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Phylogenetic position of the genus *Gonocerca* Manter, 1925 (Trematoda, Hemiuroidea), based on partial sequences of 28S rRNA gene and a reconsideration of taxonomic status of Gonocercinae Skrjabin et Guschanskaja, 1955

Sergey G. Sokolov[a], Dmitry M. Atopkin[b,c,*], Ilya I. Gordeev[d], Marina B. Shedko[b,c]

[a] A.N. Severtsov Institute of Ecology and Evolution, Russian Academy of Sciences, Leninskij pr. 33, Moscow 119071, Russia
[b] Institute of Biology and Soil Science, Far Eastern Branch of the Russian Academy of Sciences, pr-t 100-letiya Vladivostoka 159, Vladivostok 690022, Russia
[c] Department of Cell Biology and Genetics, Far Eastern Federal University, Sukhanova st. 8, Vladivostok 690051, Russia,
[d] Russian Federal Research Institute of Fisheries and Oceanography, V. Krasnoselskaya st. 17, Moscow 107140, Russia

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ABSTRACT

Adult trematodes of the genus *Gonocerca* Manter, 1925, are parasites of marine fishes. Identification of the phylogenetic positions and a revision of the taxonomic status of the subfamily Gonocercinae Skrjabin et Guschanskaja, 1955 (Derogenidae) are the main purposes of this research article. Four *Gonocerca* species were used in the study, including the type-species *G. phycidis* Manter, 1925. Molecular phylogenetic analysis, based on partial sequences of 28S rRNA gene, revealed that *Gonocerca* spp. are phylogenetically distant from other hemiuroid trematodes, including *Derogenes varicus* (Müller, 1784), representative of the type-genus of the family Derogenidae. The taxonomic rank of Gonocercinae should be raised to the family level. The generic composition of the family Gonocercidae Skrjabin et Guschanskaja, 1955 stat. nov., requires further clarification as the molecular data do not support the inclusion of the genus *Hemipera* Nicoll, 1913, in this family.

1. Introduction

The genus *Gonocerca* Manter, 1925, includes hemiuroid trematodes that are characterised by an ovary that is positioned in anterior to the testes, a vitellarium that consists of two lateral masses and lies in the same level as the ovary, eggs without filaments, a terminal part of the male duct that consists of a seminal vesicle, a tubular pars prostatica surrounded by a subglobular field of prostatic cells, and a short ejaculatory duct that opens into the genital atrium [1,2]. Definitive hosts of *Gonocerca* spp. are marine fishes. This genus includes 14 species; however, some of these have controversial taxonomic status [3–8].

Traditionally, the genus *Gonocerca* is considered as a member of the subfamily Gonocercinae Skrjabin et Guschanskaja, 1955, within the family Derogenidae Nicoll, 1910 [1,2,6,9]. Besides the type-genus, the subfamily Gonocercinae includes the genus *Hemipera* Nicoll, 1913. Skrjabin & Guschanskaja [9] reported this genus under the different name *Hemiperina* Manter, 1934. Yamaguti [10] considered *Hemiperina* Manter, 1934, as a junior synonym of the genus *Hemipera* Nicoll, 1913, an interpretation that was also used by Gibson & Bray [1] and by Gibson [2]. Olson et al. [11] were the first to reconstruct the phylogenetic relationships of the Gonocercinae using molecular data. The conclusions of these authors were based on the analysis of partial 18S rRNA and 28S rRNA gene sequences of *Hemipera manteri* (Crowcroft, 1947) (as *Hemiperina manteri* by Olson et al. [11]), a member of the Gonocercinae, and *Derogenes varicus* (Müller, 1784) the most abundant species of the type-genus of Derogenidae. Results of this study showed that *H. manteri* forms a branch basal to the rest of the Hemiuroidea, indicating polyphyly of Derogenidae. As has been shown subsequently [12–14], the species *H. manteri* and *D. varicus* are phylogenetically distant from each other. Pankov et al. [12] proposed the consideration of Gonocercinae as an independent family. This point of view is supported in our study on *Gonocerca muraenolepisi* Paruchin et Ljadov, 1979, the phylogenetic position of which was considered based on 28S rRNA gene partial sequences [8]. On the other hand, we have shown that *G. muraenolepisi* and *H. manteri* are phylogenetically unrelated [8]. In the present work, we discuss the phylogenetic position and taxonomic status of Gonocercinae, taking into consideration newly obtained molecular data on several *Gonocerca* species, including the type-species *G. phycidis* Manter, 1925.

* Corresponding author at: Institute of Biology and Soil Science, Far Eastern Branch of the Russian Academy of Sciences, pr-t 100-letiya Vladivostoka 159, Vladivostok 690022, Russia.
E-mail address: atop82@gmail.com (D.M. Atopkin).

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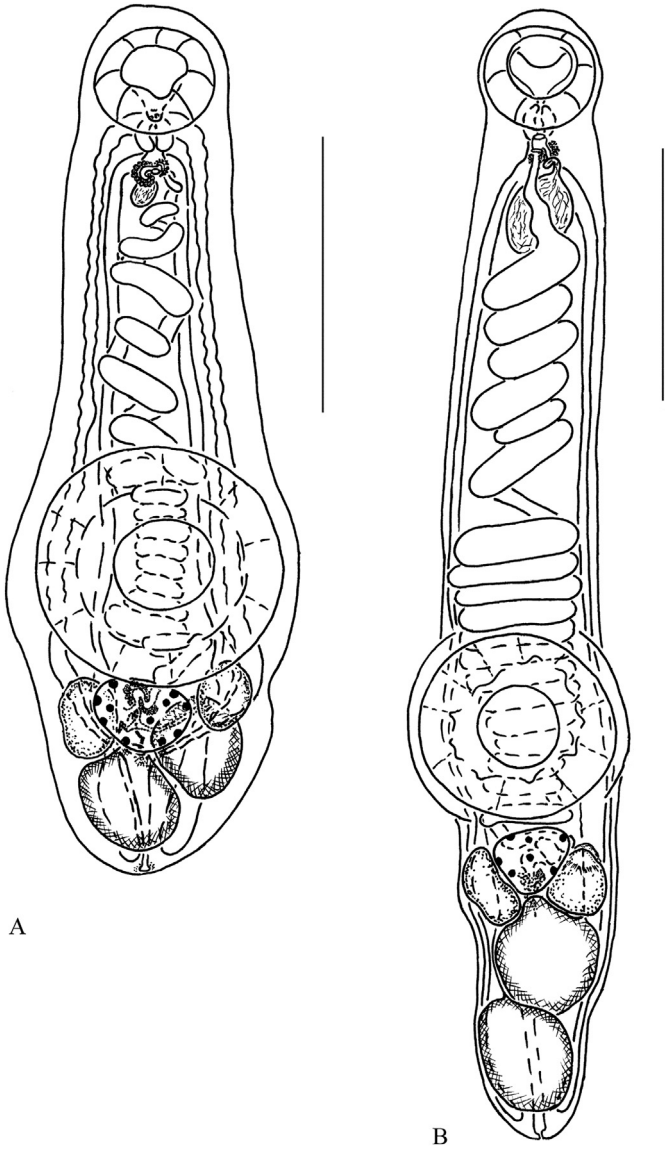

Fig. 1. *Gonocerca crassa* from *Muraenolepis marmorata*, the Ross Sea (A) and *Gonocerca phycidis* from *Pogonophryne* sp., the Ross Sea (B). Scale bars: 0.9 mm.

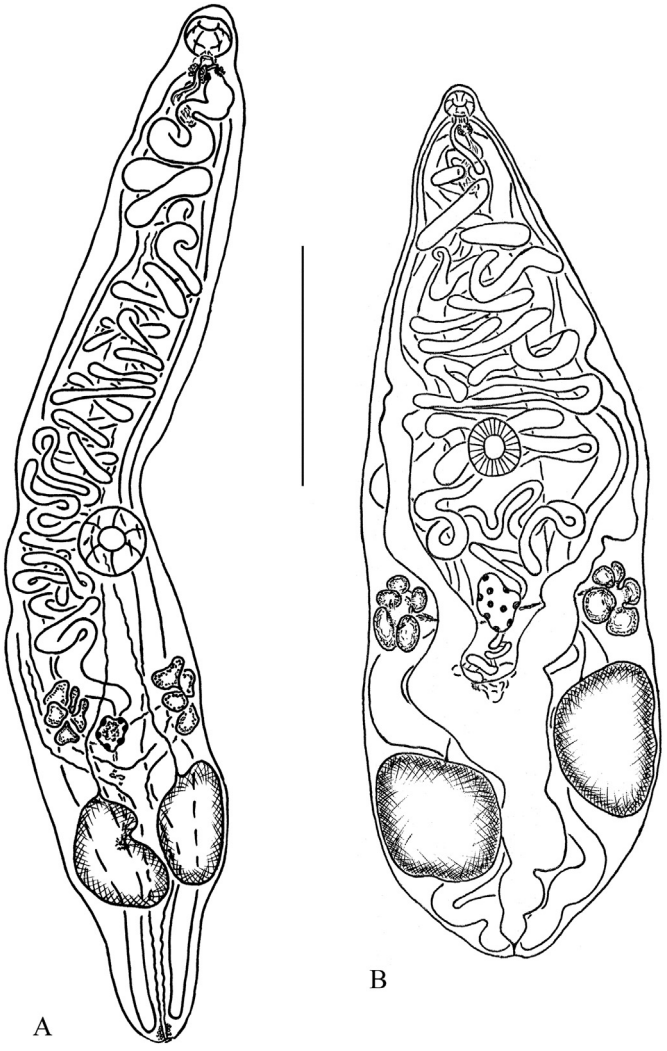

Fig. 2. *Gonocerca oshoro* from *Albatrossia pectoralis*, the Sea of Okhotsk (A) and *Gonocerca muraenolepisi* from *Muraenolepis marmorata*, the Ross Sea (B). Scale bars: A – 6 mm, B – 3 mm.

2. Materials and methods

2.1. Specimen collection

Specimens of four species of the genus *Gonocerca, G. crassa* Manter, 1934, *G. oshoro* Shimazu, 1970, *G. phycidis*, and *G. muraenolepisi* (Figs. 1 and 2), were collected during parasitological examination of fishes in the Northern Pacific and the Antarctic (Table 1). Trematodes species were identified by morphological features, following the methodologies of Manter [15–18], Shimazu [19], Gibson [20], and Sokolov et al. [8]. Voucher specimens of the studied species were deposited in the Museum of Helminthological Collections at the Centre for Parasitology of the A.N. Severtsov Institute of Ecology and Evolution, Moscow, Russia (IPEE RAS).

Table 1
Data on the studied samples of *Gonocerca* spp.

Species	Host and site of infection	Water body
G. crassa	*Muraenolepis marmorata* Günther, 1880 (Gadiformes: *Muraenolepidae*), intestine	The Ross Sea
G. muraenolepisi	*M. marmorata,* body cavity	The Ross Sea and the Amundsen Sea
G. phycidis	*Pogonophryne* sp.(Perciformes: Artedidraconidae), intestine	The Ross Sea
G. oshoro	*Albatrossia pectoralis* (Gilbert, 1892) (Gadiformes: Macrouridae), ovary	The Sea of Okhotsk

2.2. DNA extraction, amplification and sequencing

For genetic analysis, adult *Gonocerca* spp. were fixed in 96% ethanol. DNA was extracted using a “hot shot” technique [21]. Nuclear 28S rRNA gene fragment, including D1–D3 domains, was amplified using a polymerase chain reaction with the following primers: DIG12 (5′- AAG CAT ATC ACT AAG CGG-3′) and 1500R (5′- GCT ATC CTG AGG GAA ACT TCG-3′) [22]. 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 1 unit of Q5 DNA polymerase (New England Biolabs, UK). The amplification of a 1230-bp fragment of 28S rDNA was performed in a GeneAmp 9700 (Applied Biosystems) 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,

Table 2
List of taxa, incorporated into molecular analysis.

Species	Family/subfamily	n[a]	Source	GenBank accession number
Gonocerca phycidis	Gonocercidae	3	This study	KY197009—KY197011
G. crassa	Gonocercidae	1	This study	KY197012
G. oshoro	Gonocercidae	2	This study	KY197013; KY197014
G. muraenolepisi (the Ross Sea)	Gonocercidae	9	[8]	HF543941—HF543948; LN650651
G. muraenolepisi (the Amundsen Sea)	Gonocercidae	2	[8]	LN865025; LN865026
Hemipera manteri	Gonocercidae	1	[11] (as *Hemiperina manteri)*	AY222196
Derogenes varicus	Derogenidae, Derogeninae	1	[11]	AY222189
Thometrema lotzi	Derogenidae, Halipeginae	1	[13]	KC985236
Dinurus longisinus	Hemiuridae, Dinurinae	1	[11]	AY222202
Lecithochirium microstomum	Hemiuridae, Lecithochiriinae	1	[13]	KC985235
Lecithocladium. excisum	Hemiuridae, Elytrophallinae	1	[11]	AY222203
Opisthadena dimidia	Hemiuridae, Opisthadeninae	1	[11]	AY222198
Plerurus digitatus	Hemiuridae, Plerurinae	1	[11]	AY222201
Merlucciotrema praeclarum	Hemiuridae, Plerurinae	1	[11]	AY222204
Bunocotyle progenetica	Hemiuridae, Bunocotylinae	1	[12]	DQ354365
Robinia aurata	Hemiuridae, Bunocotylinae	1	[12]	DQ354367
Saturnius gibsoni	Hemiuridae, Bunocotylinae	1	[27]	KJ010542
Saturnius sp.	Hemiuridae, Bunocotylinae	1	[12]	DQ354366
Aponurus sp.	Lecithasteridae, Lecithasterinae	1	[12]	DQ354368
Lecithaster mugilis	Lecithasteridae, Lecithasterinae	2	[28]	LN865016; LN865017
L. sudzuhensis	Lecithasteridae, Lecithasterinae	1	[28]	LN865022
Machidatrema chilostoma	Lecithasteridae, Hysterolecithinae	1	[11]	AY222197
Accacoelium contortum	Accacoeliidae	1	[11]	AY222190
Copiatestes filiferus	Syncoeliidae	1	[11]	AY222188
Didymozoon scombri	Didymozoidae	1	[11]	AY222195
Didymocystis scomberomori	Didymozoidae	1	[29]	KU341979
Hirudinella ahi	Hirudinellidae	1	[13]	KC985238
Hirudinella ventricosa	Hirudinellidae	1	[13]	KC985232
Prosogonotrema bilabiatum	Sclerodistomidae	1	[11]	AY222191
Outgroup				
Otodistomum cestoides	Azygiidae	1	[11]	AY222187
Azygia longa	Azygiidae	1	[13]	KC985234
Proterometra sp.	Azygiidae	1	[13]	KC985237

[a] Number of specimens.

900F, and 1200R [22]. The PCR products were analysed using an ABI 3130xl genetic analyser at the Department of Cell Biology and Genetics, Far Eastern Federal University. The sequences have been submitted to GenBank (Table 2).

2.3. Alignments and the phylogenetic analysis

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 6.0 [23]. Phylogenetic analyses of the nucleotide sequences were performed using the Bayesian (BI) algorithm MrBayes v.3.1.2 [24] software. The best nucleotide substitution models were estimated with jModeltest v.2.1.5 software [25], using the Bayesian Information Criterion [24]. The best nucleotide substitution model for 28S rRNA gene sequence data was TVM3 + I + G – transversional model with estimates of invariant sites and gamma-distributed among-site variation [26]. The significance of the phylogenetic relationships was estimated by posterior probabilities [24]. Phylogenetic relationships were performed using our data and nucleotide sequences of 28S rDNA of Hemiuroidea and Azygioidea trematode specimens from the NCBI GenBank database (Table 2).

3. Results

All studied *Gonocerca* spp. formed a distinct clade, inside of which they were subdivided into three branches with a soft polytomy: *G. phycidis*, *G. crassa,* and *G. oshoro* + *G. muraenolepisi* (Fig. 3). Bayesian phylogenetic analysis showed a soft polytomy for *G. muraenolepisi*, obtained from the Ross Sea and the Amundsen Sea (Fig. 3). Genetic differentiation between these trematodes was 0.95% (Table 3). *G. oshoro* appears to be sister to *G. muraenolepisi,* with 0.77–1.72% genetic differentiation (Table 3).

Other hemiuroids related to *Gonocerca* spp., with the exception of *H. manteri*, formed a sister clade that subdivided on two subclades (A and B). Within each of these subclades, there were two different lineages, A1, A2, B1, and B2 (Fig. 3). Lineage A1 contained representatives of Accacoeliidae, Didymozoidae, Hirudinellidae, and Syncoeliidae, while lineage A2 contained representatives of Halipeginae and Derogeninae (both of which belong to Derogenidae), and Sclerodistomidae. Lineage B1, within subclade B, includes trematodes from five hemiurid subfamilies, Dinurinae, Elytrophallinae, Lecithochiriinae, Opisthadeninae, and Plerurinae, as well as subfamily Lecithasterinae (Lecithasteridae). Species of Bunocotylinae (Hemiuridae) and Hysterolecithinae (Lecithasteridae) were included in the B2 lineage. The phylogenetic tree topology showed obvious polyphyly of Hemiuridae and Lecithasteridae within subclade B. Trematode *Hemipera manteri* formed a sister branch related to all Hemiuroidea.

4. Discussion

The species *Gonocerca* studied form a well-supported monophyletic group. *Gonocerca phycidis* and *G. crassa*, do not form a clade, but are resolved in a three way polytomy with the group of species, "*G. oshoro* + *G. muraenolepisi*". With the latter group of species, a taxonomic problem arose, as the genetic distance between *G. oshoro* and *G. muraenolepisi* from the Ross Sea proved to be less than that noted between specimens of *G. muraenolepisi* (Table 3) from the Ross and Amundsen Seas. To resolve this problem, additional studies are needed.

Phylogenetic analysis did not reveal direct relationships between the *Gonocerca* spp. clade and representatives of any of the eight hemiuroid families deposited in GenBank, including *D. varicus* and *H.*

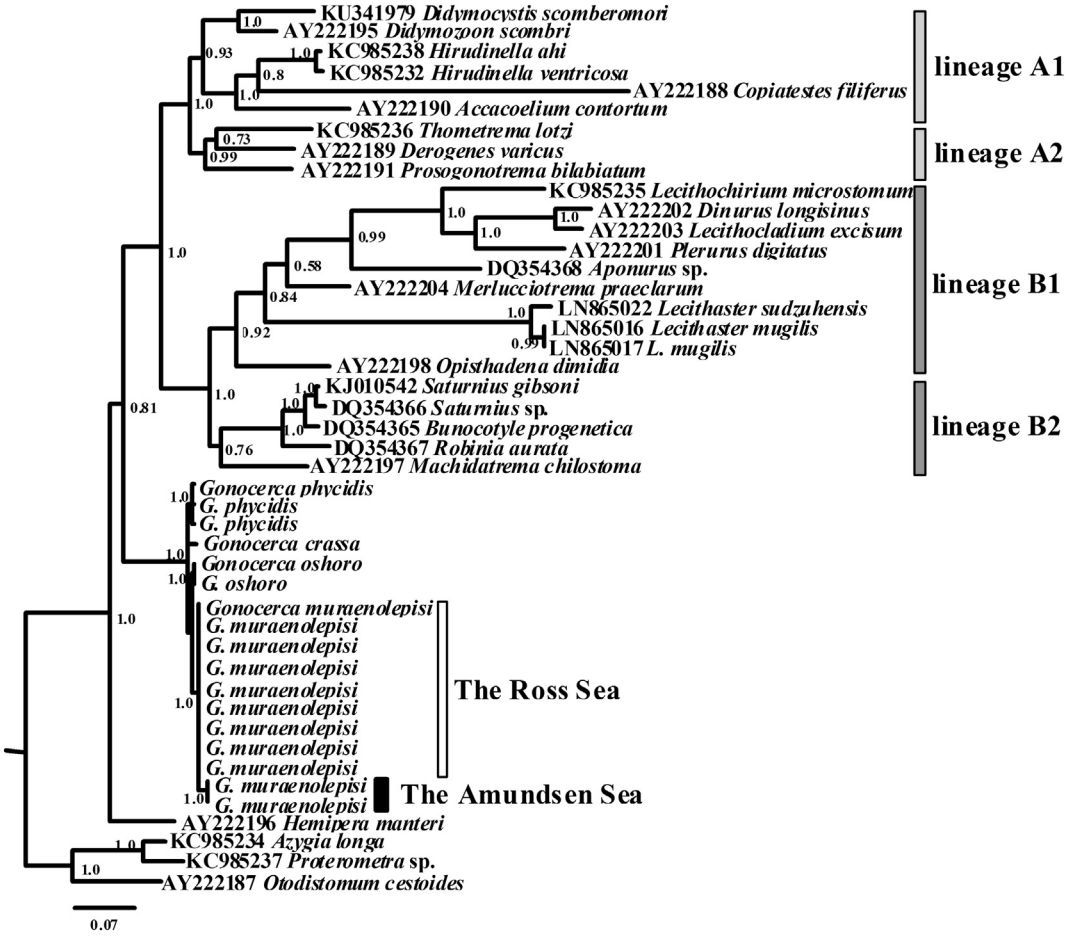

Fig. 3. Phylogenetic tree of hemiuroid trematodes based on the analysis of combined 28S rRNA gene partial sequences with Bayesian algorithm; nodal numbers indicate posterior probabilities. Sequences of representatives of family Azygiidae are used as outgroup.

Table 3
Genetic differentiation (%) of *Gonocerca* spp. by 28S rRNA gene partial sequences.

		1	2	3	4
1	*G. muraenolepisi* (the Ross Sea)				
2	*G. muraenolepisi* (the Amundsen Sea)	0.95			
3	*G. oshoro*	0.77	1.72		
4	*G. phycidis*	1.29	2.24	0.86	
5	*G. crassa*	1.63	2.58	1.37	1.37

manteri. The latter two species are phylogenetically distant from each other, corresponding to the findings of Olson et al. [11], Pankov et al. [12], Calhoun et al. [13], and Bao et al. [14]. Results of phylogenetic analyses based on molecular data correspond to deep morphological differences of the studied *Gonocerca* spp. and *D. varicus* in the position of the testes, vitellarium, and ovary, as well as the morphological features of the terminal part of the genital system. In contrast to *Gonocerca* spp., *D. varicus* is characterised by having the vitelline masses posterior to the ovary, with the testes positioned anterior to it. The terminal part of the *D. varicus* genital system consists of a genital atrium that encloses a sinus-organ linked with a sinus-sac. The sinus-sac includes a metraterm and ejaculatory duct that join to form the hermaphrodite duct in the middle of the sinus-organ. The proximal end of the ejaculatory duct is linked with a large external pars prostatica, which is surrounded by a cylindrical field of prostatic cells [20]. The most significant differences between *H. manteri* and *Gonocerca* spp. are found in the morphology of the eggs and the terminal part of the genital system. *Hemipera manteri* is characterised by a poorly developed sinus-sac and sinus-organ [30]. Eggs of this species carry a filament at the anopercular end. Differences between *H. manteri* and *D. varicus* are also related to the egg morphology (with filament and without filament, respectively), as well as in the positions of the vitellarium and gonads (in *H. manteri,* these are the same as in *Gonocerca* spp.). Morphological differences in the terminal part of the genital system of these species are not so extreme and relate primarily to the degree of development of the sinus-organ, ejaculatory duct, and pars prostatica [20,30].

In the traditional systematic of the Derogenidae dissimilarities in egg morphology and the terminal part of the genital system, observed between *Gonocerca* spp., *H. manteri* and *D. varicus*, are characteristics of the generic level of divergence, and differences in the topology of the gonads and vitellarium are differential features at the subfamilial level [1,2,6,9]. The localization of the testes posterior to the ovary and vitellarium distinguish the genera *Gonocerca* and *Hemipera* from all other derogenids and serve as the basis for their unification into subfamily Gonocercinae [1,2,6,9]. However, on the basis of molecular and morphological data we promote Gonocercinae as an independent family, Gonocercidae Skrjabin et Guschanskaja, 1955 stat. nov. The phylogenetic position of *H. manteri* disagreed with the traditional position of the genus *Hemipera* [1,2,9]. Nevertheless, we tentatively consider the genus *Hemipera* as a member of the family Gonocercidae until additional molecular data on the 28S rRNA gene of other species of this genus are obtained. The diagnosis of the family Gonocercidae agrees with the diagnosis of the subfamily Gonocercinae (see [2]). Given our findings, the traditional taxonomical composition of Derogenidae (see [1,2]) decreases to two subfamilies – Derogeninae and Halipeginae. Phylogenetic tree topology shows *Thometrema lotzi* Curran, Overstreet et Font, 2002 (Halipeginae) is sister species to *D. varicus*

with a low statistical support (Fig. 3) which does not contradict the inclusion of the Halipeginae in the Derogenidae.

The Gonocercidae, in contrast to the family Derogenidae, is associated exclusively with the marine environment. For Derogenidae there are both marine and fresh-water species, and the latter (some Halipeginae) parasitize fishes, amphibians reptiles and freshwater shrimps [2]. At the same time, biogeographical differences between gonocercids and the marine species of the family Derogenidae have not been identified. The total geographic ranges of representatives of the family Gonocercidae and of marine derogenid species covers wide areas of the World Ocean and significantly overlap [17,18,20,31–33]. According to Bray [34], the most abundant marine derogenids are the shallow-water species, but there are later findings of one of these species, *D. varicus*, in fishes caught at depths of more than 1500 m [35]. Among gonocercids there are shallow-water and eurybathyal species [34]. Many species of these two families (particularly *D. varicus* and *G. phycidis*) are generalists and share groups of definite hosts, in particular gadiform and notothenid fishes [20,32]. Unfortunately, the life-history stages of gonocercids are not known.

The divergence of Hemiuridae + Lecithasteridae and the group of aforementioned families – Accacoeliidae, Didymozoidae, Hirudinellidae, Syncoeliidae, Sclerodistomidae, and Derogenidae – into the different subclades (B and A, respectively) (Fig. 3) is consistent with the phylogenetic models of the superfamily Hemiuroidea developed by other authors [11,12,36,37]. Our data, however, add detail to the phylogenetic relationships of hemiuroid trematodes within the subclades of A (lineages A1 and A2). Also, our data, together with earlier published considerations on phylogenetic relationships of the families Hemiuridae and Lecithasteridae [11,12,36,37], indicate a need for the revision of these taxa.

Conflict of interest statement

The authors declare that they have no competing conflict of interest.

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