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Research Note

Molecular characterization of three species belongs to the Allocreadioidea, Hemiuroidea and Plagiorchioidea (Platyhelminthes: Trematoda) infecting freshwater fishes in India

A. CHAUDHARY*, S. MUKUT, H. S. SINGH

Molecular Taxonomy Laboratory, Department of Zoology, University Road, Chaudhary Charan Singh University, Meerut (U.P.), 250004, India, *E-mail: *anshu8282@rediffmail.com*

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Summary

Three species of digenetic trematodes are redescribed based on specimens collected from the intestine of freshwater fishes of Hastinapur and Meerut (U.P.), India: *Allocreadium handiai* (Pande, 1937) Madhavi, 1980 (Allocreadioidea: Allocreadiidae) from *Mystus tengara* (Hamilton, 1822) (Siluriformes: Bagridae), *Genarchopsis goppo* Ozaki, 1925 (Hemiuroidea: Derogenidae) and *Phyllodistomum chauhani* Motwani & Srivastava, 1961 (Plagiorchioidea: Gorgoderidae) from *Channa punctata* (Bloch, 1793) (Perciformes: Channidae). The three species were subjected to morphological, morphometric and molecular analyses. The morphological study revealed that *A. handiai*, *G. goppo* and *P. chauhani* can be distinguished by their congeners on the basis of their morphology. Partial nucleotide sequences of the 28S ribosomal RNA gene were obtained from the three trematode species and deposited in the GenBank. A phylogenetic reconstruction based on the 28S rRNA gene placed the three studied species within their respective families and their validity is discussed. For the first time molecular data of newly collected material of these species from India were used for confirmation of their validity and to assess their phylogenetic relationships.
Keywords: Trematoda; *Mystus tengara; Channa punctata;* morphology; DNA; India

Introduction

During a survey of the trematode parasites of freshwater fishes in Meerut and Hastinapur (U.P.), India, we have found that the tengara catfish, *Mystus tengara* (Hamilton, 1822) were commonly infected with the species of *Allocreadium* Looss, 1900 while spotted snakehead, *Channa punctata* (Bloch, 1793) was frequently infected with species of *Genarchopsis* Ozaki, 1925 and *Phyllodistomum* Braun, 1899. The host fish, *M. tengara* and *C. punctata* is an economically important food fish and available in India throughout the year. Further morphological examination has identified the specimens as *Allocreadium handiai* (Pande, 1937) Madhavi, 1980; *Genarchopsis goppo* Ozaki, 1925 and *Phyllodistomum chauhani* Motwani & Srivastava, 1961 respectively which was redescribed herein. Besides the morphological data, molecular analysis supplements the study with 28S rRNA gene analysis. This is the first molecular investigation of any species of *Allocreadium*, *Genarchopsis* and *Phyllodistomum* from India.

Many species of *Allocreadium*, *Genarchopsis* and *Phyllodistomum* were described from India, but the available descriptions are not as much of informative to clear the identification at the species level. In India, identification of these parasites is problematic due to the poor morphological descriptions and the lack of molecular data. Nevertheless, descriptions of the species belongs to these genera were based only on improper morphology. In India, for platyhelminthes taxonomy morphological differences have been widely used to discriminate between species. Though, traditional diagnostic tools are now complemented by molecular techniques

for resolving the taxonomic confusions coupled with species descriptions (Prasad *et al*., 2011; Chaudhary *et al*., 2015; Verma *et al*., 2016; Chaudhary *et al*., 2016).
In India, there is a scarcity of molecular tools in studies of trematode parasites. In the present study, we conducted morphology and molecular study of identifying *A. handiai*, *G. goppo* and *P. chauhani* infects *M. tengara* and *C. punctata* in Hastinapur and Meerut, Uttar Pradesh (UP), India.

Materials and Methods

Mystus tengara and *Channa punctata* were purchased from fish grocers of the fish market in Hastinapur (29.1700° N, 78.0200° E) and Meerut (29° 01' N, 77° 45' E), UP, India respectively during April to June 2012 and July to September 2014. Identification of host fish was carried out by ichthyologists. The fish were killed by a sharp hit on the top of the head, dissected; intestine and urinary bladder were carefully removed and placed in lukewarm water to relax the parasites, then parasites were flattened, fixed in 70 % alcohol, stained with acetocarmine, dehydrated through ascending grades of alcohol and mounted in Canada balsam. For molecular study, some samples of parasites were fixed in 95 % ethanol in vials after observation under microscope. Illustrations were made with the help of a camera lucida and light microscope (Motic SMZ-168, Xiamen, China) equipped with digital image analysis system (Motic Image Plus 2.0). All measurements given in millimeters unless otherwise stated. The specimens were deposited in the Museum, Department of Zoology, Chaudhary Charan Singh University, U.P., India and Natural History Museum, Geneva, Switzerland.
For DNA extractions, specimens preserved in 95 % ethanol were centrifuged and excess ethanol removed from the vials. Genomic DNA was isolated using the DNeasy™ Tissue Kit (Qiagen, Hilden, Germany), following manufacturer's instructions. The 28S ribosomal DNA was amplified using the primers, LSU5 (F) (5'-TAGGTCGACCCGCTGAAYTTAAGCA-3') (Bray *et al.,* 2009) and 1500R (5'-GCTATCCTGAGGGAAACTTCG-3') (Tkach *et al.,* 2003) in a 25 µl reaction mixture consist of 3 µl genomic DNA, 4 µl 1 mM deoxyribonucleotide triphosphates (dNTPs, Biotools, Spain), 0.6 µl of each primer, 2.5 µl of 10x Taq buffer (Biotools, Madrid, Spain), 0.5 µl of Taq polymerase (1U; Biotools, Madrid, Spain) and 13.8 µl of distilled water. The PCR cycling profile were performed in a Mastercycler personal-22331 (Eppendorf, Hamburg, Germany) as follows: an initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 40 s, 56 °C for 1 min, 72 °C for 1 min, completed with terminal extension at 72°C for 7 min and then stored at 4 °C. PCR products were electrophoresed in 1 % agarose gel in Tris-Acetate-EDTA buffer gel stained with 1 % ethidium bromide. Amplified DNA was purified with the Purelink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen, Germany) and was sequenced with the above primers using Big Dye Terminator vr. 3.1 cycle sequencing kit in ABI 3130 Genetic Analyzer, Applied Biosystems (Foster City, California, USA).
The sequences generated were aligned using the CLUSTAL W algorithm (Thompson *et al.,* 1994) implemented in the MEGA 6.06 (Tamura *et al.,* 2013) and assembled. A BLAST search revealed similar sequences to species of *Allocreadium*, *Genarchopsis* and *Phyllodistomum* and were downloaded from the Genbank. DNA pairwise distances were calculated using the p-distance model. Alignment of the rDNA 28S gene was then subjected to maximum likelihood (ML) and Bayesian inference (BI) analyses using MEGA 6.06 (Tamura *et al*., 2013) and Topali 2.5 (Milne *et al.,* 2009). For maximum likelihood analysis, model testing of the data was performed as a model of best fit by the Akaike Information Criterion and the GTR + I + G was chosen using MEGA 6.06. Bootstrap values based on 1,000 resampled datasets were generated. For Bayesian inference analysis, substitution model tested by the Bayesian Information Criterion and GTR + I + G was chosen. BI analysis was run for 1,000,000 generations, sampling every 100th tree and discarding 'burn in' first 25 % of the sampled tree. *Metagonimoides oregonensis* (JQ995473), *Plagiorchis elegans* (KF556678) and Echinostomatidae sp. (KU896138) were chosen as an out-group in the final alignments.

Results and Discussion

A total of 17 *A. handiai* specimens were collected from 14 infected host fish, *M. tengara* out of 22. Total of 36 specimens of host fish, *C. punctatus* were collected from which 09 were infected with *G. goppo* (13 specimens) and 08 with *P. chauhani* (11 specimens). Re-description of the parasites based on ten specimens is as follows:

Allocreadium handiai (Pande, 1937) Madhavi 1980 (Allocreadioidea: Allocreadiidae) (Fig. 1A)
Description: Body elongated, smooth, rounded at extremities 1.77 (1.50 – 1.80) long, 0.44 (0.40 – 0.55) wide. Oral sucker spherical or ovoid, 0.24 (0.20 – 0.35) long, 0.30 (0.20 – 0.40) wide. Ventral sucker spherical, pre-equatorial, equal or larger than oral sucker 0.27 (0.20 – 0.32) long, 0.25 (0.20 – 0.35) wide. Prepharynx absent. Pharynx muscular, ovoid, 0.06 (0.04 – 0.08) long, 0.06 (0.04 – 0.08) wide. Oesophagus tubular, straight, 0.08 (0.06 – 0.09) long, intestinal caeca, simple, extending up to posterior region of body. Genital pore median or submedian, intercaecal. Testes two, muscular, oval or spherical, tandem and post-equatorial. Anterior testis post-ovarian 0.30 (0.15 – 0.40) long, 0.24 (0.22 – 0.35) wide. Posterior testis smaller, close or slightly apart from anterior testis 0.37 (0.20 – 0.45) long, 0.31 (0.20 – 0.50) wide. Ovary single, oval or spherical, pre-equatorial, pre-testicular, lying between ventral sucker and anterior testis measures 0.29 (0.15 – 0.35) long, 0.23 (0.20 – 0.35) wide. Vitellaria follicular extends from behind intestinal bifurcation up to the posterior region of the body. Vitellaria mainly lateral in position anterior to ventral sucker, but behind posterior testis filling intercaecal space. Eggs oval, operculated 0.14 (0.10 – 0.16) long, 0.05 (0.02 – 0.08) wide.

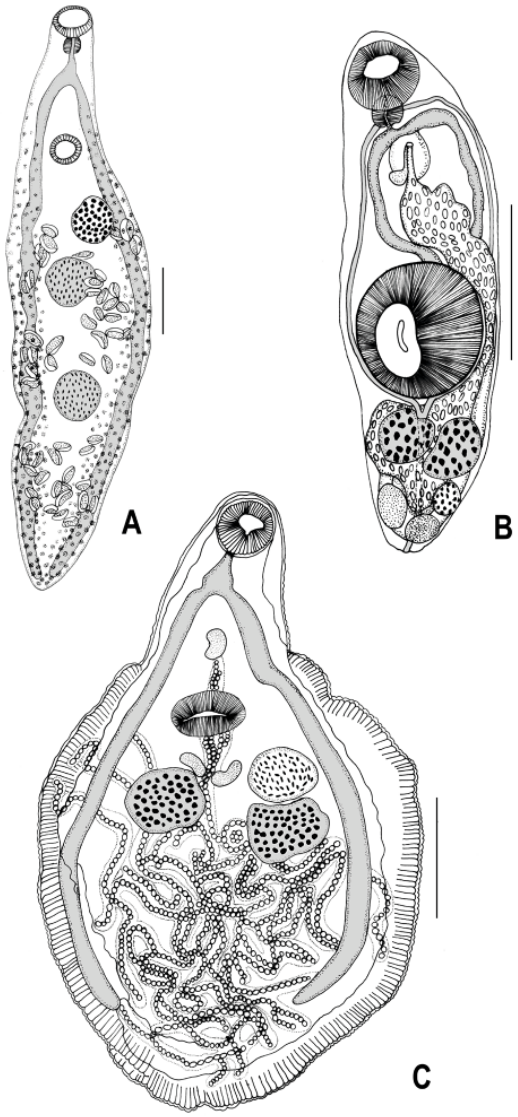

Fig. 1. Line drawings of (A) *Allocreadium handiai* (Pande, 1937) Madhavi 1980. (B) *Genarchopsis goppo* Ozaki, 1925. (C) *Phyllodistomum chauhani* Motwani and Srivastava 1961. Scale bars A), 0.10 mm; B), C), 0.50 mm

Host: *Mystus tengara* (Siluriformes: Bagridae); *Prevalence*: 64 % (14 infected out of 22); *Intensity of infection*: 1.21 (17 parasites were found from 14 infected hosts); *Site:* Intestine; *Locality:* Hastinapur (29.1700° N, 78.0200° E), Uttar Pradesh, India. Permanent slides of this parasite were deposited in the Museum of the Department of Zoology, Chaudhary Charan Singh University, Meerut, U.P., India (HS-TR/2015/01) and Natural History Museum, Geneva, Switzerland (MHNG-PLAT-94112).

Remarks: Looss (1900) erected the genus *Allocreadium* (Digenea: Allocreadiidae) with *Allocreadium isoporum* as type species. On the basis of morphology, many workers (Pande 1937, 1938; Rai 1962; Sen & Siddiqui 2010) described several species of *Allocreadium* and the number is still going on. More than 15 species have been already described in India under *Allocreadium*. It is considered that both the genera *Allocreadium* and *Polylekithum* are synonymous.

Molecular characterization: We obtained 28S sequence (1290 bp) of *A. handiai*, submitted to the GenBank under the accession number KX344072 and compared it with the sequences available in the GenBank database. All the GenBank sequences of species of *Allocreadium* that shows similarity with *A. handiai* were obtained from Russia, USA, Mexico and UK. The 28S rDNA based estimation of evolutionary divergence within the species of *Allocreadium* and *A. handiai* ranged from 0.23 – 0.24 % base substitution differences. The phylogenetic relationships based on the 28S rRNA gene of *Allocreadium* specimens were analyzed on the basis of ML and Bayesian inference and the two trees obtained generally showed similar topologies. Both methods used for the phylogenetic reconstruction produced same results as *A. handiai* clustered together with the other species of the same genus with well supported bootstrap values (100/1.00) (Fig. 2 A). The species of *Allocreadium* form a monophyletic clade with 100 % bootstrap support (Fig. 2 A). This is the first molecular data for any *Allocreadium* species reported from India.

Genarchopsis goppo Ozaki, 1925 (Hemiuroidea: Derogenidae) (Fig. 1B)

Description: Body elongated, muscular, cylindrical 1.28 (1.15 – 1.32) long, 0.43 (0.40 – 0.50) wide with a maximum width at middle or just above acetabulum, anterior and posterior ends blunt. Oral sucker ovoid, 0.25 (0.20 – 0.30) long, 0.26 (0.20 – 0.35) wide. Ventral sucker muscular, submedian, rounded 0.50 (0.40 – 0.60) long and 0.47 (0.35 – 0.65) wide. Ventral sucker larger than oral sucker. Pre-pharynx absent, pharynx well developed, oesophagus very short, intestinal caeca long, reaching far back and unite together behind or lateral to ovary. Testes two in number, larger than ovary, rounded or transversely elongated, situated behind ventral sucker. Right testis intercaecal or extracaecal and measures 0.20 (0.15 – 0.25) mm long and 0.19 (0.15 – 0.25) mm wide. Right testis also intercaecal or extracaecal or overlaps the left caecum measures 0.25 (0.20 – 0.30) long and 0.16 (0.10 – 0.30) wide. Cirrus sac absent. Ovary roughly rounded, submedian, post acetabular, intercaecal, post testicular measures 0.08 (0.5 – 0.15) long and 0.15 (0.10 – 0.25) wide. Uterus intercaecal as well as extracaecal, occupies most of pre-acetabular and also post-acetabular area. Vitellaria consist of two rounded or lobed compact glands situated near posterior extremity. Right vitellaria 0.12 (0.5 – 0.15) long and 0.10 (0.5 – 0.15) wide, left vitellaria 0.11 (0.8 – 0.20) long, 0.10 (0.5 – 0.15) wide. Eggs round, oval 0.05 (0.01 – 0.10) long and 0.02 (0.01 – 0.05) wide.

Host: *Channa punctatus* (Perciformes: Channidae); *Prevalence*: 25 % (09 infected out of 36); *Intensity of infection*: 1.44 (13 para-

sites were found from 09 infected hosts); *Site:* Intestine; *Locality:* Meerut (29° 01' N, 77° 45' E), Uttar Pradesh, India. Permanent slides of this parasite were deposited in the Museum of the Department of Zoology, Chaudhary Charan Singh University, Meerut, U.P., India (HS-TR/2015/02) and Natural History Museum, Geneva, Switzerland (MHNG-PLAT-94113).

Remarks: The genus *Genarchopsis* Ozaki, 1925 (Digenea: Derogenidae) found parasitic in freshwater fish but also present although rarely in reptiles and amphibians. Species of this genus is distributed in East, Southeast and South Asia, including India (Ozaki 1925; Yamaguti 1971; Gupta 1951; Rai 1971; Pande 1973).

Molecular characterization: The 28S sequence (1150 bp) obtained from this study of *Genarchopsis goppo* was submitted to the GenBank under the accession number KX344073. After performing a BLAST search it was found that no 28S sequence is available for any species of *Genarchopsis* on GenBank database. As a result, *G. goppo* obtained in this study was found to be closely related with the species of *Thometrema* i.e., *T. lotzi* as both belongs to the same family Derogenidae under order Azygiida (Fig. 2 B). The 28S sequence analysis yielded a generally similar tree topology by both methods, ML and BI. *G. goppo* clustered in a separate clade, distinct but its phylogeny will more clear after performing future studies as more data should be needed for the 28S gene of this group of parasites. This is the first molecular sequence from India for any species of *Genarchopsis* and first 28S sequence for *G. goppo* available on GenBank database.

Phyllodistomum chauhani Motwani and Srivastava, 1961 (Plagiorchioidea: Gorgoderidae)
(Fig. 1C)

Description: Body spatulate, divisible into a narrow tubular forebody and expanded spatulate hind end 1.87 (1.62 – 1.94) long, 1.15 (1.10 – 1.20) wide with wavy margin. Anterior part of body narrow, elongated 0.33 (0.30 – 0.45) long, 0.23 (0.20 – 0.25) wide, posterior part spatulated 0.72 (0.60 – 0.80) long, 0.64 (0.55 – 0.70) wide. Oral sucker terminal, oval, sub-spherical, mouth opening ventrally, no noticeable papillae on oral sucker 0.28 (0.25 – 0.35) long, 0.26 (0.20 – 0.30) wide. Pre-pharynx absent, pharynx oval, oesophagus short, tubular. Ventral sucker spherical 0.21 (0.15 – 0.30) long, 0.29 (0.20 – 0.35) wide, larger than oral sucker. Vitelline glands two, posterior-lateral to ventral sucker, left vitelline gland closer to ovary, right vitelline gland 0.22 (0.15 – 0.35) long, 0.15 (0.5 – 0.25) wide, left vitelline gland measures 0.16 (0.10 – 0.25) long, 0.10 (0.05 – 0.25) wide. Testes two, located at the broadest part of the body, post-equatorial, tandem and deeply lobed, present between two intestinal caeca. Anterior testis is larger than posterior one, anterior testis 0.27 (0.25 – 0.35) long, 0.21 (0.15 – 0.28) wide, posterior testis 0.25 (0.20 – 0.30) long, 0.21 (0.10 – 0.30) wide. Ovary lobed, post-equatorial, inter-caecal, just behind left vitelline gland, tandem to posterior testis 0.23 (0.15 – 0.30) long, 0.27 (0.20 – 0.35) wide. Genital pore median just below intestinal bifurcation, excretory bladder tubular, excretory pore median. Eggs oval, measures 0.05 (0.02 – 0.015).

Host: *Channa punctatus* (Perciformes: Channidae); *Prevalence*: 22 % (08 infected out of 36); *Intensity of infection*: 1.37 (11 parasites were found from 08 infected hosts); *Site:* urinary bladder; *Locality:* Hastinapur (29.1700° N, 78.0200° E), Uttar Pradesh, India. Permanent slides of this parasite were deposited in the Museum of the Department of Zoology, Chaudhary Charan Singh University, Meerut, U.P., India (HS-TR/2015/03) and Natural History Museum, Geneva, Switzerland (MHNG-PLAT-94114).

Remarks: Phyllodistomum Braun, 1899 (Trematoda: Gorgoderidae) is the largest genus that comprises more than 110 species and is one of the two largest genera of trematodes (Kudinova 1994; Cribb *et al.,* 2002). Species of *Phyllodistomum* are generally parasitic the urinary bladder of fishes, but sometimes infect amphibians and reptiles (Beilfu 1954; Rai 1964; Schell 1967; Wanson & Larson 1972; Hoffman 1999). Currently, more than 10 species of *Phyllodistomum* found from India based on morphology.

Molecular characterization: 28S rDNA sequence (1300 bp) was obtained from trematode of the genus *Phyllodistomum, i.e., P. chauhani* and submitted to the GenBank with the accession number KX344074. The pairwise distances showed differences between *P. chauhani* and other cogeners with sequence identities reaching only 0.09 – 0.16 % respectively. The ML and BI phylogenetic analysis of 28S rDNA generated a phylogenetic tree with all topologies well supported (75/0.86) (Fig. 2 C). The tree revealed that *P. chauhani* formed a well-supported clade included the *P. centropomi* (KM659384) and *P. cf. symmetrorchis* (KF013171) from Mexico and Kenya. According to our results, the genetic differences observed for 28S rDNA confirm the independent status of *P. chauhani* and indicate a distinct clade for Indian species within the genus *Phyllodistomum*.

This study is an integrative taxonomy approach used to characterize the species of Allocreadiidae, Derogenidae and Gorgoderidae trematodes that parasitize freshwater fishes in Meerut and Hastinapur, Uttar Pradesh (U.P.), India. The present study provides the first molecular identification of *A. handiai* (from the intestine of *M. tengara*), *G. goppo* (from intestine of *C. punctatus*) and *P. chauhani* (from urinary bladder of *C. punctatus*) from India that have been previously published on the basis of morphology only. Currently, the DNA based taxonomy is a reliable tool for identification of species. Molecular data related to these families from India is still in infancy, that's why this study has been performed, obtained sequences of 28S rRNA gene for the molecular phylogeny. The phylogenetic tree based on partial 28S rDNA sequences for above three species strongly supported in both analyses.

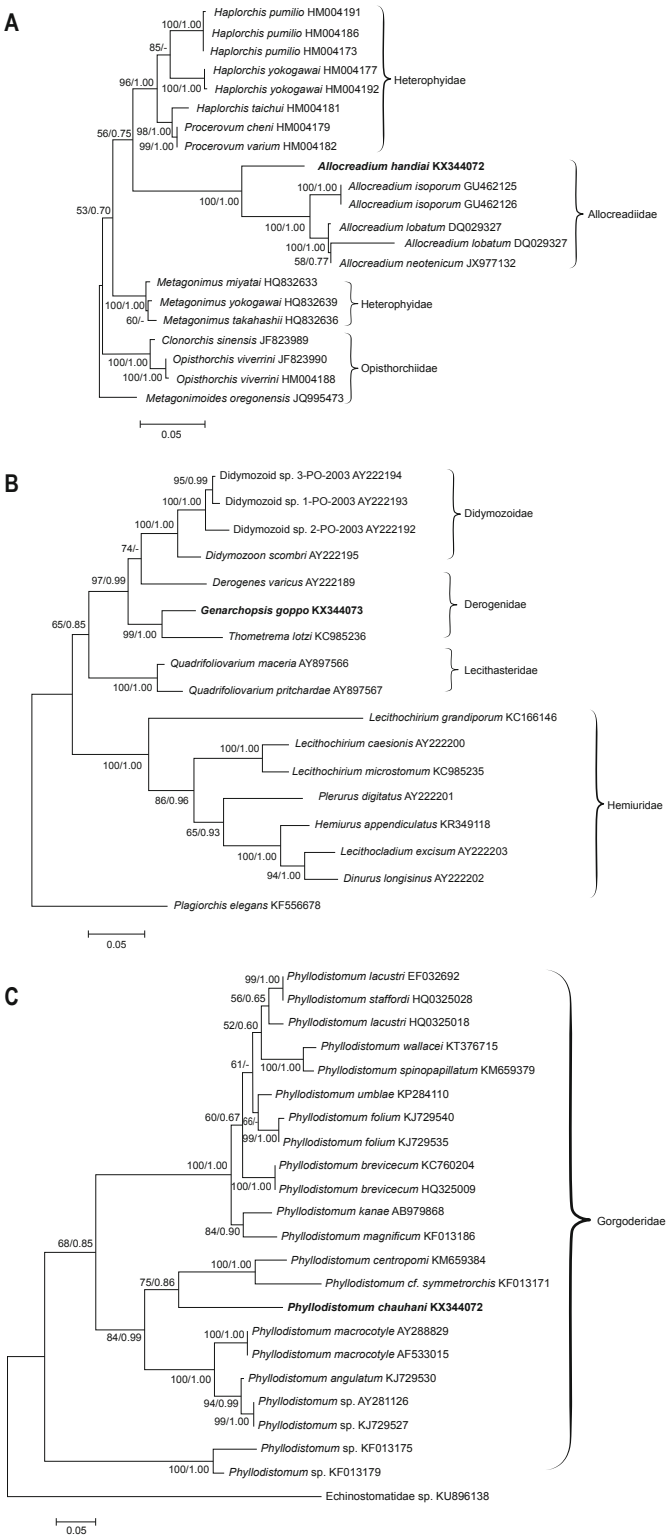

Fig. 2. Phylogenetic tree (A) *Allocreadium handiai* (B) *Genarchopsis goppo* and (C) *Phyllodistomum chauhani* showing the relative position of trematode spp. from India obtained in this study (shown in bold). Topology of tree generated by maximum likelihood (ML) analysis of the 28S rDNA sequences; bootstrap values (1000 replications) and posterior probabilities showed at nodes; numbers separated by slash near the nodes represents the bootstrap support values as: ML/BI respectively. Unsupported nodes by BI are marked with a hyphen and accession numbers are given next to species name. The scale bar indicates the expected number of substitutions per site.

Comparative analysis of the nucleotide sequences of *A. handiai* from India and other cogeners showed 0.23 – 0.24 % variable sites in the 28S gene. The genetic differentiation between the *G. goppo* and *Thometrema lotzi* were 0.07 % of the 28S rRNA gene sequence data. A phylogenetic relationship based on the 28S gene sequence data showed a clear clade generated for Indian *Phyllodistomum* species, *P. centropomi* (KM659384) and *P. cf. symmetrorchis* (KF013171) appears to be the sister species in the clade. Thus, our molecular data support the validity of *A. handiai*, *G. goppo* and *P. chauhani* from Hastinapur and Meerut, UP, India. However, for the delineation of future phylogenetic inferences, the addition of more data from different genes is required.

The analyzed sequence of *A. handiai* was interpreted and concluded that it is valid and resurrected taxonomically. The species *G. goppo* phylogenetic position based on 28S gene could be confirmed and it was identified tentatively and the taxonomic validity was confirmed. Partial sequences of the 28S ribosomal RNA of *P. chauhani* collected from the urinary bladder were compared with sequences of other congeneric species. It is thus clear that where possible, in future, descriptions or redescriptions of species should be accompanied by molecular data.

In summary, more morphological studies along with molecular analyses are required to make clear the stability of the morphological characters used for the identification. Furthermore, our analyses substantiate the requisite of getting more molecular data for other representatives of genus *Allocreadium*, *Genarchopsis* and *Phyllodistomum* from India.

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