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Morphological and Molecular Characterization of *Heterobothrium indicus* n. sp. (Monogenea: Diclidophoridae) and Its Phylogenetic Status

A.K. Verma

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ABSTRACT

Background: A new species *Heterobothrium indicus* n. sp. was isolated from the gills of *Upeneus moluccensis* (Bleeker, 1855) from the Western Coast of India in Arabian Sea region. The monogenean parasite differs from other congeners by morphological features like the presence of asymmetrical haptoral region, 4th pair of clamp smallest than other three pairs of clamps, genital atrium with 8 hooks, number of testes 29-37 and absence of isthmus.

Methods: During the survey of marine fishes at Arabian Sea region, new species of monogenean parasite was isolated from the gills of marine fish *Upeneus moluccensis*. The parasites were morphologically characterized with the help of light and phase contrast microscopy. 18S rDNA, 28S rDNA, mt COI, ITS1+5.8S and ITS2+5.8S gene regions of parasites were amplified, sequenced and compared with other diclidophorid taxa using different bioinformatics tools.

Result: Phylogenetic tree analyses (NJ, ME and MP methods) of 18S rDNA, 28S rDNA and mt COI gene regions are complementing the morphological studies and clearly suggested the placement of this new species under subfamily Choricotylinae, family Diclidophoridae.

Key words: 18S and 28S rDNA, Clamp, Diclidophoridae, *Heterbothrium indicus* n. sp., mt COI.

INTRODUCTION

The monogeneans are minute parasites mainly affecting the gills and skin of fishes. The Western Indian coast along the Arabian sea region is the biodiversity hotspot and harbors the great variety of fishes and parasites. The knowledge about the monogenean parasites of Indian marine fishes is limited only to morphological level.

The goldband goatfish *Upeneus moluccensis*, (Bleeker, 1855) is an important demersal resource in commercial landings of Maharashtra, contributing 95% in total catch of goatfishes (CMFRI, 2017). The annual catchment of goatfish at Visakhapatnam, India was 2177-3463 tonnes (average - 2859 tonnes) and *U. moluccensis* contributed up to 18% (Das, 2011). In the Mediterranean region, for trawl fishing, *U. moluccensis* is an influential economic species (Golani *et al.,* 2002). It has wide range of geographical distribution from Red Sea to New Caledonia and north to Japan and invaded the eastern Mediterranean from Red Sea through the Suez Canal.

The genus *Heterobothrium* was erected by Cerfontaine, 1895 to include type species *H. tetrodonis* (Goto, 1894) Nagibina, 1953. According to Acosta and Smit (2021) presently thirteen species of the monogenean parasite have been reported from different regions of the world. The impact of pathogenicity of *Heterobothrium okamotoi* (Oagwa, 1991) to economically valuable fishes of Japan is unmatched to other monogeneans (Ogawa, 1997).

According to Zhang *et al.* (2001), the only known parasite from *U. moluccensis* is the monogenean *Haliotrema spirale* (Yamaguti, 1968), reported from South China Sea, China. During the survey, a new species of *Heterobothrium* was observed on the gills of *Upeneus moluccensis*, (Bleeker, 1855) from Indian Western coast of Arabian Sea region, Mumbai. The parasite was morphologically as well as molecularly characterized with the help of molecular markers such as 18S rDNA, 28S rDNA and mt COI gene. The phylogenetic relationship of the worm with family members was established using the bioinformatics tools.

Department of Biosciences, Jamia Millia Islamia, New Delhi-110 025, India.

Corresponding Author: A.K. Verma, Department of Biosciences, Jamia Millia Islamia, New Delhi-110 025, India.
Email: averma@jmi.ac.in

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MATERIALS AND METHODS

Collection of hosts and parasites

Total 116 freshly dead specimens of *Upeneus moluccensis* (Bleeker, 1855) were procured at Versova dock landing centre (19°7′60N 72°47′60E), Mumbai (India) of Arabian Sea Region, during November-December, 2015 and March-April, 2016 from local fishermen using bottom trawlers along with purse seiners, multiday gillnetters and bagnetters. The fishes

were identified on the basis of database by Froese and Pauly (2015). The gills were excised and placed at 6°C in refrigerator, overnight for fixation of parasites. A total of 148 parasites were collected from the gills of fishes. For molecular work parasites were stored in eppendorfs, containing absolute alcohol at -20°C. The temporary mounts were prepared in glycerine and permanent mounts were prepared by staining with Gomori's Trichome or aceto-alum carmine stain followed by mounting in Canada Balsam or DPX resin. Infection prevalence, mean intensity and relative density of parasites were calculated according to Mergo and Cites (1986).

The study was conducted during the period of March 2015 to March 2017, at Fisheries Resources Harvest and Post-harvest Division of Central Institute of Fisheries Education (ICAR-CIFE), Mumbai and Department of Zoology, University of Lucknow, Lucknow.

Morphological methods

The worms were analysed with help of Nikon (Eclipse *80i*) phase contrast microscope at different phases equipped with DS-Fi1 (Nikon) digital camera through NIS-elements F 4.00.00 software. Illustrations and line drawings were made with the aid of a drawing tube attached to the microscope. The clamp nomenclature was followed according to Rubec and Dronen (1994). All the measurements were taken in micrometres and were represented as the range followed by mean in parentheses. The holotype and paratypes were deposited in Natural History Museum, London for accession number.

Molecular methods

The total genomic DNA was extracted from the alcohol preserved parasites, using Qiagen DNeasy tissue kit (Qiagen). For amplification of 18S rDNA, 28S rDNA, mitochondrial COI, ITS1+5.8S and ITS2+5.8S gene region primers were commercially synthesized. For 30 µl reaction volume, 1x PCR (2 mM Tris-HCl (pH 8.4), 50 mM KCl) buffer (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 200 µM of dNTP mix (Promega), 0.4 µM of each forward and reverse primer, 1U/µl Taq DNA polymerase (Invitrogen), 6 µl of DNA and 14.86 µl of Milli Q water were added in a microfuge tube and processed through PCR machine (Eppendorf).

The primers and PCR conditions for 18S and 28S ribosomal DNA were selected according to Plaisance *et al.* (2005). The primers for 18S rDNA gene - forward primer Worm A (5′ ACGAATGGCTCATTAAATCAG 3′) and reverse primer Worm B (5′ CTTGTTACGACTTTTACTTCC 3′) were used. The primers for 28S rDNA gene - forward primer Ancy 55 (5′ GAGATTAGCCCATCACCGAAG 3′) and reverse primer LSU1200R (5′ GCATAGTTCACCATCTTTCGG 3′) were used. The primers and PCR conditions for mitochondrial COI gene were according to Littlewood *et al.* (1997). The primers for mt COI gene - forward primer ASmit 1 (5′ TTTTTTGGGCATCCTGAGGTTTAT 3′) and reverse primer ASmit 2 (5′ TAAAGAAAGAACATAATGAAAATG 3′) were used. The primers and PCR conditions for internal

Table 1: Polyopisthocotylean monogenean sequences retrieved from the GenBank and analysed in the study.

Species	Host	Origin	Genbank no.	Gene	Source
***Heterobothrium indicus* n. sp.**	*Upeneus moluccensis*	India	KU900337	18S	Present study
Choricotyle australiensis	*Rhabdosargus sarba*	Australia	AF382069	18S	Olson and Littlewood, 2002
Heterobothrium okamotoi	*Takifugu rubripes*	Japan	AB162155	18S	-
Neoheterobothrium hirame	*Paralichthys olivaceus*	Japan	AB162424	18S	-
Neoheterobothrium sp. SF	*Paralichthys lethostigma*	USA	AB162425	18S	-
Mexicotyle sp. DTJL-2000	*Scomberomorus* sp.	-	AJ287539	18S	Littlewood and Olson, 2001
Paradewesia sp. DTJL-2000	*Scomberomorus* sp.	-	AJ287555	18S	Littlewood and Olson, 2001
***Heterobothrium indicus* n. sp.**	*Upeneus moluccensis*	India	KU926690	28S	Present study
Choricotyle australiensis	*Rhabdosargus sarba*	Australia	AF382046	28S	Olson and Littlewood, 2002
Diclidophora minor	*Micromesistius poutassou*	UK	AF382048	28S	Olson and Littlewood, 2002
Heterobothrium sp. n. AAA-2020 isolate 1	*Amblyrhynchotes honckenii*	South Africa	MW115856	28S	Acosta and Smit, 2021
Neoheterobothrium sp. N. LCSJ-2020	*Syacium papillosum*	Mexico	MT429192	28S	Soler-Jiménez *et al.*, 2021
Neomicrocotyle pacifica	*Caranx hippos*	Mexico	AF382043	28S	Olson and Littlewood, 2002
***Heterobothrium indicus* n. sp.**	*Upeneus moluccensis*	India	KU900340	COI	Present study
Neoheterobothrium sp. SF	*Paralichthys lethostigma*	USA	AB162616	COI	-
Paraeurysorchis sarmientoi isolate 1	*Seriolella violacea*	Chile	KJ794213	COI	Oliva *et al.*, 2014
Parapedocotyle prolatili isolate 1	*Prolatilus jugularis*	Chile	KJ794214	COI	Oliva *et al.*, 2014
Pedocotyle bravoi isolate 1	*Stellifer minor*	Peru	KJ794210	COI	Oliva *et al.*, 2014
Allodiscocotyla diacanthi isolate AEM-AD02	*Scomberoides commersonnianus*	India	KF804046	COI	Tambireddy *et al.*, 2016

(– = Not applicable).

transcribed spacer (ITS1+5.8S and ITS2+5.8S) region were according to Cable *et al.* (2005). The primers for ITS1+5.8S rDNA region - forward primer P3b (5′ TAG GTG AAC CTG CAG AAG GA TCA 3′) and reverse primer F3 (5′ TTG CTG CAC TCT TCA TC 3′) were used. The primers for ITS2+5.8S rDNA region - forward primer R1 (5′ ACT CCA TGT GGT GGA TC 3′) and reverse primer P4 (5′ GTC CGG ATC CTC CGC TTA TTG ATA TTG C 3′) were used. The PCR conditions for 18S and 28S rDNA genes were initial denaturation at 94°C for 3 min, (35 cycles of 30 sec at 94°C, 30 sec at 52°C, 2 min at 72°C) and final extension at 72°C for 10 min, followed by cooling at 4°C. The PCR conditions for mt COI gene were initial denaturation at 95°C for 5 min, (35 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C) and final extension at 72°C for 6 min, followed by cooling at 4°C. The PCR conditions for ITS1+5.8S and ITS2+5.8S rDNA regions were initial denaturation at 94°C for 2 min, 35 cycles (15 sec at 94°C, 30 sec at 60°C, 2 min at 72°C), final extension at 72°C for 6 min followed by cooling at 4°C. After amplification the PCR products were checked on 1% agarose gel electrophoresis followed by staining with ehtidium bromide and gel documentation system (Vilber Lourmat). The PCR products were purified and sequenced commercially using Big Dye Terminater version 3.1 Cycle sequencing Kit (Applied Biosystems). Five new DNA sequences of *H. indicus* n. sp. were deposited to GenBank database and sequences of 18S rDNA, 28S rDNA and mt COI gene of other Diclidophorids were retrieved from GenBank for the study (Table 1). Sequences of ITS1+5.8S and ITS2+5.8S rDNA region with accession no. KU900338 and KU900339, respectively, were not utilized in the present study due to paucity of other family member sequences.

Analyses of sequences

The similarity among the members of Diclidophoridae were obtained with help of local alignment tool BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?LINKLOC= blasthome and PAGE TYPE=BlastSearch and PROGRAM=blastn). The phylogenetic analysis for various genes was conducted using MEGA version 10.0(X) software (Kumar *et al.,* 2018). In case of 18S rDNA and mt COI gene, each data set was analyzed through neighbor joining (NJ) and minimum evolution (ME) method, using maximum composite likelihood method. The evolutionary distances were in unites of the number of base substitutions per site. In the analysis, codon positions (1st, 2nd and 3rd) and non coding sites were also included and all ambiguous positions were removed for each sequence pair (pairwise deletion option). For 28S rDNA, evolutionary history was inferred through maximum parsimony (MP) method. The tree was obtained using the subtree-prunning-regrafting (SPR) algorithm with search level 1 in which the intitial trees were obtained by the random addition of sequences (10 replicates). In the analysis, codon positions (1st, 2nd and 3rd) and non coding sites were also included. Bootstrap values were calculated on the basis of 1000 replicates for 18S rDNA, 28S rDNA and mt COI gene molecular data sets.

RESULTS AND DISCUSSION

Morphological findings

Class Monogenea Carus, 1863
Subclass Polyopisthocotylea Odhner, 1912
Order Mazocraeidea Bychowsky, 1937
Superfamily Diclidophoridea Price, 1936
Family Diclidophoridae Fuhrmann, 1928
Subfamily Choricotylinae Sproston, 1946
Genus *Heterobothrium* Cerfontaine, 1895
***Heterobothrium indicus* n. sp.**

Type host: *Upeneus moluccensis* (Bleeker, 1855) (Perciformes: Mullidae).
Type locality: Versova dock landing centre, Mumbai, Arabian Sea, India.
Site of infection: Gills.
Infection details: 148 worms collected from 41 infected fishes; examined fishes - 116; infection prevalence - 35.34%; mean intensity - 3.61; relative density - 1.27.
Etymology: The species is named after the country, because it is first time reported from the India.

Description (Fig 1, 2A-C, 3A-B, 4A-F and 5A-F)

The description is based on the 10 stained and 17 permanent mount specimens. Body elongated, dorso-ventrally flattened, differentiated into anterior long region and posterior haptor (n=27). Body length 2780-3568 (3105), width 819-925 (885). Isthmus absent between the haptor region and the rest of the body. Haptor with four pairs of clamps with short stalk in two parallel rows, anterior most pair bigger and gradually decreases in size to the last pair of clamps. Anterior most 1st pair of clamp length 84-101 (94), width 73-91 (84), 2nd pair of clamp length 83-97 (91), width 72-89 (83), 3rd pair of clamp length 80-94 (86), width 70-81 (75), 4th posterior most or terminal end pair of clamp length 71-85 (77), width 67-79 (72). Clamps diclidophorid type, closed and asymmetrical. Clamp comprised of 8 sclerites. Anterior jaw comprising three sclerites (a=a1,a2,a3,a4; c=c1,c2,c3; d=d1,d2,d3). Ventral median sclerite (a1) with unmodified symmetry (with long axis parallel to dorsal median hollow sclerite f), hollow with small nodular opening protruding as spiny processes on ventro-medial surface. a1 with extended lamellate extension (b) and fused with peripheral sclerite c1. b with proximal border (bp) located between a1 and c2. Fused dorsal sclerite a3c3 and a4d3; asymmetrical Posterior unpaired a2 and c2, paired a3c3, a4d3 asymmetrical, distal fused a3c3 having thin line; d1 and d2 fused; a4 and d3 ending blindly, d3 well developed. Posterior jaw is comprising five sclerites (f, g1, g2, i, k). f with expanded outer lateral flanges. Unmodified, g1 and g2 symmetrical, with paired g1i, g2k; diaphragm present. Posterior wall of jaw present with longitudinal and transverse sclerotised ridges of 7-9 concentric arcs and highly muscular pad in clamp cavity. Terminal lappet and larval hooks are absent in adult specimens.

Mouth sub-terminal, length 97-119 (108), width 50-57 (53). Two well developed oral suckers, length 59-67 (63),

width 49-58 (54). Pharynx short, bulb like, length 111-124 (117), width 71-82 (76) followed by long tube like oesophagus, length 88-100 (94) and width 68-79 (74). Oesophagus bifurcates into intestinal caeca at level of genital atrium. Two branches of intestinal caecum reaches up to last pair of clamp, but not united to each other and separated by close margins.

Testes post-ovarian, intercaecal, large, roughly rounded, 37-47 (42) in number, diameter 52-107 (80), occupying around 1/3rd of the body proper. Vas deferens tubular terminating into gential atrium anteriorly, 951-997 (974) long. Genital atrium cup-shaped, 53-70 (62) long by 42-53 (47) wide, located at the level of intestinal bifurcation. Atrium armed with 8 spines, each spine with pointed inner and broader outer ends, spine length 14-25 (20).

Ovary long, tubular, 194-258 (232) long by 69-83 (76) wide, located at the about middle of the body. Oviduct short, unite with genito-intestinal canal and ootype. From ootype long and thick tube like uterus arises, terminating near the genital atrium, length 998-1110 (1054), width 46-55 (51).

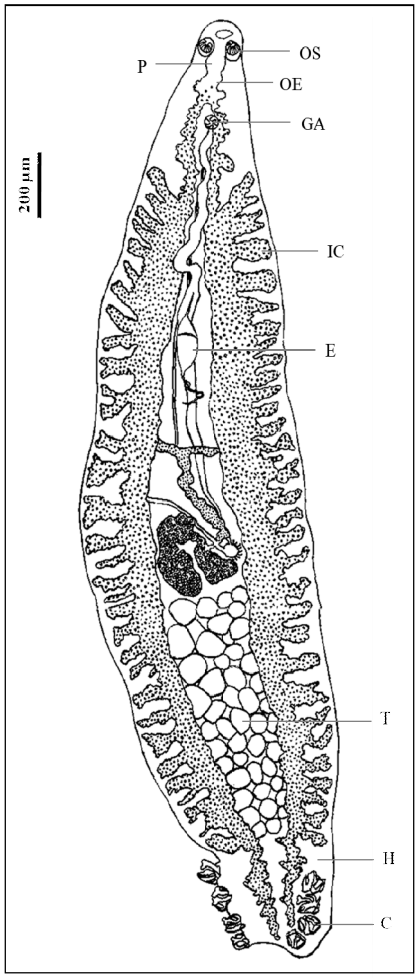

Fig 1: *Heterobothrium indicus* n. sp. composite line diagram (ventral view): OS- Oral sucker; P- Pharynx; OE- Oesophagus; GA- Genital atrium; IC- Intestinal caecaum; E- Egg; T- Testes; H- Haptor; C- Clamp.

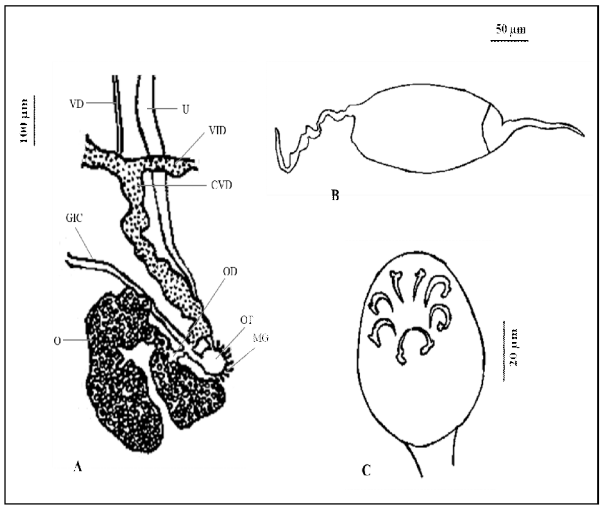

Fig 2: *Heterobothrium indicus* n. sp. line diagrams: A- Reproductive system; B- Egg and filaments; C- Genital atrium and spines. VD- Vas deferens; U- Uterus; VID- Vitelline duct; CVD- Common vitelline duct; GIC- Genito-intestinal canal; OD- Oviduct; OT- Ootype; O- Ovary; MG- Mehli's glands.

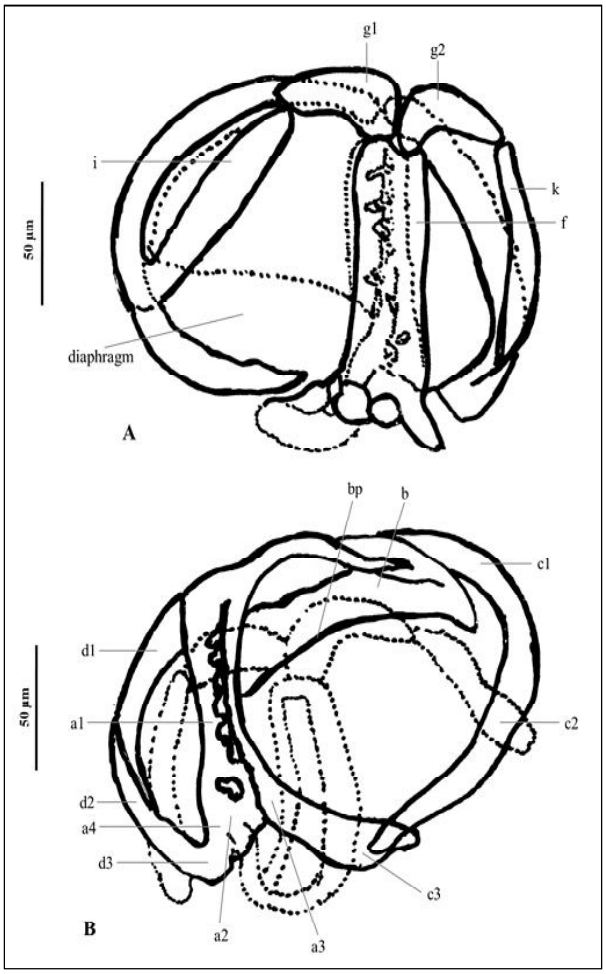

Fig 3: *Heterobothrium indicus* n. sp. detail morphology of clamp with sclerites, A- Camp (dorsal view); B- Clamp (ventral view).

Vitelline reservoir located above to ovary and give rise to common vitelline duct which bifurcated into vitelline ducts, each 90-112 (101) long. Vitelline follicles dispersed from the intestinal bifurcation to the haptoral region. Single observed egg, spindle shaped, operculated, length 217, width 91, geared with short, stout filament, length 87, while the opposite pole with longer and coiled filament, length 174.

Remarks

The last or posterior most pair of clamps as compared to other three pairs of clamps was smaller in *Heterobothrium tetrodonis*, *H. elongatum*, *H. torquigeneri*, *H. okamotoi*, *H. shinagawai* and the present new species. The overall clamp size of new species is very similar to *H. lineatus*. The genital atrium in new species is armed with 8 spines, which is similar to *H. victorwepeneri* (8-9), *H. tonkinensis* (7-9) and

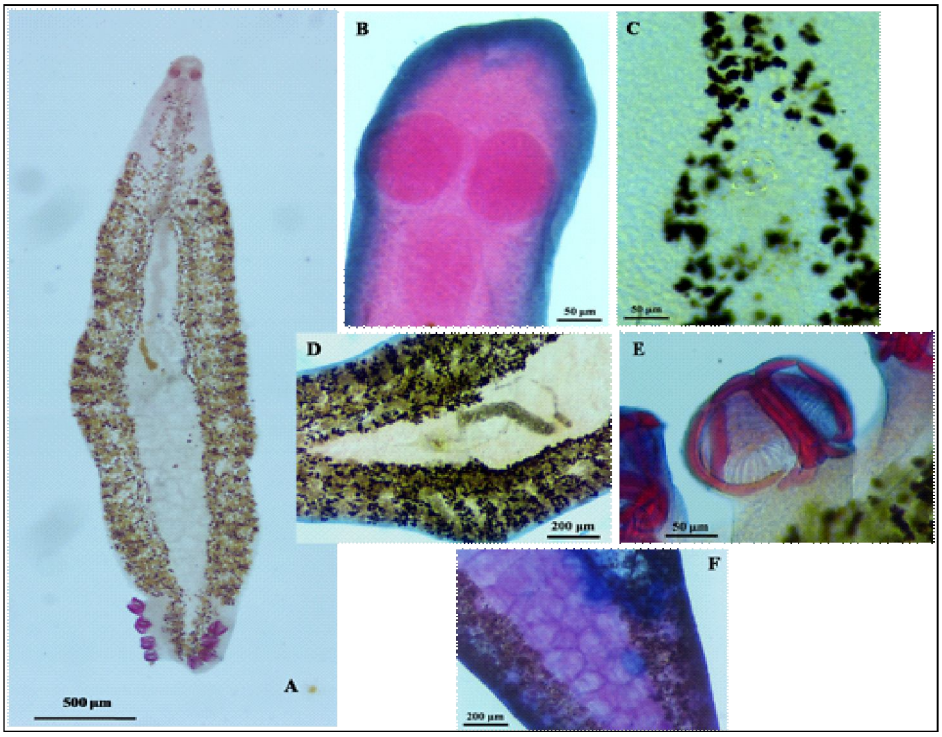

Fig 4: *Heterobothrium indicus* n. sp. digital photomicrographs.
A- Whole mount (ventral view); B- Anterior region with oral suckers, pharynx and oesophagus; C- Genital atrium and associated atrial spines; D- Mature egg; E- Clamp and associated sclerites; F- Testes.

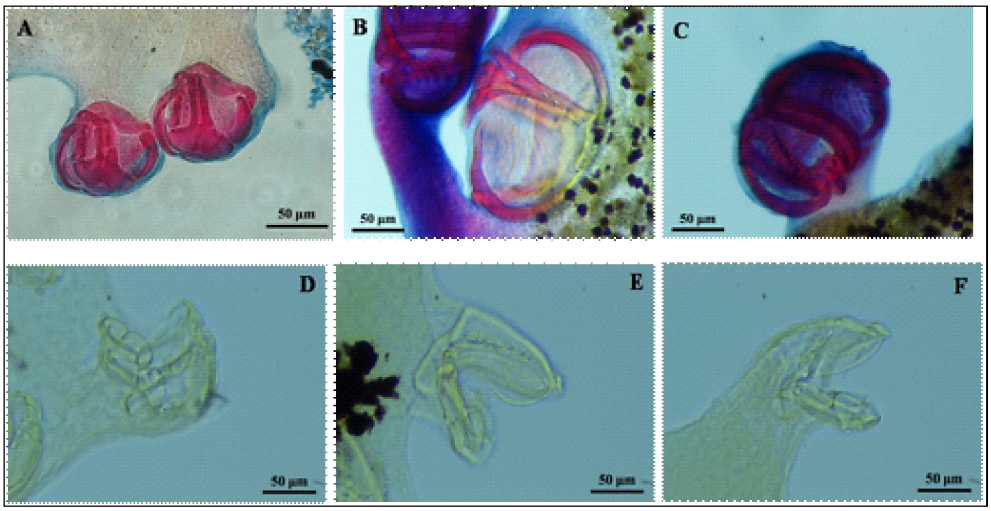

Fig 5: *Heterobothrium indicus* n. sp. digital photomicrographs of clamps.
A-C. Diclidophorid clamp in closed condition; D-F. Clamp in open state.

H. fluviatilis (6-9) while other species having higher number of atrial spines. The new species with 37-47 number of testes, was considerably similar to *H. torquigeneri* (34-44), *H.victorwepeneri* (40-50), *H. tetrodonis* (34-56) redescribed by Ogawa, 1991 and *H. ecuadori* (27-40). In contrast, the other species of *Heterobothrium* are having more number of testes except *H. fluviatilis* (6-12) and *H. lamothei* (15-26). The isthmus is absent in new species but present in *H. tetrodonis*, *H. elongatum* and *H. okamotoi*.

Polyopisthocotylean parasites are extremely host specific and are the suitable model to study the issues of host/parasite co-evolution (Šimkova *et al.,* 2006; Tambiredy *et al.,* 2016). Presently, all the species of *Heterobothrium* are highly host specific to fish family Tetraodontidae, except the new parasitic species described from *Upeneus moluccesnsis* of family Mullidae (Mladineo and Maršič-Lučič, 2006; Mandeng *et al.,* 2015).

Molecular study (Fig 6, 7, 8 and Table 1)

BLASTn searches for *Heterobothrium indicus* n. sp. for 18S rDNA expressed maximum similarity of 93.67, 93.29, 92.53 and 92% with *Diclidophora minor*, *Neoheterobothrium hirame*, *Neoheterobothrium* sp. and *Heterobothrium okmotoi*, respectively. In case of 28S rDNA the new species revealed 87.38, 87.32, 86.22 and 84.52% similarity with *Neoheterobothrium* sp., *Sauricotyle sprostoni*, *Diclidophora minor* and *Heterobothrium* sp., respectively. The mt COI gene sequence of *H. indicus* n. sp. revealed the identity of 81.38 and 80.15% with *Paradepocotyle prolatili* and *Pedocotyle bravoi*, respectively.

In case of 18S rDNA evolutionary tree, the new species form separate clade with *Choricotlye australiensis*, not with *Heterobothrium okamotoi*. The phylogram of 28S rDNA of *H. indicus* n. sp. separately claded with *Neoheterobothrium* sp., not with *Heterobothrium* sp. From the NJ/ME analysis

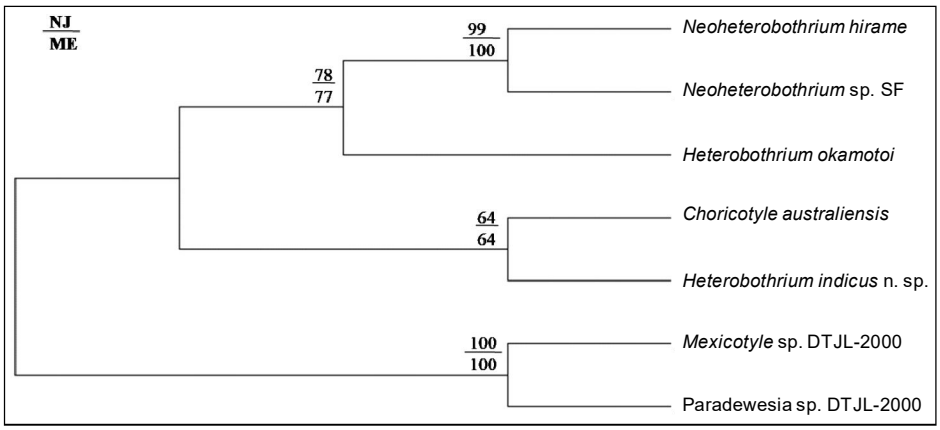

Fig 6: Phylogenetic tree based on partial 18S rDNA through neighbor joining (NJ) method. The similar tree toplogy was also obtained by minimum evolution (ME) method. The optimal tree with the sum of branch length is 8.22017808. There were a total of 1973 positions in the final dataset.

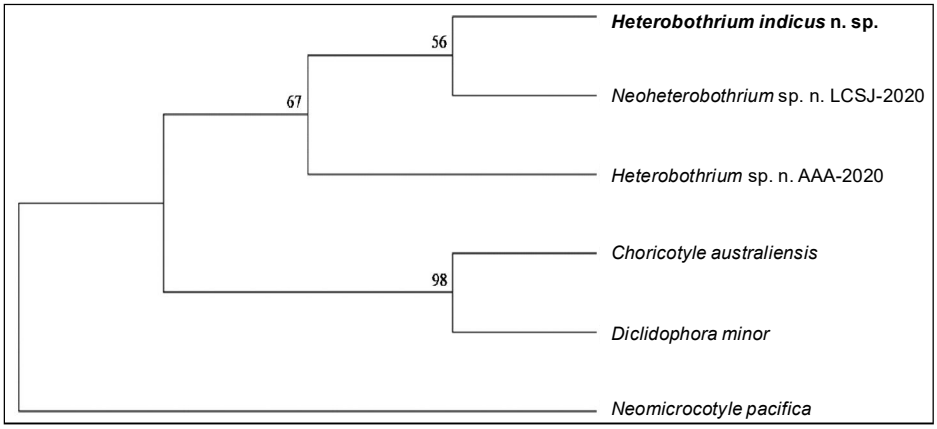

Fig 7: Phylogenetic tree based on partial 28S rDNA through maximum parsimony method. The most parsimonious tree length is 3097. The consistency index is (0.735679), the retention index is (0.345251) and the composite index is 0.279925 (0.253994) for all sites and parsimony-informative sites (in parentheses). There were a total of 1495 positions in the final dataset.

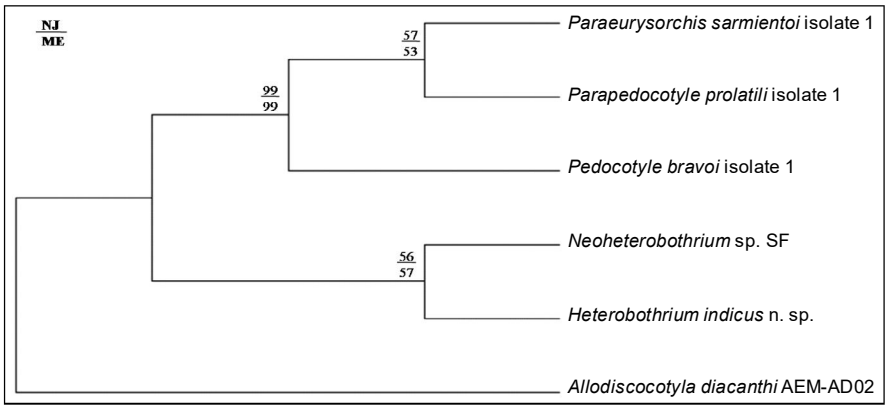

Fig 8: Phylogenetic tree based on mt COI gene through through neighbor joining (NJ) method.
The similar tree toplogy was also obtained by minimum evolution (ME) method. The optimal tree with sum of btranch length is 8.0382496. There were a total of 420 positons in the final dataset.

of mt COI gene of new monogenean forming separate clade with *Neoheterobothrium* sp. Other genes like ITS1+5.8S and ITS2+5.8S were not included in the study due to unavailability of sequences in database.

CONCLUSION

Heterobothrium indicus n. sp. is the first monogenean parasite reported from India, infesting the goatfish *Upeneus moluccensis*, while the other reported species from various parts of the world, infecting the puffer fishes of Tetraodontidae. After comprehensive analyses, the new species is allocated to subfamily Choricotylinae (Diclidophoridae) and can be differentiated with other species based on the number of atrial spines and testes. The molecular findings of *Heterobothrium indicus* n. sp. for 18S rDNA, 28S rDNA and mt COI genes revealed close proximity with species in Diclidophoridae. The more number of gene sequences of the genus *Heterobothrium* will provide better perspective of evolutionary relationships.

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