

Research Article

New Genus and Two New Species of Notocotylidae Lühe, 1909 (Digenea), from Russia: Morphomolecular Data

Anna V. Izrilskaia ^{1,2}, Yulia V. Tatonova ¹ and Yulia V. Belousova ^{3,4}

¹Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch, Russian Academy of Sciences, 100-letiya Street, 159, Vladivostok 690022, Russia

²Far Eastern Federal University, School of Medicine and Life Sciences, FEFU Campus, 10 Ajax Bay, Russky Island, Vladivostok 690922, Russia

³A. O. Kovalevsky Institute of Biology of the Southern Seas, Russian Academy of Sciences, Leninsky Avenue, 38, Moscow 119991, Russia

⁴Research Center of Freshwater and Brackish-Water Hydrobiology, Maria Fortus Street, 87, Kherson 273003, Russia

Correspondence should be addressed to Anna V. Izrilskaia; anna.kharitonova92@yandex.ru

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The study of the trematodes of the family Notocotylidae Lühe, 1909, is continued using an integrated approach based on new data. A new genus, *Pseudonotocotylus* n. gen., has been identified, and two species new to science, *Notocotylus multipapillus* n. sp. and *Pseudonotocotylus martynenkoi* n. sp., have been discovered. The life cycle and morphology of developmental stages of the new species are described, and the molecular data for nuclear (28S) and mitochondrial markers (*cox1* and *nad1*) are presented. Sequences of *cox1* and *nad1* mtDNA genes were obtained for five previously studied notocotylid species for the first time. Phylogenetic relationships within Notocotylidae were inferred from the analysis of these markers, and the results obtained reveal some subtle differences in some of the notocotylid species despite their morphological similarities. Based on the differences in gene sequences among the species, two additional criteria are proposed to divide the genus *Notocotylus* into *Notocotylus* and *Pseudonotocotylus* n. gen. The criteria are based on, first, having the Pulmonata or Prosobranchia group of snails as the first intermediate host and, second, molecular properties, as shown by substitutions specific to genus at various nucleotide locations in alignments for 28S and *cox1*.

1. Introduction

Species of the trematode family Notocotylidae Lühe, 1909, are distributed across all continents and frequently occur as components of the helminth fauna, mainly of waterfowl and rodents. Most notocotylids have a life cycle with two hosts. Their cercariae form metacercariae on the substrate after leaving or not leaving the snails. A second life cycle option has been reported for *Pseudocatropis joyeuxi* Kanev and Vassilev, 1986, and *Pseudocatropis dvoryadkini* Izrilskaia et al., 2019 [1, 2]. The family currently comprises 14 genera [1–3], but molecular data in NCBI are available for only a limited number of species from nine genera: *Notocotylus* Diesing, 1839; *Catropis* Odhner, 1905; *Pseudocatro-*

pis Kanev and Vassilev, 1986; *Quinqueserialis* (Skvorzov, 1934); *Ogmogaster* Jägerskiöld, 1891; *Ogmocotyle* Skrjabin and Shulz, 1933; *Hippocrepis* (Diesing, 1850); *Tristriata* Belopolskaia in Skrjabin, 1953; and *Paramonostomum* Lühe, 1909.

As noted in recent publications based on molecular studies and analysis of phylogenetic relationships within the Notocotylidae, using only morphological criteria is not enough to distinguish between the genera of the family [2, 4]. Furthermore, data on the morphology of the developmental stages and the molecular genetics of the worms in this family should be obtained in order to properly clarify the differences in the taxonomy and phylogenetic connections of Notocotylidae [2]. In that article, the authors proved the independence of the genus *Pseudocatropis* on the basis

of molecular data. However, information on the surface structures on the ventral side of the body, or the morphology of mature worms, suggested that *P. joyeuxi* belongs in the genus *Catatropis*.

Currently, molecular data for some of Notocotylidae species are presented in NCBI, whose taxonomic status has been determined only by to the genus or family taxonomic level. Moreover, there are a number of others whose species identification has not been confirmed by morphological data. In most cases, nuclear markers such as a fragment of the 28S rRNA gene (28S) are used to determine the species status of worms and to analyze their evolutionary relationships. Mitochondrial marker data for Notocotylidae are scarce, which makes it difficult to use them for the above purposes. A greater array of available data on this marker and the use of it along with nuclear markers can help efficiently resolve a number of taxonomic, systematic, and phylogenetic problems in the family [2, 4, 5].

Based on the findings of this study, we propose a new genus and species within the family *Notocotylidae*. The criteria thus include the life cycles and developmental stages, the nuclear and mitochondrial markers, and the assessment of these markers to ascertain the evolutionary links of the species.

2. Materials and Methods

2.1. Life Cycle and Morphology of Worms. The material for this study was adult worms retrieved from the caeca of the intestines of naturally infected Caspian gulls (*Larus cachinnans* Pallas, 1811) that were caught in the area of the Chushka Spit (Kerch Strait, Black Sea) in 2018. Six worms with morphological feature characteristic of Notocotylidae species were identified. Among these worms, three individuals were found to be representatives of a single species on the basis of their surface gland structure, and three were representatives of another species. In addition, cercariae and metacercariae of Notocotylidae were obtained from river nerites *Theodoxus fluviatilis* (Linnaeus, 1758) collected in the Black River (Khmelnyskiy Village, Crimean Peninsula).

Furthermore, several snails, *Helicorbis suffunensis* Starobogatov, 1957, and *Bithynia fuchsiana* (Möllendorff, 1888), that emitted monostome cercariae, were collected from waterbodies in the Russian Far East (Russky Island, city of Vladivostok) and in Vietnam (Nam Dinh Province), respectively. Metacercariae were obtained from each of the snails separately. Metacercariae reared from cercariae from *H. suffunensis* were fed to three ducklings, *Anas platyrhynchos* (50 cysts per animal), and those from *B. fuchsiana* were fed to one duckling (also 50 cysts). At 16 days postinfection, the caeca of ducklings were examined under a stereomicroscope for the presence of adult parasites.

Morphological measurements were made with traditional morphometric techniques. The morphology of redia, cercariae, and metacercariae was examined on live specimens. The sporocysts and metacercariae were measured live; the cercariae were fixed in a hot 4% formalin solution before measurements. Adult trematodes were fixed in 70% ethanol and then transferred to 96% ethanol for storage. Whole

mounts of adult specimens were prepared by staining with alum carmine and then dehydration in a series of graded ethanol, clearing in clove oil, and embedding in Canada balsam [6]. All measurements are given in micrometers (μm).

Euthanasia of all the animals was carried out in accordance with the decision of the Committee on the Ethics of Animal Experiments, Federal Scientific Center of East Asia Terrestrial Biodiversity (FSCEATB), Far Eastern Branch, Russian Academy of Sciences (FEB RAS) (Permit No. 1 of April 25, 2022).

2.2. Molecular Data

2.2.1. DNA Extraction, Amplification, and Sequencing. For molecular genetics, adult worms from naturally infected *L. cachinnans*, adult worms reared experimentally, and cercariae from *T. fluviatilis* were utilized.

In addition, the previously obtained DNA from *Notocotylus magniovatus* Yamaguti, 1934; *Catatropis vietnamensis* Izraïlskaia et al., 2019; and *P. dvoryadkini* [2, 7], deposited in the collection of the FSCEATB FEB RAS, were also used. Moreover, adults of *Notocotylus intestinalis* Tubangui, 1932, experimentally obtained from snails *Parafossarulus striatulus* Benson, 1842, in a previous study [7] were used for genetic analysis.

Genomic DNA was extracted from individual trematodes by the HotSHOT technique [8]. Polymerase chain reaction (PCR) was used to amplify partial sequences of the *cox1* and *nad1* genes of mitochondrial DNA, as well as partial sequences of the nuclear DNA 28S, using specific primers (see Table 1). The annealing temperatures for the 28S, *cox1*, and *nad1* markers were 55, 50, and 48°C, respectively. The efficiency and contamination of PCR were tested by setting positive and negative controls, respectively. The PCR products were sequenced by the Sanger method using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA). Nucleotide sequences were determined on an ABI 3500 genetic analyzer (Applied Biosystems, USA) at the FSCEATB FEB RAS, Russia. Both external and internal primers were used for sequencing (see Table 1).

2.2.2. Analysis of Genetic Data. Processing and alignment of consensus sequences were carried out using the FinchTV 1.4 and MEGA 5.0 software [9]. Sequences that were short in length were removed from the analysis. After alignment, the sequences 1,009, 512, and 381 bp of the 28S, *cox1*, and *nad1* markers, respectively, were analyzed. The *p*-distances between and within the species were analyzed in the MEGA software without including indels.

Phylogenetic relationships within the family Notocotylidae were assessed independently for all markers using newly obtained sequences and data for all species available in GenBank. In addition, the combined tree was constructed based on the 28S and *cox1* sequences. Members of the trematode families located left (basal) of the Notocotylidae in the phylogenetic tree inferred by [10] from nucleotide sequences of the 28S and 18S rRNA genes were selected as an outgroup in our study. The list of samples used in the study is provided in Tables S1–S3 (Supplementary Materials).

TABLE 1: List of primers for amplification and sequencing.

DNA region	Primer	Sequence 5' → 3'	Direction	Reference
28S	digl2	AAGCATATCACTAAGCGG	Forward, external	[13]
	1500R	GCTATCCTGAGGGAACTTCG	Reverse, external	[13]
	900F	CCGTCTTGAAACACGGACCAAG	Forward, internal	[13]
	1200R	CTTGGTCCGTGTTTCAAGACGGG	Reverse, internal	[13]
cox1	JB3	TTTTTTGGGCATCCTGAGGTTTA	Forward, external	[14]
	JB4.5	TAAAGAAAGAACATAATGAAAATG	Reverse, external	[15]
nad1	NDJ11	AGATTCGTAAGGGGCCTAATA	Forward, external	[14]
	NDJ2A	CTTCAGCCTCAGCATAAT	Reverse, external	[16]

Phylogenetic relationships were reconstructed using the Bayesian algorithm in the MrBayes 3.1.2. program [11] by applying a model selected as optimal on the basis of the Akaike information criterion (AIC) in the jModelTest 2.1.7 program [12]: GTR+I+G for 28S, GTR+I+G for *cox1*, TIM1+G for *nad1*, and TVM+I+G for the two genes (28S and *cox1*). The method of *a posteriori* probabilities was used for the trees constructed by the Bayesian algorithm. In the Bayesian analysis, 300,000, 3,600,000, 300,000, and 300,000 generations of the Markov chain Monte Carlo (MCMC) posterior third part were simulated for 28S, *cox1*, *nad1*, and 28S+*cox1*, respectively. The number of generations was determined to be sufficient since the SD value calculated was <0.01. The chain was sampled every 100 generations. Of the samples assessed, 25% was excluded to permit the construction of consensus trees. In the phylogenetic reconstruction based on the *nad1* marker, besides the sequences we obtained, two samples from the genus *Ogmocotyle* and sequence MT560390 for *Pseudonotocotylus intestinalis* were also represented; for this reason, this reconstruction was not used in the analysis, but we added it to Supporting Materials (see Figure S1).

3. Results and Discussion

3.1. Characteristics of *Notocotylus Sensu Lato*. In the phylogenetic reconstruction based on the 28S marker (see Figure 1), inferred from new data for *Notocotylus* species, worms that are morphologically identical in the structure of the ventral glands (attributed to the genus *Notocotylus*) appear in different clusters (cluster 1 and cluster 2), where these worms form groups isolated from other representatives in these clusters (group A (except *Notocotylus chionis* Baylis, 1928, and *Notocotylus fosteri* Kinsella and Tkach, 2005) and group B). The distances between these clusters are at the intergeneric level (3.06%). The division of *Notocotylus* into two groups on the basis of the nuclear marker and the failure to distinguish the genera of Notocotylidae, at least *Notocotylus* and *Catatropis*, on the basis of the structures on the ventral side were noted previously in other studies [2, 4]. The data obtained in the present study for the mitochondrial marker *cox1* confirm the division of *Notocotylus* representatives into two groups (see Figure S2). The distribution of species on tree based the combined 28S and *cox1* sequences replicates clusters on the phylogenetic reconstructions using

separate markers (see Figure 2). Moreover, the nucleotide sequences of *Notocotylus* representatives have unique substitutions shared by each group for both markers (see Figures S3-S4, Supplementary Materials), which is another argument in favor of generic differentiation of individuals in these groups. Thus, species of *Notocotylus* can be combined into a paraphyletic group, *Notocotylus sensu lato*.

Furthermore, worms combined into group A use in their life cycles Pulmonata snails as intermediate hosts, and those combined into group B use Prosobranchia snails (see Figure 1), which also may be evidence of their generic independence. This differentiation was also previously noted [17].

In the phylogenetic reconstruction based on the nuclear markers, the AF184259 sequence of the species *Notocotylus attenuatus* (Rudolphi, 1809) is included in group B. However, only molecular data are available for this worm, and its belonging to the species under consideration is not confirmed by any morphological characteristics of developmental stages, including adult worms. Nevertheless, *N. attenuatus* develops using Pulmonata snails [18, 19]. With a high degree of probability, the AF184259 sequence, assigned to this species and included in group B, refers to a species developing using Prosobranchia snails, and it does not actually belong to *N. attenuatus*. Based on the above-considered molecular differences between the worms of groups A and B, as well as on the involvement of first intermediate hosts from distant taxonomic groups in their life cycles, we consider the worms from groups A and B to be representatives of different genera. Taking into account that the type species develops using Pulmonata snails, we leave this priority name to the individuals included in group A of cluster 1 and assign the name *Pseudonotocotylus* n. gen. to the individuals comprising group B of cluster 2, including the AF184259 sequence, which we assigned as *Pseudonotocotylus* sp. Below are the keys for differentiating these genera.

3.2. Keys to the Differentiation of the Genera *Notocotylus* and *Pseudonotocotylus* n. gen. Based on the results of the studies and the available data on the morphology and molecular characteristics of members of the Notocotylidae, we have proposed the criteria for differentiating the species of *Notocotylus* and *Pseudonotocotylus* n. gen.: features of nucleotide sequences and intermediate hosts belonging to the group of Pulmonata or Prosobranchia snails. Molecular characteristics, expressed

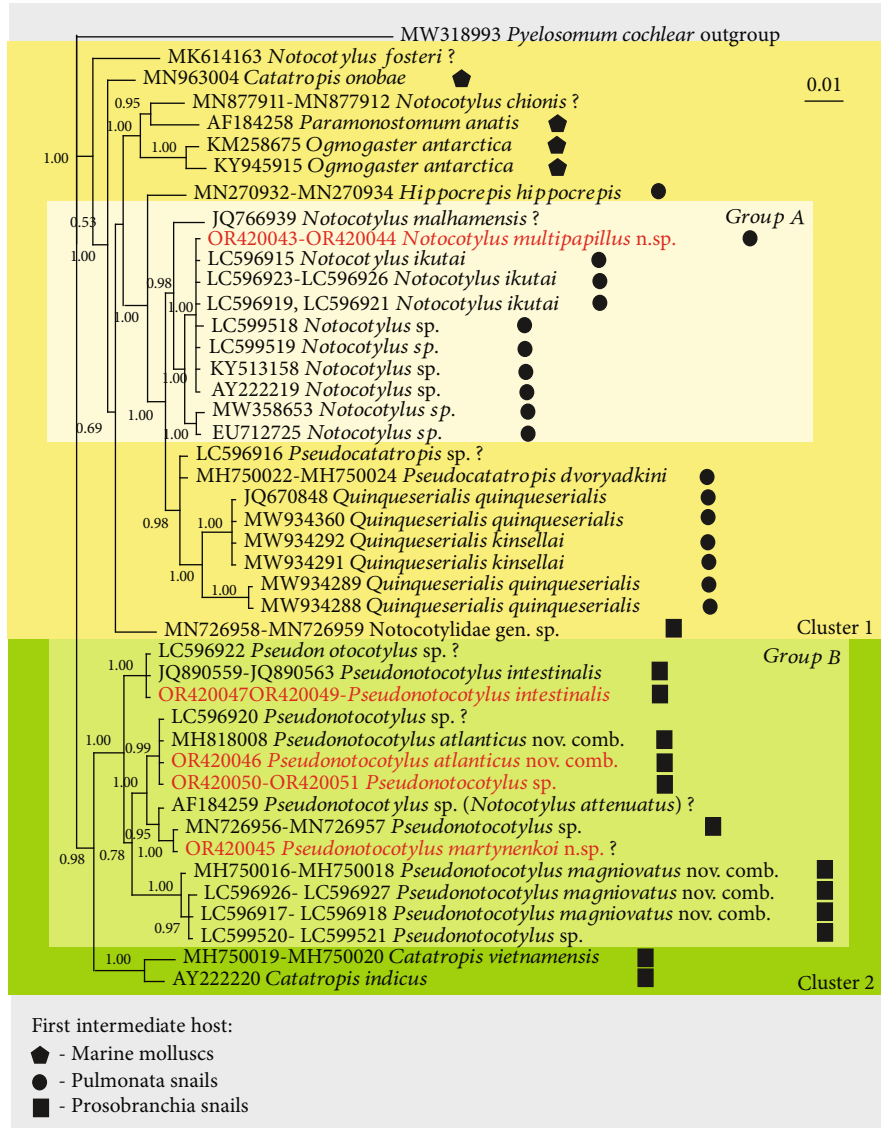


FIGURE 1: Phylogeny of the family Notocotyliidae based on 28S sequences using the Bayesian algorithm. A posterior probability of ≥ 50 was shown in the nodes. The scale bar indicates the number of substitutions per site. The sequences obtained in this study are highlighted in red. The outgroup species are in Table S1 (Supplementary Materials).

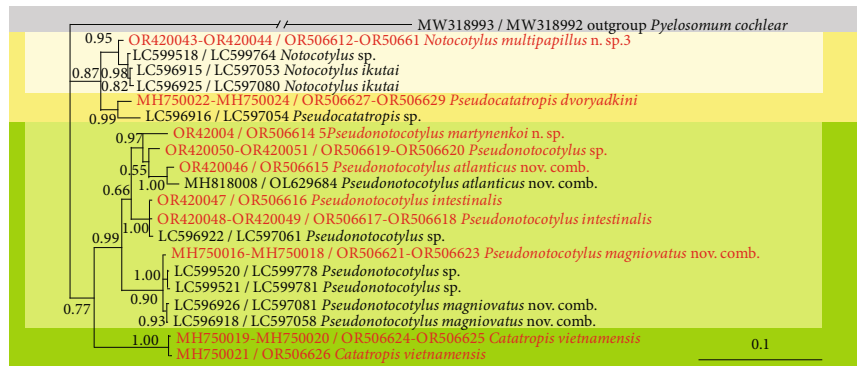


FIGURE 2: Phylogeny of the family Notocotyliidae based on the combined sequences of 28S and *cox1* using the Bayesian algorithm. A posterior probability of ≥ 50 was shown in the nodes. The scale bar indicates the number of substitutions per site. The sequences obtained in this study are highlighted in red. The outgroup species are in Tables S1 and S2 (Supplementary Materials).

in terms of genus-specific substitutions at different nucleotide positions in alignments for 28S and *cox1*, are presented in the following format: N_s , where N is the nucleotide and s is the site of this nucleotide (A is adenine, T is thymine, G is guanine, and C is cytosine) (see Figures S3-S4 and alignments for all three markers (28S, *cox1*, and *nad1*) (Supplementary Materials)).

- (i) *Notocotylus*: intermediate hosts, Pulmonata snails; molecular characteristics, C₃₀₄ for 28S and A₁₆₂ for *cox1*

Type species: *N. attenuatus*.

The genus includes species with confirmed identification and molecular data: *Notocotylus malhamensis* Boyce, Hide, Craig, Harris, Reynolds, Pickles, and Rogan, 2012; *Notocotylus ikutai* Sasaki, Kobayashi, Yoshino, Asakawa, and Nakao, 2021; and *Notocotylus multipapillus* n. sp.

In addition to the above-listed species, the genera include unidentified species with the following accession nos.: LC599518–LC599519, KY513158, AY22219, MW358653, and EU712725.

- (ii) *Pseudonotocotylus* n. gen.: intermediate hosts, Prosobranchia snails; molecular characteristics, G₄₁₆C₆₀₆G₆₁₅C₆₂₄ for 28S and A₆T₃₂₁ for *cox1*

Type species: *Pseudonotocotylus intestinalis* (syn.: *N. intestinalis*).

The genus includes species with confirmed identification and molecular data: *Pseudonotocotylus martynenkoi* n. sp., *Pseudonotocotylus atlanticus* (Stunkard, 1966) nov. comb., and *Pseudonotocotylus magniovatus* (Yamaguti, 1934) nov. comb.

In addition to the above-listed species, the genera include unidentified species with the following accession nos.: LC596922, LC596920, AF184259, MN726956–MN726957, LC599520–LC599521, and OR420050–OR420051.

Remarks: according to one of the above criteria, namely, circulation using snails from different ecological groups, the following notocotylids can be classified into genera:

Notocotylus: *N. ephemera* (Nitzsch, 1817) Harwood, 1939; *N. noyeri* Joyeux, 1922; *N. pacifier* (Noble, 1933) Harwood, 1939; *N. ralli* Baylis, 1936; *N. regis* Harwood, 1936; *N. urbanensis* (Cort, 1914) Harrah, 1922; *N. stagnicolae* Herber, 1942; and *N. thienemanni* Szidat and Szidat, 1933.

Pseudonotocotylus: *P. imbricate* (Looss, 1893) Szidat, 1935; *P. parviovatus* Yamaguti, 1934; *P. zduni* Tschiberaschwili and Dzavelidze, 1968; and *P. chinosis* Baylis, 1928.

However, the second criterion (molecular data) for these species is missing; therefore, the assignment of these parasites to any taxonomic group according to the proposed classification is premature. Moreover, in the absence of molecular data, it is also necessary to clarify the species of the listed worms.

3.3. Morphological and Genetic Features of Members of the Family Notocotylidae

3.3.1. *Pseudonotocotylus* n. gen

(1) **Diagnosis.** Body elongated, flat, with narrowed anterior and rounded posterior ends. Tegument with spines. Papillae from three discrete rows. Oral sucker small, subterminal.

Caeca simple, terminating more medially to testes without uniting. Testes lobed, symmetrical, extracaecal, at posterior end. External seminal vesicle tubular, curved. Cirrus sac median, claviform, slender anteriorly. Cirrus smooth. Genital pore immediately postbifurcal. Ovary lobed, intercaecal, intertesticular. Mehlis' gland median, preovarian. Uterus coils extending from ovary to cirrus sac. Metraterm shorter than cirrus sac. Vitelline follicles form lateral rows, pretesticular, sometimes reaching equator of body. Molecular characteristics: G₄₁₆C₆₀₆G₆₁₅C₆₂₄ for 28S and A₆T₃₂₁ for *cox1*. Adult worms inhabit the intestines of birds. Cercariae develop in freshwater Prosobranchia.

Etymology: due to the morphological similarity of adult trematodes from the above-described genus with those from the genus *Notocotylus*, the new genus was given the name *Pseudonotocotylus* n. gen. ZooBank number 2458A141-AF78-402F-9179-00472705B9FF.

Pseudonotocotylus intestinalis (Tubangui, 1932)

A total of 16 specimens of notocotylids were recovered from the caeca of duckling 16 days after the experimental infection with metacercariae, obtained from cercariae emitting from *B. fuchsiana*.

Host: *Gallus gallus* dom. [7], *Anas platyrhynchos* dom. (experimentally, this study).

Site: caeca.

Intermediate host: *Parafossarulus striatulus* (Bithyniidae Gray, 1857) [7] and *Bithynia fuchsiana* (Bithyniidae Gray, 1857) (experimentally, this study).

Locality: Nam Dinh Province, Vietnam (20° 09' N, 106° 17' E).

Remark: for *P. intestinalis*, one nucleotide sequence of each marker was obtained from the trematode isolated from *B. fuchsiana* and two from the trematodes isolated from *P. striatulus*. The individuals from different first intermediate hosts differed in both mitochondrial markers: two substitutions in *cox1* (0.5%), of which one was nonsynonymous and one synonymous for *nad1* (0.2%). No differences were found for the nuclear marker.

Pseudonotocotylus martynenkoi n. sp. Izrailskaia and Tatonova, 2023.

Definitive host: *Larus cachinnans* (naturally infected).

Site: caeca.

Type-locality: Chushka Spit (Kerch Strait, Black Sea); 45° 20' N, 36° 40' E.

Type-deposition: holotype No. 229-Tr; paratype No. 230-Tr. This material is deposited in the parasitological collection of the zoological collection (FSCEATB FEB RAS, Vladivostok, Russia) (e-mail: petrova@ibss.dvo.ru). Deposited November 22, 2021.

Etymology: the species epithet is to honor Igor Mikhailovich Martynenko, deceased (February 15, 1986, to November 8, 2021), who presented these worms. ZooBank number 5A8C1FE2-2D33-4ADB-8100-4BA17E386C67.

Adult worm (material examined: two specimens) (see Figures 3(a) and 3(b) and Table 2): body flat, elongated, ventrally concave, with tapered anterior and rounded posterior ends. Covered by spines to the middle of vitelline fields.

Ventral papillae arranged in three rows, with 13 papillae in each. Anterior papilla in lateral rows located at level of middle cirrus sac, and posterior papilla at level of anterior margin of testes. First papillae in median row located posteriorly of genital pore; posterior papillae in median row, anteriorly of ovary. Oral sucker subterminal; esophagus short; caeca extending laterally to uterine coils, between ovary and testes, and ending blindly at level of posterior margin of testes. Ventral sucker absent. Testes at posterior end of body symmetrical, deeply lobed on external margin. External seminal vesicle reaches posterior third of body. Anterior part of external seminal vesicle coiled; posterior part elongated. Cirrus sac elongated, narrowed anteriorly, and roundly expanded posteriorly, containing curved seminal vesicle, short prostatic, and cirrus with very small spines. Genital pore median, located immediately posteriorly of intestinal bifurcation. Ovary intertesticular, consisting of irregular lobes. Mehli's gland preovarian. Uterus forming 12–16 coils; metratrum reaching 1/3 length of cirrus sac. Vitellarium consisting of irregular follicles, extracaecal, located in the posterior half of body. Vitellarium anteriorly reaches level of 5th–6th uterine coils, with posterior part lying at level of anterior margins of testes. Eggs oval, smooth, operculate, with two polar filaments.

Remark: the trematode found in *L. cachinnans* shows the greatest morphological similarity to *Notocotylus seineti* Fuhrmann, 1919, whose type locality is Switzerland, and also has morphological characteristics close to those of *Notocotylus urbanensis* (Cort, 1914) from the United States. The similarity is in the number of ventral papillae in both cases. The formula of papillae for *N. seineti*, according to Fuhrmann (1919), is 12 (12) 12. The formula for *N. urbanensis*, according to Harrah (1922), is 13–14 papillae in each row. However, Herber (1935) indicates a significantly larger number of papillae for *N. urbanensis*: 15–19 (13–18) 15–19 [19]. In the publication of [19], information on the taxonomy of worms is rather contradictory, which requires additional research. Despite the existing uncertainty as regards the taxonomy of these notocotylids, the worms in our material differ from *N. seineti* and *N. urbanensis* in the location of papillae: the papillae of the lateral rows are located closer to the anterior end of the body than the papillae of the median row (in our worm) vs. the papillae of the median row are closer to the anterior end of the body than the lateral papillae (in *N. seineti* and *N. urbanensis*). Based on the above facts, we have described here this notocolid as a new species.

Pseudonotocotylus atlanticus (Stunkard, 1966) nov. comb.

Definitive host: *Larus cachinnans* (naturally infected).

Site: caeca.

Locality: Chushka Spit (Kerch Strait, Black Sea); 45° 20' N, 36° 40' E.

Remark: the adult worms that we obtained from *L. cachinnans* are identical in morphological parameters, including the number and location of the ventral glands, as well as in metric characters (see Table 2), to the trematode previously known as *N. atlanticus* (Stunkard 1966; Gonchar et al. 2019).

Pseudonotocotylus sp.

First intermediate host: *Theodoxus fluviatilis*.

Type-locality: Black River (Khmelnyskiy Village) Crimean Peninsula; 44° 32' N, 33° 39' E.

Redia (material examined: 5 specimens): body 1201 – 1277 × 260 – 297 μm, elongated, yellow-brown, with caecum long, genital pore at level of pharynx.

Cercaria (material examined: 10 specimens) (see Figure 3(c)): cercariae belonging to “Monostomi” group. Body 200 – 382 × 100 – 158 μm, oval, brown-pigmented, trioculate. Median eyespot less developed than lateral ones. Posterolateral body parts bearing adhesive pockets. Body filled with numerous cystogenous glands. Oral sucker 23 – 39 × 35 – 57 μm in diameter, subterminal. The esophagus well developed; intestinal bifurcation posteriorly of median eyespot. Caeca reaching level of excretory vesicle. Excretory vesicle sac-like, opening via excretory pore near posterior body end, between dorsal adhesive pockets. Main excretory ducts fused immediately posteriorly of oral sucker. Main excretory ducts filled with excretory granules. Tail 203–478 μm long, transparent, slightly longer than body length.

Metacercaria (material examined: 5 specimens): cyst dome-shaped, 167–270 μm in diameter. Wall 4–5 μm thick forming dome, which provides attachment of metacercariae to substrate.

3.3.2. *Phylogenetic Relationships in the Structure of Pseudonotocotylus* n. gen. According to the nuclear marker data used in this study, the genus *Pseudonotocotylus* n. gen. comprises 10 representatives of Notocotylidae, of which four have been identified as species [2, 4, 7], including *P. martynenkoi* n. sp. (see Figure 1). The type species of this genus, *P. intestinalis*, in the reconstruction based on the 28S marker together with LC596922 *Pseudonotocotylus* sp. [21] form a branch with a high *a posteriori* probability external to other species of the genus (see Figure 1). These worms do not differ in the 28S marker and most likely belong to the same species, although no morphological data are available for the LC596922 sample. In the phylogenetic reconstruction based on the mitochondrial marker, the nucleotide sequences of *P. intestinalis* obtained in this study form a branch with MT560390 of *P. intestinalis* and LC597061 *Pseudonotocotylus* sp. The genetic distances between the samples within this branch are at the intraspecific level. It is worth noting that the genetic differences (0.9%) found between OR506616 *P. intestinalis* and OR506617–OR506618 *P. intestinalis* may be associated not only with the random occurrence of mutations for these individuals but also with the adaptation to the first intermediate hosts belonging to different genera, *Bithynia* and *Parafossarulus*. Another representative of the considered genus is OR420045 *P. martynenkoi* n. sp., which forms a common branch with samples previously assigned to the genus *Notocotylus*, AF184259 and MN726956–MN726957, in the phylogenetic reconstruction based on the 28S marker (see Figure 1). The genetic distance between *P. martynenkoi* n. sp. and AF184259 is 0.4%, which corresponds to the interspecies level for the nuclear marker, while the nucleotide sequences of samples MN726956–MN726957 are identical to those of *P. martynenkoi* n. sp. However, since the 28S marker is less variable compared to the mitochondrial marker *cox1*, which does not allow species differentiation in Notocotylidae in all cases, it is premature to consider them as representatives of the same species. Moreover, the

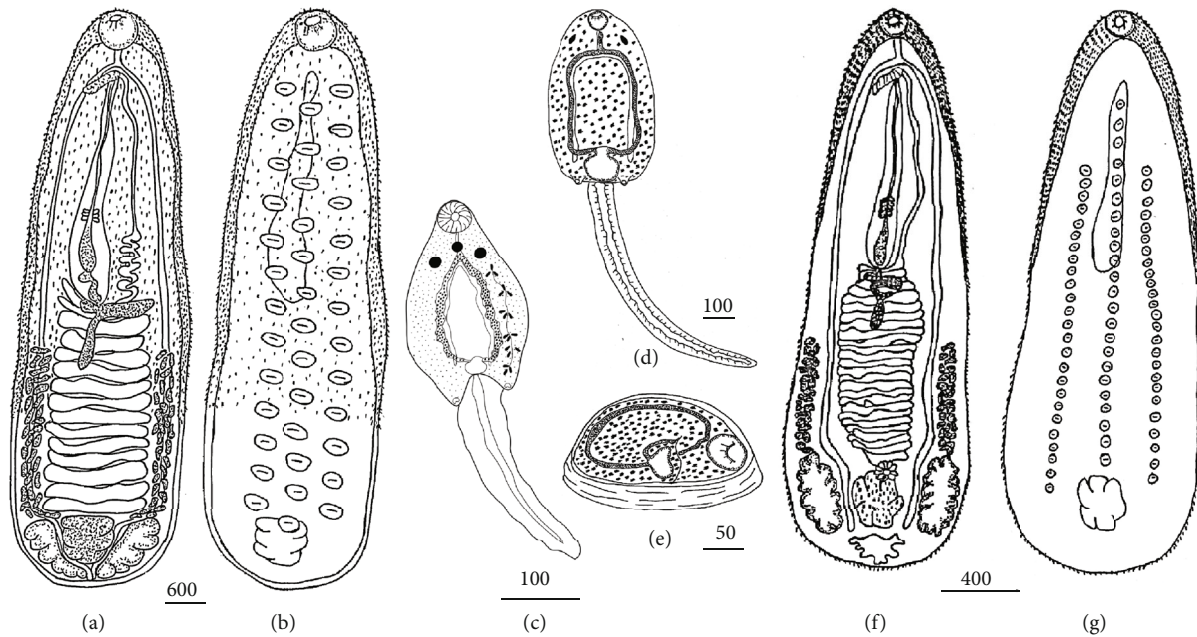


FIGURE 3: *Pseudonotocotylus martynenkoi* n. sp.: (a) adult, ventral view; (b) adult, ventral papillae. *Pseudonotocotylus* sp.: (c) cercaria. *Notocotylus multipapillus* n. sp.: (d) cercaria; (e) metacercaria; (f) adult, ventral view; (g) adult, ventral view. Scale bars in μm .

molecular characteristics for the MN726956–MN726957 notocotylida were obtained at the cercaria stage without having the morphology of mature individuals and the mitochondrial marker data available. On the phylogenetic reconstruction based on the *cox1* marker, *P. martynenkoi* n. sp. forms a separate branch external to other *Pseudonotocotylus* representatives (see Figure 2 and Figure S2). The genetic distances between *P. martynenkoi* and other *Pseudonotocotylus* representatives range from 5 to 9.5%, which corresponds to the interspecific level. Thus, the validity of *P. martynenkoi* n. sp. established at the morphological level is confirmed by analysis of both nuclear and mitochondrial markers.

As regards both the samples OR420046 *P. atlanticus* nov. comb. from our material and the cercariae OR420050–OR420051 *Pseudonotocotylus* sp. that we collected from nerite snails *T. fluviatilis*, the nucleotide sequences of the 28S marker for these worms are identical to those of MH818008 identified by [4] as *N. atlanticus* and LC596920 with unknown species identification. The listed samples form a common sister branch relative to the branch that combines *P. martynenkoi* n. sp. and specimens AF184259 and MN726956–MN726957. The minimum genetic distance between these groups is 0.4%, which corresponds to the interspecies level in the reconstruction. In contrast to *P. atlanticus* nov. comb., for which snails from the families Cochliopidae Tryon, 1866, and Hydrobiidae Troschel, 1857, play the role of the first intermediate host [5], cercariae OR420050–OR420051 *Pseudonotocotylus* sp. develop with the involvement of nerite snails *T. fluviatilis*, which is a member of the family Neritidae Rafinesque, 1815. Taking into account the known specificity of trematodes to the first intermediate host, we assume that the question as to species identification of OR420050–OR420051 *Pseudonotocotylus* sp. remains open, especially because of the lack of data on the morphology of adult worms. This also

applies to LC596920 *Pseudonotocotylus* sp. from Japan, for which molecular data for the 28S marker were obtained from adult worms, but no information on their morphology was provided. The challenge of estimating the level of relationships among these worms using only nuclear marker data, as already indicated above and noted by [5], is due to the ambiguous suitability of this marker for species differentiation among Notocotylidae representatives. The question as to the species of OR420050–OR420051 *Pseudonotocotylus* sp. and LC596920 *Pseudonotocotylus* sp., in our opinion, also remains open, especially because OR506619–OR506620 *Pseudonotocotylus* sp. forms an independent branch in the reconstruction based on the mitochondrial marker (*cox1*). The genetic distance between *P. atlanticus* nov. comb. and OR506619–OR506620 *Pseudonotocotylus* sp. is 2.7% for this marker, which corresponds to the interspecies level. It is important to note that the accessions designated as *P. atlanticus* nov. comb. and LC597059 formed two groups, of which the first included *P. atlanticus* nov. comb. from continental Europe and the second included the latter accession from the same area, as well as from Japan and the Crimean Peninsula (see Figure S2). A total of seven fixed substitutions were found between these groups at positions 22, 24, 81, 105, 132, 183, and 237 bp in the analyzed region of the *cox1* mtDNA gene. Although the substitutions are transitions that do not result in amino acid substitutions, these cause a difference between groups as high as 2.7%, which is comparable to the difference between *N. ikutai* and *N. multipapillus* n. sp. for the same marker (see below), while the differences within the groups are no greater than 0.9%. All these facts indicate isolation and, probably, the presence of two species that inhabit the same area. Although one sample (LC597059) from the second group was obtained from a Japanese trematode, we tend to agree with the suggestion by [5] that the definitive host

TABLE 2: Measurements of adult Notocotyliidae trematodes (μm).

	<i>Notocotylus multipapillus</i> n. sp. (holotype)	<i>Notocotylus multipapillus</i> n. sp. ($n = 7$)	<i>Pseudonotocotylus martynenkoi</i> n. sp. (holotype)	<i>Pseudonotocotylus martynenkoi</i> n. sp. ($n = 2$)	<i>Pseudonotocotylus atlanticus</i> nov. comb. (this study)	<i>Pseudonotocotylus atlanticus</i> nov. comb. [20]	<i>Pseudonotocotylus atlanticus</i> nov. comb. [4]	<i>Notocotylus parviovatus</i> [18]	<i>Notocotylus ralli</i> [18]
Body length	3150	2825–3625	2500	2475–2500	2500–3200	2200–3650	1850–3600	5170	3000–6500
Body width	375	375–550	725	650–725	675–800	800–1100	515–940	1190	800–1200
Oral sucker length	175	175	150	150–160	140–150	150–120	87–170	150	200–300
Oral sucker width	175	175	150	120–150	150–150	180–150	97–185	180	diameter
Cirrus sac length	1020	940–1150	950	860–950	740–820	600–840	610–1020	1625	1350–1860
Cirrus sac width	100	50–100	—	—	—	—	—	210	100–150
Metraterm length	330	320–650	—	—	—	280–370	230–470	—	—
Cirrus length	200	155–200	140	140–150	—	190	172	—	—
Cirrus width	40	40–100	40	30–40	—	28	18	—	—
Testis length	350	300–800	420	420–510	400–420	280–160	100–280	750–780	—
Testis width	175	175–350	250	180–250	220–290	480–260	290–690	270–280	—
Ovary length	175	175–575	350	250–350	250–340	190–150–	120–375	370	—
Ovary width	225	225–375	320	290–320	300–350	50–200	130–345	310	—
Number of ventral papillae	23–25	23–25	13	13	15–17	15–17	15–17	21–25	23–28

(mallard) became infected on the European coast since the first intermediate host of *P. atlanticus* nov. comb. is absent from Japan. On the other hand, when considering the presence of two species, one may assume the first intermediate host of the trematode from Japan (LC597059) to be another snail, but this is unlikely since the sample of *P. atlanticus* nov. comb. that we obtained from the same group was from a gull species whose migrations are very limited. Based on the considerations above, we suggest that the two identified groups of *P. atlanticus* nov. comb. are independent cryptic species, which can be described only on the basis of an integrated approach and additional data used.

Samples of *P. magniovatus* nov. comb. and LC599520–LC599521 *Pseudonotocotylus* sp. in the reconstruction based on the 28S marker form a separate branch with a high *a posteriori* probability. The genetic distances between these samples in the clade are at the intraspecific level (0.02%). However, the molecular data for LC599520–LC599521 *Pseudonotocotylus* sp. were obtained from the redia stage, which makes it impossible to compare the morphological features of these worms with those of *P. magniovatus* nov. comb. In the tree based on the *cox1* marker, LC599520–LC599521 *P. magniovatus* nov. comb. and the worms of unidentified species associated with them also form a separate branch and differ from other representatives of the clade by 7.2–12%. The relationship of these worms, revealed by nuclear and mitochondrial markers, may indicate that the trematodes in this group belong to the same species, *P. magniovatus* nov. comb.

3.3.3. *Notocotylus. Notocotylus multipapillus* n. sp. Izrailskaya and Tatonova, 2023.

A total of 43 specimens of notocotylids were recovered from the caeca of duckling 16 days after the experimental infection with metacercariae, obtained from cercariae emitting from *H. suifunensis*. The obtained morphological and molecular data pointed to a new species *Notocotylus multipapillus* n. sp., which is described below.

Host: Anas platyrhynchos dom. (experimentally infected).

Site: caeca.

Intermediate host: Helicorbis suifunensis.

Type-locality: Russky Island (Vladivostok), Primorsky Krai, the Russian Southern Far East; 43° 01' N, 131.54' E.

Type-deposition: holotype No. 231-Tr; paratype No. 232-237-Tr. This material is deposited in the parasitological collection of the zoological collection (FSCEATB FEB RAS, Vladivostok, Russia) (e-mail: petrova@ibss.dvo.ru). Deposited November 22, 2021. ZooBank number C4BA1ED4-CBC0-4DDA-A336-083E6702090B.

Etymology: The species epithet (*multipapillus*) means the large number of papillae found in the lateral and median rows.

Adult worm (material examined: 7 specimens) (see Figures 3(f) and 3(g) and Table 2): body flat, elongated, ventrally concave, with tapered anterior and rounded posterior ends, covered by spines. Ventral surface with three rows of papillae: 23 in median row and 25 in lateral rows. Anterior papilla in lateral row located at level of middle cirrus sac; posterior papilla at level of middle of testes. First papillae

in median row located posteriorly of genital pore; posterior papillae in median row, anteriorly of ovary. Oral sucker subterminal; esophagus short; caeca extends laterally to uterine coils, between ovary and testes, and ends blindly at level of posterior margin of testes. Ventral sucker absent. Testes at posterior end of body, symmetrical, lobed on external margin. External seminal vesicle reaching middle of body. Anterior part of external seminal vesicle coiled; posterior part elongated. Cirrus sac elongated, narrowed anteriorly, and roundly expanded posteriorly, containing curved seminal vesicle, short prostatic, and cirrus unarmed. Genital pore median, immediately posteriorly of intestinal bifurcation. Ovary intertesticular, lobed. Mehli's gland preovarian. Uterus forms 26–27 coils; metraterm reaches 1/3 length of cirrus sac. Vitellarium consists of irregular follicles, extracaecal, located in posterior half of body. Vitellarium anteriorly reaches level of 8th–9th uterine coils, with posterior part lying at level of anterior margins of testes. Eggs oval, smooth, operculate, with two polar filaments. Excretory vesicle saccular, with diverticula.

Redia (material examined: 5 specimens): body, elongated, yellow-brown, with locomotory extensions in posterior part; caecum long; genital pore at pharynx level.

Cercaria (material examined: 10 specimens) (see Figure 3(d)): cercariae belong to “Yenchingensis” group. Body 450 – 730 × 300 – 390 μm, oval, brown-pigmented, trioculate. Median eyespot less developed than lateral ones. Posterolateral body parts bearing adhesive pockets. Body filled with numerous cystogenous glands. Oral sucker 60 μm in diameter, subterminal; esophagus well developed. Caeca reach level of excretory vesicle. Excretory vesicle sac-like, opening via excretory pore near posterior body end, between dorsal adhesive pockets. Main excretory ducts fused anteriorly near or at level of intestinal bifurcation. Diverticulum of anterior arc almost equal to esophagus length. Main excretory ducts filled with excretory granules. Tail 690 – 810 × 40 – 60 μm.

Metacercaria (material examined: 5 specimens) (see Figure 3(e)): cyst dome-shaped, 200 – 205 × 200 – 210 μm in diameter. Wall 5–6 μm thick, forming dome, which provides attachment of metacercariae to substrate.

Remark: based on the morphological data of adult worms, the trematode that we found is similar to *Notocotylus parviovatus* Yamaguti, 1934, and *N. ralli* Baylis, 1936 (at least in terms of the number of papillae in the lateral and median rows) (see Table 2). However, the papillae of the lateral rows in *N. parviovatus* are located closer to the anterior end of the body than the papillae of the median row [18] unlike those in the worms in our material. In addition, *N. parviovatus* develops with the involvement of Prosobranchia snails of the genus *Bithynia* [18, 19], while in the life cycle of Far Eastern trematodes in this study, Pulmonata snails of the genus *Helicorbis* play the role of the first intermediate host. As for *N. ralli*, along with the morphological similarity to the trematodes that we obtained, it also develops with the involvement of Pulmonata snails, but belonging to two families, Lymnaeidae Rafinesque, 1815, and Planorbidae Rafinesque, 1815 [18, 19]. Taking into account the specificity of trematodes to the first intermediate host, this raises questions about reliability of

information. Moreover, *N. ralli* and the trematode we discovered are differentiated at the cercaria stage. The cercariae of *N. ralli* are from the “Monostomi” group [19], while the cercariae of the Far Eastern trematodes are representatives of the “Yenchingensis” group. The accumulated array of morphological data and information on the taxonomic affiliation of the first intermediate hosts of the trematodes under study indicates the validity of *Notocotylus multipapillus* n. sp.

3.3.4. Phylogenetic Relationships in the Structure of *Notocotylus*. Representatives of the genus *Notocotylus*, as mentioned above, except *N. chionis* and *N. fosteri*, form a separate group (group A) in the phylogenetic reconstruction based on the 28S marker (see Figure 1). *Notocotylus multipapillus* n. sp., described in the present work, forms a branch in the reconstruction based on this marker with samples of the species *N. ikutai* (LC596915, LC596919, LC596921, and LC596923-LC596926) and representatives of the genus not identified to species (LC599518 *Notocotylus* sp., LC599519 *Notocotylus* sp., KY513158 *Notocotylus* sp., and AY222219 *Notocotylus* sp.). The genetic distances between the sequences within this branch are low. Due to the low values of genetic distances, one can assume that the worm, which we referred to as *N. multipapillus* n. sp., as well as the above-listed sequences not identified as species, should be assigned to the species *N. ikutai*. However, the similarity of the sequences obtained from the samples in our study and the sequences of *N. ikutai* may be due to the low sensitivity of the 28S marker in the differentiation of the some of Notocotylidae species. The use of a mitochondrial marker (*cox1*) has shown that group A, comprising the trematodes *N. multipapillus* n. sp. and *N. ikutai*, is divided into three branches, of which the first is formed by *N. multipapillus* n. sp., while the second and third include specimens designated as *N. ikutai* and *Notocotylus* sp. (see Figure 2 and Figure S2). However, the genetic distances between *N. multipapillus* n. sp. and the samples from the other two branches vary from 1.4 (the node including accessions LC597055 and LC599769) to 2.9% (the branch containing the remaining trematodes of group A), while the distances between the branches with *N. ikutai* and *Notocotylus* sp. amount to 2.7%. Whether these values are sufficient to distinguish between certain species is to be further clarified, since, in this case, it would be worth considering the presence of fixed nucleotide substitutions, and not just the values of genetic distances. For example, the samples of *N. multipapillus* n. sp. and *Notocotylus* sp., despite the low differences, have four fixed substitutions (positions 144, 234, 372, and 375 bp), of which one is nonsynonymous, and *N. multipapillus* n. sp. differs from the *N. ikutai* branch by six fixed synonymous substitutions (positions 12, 63, 102, 144, 216, and 375 bp). In turn, *N. ikutai* differs from *Notocotylus* sp. by four fixed substitutions (positions 12, 63, 102, and 216 bp), of which one is nonsynonymous. Thus, a certain degree of isolation between these trematodes exists at the moment, but it is still impossible to judge whether these differences are at the species level. Moreover, the interpretation of molecular data in this case should generally be treated with caution. Despite the fact that, when describing *N. ikutai* [21], obtained morphometric data from experimentally grown adult worms, molecular data for this species were

from samples of trematodes recovered from naturally infected birds and similar in morphological parameters to the experimentally grown worms. Due to the probability of the existence of cryptic species, this approach may lead to the misidentification of species. Thus, we assume that there may be three separate species in group A, one of which is *N. multipapillus* n. sp. and the other two in the rest of the branches. Nevertheless, it remains unclear as to which of them should be considered the trematode *N. ikutai* and whether the trematodes referred to in NCBI as *N. ikutai* actually belong to this species. However, according to morphological data, there are differences between *N. multipapillus* n. sp. and *N. ikutai* in the number and location of papillae on the ventral side of the body [21]. *Notocotylus multipapillus* n. sp. has 23 papillae in the median row and 25 papillae in the lateral vs. 14 papillae in the median row and 15–16 in the lateral row in *N. ikutai*, respectively, and lateral rows anteriorly begin at the level of the 4th anterior papilla of the median row vs. lateral rows begin between the first and second papillae in the median row. Taking into account the above morphological and molecular data, we suggest that the trematode we found cannot be attributed to either *N. ikutai* or any other currently known representatives of the genus *Notocotylus* and is, therefore, an independent species.

Accessions LC599518–LC599519, AY222219, and KY513158 in the reconstruction based on the 28S marker are also included in the branch with *N. ikutai* and *N. multipapillus* n. sp. and, thus, can be assigned to one or the other species (see Figure 1). Due to the lack of information on adult worms for these trematodes, their species position is disputable. In addition, the positions of MW358653 *Notocotylus* sp. and EU712725 *Notocotylus* sp., forming a sister branch to the one discussed above, remain unclear. Unfortunately, molecular data for these worms were obtained at the cercaria stage, which makes it impossible to use morphological characters for species differentiation. The genetic distances between these sister branches are of greater importance than between *N. ikutai* and *N. multipapillus* n. sp. in this reconstruction (0.4%). For this reason, if we consider *N. ikutai* and *N. multipapillus* n. sp. different species, then samples MW358653 and EU712725 should also be classified as separate species, for which additional information on developmental stages, life cycles, and molecular data on other markers is needed.

3.4. Unresolved Issues in the Structure of the Family Notocotylidae. In the structure of the family Notocotylidae, the positions of *N. chionis*, *N. fosteri*, and *Catatropis onobai* Gonchar and Galaktionov, 2020, remain unclear. *Notocotylus chionis* and *N. fosteri* have three rows of papillae on the ventral side. These can both be representatives of separate genera and belong to one of the known genera. To resolve this issue, more information is needed on the morphology, life cycles, and molecular genetic characteristics of Notocotylidae species.

The position of the representatives of *Catatropis* and *Pseudocatatropis* in the reconstruction based on the *cox1* marker in the present study is consistent with that for 28S in the present and previous publication [2] and with data

by [4], which confirms the validity of the genera *Catatropis* and *Pseudocatatropis*. *Catatropis onobai* can be attributed both to the genus *Catatropis* and the genus *Pseudocatatropis* on the basis of the structures on the ventral side. However, in the reconstruction based on the 28S marker, the trematode *C. onobai* form a separate branch, which may indicate that this worm belongs to a separate genus. This may be indirectly confirmed by the involvement of brackish-water gastropods in the life cycle of *C. onobai*, while in the type species *Catatropis verrucosa* (Frölich, 1789), freshwater gastropods play the role of an intermediate host.

It should be noted that there is a sufficient congruence between the data sets based on the separate markers (see Figure 1 and Figure S2) and the combined 28S and *cox1* genes (see Figure 2 and Supplementary Materials), which consists in the same location of all representatives of the genera *Notocotylus*, *Pseudocatatropis*, *Pseudonotocotylus*, and *Catatropis* in all the phylogenetic reconstructions. In this regard, to resolve controversial issues regarding the taxonomic position of family members, it is worth using both nuclear and mitochondrial markers. The former will make it possible to clarify the generic affiliation of notocotylids, while the latter will reveal differences between cryptic species. However, data for both genes should be supported by descriptions of morphological and biological features, especially when the species are first described.

4. Conclusions

Overall, the nuclear and mitochondrial marker data obtained support the validity of *N. multipapillus* n. sp. and *P. martynenkoi* n. sp. described in this study, as well as the validity of the trematodes *P. dvoryadkini*, *P. magniovatus* nov. comb., *P. intestinalis*, and *C. vietnamensis*, for which data on the 28S marker were previously presented [2]. The analysis of phylogenetic relationships using both markers has shown that the 28S marker has low sensitivity in differentiation of some species within the Notocotylidae genera compared to the mitochondrial marker, being, however, suitable for distinguishing taxa above the species rank. The mitochondrial *cox1* gene has proven to be more effective for species differentiation within Notocotylidae. In addition, the data obtained has confirmed the importance of morphological and molecular characteristics, as well as life cycle data, collected concurrently for the identification of notocotylida species and the description of new taxa of different ranks in the family.

Data Availability

The data supporting the findings of this study are available within the article. All newly generated sequences were deposited in the GenBank database under accession numbers OR420043–OR420051, OR506612–OR506629, and OR512977–OR512994 and ZooBank numbers 2458A141-AF78-402F-9179-00472705B9FF, 5A8C1FE2-2D33-4ADB-8100-4BA17E386C67, and C4BA1ED4-CBC0-4DDA-A336-083E6702090B.

Ethical Approval

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals including birds and mammals.

Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary Materials

Supplementary 1. Figure S1: phylogeny of the family Notocotylidae based on *nad1* sequences using the Bayesian algorithm. A posterior probability of ≥ 50 was shown in the nodes. The scale bar indicates the number of substitutions per site. The sequences obtained in this study are highlighted in red. The outgroup species are in Table S3. Figure S2: phylogeny of the family Notocotylidae based on *cox1* sequences using the Bayesian algorithm. A posterior probability of ≥ 50 was shown in the nodes. The scale bar indicates the number of substitutions per site. The sequences obtained in this study are highlighted in red. The outgroup species are in Table S2. Figure S3: variable sites of nucleotide sequences of the fragment of the 28S rRNA gene for the representatives of the family Notocotylidae. Colored rectangles show fixed substitutions between genera. Figure S4: variable sites of nucleotide sequences of the partial *cox1* gene for the representatives of the family Notocotylidae. Colored rectangles show fixed substitutions between genera. Table S1: sequences analyzed in this study (28S rDNA). Table S2: sequences analyzed in this study (*cox1* mtDNA). Table S3: sequences analyzed in this study (*nad1* mtDNA).

Supplementary 2. Alignment 28S rDNA Notocotylidae with outgroup.

Supplementary 3. Alignment *cox1* Notocotylidae with outgroup.

Supplementary 4. Alignment *nad1* Notocotylidae with outgroup.

Supplementary 5. Alignment 28S rDNA plus *cox1* Notocotylidae with outgroup.

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