**Gyrodactylus colisai** n. sp. (Monogenea: Gyrodactylidae) a new monogenean from a freshwater fish, *Colisa fasciata* (Bloch & Schn.) at Meerut, U.P., India

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**Abstract.** *Gyrodactylus colisai* n. sp., found on body surface of fish, *Colisa fasciata* collected at Hastinapur, Meerut, India, is described. New species is characterized morphologically in having a club shaped head organ and absence of ventral bar membrane. 28S ribosomal DNA of this worm has also been sequenced for molecular characterization and found to be distinct from all the species of this genus, sequenced and available at GenBank. Beside this, secondary structure of 28S has also been predicted and found useful in taxonomic characterization.

**Keywords:** *Colisa fasciata*; 28S Ribosomal DNA; *Gyrodactylus colisai*; Hastinapur; Secondary RNA structure.

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**Introduction**

Monogeneans of the *Gyrodactylus* Nordmann, 1832 is one of the most species rich genus of the flatworms, parasitizing fishes (freshwater and marine) worldwide. 15 species of this genus has been reported from Indian subcontinent. Differentiation of species within the species rich and diverse *Gyrodactylus* is chiefly based on opisthaptor hard parts like marginal hooks, ventral bars, anchors and ciroos. Since the morphology of the opisthaptor of *Gyrodactylus* is also known to be affected by abiotic factors in the macro environment (Mo, 1991a; Dávidová et al., 2005), thus, morphological discrimination of the two species may not be straight forward. Factors like temperature, host and geographical location also influences the intraspecific phenotypic variation (Malmberg, 1970; Ergens, 1976; Mo, 1991a; 1991b; 1993). In addition, the large number of species and small size make the task identification more difficult and uncertain. Recently, molecular markers have been used for taxonomic identification and phylogenetic analyses in monogenean species belonging to the *Gyrodactylus* (Cunningham et al., 1995a, 1995b, 1995c; Ziętara and Lumme, 2003). These workers used 28S ribosomal gene for resolving the phylogenetic status as it is highly conserved and evolves slowly (Mollaret et al., 2000; Plaisance et al., 2005; Wu et al., 2007).

Most phylogenetic studies using molecular tools have focused on primary DNA sequence information. However, RNA secondary structures are particularly useful in systematics because they include characteristics, not found in the primary
sequence, that gives ‘morphological’ information. Several authors have emphasized that the secondary, not the primary structure (nucleotide sequence) is conserved at higher systematic level (Mai and Coleman, 1997; Michot et al., 1999; Coleman et al., 1998). Unfortunately, comparative studies of the secondary structures of these evolutionarily highly divergent regions are still rare, although such work could add significantly to the phylogenetic analysis (Coleman et al., 1998; Joseph et al., 1999; Gottschling et al., 2001).

In the present study, the main objective is to identify the Gyrodactylus sps. collected from host Colisa fasciata by comparing its morphology, using sequences of large subunit ribosomal DNA (LSU). Besides this, predictions of secondary RNA structure have also been made for this worm in order to evaluate its phylogenetic significance and comparative taxonomy.

Materials and methods

Fish Colisa fasciata (Bloch and Schn.), were collected from Hastinapur, U.P., India. Monogeneans were removed from the body surface of fish and studied as per method suggested by Malmberg (1970). Genomic DNA was extracted from ethanol-preserved parasites, using the DNeasy Tissue Kit (Qiagen). Partial 28S rDNA was amplified using the Eppendorf Master Cycler Personal in a final volume of 25 µl PCR reaction. Each amplification reaction contained 10X PCR buffer, 0.4 mM dNTP, 1 U Taq polymerase (Biotools) and 10 pM of each primer pair-universal primer, forward (5'-ACCGGCTGAATTTAAGCAT-3') and the reverse primer (5'-CTCTTCAGAGTACTTTTCAAC-3'). PCR was carried out for 35 cycles with the following amplification profile: 3 min. at 94°C, 30 s at 94°C, 45 s at 56°C and 1 min at 72°C, followed by final extension at 72°C for 10 min. PCR products were visualized using ethidium bromide on a 1.5% agarose TBE gel. Subsequently, products were purified by Chromous PCR cleanup kit (#PCR 10), according to manufacturer's instructions. Both DNA strands were sequenced using a Big Dye Terminator vr.3.1 cycle sequencing kit in an ABI 3130 Genetic Analyzer using same PCR primers. The sequence obtained in this study was submitted to NCBI GenBank with accession number GQ925912.

Computer aided alignment of the 28S region sequence was aligned using Clustal W (Thompson et al., 1994) and a square matrix based on K2P was performed using MEGA 4.0 (Tamura et al., 2007). Phylogenetic analysis was performed using minimum evolution (ME) and neighbour joining (NJ) methods. The reliabilities of phylogenetic relationships were evaluated using non-parametric bootstrap analysis (Felsenstein, 1985) for the ME and NJ trees. Bootstrap values ≥60 were considered very well supported (Hillis and Bull, 1993).

RNA secondary structure was determined using Sfold (Software for Statistical Folding and Rational Design of Nucleic Acids) in the Sribo program (Ding and Lawrence, 2003), by screening for thermodynamically optimal and suboptimal secondary structures (Default setting with T=37°C). Inferred structures were examined for stems, loops and bulges. Since, GC content is known to influence structural energy, therefore, GC percentage was determined using GC calculator (http://www.genomicsplace.com/gc_calc.html). Energy levels of presumptive secondary structure were subsequently then calculated with Mfold (Jaeger et al., 1989; Zuker et al., 1999).

Results

Type-host: Colisa fasciata (Bloch and Schn.)

Type-locality: Hastinapur (28°50'N, 77°45'E), Meerut, U.P., India.

Site of infection: Body surface (Skin)

Type material: The holotype and paratype slides have been deposited in the Museum of Department of Zoology (Voucher number HS/Monogenea/2009/07), Ch. C. S. University, U. P. India.

Etymology: Named after the host, Colisa fasciata
**Morphological description** (Based on ten specimens)

Body of worm elongated, measuring 0.19-0.20 mm in length and 0.03-0.04 mm in width (figure 1a). Both prohaptor and opisthohaptor fairly set off from body proper. Pharynx consists of an anterior part and a posterior part. Anterior part measures 0.005-0.006 mm in length and 0.01-0.02 mm in width. Posterior part measures 0.012-0.015 mm in length and 0.01-0.02 mm in width. Both parts of pharynx are of same width. Anterior part, equipped with 8 pharyngeal processes. Intestine simple, crura terminate blindly in ovarian region of body. Testis not observed. Cirrus sac elongate, oval located immediately behind pharynx, measuring 0.010-0.013 mm in diameter. Cirrus further strengthened with 2 large and 7 small spines (figure 1b). Ovary single, rounded in outline measuring 0.010-0.015 x 0.012-0.016 mm.

![Figure 1](image)

Figure 1. *Gyrodactylus colisai* n. sp.: a. Whole mount; b. Cirrus; c. Marginal hooklet; d. Haptor

Haptor oval distinctly set off from body proper and measures 0.025-0.030 mm long and 0.020-0.025 mm wide. Haptor, armed with a pair of anchors, superficial bar, deep bar and eight pairs of marginal hooklets (figure 1d). Each anchor moderately stout, made of 3 main parts, root, shaft and point. Details of measurements are: length of root 0.018-0.020 mm; length of shaft 0.020-0.025; length of point 0.010-0.015 mm. On dorsal side of anchor, between the root and shaft, site for attachment of dorsal transverse bar is apparently visible. Superficial bar, more or less straight 0.010-0.015 mm long, 0.005-0.008 mm wide, having two attachments for the anchor. Deep bar rectangular in outline with shallow depression at centre. It consist a pair of anteriorly directed processes and a central part, called true bar. Posteroid membrane absent. Length of deep bar 0.010-0.012 mm. Marginal hooklet consists of a sickle, a handle, a sickle membrane and a sicle filament loop. Proximal part of handle, bend inwardly and swollen. Marginal hook 0.030-0.035 mm long, handle 0.020-0.025 mm long, sickle length 0.010-0.015 mm, filament 0.011-0.012 mm (figure 1c).

**Molecular analysis**

Amplified fragment of 28S rDNA was 348 bp in length. Reference sequences used in this study are listed in table 1. *Merizocotyle icopae* was chosen as an out-group for phylogenetic study. Blast analysis of sequence exhibited significant difference from other monogenean species. Similarity between the sequence of *G. colisai* and other *Gyrodactylus* monogenean sequences included in alignment ranged between 83% and 92%. *G. colisai* n. sp. and *G. macracanthus* appeared to be the most closely related species is much more divergent with a well supported clade by neighbour joining and minimum evolution methods (figure 2). Both the methods gave trees with similar topology and approximate relatively bootstrapped values. Bootstrap consensus tree inferred from 500 replicates. Branches corresponding to partitions reproduced in less than 60% bootstrap replicates are collapsed. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-
parameter method and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm. The Neighbor-joining algorithm was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option).

Table 1. Reference sequences used from Genbank in the comparison and construction of phylogenetic tree

<table>
<thead>
<tr>
<th>Gyrodactylus sp.</th>
<th>Location/Source</th>
<th>Genbank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Gyrodactylus macracanthus</em></td>
<td><em>Misgurnus anguillicaudatus</em> from Australia</td>
<td>FJ971995</td>
</tr>
<tr>
<td><em>Gyrodactylus salaris</em></td>
<td><em>Salmo salar</em> from Norway</td>
<td>FJ971996</td>
</tr>
<tr>
<td><em>Gyrodactylus salaris</em></td>
<td>Norway</td>
<td>AJ542394</td>
</tr>
<tr>
<td><em>Gyrodactylus derjavini</em></td>
<td><em>Oncorhynchus mykiss</em> from Denmark</td>
<td>FJ971994</td>
</tr>
<tr>
<td><em>Merizocotyle icopae</em></td>
<td>(outgroup)</td>
<td>AF348349</td>
</tr>
</tbody>
</table>

*The asterisk is used due to same species name

Figure 2. Phylogenetic tree inferred from Minimum Evolution method

G+C content for the 28S region of rDNA of specimens at disposal of the authors is 45.1%. Minimum free energy is estimated by summing individual energy contributions from base pair stacking, hairpins, bulges, internal loops and multi-branch loops. Minimum free energy in the sample (SMFE) = -117.70 kcal/mol. Using the energy minimization approach and comparative sequence analysis, the secondary RNA structure of *G. colisai* n. sp. was predicted which is organized in five stems (figure 3). The segregation of the sequence into five separate stems is better appreciated by displaying the structure as graph plot (figure 4). The five stems were with 1 exterior loop, 5 interior loop, 5 bulge loop, 7 hairpin loop and 4 multi loop.

\[ \Delta G^\circ = -117.70 \]

Figure 3. Predicted 28S RNA secondary structure of *G. colisai* n. sp.
Each residue is represented on the abscissa and semi-elliptical lines connect bases that pair with each other.

To aid in the characterization of the sampled structural space, we introduced the centroid structure as an efficient means to characterize the structure and present a procedure for its identification. In a centroid diagram, bases are positioned along a circle, in a clockwise orientation and arc connecting two bases across the circle indicates pairing between the bases. Lack of pseudoknots in the secondary structure is reflected by the absence of intersecting lines in the centroid structure (figure 5).

From the two-dimensional histogram, the patterns of base pair frequencies are nearly identical for the sample (figure 6). Probability profiles \(W=4\) for the sample are also computed (figure 7).

![Figure 4](image1.png)

**Figure 4.** Bar diagram showing distribution of various loops in 28S region of *G. colisai* n. sp.

![Figure 5](image2.png)

**Figure 5.** Centroid structure as a representative of predicted structure of *G. colisai* n. sp.

![Figure 6](image3.png)

**Figure 6.** The two-dimensional histogram displays nearly identical patterns of base pair probabilities

![Figure 7](image4.png)

**Figure 7.** Statistical reproducibility for *G. colisai* n. sp. is illustrated by probability profile

On a profile for fragment width \(W\), the probability that \(W\) consecutive bases are all unpaired is plotted against the first base of the segment. This approach was shown to make substantially better predictions than the MFE structure. Significance of assigning probability as a measure of confidence in prediction is also highlighted. A single-stranded region predicted by both the MFE structure and the ss-count statistic from mfold has low probabilities on the probability profile \(W=4\) bases, as described in Ding and Lawrence, 2001. The ss-count statistic gives the propensity of a base to
be unpaired, as measured by the frequency with which it is unpaired in a group of the optimal and suboptimal foldings within a specified increment of the MFE. At nucleotide position i, the probability that nucleotide i, i+1, i+2, i+3 (i.e. fragment width W=4) are all single stranded is plotted against i. This probability is computed by MFE structure and by ss-count from mfold for the nucleotides 1-348.

Discussion

The morphological features of the present specimen, place it within the *Gyrodactylus* Nordmann, 1832 and the *G. neonephrotus anguillae* sp. Group (Malmberg, 1970). A list of fifteen species of this genus reported from India is given in the form of table 2. The specimens at disposal of the authors is different in morphological details of marginal hooklets, anchors, transverse bar, cirrus, presence of club shaped head organs and absence of a ventral bar membrane. It differs from *G. elegans indicus* Tripathi, 1957 in having different shape and size of anchors and hooks. *G. medius* (Kathariner, 1894) Tripathi, 1957 and *G. colisai* shows differences in body size, length of anchors, dorsal bar and marginal hooklets. It is also different from *G. hyderabadensis* Venkatanarsaiah, 1979 in having different shape of dorsal bar, anchors, number of spines and spinelets in the cirrus. It was also found that *G. colisai* shows differences in shape of anchor and deep bar when compared with *G. eutheraponsis* Venkatanarsaiah and Kulkarni, 1980. However, *G. recurvensis* Rukmini & Madhavi, 1989 differs from present species on account of having different shape and size of hard parts like narrow dorsal bar and a 'W' shaped ventral bar, that shape were not found in the present specimen. Differences were found from *G. raipurensis* Dubey et al., 1990 in having strong inward curvature of superficial root of anchors, to the extent that length of their main part is almost equal to total length of anchors which is different in the present form. Dubey et al. (1990) also described *G. gussevi*, which is different from *G. colisai* in body size and shape of hooks. They also described *G. mizellei* in the same year, although validity of this species is doubtful, between *G. mizellei* and *G. gussevi* but like *G. gussevi*, *G. mizellei* also differs from *G. colisai* in body size and structure of hooks. *G. neonephrotus malmbergi* Singh and Agrawal, 1994, shows differences from *G. colisai* in having alae at the base of anchor root, shape of wings at the base of anchor and shape of hooklets. *G. chauhani* Agrawal and Bhatnagar, 1997 shows differences from *G. colisai* in having different shape of superficial and deep bars, anchors and hooks.

Table 2. List of various species of *Gyrodactylus* Nordmann, 1832 reported from India, their host and geographical origin

<table>
<thead>
<tr>
<th>Gyrodactylus sp.</th>
<th>Host species</th>
<th>Locality</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. elegans indicus</em> Tripathi, 1957</td>
<td><em>Labeo rohita, Labeo bata, Cirrhinus cirrhosus, Cariza, Catla catla</em></td>
<td>Lucknow, Bengal</td>
</tr>
<tr>
<td><em>G. medius</em> (Kathariner, 1893)</td>
<td><em>Labeo rohita, Cyprinus carpio, Carassius auratus</em></td>
<td>Karnataka</td>
</tr>
<tr>
<td>Tripathi, 1957</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>G. hyderabadensis</em> Venkatanarsaiah, 1979</td>
<td><em>Channa sp., C. punctata, Eutherapon therops</em></td>
<td>Hyderabad, Meerut</td>
</tr>
<tr>
<td><em>G. recurvensis</em> Rukmini and Madhavi, 1989</td>
<td><em>Heteropeuceus fasciatus, Heteropeuceus siamensis</em></td>
<td>Visakhapatnam, Kondakaria, Chilka</td>
</tr>
<tr>
<td><em>G. raipurensis</em> Dubey et al., 1990</td>
<td><em>Channa gachua, C. punctatus</em></td>
<td>Raipur</td>
</tr>
<tr>
<td><em>G. gussevi</em> Dubey et al., 1990</td>
<td><em>Heteropeuces fasciatus</em></td>
<td>Raipur</td>
</tr>
<tr>
<td><em>G. mizellei</em> Dubey et al., 1990</td>
<td><em>Mystus horai</em></td>
<td>Meerut</td>
</tr>
<tr>
<td><em>G. neonephrotus malmbergi</em> Singh and Agrawal, 1994</td>
<td><em>Heteropeuces malmbergi</em></td>
<td>Meerut</td>
</tr>
<tr>
<td><em>G. chauhani</em> Agrawal and Bhatnagar, 1997</td>
<td><em>Colisa fasciatus</em></td>
<td>Lucknow</td>
</tr>
<tr>
<td><em>G. vivekanensis</em> Shuda, 2001</td>
<td><em>Puntius sophore</em></td>
<td>Sultanpur</td>
</tr>
<tr>
<td><em>G. baughi</em> Agrawal et al., 2004</td>
<td><em>Puntius ticto</em></td>
<td>Lucknow</td>
</tr>
<tr>
<td><em>G. punti</em> Agrawal et al., 2004</td>
<td><em>Puntius sophore, Puntius ticto</em></td>
<td>Lucknow</td>
</tr>
<tr>
<td><em>G. medius orientalis</em> Singh et al., 2006</td>
<td><em>Channa punctatus, C. striatus</em></td>
<td>Meerut</td>
</tr>
<tr>
<td><em>G. medius malambergi</em> Singh et al., 2006</td>
<td><em>Carassius sp.</em></td>
<td>Meerut</td>
</tr>
<tr>
<td><em>G. medius exotica</em> Singh et al., 2006</td>
<td><em>Carassius sp.</em></td>
<td>Meerut</td>
</tr>
</tbody>
</table>
Shukla (2001) described *G. vivekanensis* which differs from *G. colisai* on the basis of differences in structure of anchors and ventral bar. Agrawal et al. (2004) described two new species of *Gyrodactylus* viz., *G. baughii* which shows differences in shape bars and male copulatory organ with *G. colisai*. Moreover, *G. punti* also differs from *G. colisai* in shape and size of anchors, deep bar and superficial bar.

Singh et al. (2006) described three new species of *Gyrodactylus* i.e. *G. medius orientalis*, *G. medius malambergi* and *G. medius exotica* from Meerut (U.P., India). *G. medius orientalis* different from *G. colisai* in having ventral bar membrane, four spinelets in the cirrus and different shape of dorsal transverse bar. *G. medius malambergi* shows differences with the present specimen in having well marked sigmoid curve and inwardly directed root of the anchor, one large spine, a row of six small spinelets and short ventral bar membrane. *G. medius exotica* and *G. colisai* different in anchor roots, anchor folds and differences in the spinelets of cirrus.

The morphological and morphometric diagnosis of *Gyrodactylus* species is evidently difficult (Bakke et al., 2007). The best evidence of this difficulty is the ratio of described and named species to the estimated global number of species. Bakke et al. (2002) predicted that the real number of *Gyrodactylus* species might be more than 20000, yet only 470 names are considered valid and available. It is difficult to manage 20000 species in a morphological archive based on subtle differences in the opisthaptoral hard parts. Thus, additional and more informative characters are needed and available in the form of DNA sequences. Present work demonstrated that both morphological and molecular markers are needed to understand the evolution and even taxonomy of parasites in this genus.

DNA taxonomy use nucleotide sequence data to achieve comprehensive species descriptions that facilitate reliable species diagnostics and rapid assessment of biodiversity, which is of great importance for parasitologists. Beside the morphological features, molecular data further supports that *G. colisai* is a new species, fits in the *G. neonephotrotus anguillae* sp. group of *Gyrodactylus* (Malmberg, 1964). Unfortunately, no sequence data are available for any species of *Gyrodactylus* from India. Analysis of the sequence of 28S rDNA region clearly allowed discrimination at the species level from species reported from foreign land which sometimes creates problem in species identification, using morphological structures.

The combined approach of morphological and molecular biology clears the difficulties in species identification. In the case of monogenea, the rDNA have proven to be the reliable markers for determination, especially for species in which identification based on morphology is difficult. 28S rDNA sequences are the most frequently used DNA markers for studying monogenean evolution. Alignment of the sequence with other *Gyrodactylus* species clearly revealed that the 28S rRNA region is highly conserved, even across distantly related phyla as also reported by Mollaret et al. (2000); Plaisance et al. (2005); Wu et al. (2007).

Neighbour joining (NJ) and minimum evolution (ME) analysis, showed that *G. colisai* and *G. macrurcanthus* are closely related species. Genetic relatedness with specimens documented from other parts of the world provides further clues to the understanding of the evolution of the *Gyrodactylus* species. Besides this, our analyses strongly indicate that 28S rDNA in monogenean, with careful readjustment under guidance of the secondary structure, is very much applicable to different levels of phylogenetic analyses from populations to genera. The position of *G. colisai* in the phylogenetic trees reconstructed in BLAST confirms its placement within Monogenoidea and it also shows that the position of *G. colisai* in the molecular phylogenetic tree corresponds well to the morphological similarity with the representative of *Gyrodactylus*. This approach can be further fine-tuned in resolving ambiguities using differences at the RNA structural level for identification of sibling species complexes.

Moreover, we also inferred the secondary structures of the long subunit (LSU) in *G. colisai*. The structure prediction method we proposed, presents a promising approach to
reconstruct secondary structures of non-coding genes in taxa that have not been studied so far but has significant taxonomic and phylogenetic value as also advocated by Coleman et al. (1998); Joseph et al. (1999); Gottschling et al. (2001). The consideration of taxon-specific secondary structure models helps to improve the inference. 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 meaningful dimensions to our understanding of the relationships among the sequence features and structural parameters that come into play in determining the structural energy. The present study demonstrates use of secondary structure on phylogenetic analyses of rRNA sequences. This effect was remarkable in sequence alignment and tree reconstruction of both simulated and empirical data.

In conclusion, we can say that the integration of morphological and molecular data seems the best approach to resolve the difficulties in species identification. This work has confirmed the existence of *G. colisai* n. sp. from skin of *Colisa fasciata*. Further work on the systematics of gyrodactyliids might provide important clues about the evolution of this group of parasites in Indian subcontinent.

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