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A Phylogenetic Re-Evaluation of the Stenakrine Opecoelids (Trematoda, Digenea: Opecoeloidea) with Some Taxonomic Novelties †

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Abstract: The Opecoeloidea is a large group of xiphidiate digeneans parasitizing marine and freshwater fishes. According to the current taxonomic model, this superfamily contains only one family with numerous subfamilies. This study is devoted to the members of the Stenakrinae. Based on phylogenetic analysis of concatenated sequences of 18S and 28S rRNA genes of stenakrine opecoelids *Caudotestis dobrovolski*, *C.* cf. *dobrovolski*, *Hexagrammia zhukovi*, *Stenakron vetustum*, as well as the deep-sea xiphidiate digenean *Zdzitowieckitrema incognitum*, which so far has had an ambiguous phylogenetic status, we erect a new opecoeloid family, the Zdzitowieckitrematidae fam. nov. The genera *Holsworthotrema* and *Scorpidotrema* are removed from the Stenakrinae to the Scorpidotrematinae subfam. nov. within the Opecoelidae. We also remove the Stenakrinae from the Opecoelidae and recognize it as a separate family within the Opecoeloidea. The Stenakridae stat. nov. is a sister taxon to a well-supported Opecoelidae. The Zdzitowieckitrematidae occupies a sister position relative to the stenakrids and the opecoelids taken together. All three families are clearly phylogenetically distinct, however convincing morphological differences are revealed only between the Zdzitowieckitrematidae and the Stenakridae and between the Opecoelidae and the Stenakridae.

Keywords: 18S; 28S; *Biospeedotrema*; *Hexagrammia*; *Stenakron*; *Zdzitowieckitrema*; *Holsworthotrema*; *Scorpidotrema*; Opecoelidae; Liparidae; North Pacific

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1. Introduction

The Opecoeloidea Ozaki, 1925 is a species-rich superfamily of xiphidiate digeneans with the type and only family Opecoelidae Ozaki, 1925 [1,2]. The family has a complex taxonomic structure. According to the modern concept, which is based on phylogenetic and morphological data, 11 subfamilies and several early-branching clades are distinguished within the Opecoelidae [3–6]. Nomenclatural formalization at the subfamily level for these early branching clades is clearly required.

One of the controversial subfamilies within the Opecoelidae is the Stenakrinae Yamaguti, 1970. It is a relatively small group, comprising eight genera: *Biospeedotrema* Bray, Waeschenbach, Dyal, Littlewood and Morand, 2014, *Caudotestis* Issaitschikov, 1928, *Hexagrammia* Baeva, 1965, *Holsworthotrema* Martin, Huston, Cutmore, and Cribb, 2019, *Neonotoporus* Srivastava, 1942, *Pseudopecoelina* Yamaguti, 1942, *Scorpidotrema* Aken'Ova and Cribb, 2003, and *Stenakron* Stafford, 1904 [7–9]. Originally, the Stenakrinae was erected as a subfamily in the Fellodistomidae Nicoll, 1909 [10], but subsequently began to be regarded as a subfamily of the Opecoelidae [3,4,7–9,11,12]. Notably, members of the Stenakrinae

originally placed outside of this subfamily were considered as fellodistomid, opecoelid, or allocreadiid concepts [13–17].

Phylogenetic analysis do not support the monophyly of the Stenakrinae. For instance, *Biospeedotrema* and *Caudotestis* are grouped into one clade with *Zdzitowieckitrema incognitum* Sokolov, Lebedeva, Gordeev, and Khasanov, 2019, which is a deep-sea xiphidiate digenean without any clear family affiliation [18,19]. Taxonomic status of this clade is still obscure [18]. At the same time, it has been confirmed that *Holsworthotrema* and *Scorpidotrema* are Opecoelidae [6,9,19]. In the family wide phylogenetic analyses of [9,18], these two genera formed a clade interpreted to be distinct at the level of subfamily which we previously [18] named as the Stenakrinae sensu Martin, Huston, Cutmore and Cribb, 2019. There are no published sequence data available for other Stenakrinae, including the representatives of the genus type *Stenakron*. Morphologically, *Hexagrammia* and *Stenakron* are similar to *Biospeedotrema* and *Caudotestis*, while *Neonotoporus* and *Pseudopecoelina* are closer to members of the Stenakrinae sensu Martin Huston, Cutmore, and Cribb, 2019 [18]. However, conclusions about the systematic position of opecoelid-like digeneans based on morphological evidence are not always reliable [9,11,18–21].

Concatenation of the 18S and the 28S rRNA gene sequences is one of the most reliable methods of phylogenetic analysis for divergence events related to deeper nodes [22–25]. Phylogenetic inferences generated by these datasets can be more accurate due to an increased number of sites for a given set of taxa [26]. The aim of this study was to assess the phylogenetic position of the genera *Biospeedotrema*, *Caudotestis*, *Hexagrammia*, *Holsworthotrema*, *Scorpidotrema*, *Stenakron*, and *Zdzitowieckitrema* Sokolov, Lebedeva, Gordeev, and Khasanov, 2019 on the basis of concatenated 18S+28S rRNA gene sequences. For this purpose, we used newly obtained sequences of *Stenakron vetustum* Stafford, 1904 (type species of type genus for the Stenakrinae), *Caudotestis dobrovolski* Sokolov, Lebedeva, Shchenkov, and Gordeev, 2020, *C.* cf. *dobrovolski*, *Hexagrammia zhukovi* Baeva, 1965, and *Z. incognitum*, as well as data from GenBank NCBI.

2. Materials and Methods

2.1. Sample Collection

Caudotestis cf. *dobrovolski* and *S. vetustum* were found in the intestine of five specimens of Falcate snailfish *Careproctus cypselurus* (Jordan and Gilbert, 1898) (Liparidae) caught at a depth of 200 m in the coastal area of the southern part of Sakhalin Island (46°30′1″ N; 143°47′6″ E) in October 2021. Trematodes were initially relaxed in fresh water and fixed in 70% ethanol, and after a few minutes transferred into 96% ethanol. *Hexagrammia zhukovi* was collected from the intestine of five specimens of Rock greenling *Hexagrammos lagocephalus* (Pallas, 1810) (Hexagrammidae) caught at a depth of 20 m in the coastal area of the southwestern part of Iturup Island (44°42′4″ N; 147°11′7″ E) in August-September 2021. The host individuals were frozen and dissected later; specimens of *H. zhukovi* were washed in fresh water and preserved in 96% ethanol.

2.2. Morphological Study

Trematode specimens were identified using morphological study (*S. vetustum and H. zhukovi*) or combined morphological study and genetic sequencing (*Caudotestis* cf. *dobrovolski*). For morphological study, trematode specimens were stained with acetocarmine, dehydrated in a graded ethanol series, cleared in dimethyl phthalate and mounted in Canada balsam. In morphological descriptions, the measurements reported as the range are followed by the average in brackets (for n > 1). All measurements are in micrometers. Drawings were made with the aid of a camera lucida. Specimens of *C. dobrovolski* and *Z. incognitum* have been described in our previous publications [18,19].

Paragenophores were deposited in the Museum of Helminthological Collections at the Center of Parasitology of the Severtsov Institute of Ecology and Evolution (IPEE RAS; Moscow, Russia).

2.3. DNA Amplification and Sequencing

We obtained partial sequences of the 18S rRNA gene for all five digenean species mentioned above, as well as the 28S rRNA gene for *C.* cf. *dobrovolski*, *H. zhukovi, S. vetustum,* and *Z. incognitum*. For *C. dobrovolski,* the sequence of the 18S rRNA gene was obtained from the same specimen from which the 28S rRNA gene sequence (MN437379) had previously been obtained [18]. In addition, partial sequences of the nd1 mitochondrial DNA gene were obtained for *C.* cf. *dobrovolski*. Unfortunately, attempts to obtain an amplicon of the same gene from *C. dobrovolski* were unsuccessful. In order to obtain the sequences, small fragments of single specimens of each species were dried of ethanol in a dry block heater for 1.5 h at 35 °C, digested with a mixture of 49 µL 0.1 % Chelex-100 and 1 µL Proteinase K (concentration 10 mg/mL), and incubated for one hour at 55 °C followed by 20 min at 95 °C. After that, the water solution of the total DNA was placed into a sterile 500 µL tube and frozen.

The partial sequence of 18S rRNA gene was amplified with WormA (5′-GCGAATGG CTCATTAAATCAG-3′) and WormB (5′-ACGGAAACCTTGTTACGACT-3′) primers [27] using the following PCR parameters: initial denaturation at 95 °C (3 min); 35 cycles of 20 s at 95 °C; 20 s at 50.1 °C; 180 s at 72 °C; 5 min at 72 °C for final extension.

The D1–D3 domains of 28S rRNA gene (approximately 1200 bp long) were amplified using the dig12 (5′-AAG CAT ATC ACT AAG CGG-3′) and L0 (5′-GCT ATC CTG AGR GAA ACT TCG-3′) primers [28]. The thermal cycle parameters were as follows: initial denaturation at 95 °C (3 min); 35 cycles of 20 s at 95 °C; 20 s at 49,4 °C; 130 s at 72 °C; 5 min at 72 °C for final extension.

All amplicons were sequenced directly with the same pair of primers using the equipment of the Research Park of St. Petersburg State University (Centre for Molecular and Cell Technologies). The newly obtained sequences from both forward and reverse primers were assembled using Chromas Pro 1.7.4. After assembling and trimming of low quality parts of contigs, the sequence lengths were: 1262–1268 bp for 28S rDNA and 1741–1769 bp for 18S rDNA.

The partial sequences of the nd1 gene were generated with NDJ1 (5′-AGA TTC GTA AGG GGC CTA ATA-3′) and ND1J2A (5′-CTT CAG CCT CAG CAT AAT C-3′) primers [29]. PCR parameters were as follows: initial denaturation at 95 °C (3 min); 35 cycles of 20 s at 95 °C; 20 s at 49.7 °C; 35 s at 72 °C; 5 min at 72 °C for final extension. Contigs were assembled and trimmed in ChromasPro software. The final length of newly obtained sequences was 455 bp in both specimens of *C.* cf. *dobrovolski*. Attempts to amplify another mitochondrial DNA marker suitable for DNA barcoding, cox1, were unsuccessful in this species.

2.4. Phylogenetic Analysis

To assess the phylogenetic affinities of nominal stenakrine taxa, maximum likelihood and Bayesian inference analyses were performed against a concatenated 18S + 28S alignment including the novel data as well as other relevant sequences from GenBank. Sequences for general alignment of the main subfamilies/clades, including the Opecoelidae, other xiphidiate families belonging to the Gorgoderoidea, Haploporoidea, Troglotrematoidea, Microphalloidea, Plagiorchioidea and Brachycladioidea, as well as non-xiphidiate taxa including from the Monorchiata, Lepocreadiata, Opisthorchiata, Apocreadiata, Gymnophallata, and Hemiurata were downloaded from GenBank database [3,8,9,11,18,20,22,27,30–44] with custom R script [45] based on the "ape" package [46]. To concatenate sequences of 18S and 28S rRNA genes "catfasta2phyml.pl" script [47] was used with "-f" parameter. Final length of alignment was 2999 bp. The full list of sequences used in phylogenetic reconstructions is provided in Supporting Information, Table S1. A representative species of the Hemiurata was selected as the outgroup based on the findings of Olson et al. [22] and Littlewood et al. [1]. The sequences involved in the phylogenetic analyses were aligned using MUSCLE algorithm as implemented in SeaView software [48]. Evolutionary model for maximum likelihood (ML) and Bayesian inference (BI) analyses was chosen with Mr-

Modeltest v. 2.4 [49]. The best fitted model was GTR + G + I. maximum likelihood analysis was performed in RaxML through the Cipres portal [50] with non-parametric bootstrap with 1000 pseudoreplicates. Bayesian analysis was performed using MrBayes 3.2.7 at Cipres portal with gamma correction for intersite rate variation. Trees were run as two separate chains (default heating parameters) for 15,000,000 generations. The quality of the chains was estimated using built-in MrBayes tools and additionally with Tracer 1.6 package [51]. Based on the estimates by Tracer software, the first 20,000 generations were discarded for burn-in.

3. Results

3.1. Phylogeny

In BI and ML analyses based on combined 18S and 28S rRNA gene sequences, *Stenakron* was a well-supported sister taxon to the polytomic group comprising *Caudotestis* spp. and the moderately supported *Hexagrammia* + *Biospeedotrema* clade (Figure 1). In turn, the clade of *Biospeedotrema*, *Caudotestis*, *Hexagrammia*, and *Stenakron* has a sister relationship with the well-supported Opecoelidae clade. However, the two clades mentioned were separated from each other by long branch lengths. Here and below, we interpreted the clade of the stenakrine genera *Biospeedotrema*, *Caudotestis*, *Hexagrammia*, and *Stenakron* at family level and named it the Stenakridae stat. nov. *Holsworthotrema chaoderma* Martin, Huston, Cutmore and Cribb, 2019, *Holsworthotrema enboubalichthys* Martin, Huston, Cutmore, and Cribb, 2019, and *Scorpidotrema longistipes* Aken'Ova and Cribb, 2003, which had been considered to belong to the Stenakrinae prior to our study, have no direct phylogenetic relationships with the true stenakrids and formed a distinct clade at the subfamily level within the Opecoelidae. However, *Holsworthotrema* appeared as a paraphyletic assemblage.

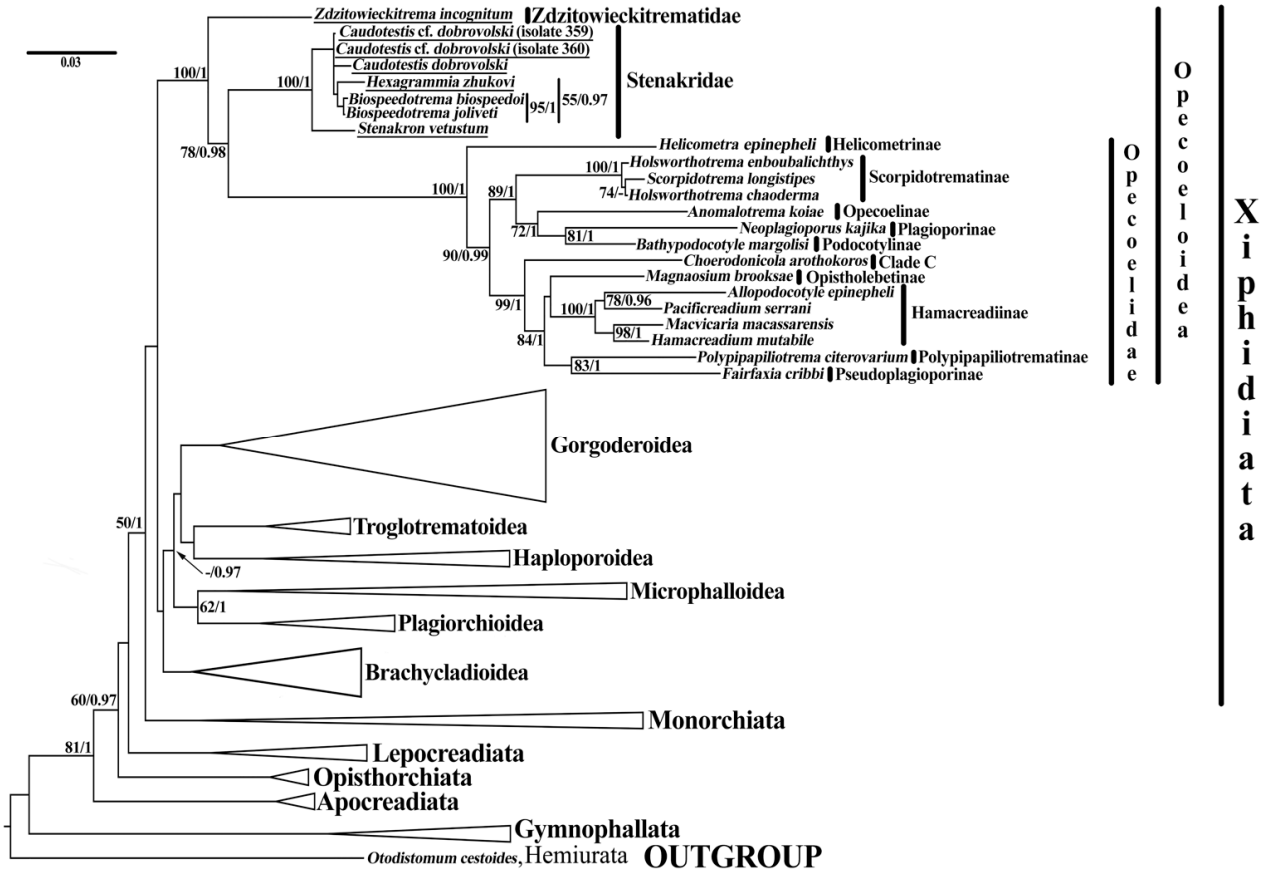

Figure 1. Phylogenetic relationships of opecoeloid digeneans based on concatenated dataset of 18S + 28S rRNA gene sequences. The bootstrap (≥50) and posterior probability (≥0.90) values are given near the nodes for ML and BI analyses, respectively. Newly obtained sequences are underlined.

Zdzitowieckitrema occupied a sister position to the Stenakridae + Opecoelidae clade. *Zdzitowieckitrema* was also separated from the Stenakridae and the Opecoelidae by long branch lengths. This position warrants the family level, and we establish the Zdzitowieckitrematidae fam. Nov. Thus, the zdzitowieckitrematids, the stenakrids, and the opecoelids are three lineages of a large and well-supported Opecoeloidea clade.

The Opecoeloidea was nested within the poorly supported clade of Xiphidiata *sensu* Olson, Cribb, Tkach, Bray, and Littlewood, 2003 (Xiphidiata *sensu stricto* + Haploporoidea) as a sister taxon to the group that included all the other xiphidiate superfamilies.

ML analyses produced similar results, but the Stenakridae + Opecoelidae and the *Hexagrammia* + *Biospeedotrema* clades had, respectively, a moderate and a low support (Figure 1).

3.2. Taxonomy

We provide descriptions of *C.* cf. *dobrovolski*, *H. zhukovi*, and *S. vetustum* used in our phylogenetic analyses. In the light of our findings, we provide an amended diagnosis of the Stenakrinae comb. nov., and establish a new subfamily Scorpidotrematinae subfam. Nov. and a new family Zdzitowieckitrematidae fam. nov.

Superfamily Opecoeloidea Ozaki, 1925.

Family Stenakridae Yamaguti, 1970 stat. nov.

Diagnosis (modified from subfamily diagnosis of Cribb [7]): Body elongate to fusiform. Tegument unarmed. Oral sucker unspecialized or cup-shaped. Ventral suckers unspecialized. Pharynx present. Intestine bifurcates in forebody. Caeca blind, terminate at different levels of hindbody but not near posterior end of body. Testes two, tandem, oblique or opposite, in hindbody. Cirrus-sac elongate and narrow or relatively short and stout, subcylindrical or clavate. Internal seminal vesicle unipartite; pars prostatica tubular; cirrus unarmed. Genital pore median or slightly submedian. Ovary pretesticular or at level of anterior testis. Muscular ovicapt indistinct. Seminal receptacle uterine. Uterus strictly pretesticular or descends to posterior testis or into post-testicular region but not to posterior end of body. Vitellarium follicular, in lateral fields, enters forebody, penetrates into hindbody to testes or just posterior to testes but not to posterior end of body. Excretory vesicle I-shaped. Adults parasitize marine fish of many families; cosmopolitan. Type genus *Stenakron* Stafford, 1904.

Other genera: *Biospeedotrema* Bray, Waeschenbach, Dyal, Littlewood, and Morand, 2014, *Caudotestis* Issaitschikov, 1928, *Hexagrammia* Baeva, 1965.

Remarks.

The Stenakridae stat. nov. differs from the Opecoelidae by the combination of the following features: the presence of the cirrus-sac, the absence of the canalicular seminal receptacle and the fact that the fields of vitelline follicles end at a significant distance from the posterior end of the body. Each of these features may be present in members of different opecoelid genera. For example, the absence of the canalicular seminal receptacle is also typical for opecoelines, and *Shimazuia* Cribb, 2005 (Pseudoplagioporinae) is defined for relatively short fields of vitelline follicles [7]. However, all together they do not occur in any subtaxon of the Opecoelidae (compare with [3,4,7,9,11]). The Stenakridae stat. nov. differs from the Zdzitowieckitrematidae fam. nov. in the absence of the canalicular seminal receptacle and the position of the posterior border of fields of vitelline follicles (ending not near posterior end of the body vs. nearly to the posterior end).

Genus *Stenakron* Stafford, 1904.

Stenakron vetustum Stafford, 1904 Figure 2.

Type hosts: Yellowtail flounder, *Myzopsetta ferruginea* (Storer, 1839) (Pleuronectiformes: Pleuronectidae).

Other host (in present study): Falcate snailfish, *Careproctus cypselurus* (Jordan and Gilbert, 1898) (Perciformes: Liparidae).

Type locality: Northwest Atlantic near Woods Hole, Massachusetts.

Other locality (in present study): The coastal area of the southern part of Sakhalin Island (the Sea of Okhotsk, Northwestern Pacific), (46°30′1″ N; 143°47′6″ E).

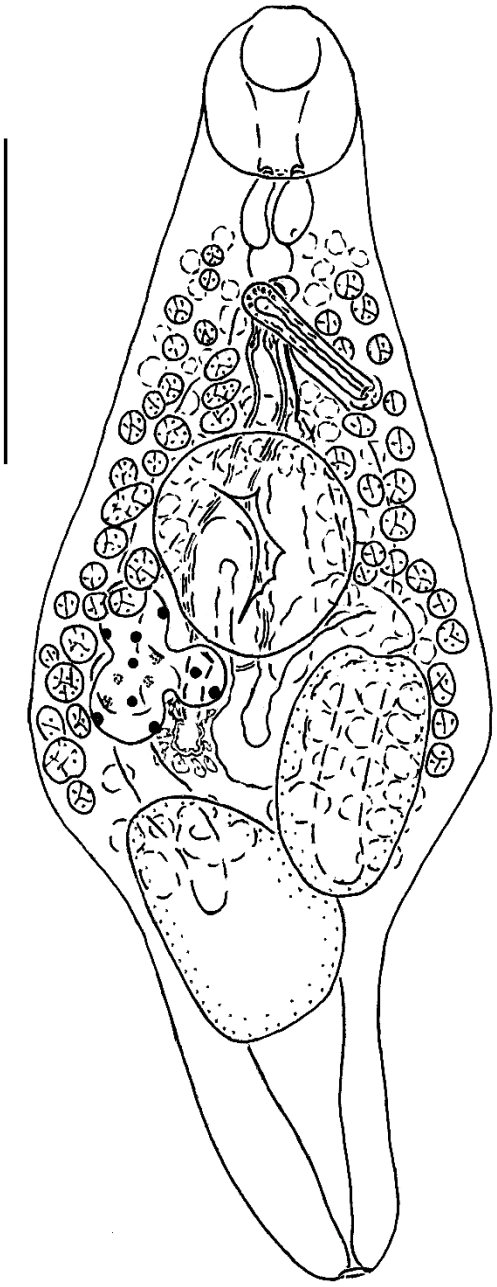

Figure 2. *Stenakron vetustum* Stafford, 1904 from intestine of Falcate snailfish *Careproctus cypselurus* (Jordan and Gilbert, 1898) (Liparidae), the Sea of Okhotsk. Scale bar = 400 μm.

Site of infection: Intestine.

Prevalence and intensity infection: In one of five host specimens; two trematode individuals.

Specimens deposited: One paragenophore IPEE RAS 14320.

Sequences deposited: Partial sequences of the 18S rRNA and 28S rRNA genes from one *S. vetustum* specimen are deposited in GenBank NCBI as OP094618 and OP094624, respectively (Supporting Information, File S1).

Description (based on one whole mount adult specimen—paragenophore): Body fusiform, length 1568, maximum width 560 in area just posterior to mid-body. Forebody 33% of body length. Tegument smooth. Oral sucker suboval, 201 × 187. Mouth subtermi-

nal. Ventral sucker suboval, sessile, 277 × 239. Oral sucker to ventral sucker width ratio 1:1.28. Prepharynx absent. Pharynx 90 × 90. Esophagus twisted, about 138 long. Intestinal bifurcation in area just posterior to border between third and posterior quarter of forebody. Caeca blind, terminate in area just posterior to border between anterior and middle third of hindbody. Post-caecal region 28.6% of body length. Testes two, entire, suboval, oblique, partly overlapping, in posterior half of body; anterior testis strongly sinistro-submedian, 305 × 215, posterior testis strongly dextro-submedian 318 × 180. Post-testicular region 19.6% of body length. Cirrus-sac elongate-clavate, 464 × 125, slightly protrudes beyond posterior margin of ventral sucker; contains long, rectilinear seminal vesicle, short tubular pars prostatica surrounded by field of prostate cells, ejaculatory duct and unarmed cirrus. Vas deferens absent; vasa efferentia directly joining seminal vesicle. Genital pore median, at midlevel of esophagus. Ovary four-lobed, strongly dextro-submedian, antero-dextral to anterior testis and at level of posterior margin of ventral sucker 194 × 166. Oötype with Mehlis' gland near postero-sinistral margin of ovary. Laurer's canal not observed. Canalicular seminal receptacle absent. Uterine seminal receptacle evident. Uterus passes posteriorly as far as posterior testis. Muscular metraterm tubular, runs sinistro-subdorsally to cirrus sac. Eggs deformed in balsam; length of two least-deformed eggs 75 and 78. Vitellarium follicular; follicles in two longitudinal lateral fields extend from level of posterior edge of pharynx to level of posterior margin of anterior testis, overlap caeca dorsally and partly ventrally, confluent dorsally at level of anterior margin of ventral sucker. Excretory pore terminal. Excretory vesicle traced as far as posterior testis, probably extending further.

Remarks.

The lobate structure of the ovary, the median position of the genital pore, the absence of the canalicular seminal receptacle, the fields of vitelline follicles extending to the testes level, and the short caeca indicate that described digenean belongs to *Stenakron* (compare with Cribb [7]). This genus is currently composed of four species: *Stenakron mancopsetti* Gaevskaya and Kovaleva, 1977, *Stenakron skrjabini* (Issaitchikov, 1928), *S. vetustum* (=*Rhodotrema problematicum* Issaitschikov, 1928, *Rhodotrema quadrilobata* Bazikalova, 1932, *Rhodotrema quinquelobata* Layman, 1930), and *Stenakron vitellosum* (Manter, 1934) [7,12,52]. Our specimen corresponds to the description of *S. vetustum* in several key features, namely, the body shape, the position of the genital pore, the arrangement of the cirrus-sac in relation to the ventral sucker and the fields of vitelline follicles relative to the testes, and egg size (compare with [14,15,53–58]). Previously, this species was repeatedly recorded in many fish species of the Northwestern Pacific, including representatives of the genus *Careproctus* Krøyer, 1862 [53,54,58–60].

Genus *Caudotestis* Issaitschikov, 1928.

Caudotestis* cf. *dobrovolski Sokolov, Lebedeva, Shchenkov, and Gordeev, 2020 Figure 3.

Type host: *Liparis* sp. (Perciformes: Liparidae).

Other host (in present study): Falcate snailfish, *Careproctus cypselurus* (Jordan and Gilbert, 1898) (Perciformes: Liparidae).

Type locality: Northwestern Pacific in the environs of Simushir Island.

Other locality (in present study): The coastal area of the southern part of Sakhalin Island (the Sea of Okhotsk, Northwestern Pacific), (46°30′1′′ N; 143°47′6′′ E).

Site of infection: Intestine.

Prevalence and intensity infection: In one of five host specimens; 15 trematode individuals.

Specimens deposited: 13 paragenophores IPEE RAS 14322–14329.

Sequences deposited: Partial sequences of the nd1 (OP087398, OP087399), 18S rRNA (OP094616, OP094617) and 28S rRNA (OP094622, OP094623) genes from two *Caudotestis* cf. *dobrovolski* specimens are deposited in GenBank NCBI.

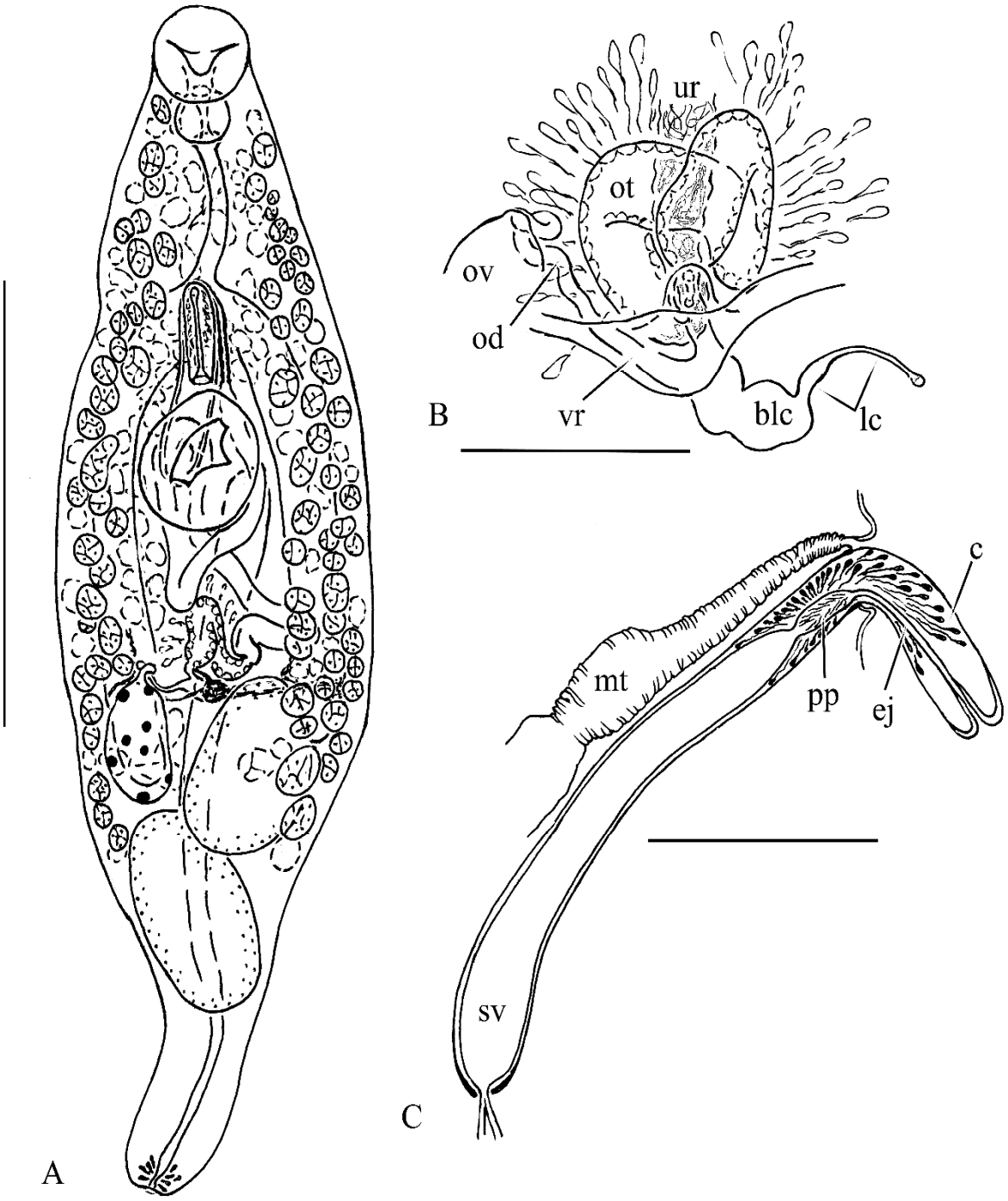

Figure 3. *Caudotestis* cf. *dobrovolski* Sokolov, Lebedeva, Shchenkov, and Gordeev, 2020 from intestine of Falcate snailfish *Careproctus cypselurus* (Jordan and Gilbert, 1898) (Liparidae), the Sea of Okhotsk. (**A**), whole mounted specimen, ventral view; (**B**), ovarian complex, ventral view; (**C**), terminal genitalia, lateral view. Abbreviations: blc, proximal dilation of Laurer's canal; c, evaginated cirrus; ej, ejaculatory duct; lc, Laurer's canal; mt, metraterm; od, oviduct; ot, oötype with Mehlis' gland cells; ov, ovary; pp, pars prostatica with field of prostatic cells; sv, seminal vesicle; ur, uterine seminal receptacle; vr, vitelline reservoir. Scale bars (**A**) = 1000 μm; (**B**,**C**) = 200 μm.

Description (based on 13 whole mount adult specimens—paragenophores). Body fusiform, length 1946–2933 (2374), maximum width 490–742 (661) in posterior quarter of anterior half or middle of body. Forebody 28.8–33.9 (32.3)% of body length. Tegument smooth. Oral sucker trapezoid or round, 159–228 × 180–256 (198 × 208). Ventral sucker

round to subglobular or almost trapezoid, sessile, 208–312 × 215–298 (266 × 258). Oral sucker to ventral sucker width ratio 1:1.13–1.37 (1:1.24). Mouth subterminal. Prepharynx absent. Pharynx 76–132 × 76–132 (108 × 113). Esophagus 235–381 (291) long. Intestinal bifurcation at about two-thirds to three-quarters of forebody. Caeca blind, terminate in second quarter of hindbody. Post-caecal region 33.1–40.4 (36.6)% of body length. Testes two, entire, occasionally indented, oval to ovoid or round, often oblique (in eight specimens), less frequent strictly or almost tandem (in five specimens), partly overlapping to contiguous or separated, in second and third quarters of hindbody and penetrating into posterior or anterior quarters; anterior testis sinistro-submedian to median, 291–450 × 222–298 (392 × 263), posterior testis dextro-submedian to median, 367–498 × 222–291 (442 × 255). Post-testicular region 5.6–16.3 (11.5)% of body length. Cirrus-sac elongate, narrow 415–623 × 69–104 (488 × 86), often reaches to third or posterior quarters or posterior margin of ventral sucker (nine specimens), sometimes to midlevel of sucker (in one specimen) or extends into hindbody up to distance equal to one-fourth of ventral sucker length (in three specimens), opens into common genital atrium; contains long, rectilinear seminal vesicle, tubular pars prostatica surrounded by field of prostate cells, ejaculatory duct and unarmed cirrus. Vas deferens absent; vasa efferentia directly joining seminal vesicle. Genital pore median, at level of intestinal bifurcation or just anterior to this level. Ovary entire, occasionally indented, oval to ovoid or pyriform, dextro-submedian, antero-dextral or strictly dextral to and separated from or contiguous with anterior testis, 201–312 × 125–159 (241 × 140). Oviduct relatively long, receives Laurer's canal and common vitelline duct and then forms oötype. Oötype long, tubular, twisted. Mehlis' gland extensive. Laurer's canal bulbous proximally, opens at level of ovary or slightly postovarian, postero-sinistral to oötype. Canalicular seminal receptacle absent. Uterine seminal receptacle evident. Uterus entirely pretesticular or extends posteriorly no further than midlevel of anterior testis. Metraterm tubular, muscular, runs sinistro-subdorsally to cirrus sac. Eggs deformed in balsam; length of least-deformed eggs 81–104 (89). Vitellarium follicular; follicles in two lateral fields extending from oral sucker or level of pharynx or level of anterior quarter of esophagus to level of anterior testis or level just posterior to anterior testis, overlap or encroach over ventral and dorsal surfaces of caeca, confluent dorsally along entire length of forebody or only as narrow band in space between intestinal bifurcation and ventral sucker. Common vitelline reservoir median, at level of ovary. Excretory pore terminal. Excretory vesicle claviform, extends to level of just posteriorly to posterior margin of ventral sucker or further anteriorly, but not further than midlevel of ventral sucker, posterior end surrounded by numerous glands.

Remarks.

Caudotestis was originally erected as a subgenus of *Lebouria* Nicoll, 1909 [14]. Yamaguti [61] elevated *Caudotestis* to the rank of genus but later downgraded it to a subgenus of *Plagioporus* Stafford, 1904 [62,63]. Bray [12], Gibson and Bray [64], and Cribb [7] recognized it as a distinct genus. To date, *Caudotestis* comprises eight species of opecoelid trematodes parasitizing marine fishes [18] (present study). According to WoRMS [52], this genus contains another 11 nominal species originally described in the subgenus *Caudotestis* of the genus *Plagioporus* or subsequently transferred to it: *Plagioporus* (*Caudotestis*) *azurionis* Yamaguti, 1951, *Plagioporus* (*Caudotestis*) *dorosomatis* Yamaguti, 1951, *Plagioporus* (*Caudotestis*) *fusiformis* (Price, 1934), *Plagioporus* (*Caudotestis*) *neopercis* (Yamaguti, 1938), *Plagioporus* (*Caudotestis) pachysomus* Manter, 1954, *Plagioporus* (*Caudotestis*) *parapercis* Yamaguti, 1959, *Plagioporus* (*Caudotestis*) *rhabdosargi* Wang, 1982, *Plagioporus* (*Caudotestis*) *seychellensis* Toman, 1992, *Plagioporus* (*Caudotestis*) *spari* Yamaguti, 1951, *Plagioporus* (*Caudotestis*) *trachuri* (Pogoril'tseva, 1954), *Plagioporus* (*Caudotestis*) *tyrrhenicus* Paggi and Orecchia, 1976. However, the inclusion of these species in the genus *Caudotestis* was entirely formal (based on raising the rank of the subgenus *Caudotestis* to the generic status), without a critical assessment of their morphology. None of these 11 species fit the modern diagnosis of *Caudotestis* (compare with Cribb [7]). Suffice it to mention that *P*. (*C*.) *azurionis, P.* (*C*.) *dorosomatis*, *P*. (*C*.) *fusiformis, P.* (*C*.) *neopercis*, *P*. (*C) pachysomus*, *P*. (*C*.) *parapercis*, *P*. (*C*.) *rhabdosargi, P.* (*C*.) *seychellensis*,

P. (*C.*) *spari* and *P.* (*C.*) *tyrrhenicus* have a canalicular seminal receptacle [62,65–71]. No information is available on the seminal receptacle in *P.* (*C.*) *trachuri*, but the fields of vitelline follicles extending nearly to the posterior end of the body are typical of this species [72]. Machida [73] considers *P.* (*C.*) *azurionis* as a member of *Neolebouria* Gibson, 1976.

Caudotestis cf. *dobrovolski* corresponds to the diagnosis of the genus *Caudotestis*: the cirrus-sac is elongated and narrow, the seminal receptacle is uterine and not canalicular, the ovary is entire or nearly so, the caeca are blind, the genital pore is median, the testes are oblique or tandem and not strikingly elongated, the fields of vitelline follicles end at the level of the anterior testis or so, the uterine coils do not pass posteriorly beyond the anterior testis level [7,8].

Caudotestis cf. *dobrovolski* is very similar to *C. dobrovolski* described from liparid and cyclopterid fishes in the environs of Simushir Island. In fact, these trematodes differ from each other only in size of gonads (length of the anterior and posterior testes and ovary as % of the body length, averaging 16.58, 18.7, and 10.18 vs. 22.21, 20.73, and 11.6, respectively), oral and ventral suckers (length of both sucker as % of the body length, averaging 8.40 and 11.23 vs. 10.93 and 12.53, respectively) and pharynx (length of the pharynx as % of the body length, averaging 4.55 vs. 6.44). Sequences of the 28S rRNA gene of *C.* cf. *dobrovolski* were similar to those of *C. dobrovolski* deposited in GenBank NCBI under accession numbers MN437379 and MN437380: p-distance 0–0.08%. Nevertheless, *C. dobrovolski* has a much longer branch than *C.* cf. *dobrovolski* on our tree (Figure 1)due to the noticeable differences in the sequences of the 18S rRNA gene in these species (Supporting Information, File S1). The difference in 18S rRNA gene sequences between *C.* cf. *dobrovolski* and *C. dobrovolski* is mostly determined by the presence of an extended gap in the interval of 1745–1796 bp of alignment in the second species. However, the 18S rRNA sequence of *C. dobrovolski* was obtained after long-term (more than three years) storage of its total DNA in an aqueous solution. This gap may be a consequence of a deteriorated quality of the DNA. The morphological similarity between *C.* cf. *dobrovolski* and *C. dobrovolski* combined with the low degree of their differences by 28S rRNA gene, the proximity of the localities of their findings and the phylogenetic affinity of the hosts seem to suggest that these trematodes are conspecific, but some doubts remain. So, we identified our specimens as *C.* cf. *dobrovolski* and provided the data on the nd1 gene sequences, which can be used in the future to reassess its taxonomic relationships relative to *C. dobrovolski*.

Six other congeners strikingly differ from *C.* cf. *dobrovolski* in the following features. *Caudotestis nicolli* Issaitschikov, 1928 and *Caudotestis ventichthysi* Bray, Waeschenbach, Dyal, Littlewood, and Morand, 2014 have smaller testes, which are comparable to or smaller than the ventral sucker [8,12,14]. Furthermore, the fields of vitelline follicles in *C. nicolli* are confluent dorsally in the hindbody [12,14]. In addition, *C. ventichthysi* has relatively shorter caeca (reach to the posterior margin of the ventral sucker) and a short excretory vesicle (reaches to the ovary), and a very large ventral sucker (slightly less than the maximum body width) [8]. *Caudotestis opisthorchis* (Poljansky, 1955) has a large oral sucker in comparison with the ventral sucker (1:0.73–0.89), a distinctly short excretory vesicle (reaches to the middle of posterior testis), and often tandem testes [12,60,74,75]. *Caudotestis patagonensis* Cantatore, Lancia, Lanfranchi, and Timi, 2012 has relatively short fields of vitelline follicles (never extending posteriorly to the anterior margin of the anterior testis), relatively short caeca (*idem*) and a short excretory vesicle (reaches to the ovary), but a relatively long cirrus sac (always extends into hindbody) [76]. *Caudotestis glacialis* (Zdzitowiecki, 1989) has a very long excretory vesicle (extends to the level between the posterior edge of the pharynx and the genital pore), always tandem testes, an ovary that is often comparable in length to the testes, and fields of vitelline follicles that are confluent dorsally in the hindbody [77]. *Caudotestis kerguelensis* (Prudhoe and Bray, 1973) has a shorter body (450–1830 μm), a smaller ovary (60–200 × 65–150) and a sinistro-submedian genital pore [78].

Genus *Hexagrammia* Baeva, 1965.

Hexagrammia zhukovi Baeva, 1965 Figure 4.

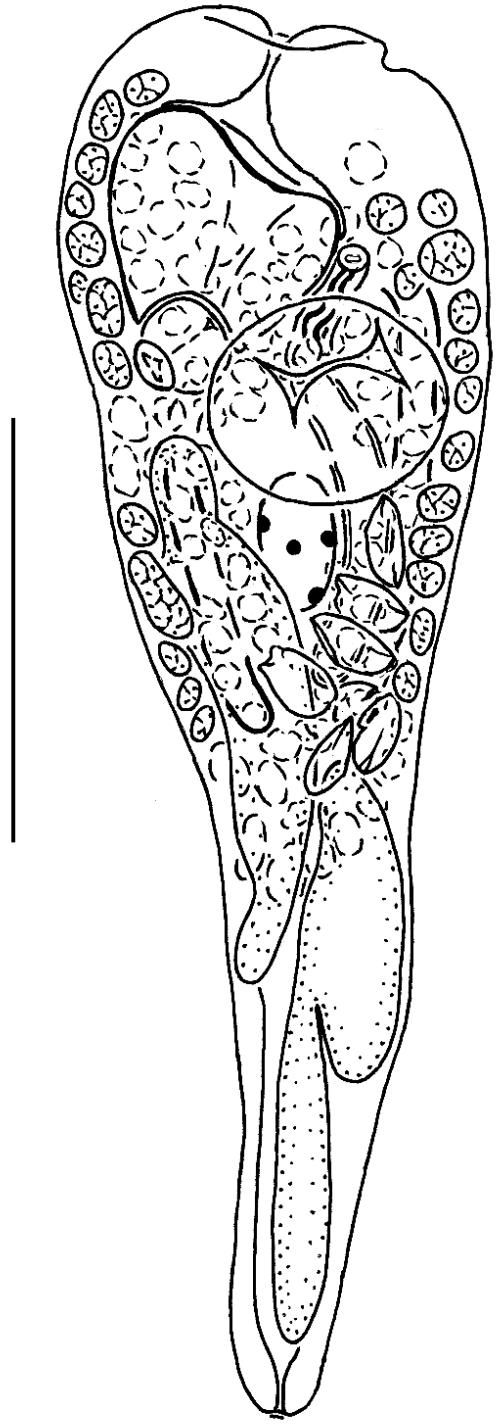

Figure 4. *Hexagrammia zhukovi* Baeva, 1965 from intestine of Rock greenling *Hexagrammos lagocephalus* (Pallas, 1810) (Hexagrammidae), the Sea of Okhotsk. Trematode specimen with invaginated oral sucker. Scale bar = 400 μm.

Type host: Atka mackerel, *Pleurogrammus monopterygius* (Pallas, 1810) (Perciformes: Hexagrammidae).

Other host (in present study): Rock greenling, *Hexagrammos lagocephalus* (Pallas, 1810) (Perciformes: Hexagrammidae).

Type locality: Avacha Bay, Northwestern Pacific.

Other locality: The coastal area of the south-western part of Iturup Island (the Sea of Okhotsk, Northwestern Pacific).

Site of infection: Intestine.

Prevalence and intensity infection: In one of five host specimens; five trematode individuals.

Specimens deposited: One paragenophore IPEE RAS 14321.

Sequences deposited: Partial sequences of the 18S rRNA and 28S rRNA genes from one *H. zhukovi* specimen are deposited in GenBank NCBI as OP094620 and OP094626, respectively.

Description (based on whole mount adult specimen with invaginated oral sucker and three badly damaged specimens—paragenophores): Body elongate, length of one entire specimen including invaginated oral sucker about 1456, maximum width 392. Tegument smooth. Oral sucker cup-shaped, 215–228 × 215–228 (222 × 222). Ventral sucker suboval, sessile, 142–201 × 180–222 (171 × 201). Oral sucker to ventral sucker width ratio 1:0.78–1.03 (1:0.91). Mouth terminal. Prepharynx absent. Pharynx 69–80 × 90–97 (74 × 93). Esophagus and intestinal bifurcation indistinct (not visible). Caeca blind, terminate in anterior third of hindbody. Testes two, exceptionally long and relatively narrow, rectilinear or curved, entire to slightly indented, occasionally distinctly indented, oblique, separated or slightly overlapped, left testis anterior to right testis or vice versa; anterior testis extends into ventral sucker area to level of posterior third or middle of sucker, 374–505 × 97–111 (427 × 102), posterior testis entirely in hindbody or also extends into ventral sucker area, 332–533 × 83–104 (427 × 92). Cirrus-sac elongate, narrow, 374–415 × 62 (395 × 62), extends into hindbody; contains long, rectilinear seminal vesicle, short pars prostatica surrounded by field of prostate cells, ejaculatory duct, and invaginated cirrus. Genital pore median, at some distance anteriorly to ventral sucker. Ovary entire, oval to pyriform, dextro-submedian, occasionally median, at level of anterior portion of anterior testis, extends into ventral sucker area, separated or partly overlapped by anterior or posterior testis 138–159 × 83–97 (152 × 90). Laurer's canal and oötype with Mehlis' gland not observed. Canalicular seminal receptacle absent. Uterine seminal receptacle evident. Uterus extends posteriorly into anterior third of hindbody. Metraterm not observed. Eggs operculate, with small knob at anopercular end in some specimens, deformed in balsam; length of least-deformed eggs 104–110 (106). Vitellarium follicular; follicles in two longitudinal lateral fields extend from oral sucker level to near midlevel of hindbody, overlap caeca dorsally, extracaecal ventrally except some follicles encroaching over ventral surface of caeca, confluent dorsally in forebody, anterior part or ventral sucker area and post-ovarian space of hindbody. Excretory pore terminal. Excretory vesicle observed only to midlevel of hindbody.

Remarks.

The studied trematode specimens from *H. lagocephalus* undoubtedly belong to *Hexagrammia*, as evidenced by the shape of the body, the oral sucker, the ovary, and the testes, the absence of the canalicular seminal receptacle, and a short caeca and fields of vitelline follicles [7]. Two currently known *Hexagrammia* spp., *H. zhukovi* and *Hexagrammia longitestis* Schell, 1973, can be differentiated only by the length of the body (1.7–2.8 vs. 0.8–1.3 mm) and the morphology of the eggs (length: 87–99 vs. 78–85 μm; knob at anopercular end: presence vs. absence) [13,58,79]. According to Schell [79], these two species can be differentiated by several more features related to the length of the fields of vitelline follicles, the cirrus sac and the position of the genital pore, but this assumption was made without taking into account the data published by Zhukov [58]. Our specimens are similar to *H. zhukovi.* Previously, this species was recorded in hexagrammids of the Northwestern Pacific, including *Hex. lagocephalus* [13,58]. *Myoxocephalus brandtii* (Steindachner, 1867) (Cottidae) has also been recorded as a host of *H. zhukovi* [58].

Superfamily Opecoeloidea Ozaki, 1925.

Family Zdzitowieckitrematidae fam. nov.

urn:lsid:zoobank.org:act:357137F5-0ACF-4AB7-B421-CD064BF4DF78

Diagnosis (based on diagnosis of *Zdzitowieckitrema* in [19]): Body foliate. Tegument unarmed. Oral and ventral suckers unspecialized. Prepharynx present. Intestine bifurcates

in forebody. Caeca blind, terminate posteriorly to testes but not near posterior end of body. Testes two, slightly oblique or nearly tandem, in hindbody. Cirrus-sac elongate and narrow. Internal seminal vesicle bipartite; pars prostatica tubular; cirrus unarmed. Genital pore slightly submedian. Ovary pretesticular. Muscular ovicapt large. Seminal receptacle canalicular. Uterus preovarian. Vitellarium follicular, in lateral fields, extends from level anterior to intestinal bifurcation to posterior end of body. Excretory vesicle I-shaped. Adults parasitize marine deep-sea fishes of the Muraenolepididae Regan, 1903; Antarctic. Type and only genus *Zdzitowieckitrema* Sokolov, Lebedeva, Gordeev, and Khasanov, 2019.

Remarks.

The newly described family differs from the Opecoelidae by the combination of the following features: an unspecialized oral sucker, two tandem testes, an entire ovary, an elongate and narrow cirrus-sac, the presence of the canalicular seminal receptacle, the fields of vitelline follicles extending nearly to the posterior end of body. As in the case of the stenakrines, each of the listed features may be present in members of different opecoelid genera (compare with [7]). The differences between the Zdzitowieckitrematidae fam. nov. and the Stenakridae are discussed above.

Superfamily Opecoeloidea Ozaki, 1925.

Family Opecoelidae Ozaki, 1925.

Subfamily Scorpidotrematinae subfam. nov.

urn:lsid:zoobank.org:act:7AB0D584-C54C-4081-9BDF-6B89DDDF9E26.

Diagnosis (based on diagnoses of *Scorpidotrema*, *Holsworthotrema*, *Neonotoporus*, and *Pseudopecoelina* in [7,9]): Body elongate. Tegument unarmed. Oral sucker unspecialized or distinctly funnel-shaped. Ventral sucker sessile or distinctly pedunculate. Pharynx present. Intestine bifurcates in forebody. Caeca terminate posteriorly to testes, blind or unite with excretory vesicle to form uroproct. Testes two, tandem, oblique or almost opposite, in hindbody. Cirrus-sac elongate to exceptionally long, convoluted or curved, narrow or relatively stout. Internal seminal vesicle unipartite; pars prostatica vesicular or indistinct; cirrus unarmed. Genital pore submedian to almost marginal. Ovary pretesticular. Ovicapt indistinct. Seminal receptacle uterine. Uterus strictly preovarian or passes posteriorly as far as posterior testis. Vitellarium follicular, in lateral fields, restricted to hindbody or enters forebody, extends to posterior end of body. Excretory vesicle I-shaped. Adults parasitize marine fishes of many families; cosmopolitan. Type genus *Scorpidotrema* Aken'Ova and Cribb, 2003.

Other genera: *Holsworthotrema* Martin, Huston, Cutmore, and Cribb, 2019, *Neonotoporus* Srivastava, 1942, *Pseudopecoelina* Yamaguti, 1942.

Remarks.

The Scorpidotrematinae subfam. nov. differs from all other opecoelid subfamilies by the combination of the presence of the cirrus-sac and the absence of the canalicular seminal receptacle (compare with [3,4,7,9,11]).

4. Discussion

The data obtained in this study support our earlier conclusion [18] that the Stenakrinae are polyphyletic, because *Biospeedotrema*, *Caudotestis*, *Hexagrammia*, and *Stenakron* branched outside the Opecoelidae (Figure 1), whereas *Holsworthotrema* and *Scorpidotrema* formed a separate subclade within the Opecoelidae. In previous reconstructions of the phylogeny of xiphidiate digeneans based on 28S or 18S rRNA gene sequences and taking into account *Caudotestis* and/or *Biospeedotrema* spp., the lineage of these *Stenakron*-like digeneans resolved as sister to *Zdzitowieckitrema* [18,19] or occupied a separate position within the Xiphidiata [2]. However, in the latter case, the authors [2] did not take *Zdzitowieckitrema* into account. The results of our phylogenetic analyses clearly demonstrate the sister relationship of *Zdzitowieckitrema* with the large clade that includes both the clades of the Stenakridae (*Biospeedotrema*, *Caudotestis*, *Hexagrammia*, and *Stenakron*) and the Opecoelidae. *Zdzitowieckitrema*, the Stenakridae clade and the Opecoelidae clade are characterized by long branches, probably reflecting the long evolutionary separation of these trematodes.

Morphological autapomorphies of the Stenakridae clade at the level of adult worms are not obvious. However, *Biospeedotrema*, *Caudotestis*, *Hexagrammia,* and *Stenakron* have a combination of features that distinguish them from all opecoelids. *Zdzitowieckitrema* is morphologically distinct from these genera (e.g., by the presence of the canalicular seminal receptacle). At the same time, the distinction of *Zdzitowieckitrema* from opecoelids is not strong enough, as evidenced by the large number of characters necessary to tell them apart. The current biological classifications are seriously flawed because they fail to standardize criteria for taxonomic ranking [80]. In this regard, the choice of the rank of basal groups (family/subfamily) is largely subjective. We believe that early branching and late-diverging lineages must have a different taxonomic rank. As a result, we propose to consider the group of genera *Biospeedotrema*, *Caudotestis*, *Hexagrammia*, and *Stenakron* as the family Stenakridae stat. nov., and erect the Zdzitowieckitrematidae fam. nov for the genus *Zdzitowieckitrema.* The Zdzitowieckitrematidae fam. nov. and the Stenakridae stat. nov., as well as the Opecoelidae, are representatives of the Opecoeloidea. To note, the idea of the family status of the Stenakrinae was first proposed by Bray et al. [11] but could not be implemented at that time due to the lack of molecular data for *Stenakron*. In this study we finally filled this gap. Our analyses indicates the monophyly of stenakrids with an entire ovary (*Biospeedotrema*, *Caudotestis*, *Hexagrammia*) and the presence of a nearest common ancestor for *Biospeedotrema* and *Hexagrammia*.

Cercarial morphology might reflect phylogenetic relationships and evolutionary history of trematodes better than adult morphology alone [81]. Among stenakrids, the life cycle has been described only for *S. vetustum* [82,83]. Its cercariae localized in the sporocysts; the larvae have a short tail (the Microcercous cercariae) and a thin-walled excretory vesicle, and are devoid of the stylet [18,83]. These cercariae differ significantly from both the cotylomicrocercous (typical of most opecoelids) and "long-tail" (described only in *Helicometra gibsoni* Meenakshi, Madhavi, and Swarnkumari, 1993 see [84]) cercariae of the opecoelids at least in the morphology of the excretory vesicle: thin-walled vs. thick-walled. In addition, the cercariae of *S. vetustum* differ from most opecoelid cercariae in the absence of the stylet. Cercariae devoid of the stylet have only been described in two opecoelid species, *Plagioporus sinitsini* Mueller, 1934 (Plagioporinae) and *Choerodonicola arothokoros* Martin, Cribb, Cutmore, and Hutson, 2018 (Clade C) [38,85]. However, the cercariae of these species, in contrast to those of *S. vetustum*, have a thick-walled excretory vesicle typical of the opecoelid cercariae. The lack of data on the fine morphology of the tail of the cercariae of *S. vetustum* does not allow a detailed comparison of this organ with the cup-shaped tail of opecoelid cotylomicrocercous cercariae. With some degree of probability, it can be assumed that the morphological type of cercariae typical for *S. vetustum* is also typical for other stenakrid digeneans. The differences between the stenakrids and the opecoelids in the cercarial morphology probably indicate a strong divergence of these opecoeloid groups in the larval phase of development of their hermaphroditic generation.

The life history of the only member of the Zdzitowieckitrematidae, *Z. incognitum,* is unknown. As noted above, the Zdzitowieckitrematidae has insubstantial morphological difference from the Opecelidae, in contrast to the relatively large genetic distinction between these digenean groups. However, the basal position of the Zdzitowieckitrematidae in relation to both opecoelids and stenakrids allows us to hypothesize that the strong morphological differences between the Zdzitowieckitrematidae and the Opecoelidae may become evident in their cercariae when studied. Overall, considering the totality of the arguments, we can conclude that the phylogenetic evidence for our updated concept of the Opecoeloidea is the most compelling.

Based on the above circumstances, we erected the Scorpidotrematinae subfam. nov. for the rest of the former stenakrines. One of the genera of this subfamily, *Holsworthotrema*, does not form a monophyletic assemblage in our phylogram. However, taking into account the low support of the relevant key node, we refrain from making taxonomic decisions on this issue. According to our earlier data based on the analysis of 28S r RNA gene sequences, the Scorpidotrematinae subfam. nov. (=Stenakrinae sensu Martin, Huston, Cutmore,

and Cribb, 2019) has phylogenetic affinity to the opecoelid CladeD, which contains the genera *Abyssopedunculus* Martin, Huston, Cutmore, and Cribb, 2019, *Mesobathylebouria* Martin, Huston, Cutmore, and Cribb, 2019 and *Tellervotrema* Gibson and Bray, 1982 [6,86]. Considering that the canalicular seminal receptacle is typical both for the basal family of the Opecoeloidea (Zdzitowieckitrematidae) and for the basal subfamily of the Opecoelidae (Helicometrinae), we suggest that the absence of this organ in some opecoelids, in particular scorpidotrematines, is the result of a secondary loss. The same hypothesis applies to the stenakrids.

The topology of our ML and BI trees allows us to touch on another taxonomic problem of Xiphidiata. The results of our analysis suggest that members of the Haploporoidea branch belong within the Xiphidiata clade. This hypothesis agrees with the findings of Olson et al. [22] and Litlewood et al. [1] that the Haploporoidea belongs to the Xiphidiata. Pérez-Ponce de León and Hernández-Mena [2], on the basis of phylogenetic results derived from the analysis of 28S rDNA gene sequences, considers haploporoids within the concept of the suborder Haploporata. However, it appears that the systematic position of the Haploporoidea needs to be clarified.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14110949/s1, Table S1. Trematode species involved in phylogenetic analyses. File S1. Phylogenetic data matrix in NEXUS format.

Author Contributions: Conceptualization, morphological study, interpretation of phylogenetic data, and writing—original draft preparation, S.G.S.; samples collection, I.I.G. and E.V.F.; phylogenetic analyses, S.V.S.; writing—editing, S.G.S. and I.I.G. All authors have read and agreed to the published version of the manuscript.

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