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PCR-based molecular characterization, phylogenetic analysis and secondary structure of the 28S rDNA of *Thaparocleidus wallagonius* (Monogenea: Dactylogyridae) – the most primitive species of this genus from India

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Abstract:
Species of the monogenean genus *Thaparocleidus* are specific to freshwater siluriform fish. The infection caused by these gill parasites are a major health problem to fish. But, to focus the control strategies of these parasites, first it is important to establish an accurate discrimination by molecular methods. In the present study, phylogenetic and structural analysis of 28S region of ribosomal DNA of *T. wallagonius* species collected from fish *Wallago attu* from Meerut (U.P.), India, was carried out. In the first step, we amplified, sequenced 28S region of ribosomal DNA of *T. wallagonius* to establish the phylogenetic relationship with other species of this genus. *T. wallagonius* found on gill filaments of fish *W. attu*, is the most primitive parasite of this genus from India, was unequivocally discriminate from other species of the same genus in this study. A secondary-structure model of the large subunit rDNA was also predicted using a combined comparative and thermodynamic approach. Molecular morphometric and phylogenetic relationship of *T. wallagonius* are discussed in detailed that based on molecular analysis using bioinformatic tools.

Key words: Monogenea, 28S, secondary structure, India, ribosomal DNA

Background:
During the general survey of economically important freshwater fish of the Meerut region, the important and commonly available fish, namely *Wallago attu* were found harbouring with the species of the genus *Thaparocleidus* **[1]**. *Thaparocleidus* **[1]** was proposed by Jain **[2]** for *T. wallagonius* **[1]** from the gills of *W. attu* (Bloch & Schneider, 1801) collected in the Gomti River at Lucknow, India. Later, Gusev **[3]** proposed *Silurodiscoides*, to which species of *Thaparocleidus* were transferred. However, Lim **[4]** pointed out that *Thaparocleidus* is a senior synonym of *Silurodiscoides* and listed 80 species of *Thaparocleidus* with emphasized the need to ascertain the status of some species from Indian fish and suggested that a detailed redescription of *T. wallagonius*, the type-species, was required. Taxonomic revisions of *T. wallagonius* have been done based on morphological criteria **[5]**. It is hard to differentiate the species, strains and populations from any stage in their life history only on the basis of morphology. Molecular methods also enable in distinguished morphologically similar parasites. PCR-based techniques utilizing the 28S rDNA sequences have proven to be a reliable tool to identify the helminth species and their phylogenetic relationships. Moreover, in platyhelminth systematics, rDNA genes, in general, have been used successfully **[6-8]** and 28S rDNA, in particular, to estimate the relationships existing among the platyhelminthes **[9]**. In view of this, study on the molecular characterization and phylogeny of *T. wallagonius* was achieved by the help of ribosomal DNA (rDNA).

RNA secondary structures are particularly useful in systematics because they include characteristics, not found in the primary sequence, that give 'morphological' information **[10]**. The derivation of reliable secondary structure models for 28S region would represent a major step towards a detailed understanding of their biological roles which provides a powerful tool for identifying biologically relevant folding patterns in RNA molecules **[11-15]**. For the present study, we describe the molecular characterization and discrimination of the species *T. wallagonius* by using 28S rDNA region and to estimate the genetic variation, phylogenetic relationship with other species of the genus *Thaparocleidus*. We also aimed prediction of the secondary structure to substantiate the findings. In this paper, we provide first molecular confirmation of the presence of *T. wallagonius* species from Meerut (U.P.), India.

Figure 1: Pairwise alignment of 28S rDNA of *T. wallagonius.*

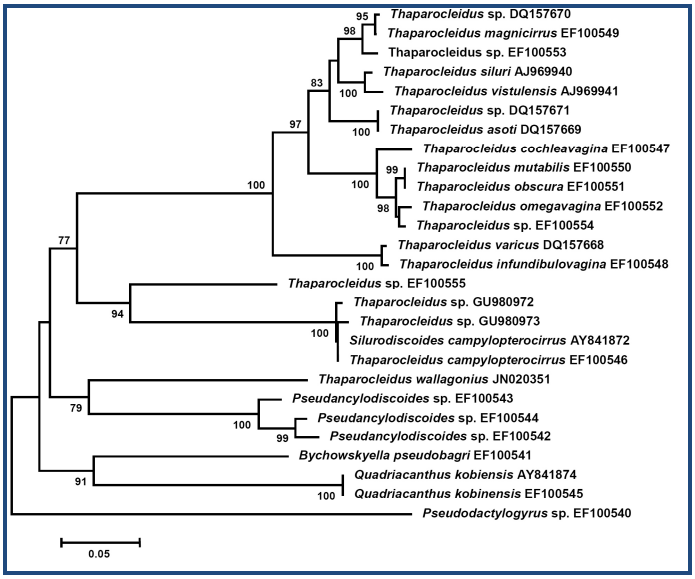

Figure 2: Phylogenetic positioning of *T. wallagonius* based on 28S sequences using neighbour joining (NJ).

Methodology:

Parasite material and DNA isolation

The economically important food fish of India *Wallago attu* (Bloch & Schneider, 1801) belong to the order Siluriformes and family Siluridae were collected from Meerut (29°01'N and 77°45'E), U.P., India and examined. Monogenea, *T. wallagonius* were obtained in live form from gill filaments of *W. attu*. The worms were mounted as per method suggested by Malmberg **[16]**. Mounted voucher specimen of *T. wallagonius* species were deposited in the Museum of Department of Zoology, Ch. C.S. University, Meerut (U.P.), India under the voucher no. HS/monogenea/2011/01. Monogenean was examined with a microscope for species identification and then destroyed in the process of DNA extraction.

DNA amplification, Sequencing and its Analysis

Monogenean DNA was extracted from the whole specimen using DNeasy Tissue kit (Qiagen). A fragment of 866 bp of 28S gene was amplified using the specifically designed primers forward (5'-TCAGTAAGCGGAGGAAAAGAA-3') and reverse (5'-CAAAACCACAGTTCTCACAGC-3'). Each PCR reaction was performed in a final volume of 25 ml containing 3 ml of lysate, 10 X polymerase chain reaction (PCR) buffer, 1U Taq polymerase (Biotools, Madrid, Spain), 0.4mM deoxyribonucleotide triphosphates (dNTP) and 10 pM of each primer pair. PCR products were examined on 1.5% agarose–TBE (Tris–borate–EDTA) gels, stained with ethidium bromide and visualized under ultraviolet light. Amplification products were purified by a Chromous PCR clean up kit (#PCR 10, Chromous Biotech). Gel-purified PCR products were sequenced using a Big Dye Terminator version 3.1 cycle sequencing kit in ABI 3130 genetic analyser (Applied Biosystems) with the same primers. The DNA sequence were put to further analysis by using various Bioinformatics tools including similarity search BLAST and phylogenetic prediction by CLUSTALW for query DNA sequence. The Nucleotide sequence data reported in this paper have been submitted to the GenBank with the accession number JN020351.

Molecular Phylogenetic Analysis and secondary structure

The sequences were aligned using ClustalW multiple alignment **[17]** with the default gap and extension penalties used by this program. 28S sequences were entered in the MEGA 5.0 **[18]** for construction of the phylogenetic trees. The phylogenetic trees of the *T. wallagonius* were constructed using distance method, neighbour-joining (NJ) and character state method, maximum-parsimony (MP). Branch support was given using 1000 bootstrap replicates. Secondary structures of 28S sequences of *T. wallagonius* were predicted by the online MFold package **[19]**. MFold is the most widely used algorithms for RNA secondary structure prediction that are based on a search for the minimal free energy state.

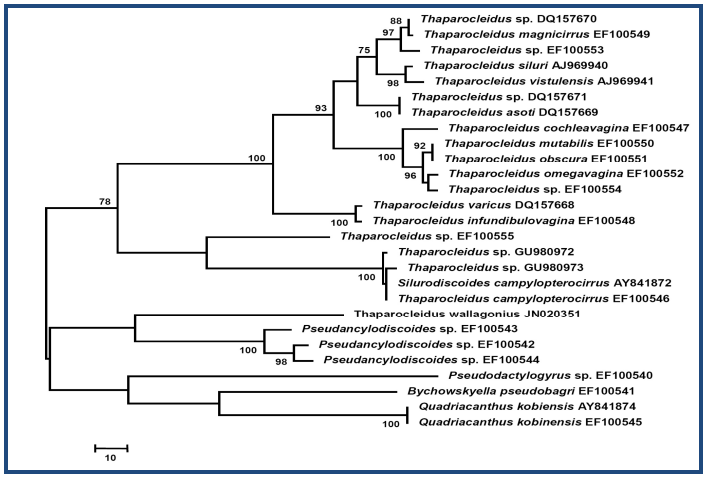

Figure 3: Phylogenetic positioning of *T. wallagonius* based on 28S sequences using maximum parsimony (MP).

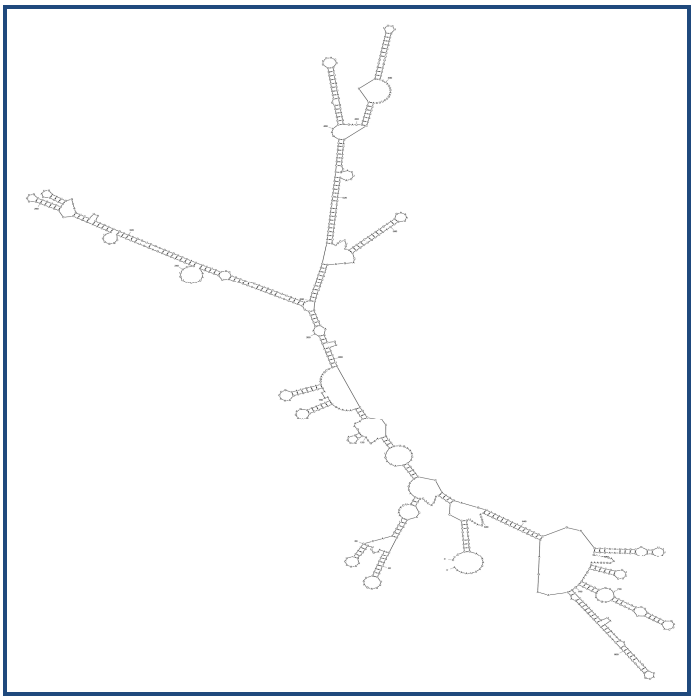

Figure 4: Schematic representation of 28S rRNA predicted secondary structure for *T. wallagonius*.

Results:

The 28S rDNA region of the *T. wallagonius* was successfully amplified using primers as mentioned above **(Figure 1).** The nucleotide sequence of 866 bp obtained from the PCR amplification was put to BLAST and compared with other available *Thaparocleidus* species sequences from GenBank. The BLAST hits result showed that the sequence of the *T. wallagonius* is closer to the species of *Thaparocleidus* within range of 91-98%. Phylogenetic trees were obtained by comparing the sequences of *T. wallagonius* and available 28S sequences of other *Thaparocleidus* species. Phylogenetic analyses using the various methods like distance method, neighbour-joining (NJ) **(Figure 1)** and character state method, maximum-parsimony (MP) **(Figure 2)** showed that the topology is similar among the trees obtained with significant bootstrap support for the clades. In the bootstrap test, the values of 70% and above show the phylogenetic accuracy and indicate reliable grouping among different members of *Thaparocleidus* species. Phylogenetic trees constructed in MEGA also revealed a close relationship of *T. wallagonius* with isolates of *Pseudancylodiscoides* because this genus is morphologically similar to *Thaparocleidus. Pseudancylodiscoides* has been considered a synonym of *Thaparocleidus* **[4]** but now this genus is considered valid and well differentiated from *Thaparocleidus*.

Secondary structure analysis

Secondary structure was reconstructed from the 28S sequence with highest negative free energy ΔG= -324.90 Kcal/mol of *T. wallagonius* to provide the basic information for phylogenetic analysis **(Figure 4)**. The secondary structural features of 28S region as shown in the figure were analyzed based on conserved stems and loops. In the structure of *T. wallagonius* the orders of preference of loops in their number were interior loop, hairpin loop, bulge loop, multi loop and exterior loop **(Figure 5)**. The stems stabilize RNA secondary structure and the different features of the structure are: G+C content (%) = 49.3; number of GC = 427; AU = 439 and GU = 461. The topology based only on the predicted RNA secondary structure of the 28S region resolved most relationships among the species studied.

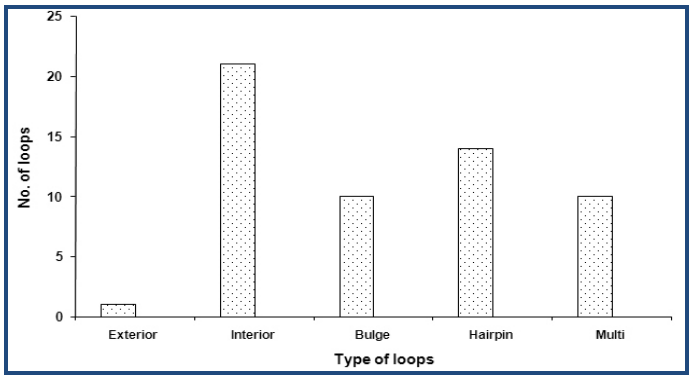

Figure 5: Distribution of different loops in the 28S region of *T. wallagonius*

Discussion:

The demand for fish *Wallago attu* as important food fish and as valuable protein source has been increased. Among the parasites infecting fishes, the monogeneans constitute a group, which plays an important role as pathogens of severe diseases **[20]** because the monogeneans affect those organs and tissues which are vital to normal functioning *i.e.* the gill and skin, the organ of respiration. This negative effect of monogeneans on the fish host may vary between species and also bacterial or viral organisms may get entry into the host through the injuries infected by monogenean anchors and feeding apparatus **[21, 22]**. Delimiting species of *Thaparocleidus* monogeneans is often difficult, owing to their limited morphological characters and this may have resulted in a gross under estimation of the true number of species. The taxonomy of *Thaparocleidus* species has been based mainly on morphological data and morphological differences found on stained and mounted adult specimens have been widely used to discriminate between the species. Of the 92 nominal species attributed to the genus *Thaparocleidus* and its synonyms, there are 15 species that are considered species inquirendae/nomina nuda and synonyms and 77 that are tentatively considered valid. At least 16 nominal species of *Thaparocleidus* have been described from *W. attu* in India **[23, 5].** So, it is difficult to ascertain from the morphological descriptions, even though many are detailed, whether the differences noted are significant or the result of environmental or other variation. The original description of *T. wallagonius* is incomplete and it needs a detailed redescription **[4, 5].** About fifteen species were considered as synonym of *T. wallagonius* because there is no significant structural difference was found in the copulatory complex and haptoral armature **[24]**. But the DNA based identification used during this study has enabled the recognition and validation of *T. wallagonius*, which has implications for our understanding of their species diversity estimates.

In the present study, primary sequence analysis revealed the validity of *T. wallagonius* and is strongly supported by molecular evidence inferred from rDNA 28S sequence analysis. With phylogenetic analysis, as general rule, if the bootstrap value for a given interior branch of a phylogenetic tree is 70% or higher, then the topology at that branch is considered reliable.

The present findings show the bootstrap value to be >70% for the tree obtained. The *T. wallagonius* species genetically well distinct from the other species of *Thaparocleidus* previously recognized with the same ribosomal marker, available at the National Center for Biotechnology Information (NCBI). The tree topologies derived from the phylogenetic analysis are in agreement where they depicted *T. wallagonius* as genetically closely related with the sibling-species, including species of genus *Pseudancylodiscoides.* Besides molecularly, *Thaparocleidus* is morphologically similar to *Pseudancylodiscoides* and can be differentiated on the basis of having a divided ventral bar, the parts of which are well separated and larval type marginal hooks **[23]**. However, there was some species of the genus *Pseudancylodiscoides* with similar characteristics described under *Thaparocleidus.*

In phylogenetic studies involving secondary-structure analysis as a tool, RNA folding is used for refining the alignment. The molecules measurable structural parameters are used directly as specific characters to construct a phylogenetic tree. These structures are inferred from the sequence of the nucleotides, often using energy minimization **[25]**. Molecular morphometrics has been found to be the most powerful tool in comparison to classical primary sequence analysis, because in the study of phylogenetics only the size variations of homologous structural segments are considered, whereas molecular morphometrics infers the folding pattern of an RNA molecule. Moreover, the secondary structure analysis also confirmed the results obtained from primary sequence analysis. Different RNA folding algorithms also take into account the structural energy as the major determinant in furnishing RNA secondary-structure models and conformation, which will definitely add significant dimensions to our understanding of the relationships among the sequence features and structural parameters that come into play in determining the structural energy. This approach can be further fine-tuned for resolving ambiguities, using differences at the RNA structural level for identification of sibling-species complexes. Therefore, structural model-based analyses of DNA sequence data have become increasingly important for phylogenetic inference. Application of the secondary-structure model of rRNA to phylogenetic analysis leads to trees with less resolved relationships among clades and probably eliminates some artefactual support for misinterpreted relationships in the highly diverse genus like *Thaparocleidus.* The highly resolved topology in tree parts suggests that a deep phylogenetic signal has been retained in the 28S sequences of extant species. However, incorporating secondary structure information allows improved estimates of phylogeny among several *Thaparocleidus* species.

Conclusion:
The present identification of the *T. wallagonius* species with 28S sequence and secondary structure analysis is consistent with investigations made using traditional approaches, i.e. by morphology. RNA secondary structure analysis could be a valuable tool for distinguishing species and completing their systematic, because the 28S secondary structure contains more information than the usual primary sequence alignment.

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