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A New Record of *Cornudiscoides proximus* Gussev, 1976, a Freshwater Monogenean and its Phylogeny, Inferred from Partial Sequence of 28S rDNA

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Abstract.- The present communication deals with the study of a monogenean *Cornudiscoides proximus* Gussev, 1976 collected from the gill filaments of *Mystus vittatus* (Bloch, 1794) from Hastinapur, U.P., India. Gussev (1976) described this monogenean from the gill filaments of the same host at Lucknow. Agrawal and Vishwakarma (1996) re-described this species in detail from the type host and locality and added *M. tengra*, as an additional host. Besides recording the species in a new locality, phylogenetic analysis based on partial 28S rDNA sequence of *C. proximus* Gussev, 1976 was also conducted, using neighbour joining and maximum parsimony in order to investigate the validity of this species in the genus. *Cornudiscoides* Kulkarni, 1969.

Keywords- *Cornudiscoides*, 28S rDNA gene, Dactylogyridea, *Mystus vittatus*, Bagridae, Siluriformes, Dactylogyridae

INTRODUCTION

Monogeneans are obligate parasites of aquatic and semi-aquatic organisms because they are unable to withstand desiccation (Bychowsky, 1957). Fish form the main host group for the majority of known monogeneans (Euzet and Combes, 1980; Lim, 1998). Siluriforms are important both as food and source of income. During a general survey of siluriform fishes of the Hastinapur, we came across specimens of *Mystus vittatus* (Bloch, 1794), infected with dactylogyrid monogeneans belonging to the genus *Cornudiscoides* Kulkarni, 1969. On subsequent study, this parasite was found to *C. proximus* Gussev, 1976. Agrawal and Vishwakarma (1996) re-described this species from the type host and locality and synonymized *C. raipurensis* Dubey *et al*., 1992 and *Neomurraytrema shuklai* Agrawal and Singh, 1985 with it. The present worm is recorded on the type host from a new locality only. Besides, morphology and morphometry, 28S rDNA sequence was worked that provided a very effective and useful tool in distinguishing monogeneans at the species level.

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Evolution of rDNA is relatively independent of changes in morphology, and analyses of genetic data have been shown to provide good phylogenetic resolution (Nadler, 1992). In addition, the analysis of rDNA nucleotide sequences has recently been used to assess phylogenetic relationships among taxa of both higher and lower organisms (Hillis and Dixon, 1991; Sidow and Thomas, 1994). Therefore, during the course of study, phylogenetic relationship of *C. proximus* Gussev, 1976 were investigated using nucleotide sequences of the 28S rDNA region.

MATERIALS AND METHODS

Parasites were collected from the gills of *Mystus vittatus* (Bloch, 1794) from Hastinapur (29°01'N and 77°45'E), U.P., India as per method suggested by Malmberg (1970). They were studied. All the measurements are given in µm.

For genomic DNA extraction, specimens of Monogenea were fixed in either 95 or 100% ethanol. DNA was extracted from one parasite using the Qiagen DNeasy Tissue Kit as per the manufacturer's instructions. Polymerase chain reaction (PCR) for the amplification of ribosomal DNA was undertaken using the universal primers, forward (5'-ACCCGCTGAATTTAAGCAT-3') and the reverse primer (5'-CTCTTCAGAGTACTTTTCAAC-3'). A total volume of 25 µl was used for the PCR reaction. Each reaction contained 10X PCR buffer, 3 µl

template DNA, 1 U Taq polymerase (Biotools), 0.4 mM dNTP, 10 pM of each primer pair, and Milli-Q water. The PCR assay was carried out in a thermocycler (Eppendorf Mastercycler Personal) under the following conditions: after an initial denaturation at 94°C for 3 min (initial denaturation), 35 cycles of 94 ^{0}C for 30 sec (denaturation), 56°C for 45 sec (annealing), 1 min for 72°C (extension) and a final extension for 10 min at 72°C. An aliquot (3 µl) of amplicon was checked on 1.5% agarose-TBE gels, stained with ethidium bromide and visualized under ultraviolet light.

The amplification product was purified by Chromous PCR clean up Kit (#PCR 10) according to manufacturer's instructions. Both DNA strands were sequenced using a Big Dye Terminator version 3.1 cycle sequencing kit in an ABI 3130 Genetic Analyzer using the same primers.

Sequences were uploaded on NCBI to search for the most similar reference sequences and positions of the 28S gene were determined with the help of BLAST (available at www.ncbi.nlm.nih.gov). Subsequently, nucleotide sequences of various species were aligned using the alignment tool Clustal W (Thompson *et al.*, 1994). Data were analyzed using maximum parsimony (MP) and neighbor joining (NJ) methods by using MEGA version 4.0 (Tamura *et al*., 2007). The long subunit of rDNA gene sequence of *C. proximus* Gussev, 1976 extracted in this study was 362 base pairs and is deposited in GenBank under the accession no. GQ925913.

RESULTS

Cornudiscoides proximus Gussev, 1976

Type host	*Mystus vittatus* (Bloch, 1794)
Type locality	Lucknow
Additional Locality	Raipur, Gorakhpur, Hastinapur, Meerut, U.P., India
Site of infection:	Gills

Type material

The paratype slides have been deposited in the museum of Department of Zoology (Voucher numbers. HS/Monogenea/2009/03), Ch. C.S. University, Meerut, U.P., India.

Redescription (Based on 15 specimens.)

Body elongate 475 (450-500) long, 85 (80-90) wide (Fig. 1). Body divided into anterior region, prohaptor, body proper and haptor. Anterior end equipped with four pairs of anterior gland duct endings and two pairs of eye spots. Anterior eye spots smaller. Pharynx spherical,, muscular, 25 (20-30) in diameter. Intestine simple, bifurcated, crura uniting posteriorly, slightly anterior to haptor.

Testis elongate- oval, inter-caecal, post-equatorial, 95 (90-100) long, 45 (40-50) wide. From the anterior border of testis a fine vas deferens arises, looped around left intestinal caeca and dilate to form a fusiform seminal vesicle. Seminal vesicle at level of vagina on opposite side of body, 55 (50-60) long 25 (20-30) wide, opening at base of male copulatory complex. The male copulatory complex 34 (33-35) long, comprising male copulatory tube and accessory piece. Copulatory tube curved, short, with slightly widened base. Accessory piece comprising two parts, one with groove in which copulatory tube glides, other with two claw like processes. Ovary globular, post-equatorial 45 (40-50) long, 55 (50-60) wide. Vagina dextral, funnel shaped provided with short tube.

Haptor is bilobed and distinctly set off from the body proper. The haptor measures 75 (70-80) in length and 85 (80-90) in width. The armature of the haptor comprises of two unequal pairs of anchor (dorsal and ventral), an unpaired dorsal transverse bar, a paired ventral transverse bar and seven pairs of hooks. The dorsal anchor is with well defined base, strong shaft and long recurved point approaching each other measuring 43 (40-46) in length. The base is divided into long inner root, measures 13 (12-14) and short and stumpy outer root which is not more than 2.5 (2-3). The shaft is more or less cylindrical and narrows into strongly recurved points, measures 22.5 (20-25) in length. Each dorsal anchor is attached with a small conical patch, measuring 4 (3-5). The dorsal transverse bar is straight, with widened end and slightly flattened center measuring 14 (13-15). The ventral anchors are 25 (20-30) in length, with broad base, divisible into curved inner root of 1.5 (1-2) and outer root of 3 (2-4) in length. The inner length of ventral anchor 15 (14-16), outer length 13 (12-14) and straight

Table I.- Comparative measurements of *C. proximus* Gussev, 1976.

Body features	Gussev (1976) (in µm)	Agrawal and Vishwakarma (1996) (in µm)	Present specimen (in µm)
Body:			
Length	500	280-480	450-500
Width	100	60-100	80-90
Pharynx		12-30	20-30
Haptor		55-125x40-145	70-80 x 80-90
Dorsal anchor:			
Total length	34-38	20-40	40-45
Outer length	25-28	18-36	
Main part	9-11		
Inner root	20-24	8-15	12-14
Outer root		2-4	2-3
Recurved point		12-25	20-25
Patch		3-9	3-5
Ventral anchor:			
Total length	33-36		20-30
Inner length		12-23	14-16
Outer length		8-18	12-14
Length of root	33-5.5		
Inner root		4-6	1-2
Outer root		2-5	2-3
Straight point		8-15	9-10
Dorsal bar	2.2-3.3 x 25-28	15-38	13-15
Ventral bar	2.2-3.3 x 34-38	22-40	30-36
Filament	11-12		22-23
Hooks		8-12, 15-30	3-5, 14-16
Testis		70-80 x 32-45	90-100 x 40-50
Ovary		40-65 x 35-50	40-50 x 50-60
Copulatory complex:			
Length	29-32	25-38	33-35
Diameter of initial part	2		
Medial part	1		
Accessory piece	24-30		
Vagina	8 x 2.5		
Seminal vesicle			50-60 x 20-30

point 9 (8-10). The paired ventral transverse bar 33 (30-36), stick shaped, wide in the middle, both halves, connected by long thin filament of 23 (22-24) in length. The hooks with a protruding heel, a long handle and sickle filament loop. Hooks of two types: 3rd pair 15 (14-16) and others 4 (3-5).

A comparative measurement of *C. proximus* Gussev, 1976, described by earlier earlier worlers and obtained in the present study are given in Table I.

Phylogenetic analysis

28S rDNA sequence was aligned using the

Table II.- Genbank reference sequences used in this study, their geographical origins as well as accession numbers.

Species	Location / source	Accession No.
Cornudiscoides proximus	India	GQ925913*
Thaparocleidus infundibulovagina	China	EF100548
Pseudancylodiscoides sp.HSY1	China	EF100542
Thaparocleidus varicus	China	DQ157668
Thaparocleidus magnicirrus	China	EF100549
Thaparocleidus sp.NY1	China	DQ157670
Bifurcohaptor sp.	India	GU830881

*Species sequenced in the present study

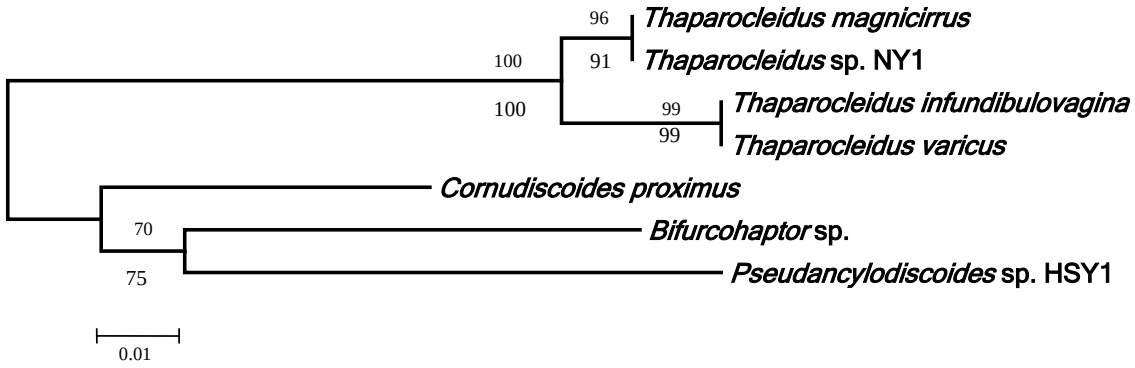

Fig. 1. Phylogenetic relationship of the species *C. proximus* inferred from the 28S region. Bootstrap values (as percentages) are shown at internal nodes. Above branch, NJ bootstrap values and below branch MP bootstrap values are shown. The scale bar indicates the proportion of sites changing along each branch.

Table III.- Kimura 2- parameter distances comparison of sequence differences (in %) in the 28S among different species.

	C. proximus	***B.* sp.**	***P.* sp.HSY1**	***T. magnicirrus***	***T. infundibulovagina***	***T. varicus***	***T.* sp.NY1**
C. proximus							
B. sp.	0.0994						
P. sp.HSY1	0.1209	0.1203					
T. magnicirrus	0.1277	0.1525	0.1616				
T. infundibulovagina	0.1371	0.1661	0.1709	0.0280			
T. varicus	0.1371	0.1661	0.1709	0.0280	0.0000		
T. sp.NY1	0.1277	0.1525	0.1616	0.0000	0.0280	0.0280	

clustal W to perform the phylogenetic analysis. The reference sequence which shows close similarity to *C. proximus* used in this study is listed in Table II. Pairwise comparisons were made by using Kimura-2 parameter model (Kimura, 1980) shown in Table III. The phylogenetic reconstructions inferred from analysis of the 28S rDNA sequences showed great resolution for the species of the monogeneans.

During the study in all 06 species of different monogeneans exhibit 90% nucleotide similarity with *C. proximus*. The sequences appeared to be the most closely related and is much more divergent with a well supported clade by neighbour joining (NJ) and maximum parsimony (MP) with a high degree of confidence. Bootstrap values, indicating the robustness of the internal nodes, were set at 1000 replications. Both the methods gave trees with similar topology and approximate relatively bootstrapped values among the tree obtained therefore, only the NJ tree is presented (Fig. 1). These sequences were aligned with the 28S rDNA genes and revealed clear differences in nucleotide sequences among different species (Fig. 2).

DISCUSSION

Genus *Cornudiscoides* of the subfamily Ancylodiscoidinae was established by Kulkarni (1969). The different species of this genus reported from India are listed in Table IV. This genus is found only on bagrids of India, Sri Lanka, Peninsular Malaysia and Thailand (Gussev, 1963, 1976; Lim, 1987; Dubey *et al*., 1992; Lerssuthichawal, 1999; Pandey and Agrawal, 2008).

Species of *Cornudiscoides* Kulkarni, 1969 exhibit morphological similarities with species in the following genera: *Bifurcohaptor* Jain, 1958; *Hargitrema* Tripathi, 1959; *Pseudancylodiscoides* Yamaguti, 1961; *Thaparocleidus* Jain 1952; *Tribaculocauda* Tripathi, 1959; *Wallagotrema*

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C.proximus          ---------T TTGAAATATG GGACTAACCA GGATTTTCCT AGTAACGGCG [50]
P.sp.HSY1           ---------- ---------- ---------. .....C.... .......... [50]
T.infundibulovagina ---------- ---------- ---------. .....C..T. .......... [50]
T.varicus           ---------- ---------- ---------. .....C..T. .......... [50]
T.magnicirrus       ---------- ---------- ---------. .....C.... .......... [50]
T.sp.NY1            ---------- ---------- ---------. .....C.... .......... [50]
B.sp.               ATCAGTAAGC GGAGG.A.A. AA........ .....C...C .......... [50]

C.proximus          AGTGAACGGA AATTAGCCCA GCACCGAAGC CTATCTGCAT TTGTGGACAG [100]
P.sp.HSY1           .......... G......... ..G....... .....C.TG. ...C...... [100]
T.infundibulovagina .......... G......... ..G....... .......... ....A..... [100]
T.varicus           .......... G......... ..G....... .......... ....A..... [100]
T.magnicirrus       .......... G......... ..G....... .......... .......... [100]
T.sp.NY1            .......... G......... ..G....... .......... .......... [100]
B.sp.               .......... G......... ..G....... .......... .......... [100]

C.proximus          GGCAATGTGG TGTTTAGACT TGAACCTGGG GACACTTATC TACTCGAAGT [150]
P.sp.HSY1           .......... C......... A...T..... AGTG...G.T .......... [150]
T.infundibulovagina .......... C......GTG CA.G...... ...G..AT.. .......... [150]
T.varicus           .......... C......GTG CA.G...... ...G..AT.. .......... [150]
T.magnicirrus       .......... C......G.G CA.G...... ...G..AT.. .......... [150]
T.sp.NY1            .......... C......G.G CA.G...... ...G..AT.. .......... [150]
B.sp.               .......... C...G..... .......... ..TG...G.. ..TC...... [150]

C.proximus          CCAACTCCGA ATATGGCTTG GATTTGTTCC ATAGAGGGTG AAAGACCCGT [200]
P.sp.HSY1           ...G...... .......... .......... .G........ .T..C..... [200]
T.infunibulovagina  ...G...... TC........ .......C.. .......... ....G..... [200]
T.varicus           ...G...... TC........ .......C.. .......... ....G..... [200]
T.magnicirrus       ...G...... TC........ .......C.. .A........ ....G..... [200]
T.sp.NY1            ...G...... TC........ .......C.. .A........ ....G..... [200]
B.sp.               .......... .......... .......... .......... ....T..... [200]

C.proximus          ACGGGTAGAT TATAT-GTTT T--GAAGTGT TCCTTAGATG TTCTTGTCTT [250]
P.sp.HSY1           ...A.....C CTG..-.... .T-A...CAC G........A .......... [250]
T.infundibulovagina ...A.....A AT.G.-..A. C--AT..... C......... C.TG..CT.. [250]
T.varicus           ...A.....A AT.G.-..A. C--AT..... C......... C.TG..CT.. [250]
T.magnicirrus       ...A.....A GT.G.-..A. C--TT..... C......C.. C.TG..C... [250]
T.sp.NY1            ...A.....A GT.G.-..A. C--TT..... C......C.. C.TG..C... [250]
B.sp.               ....A..... .TA..T..C. .TAT...CA. G......... ...AG..... [250]

C.proximus          GGAGTCGGAT TGCTTGAGAA TGCAGTCCAA AGTGGGTGGT AAACTCCATC [300]
P.sp.HSY1           A......... .......... .......... .......... .......... [300]
T.infundibulovagina .......... .......... .......... .......... .......... [300]
T.varicus           .......... .......... .......... .......... .......... [300]
T.magnicirrus       .......... .......... .......... .......... .......... [300]
T.sp.NY1            .......... .......... .......... .......... .......... [300]
B.sp.               A......... .......... .......... .......... .......... [300]

C.proximus          CAAGGCTAAA TACCTGCACG AATCCGATAG TAGACAAGTA CCGCGAGGGA [350]
P.sp.HSY1           .......... .......... .G........ .......... .......... [350]
T.infundibulovagina .......... ...TG..... .......... .......... .......... [350]
T.varicus           .......... ...TG..... .......... .......... .......... [350]
T.magnicirrus       .......... ...TG..... .......... .......... .......... [350]
T.sp.NY1            .......... ...TG..... .......... .......... .......... [350]
B.sp.               .....A.... ...T-A.... .-..G.---- ---------- ---------- [350]

C.proximus          AAGTTGAAAA GTACTCTGAA GAGA [374]
P.sp.HSY1           .......... .......... .... [374]
T.infundibulovagina .......... .......... .... [374]
T.varicus           .......... .......... .... [374]
T.magnicirrus       .......... .......... .... [374]
T.sp.NY1            .......... .......... .... [374]
B.sp.               ---------- ---------- ---- [374]
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Fig. 2. Alignment of 28S sequences for comparative purposes of different species from different geographical locations showed nucleotide identical to *C. proximus*. Dots indicate identity with the first sequence and dashes are inferred insertion-deletion events.

Table IV.- List of *Cornudiscoides* sp. reported from India and their hosts

Cornudiscoides sp.	Host species
C. heterotylus Kulkarni, 1969	*Mystus tengara*
C. megalorchis Kulkarni, 1969	*Mystus tengara*
C. microtylus Kulkarni, 1969	*Mystus tengara*
C. jaini (Gusev, 1963) Gusev, 1976	*Mystus keletius*
C. geminus Gusev, 1976	*Mystus vittatus*
C. proximus Gusev, 1976	*Mystus vittatus/tengra*
C. raipurensis Dubey, Gupta & Agarwal, 1993	*Mystus vittatus*
C. vittati Dubey, Gupta & Agarwal, 1993	*Mystus vittatus*
C. tukarami Agrawal & Vishwakarma, 1996	*Mystus bleekeri*
C. gomtiai Agrawal & Vishwakarma, 1996	*Mystus vittatus*
C. susanae Agrawal & Vishwakarma, 1996	*Mystus bleekeri*
C. bleekerai Agrawal & Vishwakarma, 1996	*Mystus bleekeri*
C. gussevi Agrawal & Vishwakarma, 1996	*Mystus vittatus*
C. agarwali Agrawal & Vishwakarma, 1996	

Tripathi, 1959 and *Indocotylus* Kulkarni, 1969. Kulkarni (1969) on the basis of morphology, differentiated this genus from closely related genera, *Hargitrema* and *Bifurcohaptor*. *Thaparocleidus*, in having a divided ventral bar or a ventral bar with thin long medial region, a pair of long modified hooks, large dorsal anchors and smaller ventral anchors which are located on the lobes of the bilobed haptor. It is similar to *Bifurcohaptor* in having a divided ventral bar and ventral anchors disposed on separate lobes of the haptor. It differs from *Bifurcohaptor* in the shape of the dorsal bar and the presence of a pair of modified needle-like hook. It is similar to *Pseudancylodiscoides* in possessing a divided ventral bar, but differs in having a pair of modified needle-like marginal hooks. All these differences are based on the traditional identification of parasite species by morphology and morphometrics.

Sclerotized parts of haptors and reproductive organs have been used as the most important characters for species and genus determination within the diversified monogeneans, and these characters have also been used as key features in the phylogenetic analyses among genera (*e.g.*, Lim *et al*., 2001; Pouyaud *et al*., 2006; Wu *et al*., 2008). Thus recent molecular phylogenetic analyses based on ribosomal deoxyribonucleic acid (DNA) sequences have revealed some hidden taxonomic problems. Depending on the level of investigation, researchers have chosen different regions: large subunit ribosomal DNA (LSUrDNA), small subunit (SSUrDNA), or ITS region. All these molecular tools are reliable and may function as aiding kit in the prevailing taxonomical identification processes with the morphological identification. In conclusion, this work indicates that modern identification and understanding of *Cornudiscoides* species should be necessarily accompanied with DNA analyses.

Based on the genetic distances (Table III), *C. proximus* was found to be more closely related to a *Bifurcohaptor* spp than *Pseudancylodiscoides* spp. and three nominal *Thaparocleidus* spp. and one species identified only to this genus. Therefore, based on the morphological and molecular data we also agree with the position of *Cornudiscoides proximus* in the Ancyrocephalinae established by Kulkarni (1969). *C. proximus* is the first species of genus *Cornudiscoides* to have been sequenced and it indicates similarity to other species of Ancyrocephalinae. However, this similarity might be revised in future as and when new sequences become available.

In conclusion, molecular phylogenetic studies are still at an early stage in terms of limited amount of DNA sequence data compared with the larger amount of information available from morphological taxonomy. Therefore, more study is needed as more phylogenetic data becomes available.

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