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One known and an unknown species of the genus *Thaparocleidus* Jain, 1952, infecting *Sperata aor* (Hamilton, 1822): comparison with species from China, on molecular basis

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Abstract:
Sperata aor commonly called as long- whiskered cat fish or "Bada Tengan" in local fish markets harboured one new and one previously known species of genus *Thaparocleidus* Jain, 1952, along with two species of *Cornudiscoides* Kulkarni, 1969, infesting gills. *Thaparocleidus aori* (Rizvi, 1971) Lim, 1996, was earlier described by Rizvi therefore was briefly recorded in the present study, except the egg. The newly found species *Thaparocleidus susanae* n.sp was characterized by the structure of its peculiar copulatory organ. Phylogenetic relationship of the two species under study, along with 14, reterived from GenBank was established using the sequences of 28S rDNA region (*Dactylogyrus* Diesing, 1850 taken as an out group).

Keywords: *Thaparocleidus*, 28S rDNA, Phylogenetic analysis.

Background:
Sperata aor, a freshwater, demersal, potamodromous cat fish **[1, 2]**, hosts two ancylodiscoidins *Thaparocleidus* and *Cornudiscoides*, distributed from Pakistan, India, Nepal, Bangladesh upto upper Myanmar. So far, three oioxenous species of the genus *Thaparocleidus* had been reported from *S. aor., T. aori* (Rizvi, 1971) Lim, 1996; *T. mystusi* (Rizvi, 1971) Lim, 1996 and *T. speratai* Agrawal *et al.,* 2004. An unknown species *T. susanae* n.sp. was collected along with *T. aori* and their morphology as well as molecular data were analyzed. Their phylogenetic analysis had been done along with 14 species (sequences retrieved from GenBank) from China, the neighboring country to ascertain their relationship. Max identity between obtained sequences of *Thaparocleidus* is 87% (on line BLAST: Basic Local Alignment Search Tool) set at default value.

Methodology:
Fishes were collected from River Gomti at Lucknow. Live hosts were also bought from fish markets and maintained in glass aquaria. Hosts were identified by Fishbase **[2]**. Gills of freshly dead hosts were examined fresh as well as fixed (3% formaline diluted with lukewarm water). Parasites were dislodged with micro needles in glass petri-dishes and studied under a phase contrast microscope (Olympus BX 51). The methods for staining, mounting and illustrating the dactylogyrids were as described by Kritsky *et al.,* **[3]** and numbering of hooks was that of Kulweic **[4]**. Measurements were taken in µm using a calibrated micrometer following the procedure and terminology of Gusev **[4]**; means are followed by the range and the number (n) of specimens measured in parentheses. Unstained Glycerine mounts, sealed with sealant, were used for measurements of soft as well as hard parts. Measurements and illustrations were

made with the help of an image taken with camera (Olympus-Photometrics coolsnap) attached with microscope, using Image-ProExpress 6.0 (for image analysis).

Molecular analysis

DNA isolation

Single parasite was collected in absolute ethanol for DNA extraction. Total DNA was extracted from the collected parasite using Qiagen's Dneasy Blood and Tissue Kit (Cat. No. 69504) by following protocol as per DNA extraction kit with slight modifications.

Polymerase chain reaction (PCR)

Partial 28S rDNA region of *T. aori* and *T. susanae. sp.* was amplified in an Eppendorf Master Cycler Personal (PCR machine: Polymerase chain reaction machine) using forward (5'ACCCGCTGAATTTAAGCAT-3') and reverse(5'CTCTTCAGAGTACTTTTCAAC-3') primers. The reaction volume was 25µl, (PCR) buffer (10X), 0.5µl dNTPs (10mM), 0.5µl forward primer (19.6 nMol.), 0.5µl reverse primer (31.9 nMol.), 0.5µl Taq polymerase (5 Units), 1µlMgCl2 (25mM) 5µl genomic DNA and 15µl miliQ water. PCR conditions were 95 ˚C for 4 min (initial denaturation), followed by 35 cycles of 95 ˚C for 1 min (denaturation), 55 ˚C for 45 sec (annealing), 72 ˚C for 1 min (extension) and 72 ˚C for 10 min (final extension). PCR products were checked on 1.5 % agarose gels in TAE buffer stained with ethidium bromide (EtBr) and visualized under UV light. Amplicons were sequenced with the same primers using automated sequencer (Inst Model/Name: 3730xl/SYNGENE-373XL/-140362-004 of Applied Biosystems).

Data analysis

Sequencing products were subjected to BLAST (Basic Local Alignment Search Tool) for homology search. Multiple sequence alignment was performed using Clastal W **[5]**. The sequence of query species (*T. susanae* n. sp. and *T. aori*) was compared with retrieved sequences **Table 1 (see supplementary material)** to infer phylogenetic relationship among them. Sequence data (obtained /retrieved) were analyzed using minimum evolution and neighbor- joining methods of MEGA 5 (Molecular Evolutionary Genetics Analysis-5 **[6]** for generating phylogenetic tree among them. The robustness of the inferred phylogeny was assessed using bootstrap value at 1,000 replications. Genetic relatedness among the analyzed monogenes is due to conserved as well as identical regions. Sequence (partial 28S rDNA) of *T. susanae* n. sp. and *T. aori* were submitted to Genbank under accession numbers KC962228 and KC962227 respectively.

Thaparocleidus susanae n. sp.

Figure 1 & Figure 2 (A-H) Type host: *Sperata aor*; Site: Gills; Present record and locality: River Gomti, Lucknow; UttarPradesh; No. of hosts examined: 2; No. of hosts found infected: 2; No.of specimens collected: 60; Prevelance and intensity: The prevelance of *T. susanae* n.sp. was 40% and intensity reached upto 70 to 80 parasites per fish host. Gene sequences: sequence of partial 28S rDNA was submitted in NCBI under accession no. KC962228. Specimens studied: 5 paratype specimens (accession no. w9311-15/1) were submitted in Helminthological collection of ZSI (Zoological survey of India).

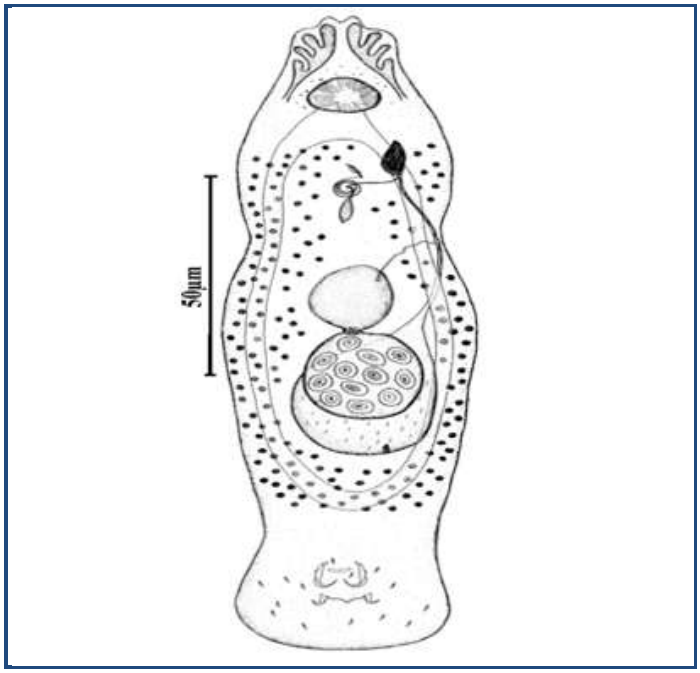

Figure 1: *Thaparocleidus susanae n.* sp. (whole mount: ventral view).

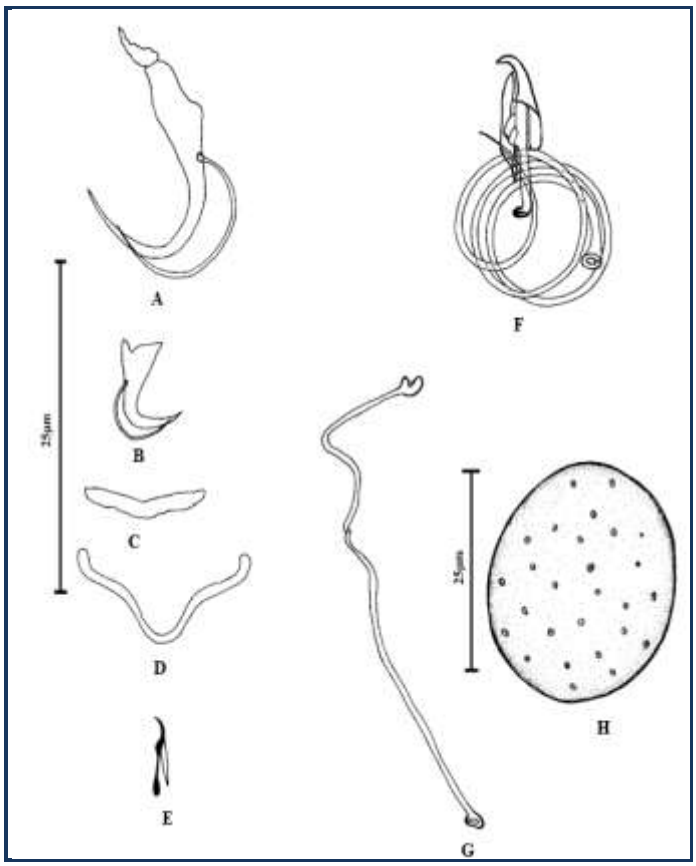

Figure 2: (A) Dorsal anchors**, (B)** Ventral anchors, **(C)** Dorsal bar, **(D)** Ventral bar, **(E)** hook, **(F)** Copulatory complex, **(G)** Vaginal armature, **(H)** Egg**.**

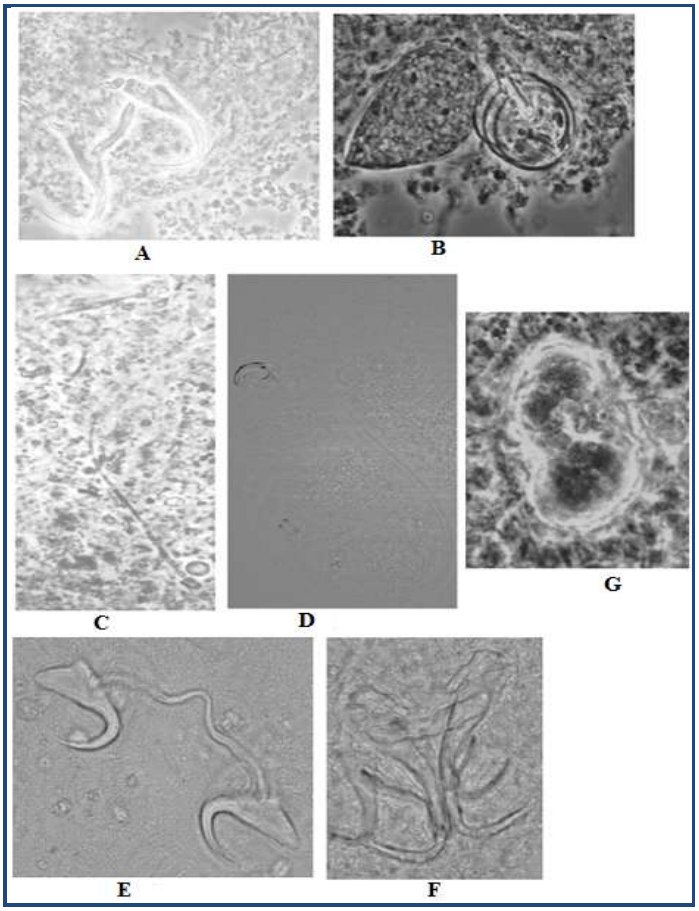

Figure 3: A) Photomicrograph showing dorsal anchor, dorsal patch, dorsal bar and hooks of *T. susanae* n.sp; **B)** Photomicrograph showing copulatory complex of *T. susanae* n.sp; **C)** Photomicrograph showing vaginal armature of *T. susanae* n.sp; **D)** Photomicrograph showing copulatory complex of *T. aori;* **E)** Photomicrograph showing ventral anchors and ventral bar of *T. aori;* **F)** Photomicrograph showing dorsal anchors and dorsal bar of *T. aori;* **G)** Photomicrograph showing egg of *T. aori.*

Etymology

The species was named in honour of Prof. L. H. S. Lim, University of Kuala Lumper, Malayasia for her outstanding contributions.

Description

Body 1043 (885-1299; n=10) long, maximum width at mid length 153 (113-182; n=10). Cephalic region well developed; cephalic lobes well developed and 2 pairs; accessory granules present; pharynx spherical, width 54 (44-66; n=10) diameter; oesophagus short to nonexistent; intestinal caeca united posteriorly. Testis 165 (138-195; n=10) long, maximum width at mid length of testis, 57 (37-68; n=10); vas deferens coils left intestinal caecum; seminal vesicle 42 (37-50; n=10) long, highly muscular sigmoid dilation of vas deferens; fusiform prostatic reservoir present. Copulatory tube **(Figure 2F & Figure 3B)** sclerotised, with 3 clockwise coils, 198 (193-198; n=10) long; accessory piece pointed angle shaped, 22 (21-27; n=10) long. Ovary round to oval, 118 (90-120; n=10) long, maximum width at mid length of ovary 85 (56-110; n=10), inter-caecal. Vaginal tube **(Figure 2G & Figure 3C)** 80 (59-95; n=10) long; vitellaria dense throughout the trunk except in the regions of reproductive organs.

Haptor 105 (71-136; n=10) long, 112 (84-165; n=10) wide. Dorsal anchor **(Figure 2A & 3A)**: outer length 24 (24-25; n=10), inner length 29 (26-31; n=10), recurved point 16 (15-19; n=10); dorsal patch **(Figure 3A)** 5 (3-7; n=10). Ventral anchor **(Figure 2B)**: outer length 13 (12-15; n=10), inner length 15 (14-15; n=10), recurved point 5 (4-6; n=10). Dorsal bar **(Figure 2C & Figure 3A)** 18 (16-21; n=10). Ventral bar **(Figure 2C)** 27 (14-33; n=10) long. Seven pairs hooks, **(Figure 2E & Figure 3A)** similar in shape and size 11 (10-13; n=10) long. Egg **(Figure 2H)** nonpolar round to oval, 51 (41-56; n=5) long, width at mid length 48 (39-59; n=5).

Remarks

This species was characterized by structure of copulatory complex having a pointed "angle" shaped accessory piece, attached distaly to a copulatory tube with three clockwise coiles and long vaginal tube having a funnel like, lightly sclerotised opening. The present species resemblse with *T. mystusi* in comparative morphology of vaginal apparatus, dorsal anchors, dorsal bar and hooks but differs in the structure of copulatory complex, copulatory tube without coiling and accessory piece being pitcher shaped, also the ventral bar is longer, its middle region being fine and thin in *T. mystusi* which was smaller and of same width throughtout its length in *T. susanae* n. sp. Similarly, the ventral anchor has a sharp and pointed inner root end in *T. mystusi* while in *T. susanae* n. sp the two roots are short, pointed and of almost smiliar length. It also resembles with *T. speratai* in comparative morphology of vaginal apparatus, ventral bar and hooks but chiefly differs in the structure of copulatory complex which had single, horse shoe shaped accessery piece attached diataly to copulatory tube. It also differs from *T. aori* in the structure of copulatory complex, vaginal armature, ventral anchors, ventral bar, dorsal bar and dorsal anchor. Therefore this species, regarded as a new species, named in honour of Prof. L. H. S. Lim, for her outstanding contributions.

Thaparocleidus aori (Rizvi, 1971) Lim, 1996

Syn: *Ancylodiscoides aori* Rizvi, 1971**,** *Silurodiscoides aori* (Rizvi, 1971) Gusev, 1976 *Parancylodiscoides aori* (Rizvi, 1971) Abha, Dubey, Gupta and Agrawal, 1992**;** Type host: *Sperata aor* (Hamilton, 1822)**;** Infection Site: Gills**;** Type locality; Sindh (http://en.wikipedia.org/wiki/Administrative_units_of_Pakistan)**;** Present record and locality: River Gomti, Lucknow; UttarPradesh**;** No. of hosts examined: 2**;** No. of hosts found infected: 2**;** Prevelance and intensity: The prevelance of *T. aori* was 35% and intensity reached upto 60 to 70 parasites per fish host. Gene sequences: sequence (partial) of 28S rDNA was submitted in NCBI under accession no KC962227. Specimens studied: 5 paratype specimens (accession no. w9316-20/1) were submitted in Helminthological collection of ZSI (Zoological survey of India).

Measurements (values as per Agrawal et al., 2004 [7] given in square brackets)

Body 671 (506-795; n=10) [630 (610-670)] long, maximum width at mid length 102 (73-203; n=10) [130 (110-145)]. Cephalic region

well developed; cephalic lobes well developed and 2 pairs; eye spots 2 pairs, posterior pair larger, accessory granules present; pharynx spherical, width 40 (26-60; n=10) [32 (28-40)] diameter; oesophagus short to nonexistent; intestinal caeca united posteriorly.

Testis 133 (94-154; n=10) [56 (50-62)] long, maximum width at mid length of testis, 60 (44-74; n=10) [43 (41-60)]; vas deferens coils left intestinal caecum; seminal vesicle highly muscular sigmoid dilation of vas deferens; prostatic reservoir opens at base of copulatory tube. Copulatory complex **(Figure 3D)** consists of a copulatory tube 150 (133-159; n=10) [102 (97-110)] long, proximally articulated with accessory piece; accessory piece made of three parts, part I 11 (10-12; N=10) [12 (11-13)], part II 8 (7-9; n=10) [12 (7-9)] and part III 6 (5-8; n=10) [8 (7-9)] long. Ovary round to oval, 110 (65-159; n=10) [101 (92-110)] long, maximum width at mid length of ovary 81 (55-98; n=10) [38 (30-50)], inter-caecal. Vaginal opening funnel shaped, vaginal tube 68 (60-77; n=10) long; vitellaria dense throughout the trunk except in the regions of reproductive organs.

Haptor 120 (70-150; n=10) [134 (110-145)] long, 93 (80-105; n=10) [120 (105-130)] wide. Dorsal anchor **(Figure 3F):** outer length 43 (40-46; n=10) [28 (22-23)], inner length 47 (48-56; n=10) [36 (30-42)], recurved point 20 (18-23; n=10) [19(19-22)]; dorsal patch **(Figure 3F)** 13 (9-16; n=10) [20(18-22)]. Ventral anchor **(Figure 3E):** outer length 21 (18-23; n=10) [19 (16-24)], inner length 23 (22-26; n=10) [24 (22-28)], recurved point 10 (8-12; n=10) [15 (13-18)]. Dorsal bar **(Figure 3F)** 33 (28-38; n=10) [29 (27-24)] long. Ventral bar **(Figure 3E)** 37 (26-53; n=10) [32 (28-35)] long. Seven pairs hooks similar in shape and size 12 (11-12; n=10) [12 (12-14)] long. Egg, **(Figure 3G)** unipolar round to oval, 50 (42-56; n=10) long, width at mid length 18 (17-20; n=10), polar filaments 8 (7-8; n=10) long.

Remark:

Rizvi **[8]** described *T. aori* from *S. aor* as *A. aori* form Sind; Pakistan. Later on Gusev **[4]** described it as *S. aori* with three forms (one typical form and two atypical forms) and transferred it under genus *Silurodiscoides* Gusev, 1976 (focued on hard parts only). Lim **[9]** considered *A. aori* and *S. aori* as junior subjective synomyms of *Thaparocleidus* Jain, 1952. We have recorded the species briefly with addition of egg strcuture.

Discussion:

In monogenes sequences of ribosomal subunits were widely used to infer phylogenetic relationships at the level of families and sub families **[10-12]** and also to investigate evolutionary association between the parasites and their hosts **[12, 13]**.

In the present study, phylogenetic analysis using 28S genes showed a well resolved grouping of two Indian species of the genus *Thaparocleidus* under study and 14 from China. In **(Figure 4 A & B)** shows the phylogenetic relatedness and evolutionary pattern of 16 species of the genus *Thaparocleidus* and three species of *Dactylogyrus,* using Neighbour-joining method and Minimum Evolution method of MEGA5. They probably originated from same ancestor, forming two clusters and *Dactylogyrus* as an out group. Further, cluster "A" had two lineages for the genus *Thaparocleidus*. Lineage one includes *T. mutabilis*, *T.* sp. NY1, *T. omegavagina*, *T.* sp. 2 XW, *T. cochleavagina*, *T. magnicirrus*, *T. obscura*, *T. infundibulovagina*, *T. varicus*, *T.* sp. 1 XW, *T.* sp. NY2, *T. asoti*, *T.* sp. BDY, *T. campylopterocirrus* (all from China) and lineage two includes *T. aori* and *T.susanae* n.sp. (from India), while cluster "B" had *D. petruschewskyi*, *D. lamellatus*, *D. inversus* (from China/Iran). Hence molecular analysis also revealed inter-species differences, further confirming their validity, earlier based on conventional morphological studies.

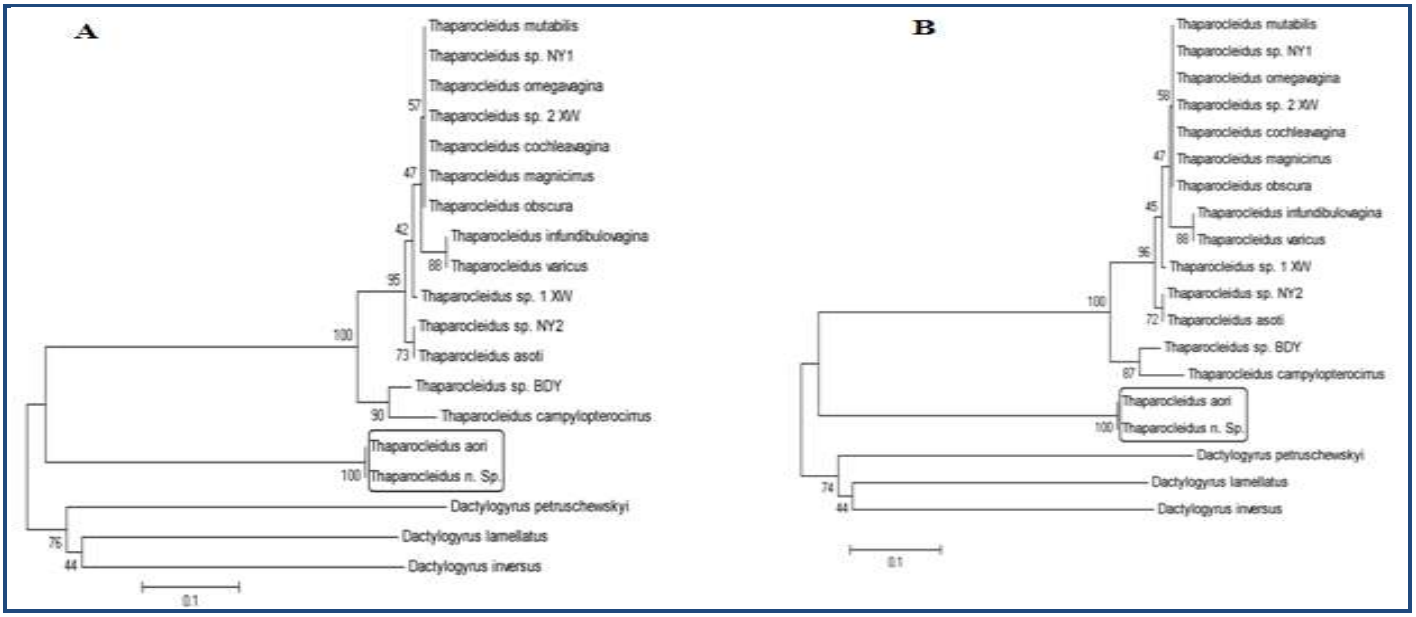

Figure 4: A) Neighbour-joining method**; B)** Minimum Evolution method for predicting phylogenetic tree of partial 28S rDNA region of I6 sp. of genus *Thaparocleidus* and 3 sp. of *Dactylogyrus* (as outgroup). using (MEGA 5) (within rectangle query species *T. aori* and *T. susanae* n.sp.).

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Supplementary material:

Table 1: Genbank reference sequence used in this study, with their respective information

S. no.	Parasite	Host	Accession no.	Country
1	*Thaparocleidus susanae* n.sp.	*Sperata aor*	Submitted to GenBank KC962228	India
2	*Thaparocleidus aori*	*Sperata aor*	Submitted to GenBankKC962228	India
3	*Thaparocleidus* sp. BDY	-	EF100555	China
4	*Thaparocleidus* sp. 1 XW	-	EF100553	China
5	*Thaparocleidus obscura*	-	EF100551	China
6	*Thaparocleidus magnicirrus*	-	EF100549	China
7	*Thaparocleidus cochleavagina*	-	EF100547	China
8	*Thaparocleidus* sp. 2 XW	-	EF100554	China
9	*Thaparocleidus omegavagina*	-	EF100552	China
10	*Thaparocleidus mutabilis*	-	EF100550	China
11	*Thaparocleidus varicus*	-	DQ157668.1	China
12	*Thaparocleidus* sp. NY2	-	DQ157671.1	China
13	*Thaparocleidus* sp. NY1	-	EF 100648	China
14	*Thaparocleidus infundibulovagina*	-	DQ 1577670	China
15	*Thaparocleidus asoti*	-	DQ157669.1	China
16	*Thaparocleidus campylopterocirrus*	*Pangasius hypophthalmus*	AY841872.1	China
17	*Dactylogyrus petruschewskyi*	-	AY548927.1	China
18	*Dactylogyrus lamellatus*	-	JX524549.1	Iran
19	*Dactylogyrus inversus*	*Lateolabrx japonicus*	AY548928.1	China