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Morphological and molecular characterization of six Indian *Tetracotyle* type metacercariae (Digenea: Strigeidae Railliet, 1919), using ribosomal and mitochondrial DNA

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ABSTRACT

Strigeids have a cup-shaped fore body, containing a holdfast organ with two lobes and hind body. The species diversity of strigeids remains incomplete, especially in the Indian sub-continent. Here, we described six *Tetracotyle* type metacercariae (*T. muscularis*, *T. fausti*, *T. lucknowensis, T. xenentodoni*, *T. mathuraensis* and *T. glossogobii*) from five fresh water fish, collected at Lucknow (India). Next, we characterized these metacercariae using ribosomal (18S, 28S, ITS2) and mitochondrial DNA (COI) to determine their systematic and phylogenetic position. Molecular identification using inter-specific variation range for all four molecular markers revealed 1.9–4.9% (18S); 3.3–8.8% (28S); 6.8–12.9% (ITS2), and 3.5–9.4% (COI) among six *Tetracotyle* type metacercariae. In phylogenetic analysis, constructed by neighbour-joining (NJ) and maximum likelihood (ML) methods, *T. fausti, T. glossogobii*, *T. xenentodoni*, *T. lucknowensis* and *T. mathuraenis* nested as sister groups with the member of strigeids for all four markers used; *T. muscularis*, however, formed a basal clade. Furthermore, phylogenetic placement states the monophyly of the *Tetracotyle* type of metacercariae in all the markers (18S, 28S, COI), except ITS2.

1. Introduction

The strigeids are chiefly characterized by bipartite body consisting of a cup-shaped fore body, containing a holdfast organ with two lobes (ventral and dorsal) and hind body [29]. Dubois [14] distinguished the diplostomoids by their host groups (birds and mammals), of which strigeids are mainly restricted to birds. The Family Strigeidae Railliet, 1919 is comprised of twelve genera, having approximately 110 nominal species viz, *Parastrigea* Szidal, 1928; *Apharyngostrigea* Ciurea,1927; *Strigea* Abildgaard,1790; *Nematostrigea* Sandground, 1934; *Ophiosoma* Szidat, 1928; *Cardiocephaloides* Szidat, 1959; *Schwartzitrema* Pérez Vigueras, 1941; *Pseudapatemon* Dubois,1936; *Cotylurus* Szidat, 1928; *Ichthyocotylurus* Odening, 1969; *Apatemon* Szidat, 1928; *Australapatemon* Sudarikov, 1959 [29]. The generic level identification of metacercariae of Diplostomoids is quite difficult due to intra-specific plasticity and inter-specific or inter-generic homogeneity in morphological features. Therefore, little is known about the true diversity of Diplostomoids. The distinction among larval *Tetracotyle* type species or other strigeids may be questionable, due to their phenotypic resemblance, a limited number of morphological characters and relatively small size. Although, Niewiadomska [28] enlisted the morphological characters to distinguish the *Tetracotyle* type metacercariae, often used in practice. The larvae of '*Tetracotyle*' type involve gastropod first intermediate hosts and fish-eating bird as final hosts [29]. However, the generic placement of thirty-five species of *Tetracotyle* type metacercariae described form Indian freshwater fish remains unknown [33].

As an alternative to classical morphological approaches, the application of PCR and sequencing-based molecular techniques has been efficiently used in the identification and discrimination at all stages during the life cycle of trematode in the diverse hosts [12,13,17]. The ribosomal DNA is chiefly helpful in resolving the phylogeny because it contains regions with varying rates of evolution, from highly conserved (18S and 28S) as well as (ITS1 & ITS 2) variable domains [23,27,41]. The internal transcribed spacer regions (ITS1 and ITS2) verified as promising genetic marker having a broad range of application for phylogenetic analyses at generic and species level [11,19,30]. The mt DNA (COI) has also been used for recognition of host-parasite interaction, cryptic diversity, evaluation of genetic variation and inferring phylogenies [10,25,37,42,43].

Here we present data from ongoing survey work using first molecular data to distinguish and identify species diversity of *Tetracotyle* type of metacercariae from India. During the winter (September to

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December) of 2015, we collected six *Tetracotyle* type metacercariae (*T. muscularis* Chakrabarti, 1970a; *T. fausti* Rai and Pande, 1969; *T. lucknowensis* Pandey, 1971; T. *xenentodoni* Chakrabarti, 1970b; *T. mathuraensis* Rai and Pande, 1969 and *T. glossogobii* Chakrabarti, 1970c) from five fresh water fish. Here we aimed to [29] reassessment of morphological features of nominal species of *Tetracotyle* and to validate them by using both ribosomal (18S, 28S, and ITS2) and mitochondrial COI gene sequences; and [14] to determine the systematic position of six *Tetracotyle* type metacercariae within Strigeidae.

2. Materials and methods

2.1. Collection and examination of fish

During the winter (September to December) of 2015, 300 live specimens of five freshwater fish, namely *Heteropneustes fossilis* Bloch, 1794, *Mastacembelus armatus* Lacepède, 1800, *Xenentodon cancila* Hamilton, 1822, *Channa punctatus* Bloch, 1793 and *Glossogobius giuris* Ham. 1822 were collected from the river Gomti at Lucknow, the state of Uttar Pradesh, India (26˚.50′ N, 80˚.50′ E). Fish were sacrificed by spinal severance and examined afresh under a stereomicroscope to detect infection with strigeids. Worms recovered from the gills, body muscles, cranial cavity, and visceral organ, liver, were observed in the light microscope (Olympus BX-51), and identified according to Pandey and Agrawal [33]. After identification, they were fixed in 70% ethanol for whole mount preparation and absolute ethanol for DNA isolation. The prevalence and intensity of infestation were recorded (Table 1).

2.2. Morphological study

Permanent mounts were prepared by staining the worms fixed for the whole mount in aceto-alum carmine, dehydrated in graded alcohol series (Successively in 50%, 70%, 90% and 100%), cleared in Xylol and mounted on glass slides using DPX. Figures of mounted worms were drawn by means of drawing tube, attached to a phase–contrast light microscope (Olympus BX-51, Tokyo, Japan). All morphometric measurements (in mm) were taken with the aid of ocular micrometer. Voucher specimens were deposited as permanent mounts at the Helminthological collection of the Zoological Survey of India, Kolkata as indicated in the respective parasitic records.

2.3. DNA isolation, amplification

Genomic DNA for molecular analysis was extracted from single ethanol-fixed specimens of each *Tetracotyle* type metacercariae (*T. muscularis*; *T. fausti*; *T. lucknowensis*; *T. xenentodoni*; *T. mathuraensis* and *T. glossogobii*) by using Qiagen's DNeasy Tissue Kit (Qiagen Hilden, Germany), according to manufacturer's instruction. DNA samples were stored at -20˚C for further use. For PCR- amplification, universal primers of different rDNA marker gene regions: 18S, 28S, internal transcribed spacer 2 (ITS2) and the mitochondrial cytochrome oxidase subunit I (COI) were employed. Description of ribosomal and mitochondrial gene primers used in this study:

18S Euk A (Forward): 5'AACCTGGTTGATCCTGCCAGT-3' and Euk B (Reverse): 5'TGATCCTTCTGCAGGTTCACCTA-3' [18]

28S (Forward): 5'ACCCGCTGAATTTAAGCAT-3' and (Reverse): 5' CTCTTCAGAGTACTTTTCAA-3' [26]

ITS2 3S (Forward): 5'GGTACCGTGGATCACTCGGCTCGTG-3' and A28 (Reverse): 5'GGGATCCTGGTTAGTTTCTTTTCCTCCGC-3' [6]

COI JB3 (Forward): 5'TTTTTTGGGCATCCTGAGGTTTAT-3' and JB4.5 (Reverse): 5'TAAAGAACATAATGAAAATG-3' [5]

Each PCR amplification reaction was performed in a total volume of 12.5 µl, containing 10X buffer (100 mM Tris, pH 9.0), 50 mM KCl and 15 mM $MgCl_2$, 2.5 U Taq Polymerase, 10 mM of each deoxynucleotide triphosphates (dNTP's) and 3µl DNA. The thermocycling program consisted: initial denaturation at 94 ˚C for 5 min, annealing for 18S at 58˚C (1.10 min), 28S at 54 ˚C (1min), ITS-2 at 57 ˚C (1.10 min) and COI at 56 ˚C (1.10 min), final extension at 72 ˚C for 10 min. PCR products were checked on 2% agarose gel in TAE buffer, stained with Ethidium Bromide (EtBr) and visualized under UV light. The purified PCR product was subjected to the forward direction, using an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA).

2.4. Sequence analysis

Nucleotide sequence similarity search analysis was performed by Basic Local Alignment search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast). The sequences of each gene (18S, 28S, ITS2 and COI) were aligned using (http://www.ebi.ac.uk/clustalw/) Clustal W [40] under following parameters: gap opening 10.00 and gap extension 0.50 and corrected manually with BioEdit software version 7.0.9.0 [21]. GC percentage was calculated by using GC calculator (http:www.genomicsplace.com/gc_calc.html) to count the bases and determines the% G~C content.

2.5. Phylogenetic analysis

Alignments were trimmed to the shortest available sequence prior to phylogenetic analyses (18S: 596 nt; 28S: 383 nt; ITS2: 532 nt; COI: 555 nt). Phylogenetic trees were generated using MEGA 6 [39], and analysed using the Neighbour-joining (NJ) and Maximum likelihood (ML) method for each data set (18S, 28S, ITS2, and COI). Potential species were distinguished by clustering in Neighbour-joining (NJ) method of best nucleotide substitution model estimated by "Kimura 2 parameter model". More complicated models may sometimes yield inconsistent results when large numbers of sequences were compared. The best model (General time reversible model) for ML analysis was selected with a gamma distribution of rates and proportion of invariant sites (GTR + G + I), which provide the best fit to the data. All positions containing gaps and missing data were eliminated. The reliability of internal branches in all the trees was estimated by using the bootstrap method, 1000 replicates.

3. Results

3.1. Morphology

Based on their morphological features, the strigeid metacercariae recovered were identified as six *Tetracotyle* type metacercariae: *T. muscularis*, *T. fausti*, *T. lucknowensis*, *T. xenentodoni*, *T. mathuraensis* and

Table 1
Prevalence, mean intensity and abundance of *Tetracotyle* type metacercariae

Name of metacercariae	No. of examined fish	No. of infested	fish Prevalence	No. of metacercariae	Mean intensity	Abundance
T. muscularis	50	23	46%	118	5.2	2.36
T. fausti	35	16	45%	97	6.06	2.77
T. xenentodoni	50	40	80%	176	4.4	3.52
T.lucknowensis	50	28	56%	166	4.2	3.32
T.mathraensis	50	27	54%	85	3.22	1.70
T. glossogobii	65	48	73%	342	7.1	5.26

Table 2
A comparison of measurements based on 10 specimens (presented in mm) of *T.muscularis*, *T. fausti*, *T.lucknowensis* with their original description. (Abbreviations used in the given table, L = Length, W = Width).

Metacerc aria species		*T.muscularis*	*T.muscularis*	*T.fausti*	*T.fausti*	*T.lucknowensis*	*T.lucknowensis*
Host		Present Study	Chakrabarti,1970	Present Study	Rai & Pande,1969	Present Study	Pandey, 1971
Source		*H.fossilis*	*H.fossilis*	*M.armatus*	*M.armatus*	*X.cancila*	*X.cancila*
Characters		Liver	Liver	Liver	Liver	Visceral organ	Visceral organ
Cyst	L	0.94–1.02	0.80–0.92	0.81–0.84	–	0.70–0.71	0.60–0.67
	W	0.51–0.57	0.42–0.48	0.50–0.54	–	0.59–0.55	0.37–0.45
Fore- body	L	0.80–0.82	0.62–0.85	0.78–0.80	0.59–0.66	0.54–0.57	0.48–0.67
	W	0.61–0.63	0.58–0.69	0.53–0.57	0.34–0.50	0.62–0.64	0.36–0.48
Hind- Body	L	0.21–0.22	0.35–0.44	0.19–0.22	0.17–0.27	0.16–0.19	–
	W	0.26–0.28	0.31–0.40	0.22–0.25	0.13–0.19	0.24–0.26	–
Oral -Sucker	L	0.08–0.10	0.07–0.10	0.11–0.12	0.05–0.06	0.05–0.06	0.07–0.09
	W	0.09–0.10	0.07–0.10	0.10–0.12	0.06–0.08	0.13–0.14	0.07–0.09
Ventral Sucker	L	0.08–0.09	0.06–0.09	0.11–0.11	0.09–0.10	0.08–0.10	0.09–0.12
	W	0.07–0.09	0.06–0.09	0.10–0.12	0.11–0.14	0.08–0.10	0.08–0.09
Pseudo Suckers	L	0.08–0.10	–	0.07–0.08	0.16–0.19	0.20–0.22	–
	W	0.08–0.10	–	0.06–0.08	0.06–0.09	0.14–0.16	–
Pharynx	L	0.03–0.04	0.03–0.05	0.02–0.03	0.030–0.035	–	–
	W	0.03–0.04	0.03–0.04	0.02–0.03	0.028–0.040	–	
Hold fast	L	0.40–0.42	0.16–0.22	0.20–0.22	0.14–0.27	0.26–0.28	–
	W	0.22–0.25	0.15–0.20	0.23–0.25	0.19–0.31	0.42–0.45	
Anterior testis	L	0.04–0.06	0.06–0.08	–	–	–	
	W	0.04–0.05	0.07–0.09	–	–	–	
Posterior	L	0.05–0.06	0.02–0.04	–	–	–	
	W	0.06–0.08	0.02–0.04	–	–	–	

Table 3
A comparison of measurements based on 10 specimens (presented in mm) of *T. xenentodoni, T.mathuraensis, T.glossogobii* with their original description (Abbreviations used in the given table, L = Length, W = Width).

Metacercaria species		*T.xenentodoni*	*T.xenentodoni*	*T.mathuraensis*	*T.mathuraensis*	*T.glossogobii*	*T.glossogobii*
		Present Study	Chakrabarti,1970	Present Study	Rai & Pande,1969	Present Study	Chakrabarti,1970
Host		*X. cancila*	*X.cancila*	*C.punctata*	*C.punctata*	*G.giuris*	*G.giuris*
Source		Visceral organ	Visceral organ	Gills	Gills	Cranial cavity& Nervous tissue	Cranial cavity &Nervous tissue
Characters							
Cyst	L	1.25–1.28	1.14–1.64	1.21–1.32	1.70–2.20	0.42–0.44	0.60–0.81
	W	0.61–0.63	0.54–0.78	0.81–0.83	0.71–0.85	0.30–0.32	0.37–0.40
Fore- body	L	1.10–1.15	1.58–1.92	1.10–1.13	0.58–1.06	0.26–0.28	0.45–0.91
	W	0.59–0.62	0.68–1.14	0.80–0.82	0.61–0.85	0.27–0.30	0.50–0.79
Hind- Body	L	0.31–0.32	–	0.29–0.30	0.50–0.80	0.12–0.15	0.28–0.61
	W	0.37–0.39	–	0.51–0.53	0.32–0.45	0.19–0.21	0.22–0.79
Oral -Sucker	L	0.11–0.13	0.09–0.13	0.13–0.16	0.04–0.07	0.04–0.06	0.07–0.10
	W	0.09–0.10	0.12–0.17	0.08–0.10	0.05–0.09	0.06–0.09	0.07–0.08
Ventral Sucker	L	0.12–0.14	0.08–0.12	0.17–0.19	0.08–0.17	0.08–0.09	0.11–0.13
	W	0.09–0.12	0.08–0.12	0.15–0.17	0.08–0.17	0.06–0.08	0.12–0.15
Pseudo Suckers	L	0.17–0.19	–	0.17–0.19	0.16–0.21	0.07–0.09	–
	W	0.12–0.14	–	0.09–0.10	0.07–0.08	0.06–0.07	–
Pharynx	L	0.08–0.09	0.05–0.07	0.07–0.08	0.05–0.06	0.02–0.03	0.02–0.04
	W	0.03–0.04	0.05–0.07	0.06–0.07	0.03–0.06	0.02–0.03	0.01–0.03
Hold fast	L	0.30–0.33	0.21–0.28	0.35–0.40	0.27–0.39	0.12–0.15	–
	W	0.28–0.30	0.26–0.30	0.32–0.35	0.25–0.30	0.19–0.21	–
Anterior testis	L	–	–	–	–	0.02–0.04	0.04–0.08
	W	–	–	–	–	0.06–0.08	0.07–0.13
Posterior	L	–	–	–	–	0.04–0.05	0.04–0.07
	W	–	–	–	–	0.07–0.08	0.08–0.17

T. glossogobii. Measurements of each metacercariae are based on 10 specimens, given in mm in Table 2 & Table 3.

3.1.1. Tetracotyle muscularis Chakrabarti, 1970 (Fig. 1)

Host and locality: Stinging catfish *Heteropneustes fossilis* (Bloch, 1974) collected from the River Gomti, Lucknow, India (26.50° N, 80.50° E).

Site of infection: Body muscles

Type host and locality: *Heteropneustes fossilis* (Bloch, 1974), collected from the Tulsipur, Gonda, India (27.55° N, 82.42° E)

Voucher specimens deposited: W10006/1

Description (Based on 10 specimens): Metacercarial cysts rounded. Larvae with the bipartite body. Fore body: aspinose, foliaceous; oral sucker terminal, circular; ventral sucker post-equatorial; pseudo-suckers well developed, located anterolaterally; hold fast organ post acetabular, sub-circular, strongly muscular, hold fast gland close behind

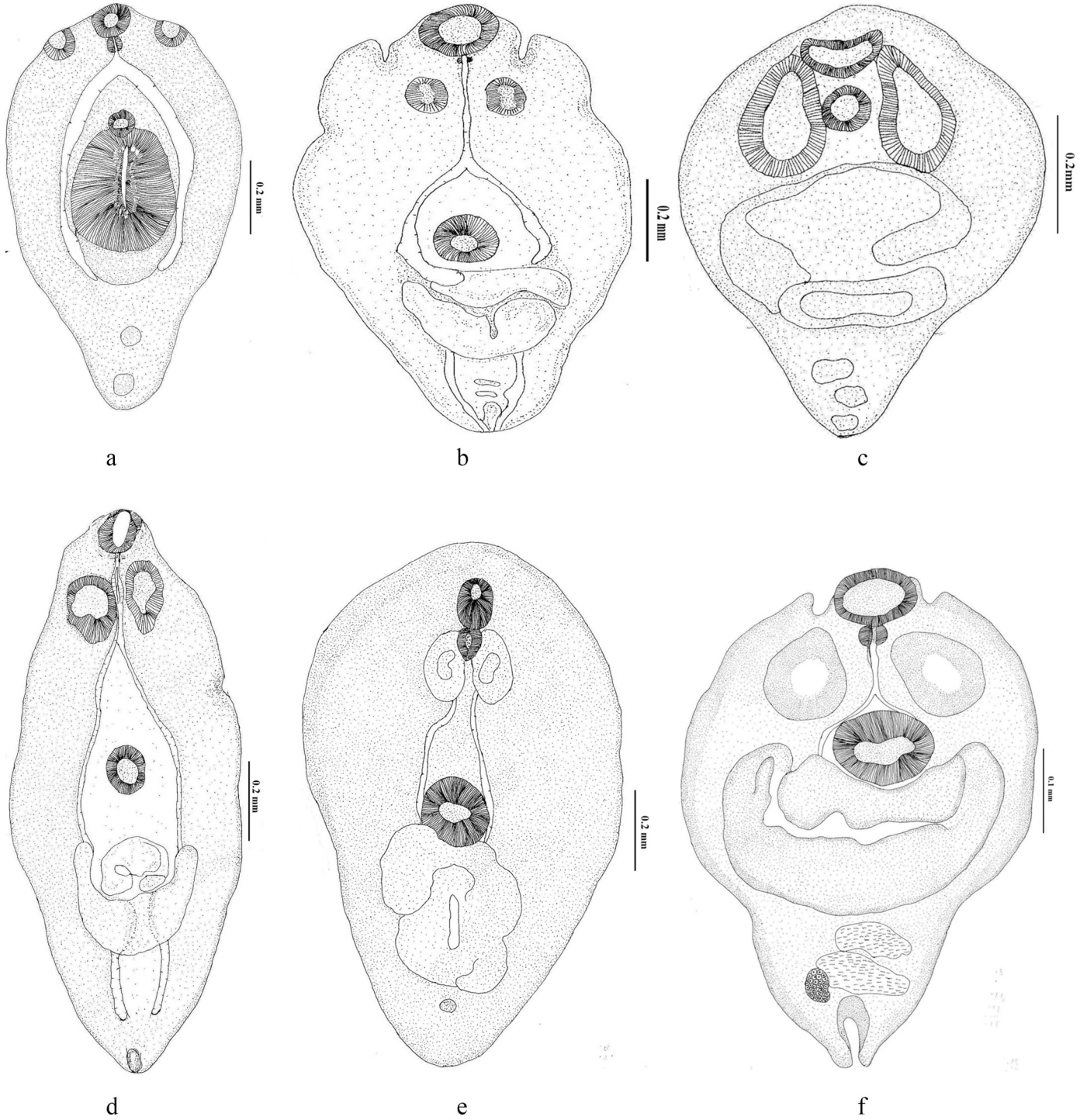

Fig. 1. (a–f) The morphology of (a) *T. muscularis* (b) *T. fausti* (c) *T. lucknowensis* (d) *T. xenentodoni* (e) *T. mathuraensis* (f) *T. glossogobii*

hold fast organ; pharynx sub-globular; pre-pharynx absent; oesophagus short; intestinal caeca extending up to hold fast gland. Hind body: small, conical, with genital rudiments, testes two, a smaller mass representing anterior testis, posterior testis mass larger than anterior mass; excretory bladder "V" shaped with the terminal pore.

Remarks: The comparative morphology of our specimens was nearly identical with the original description. The measurements of our specimens were consistently larger than those reported by Chakrabarti [7], which could possibly be due to intra and inter-specific variability caused by variation in environmental or genetic factors. Measurements of our specimens and their comparison with the original description are presented in Table 2.

3.1.2. *Tetracotyle fausti* Rai and Pande, 1969 (Fig. 1b)

Host and locality: Spiny eel *Mastacembelus armatus* (Lacepède 1800), collected from the River Gomti, Lucknow, India (26.50° N, 80.50° E).

Site of infection: Liver & Ovary

Type host and locality: Mastacembelus armatus (Lacepède 1800), collected from the Gorakhpur India (26.75° N, 83.36° E)

Voucher specimens deposited: W10007/1

Description (Based on 10 specimens): Metacercarial cysts round, transparent. Larvae with the bipartite body. Fore body: aspinose, cup-

shaped; oral sucker terminal; pharynx small; Oesophagus short; intestinal caeca extending nearly up to two third of body; ventral sucker post-equatorial; hold fast organ post–acetabular; lateral pseudo-suckers. Hind body cylindrical; genital rudiments two, linearly placed; excretory pore terminal.

Remarks: The comparative morphology of our specimens was nearly identical with original description, with minor differences. The measurements of our specimen were consistently larger than those reported by Rai and Pande [36], which could possibly be due to intra and inter-specific variability caused by variation in environmental or genetic factors. Measurements of our specimens and their comparisons with the original description are presented in Table 2.

3.1.3. *Tetracotyle lucknowensis Pandey, 1971 (Fig. 1c)*

Host and locality: Freshwater garfish *Xenentodon cancila* (Ham. 1822), collected from the River Gomti, Lucknow, India (26.50° N, 80.50° E).

Site of infection: Visceral Organ

Type host and locality: Channa punctatus (Bloch, 1793) collected from Fish market, Lucknow, India (26.50° N, 80.50° E)

Voucher specimens deposited: W10008/1

Description (Based on 10 specimens): Metacercarial cysts round, transparent. Larvae with the bipartite body. Fore body: aspinose, oval; oral sucker, circular, sub-terminal; ventral sucker pre-equatorial, smaller than oral sucker, pseudo-suckers large, prominent, oval, covering almost entire antero-lateral region of body, extending beyond ventral sucker; hold fast organ, close to ventral sucker; hold fast gland present; pharynx not observed; oesophagus short. Intestinal caeca thin walled extending up to the hind end of the body. Hind body: small lodging three acetabular masses; excretory bladder "V" shaped with the terminal excretory pore.

Remarks: The comparative morphology of our specimens was nearly identical with original description, with minor differences. The measurements of our specimens were consistently larger than those reported by Pandey [32], which could possibly be due to intra and inter-specific variability caused by variation in environmental or genetic factors. Measurements of our specimens and their comparisons with the original description are presented in Table 2. The freshwater garfish is a new host record for *T. lucknowensis*.

3.1.4. *Tetracotyle xenentodoni Chakrabarti, 1970 (Fig. 1d)*

Hosts and localities: Freshwater garfish *Xenentodon cancila* (Ham, 1822), collected from the River Gomti, Lucknow, India (26.50° N, 80.50° E).

Site of infection: Visceral Organ

Type host and locality: Xenentodon cancila (Ham, 1822), collected from Fish market, Lucknow, India (26.50° N, 80.50° E)

Voucher specimens deposited: W10009/1

Description (Based on 10 specimens): Metacercarial cysts round, transparent. Larvae with the bipartite body. Fore body: aspinose, oval to elongate; oral sucker rounded, terminal; ventral sucker equatorial, smaller than oral sucker; pseudo-suckers large, close to pharynx; pre-pharynx small; pharynx sub-globular; oesophagus short, intestinal caeca extending up to the hind end of the body; hold fast organ post-acetabular and strongly developed. Hind body small: gonads incipient; excretory bladder "V" shaped with the terminal excretory pore.

Remarks: The comparative morphology of our specimens was nearly identical with original description, except spinose body as described by Chakrabarti [8]. The measurements of our specimens were consistently larger than those reported by Chakrabarti [8], which could possibly be due to intra and inter-specific variability caused by variation in environmental or genetic factors. Measurements of our specimens and their comparisons with the original description are presented in Table 3.

3.1.5. *Tetracotyle mathuraensis Rai and Pande, 1969 (Fig. 1e)*

Host and locality: Spotted snakehead *Channa punctatus* (Bloch, 1793), collected from the River Gomti, Lucknow, India (26.50° N, 80.50° E).

Site of infection: Gills

Type host and locality: Channa punctatus (Bloch, 1793), Mathura (27.29° N, 77.40° E)

Voucher specimens deposited: (W10010/1)

Description (Based on 10 specimens): Metacercarial cysts round, transparent. Larvae with the bipartite body. Fore body: aspinose, cup-shaped; oral sucker sub–terminal; pharynx small; oesophagus short, dividing into two intestinal caeca; pseudosuckers close to pharynx; ventral sucker larger than oral sucker situated in middle of body; hold fast organ with one central and two lateral lobes, forming its anterior part, posterior glandular region. Hind body conical: posterior region cylindrical, smaller, with incipient gonads, excretory pore terminal.

Remarks: The comparative morphology of our specimens was nearly identical with original description, with minor differences as described by Rai and Pande [36], which could possibly be due to intra and inter-specific variability caused by variation in environmental or genetic factors. Measurements of our specimens and their comparisons with the original description are presented in Table 3.

3.1.6. *Tetracotyle glossogobii Chakrabarti, 1970c (Fig. 1f)*

Host and locality: Tank goby *Glossogobius giuris* (Ham, 1822), collected from the River Gomti, Lucknow, India (26.50° N, 80.50° E).

Site of infection: Cranial cavity & Nervous tissue

Type host and locality: Glossogobius giuris (Ham, 1822), collected from Lucknow, India (26.50° N, 80.50° E).

Voucher specimens deposited: (W10011/1)

Description (Based on 10 specimens): Metacercarial cysts round, transparent. Larvae with the bipartite body. Fore body large: aspinose; oral sucker terminal; ventral sucker larger than oral sucker; pseudo–-sucker well developed; hold fast organ lobed, behind ventral sucker; pre-pharynx present; pharynx elongate to oval; oesophagus long, intestinal caeca thin walled extending up to gonads. Hind body: conical; rudiments of testes asymmetrical, tandem, anterior testis small, posterior testis larger, transversely elongate to oval; ovarian primordium, oval; excretory bladder "V" shaped with the terminal excretory pore.

Remarks: The comparative morphology of our specimens was nearly identical with original description, except spinose body as described by Chakrabarti [9], which could possibly be due to intra and inter-specific variability caused by variation in environmental or genetic factors. Measurements of our specimens and their comparisons with the original description are presented in Table 3.

3.2. *Molecular analysis*

In the present study, nucleotide sequences were submitted to NCBI/GenBank database under the accession numbers given in Table 4. The amplicon size and G + C content of different markers for six *Tetracotyle* type metacercariae are given in Table 5. Guanine and cytocine (G + C)

Table 4
The deposited Genbank accession no. of 18S, 28S, ITS2 r DNA and mt COI DNA sequences of *Tetracotyle* type metacercariae in the present study.

Name of Metacercariae	GenBank accession number			
	28S	18S	ITS2	mtCOI
T. muscularis	KY412569	KY412568	KY412570	KX246270
T. fausti	KY438967	KY438966	KY438968	KY064067
T. xenentodoni	KU307194	KU307193	KU316948	KU878578
T.lucknowensis	KU365155	KU365156	KU878576	KU878577
T.mathraensis	KY449057	KY449056	KY449058	KY461002
T. glossogobii	KY432868	KY432869	KY432870	KY432871

Table 5
Amplicon size (bp) and G + C% of 18S, 28S, ITS2 and COI gene sequences used in present study.

Name of metacercariae	Amplicon size (bp) with G + C%							
	18S	G + C%	28S	G + C%	ITS2rDNA	G + C%	mtCOI	G + C%
T.muscularis	549	49	373	51	505	49	433	31
T.fausti	625	52	364	52	506	50	437	34
T.lucknowensis	342	48	361	52	531	48	454	34
T.xenentodoni	596	46	359	52	532	46	555	37
T.mathuraensis	621	49	358	51	529	48	430	30
T.glossogobii	535	50	383	52	499	55	424	36

Table 6
An analysis of inter-specific variation (Ts- Transition, Tv-Tansversion) among Six *Tetracotyle* type metacercariae used in this study.

Metacercariae	18s Ts/Tv	28S (Ts/Tv)	ITS2 (Ts/Tv)	COI (Ts/Tv)
T.lucknowensis and *T.xenentodoni*	1.9% (1/1)	3.8% (4/5)	7% (10/12)	4.3% (7/14)
T.fausti and *T.glossogobii*	2.1% (3/2)	3.2% (1/0)	6.9% (6/10)	3.5% (9/3)
T.glossogobi and *T. mathuraensis*	4.5% (16/18)	6.6% (10/14)	12% (23/42)	9.4% (21/26)
T. mathuraensis and *T.fausti*	4.2% (9/12)	7.1% (12/17)	9.9% (22/30)	7.9% (13/18)
T. mathuraensis and *T.muscularis*	4.8% (12/18)	8.8% (14/18)	12% (26/40)	8.4% (15/17)
T. mathuraensis and *T.lucknowensis*	4.1% (8/13)	6.6% (13/16)	11.6% (20/43)	8.9% (28/31)
T.glossogobi and *T. mascularis*	3.0% (10/13)	4.0% (10/12)	9.0% (20/26)	5.8% (10/14)
T.glossogobi and *T. lucknowensis*	3.9% (11/14)	6.4% (12/13)	8.2% (20/24)	6.2% (16/19)
T.glossogobi and *T.xenentodoni*	4.0% (12/13)	7.2% (14/15)	10.4% (22/34)	8.3% (17/23)
T.fausti and *T.muscularis*	3.9% (8/11)	5.9% (6/10)	8.5% (18/26)	6.3% (14/17)
T.fausti and *T. lucknowensis*	3.3% (6/11)	3.5% (4/5)	7.8% (16/18)	5.6% (13/21)
T.fausti and *T.xenentodoni*	3.2% (6/12)	3.7% (6/7)	6.8% (18/17)	5.8% (17/19)
T.muscularis and *T. lucknowensis*	4.6% (7/9)	5.6% (7/14)	10.1% (29/31)	7.6% (18/22)
T.muscularis and *T.xenentodoni*	4.9% (7/10)	5.6% (8/12)	12.9% (28/47)	7.8% (19/21)

content of six *Tetracotyle* were found more or less similar for each metacercariae. This GC richness contributes to physical attributes of RNA structures, as there is a correlation between GC content and optimal growth temperature. The metacercariae showing lowest divergence are given here, while others are listed in Table 6. Pairwise alignment of 18S sequences of *T. xenentodoni* and *T. lucknowensis*, revealed the inter-specific variation (1.9%) in which 1 transitions and 1 transversion at two nucleotide sites were found and 2.1% (3 transition and 2 tranversion at five nucleotide sites) inter-specific variation between *T. fausti* and *T. glossogobii.* For the 28S sequences, the pairwise alignment revealed 3.8% (4 transitions and 5 transversions at 9 nucleotide sites) inter-specific variation between *T. xenentodoni* and *T. lucknowensis* and 3.2% (1 transitions and no transversion at 1 nucleotide sites) inter-specific variation between *T. fausti* and *T.glossogobii*. For the ITS2 sequences, the pairwise alignment revealed the inter-specific variation of 7% (10 transitions and 12 transversions at 22 nucleotide sites) between *T. xenentodoni* and *T. lucknowensis*; 6.9% (6 transitions and 10 transversions at 16 nucleotide sites) inter- specific variation between *T. fausti* and *T.glossogobii*. For the COI gene, the pairwise alignment revealed 4.3% (7 transition and 14 transversions at 21 nucleotide sites), inter-specific variation between *T. xenentodoni* and *T. lucknowensis*; 3.5% (9 transition and 3 transversions at 12 nucleotide sites) inter-specific between *T. fausti* and *T. glossogobii*. The variable nucleotide sites of alignments of 18S, 28S, ITS2 and COI in total are as follows: 549/2426 positions were variable in 18S (6.4% singleton sites and 16% parsimony informative sites), 896/1272 in the 28S alignment (27% singleton sites and 42% parsimony informative sites), 763/1349 in the ITS2 alignment (31% singleton sites and 25% parsimony informative sites) and 446/1278 in the COI alignment (5.6% singleton sites and 29% parsimony informative sites).

3.3. Phylogenetic analysis

The phylogenetic trees were constructed for each data set (18S, 28S, ITS2 and mt COI) showed similar topologies using NJ and ML methods. The sequences of *T. fausti, T. glossogobii,* and *T. xenentodoni*, *T. lucknowensis* are nested together, while *T. mauthraensis* form sister clade with it. *T. muscularis* formed a basal clade (Fig. 2–d). In all the trees, *Tylodelphys* sp. (Diplostomidae, Poirier 1886) is employed as an out group.

4. Discussion

The *Tetracotyle* type of metacercariae are encountered in the survey of various fresh water sympatric fish hosts from river Gomti and are also evidenced by previous Indian workers [1,2,34,35]. Although, thirty-five species are recognized by Pandey and Agrawal [33] from India. Regardless of 29 species, which have been omitted in our study, as selected six metacercariae to share a morphological resemblance, similar host and adjoining localities with their original description. For the first time, we have attempted the comprehensive analysis of six *Tetracotyle* type metacercariae, using ribosomal and mitochondrial DNA sequences to show the genetic variation. Earlier workers have used ribosomal RNA and mt COI gene sequences for strigeids to support the morphological study [3,4,20,22,24]. The genetic divergence estimated among six *Tetracotyle* type of metacercariae (Table 6) ranged from 1.9% to 4.9% for 18S. However, it ranged from 3.3% to 8.8% for 28S. These ranges are comparable with other strigeids using the same gene, 0.0–1.2% in *Australapatemon* from Central Alberta [20]. On the second internal transcribed spacer ITS2, the inter-specific divergence ranged from 6.8–12.9%. These ranges are comparable with other strigeids, 1.3–1.87% for *Ichthyocotylurus* spp, from Finland [3]; 0.5–1.48% for *Parastrigea* spp. from Mexico [22]; 0.9–2.1% in *Apatemon* sp., 1.7% in *Apharyngostrigea*, 1.9% in *Australapatemon*, 0.8–1.7% in *Parastrigea* from Canada [4]; 0.4–1.9% in *Australapatemon* from Central Alberta [20]. While in COI, the range of inter-specific variability was found 3.5–9.4% which is completely within the range of other strigeids, 9.56–12.84% for *Ichthyocotylurus* spp. from Finland [3]; 0.31–11.47% for *Parastrigea* spp. from Mexico [22]; 6.7–14.4% in *Australapatemon* from Central Alberta [20]. Sequence comparison across the ITS2, COI, 28S and 18S indicate that the highest inter-specific divergence among six putative metacercariae of the *Tetracotyle* type which we analyzed

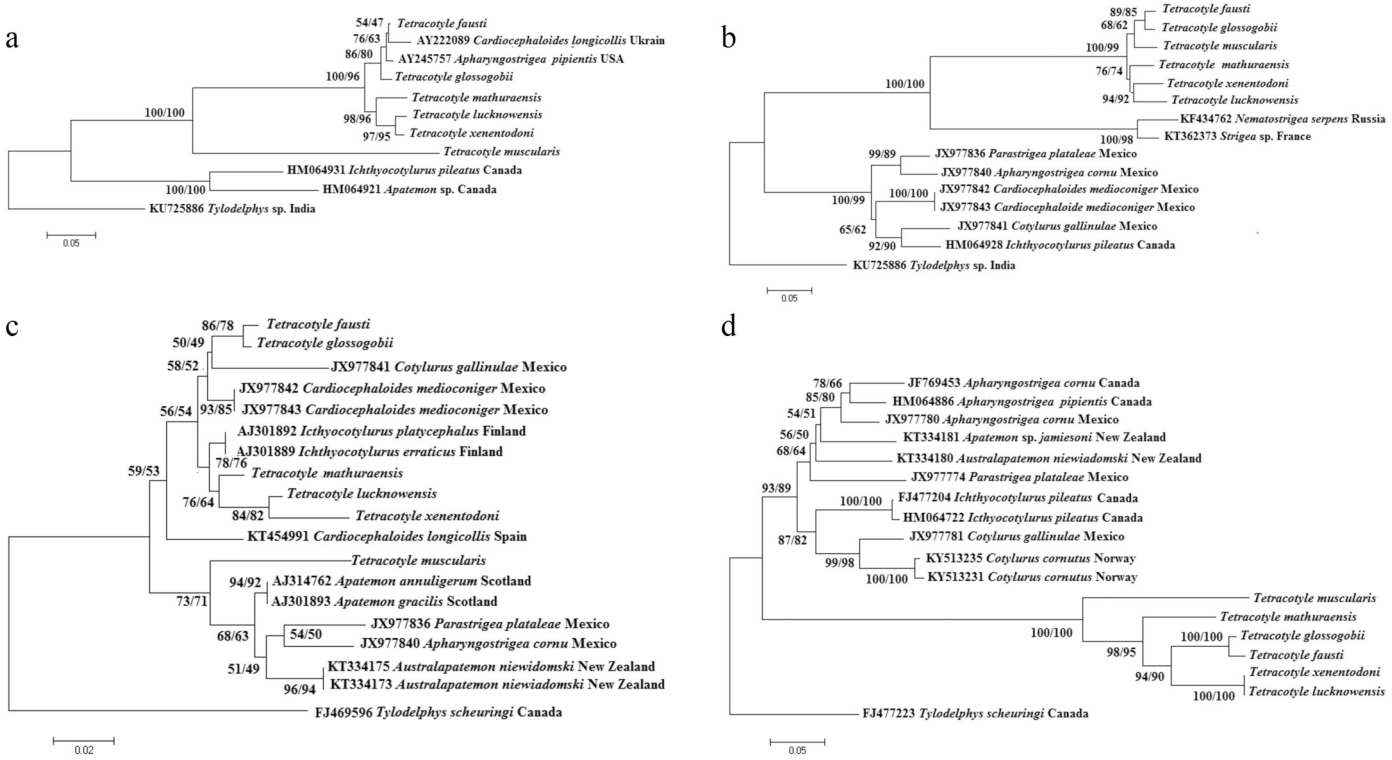

Fig. 2. (a) NJ and ML tree from the phylogenetic analysis of 18S r RNA gene (partial sequence) dataset of six *Tetracotyle* type metacercariae using a member of the family strigeidae. Numbers preceding the taxa are GeneBank accession numbers. The number of the internodes are NJ bootstrap values (above) and ML bootstrap (below). *Tylodelphys* sp. (KU725886) was used as an outgroup. (b) NJ and ML tree from the phylogenetic analysis of 28S r RNA gene (partial sequence) dataset of six *Tetracotyle* type metacercariae using a member of the family strigeidae. Numbers preceding the taxa are GeneBank accession numbers. The number of the internodes are NJ bootstrap values (above) and ML bootstrap (below). *Tylodelphys* sp. (KU725886) was used as an outgroup. (c) NJ and ML tree from the phylogenetic analysis of ITS2 r RNA gene (partial sequence) dataset of six *Tetracotyle* type metacercariae using a member of the family strigeidae. Numbers preceding the taxa are GeneBank accession numbers. The number of the internodes are NJ bootstrap values (above) and ML bootstrap (below). *Tylodelphys* sp. (FJ469596) was used as an outgroup. (d) NJ and ML tree from the phylogenetic analysis of mt COI (partial sequence) dataset of six *Tetracotyle* type metacercariae using a member of the family strigeidae. Numbers preceding the taxa are GeneBank accession numbers. The number of the internodes are NJ bootstrap values (above) and ML bootstrap (below). *Tylodelphys* sp. (FJ477223) was used as an outgroup.

occurs in ITS2 (12.9%), COI (9.4%), and 28S (8.8%) followed by 18S (4.9%). With exception of ITS2, these inter-specific values are low for being different species within the strigeidae, shows the high variability of ITS2 gene and conservedness of other genes (18S; 28S and COI). The four molecular markers preferred for this study produced somewhat conflicting results in terms of the phylogenetic position of six tested *Tetracotyle* type metacercariae with respect to the other genera of Strigeidae. In phylogeny of 18S, *T. fausti* and *T. glossogobii* consistently nested as sister groups of *Cardiocephaloides longicollis* and *Apharyngostrigea pipientis*. However, *T. mathuraensis*, *T. xenentodoni,* and *T. lucknowensis* formed a second sub clade with these strigeids. While *T. muscularis* formed as a basal clade. In phylogeny of 28S, all the six *Tetracotyle* species are identified as sister taxa of *Nematostrigea serpens* and *Streigea* sp. In ITS2 tree, *T. fausti* and *T. glossogobii* nests with *Cotylurus gallinulae* and *Cardiocephaloides medioconiger* but *T. mathuraensis, T. lucknowensis* and *T. xenentodoni* nests with *Icthyocotylurus* sp. Whereas, *T. muscularis* formed as basal clade within other strigeids. In phylogeny of COI, all six species of *Tetracotyle* formed a separate subgroup with all other strigeids. The present study points out the closeness of six *Tetracotyle* type metacercariae although, they are found in different species of fish hosts. Despite this, we find it difficult to place *Tetracotyle* type of metacercariae with any specific strigeids genera. Resolution of phylogenetic placement of *Tetracotyle* spp. should probably await further studies elucidating its parasite life cycle in natural and aquaculture conditions. Phylogenetic placement states the monophyly of the *Tetracotyle* type of metacercariae in all markers (18S, 28S, COI), except ITS2. Monophyly of strigeids is also recommended by many workers [15,16,22,38]. We also recommend monophyly of *Tetracotyle* type of metacercariae, except *T. muscularis*, which apparently depicts its paraphyletic status. Paraphyly of strigeids has also been reported in few previous studies, based on 18S and 28S [31,44]. We can, therefore, conclude that these comparative characteristics are justified and valid which allowed us to confidently separate and confirm the validity of species of *Tetracotyle*. In a broader context, molecular markers can be utilized to augment taxonomy and species delimitation by corroborating evidence for systematic.

5. Conclusion

In summing up, on the basis of morphological criteria, all species of *Tetracotyle* type metacercariae are distinct species but are closely related. The molecular results of 18S, 28S, ITS2 r DNA, and COI gene sequences revealed a close association between *T. xenentodoni* and *T. lucknowensis* or *T. fausti* and *T.glossogobii*. We found that *T. fausti*, *T. glossogobii*, *T. xenentodoni*, *T. lucknowensis* and *T. mathuraenis* nested as sister groups with the other member of strigeids. Moreover, the generic placement of *Tetracotyle* type of metacercariae is difficult to assign any specific strigeids genera. The phylogenetic studies of *Tetracotyle* spp. should probably need to explore its parasitic life cycle in natural conditions. In addition, the molecular genetics analysis together with the experimental study of intra and inter species variability and life cycle would provide a more comprehensive strategy for solving the taxonomy and phylogeny of *Tetracotyle* metacercariae.

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References

[1] N. Agrawal, S. Khan, Studies on some *Tetracotyle* Fillipi (1859) metacercariae from fishes of Lucknow, Proc. Natl. Acad. Sci. 91 (1982) 515–521.
[2] S.C. Baugh, K.K. Chakrabarti, On a triple infection of strigeid metacercaria in an Indian freshwater fish *Xenentodoni cancila*, Angew. Parasitol. 18 (1977) 84–94.
[3] A.S. Bell, C. Sommerville, E.T. Valtonen, A molecular phylogeny of the genus *Ichthyocotylurus* (Dignea, Strigeidae), Int. J. Parasitol. 31 (2001) 833–842.
[4] R. Blasco-Costa, B. Presswell Poulin, Species of *Apatemon* Szidat, 1928 and *Australapatemon* Sudarikov, 1959 (Trematoda: Strigeidae) from New Zealand: linking and characterising life cycle stages with morphology and molecules, Parasitol. Res. 115 (2016) 271–289.
[5] J. Bowles, M. Hope, W.U. Tiu, X. Liu, D.P. Mcmanus, Nuclear and mitochondrial genetic markers highly conserved between Chinese and Philippine *Schistosoma japonicum*, Acta Trop. 55 (1993) 217–229.
[6] J. Bowles, D. Blair, D.P. Mcmanus, A molecular phylogeny of the human schistosome, Mol. Phylogenet. Evol. 4 (1995) 103–109.
[7] K.K. Chakrabarti, A double metacercarial infection in an Indian fresh water fish, *Heteropneustis fossilis* (Bloch), Rev. Biol. Trop. 17 (1970) 91–96.
[8] K.K. Chakrabarti, Two new species of Strigeid metacercariae from an Indian fresh water fish, *Xenentodon cancilla* (Ham.), Proc. Helminthol. Soc. Wash. 37 (1970) 5–10.
[9] K.K. Chakrabarti, A new strigeid metacercarcaria, *Tetracotyle glossogobii* sp.n., from an Indian freshwater fish, *Glossogobius giuris* (Ham.), Helminthologia 11 (1970) 77–82.
[10] T. Chontananarth, C. Wongsawad, S. Chomdej, D. Krailas, J.Y. Chai, Molecular phylogeny of trematodes in family Heterophyidae based on mitochondrial cytochrome c oxidase subunit, Asian Pac. J. Trop. Med. 7 (2014) 446–450.
[11] K. Choudhary, A.K. Verma, S. Swaroop, N. Agrawal, Review on "A Review on the Molecular Characterization of Digenean Parasites using Molecular Marker with Special Reference to ITS region", Helminthologia 52 (2015) 167–187.
[12] K. Choudhary, A. Tripathi, K.C. Pandey, N. Agrawal, Morphological and molecular characterisation of three Indian *Neascus*-type metacercariae (Digenea: Diplostomidae), Parasitol. Int. 60 (2017) 652–659.
[13] T.H. Cribb, G.R. Anderson, R.D. Adlard, R.A. Bray, A DNA based demonstration of a three-host life –cycle for the Bivesiculidae (Platyhelminthes: Digenea), Int. J. Parasitol. 28 (1998) 1791–1795.
[14] G. Dubois, Monographie des Strigeida (Trematoda), Mémoires de la Société Neuchateloise des Sciences Naturelles 6 (1938) 1–535.
[15] G. Dubois, Synopsis des Diplostomatidae (Trematoda), Mémoires de la Société Neuchateloise des Sciences Naturelles 10 (1968) 1–258.
[16] G. Dubois, Synopsis des Diplostomatidae (Trematoda), Mémoires de la Société Neuchateloise des Sciences Naturelles 10 (1970) 259–723.
[17] R. Dzikowski, M.G. Levy, M.F. Poore, J.R. Flowers, I. Paperna, *Clinostomum complanatum* and *Clinostomum marginatum* (Rudolphi,1819) (Digenea:Clinostomidae) are seprate species based on differences in ribosomal DNA, J. Parasitol. 90 (2004) 413–414.
[18] B. Díez, C. Pedrós- Alió, R. Massana, Study of genetic diversity of eukaryotic picoplankton in different oceanic region by small – subunit rRNA gene cloning and sequencing, Appl. Environ. Microbiol. 67 (2001) 2932–2941.
[19] S. Ghatani, J.A. Shylla, V. Tandon, A. Chatterjee, B. Roy, Molecular characterization of pouched amphistome parasites(Trematoda: Gastrothylacidae) using ribosomal ITS2 sequence and secondary structure, J. Helminthol. 86 (2012) 117–124.
[20] M.A. Gordy, S.A. Locke, T.A. Rawlings, A.R. Lapierre, P.C. Hanington, Molecular and morphological evidence for nine species in North American *Australapatemon* (Sudarikov, 1959): a phylogeny expansion with description of the zygocercous *Australapatemon mclaughlini* n. sp, Parasitol. Res. 116 (2017) 2181–2198.
[21] T.A. Hall, BioEdit: a user- friendly biological sequence alignment editor and analysis program for Window 95/98/NT, Nucleic Acids Symp. Ser. 41 (1999) 95–98.
[22] D. Hernández-Mena, L. García-Prieto, M. García-Varela, Morphological and molecular differentiation of *Parastrigea* (Trematoda: Strigeidae) from Mexico, with the description of a new species, Parasitol. Int. 63 (2014) 315–323.
[23] D.M. Hillis, M.T. Dixon, Ribosomal DNA: molecular evolution and phylogenetic inference, Q. Rev. Biol. 66 (1991) 411–453.
[24] S.A. Locke, J.D. Mclaughlin, A.R. Lapierre, P.T.J. Johnson, D.J. Morcogliese, Linking larvae and adults of *Apharyngostrigea cornu, Hysteromorpha triloba,* and *Alaria mustelae* (Diplostomoidea: Digenea) using molecular data, J. Parasitol. 97 (2011) 846–851.
[25] O. Miura, A.M. Kuris, M.E. Torchin, R.F. Hechinger, E.J. Dunham, S. Chiba, Molecular-genetic analyses reveal cryptic species of trematodes in the intertidal gastropod, *Batillaria cumingi* (Crosse), Int. J. Parasitol. 35 (2005) 793–801.
[26] L.H.S. Mollaret, J.L. Justine Lim, Phylogenetic position of the monogeneans *Sundanonchus, Thaparocleidus*, and *Cichlidogyrus* inferred from 28S rDNA sequences, Int. J. Parasitol. 30 (2000) 659–662.
[27] C.J. Mwita, G. Nkwengulila, Phylogenetic relationships of the metazoan parasites of the clariid fishes of Lake Victoria inferred from partial 18S rDNA sequences, Tanz. J. Sci. 36 (2010).
[28] K. Niewiadomska, An analysis of criteria for generic differentiation within the order Strigeidida (La Rue, 1926), Acta Parasitol. 18 (1970) 277–289.
[29] K. Niewiadomska, Family Strigeidae Railliet, 1919, in: D.I. Gibson, A. Jones, R.A. Bray (Eds.), Keys to the Trematoda, Vol. 1 CABI Publishing., The Natural History Museum, Wallingford, 2000, pp. 231–241.
[30] M.J. Nolan, T.H. Cribb, The use and implications of ribosomal DNA sequencing for the discrimination of digenean species, Adv. Parasitol. 60 (2005) 101–163.
[31] P.D. Olson, V.V. Tkach, Advances and trends in the molecular systematic of the parasitic platyhelminthes, Adv. Parasitol. 60 (2005) 165–243.
[32] K.C. Pandey, Studies on metacercaria of fresh water fishes of India VIII. On the morphology of *Tetracotyle lucknowensis* n.sp. from *Channa punctatus* (Bloch), Proc. Ind. Acad. Sci. 44 (1971) 1–5.
[33] K.C. Pandey, N. Agrawal (Eds.), Metacercarial Fauna of India, 1st ed., ZSI Publishing, Kolkata, India, 2013.
[34] K.C. Pandey, N. Pandey, Studies on two new strigeid metacercariae from *Xenentodon cancila* (Ham.), Ind, J. Helminthol. (N.S.) 17 (2000) 29–32.
[35] K.C. Pandey, S.K. Tewari, Studies on some new strigeid metacercaria, PartII. J. Adv. Zool. 4 (1983) 115–122.
[36] P. Rai, B.P. Pande, On the morphology and pathogenic significance of the strigeoid metacercariae in some Indian freshwater fishes. I. *Tetracotyle* and *Neascus*, Ind. J. Ann. Sci. 39 (1969) 355–359.
[37] S. Rajvanshi, K. Choudhary, N. Agrawal, Threading: a novel insilico indagation method for genetic characterization of some diplostomoid metacercariae (Digenea: Diplostomidae Poirier, 1886), Exp. Parasitol. 171 (2016) 71–76.
[38] W.L. Shoop, Systematic analysis of the Diplostomidae and Strigeidae (Trematoda), J. Parasitol. 75 (1989) 21–32.
[39] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: molecular evolutionary genetics analysis version 6.0, Mol. Phylogenet. Evol. 30 (2013) 2725–2759.
[40] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, positions-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.
[41] V.V. Tkach, J. Pawlowski, J. Mariaux, Phylogenetic analysis of the suborder Plagiorchiata (Platyhelminthes, Digenea) based on partial lsr DNA sequences, Int. J. Parasitol. 30 (2000) 83–93.
[42] R. Vilas, C.D. Criscione, M.S. Blouin, A comparison between mitochondrial DNA and the ribosomal internal transcribed regions in prospecting for cryptic species of platyhelminth parasites, Parasitology 13 (2005) 839–846.
[43] J. WanZhong, Y. HongBin, N. HongWei, L. ZhongZi, L. HongMin, C. Ping, C. XuePeng, Advances in the study of helminth mitochondrial genome and their associated applications, Chin. Sci. Bull. 57 (2012) 54–67.
[44] O. Zazornova, A. Sysoev, Phylogenetic relations between species of the genus *Cotylurus* and its position in the system of the trematode family strigeidae, Parazitologiia 27 (1993) 69–76.