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Author for correspondence:
Ilya I. Gordeev, E-mail: gordeev_ilya@bk.ru

Phylogenetic analysis of the superfamily Hemiuroidea (*Platyhelminthes*, *Neodermata*: Trematoda) based on partial 28S rDNA sequences

Sergey G. Sokolov¹, Dmitry M. Atopkin^{2,3}, Misako Urabe⁴ and Ilya I. Gordeev^{5,6}

¹A.N. Severtsov Institute of Ecology and Evolution RAS, Moscow, Leninskiy Av., 33, 119071, Russia; ²Far Eastern Branch of the RAS, Federal Scientific Center of the East Asia Terrestrial Biodiversity, Prospect 100-letija, 159, Vladivostok 690022, Russia; ³Department of Cell Biology and Genetics, Far Eastern Federal University, Vladivostok, October Str., 27, 690000, Russia; ⁴University of Shiga Prefecture, Hikone, 522-8533, Shiga, Japan; ⁵Russian Federal Research Institute of Fisheries and Oceanography, Moscow, V. Krasnoselskaya Str., 17, 107140, Russia and ⁶Faculty of Biology, Lomonosov Moscow State University, Moscow, Leninskiye Gory 1/12, 119234, Russia

Abstract

In the present paper, the phylogenetic relationships between genera, subfamilies and families of the Hemiuroidea are explored. Twelve new sequences of 28 rDNA and data taken from GenBank (NSBI) on 43 species affiliated to 34 genera were included in the analysis. Most of the hemiuroidean trematodes form two highly supported clades (A and B), which are sister groups to each other. *Hemipera manteri* joined with *Gonocerca* spp. with moderate statistical support. This clade is basal relative to the clades A and B. Clade A is polytomic and contains representatives of the families Accacoeliidae, Syncoeliidae, Didymozoidae, Hirudinellidae and Sclerodistomidae, and derogenid subfamilies Derogeninae and Halipeginae. At the same time, the Syncoeliidae, Hirudinellidae and Accacoeliidae form a well-supported monophyletic group. The phylogenetic relationship between Derogeninae and Halipeginae is poorly resolved. Clade B unites the isoparorchiid, bunocotylid, lecithasterid and hemiurid trematodes. Our data re-establishes the family Bunocotylidae, which consists of two subfamilies, Opisthadeninae and Bunocotylinae, and the *Machidatrema chilostoma* + *Hysterolecithoides frontilatus* group. The Bunocotylidae is the sister group to the Hemiuridae + Lecithasteridae group and the Isoparorchiidae is a basal relative to the representatives of these three hemiuroid families.

Introduction

The superfamily Hemiuroidea Looss, 1899 is a large group of trematodes that are mainly parasitic in marine fish. The taxonomic structure of the hemiuroidean trematodes has been repeatedly subject to change. The modern version of the systematics of the Hemiuroidea is based on the taxonomic model proposed by Gibson and Bray (1979), with adjustments at the levels of families, subfamilies and genera proposed by several authors (see Gibson, 2002a, 2002b, 2002c, 2002d, 2002e, 2002f, 2002g, 2002h, 2002i, 2002j, 2002k, 2002l; Pozdnyakov and Gibson, 2008). In recent years, a number of changes have been made related to the description of new genera and subfamilies (Bray and Nahhas, 2002; Pankov *et al.*, 2006; Bursey *et al.*, 2008; Bilqees *et al.*, 2009, 2010; Justo and Kohn, 2012; Urabe and Shimazu, 2013), as well as a change in the taxonomic rank of previously described subfamilies (Sokolov *et al.*, 2018).

The analysis of nucleotide sequences has great potential for studying phylogenetic relationships and is widely used in the modern era in the taxonomy of all groups of organisms (Littlewood and Bray, 2001; Brown, 2002; Olson *et al.*, 2003; Patwardhan *et al.*, 2014; Timothy *et al.*, 2015). The first study on the molecular phylogeny of the Hemiuroidea was published by Blair *et al.* (1998). The analysis of the V4 domain sequences (18S rDNA) performed by these authors revealed that phylogenetic connections of hemiuroidean trematodes were not reflected in the taxonomic scheme by Gibson and Bray (1979). An important result of this work was the statement of the monophyly of the Hemiuridae *sensu* Gibson and Bray, 1979 + Lecithasteridae *sensu* Gibson and Bray, 1979, in which some groups of the Lecithasteridae were paraphyletic. Further research of 28S rDNA or both 28S and 18S rDNA revealed a serious discrepancy between phylogenetic and current taxonomic models of derogenids, hemiurids and lecithasterids (see Gibson, 2002a, 2002d, 2002h; Olson *et al.*, 2003; Pankov *et al.*, 2006; Calhoun *et al.*, 2013; Marzoug *et al.*, 2014; Sokolov *et al.*, 2016, 2018; Atopkin *et al.*, 2017).

In the present paper, we explore the phylogenetic relationships between hemiuroidean genera, subfamilies and families to establish a basis for further taxonomic studies of this superfamily. Pankov *et al.* (2006) noted that ‘analyses based on the V4 domain of the *ssrRNA* gene has added little to and has not improved an earlier phylogenetic study of the Hemiuroidea’. Because of this reason, we did not perform phylogenetic analysis by means of 18S rDNA nucleotide sequences and used most representative data on 28S rDNA sequences only.

Material and methods

Phylogenetic tree constructions were performed using our data and the nucleotide sequences of 28S rDNA of hemiuroidean trematode specimens from the NCBI GenBank database (Table 1). The following species were originally studied: *Allognathopoda problematica* (Faust, 1924), cercaria [ex *Semisulcosipra reiniana* (Brot, 1876), midgut; agricultural canal near Hino River, Yasu, Japan], *Genarchopsis chubuensis* Shimazu, 2015, adult [ex *Rhinogobius flumineus* (Mizuno, 1960), stomach; agricultural canal near Ane River, Nagahama, Japan], *Hemiurus luehei* Odhner, 1905, adult [ex *Ophidion rochei* Müller, 1845, stomach; the Black Sea near Sevastopol, Russia], *Pulmovermis cyanovitellosus* Coil and Kuntz, 1960, adult [ex *Laticauda semifasciata* (Reinwardt in Schlegel, 1837), lung; Ishigaki Island, Japan], *Brachyphallus crenatus* (Rudolphi, 1802), adult [ex *Salvelinus leucomaenis* (Pallas, 1814), stomach; the Sea of Okhotsk, Siglan Bay], *Dinosoma synaphobranchi* Yamaguti, 1938, adult [ex *Antimora microlepis* Bean, 1890, stomach; the Sea of Okhotsk near south-eastern Sakhalin], *Lecithaster micropsi* Zdzitowiecki, 1992, adult [ex *Dissostichus mawsoni* Norman, 1937, intestine; the Amundsen Sea and *Muraenolepis marmorata* Günther, 1880, intestine; the Ross Sea], *Lecithophyllum botryophoron* (Olsson, 1868), adult [ex *Oneirodes thompsoni* (Schultz, 1934), intestine; Simushir Island area and *A. microlepis*, stomach; the Sea of Okhotsk near south-eastern Sakhalin], *Genolinea anura* (Layman, 1930), adult [ex *Pleurogrammus monopterygius* (Pallas, 1810), intestine; Simushir Island area], *Hysteroleithoides frontilatus* (Manter, 1969), adult [ex *Siganus fuscescens* (Houttuyn, 1782), intestine; the South China Sea near Nha Trang, Vietnam], *Isoparorchis eurytremus* (Kobayashi, 1915), adult [ex *Silurus asotus* Linnaeus, 1758, air bladder; Shin-asahi, Takashima, Japan], *Philopinna higai* Yamaguti, 1936, adult [ex *Sarcocheilichthys variegatus* (Temminck and Schlegel, 1846), fin; Lake Biwa, Takashima, Japan].

Species identification was performed according to different authors (Skrjabin and Guschanskaja, 1955a; Telford, 1967; Yamaguti, 1971; Nikolaeva *et al.*, 1975; Gibson, 1996; Bray and Cribb, 2000; Kuramochi, 2001; Sokolov and Gordeev, 2013; Urabe and Shimazu, 2013; Shimazu, 2015a, 2015b). Specimens destined for molecular analysis were fixed in 96% ethanol and stored at +4 °C.

Genomic DNA of the first part of species – *B. crenatus*, *D. synaphobranchi*, *G. anura*, *H. luehei*, *H. frontilatus*, *L. botryophoron*, *L. micropsi* – was extracted using a 'hot shot' technique (Truett, 2006). Nuclear 28S rDNA fragment, including D1–D3 domains, was amplified using a polymerase chain reaction (PCR) with the following primers: 28S-A (5'-TCG ATT CGA GCG TGA WTA CCC GC-3') (Matejusova and Cunningham, 2004) and 1500R (5'-GCT ATC CTG AGG GAA ACT TCG-3') (Tkach *et al.*, 2003). The initial PCR reaction was carried out in a total volume of 25 µL containing 0.25 mM of each primer pair, 5 µL DNA in water, 1 × Q5 polymerase buffer, 2.5 mM dNTP and one unit of Q5 DNA polymerase (New England Biolabs, Massachusetts, UK). The amplification of a 1230 bp fragment of 28S rDNA was performed in a GeneAmp 9700 (Applied Biosystems, Massachusetts, USA) with a 1 min denaturation hold at 98 °C; 35 cycles of 10 s at 98 °C, 5 s at 55 °C and 20 s at 72 °C; followed by a 2 min extension hold at 72 °C. Negative and positive controls, using both primers, were used. The PCR products were directly sequenced using an ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit, as recommended by the manufacturer, with the internal sequencing primers 300F, ECD2, 900F and 1200R (Tkach *et al.*, 2003). The PCR products were analysed using an ABI 3130xl genetic analyser at the Department of Cell Biology and Genetics, Far Eastern Federal University. The voucher specimens of the studied species are deposited in the

parasitological collection of the Zoological Museum of Federal Scientific Centre of the East Asia Terrestrial Biodiversity, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia: *B. crenatus* (without a number), *D. synaphobranchi* (# AM14.1, AM11, AM3), *G. anura* (# 754); *H. frontilatus* (without a number), *H. luehei* (without a number), *L. botryophoron* (# AM1.1, AM1.x, 805), *L. micropsi* (# 654, 676, 700TOA).

The rest of the five species, namely *A. problematica*, *G. chubuensis*, *P. cyanovitellosus*, *I. eurytremus* and *P. higai* were studied in the following way. Extraction was performed with a Wizard® SV Genomic DNA Purification System (Promega). A nuclear 28S rDNA fragment, including D1–D3 domains, was amplified using a PCR with the following primers: LSU-5 and 1500R (Olson *et al.*, 2003). The initial PCR reaction was carried out in accordance with Olson *et al.* (2003). The amplification of a 28S rDNA fragment was performed in MyCycler™ (Bio-Rad): 40 cycles of 10 s at 94 °C, 30 s at 50 °C and 60 s at 72 °C. The PCR products were sequenced by the FASMAC Sequencing Service (Kanagawa). The voucher specimens of these species (collected from the same host species at the same or close sampling localities) are deposited in the National Museum of Nature and Science, Tokyo (catalogue numbers: *A. problematica* – NSMT-PI 5851–5853; *G. chubuensis* – NSMT-PI 6347; *P. cyanovitellosus* – NSMT-PI 6348; *I. eurytremus* – NSMT-PI 5861–5868; *P. higai* – NSMT-PI 5391). All sequences have been submitted to GenBank (Table 1).

Ribosomal DNA sequences were assembled with SeqScape v.2.6 software provided by Applied Biosystems. Alignments and estimation of the number of variable sites and sequence differences were performed using the MEGA 7.0 (Kumar *et al.*, 2016). Alignment of nucleotide sequences was performed using Clustal W algorithm with gap opening penalty and gap extension penalty values, which were 15 and 5, respectively. Phylogenetic analyses of the nucleotide sequences were performed using the Bayesian (BI) algorithm MrBayes v.3.6.2 (Huelsenbeck *et al.*, 2001) software. The best nucleotide substitution models were estimated with jModeltest v.2.1.5 software (Darriba *et al.*, 2012), using the Bayesian Information Criterion (Huelsenbeck *et al.*, 2001). The best nucleotide substitution model for 28S rDNA sequence data was TVM + I + G, a transversional model with estimates of invariant sites and γ -distributed among-site variation (Darriba *et al.*, 2012). The significance of the phylogenetic relationships was estimated by posterior probabilities (Huelsenbeck *et al.*, 2001).

Results

The length of the 28S rDNA locus of the species was studied and the outgroup species in the alignment was 1224 base pairs (bp), including 737 variables and 616 parsimony-informative sites. These data were used for phylogenetic relationship reconstructions. Most of the studied hemiuroidean trematodes were within two highly supported clades, A and B, which were sister groups to each other. *Hemipera manteri* (Crowcroft, 1947) was joined to *Gonocerca* spp. with moderate statistical support. This clade is basal relative to the clades A and B.

Clade A is polytomic and contains representatives of the families Accacoeliidae, Syncoeliidae, Didymozoidae, Hirudinellidae and Sclerodistomidae, and derogenid subfamilies Derogeninae and Halipeginae. At the same time, members of the Syncoeliidae, Hirudinellidae and Accacoeliidae families form a well-supported monophyletic group. Families Hirudinellidae and Didymozoidae, and subfamily Halipeginae are presented on the tree by more than one species and form well-supported monophyletic groups. The phylogenetic relationship between Derogeninae and Halipeginae is poorly resolved.

Clade B unites the isoparorchiid, bunocotyloid, lecithasterid and hemiurid trematodes. The Bunocotyliidae is the sister group to the

Table 1. List of taxa, incorporated into molecular analysis: systematic position according to Gibson (2002a, 2002b, 2002d, 2002f, 2002j, 2002l); Pozdnyakov and Gibson (2008); Pankov *et al.* (2006); Urabe and Shimazu (2013) and Sokolov *et al.* (2018) with correction according to our data

Species	Family/subfamily	Number of specimens	Source	GenBank accession number
<i>Gonocerca crassa</i>	Gonocercidae	1	Sokolov <i>et al.</i> (2018)	KY197012
<i>Gonocerca muraenolepisi</i>	Gonocercidae	3	Sokolov <i>et al.</i> (2016)	HF543941, LN650651, LN865025
<i>Gonocerca oshoro</i>	Gonocercidae	1	Sokolov <i>et al.</i> (2018)	KY197013
<i>Gonocerca phycidis</i>	Gonocercidae	1	Sokolov <i>et al.</i> (2018)	KY197009
<i>Hemipera manteri</i>	Gonocercidae	1	Olson <i>et al.</i> (2003) (as <i>Hemiperina manteri</i>)	AY222196
<i>Derogenes varicus</i>	Derogenidae, Derogeninae	1	Olson <i>et al.</i> (2003)	AY222189
<i>Allogenarchopsis problematica</i>	Derogenidae, Halipeginae	1	This study	MH628313
<i>Genarchopsis chubuensis</i>	Derogenidae, Halipeginae	1	This study	MH628311
<i>Thometrema lotzi</i>	Derogenidae, Halipeginae	1	Calhoun <i>et al.</i> (2013)	KC985236
<i>Aphanurus mugilis</i>	Hemiuridae, Aphanurinae	1	Atopkin <i>et al.</i> (2017)	LT607807
<i>Dinurus longisinus</i>	Hemiuridae, Dinurinae	1	Olson <i>et al.</i> (2003)	AY222202
<i>Hemiurus luehei</i>	Hemiuridae, Hemiurinae	1	This study	MH628316
<i>Lecithochirium microstomum</i>	Hemiuridae, Lecithochiriinae	1	Calhoun <i>et al.</i> (2013)	KC985235
<i>Lecithocladium excisum</i>	Hemiuridae, Elytrophallinae	1	Olson <i>et al.</i> (2003)	AY222203
<i>Plerurus digitatus</i>	Hemiuridae, Plerurinae	1	Olson <i>et al.</i> (2003)	AY222201
<i>Pulmovermis cyanovitellosus</i>	Hemiuridae, Pulloverminae	1	This study	MH628314
<i>Brachyphallus crenatus</i>	Hemiuridae, subfamily unknown	1	This study	MH628299
<i>Dinosoma synaphobranchi</i>	Hemiuridae, subfamily unknown	3	This study	MH628302–MH628304
<i>Aponurus laguncula</i>	Lecithasteridae, Lecithasterinae	1	Claxton <i>et al.</i> (2017)	KU527430
<i>Aponurus</i> sp.	Lecithasteridae, Lecithasterinae	2	Pankov <i>et al.</i> (2006) Carreras-Aubets <i>et al.</i> (2011)	DQ354368 HQ713441
<i>Lecithaster mugilis</i>	Lecithasteridae, Lecithasterinae	1	Besprozvannykh <i>et al.</i> (2017)	LN865016
<i>L. sudzuhensis</i>	Lecithasteridae, Lecithasterinae	1	Besprozvannykh <i>et al.</i> (2017)	LN865022
<i>L. gibbosus</i>	Lecithasteridae, Lecithasterinae	1	Olson <i>et al.</i> (2003)	AY222199
<i>L. micropsi</i>	Lecithasteridae, Lecithasterinae	2 (ex <i>M. marmorata</i>) 1 (ex <i>D. mawsoni</i>)	This study	MH628306–MH628307 MH628305
<i>Lecithophyllum botryophoron</i>	Lecithasteridae, Lecithasterinae	1 1 (ex <i>O. thompsoni</i>) 2 (ex <i>A. microlepisi</i>)	Olson <i>et al.</i> (2003) (as <i>Lecithophyllum botryophorum</i>) This study	AY222205 MH628309 MH628300–MH628301
<i>Merlucciotrema praeclarum</i>	Family unknown	1	Olson <i>et al.</i> (2003)	AY222204
<i>Genolinea anura</i>	Bunocotylidae, Opisthadeninae	1	This study	MH628308
<i>Opisthadena dimidia</i>	Bunocotylidae, Opisthadeninae	1	Olson <i>et al.</i> (2003)	AY222198
<i>Hysteroleclithoides frontilatus</i>	Bunocotylidae, subfamily unknown	1	This study	MH628310
<i>Machidatrema chilostoma</i>	Bunocotylidae, subfamily unknown	1	Olson <i>et al.</i> (2003)	AY222197
<i>Bunocotyle progenetica</i>	Bunocotylidae, Bunocotylineae	1	Pankov <i>et al.</i> (2006)	DQ354365
<i>Robinia aurata</i>	Bunocotylidae, Bunocotylineae	1	Pankov <i>et al.</i> (2006)	DQ354367
<i>Saturnius gibsoni</i>	Bunocotylidae, Bunocotylineae	1	Marzoug <i>et al.</i> (2014)	KJ010542

(Continued)

Table 1. (Continued.)

Species	Family/subfamily	Number of specimens	Source	GenBank accession number
<i>Saturnius minutus</i>	Bunocotylidae, Bunocotylinae	1	Pankov <i>et al.</i> (2006) (as <i>Saturnius</i> sp.)	DQ354366
<u><i>Isoparorchis eurytremus</i></u>	Isoparorchidae	1	This study	MH628315
<i>Accacoelium contortum</i>	Accacoeliidae	1	Olson <i>et al.</i> (2003)	AY222190
<i>Copiatestes filiferus</i>	Syncoeliidae	1	Olson <i>et al.</i> (2003)	AY222188
<i>Didymocystis scomberomori</i>	Didymozoidae	1	Schrandt <i>et al.</i> (2016)	KU341979
<i>Didymozoid</i> sp.	Didymozoidae	2	Olson <i>et al.</i> (2003)	AY222192, AY222194
<i>Didymozoon scombri</i>	Didymozoidae	1	Olson <i>et al.</i> (2003)	AY222195
<u><i>Philopinna higai</i></u>	Didymozoidae	1	This study	MH628312
<i>Hirudinella ahi</i>	Hirudinellidae	1	Calhoun <i>et al.</i> (2013)	KC985238
<i>Hirudinella ventricosa</i>	Hirudinellidae	1	Calhoun <i>et al.</i> (2013)	KC985232
<i>Prosogonotrema bilabiatum</i>	Sclerodistomidae	1	Olson <i>et al.</i> (2003)	AY222191
Outgroup				
<i>Otodistomum cestoides</i>	Azygiidae	1	Olson <i>et al.</i> (2003)	AY222187
<i>Azygia longa</i>	Azygiidae	1	Calhoun <i>et al.</i> (2013)	KC985234
<i>Proterometra</i> sp.	Azygiidae	1	Calhoun <i>et al.</i> (2013)	KC985237

Underlines texts indicates the species studied during this study.

Hemiuridae + Lecithasteridae group and the Isoparorchidae is basal relative to representatives of these three Hemiuroidea families. Within the Bunocotylidae, there are three well-supported groups of the species: *Opisthadena dimidia* Linton, 1910 + *G. anura*, *Machidatrema chilostoma* (Machida, 1980) + *H. frontilatus* and [*Bunocotyle progenetica* Chabaud and Buttner, 1959 + *Saturnius* spp.] + *Robinia aurata* Pankov, Webster, Blasco-Costa, Gibson, Littlewood, Balbuena and Kostadinova, 2006. The Hemiuridae + Lecithasteridae group contains three well-supported lineages. The first one unites representatives of the genera *Aphanurus* Looss, 1907, *Dinurus* Looss, 1907, *Lecithocladium* Lühe, 1901, *Brachyphallus* Odhner, 1905, *Dinosoma* Manter, 1934, *Plerurus* Looss, 1907, *Pulmovermis* Coil and Kuntz, 1960, *Lecithochirium* Lühe, 1901 and *Hemiurus* Rudolphi, 1809. The second lineage contains *Merlucciotrema praeclarum* (Manter, 1934) and *Lecithaster* spp., and the third contains *L. botryophoron* and *Aponurus* spp.

Discussion

Bunocotylids

Gibson and Bray (1979) established the Bunocotylidae and considered this family as a group of trematodes closely relevant to the Hemiuridae, but differing from the latter due to a secondary loss of an ecsoma. These authors divided the Bunocotylidae into four subfamilies: Bunocotylinae, Opisthadeninae, Aphanurinae and Theletrinae.

Brooks *et al.* (1985, 1989) concluded that bunocotylids are the sister group of hemiurids based on the cladistic analysis of their morphological features. In the phylogenetic reconstruction of these authors, a terminal clade consisting of bunocotylids and hemiurids was combined sequentially with derogenids, lecithasterids, dictysarcids, didymozoids, sclerodistomids and isoparorchids into a large monophyletic group. Eventually, Brooks *et al.* (1985, 1989) proposed a new taxonomic model of the Hemiuridae that includes all of the above groups. Gibson (1996) did not adopt the system of the Hemiuridae proposed

by these authors and considered hemiurid and bunocotylid trematodes in accordance with the taxonomic concept of Gibson and Bray (1979).

León-Règagnon *et al.* (1996, 1998) and León-Règagnon (1998) recognized the Bunocotylidae *sensu* Gibson and Bray, 1979 as the subfamily of the Hemiuridae. In designation of the taxonomic rank of bunocotylids, these authors referred to Brooks *et al.* (1985). In the cited publication, however, the taxon Bunocotylinae is not mentioned. León-Règagnon (1998) established a new genus, *Machidatrema*, and affiliated it with this subfamily. The genus *Machidatrema*, according to the author, includes four species. One of these, *M. frontilatum* (Manter, 1969), previously belonged to the family Lecithasteridae, genus *Hysterolecithoides* Yamaguti, 1934. Later, Bray and Cribb (2000) carried out a revision of the genera *Machidatrema* and *Hysterolecithoides*. Both genera were placed by them into the subfamily Hysterolecithinae of the family Lecithasteridae and species *M. frontilatum* was returned to genus *Hysterolecithoides*.

The molecular analysis performed by Blair *et al.* (1998) did not support the taxonomic model of the Hemiuridae proposed by Brooks *et al.* (1985, 1989). However, bunocotylid species were not studied in the paper of Blair *et al.* (1998).

In the systematics of the Hemiuroidea proposed by Gibson (2002a), the Hemiuridae *sensu* Gibson and Bray, 1979 and Bunocotylidae *sensu* Gibson and Bray, 1979 were united within one family: Hemiuridae, with the preservation for all four subfamilies of bunocotylids of similar ranks within the Hemiuridae. The taxonomy of the Hemiuridae proposed by Gibson (2002a) is now generally accepted.

The result of our analysis united *M. chilostoma*, *H. frontilatus* and representatives of the subfamilies Opisthadeninae and Bunocotylinae (including members of their type genera) into a well-supported monophyletic group, occupying a sister position to the Hemiuridae + Lecithasteridae group (Fig. 1). As noted above, (León-Règagnon, 1998; Bray and Cribb, 2000), they hold opposite points of view on subfamilial/familial affiliation of *M. chilostoma* and *H. frontilatus*: the subfamily Bunocotylinae of the family Hemiuridae s. lato and the subfamily

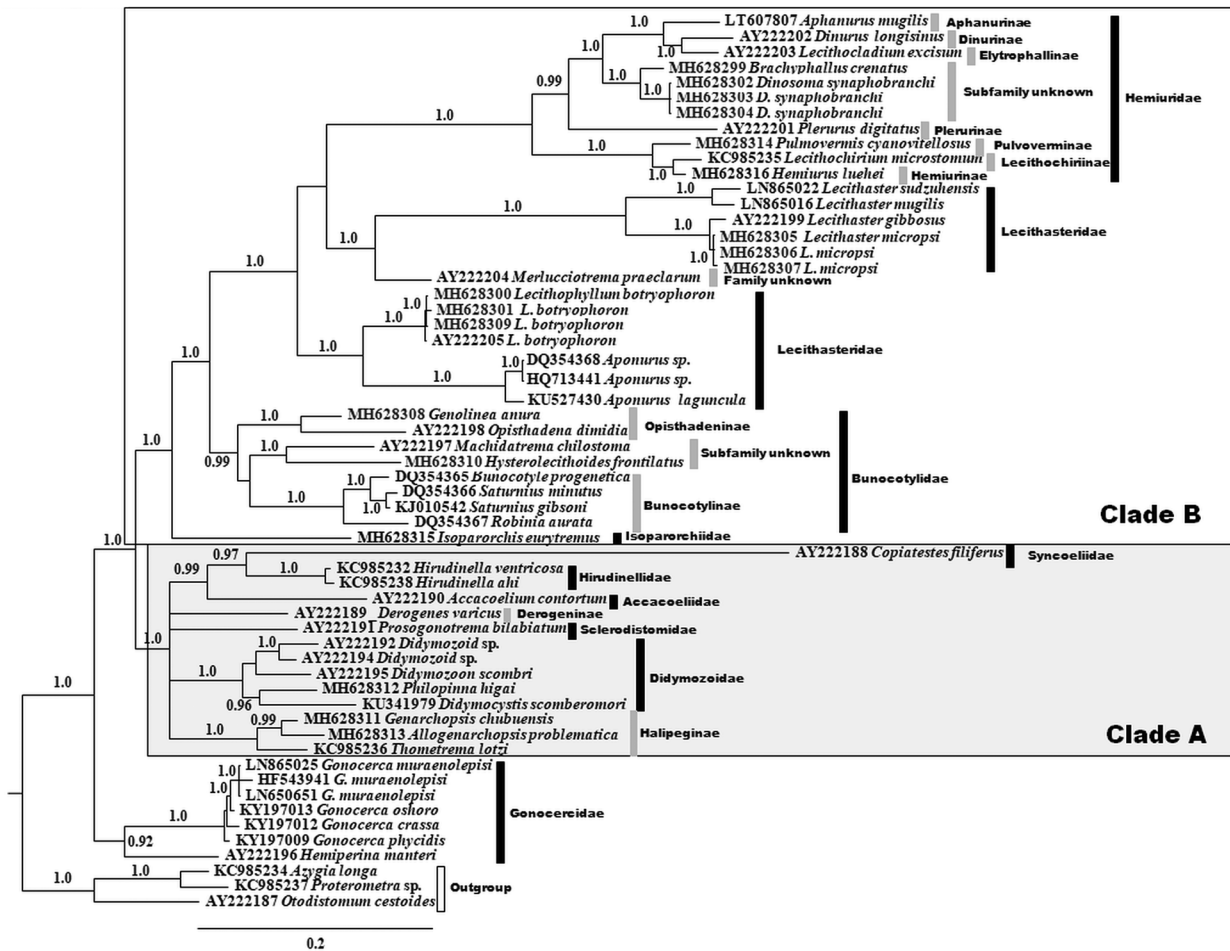


Fig. 1. Phylogenetic relationships of the superfamily Hemiuroidea obtained with Bayesian algorithm based on partial 28S rDNA sequences. Nodal numbers are posterior probabilities that indicate statistical support of phylogenetic relationships.

Hysterolecithinae of the family Lecithasteridae. It should be noted that previous phylogenetic reconstructions of hemiuroids, created using partial sequences of 28S rDNA or a combination of 18S and 28S rDNA partial sequences, highly supported clustering of *M. chilostoma* with representatives of the Opisthadeninae and Bunocotyliinae was not observed (Pankov *et al.*, 2006; Marzoug *et al.*, 2014; Bao *et al.*, 2015; Atopkin *et al.*, 2017; Faltýnková *et al.*, 2017; Sokolov *et al.*, 2018).

Our findings resurrect the family Bunocotyliidae and it is now possible to establish two subfamilies: Opisthadeninae and Bunocotyliinae, and the *M. chilostoma* + *H. frontilatus* group. The phylogenetic connections between these two subfamilies and the group were poorly resolved (Fig. 1). It is interesting that the Aphanurinae, which is affiliated to Bunocotyliidae according to Gibson and Bray (1979), does not show phylogenetic proximity to bunocotyliid trematodes (Fig. 1; see also Atopkin *et al.*, 2017).

Family Bunocotyliidae Dollfus, 1950 emend

Diagnosis [based on Gibson (1996), with changes]. Hemiuroidea. Body usually small, fusiform to elongate. Distinct ecsoma absent, but vestige may remain. Body surface without crenulate plications. Ridges or flanges around body can be present at the level of oral sucker, posterior margin of ventral sucker and close to posterior extremity. Ventral sucker normally inside anterior half of worm. Pharynx well developed. Oesophagus normally short. Gut caeca normally end blindly near posterior extremity or occasionally form cyclocoel. Testes two, pre-ovarian in hindbody, tandem to symmetrical. Seminal vesicle saccular or tubular, in forebody or hindbody. Pars prostatica tubular or vesicular, short or long,

may extend into hindbody. Ejaculatory duct present or absent. Sinus sac present, occasionally absent. Hermaphroditic duct present, within sinus sac when latter present, may extend to form temporary sinus-organ. Genital pore median, at the level of pharynx or posterior to it. Ovary oval, rarely bilobed, between testes and vitellarium. Laurer's canal and canalicular seminal receptacle absent. Juel's organ and uterine seminal receptacle present or absent. Blind seminal receptacle present or absent. Uterus normally almost entirely in hindbody, mainly pre- to mainly post-ovarian. Eggs numerous, small, without filaments. Vitellarium one or more entire, occasionally irregular masses, posterior or postero-lateral to ovary. Excretory arms extend to forebody, united or blinded. Parasitic mainly in stomach of marine teleosts.

Type genus: *Bunocotyle* Odhner, 1928

The family Bunocotyliidae differs from its sister cluster, the Hemiuridae + Lecithasteridae group, by the following combination of features: (i) body surface without crenulate plications, (ii) distinct ecsoma absent, (iii) genital pore at the level of pharynx or posterior to it, (iv) sinus-sac, if present, contains only a hermaphroditic duct that can form a temporary sinus-organ, (v) vitellarium is represented by one or more entire (rarely irregular) masses. If there are more than two vitelline masses, then these are Juel's organ and a uterine seminal receptacle.

Notice that an ecsoma is completely absent in *Machidatrema* spp., *Hysterolecithoides* spp., opisthadenines, and the majority of bunocotyliines (Gibson and Bray, 1979; León-Régagnon *et al.*, 1998; Bray and Cribb, 2000; Blasco-Costa *et al.*, 2008). There is a vestigial ecsoma in bunocotyline species *R. aurata*, however

see Pankov *et al.* (2006). According to the evolutionary model of Gibson and Bray (1979) and León-Régagnon *et al.* (1998), the absence of an ecsoma is plesiomorphic for hemiuroidean trematodes and the presence of this structure is an apomorphic feature of this hemiurid ancestor. These authors assumed that bunocotylids had evolved from a hemiurid ancestor and considered the absence of an ecsoma in bunocotylids as a secondary loss. Given the basal position of bunocotylids in the (Hemiuridae + Lecithasteridae) + Bunocotylidae subclade; however, we hypothesize that the absence of ecsoma is plesiomorphy for the Bunocotylidae. In the light of this hypothesis, the ecsoma of hemiurids and the vestigial ecsoma of *R. aurata* are homoplasies. This conclusion is consistent with the assumption of Atopkin *et al.* (2017) regarding the primordial nature of ecsoma in hemiurid trematodes.

Cercariae are described for the species that belong to the genera *Bunocotyle*, *Hemiurus*, *Brachyphallus*, *Lecithochirium*, *Lecithocladium*, *Lecithaster* Lühe, 1901 and *Lecithophyllum* Odhner, 1905 (e.g. Chabaud and Buttner, 1954; Køie, 1989, 1990, 1995; Køie *et al.*, 2002) from the (Hemiuridae + Lecithasteridae) + Bunocotylidae subclade. The similarity and difference of the cystophorous cercariae of the hemiuroids manifests itself primarily through the morphology of the caudal vesicle (= caudal cyst). The cercariae of the genus *Bunocotyle* have a single appendage on the caudal vesicle: the caudal filament, which is probably one of the variants of an excretory appendage (see Chabaud and Buttner, 1954). This feature makes them comparable to cercariae of the genera *Hemiurus*, *Brachyphallus*, *Lecithocladium* and *Lecithaster* (see Hunninen and Cable, 1943; Køie, 1989, 1995). The caudal filament of the *Bunocotyle*'s cercariae, however, is very long, slightly motile and deprived of additional appendages (e.g. membranous folds and furcae), which distinguish it from immotile excretory appendages in *Lecithaster* spp. cercariae and motile appendages in cercariae of the other three genera. To date, cercariae are described only for a limited number of hemiuroidean subfamilies and families (see e.g. Littlewood and Bray, 2001). In this connection, adequate phylogenetic and taxonomic interpretation of hemiuroids' cercarial morphology is not yet feasible.

Hemiuridae + Lecithasteridae group

The monophyly of the group is convincingly demonstrated with good support. The family Lecithasteridae in this group is represented by the genera attributable to the nominative subfamily: *Lecithaster*, *Lecithophyllum* and *Aponurus* Looss, 1907 (see Gibson and Bray, 1979; Gibson, 2002d). Genera *Lecithophyllum* and *Aponurus* are sister taxa in our tree (Fig. 1). *Merluciotrema praeclarum* is the nearest neighbour to the genus *Lecithaster*. Gibson and Bray (1979), Bray (1996) and Gibson (2002a) place *M. praeclarum* into the hemiurid subfamily Plerurinae. The results of our analysis, however, are the same as the previously published data (Olson *et al.*, 2003; Pankov *et al.*, 2006; Marzoug *et al.*, 2014; Bao *et al.*, 2015; Atopkin *et al.*, 2017; Faltýnková *et al.*, 2017; Sokolov *et al.*, 2018), and show that this parasite is phylogenetically distant from plerurines (Fig. 1). The closeness of *M. praeclarum* to *Lecithaster* spp. is consistent with the opinion of Skrjabin and Guschanskaja (1955b) regarding the belonging of this species (as *Musculovesicula praeclarus* in these authors) to Lecithasteridae. The point of view of these authors was based on the morphology of vitellarium, which in this species, as in many lecithasterids, is represented by an unpaired star-shaped mass. The relationships between subgroups of *M. praeclarum* + *Lecithaster* spp. and *L. botryophoron* + *Aponurus* spp. are poorly resolved on our tree (Fig. 1).


The Hemiuridae *sensu* Gibson, 2002 remaining after exclusion of bunocotylids and *M. praeclarum* form a monophyletic subgroup (Fig. 1). Taking into account the research of Faltýnková *et al.* (2017), the following species are also integrated into this subgroup: *Glomicirrus macrouri* (Gaevskaia, 1973) (Glomicirrinae), *Elytrophalloides oatesi* (Leiper and Atkinson, 1914) (Elytrophallinae), *Ectenurus lepidus* Looss, 1907 (Dinurinae) and *Lecithochirium caesionis* Yamaguti, 1942 (Lecithochiriinae). Our analysis does not support the monophyly of two hemiurid subfamilies: Plerurinae *sensu* Gibson, 2002 and Lecithochiriinae *sensu* Gibson, 2002. *Dinosoma synaphobranchi* (Plerurinae) turned out to be close to *B. crenatus* (Lecithochiriinae) and the subgroup formed by them does not have direct phylogenetic connections with representatives of the type genera of the corresponding subfamilies *Plerurus digitatus* (Looss, 1899) and *Lecithochirium microstomum* (Chandler, 1935). Note that earlier, Skrjabin and Guschanskaja (1955a) united genera *Dinosoma* and *Brachyphallus* in subfamily *Brachyphallinae*, although the genus *Dinosoma* was placed in it as a member of the tribe Plerurea.

Other hemiuroids

Our research of phylogeny revealed that isoparorchiids share a recent common ancestor with the (Hemiuridae + Lecithasteridae) + Bunocotylidae subclade (Fig. 1). In the previous molecular genetic studies based on 18S rDNA, the phylogenetic positions of the Isoparorchiidae were poorly resolved (Blair *et al.*, 1998; Pankov *et al.*, 2006; Bao *et al.*, 2015). The morphological features of adult isoparorchiids show that these trematodes are close to the members of our clade A (Gibson and Bray, 1979; Brooks *et al.*, 1985; Blair *et al.*, 1998). Isoparorchiid cercariae have a unique morphology, combining the presence of the delivery tube, multiple filaments on the caudal vesicle and the absence of an excretory appendage (Ito, 1953; Besprozvannykh and Ermolenko, 1989). This combination of characteristics distinguishes them from cercariae with filaments on the caudal vesicle, which are characteristic of some members of clade A (*A. problematica*) and the (Hemiuridae + Lecithasteridae) + Bunocotylidae subclade (*Lecithochirium* spp.) (see Arvy, 1963; Matthews, 1981; Køie, 1990; Urabe and Shimazu, 2013).

Highly supported inter-family relations within clade A have so far been identified only for Syncoeliidae, Hirudinellidae and Accacoeliidae (Fig. 1; see also Atopkin *et al.*, 2017; Sokolov *et al.*, 2018). The close phylogenetic relationship between these families was first revealed by molecular genetic studies by Calhoun *et al.* (2013). These connections were not strongly supported in the publication of these authors, however.

Our study confirms the previously obtained data regarding the basal position of Gonocercidae compared with the rest of the hemiuroidean trematodes (Sokolov *et al.*, 2016, 2018). At the same time, it cannot yet be reliably confirmed whether genus *Hemiperina* Nicoll, 1913 belongs to this family (Fig. 1; see also Sokolov *et al.*, 2018).

Author ORCIDs.  Ilya I. Gordeev <http://orcid.org/0000-0002-6650-9120>

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