PHYLOGENETIC POSITION OF THE MONOGENEAN MALAYANODISCOIDES BIHAMULI LIM ET FURTADO, 1986 INFERRED FROM SEQUENCE AND SECONDARY STRUCTURE OF 28S rDNA

ANSHU CHAUDHARY* and HRIDAYA SHANKER SINGH

Abstract. The present study is a brief description of the morphology and molecular phylogeny of Malayanodiscoides bihamuli Lim and Furtado, 1986 infecting gill filaments of fish Notopterus chitala (Hamilton). The phylogenetic study was made of M. bihamuli and closely related taxa using DNA sequence data obtained from 28S rDNA region. Morphology as well as molecular phylogeny strongly supports the establishment of genus Malayanodiscoides and also demonstrates its close relationship with other genera of class Monogenea. In addition, the 28S secondary structure model was predicted and found to serve as a useful tool for reconstruction of optimal alignment and can be used as an additional source of data incorporating structural parameters of molecules for the study of phylogeny. Application of the secondary 28S structure data allows a more resolved and realistic picture of relationships. Results also demonstrate the phylogenetic utility of the 28S sequence secondary structure data for inferences at higher taxonomic levels.

Keywords: Malayanodiscoides, monogenea, 28S rDNA gene, ribosomal DNA, Notopterus chitala

INTRODUCTION

Genus Malayanodiscoides was established for the worms collected from gill filaments of Notopterus notopterus on the Malaysian peninsula by LIM and FURTADO (1986) with M. bihamuli as a type-species. Subsequently, AGRAWAL et al.
(1998) described another species under the genus *viz.*, *M. indicus* from *N. notopterus* near Lucknow, Uttar Pradesh, India. Lim et al. (2001), on the basis of morphological features, synonymize *M. indicus* Agrawal et al. (1998) with *M. bihamuli* Lim and Furtado (1986).

Since its inception, the establishment of the genus has been surrounded with controversies. This genus was differentiated initially from an Ancylocephalid monogenean, *Thaparocleidus* Jain (1952) on account of a sac like seminal vesicle and onchium between the first pair of marginal hooks. Presence of such a sclerite is not exclusive to *Malayanodiscoides* but is also reported from other genera like *Bagrobdella* Paperna, 1969, *Protoancylodiscoides* Paperna, 1969, and *Bychowskyella* Akherov, 1952. In addition, this genus was placed, based on the basis of the presence of a sac like seminal vesicle, in the family Ancylodiscoididae, although it is recovered from a Notopterid fish, *Notopterus* species. Thus, inclusion of this genus in Ancylocephalid has been doubted (Lim et al. 2001).

Morphological characters traditionally used in taxonomy on several occasions are not likely to be informative to infer phylogeny. Besides this, issues of phenotypic plasticity (e.g. convergence), potential flaws of particular morphological characters in their application to phylogenetic inferences which may include character linkage, exceeding interspecific variability, nonhomology, and artifacts in interpreting characters. However, it is found that in monogeneans, the nucleotides contain a sufficient phylogenetic signal to resolve relationships (Olson et al. 2003). Sequences of partial large subunit (28S rDNA) have been successfully used to study phylogenetic relationships of the monogeneans at higher levels (Mollaret et al. 1997, Littlewood et al. 1998, Mollaret et al. 2000b, Jovelin and Justine 2001) and generic levels (Mollaret et al. 2000a, Chisholm et al. 2001, Justine et al. 2002, Olson and Littlewood 2002, Whittington et al. 2004, Wu and Worheide 2005). A general goal of the present work is to develop a phylogenetic framework for *Malayanodiscoides* Lim and Furtado (1986) and to analyze 28S rRNA gene for inferring the phylogenetic position of this genus within the vast class Monogenea.

Moreover, during the study, the secondary structure was predicted for fragments of 28S for this species. RNA molecules are key elements in some of the cells most fundamental processes, including catalysis, RNA splicing, and regulation of transcription and translation. To a large degree, the function of a structural RNA molecule is determined by its structure. As ribosomal RNA (rRNA) sequences are highly structured, with large regions exhibiting conserved base pairing patterns, incorporating this information in sequence alignment and tree reconstruction might help to reduce errors associated with these problems (Jow et al. 2002, Kjer 2004, Telford et al. 2005, Dohrmann et al. 2006, 2008, Erpenbeck and Worheide 2007, Kjer and Honeycutt 2007, Voigt et al. 2008, von Reumont et al. 2009). Since the rRNA molecule forms secondary structures mediated by base pairings, knowledge of secondary structure allows for the application of a more sophisticated model, consequently generating a picture of relationships that is arguably more realistic.
Materials and Methods

Parasites, DNA extraction and amplification
Monogenean *Malayanodiscoides bihamuli* were collected from gills of *Notopterus chitala* (HAMILTON, 1822) near Hastinapur (29°01’N and 77°45’E), Meerut, U.P., India, as per the method suggested by MÅLMBERG (1970). Morphological studies were made as suggested by MÅLMBERG (1970). For the molecular study, each monogenean was examined with a microscope for species identification and fixed in either 95% or 100% ethanol for genomic DNA extraction or destroyed in the process of DNA extraction. Monogenean DNA was extracted from the whole specimen using Qiagen DNeasy Tissue Kit (Qiagen) as per the manufacturer’s instructions. 28S rDNA region was amplified with the universal forward primer, (5’-ACCCGCTGAATTAAAGCAT-3’) in combination with reverse primer (5’-CTCTTCAGAGTACTTTTCAAC-3’). Each amplification reaction was performed in a final volume of 25 µl containing 3 µl of lysate, 10X PCR buffer, 1 U Taq polymerase (Biotools), 0.4 mM dNTP and 10 pM of each primer pair in a thermocycler (Eppendorf Mastercycler Personal). PCR amplification was performed using the following conditions: after an initial denaturation at 94°C for 3 min, 35 cycles at 94°C for 30 sec (denaturation), 56°C for 45 sec (annealing), 72°C for 10 min (elongation). Resultant PCR products were examined on 1.5% agarose-TBE gels, stained with ethidium bromide and visualized under ultraviolet light.

DNA sequencing and phylogenetic analyses
Amplification products were purified by Chromous PCR clean up Kit (#PCR 10) according to manufacturer’s instructions. Gel purified PCR products were sequenced using a Big Dye Terminator version 3.1 cycle sequencing kit in an ABI 3130 Genetic Analyzer with the same primers. Sequences of closely related monogeneans were aligned using the program ClustalW (THOMPSON et al. 1994). These are: *Bothitrema bothi* AF387508; *Monocotyle* sp. AF387511; *Triloculotrema* sp. AF387512; *Calicotyle affinis* AF382061; *Pseudodactylogyrus anguillae* AJ969950; *Pseudodactylogyrus bini* AJ969949; *Neocalceostoma* sp. AF387510; and *Thaparocleidus siluri* AJ969940. The results of ClustalW analysis is graphically represented by using GeneBee server (BRODSKY et al. 1992). Phylogenetic trees were reconstructed using MEGA version 5 (TAMURA et al. 2011). Phylogenetic analyses were performed based on neighbor-joining (NJ) and maximum-parsimony (MP) methods. In reconstructing the NJ tree, the Kimura-2-parameter model was used to estimate the distances. To obtain the most-parsimonious tree, the Max-Mini Branch-and-bound search strategy was used in MP method. Robustness of the inferred phylogeny was assessed using a bootstrap procedure with 1,000 replications.

Prediction of secondary structures
RNA secondary structure was determined using Sfold (Software for Statistical Folding and Rational Design of Nucleic Acids) in the Sriba program based on a statistical sample of Boltzmann ensemble for secondary structures (DING...
and Lawrence 2003). Subsequently, inferred structure was examined for stems, loops, and bulges. Since GC content is known to influence structural energy, GC percentage was determined using GC calculator (http://www.genomicsplace.com/gc_calc.html). All non-DNA characters were stripped before computing. Energy levels of presumptive secondary structure were then calculated with Mfold (Jaeger et al. 1989, Zuker et al. 1999). 28S sequences with secondary-structure formats of M. bihamuli and T. siluri were aligned using the MARNA web server (Siebert and Backofen 2005) based on both the primary and secondary structures.

RESULTS

Malayanodiscoides bihamuli Lim and Furtado, 1986

Host- Notopterus chitala (Hamilton, 1822)
Locality- Hastinapur, (29°01’N and 77°45’E), Meerut, U.P., India
Site of infection- Gills

The 108 slides were deposited in the museum of the Department of Zoology (Voucher number HS/Monogenea/2009/08), Ch. C.S. University, Meerut, U.P., India. 298 bp fragment of 28S rDNA gene sequence of Malayanodiscoides bihamuli obtained in this study was deposited in GenBank under the accession number GU830882.

Morphology

Body elongated with a narrow anterior end and broad posterior end measuring 200-250 x 30-35 µm. Cephalic region is equipped with four pairs of head organs and two pairs of eye spots along with scattered accessory granules. Pharynx round measuring 15-12 µm. Oesophagus very short, intestinal caeca united and crura confluent posteriorly, anterior to the haptor. Ovary is elongated oval, post-equatorial, ventral, intercaecal measuring 45-50 x 15-20 µm. Vagina 5-10 µm long, dextral, funnel shaped which leads to ootype complex through vaginal tube. Vitelline glands are densely distributed through out the body, from behind the pharynx up to haptor. Testis measures 25-30 x 10-20 µm single, pear-shaped, intercaecal, pre-equatorial, pre-ovarian. Vas deferens arises from anterior end of testis, loops between intestinal caeca, dilate to form a pear-shaped seminal vesicle which opens at base of copulatory organ. Male copulatory complex comprises of a tube which tapers and ends in a hook measuring 50-55 µm in length. Tube has a bulbous structure in middle region, a broad flat accessory piece. Opisthohaptor is distinctly set off from the body proper, round structure. Armature of the haptor comprised of dorsal and ventral anchors, dorsal and ventral bars and seven pairs of marginal hooklets. Dorsal anchors, inner length 40-45 µm, outer length 43-45 µm, inner root long 20-25 µm, outer root 4-7 µm, length of patches 50-10 µm, recurved point 10-15 µm long. Ventral anchors, inner length 35-40 µm, outer length 40-45 µm, inner root 15-20 µm long, outer root 3-5 µm, recurved point 15-20 µm long. Dorsal and ventral bar “V” shaped 30-35 µm and 35-40 µm in length respectively. Well developed sleeve
sclerite is found at the shaft of dorsal and ventral anchors. Marginal hooklets are 7 pairs, of which pair no. 1 measures 15-20 µm, ventral hooks pair no. 7 with boat shaped bar measures 20-25 mm and five pairs similar, 15-20 µm. Comparison of various body parts is given in the table 1.

Table 1 Comparative measurements of *Malayanodiscoides bihamuli*.

<table>
<thead>
<tr>
<th>Body features</th>
<th>Agrawal, Vishwakarma, 1998 (in µm)</th>
<th>Present specimen (in µm)</th>
</tr>
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<tbody>
<tr>
<td>Body:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length</td>
<td>430-770</td>
<td>0.200-250</td>
</tr>
<tr>
<td>Width</td>
<td>-</td>
<td>30-35</td>
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<tr>
<td>Pharynx</td>
<td>40-45</td>
<td>15-20</td>
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<tr>
<td>Haptor:</td>
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<td></td>
</tr>
<tr>
<td>Length</td>
<td>-</td>
<td>45-50</td>
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<tr>
<td>Width</td>
<td>-</td>
<td>40-45</td>
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<tr>
<td>Dorsal anchor:</td>
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<tr>
<td>Inner length</td>
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<td>40-45</td>
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<td>Outer length</td>
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<td>20-25</td>
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<tr>
<td>Outer root</td>
<td>2-5</td>
<td>4-7</td>
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<tr>
<td>Recurved point</td>
<td>25-35</td>
<td>20-25</td>
</tr>
<tr>
<td>Patch</td>
<td>5-10</td>
<td>5-10</td>
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<tr>
<td>Ventral anchor:</td>
<td></td>
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<tr>
<td>Inner length</td>
<td>30-40</td>
<td>35-40</td>
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<tr>
<td>Outer length</td>
<td>35-45</td>
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<tr>
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</tr>
<tr>
<td>Length</td>
<td>60-84</td>
<td>45-50</td>
</tr>
<tr>
<td>Width</td>
<td>-</td>
<td>15-20</td>
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<tr>
<td>Vagina</td>
<td>-</td>
<td>5-10</td>
</tr>
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**Phylogenetic Analysis**

Amplified sequence of 28S rDNA of *Malayanodiscoides bihamuli* is 298 bp long. Analysis with ClustalW shows alignment of *M. bihamuli* and other monogenean genera individually (Fig. 1). The multiple sequence alignments exhibit the variation in the alignment very clearly. The smallest K2P distance
Fig. 1 Alignment of 28S sequences of monogenean species.

Table 2 Estimates of evolutionary divergence between sequences using the Kimura 2-parameter model.

<table>
<thead>
<tr>
<th></th>
<th>GU830882</th>
<th>AJ969940</th>
<th>AF387510</th>
<th>AJ969950</th>
<th>AJ969949</th>
<th>AF387508</th>
<th>AF382061</th>
<th>AF387511</th>
<th>AF387512</th>
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<tbody>
<tr>
<td>M. indicus</td>
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<tr>
<td>T. siluri</td>
<td>0.17</td>
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<td></td>
<td></td>
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<tr>
<td>N. sp.</td>
<td>0.19</td>
<td>0.16</td>
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<tr>
<td>P. anguillae</td>
<td>0.33</td>
<td>0.24</td>
<td>0.21</td>
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<tr>
<td>P. bini</td>
<td>0.34</td>
<td>0.25</td>
<td>0.21</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>B. bothi</td>
<td>0.34</td>
<td>0.27</td>
<td>0.27</td>
<td>0.30</td>
<td>0.31</td>
<td></td>
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<tr>
<td>C. affinis</td>
<td>0.36</td>
<td>0.23</td>
<td>0.28</td>
<td>0.29</td>
<td>0.25</td>
<td></td>
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<tr>
<td>M. sp.</td>
<td>0.38</td>
<td>0.24</td>
<td>0.28</td>
<td>0.29</td>
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<tr>
<td>T. sp.</td>
<td>0.40</td>
<td>0.27</td>
<td>0.31</td>
<td>0.30</td>
<td>0.31</td>
<td>0.25</td>
<td>0.10</td>
<td>0.12</td>
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(0.17) was found between sequence of *M. bihamuli* and *Thaparocleidus siluri* (AJ969940), the longest between *M. bihamuli* and *Triloculotrema* sp. (AF387512) as shown in Table 2. Both NJ and MP analysis inferred from 28S rDNA sequences gave similar topology, thus only the NJ phylogenetic tree is given (Fig. 2). Bootstrap values below 50% were not considered. The most important finding is that *M. bihamuli* clustered in the same clade with *Thaparocleidus* in both analyses. In addition, in the NJ and MP tree, *M. bihamuli* is found to be closest to *T. siluri* (Zandt, 1924), the species of the genus *Thaparocleidus* Jain, 1952 (Fig. 2).

Fig. 2 Neighbor Joining (NJ) bootstrap consensus tree inferred by MEGA 5 for *Malayanodiscoides bihamuli* based on 28S rDNA. The NJ tree was obtained by the bootstrap method with 1000 heuristic search replicates. Bootstrap values are shown at the nodes: NJ and MP values are shown above and below the nodes, respectively.

**Secondary structure prediction**

GC content of 28S rDNA fragment of the monogenean, *M. bihamuli* is 46.6%. With the help of primary sequences, the secondary structure is drawn (Fig. 3). In this figure, each residue is shown by a base pair, the hydrogen bonds and backbone of the rRNA secondary structure presented as dots in between the base pair. The highest negative free energy is -98.00 Kcal. Moreover, it shows that the five different types of loops are present *i.e.*, exterior, hairpin, multi, interior and bulge (Fig. 4). Centroid structure of *M. bihamuli* has also been worked out, showing minimum total base-pair distance (Fig. 5). Analysis of this centroid shows an absence of intersecting lines, which proves that pseudoknots are absent in the secondary structure. Besides this, secondary structure of *M. bihamuli* base pair frequencies are nearly similar in the two dimensional histogram (Fig. 6).

The 3D energy landscape plot of the secondary structure of *M. bihamuli* rRNA (Fig. 7) exhibits the presence of two optimal numbers of clusters displayed with the help of red and blue colored dots. Minimum free energy (MFE) structure and ensemble centroid, light blue in color, form the largest cluster with a
Fig. 3 Predicted 28S RNA secondary structure and their structure formation enthalpy.

$$\Delta G^\circ = -98.00$$
Fig. 4 Distribution of different types of loops of *Malayanodiscoides bihamuli* large subunit sequence 28S.

Fig. 5 Centroid for the 28S rDNA sequence.
probability of 0.681. The coordinates are given for the MFE structure (-19.16, -16.93, -98.30), the ensemble centroid (-6.05, 7.21, -90.90), the centroid of cluster 1 (-13.99, 7.92, -96.90), and the centroid of cluster 2 (26.68, 8.16, -86.24).

Reconstruction of optimal alignment using secondary structure information of *M. bihamuli* and *T. siluri* 28S sequences were made (Fig. 8). The consensus structure is as a string of dots and brackets on top of the alignment. Compatible base pairs are colored. The hue shows sequence conservation of the
The alignment is shown together in the form of the predicted consensus structure because the accuracy of secondary structure prediction from single species sequences is limited, the consensus structure is formed from the related RNA sequences (Fig. 9). The presence and absence of stems and loops are therefore good phylogenetic indicators for relatively closely related taxa. Considering our findings, the presence and absence of these structures appears to provide strong phylogenetic information at taxonomical levels. We reported the secondary structure of a monogenean 28S rDNA sequences and suggest that these predictions of secondary structures that are specific for some monogeneans may be in structures of all diverging monogeneans. Relating secondary structure to sequence information will allow the phylogenetic signal of the large numbers of 28S rRNA sequences of monogenea that are currently available in Genbank to be considerably increased. 28S rDNA secondary structures will facilitate the inclusion of secondary structure information in phylogenetic analyses.

Fig. 7 3D energy landscape plot. Cluster size is according to the descending order of clusters and red asterisk shows the cluster containing MFE structure.
Fig. 8 Alignment with color annotation showing conservation of base pairs and annotated column identity between *Malayanocephaloides* and *T. siluri* (Base pairs are colored using the same color code, such that the hue shows sequence conservation and saturation shows structural conservation).
DISCUSSION

Taxonomic position of *M. bihamuli* Lim and Furtado, 1986 is based on only morphological features. Moreover, inclusion of this genus in Ancylocephalid has been doubted (Lim et al. 2001). In the present work, *M. bihamuli* phylogenetic sequence analysis and derived tree topology show a close relationship with the genus *Thaparocleidus* Jain, 1952 from which it was initially differentiated morphologically. This confirmed the description of new genus *Malayanodiscoides* by Lim and Furtado (1986). Both the taxa show the formation of the one clade from which, the two species *M. bihamuli* and *T. siluri* (Zandt, 1924) formed separate subclades and confirmed the establishment of species *M. bihamuli* by Lim and Furtado (1986) which is in agreement with the findings of Mollaret et
RNA molecules have versatile functions inside a cell ranging from catalytic processes to complex patterns in gene regulation (Fedor and Williamson 2005, Amaral et al. 2008, Sharp 2009). During the study, it was found that for monogeneans systematics, RNA secondary structures can be used as an important tool for the differentiation of species because in comparison to primary sequences, the secondary structure contains more information in the form of stems, loops, and minimum free energy (MFE). Little is known about the function and the secondary structure of monogeneans 28S SRNA. Free energy minimization is computed for a specific, selectable temperature. A study by Armbruster (2001) showed slight alterations of the temperature can have a strong influence on the prediction of structure. The minimum free energy is estimated by summing individual energy contributions from base pair stacking, hairpins, bulges, internal loops, and multi-branch loops. The RNA secondary structure consists of stems and loops. Moreover, MDS (multidimensional scaling) is a technique for representing high-dimensional objects in typically two dimensions (Kruskal and Wish 1977). In RNA secondary structures, base pair distances are used as an input to MDS, and members of all other clusters are plotted as small circles (Chan et al. 2005). RNA molecular structure helps to elucidate an understanding of their functions. A common structure that is conserved is expected when the RNA structure is under selective pressure (Bernhart and Hofacker 2009). The consensus structure is found to be closely linked with molecular functions, so the prediction of these structures is very useful in evolutionarily conserved RNA molecules. The structure prediction method we propose presents a promising approach to reconstruct secondary structures of the 28S gene in monogeneans that have not been studied so far. The consideration of taxon specific predicted secondary structures helps to improve the inference of phylogenetic relationships and can provide more realistic values of tree robustness.

In the future, this study needs to be elaborated in order to form a better understanding of the systematics of parasites as well as their evolutionary processes. Earlier studies such as Jow et al. (2002), Kjer (2004), Telford et al. (2005), Dohrmann et al. (2006, 2008), Erpenbeck et al. (2007), Kjer and Honeycutt (2007), Voigt et al. (2008) and von Reumont et al. (2009) also applied the approach of ribosomal DNA to study mammalian evolution, insect phylogeny, phylogeny of bilateria, phylogeny and evolution of sponges, phylogeny of
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eutheria, molecular evolution of metazoans, and phylogenetic association of arthropods, respectively. 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. Molecular morphometrics has been found to be one of the most powerful tools 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. Therefore, with the help of this, homologous recognizable characters are easily seen by finding the same pattern in the secondary structures, as also advocated by PRASAD et al. (2009). Application of the secondary structure model of rRNA to phylogenetic analyses leads to trees with resolved relationships among clades and probably eliminates some artifactual support for misinterpreted relationships. The highly resolved topology in parts of the tree 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 monogenean species. The present identification of the M. bihamuli 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 because the secondary structure contains more information than the usual primary sequence alignment.

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