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Phylogenetic Studies on *Thaparocleidus* Jain, 1952, Infecting *Wallago attu* Bloch and Schneider, 1801 Inferred from 28S rDNA Sequences in India

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Abstract: ***Five species of the genus Thaparocleidus Jain, 1952 (Ancylodiscoidinae) namely T. wallagonius Jain, 1952, T. yogendraii Agrawal, 1981, T. gomtius Jain, (1952) Lim, 1996, T. indicus (Kulkarni, 1969) Lim, 1996 and T. sudhakari (Gusev, 1976) Lim, 1996, gill parasites had been collected and restudied in detail, from siluriform fish host, Wallago attu Bloch and Schneider, 1801. All were oioxenous species. T. yogendraii, T. gomtius, T. indicus and T. sudhakari had been collected for molecular study from freshly killed fish hosts (W. attu). We could not collect T. wallagonius (type species) for DNA isolation (rare species). Obtained sequences of T. yogendraii, T. gomtius, T. indicus, and T. sudhakari had been compared with 18 retrieved sequences of Thaparocleidus sp. (Table 1), using Tetraonchus monenteron (as an out group). The phylogenetic tree of the genus Thaparocleidus sp. had been reconstructed using 28S rDNA region. Phylogenetic trees confirmed monophyly of the genus Thaparocleidus. Secondary structures of obtained sequences had been used to predict the genetical distinction of species, based on highest negative free energy and topology of secondary RNA structure.***

Keywords*:* *Thaparocleidus* Jain, 1952, *Wallago attu*, 28S r DNA, Phylogenetic analysis, Secondary structure

1. Introduction

Wallago attu, known as the freshwater shark, is a demersal and potamodromous ‘‘sheatfish’’ found inhabiting in standing and running inland water bodies of India, Bangladesh, Pakistan, Nepal, Burma, Sri-Lanka, and other Asian countries including Thailand, Vietnam, Kampuchea, Malay Peninsula, Indonesia and Afghanistan [4, 6, 7, 15). *W. attu* or ‘‘Padhan’’ as it is known locally in India, has been studied extensively in India as well as in the rest of the Asian countries for helminth parasites. The freshwater shark *W. attu* in India, is indisputably having greatest monogenes diversity (5 species of *Thaparocleidus*, 4 species of *Bychowskyella* Akhmerov, 1952, 2 species of *Mizelleus* Jain, 1957, 4 species of *Hamatopeduncularia* Yamaguti, 1953, one species of *Rhamnocercus* Monaco et al., 1954, one species of *Cosmetocleithrum* Kritsky et al., 1986 and 2 species of *Neocalceostoma* Tripathi, 1959 [13]. Number of *Thaparocleidus* sp. varies 4-5 namely *T. wallagonius, T. yogendraii, T. gomtius, T. indicus* and *T. sudhakari.* per host (*T. wallagonius* is rare species). We have collected *Bychowskyella* sp. and *Mizelleus* sp. along with *Thaparocleidus* from gills of *W. attu.*

Genus *Thaparocleidus* has greatest species diversity amongst the member of Ancylodiscoididae Gusev, 1961 [11] and mentioned 16 species of the genus *Thaparocleidus* from India, parasitizing on *W. attu* [10, 11]. Of above 16 species only five species have been found valid, morphologically, which is further confirmed using molecular tool (rest are either synonymised or transferred into other genera). It is worthy to mention that all being oioxenous species (strictly host specific). Pandey et al. [14], redescribed *T. wallagonius* (originally *T. yogendraii*), *T. indicus, T. gomtius, T. malabaricus, T. seenghali* and *T. pusillus* along with reproductive biology and behavioural aspects. Later on *T. yogendraii* Agrawal, 1981 was redescribed by Rajvanshi & Agrawal [16].

Earlier workers [12, 17, 18, 19] had mentioned, the monophyletic origin of the genus *Thaparocleidus*. Chaudhary & Singh [2] gave phylogenetic utility of the ribosomal subunit (28S) in *T. parvulus* (Gusev, 1976) Lim, 1996, parasitizing gill filaments of *Mystus vittatus.* Chaudhary & Singh [3] later described two new species of the genus *Thaparocleidus* Jain, 1952 viz., *T. longiphallus* (on *W. attu)* and *T. siloniansis* (on *Silondia silondia*) in India, giving morphological and molecular phylogenetic evidences. The aim of this paper is to validate species of the genus *Thaparocledius* infecting *W. attu* in India, using 28S ribosomal region (partial).

2. Materials and Methods

2.1 Parasite collection and identification

About two hundred specimens of *W. attu* (type host) were collected from the River Gomti, local fish markets of Quaiserbaugh at Lucknow (26.8470° N, 80.9470° E) and various water bodies of Haidergarh at Barabanki (26.9200° N, 81.2000° E), Hardoi (27.4167° N, 80.2500° E), Sidhauli at district Sitapur (27.5700° N, 80.6800° E), State of Uttar Pradesh, India for investigation, during 2009–2013. Live hosts were also bought from fish markets and maintained in glass aquaria. Hosts were identified by Fishbase [4]. 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., [9]. Measurements were taken in µm using a calibrated micrometer following the procedure and terminology of Gusev [5], 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 a image taken with camera (Olympus- Photometrics coolsnap) attached with microscope, using Image-ProExpress 6.0 (for image analysis).

2.2 DNA Isolation and Amplification

Genomic DNA was extracted from ethanol-preserved parasites using Qiagen's Dneasy Blood and Tissue Kit, Hilden, Germany with slight modifications in manufacturer's instructions (as per requirements). Partial 28S rDNA region of *T. yogendraii, T. gomtius, T. indicus, and T. sudhakari* 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 [21]. The reaction volume was 25µl, containing 2µl polymerase chain reaction (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), 1µl MgCl2 (25 mM), 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. Sequencing was carried out by Xcelris Labs Limited, Ahemadabad using Big Dye ® Terminator v3.1 cycle sequencing kit as per manufacturer's instructions.

2.3 Data analysis

Sequencing products were subjected to BLAST (Basic Local Alignment Search Tool) for homology search. Multiple sequence alignment was performed using Clastal W [20]. The sequences of query species (*T. wallagonius* (retrieved), *T. gomtius, T. indicus, T. sudhakari, yogendraii*) were compared with other retrieved sequences (Table 1) to infer phylogenetic relationship among species of genus *Thaparocleidus*. Sequence data (obtained /retrieved) was analyzed using neighbor- joining and minimum evolution methods of MEGA 5 [22] for generating phylogenetic tree among them. The robustness of the inferred phylogeny was assessed using bootstrap value at 1,000 replications. Species differentiation has been confirmed using secondary structures of partial 28S rDNA region of each species, by Mfold separately [24]. The topology (geometry of base pairing) and highest negative free energy of secondary structure seems to be species specific. Thus, secondary structures and its various parameters can be used as an important tool for species discrimination.

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

S. no.	*Parasite*	*Host*	*Accession no.*	*Country*
1	*Thaparocleidus asoti*	*Silurus astus*	DQ157669	Rongchang, Chongqing, China
2	*Thaparocleidus campylopterocirrus*	*Pangasius hypophthalmus*	EF100546	Panyu, Guangdong China
3	*Thaparocleidus* sp. BDY	*Pangasius sutchi*	EF100555	Panyu, Guangdong, China
4	*Thaparocleidus* sp. 1 XW	*Silurus astus*	EF100553	Rongchang, Chongqing, China
5	*Thaparocleidus obscura*	*Silurus astus*	EF100551	Rongchang, Chongqing, China
6	*Thaparocleidus magnicirrus*	*Silurus astus*	EF100549	Kaiping, Guangdong, China
7	*Thaparocleidus cochleavagina*	*Silurus astus*	EF100547	Kaiping, Guangdong China
8	*Thaparocleidus* sp. 2 XW	*Silurus astus*	EF100554	Wuhua, Guangdong, China
9	*Thaparocleidus omegavagina*	*Silurus astus*	EF100552	Kaiping, Guangdong, China
10	*Thaparocleidus mutabilis*	*Silurus astus*	EF100550	Kaiping, Guangdong, China
11	*Thaparocleidus varicus*	*Silurus astus*	DQ157668	Rongchang, Chongqing, China
12	*Thaparocleidus* sp. NY2	-	DQ157671	China
13	*Thaparocleidus* sp. NY1	-	DQ157670	China
14	*Thaparocleidus infundibulovagina*	*Silurus astus*	EF100548	Rongchang, Chongqing, China
15	*Thaparocleidus vistulensis*	*Silurus glandis*	AJ969941	River Morava, CR
16	*Thaparocleidus siluri*	*Silurus glandis*	AJ969940	River Morava, CR
17	*Thaparocleidus wallagonius*	*Wallago attu*	JN020351	India
18	*Thaparocleidus indicus*	*Wallago attu*	JX960419	India
19	*Thaparocleidus sudhakari*	*Wallago attu*	JX984666	India
20	*Thaparocleidus gomtius*	*Wallago attu*	KC96229	India
21	*Thaparocleidus yogendraii*	*Wallago attu*	JX984665	India
22	*Thaparocleidus* sp. 1 HS	*Wallago attu*	GU980972	India
23	*Thaparocleidus parvulus*	*Mystus vittatus*	GU014844	India
24	*Tetraonchus monenteron*	*Esox lucius*	AJ969953	River Morava, CR

3. Results

3.1 Phylogeny

The sequence length of species under study varied from 351 (*T. sudhakari*) to 372 (*T. indicus*). G (Guanosine) and C (cytosine) content ranged between 45% and 50.1%. The phylogenetic trees (Figure 1.1 & 1.2) generated by neighbor-joining (NJ) and minimum evolution (ME) methods of MEGA 5 [22]. An estimate of average evolutionary divergence of sequence pairs is 0.55 (Maximum Composite Likelihood model). Estimate of evolutionary divergence ranged 0.10-0.78 between sequence. The evolutionary

distances were computed using p-distance method in the units of the number of base differences per site. Codon position included were 1st+2nd+3rd+Noncoding. Gaps and missing data were eliminated. Minimum Evolution was also based on p-distance method. The ME tree was constructed using close-neighbor- interchange (CNI) algorithm.

The phylogenetic trees showed four clusters for the genus *Thaparocleidus*. All the Indian species formed a distinct cluster than (16 retrieved) species of other countries. All the retrieved species of other countries were clustered in group one. *T. indicus, T. gomtius* and *T.* 1 HS were clustered in group two. *T. wallagonius, T. yogendraii* and *T. sudhakari* were in group three and *T. parvulus* was in group four. *T. sudhakari,* formed a sister clad with *T. wallagonius* and *T. yogendraii* (sister species). However *T. indicus* and *T. gomtius,* of group two formed sister clad with *T.* sp. 1. In Neighbor-Joining method of phylogeny, *T. indicus* showed 34% bootstrap value with *T. gomtius, T.* 1 HS had 16% bootstrap value with *T. indicus* and *T. gomtius* (group two). However, *T. wallagonius* and *T. yogendraii* showed 51% bootstrap value with each other. *T. sudhakari* had 19% bootstrap value with *T. wallagonius* and *T. yogendraii* (group three). In Minimum Evolution method, almost similar results had been obtained. *T. gomtius,* formed sister clad with *T. indicus*; *T. yogendraii* with *T. wallagonius* and *T. sudhakari.* The NJ and ME methods had proved that all the five are genetically distinct and valid species. Both methods of phylogeny (NJ and ME), confirmed *Thaparocleidus* to be of monophyletic origin [12, 17, 18] and all species, evolved from the same ancestral node. The species described from China, Czeck Republic and India formed distinct clusters, proved to be of different origin.

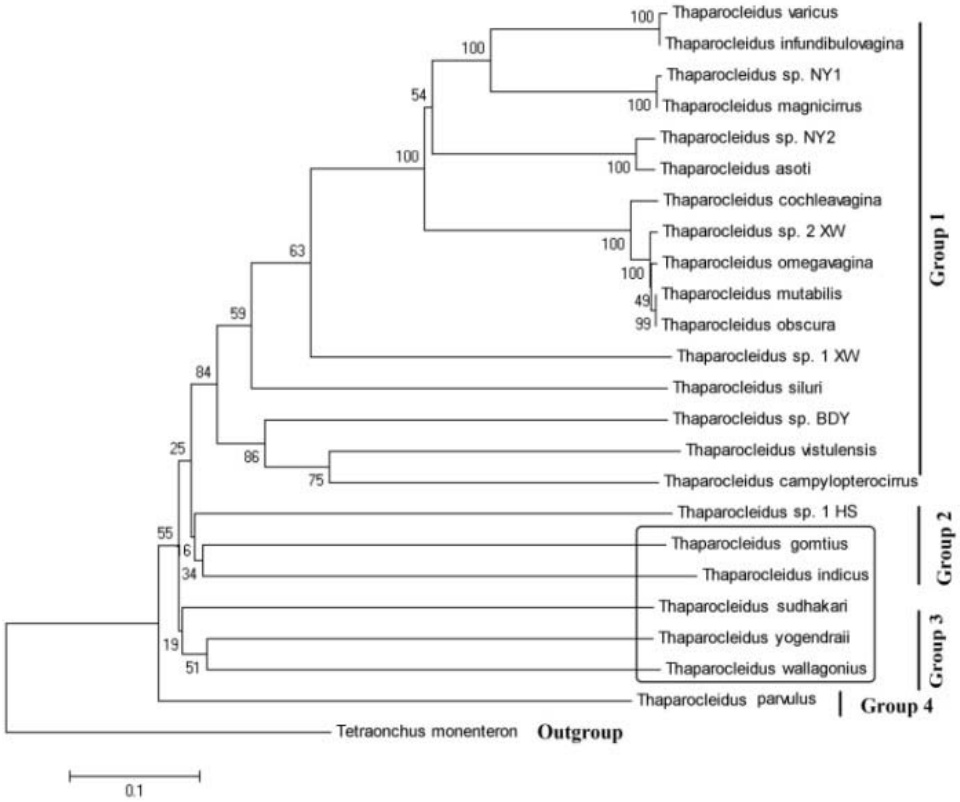

Figure 1.1: Neighbour-joining method (based on p-distance method)

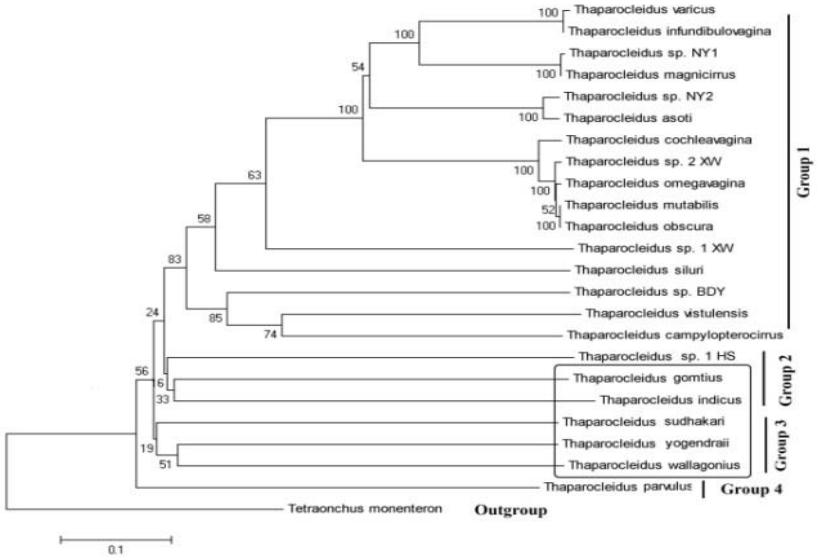

Figure 1.2: Minimum evolution method (based on p-distance method)

3.2 Secondary Structures

The secondary structures of *T. yogendraii* (Figure 1.3), *T. sudhakari* (Figure 1.6), *T. indicus* (Figure 1.5) *and T. gomtius* (Figure 1.4), generated by Mfold showed the distinction between the species using highest negative free energy and pattern of loops formation. We are not focusing on secondary structures of *T. wallagonius* [23]. Secondary structures of sequence of the Indian *Thaparocleidus* sp. (under study) proves that all are genetically distinct. Secondary structure of *T. wallagonius* had highest negative free energy ΔG= -324.90 Kcal/mol [23]. However, *T. indicus, T. gomtius, T. sudhakari* and *T. yogendraii* has -99.20 Kcal/mol, -116.40 Kcal/mol, -101.20 Kcal/mol and -107.20 Kcal/mol, negative free energy respectively. In secondary structure of *T. wallagonius,* the order of loop preference (in number), was interior loop, hairpin loop, bulge loop, multi loop and exterior loop [23]. In *T. yogendraii*, it is hairpin loop, interior loop, bulge loop, multi loop and exterior loop. In *T. gomtius,* order of loops is hairpin loop, (interior loop and bulge loop are equal in number), multi loop and exterior loop but in *T. sudhakari,* it is hairpin loop, interior loop, multi loop, bulge loop and exterior loop. In *T. indicus*, the loop preference is hairpin loop, interior loop, bulge loop, multi loop, and then exterior loop.

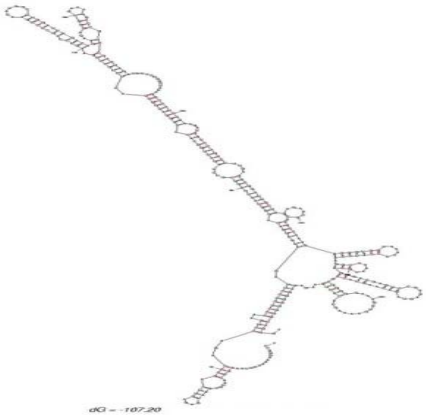

Figure 1.3: Secondary structure of *T. Yogendraii*

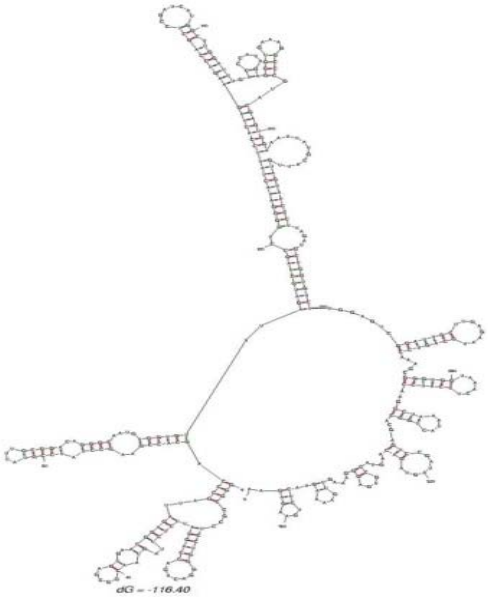

Figure 1.4: Secondary structure of *T. gomtius*

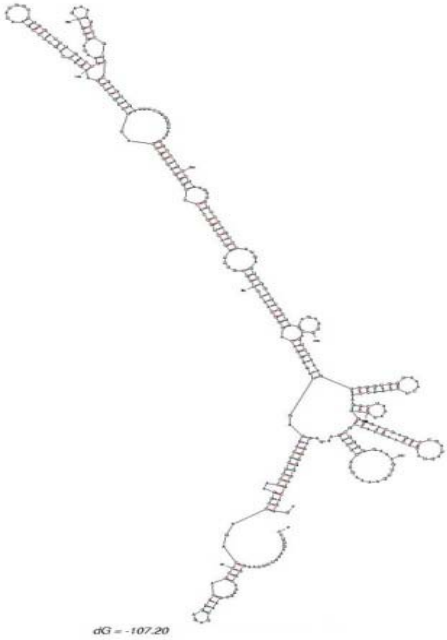

Figure 1.5: Secondary structure of *T.indicus*

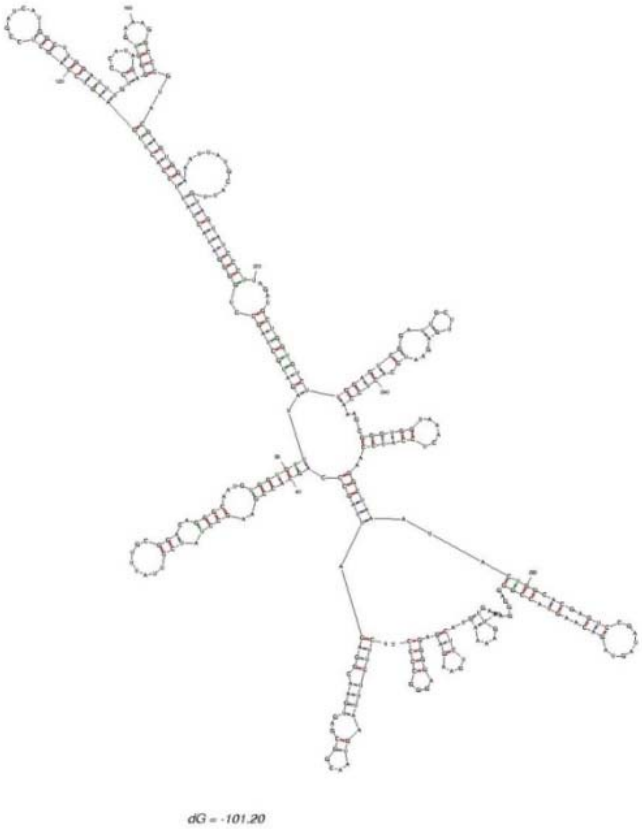

Figure 1.6: Secondary structure of *T. Sudhakari*

4. Discussion and Conclusions

Discrimination between species of *Thaparocleidus* relies chiefly on the structure of sclerotized parts of haptor and copulatory organs. Sometimes, species identification could be difficult due to slight structural variations (ex. *T. suhakari, T. gomtius* and *T. indicus*). Thus, intraspecific relationship of five species of genus *Thaparocleidus*, infecting gills of freshwater shark *W. attu* in India, is evaluated for the first time using molecular data. Our study shows that *T. wallagonius, T. yogendraii T. indicus, T.*

gomtius and *T. sudhakari* are strictly host specific species and supports monophyly of the genus *Thaparocleidus*.

Molecular characterization, using molecular markers further strengthens our observations that all the five species, under study, are genetically distinct. Number, position and type of loops formed in secondary structures of different species of *Thaparocleidus* are quite distinct. Hence, it confirms the identification of species and outcomes of primary sequence [3]. It is proved morphologically as well as molecularly that all the five *Thaparocleidus* species, from *W. attu i.e T. wallagonius, T. yogendraii, T. gomtius, T. indicus* and *T. sudhakari* are valid species and evolved from common ancestor. Thus, the agreement between molecular and morphological intraspecific similarities observed in *Thaparocleidus* supports the validity of morphometric characters used for species identification.

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