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Phylogenetic Positions of the Bothitrematidae and Neocalceostomatidae (Monopisthocotylean Monogeneans) Inferred from 28S rDNA Sequences

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ABSTRACT: A molecular phylogeny was inferred from newly obtained partial (D1 domain) 28S rDNA gene sequences of *Bothitrema bothi* (Bothitrematidae), *Neocalceostoma* sp. (Neocalceostomatidae), *Bravohollisia* sp. (Ancyrocephalidae), and other already available sequences of Ancyrocephalidae, Anoplodiscidae, Pseudodactylogyridae, and Sundanonchidae, with the Diplectanidae as outgroup. *Bothitrema*, *Anoplodiscus*, and *Sundanonchus* formed a very robust clade that was the sister group to a group that included all other species examined. In this latter group, *Neocalceostoma* and *Thaparocleidus* were basal to a clade in which the Ancyrocephalidae and Pseudodactylogyridae were sister groups. Molecular results that suggest inclusion of the families Bothitrematidae, Anoplodiscidae, and Sundanonchidae in the same group partially contradict a previous morphological analysis of Boeger and Kritsky in which the first 2 were placed in the Gyrodactylidea and the third in the Dactylogyridea.

KEY WORDS : Molecular phylogeny, rDNA sequences, Monogenea

The Monogenea has been the subject of several major molecular phylogenetic analyses in recent years. These have used sequences of the 28S rDNA D1 (Mollaret et al., 1997; Mollaret, Jamieson and Justine, 2000), D2 (Jovelin and Justine, 2001) or D3-D6 domains (Litvaitis and Rohde, 1999), 18S rDNA (Sinnappah et al., 2001), or a combination of 28S and COI (Littlewood et al., 1997) or of 18S and 28S (Littlewood et al., 1998, 1999; Olson and Littlewood, 2002). The domain D1 of 28S rDNA is poorly informative within the Polyopisthocotylea (Mollaret, Jamieson, and Justine, 2000), and better results were obtained with the D2 domain (Jovelin and Justine, 2001). In contrast, the Monopisthocotylea are relatively fast-evolving organisms, and the D1 domain used alone has revealed good resolving power within this group (Mollaret, Jamieson, and Justine, 2000; Mollaret, Lim, and Justine, 2000), although results concerning higher taxonomic rank obtained only

with this domain are probably of limited value (Olson and Littlewood, 2002).

In the present article, we used the D1 domain to infer the phylogenetic positions of *Bothitrema bothi* (MacCallum, 1913) Price, 1936 (Bothitrematidae), a relatively obscure monogenean of the family Bothitrematidae, and *Neocalceostoma* sp., a member of the recently proposed family Neocalceostomatidae. A new sequence of *Bravohollisia* sp. (Ancyrocephalidae) was also added to the database.

Materials and Methods

New sequences (accession numbers in Table 1) obtained for this study are from *B. bothi* from the windowpane *Scophthalmus aquosus* (Mitchill, 1815), New Jersey, U.S.A., collected by S.S.H., and from *Bravohollisia* sp. (Ancyrocephalidae) from a grunt *Pomadasy hasta* (Bloch, 1790), and *Neocalceostoma* sp. (Neocalceostomatidae) from the veined catfish *Arius venosus* (Valenciennes 1840), both collected by I.M. and L.H.S.L. in Malaysia. Nucleotide sequences data reported in the present article will be available in the GenBank database under accession numbers AF387508–A387510. Alignment data will be available

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Table 1. Taxonomic listing of monopisthocotylean monogeneans used in the analysis.

Species	GenBank accession number
Ancyrocephalidae	
<i>Tetrancistrum</i> sp.	AF026114
<i>Haliotrema chrysotaeniae</i> Young, 1968	AF026115
<i>Ligophorus mugilinus</i> (Hargis, 1955) Euzet et Suriano, 1977	AF131710
<i>Thaparocleidus siamensis</i> (Lim, 1990) Lim, 1996	AF218124
<i>Cichlidogyrus</i> sp.	AF218123
<i>Pseudohaliotrema sphincteroporos</i> Yamaguti, 1953	AF382058
<i>Bravohollisia</i> sp.	AF387509*
Pseudodactylogyridae	
<i>Pseudodactylogyrus</i> sp.	AF382057
Neocalceostomatidae	
<i>Neocalceostoma</i> sp.	AF387510*
Anoplodiscidae	
<i>Anoplodiscus cirrusspiralis</i> Roubal, Armitage et Rohde, 1983	AF382060
Sundanonchidae	
<i>Sundanonchus micropeltis</i> Lim et Furtado, 1985	AF218122
Bothitrematidae	
<i>Bothitrema bothi</i> (MacCallum, 1913) Price, 1936	AF387508*
Diplectanidae	
<i>Acleotrema</i> sp.	AF026118
<i>Furnestinia echeneis</i> (Wagener, 1857) Euzet et Audoin, 1959	AF131711

* New sequences.

on request to the authors or from <http://www.mnhn.fr/mnhn/bpph/Data/IndexData.html>.

Nucleotide sequence data reported were obtained by use of the methods detailed by Jovelin and Justine (2001) and only summarized herein. DNA extraction was performed on parasites kept in 90% ethanol by use of standard CTAB protocol (Winnepenninckx et al., 1993). The partial domain C1, full domain D1, and partial domain C2 of the 28S rDNA were amplified by use of the universal primers C1' (5'-ACCCGCTGAA-TTTAAGCAT-3') and reverse C2 (5'-CTCTCTYTYC-AAAGTCTTTTC-3'). PCR amplifications were processed with 5 µl DMSO, 26.4 µl mix dNTP at 6.6 mM, 60 pM of each primer, 10 µl 10× Buffer (Quantum), 3 U QBiotaq polymerase (Quantum, now Qbiogene, Inc., Illkirch, France), and 5 µl template DNA made up to 50 µl with water. PCR conditions were: hot start (95°C/4 min) followed by 35 cycles of 95°C/30 sec, 55°C/30 sec, and 72°C/30 sec. PCR products (5 µl) were first checked for size by gel electrophoresis with the molecular weight marker XIV (Boehringer Mannheim Corp., Meylan, France) on 1.5% agarose gel and were gel-purified on 1.5% agarose by use of the QIAquick PCR purification kit (Qiagen S.A., Courtaboeuf, France). The partial domain C1, full domain D1, and partial domain C2 were sequenced with an automated sequencer (Beckman Coulter, Inc., Palo Alto, California, U.S.A.) by use of the DNA CEQTM 2000 Kit from Beckman and following their concentrations under the following conditions: 40 cycles of 96°C/40 sec, 50°C/40 sec, and 60°C/4 min. The primers used here allowed the entire sequence to be confirmed from both strands.

Previous alignments (Mollaret, Jamieson, and Justine, 2000; Mollaret, Lim, and Justine, 2000) were up-

dated with the addition of our 3 new sequences and a selection of newly available sequences (Olson and Littlewood, 2002), with a total of 34 taxa of monopisthocotylean monogeneans. Sequence alignment was performed by eye with BioEdit (Hall, 1999) on a PC computer or SeqAl version 1a1 (Rambaut, 1996) on a Macintosh computer, by minimizing the insertion of gaps. Gaps were treated as a fifth base in the analysis. Base positions that could not be aligned unambiguously by eye were removed prior to phylogenetic analysis. Autapomorphies were removed from the matrix. Preliminary phylogenetic analyses revealed 3 monophyletic groups within the monopisthocotylean monogeneans, sustained by low bootstrap values: the Monocotylidae, the Capsalidae + Udonellidae, and a clade that contained the Ancyrocephalidae, Pseudodactylogyridae, Anoplodiscidae, Sundanonchidae, Bothitrematidae, Diplectanidae, and Neocalceostomatidae; the Diplectanidae were the sister group to the other taxa within this clade. In the present analysis, concentrating on the Bothitrematidae and closely related families (Table 1), the Diplectanidae were used as the outgroup for an analysis of the other taxa. This hypothesis is compatible, for the Ancyrocephalidae and other families, with a cladistic hypothesis based on morphology (Boeger and Kritsky, 2001) in which all these families belong to the monophyletic Dactylogyridea, but this does not extend to the Bothitrematidae and Anoplodiscidae. The hypothesis is compatible with an analysis based on 18S rDNA, in which *Anoplodiscus*, *Sundanonchus*, *Pseudodactylogyrus*, and *Pseudohaliotrema* are in the same clade (Olson and Littlewood, 2002). In addition, the families included in the analysis all belong to the Monoaxonematidea Justine, 1991, a group characterized by the presence of spermatozoa with a single ax-

sp. was the sister group to a clade that included all the Ancyrocephalidae. The least robust node of the parsimony tree was the node that united *Neocalceostoma* and the Pseudodactylogyridae and Ancyrocephalidae; indeed, NJ and ML analyses and their bootstraps produced a slightly different tree (Fig. 1b), in which *Neocalceostoma* and *Thaparocleidus* were united in the same clade, with relatively high bootstraps; otherwise, the tree topology was the same.

Discussion

The classification adopted here for discussing our results uses family ranks for the Ancyrocephalidae and Pseudodactylogyridae. An alternative is to consider these, and others, as subfamilies of the Dactylogyridae (Kritsky and Boeger, 1989).

As expected, *Bravohollisia* was found within a clade that included *Cichlidogyrus*, *Haliotrema*, *Ligophorus*, *Pseudohaliotrema*, and *Tetrancistrum*. This clade corresponds to the Ancyrocephalidae, a “catch-all” group for many species, and detailed phylogenetic hypotheses within this group will need many more sequences. However, good support was found for a group that included all species belonging to the Ancyrocephalidae. Two clades were found within the family: 1 with *Pseudohaliotrema*, *Tetrancistrum*, and *Bravohollisia*, and 1 with *Cichlidogyrus*, *Ligophorus*, and *Haliotrema*; *Cichlidogyrus* sp. is the single species studied from freshwater fishes, whereas all other species are from marine fishes. *Pseudodactylogyrus* (Pseudodactylogyridae; see Le Brun et al., 1986) was found to be the sister group to the Ancyrocephalidae (Ancyrocephalinae).

Depending on the method used (MP vs. NJ or ML), a sister-group relationship or paraphyletic close relationships, were found between *Thaparocleidus* and *Neocalceostoma*. The family Neocalceostomatidae, to which *Neocalceostoma* belongs, was recently proposed to group several species previously included in the Calceostomatidae (Lim, 1995). *Thaparocleidus* has been classified within the Ancylo-discoidinae (Lim, 1990, 1996), a subfamily of the Ancyrocephalidae, and a recent morphological analysis supports raising the Ancylo-discoidinae to family status (Lim et al., 2001). Our results support the separation of the Ancylo-discoidinae from the Ancyrocephalidae (Ancyrocephalinae).

The main result of our analysis is the very

robust sister-group relationships among the Bothitrematidae (*B. bothi*), Sundanonchidae (*Sundanonchus micropeltis*) and Anoplodiscidae (*Anoplodiscus cirrusspiralis*). The family Bothitrematidae has been classified within the Tetraonchidea (Bychowsky, 1961), and the family Sundanonchidae, after some initial instability, was also classified within the Tetraonchidea (Kritsky and Lim, 1995). The oncomiracidium of *B. bothi* (S.S.H., unpublished observations) has 2 pairs of eyes, with the posterior pair fused and a circle of 16 hooks in the haptor; the intestine is single in both the oncomiracidium and adult. Thus, the family Bothitrematidae shares the fused posterior eyes and single intestine with the Sundanonchidae and Tetraonchidae. Our molecular results support these relationships.

In a recent morphological analysis (Boeger and Kritsky, 2001), the Anoplodiscidae and Bothitrematidae were relatively close groups but were included within the Gyrodactylidae; the Sundanonchidae was, with the Tetraonchidae, within the Tetraonchidea. This morphological hypothesis is not compatible with our molecular results based on 28S D1 nor with an analysis based on 18S complete sequences, in which *Sundanonchus* and *Anoplodiscus* are regularly found to be sister taxa (Olson and Littlewood, 2002). Inclusion of the Anoplodiscidae in the same group as Dactylogyridae revives the hypothesis of a monophyletic Monoaxonematidea, characterized by spermatozoa with a single axoneme (Justine, 1991). The sperm structure known as “Type 4,” with a single axoneme and no microtubules (Justine, Lambert, and Mattei, 1985), is now known in the Anoplodiscidae, Diplectanidae, Ancyrocephalidae, Pseudodactylogyridae, Sundanonchidae, Tetraonchidae, Tetraonchoididae, Amphibdellatidae, and Calceostomatidae, but molecular data are lacking for the latter 4 families.

Recent comparative analyses of D1 and 18S sequences have shown that the domain D1 is not the best choice for resolving higher-rank phylogenetic relationships within the Monogenea (Olson and Littlewood, 2002). This is certainly true for the Polyopisthocotylea, in which domain D1 cannot resolve the higher branches of the tree (Jovelin and Justine, 2001; Mollaret, Jamieson, and Justine, 2000). However, the present study shows that such short sequences may be useful instruments for understanding relation-

ships among at least certain groups within the Monopisthocotylea.

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