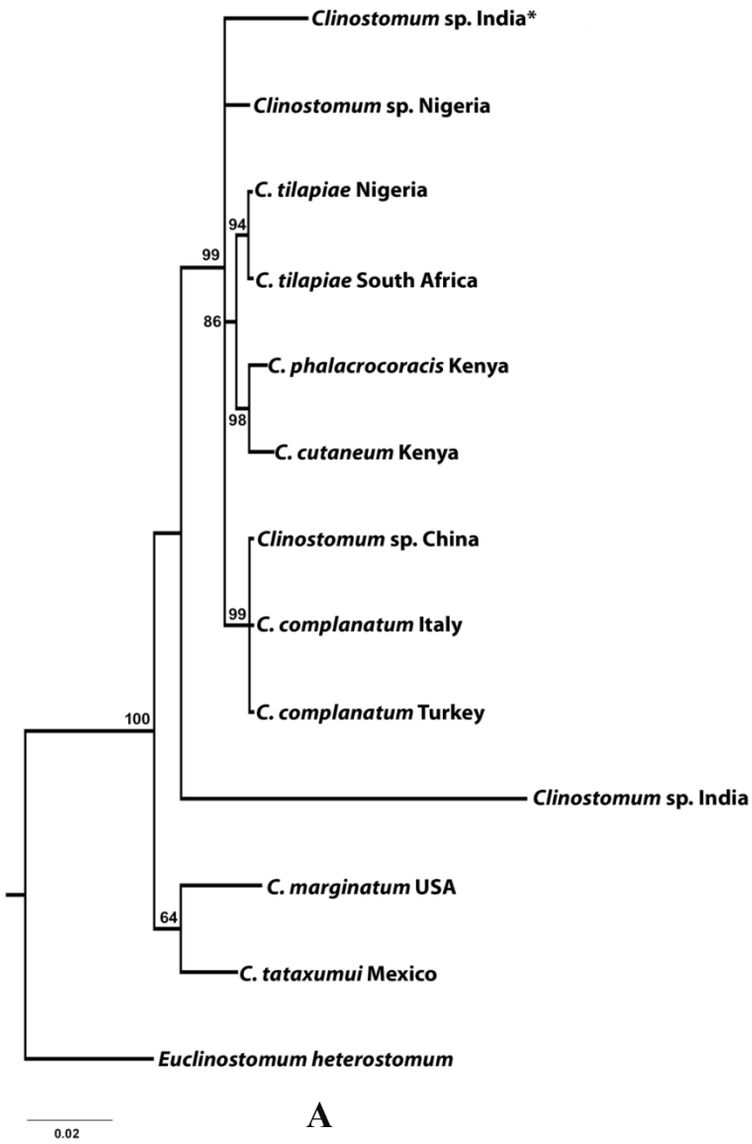


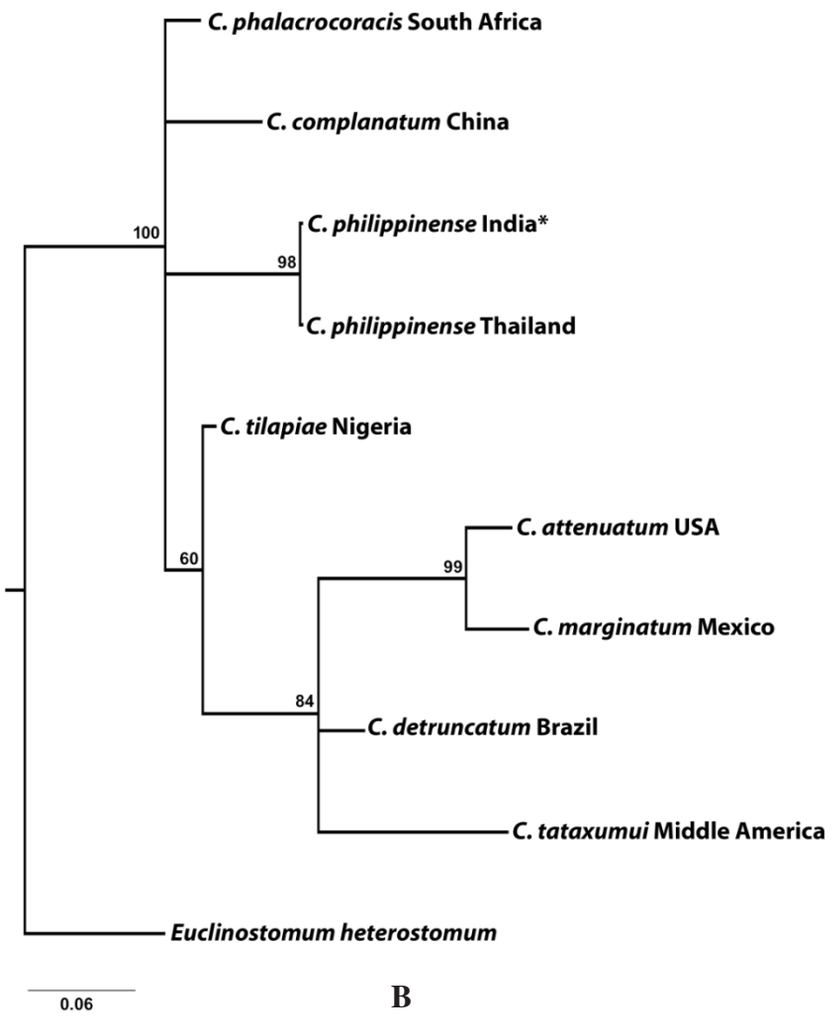
Fig. 3. Phylogenetic tree of *Clinostomum* species inferred via Bayesian Inference in MrBayes using, (A) rDNA-ITS2, (B) mtCO1 gene regions. Numbers against the nodes indicate Bayesian posterior probability values.

the nodes were strongly supported by high Bayesian posterior probabilistic (Bpp) values. In the ITS2 inferred phylogeny our species did not show any sort of association with any other species of *Clinostomum* erected separately (Fig. 3A). The CO1 inferred phylogeny, however, depicted our species to be closely related to *C. phillipinense* (Thailand) which is supported by strong nodal Bpp value of 98% (Fig. 3B).

Annotation of the ITS2 sequence of the species in the present study revealed the precise length of the ITS2 region in different species of *Clinostomum* which ranged from 278-284bp (Table 5). Folding of the primary transcripts of ITS2 revealed the hallmark four helix model of ITS2 secondary structure with the longest arm being represented by IIIrd helix (Fig. 4).

Discussion

Inadequate reliable morphological characters among digenetic metacercariae recovered from fishes often makes identification difficult and tedious, resulting in misidentification of the species which causes problems for taxonomists to explain their complex life cycles [37]. In the present study, the *Clinostomum* metacercariae obtained from *T. fasciata* was characterized and identified using light



B

and scanning electron microscope and, molecular tools. The light and scanning electron micrographs revealed metacercariae as a member of the genus *Clinostomum,* however, the species identity of the same could not be revealed. The metacercariae of *Clinostomum* species are known to have similar morphology even though if there are some distinct features, it is not enough to discriminate up to the species level [38]. Scanning electron microscopic images in the present study showed considerable differences in morphological features from other species of *Clinostomum* where SEM images have already been published [9,39,40]. In India, the *Clinostomum* species of common occurrence is represented by *C. complanatum* [5,39,41] which have distinctly different surface topographical features from the one we studied. The other species commonly found in the Asian countries is *C. philippinense,* which also lacked the SEM studies [42]. Thus, we compared the SEM micrographs of our species with other described species of *Clinostomum* and found a significant difference in its morphology and surface microtopography. For example, SEM images showed the presence of an excretory pore surrounded by well-developed spines, thin thread like structure bisecting the ventral sucker internally, antero-lateral spination of the tegument and presence of genital pore in the ventral surface of the body which were found to be

Table 5. Length of ITS2 region in different species of *Clinostomum*

Species	Length of annotated ITS (bp)
*C. philippinense**	**284**
C. tilapiae	283
C. phalacrocoracis	283
C. marginatum	284
C. complanatum	278
C. cutaneum	283

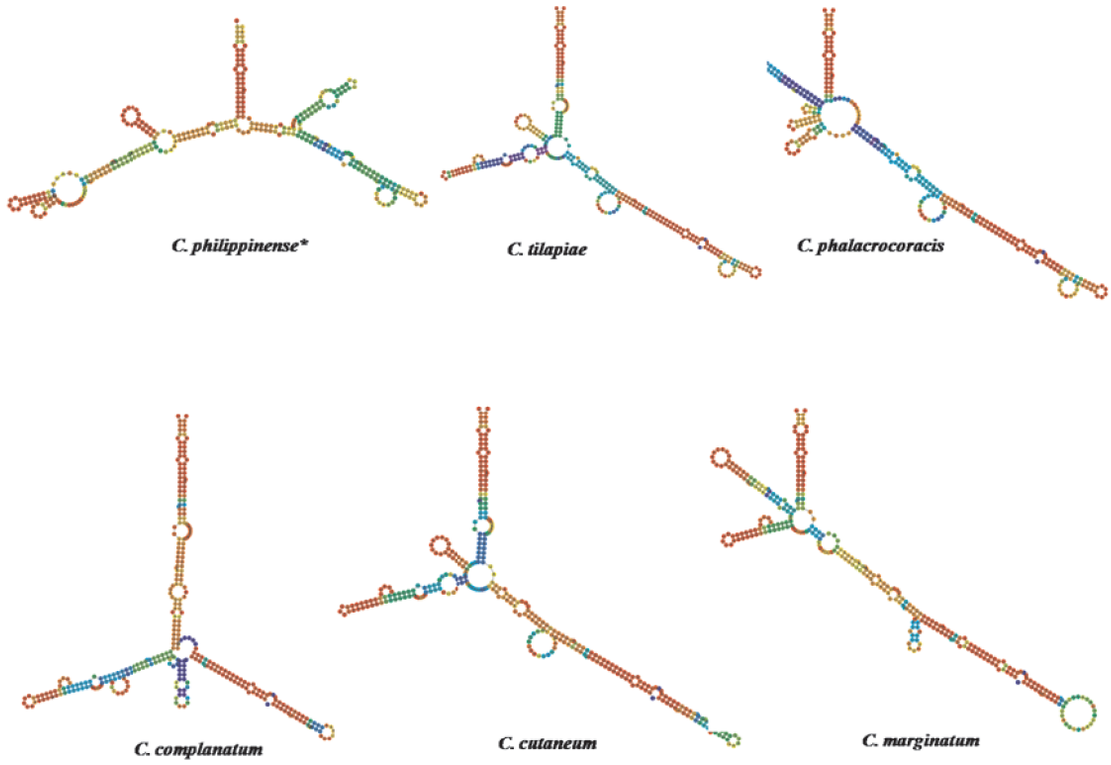


Fig. 4. Predicted secondary structure of the annotated ITS2 region of *Clinostomum* sp. generated via RNA fold

absent and/or different in other species such as *C. cutaneum* and *C. complanatum* [9,39,40]. In spite of having differences in size, shape and tegumental structures it was not possible to ascertain the identity of the *Clinostomum* metacercaria up to the species level.

Alternatively, addition of molecular tools to morphological studies has proven to be of immense value in identification and discrimination of *Clinostomum* species complex [9,14,43]. Though, the ITS2 gene in this genus was not able to produce additional information helpful in the species identity of the recovered metacercaria, the mtCO1 gene provided reliable outcomes that revealed the close relatedness of our species to *C. philippinense*, both in sequence as well as phylogenetic analysis. Effectiveness of mtCO1 gene in discriminating species of *Clinostomum* also has been shown in earlier investigations made by various authors [15,16]. Though, in our investigation the ITS2 primary sequence did not prove to be of much use in species level identification, the prediction of secondary structure generated from the primary sequence data of ITS2 can be advantageous in species delimitation and identification [24,26]. Secondary structures are conserved and offers additional information for species identification prediction [44]. The utility of secondary structures has been extended towards evolutionary studies of nematodes and trematodes [25,45,46]. Therefore, we generated the secondary structure for all the species of *Clinostomum* where ITS2 sequence could be successfully annotated.

Through light microscopic, scanning electron microscopic studies and molecular tools, the metacercariae collected from freshwater fish *T. fasciata* is identified as the larval stage of *C. philippinense*. A novel description of ITS2 secondary structure information which otherwise is lacking in this group of digenetic trematode has also been added which in future can be valuable information for comparative studies. Finally, knowledge of the parasitic infection in fishes such as *T. fasciata* which is an ornamental and fish of food value can help us to control the spread of infection.

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