



Developmental stages of *Notocotylus magniovatus* Yamaguti, 1934, *Catatropis vietnamensis* n. sp., *Pseudocatatropis dvoryadkini* n. sp., and phylogenetic relationships of Notocotylidae Lühe, 1909

Anna V. Izrail'skaia^{1,2} · Vladimir V. Besprozvannykh¹ · Yulia V. Tatonova¹  · Hung Manh Nguyen³ · Ha Duy Ngo³

Received: 12 September 2018 / Accepted: 13 December 2018 / Published online: 8 January 2019
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Abstract

Data on the life cycles and morphology of the developmental stages of *Notocotylus magniovatus*, *Catatropis vietnamensis* n. sp., and *Pseudocatatropis dvoryadkini* n. sp. were obtained. The *Pseudocatatropis* genus was restored based on our results. For the studied trematodes, the snails *Parajuga* spp., *Helicorbis suffunensis* (Russia), and *Melanooides tuberculata* (Vietnam) serve as first intermediate hosts. It has been established that *C. vietnamensis* n. sp. differs from *Catatropis harwoodi* and *Catatropis pakistanensis* in the length of the ridge and metraterm and the location of the anterior papillae. In the life cycle of *P. dvoryadkini* n. sp., as in *Pseudocatatropis joyeuxi*, cercariae do not leave the first intermediate host. Both species are very similar in morphometric features, despite the fact that they share no common first intermediate hosts in their life cycles, and the areas of the European and Asian populations of flukes do not overlap. In phylogenetic trees and genetic distances based on the nucleotide sequences of the 28S gene and the ITS2 region of ribosomal DNA, *Notocotylus attenuatus*, *Notocotylus intestinalis*, and *Notocotylus magniovatus* are combined into one systematic group, while *C. vietnamensis* n. sp. and *Catatropis indicus* form another group. A third group includes members of different genera: *P. dvoryadkini* n. sp., and *Notocotylus malhamensis*, as well as three unclassified *Notocotylus* spp. The presence in the last group of flukes with three rows of papillae and a median ridge and lateral papillae indicates that these systematic criteria are not determinant in establishing membership of the parasitic worms to one or another genus of Notocotylidae.

Keywords Notocotylidae · *Catatropis vietnamensis* n. sp. · *Pseudocatatropis dvoryadkini* n. sp. · Life cycle · 28S gene · ITS2 region

Introduction

The trematode genera *Notocotylus* Diesing, 1839 and *Catatropis* Odchner, 1905 (Notocotylidae Lühe, 1909)

include cosmopolitan species, found primarily in birds, and to a lesser extent, in mammals, particularly rodents. The first genus includes from 41 to 63 valid species according to different sources (Cribb 1991; Kinsella and Tkach 2005; Boyce et al. 2012), and the second genus includes 17 species (Schuster and Wibbelt 2012). Many of these parasites are found in East Asia, including southern Russian Far East and Vietnam. Among the members of the genera *Notocotylus* and *Catatropis*, 10 and 4 species, respectively, were found in the Russian southern Far East, whereas four and one species, respectively, were described in Vietnam. Of these flukes, *Notocotylus intestinalis* Tubanguui, 1932 and *Notocotylus imbricatus* Looss, 1894 are common to both regions (Besprozvannykh 2010; Besprozvannykh et al. 2012; Le et al. 2013). To date, genetic data have only been obtained for *Notocotylus attenuatus* (Rud., 1809), *Notocotylus malhamensis* Boyce, Hide, Craig, Harris, Reynolds, Pickles, Rogan, 2012, *N. intestinalis*, and three unidentified

Section Editor: David Bruce Conn

✉ Yulia V. Tatonova
ytatonova@gmail.com

- ¹ Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch, Russian Academy of Sciences, 100-letiya Street, 159, Vladivostok, Russian Federation 690022
- ² Far Eastern Federal University, Sukhanova, 8, Vladivostok, Russian Federation 690091
- ³ Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Street, Cau Giay Dist, Hanoi, Vietnam

Notocotylus species, as well for *Catatropis indicus* Srivastava, 1935 (Tkach et al. 2001; Olson et al. 2003; Boyce et al. 2012; Besprozvannykh et al. 2013; Hanelt 2009; Soldanova et al. 2017). The species status of the remaining flukes is based on morphometric data. We obtained adult Notocotylidae flukes, *Notocotylus magniovatus* Yamaguti, 1934, *Catatropis vietnamensis* n. sp., and *Pseudocatatropis dvoryadkini* n. sp. to conduct experimental studies. For all flukes, morphometric data were obtained for developmental stages, and genetic studies were performed using nucleotide sequences of the 28S gene (*28S*) and ITS2 region (*ITS2*) of rDNA. In addition, new genetic data (sequences of *ITS2*) were obtained for *N. intestinalis*, which was previously described by Besprozvannykh et al. (2013). The results of these studies are presented below.

Material and methods

Morphology and life cycles

The material for this study was freshwater snails *Melanooides tuberculata* Müller, 1774 from Vietnam, as well as *Parajuga subtegulata* Prozorova et Starobogatov, 2004 and *Helicorbis suffunensis* Starobogatov, 1957 from the Russian southern Far East, which were infected by representatives of Notocotylidae Lühe, 1909. The first two snails were placed separately in Petri dishes containing water, from which cercariae emerged and were encysted on the bottom and walls of the Petri dishes. Metacercariae, which were formed from cercariae emitted from *M. tuberculata* and *P. subtegulata*, were fed to two ducklings and two chickens (50 metacercariae for each bird). On day 16 after infection, in the caeca of the intestine of one of the exposed ducklings, nine flukes were found which had no eggs in the uterus. On day 22, five mature flukes were obtained in the caeca of the intestine of the second duckling. On day 16 after infection, adult flukes were found in the caeca of the intestine of both chickens. The number of trematodes was 6 and 35 worms, respectively. Crushed *H. suffunensis* contained rediae, cercariae, and metacercariae. At least 500 metacercariae were detected in this snail. Metacercariae were fed to two ducklings (50 specimens for each animal). On day 16, adult flukes were found in the caeca of the intestine (20 and 30 parasites, respectively).

The rediae and metacercariae were measured in live specimens, while cercariae were fixed in 4% hot formalin before measurements. Adult flukes were fixed in 70% ethanol and stored in 96% ethanol. Whole-mounts of flukes were prepared by staining with alum carmine, dehydrated in a graded ethanol series, cleared in clove oil, and mounted in Canada balsam. All measurements were made in millimeters (mm).

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Euthanasia of laboratory animals was carried out in accordance with the Committee on the Ethics of Animal Experiments of the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Russia.

Molecular data

Experimentally obtained adult flukes, for three samples of each species, were used in the analysis: *N. magniovatus* (Russia), *C. vietnamensis* n. sp. (Vietnam), and *P. dvoryadkini* n. sp. (Russia). In addition, five *N. intestinalis* samples, for which 28S data have been previously published (Besprozvannykh et al. 2013), were used to obtain the sequences of *ITS2*. DNA isolation was performed by the HotSHOT method (Truett et al. 2000). The partial sequences of 28S and complete sequences of *ITS2* were amplified by polymerase chain reaction (PCR) using specific primers: digl2 (5'-AAG-CAT-ATC-ACT-AAG-CGG-3') and 1500R (5'-GCT-ATC-CTG-AGG-GAA-ACT-TCG-3') (Tkach et al. 2003) for 28S and BD1 (5'-GTC-GTA-ACA-AGG-TTT-CCG-TA-3') (Luton et al. 1992), 28S4R (5'-TAT-TTA-GCC-TTG-GAT-GGA-GTT-TAC-C-3') (Besprozvannykh et al. 2018). The annealing temperature for both markers was 55 °C. Control of the efficiency and contamination of PCR was carried out by setting positive and negative samples, respectively. PCR products were sequenced by Sanger terminator synthesis using the BigDye Terminator Cycle Sequencing kit from Applied Biosystems, USA. Nucleotide sequences were determined with the genetic analyzer ABI 3130 on the basis of the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Russia. 28S and *ITS2* were sequenced with the primers used for amplification, while for 28S, the internal primer 900F (5'-CCG-TCT-TGA-AAC-ACG-GAC-CAA-G-3') (Tkach et al. 2003) was also used.

The processing and alignment of consensus sequences were carried out using FinchTV 1.4 and MEGA 5.0 programs (Tamura et al. 2011). Genetic *p*-distances and nucleotide composition were also determined in MEGA.

The reconstruction of phylogenetic relationships was performed using the rDNA data of other trematodes belonging to Notocotylidae presented in the NCBI database (GenBank). Members of the trematode families located to the left (basal) of the Notocotylidae on a phylogenetic tree constructed by Olson et al. (2003) using nucleotide sequences of the 28S and 18S rRNA genes were selected as an outgroup in our study. The list of samples used in the work is presented in Table 1.

Phylogenetic relationships were reconstructed using the Bayesian algorithm in the program MrBayes 3.1.2. (Ronquist and Huelsenbeck 2003) and the maximum likelihood algorithm in the PhyML 3.1 program using the model chosen as optimal in the program jModeltest 2.1.7 (Darriba et al. 2012): TPM3uf+G and TPM3+G for 28S and *ITS2*, respectively. In the reconstruction of trees, the information

Table 1 Sequences analyzed in this study

Species	References	Accession number in the NCBI database	
		28S rRNA gene	ITS2 region
<i>Notocotylus intestinalis</i>	Besprozvannykh et al. 2013	JQ890559–JQ890563	<i>MN750025–MN750029</i>
<i>Notocotylus magniovatus</i>	–	<i>MN750016–MN750018</i>	<i>MN750016–MN750018</i>
<i>Pseudocatatropis dvoryadkini</i> n. sp.	–	<i>MN750022–MN750024</i>	<i>MN750022–MN750024</i>
<i>Catatropis vietnamensis</i> n. sp.	–	<i>MN750019–MN750021</i>	<i>MN750019–MN750021</i>
<i>Ogmocotyle sikae</i>	Unpublished data	–	KR080167 Outgroup
<i>Ogmocotyle capricorni</i>	Unpublished data	–	AB367788 Outgroup
<i>Notocotylus attenuatus</i>	Tkach et al. 2001	AF184259	–
<i>Catatropis indicus</i>	Olson et al. 2003	AY222220	–
<i>Notocotylus</i> sp. 1	Soldanova et al. 2017	KY513158	–
<i>Notocotylus</i> sp. 2	Hanelt 2009	EU712725	–
<i>Notocotylus</i> sp. 3	Olson et al. 2003	AY222219	–
<i>Notocotylus malhamensis</i>	Boyce et al. 2012	JQ766939	JQ766940
<i>Ogmogaster antarctica</i>	Fraija-Fernandez et al. 2015	KM258675	–
<i>Paramonostomum anatis</i>	Tkach et al. 2001	AF184258	–
<i>Heronimus mollis</i>	Olson et al. 2003	AY116878 Outgroup	–
<i>Azygia longa</i>	Calhoun et al. 2013	KC985234 Outgroup	–

Accession numbers in italics are newly determined sequences

criterion Akaike (AIC) was used. The statistical significance of phylogenetic connections for trees constructed using the maximum likelihood algorithm was established using the bootstrap method. The method of posterior probabilities was used for trees constructed with the Bayesian algorithm. In the Bayesian analysis, 300,000 and 100,000 generations of the Markov chain Monte Carlo posterior part third (MCMC) were simulated for 28S and ITS2, respectively. In addition, a phylogenetic UPGMA reconstruction was constructed in the MEGA 5.0 program using *p*-distances based on the sequences of 28S.

Results

Morphological description

Cem. Notocotylidae Luhe, 1909.

Notocotylus magniovatus Yamaguti, 1934.

Host: *Gallus gallus dom.* (experimental).

Site: Caeca of the intestine.

Intermediate host: *Parajuga subtegulata*.

Site: Digestive gland.

Locality: Chernyatino village (43°95'N, 131°47'E), the Razdolnaya River, Primorsky Region, Russia.

Adult worm (material examined: 10 specimens) (Figs. 1a–c and 3a; Table 2).

Body flat, elongate, ventrally concave, with tapered anterior and rounded posterior ends. Ventral surface of

anterior half of body covered by spines. Eyespot pigment dispersed at level of oral sucker and esophagus. Ventral papillae arranged in three rows, 14–15 papillae in each row. Anterior papilla in median row located at level of border between first and second thirds of cirrus sac, posterior papilla at level of middle ovary. First papillae in lateral rows located at level between first and second papillae of median row. Posterior papillae in lateral rows at level of posterior ends of ovary and testes. Oral sucker subterminal, esophagus short, caeca extends laterally to uterine coils, between ovary and testes, and ends blindly immediately before excretory vesicle. Testes at posterior end of body, symmetrical, deeply lobed on external margin. External seminal vesicle reached posterior third of body. Anterior part of external seminal vesicle coiled, posterior part elongate. Cirrus sac elongated, narrowed anteriorly and roundly expanded posteriorly, contains curved seminal vesicle, short prostatic and cirrus with minute spines. Genital pore median, immediately posterior to intestinal bifurcation. Ovary intertesticular, consisting of three irregular lobes. Mehli's gland preovarian. Uterus forms 14–15 coils. Metraterm does not reach half of cirrus sac length. Vitellarium consisting of irregular follicles, extracaecal, located in posterior half of body. Vitellarium anteriorly reaches level of 12–13th uterine coils, posterior part lies at level of anterior margins of testes. Eggs oval, smooth, operculate, with two polar filaments. Excretory vesicle saccular with diverticulum.

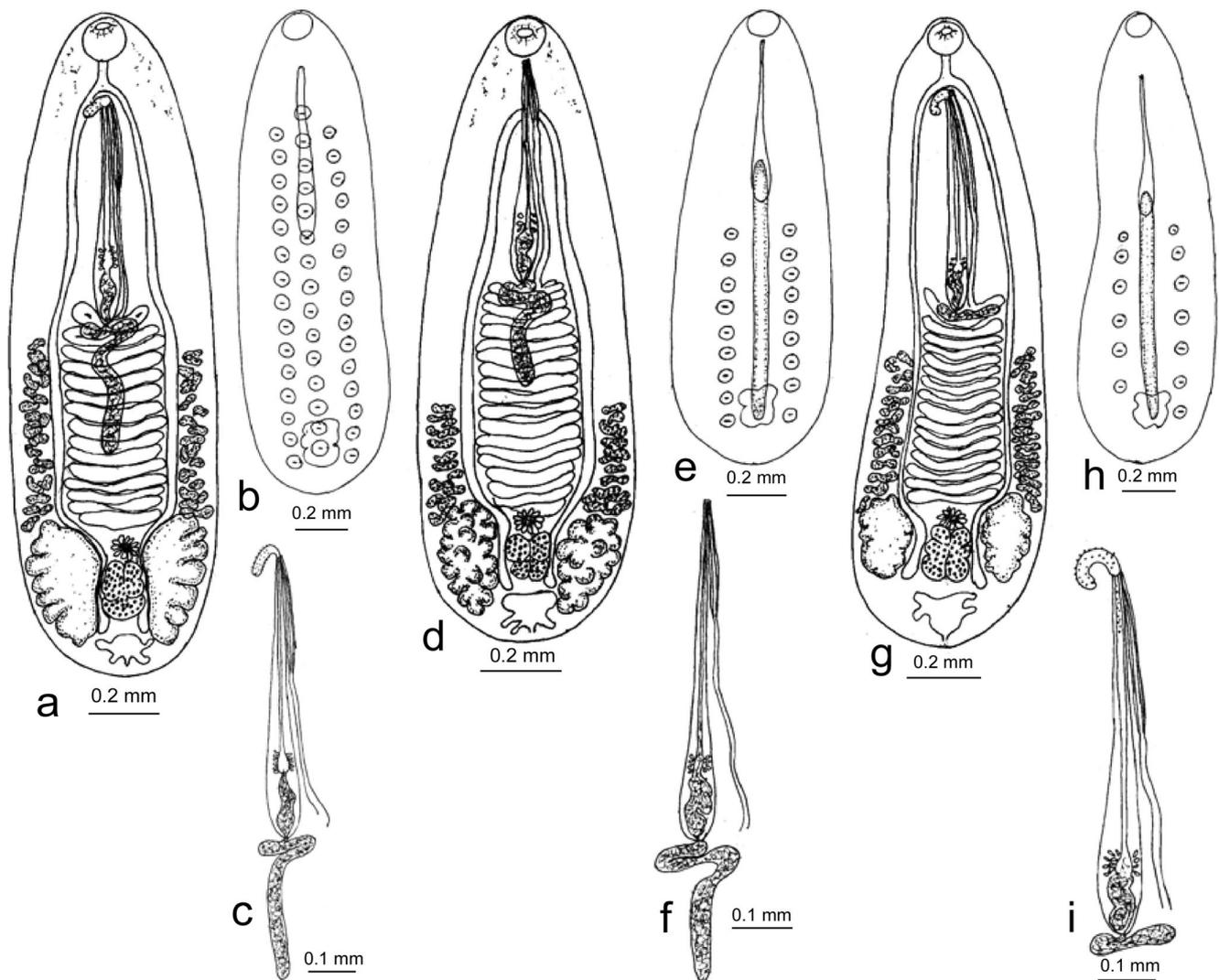


Fig. 1 Adult worms. *Notocotylus magniovatus* Yamaguti, 1934: **a** Ventral view. **b** Ventral papillae. **c** Terminal genitalia. *Catatropis vietnamensis* n. sp.: **d** Ventral view. **e** Ventral ridge and papillae. **f** Terminal genitalia.

Pseudocatatropis dvoryadkini n. sp.: **g** Ventral view. **h** Ventral ridge and papillae. **i** Terminal genitalia

Redia (material examined: 5 specimens)

Body 0.93–1.20 × 0.34–0.40, elongate, yellow-brown, posterior part with locomotory extensions, pharynx 0.10–0.11 in diameter, caecum long, birth pore at pharynx level.

Cercaria (material examined: 15 specimens) (Figs. 2a and Fig. 3b; Table 3)

Cercariae belonging to “Yenchingensis” group. Body 0.220–0.260 × 0.140–0.150, oval or triangular, brown pigmented, tri-oculate. Median eyespot less developed than lateral ones. Posterior-lateral body parts bearing adhesive pockets. Body filled with numerous cystogenous glands. These glands absent around ocelli and oral sucker. Oral sucker 0.028–0.033 × 0.033–0.039, subterminal, esophagus well developed, intestinal bifurcation posterior to median eyespot. Caeca

reached level of excretory vesicle. Excretory vesicle sac-like, opening by 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 length of esophagus. Main excretory ducts filled with excretory granules. Tail 0.280–0.390 × 0.050–0.056.

Metacercaria (material examined: 10 specimens) (Figs. 2b and 3c; Table 3)

Cyst dome-shaped, 0.156–0.169 in diameter. Width of wall 0.056–0.061, forming dome, which provides attachment of metacercariae to substrate.

Catatropis vietnamensis n. sp.

Host: *Anas platyrhynchos* dom. (experimental).

Site: Caeca of the intestine.

Table 2 Measurements of adult Notocotylidae flukes

Features	<i>Notocotylus magniovatus</i>		<i>Catantropis vietnamensis</i> n. sp.		<i>Pseudocatantropis dvoryadkini</i> n. sp.	
	<i>n</i> = 10	Holotype	<i>n</i> = 5	Holotype	<i>n</i> = 10	Holotype
Body	1.617–1.925 × 0.493–0.570	1.602 × 0.524	1.463–1.602 × 0.524–0.554	1.525 × 0.539	1.525–1.728 × 0.446–0.539	1.525 × 0.539
Oral sucker	0.096–0.104 × 0.108–0.112	0.092 × 0.115	0.089–0.092 × 0.108–0.115	0.081 × 0.089	0.077–0.081 × 0.081–0.092	0.077–0.081 × 0.081–0.092
Esophagus length	0.059–0.069	0.119	0.096–0.119	0.104	0.092–0.104	0.092–0.104
Testis left	0.231–0.385 × 0.139–0.196	0.273 × 0.181	0.208–0.281 × 0.173–0.181	0.219 × 0.116	0.200–0.250 × 0.116–0.162	0.200–0.250 × 0.116–0.162
Testis right	0.262–0.377 × 0.135–0.212	0.293 × 0.154	0.270–0.293 × 0.154–0.169	0.231 × 0.131	0.193–0.239 × 0.127–0.154	0.193–0.239 × 0.127–0.154
Cirrus sac	0.524–0.616 × 0.058–0.077	0.597 × 0.054	0.508–0.597 × 0.046–0.077	0.539 × 0.081	0.492–0.616 × 0.069–0.084	0.492–0.616 × 0.069–0.084
Ovary	0.135–0.177 × 0.116–0.123	0.135 × 0.123	0.119–0.158 × 0.116–0.123	0.116 × 0.119	0.116–0.169 × 0.108–0.119	0.116–0.169 × 0.108–0.119
Mehli's gland	0.081–0.096 × 0.084–0.119	0.081 × 0.103	0.062–0.081 × 0.085–0.103	0.085 × 0.100	0.058–0.085 × 0.069–0.100	0.058–0.085 × 0.069–0.100
Metraterm	0.204–0.250	0.192	0.177–0.192	0.281	0.265–0.327	0.265–0.327
Vitellarium fields	0.377–0.554	0.270 left, 0.327 right	0.270–0.385	0.270 left, 0.327 right	0.347–0.462	0.347–0.462
Distance from the anterior end body to median papillae or ridge	0.277–0.308	0.570	0.539–0.570	0.700	0.700–0.770	0.700–0.770
Distance from the anterior end body to vitellarium	0.847–0.932	1.00	1.00–1.09	0.907	0.886–0.907	0.886–0.907
Eggs	0.022–0.023 × 0.012–0.013		0.019–0.023 × 0.0077–0.0116		0.019–0.023 × 0.0116–0.0154	0.019–0.023 × 0.0116–0.0154

Intermediate host: *Melanoides tuberculata*.
 Site: Digestive gland.
 Locality: Nam Dinh Province, Vietnam (20°09'N, 106°17' E).

Etymology: The specific name refers to Vietnam, the type locality.

Adult worm (material examined: 5 specimens) (Figs. 1d–f and 3d; Table 2)

Body flat, elongate, ventrally concave, with tapered anterior and rounded posterior ends. Ventral surface of anterior half of body covered by scale-like spines. Eyespot pigment dispersed at level of oral sucker and esophagus. Ventral papillae in median row united in continuous ridge and arranged from level of internal seminal vesicle to level of posterior end of ovary. Two lateral rows with 9 papillae in each. First pair of papillae in lateral rows located posterior to anterior border of ridge at level of 15th uterine coil. Posterior papillae in lateral rows at level of posterior half of testes. Oral sucker subterminal, esophagus short, caeca extends laterally to uterine coils, between ovary and testes and ends blindly at level of posterior edge of ovary. Testes at posterior end of body, symmetrical, with numerous deep lobes. External seminal vesicle almost reaches posterior third of body. Anterior part of external seminal vesicle coiled, posterior part elongate. Cirrus sac elongated, narrowed anteriorly and roundly expanded posteriorly, contains curved internal seminal vesicle, short prostatic and unarmed cirrus. Genital pore median, immediately after posterior end of oral sucker. Ovary intertesticular, consisting of four lobes. Mehli's gland preovarian. Uterus forms 15–18 coils, metraterm reaches 1/3 length of cirrus sac. Vitellarium consisting of irregular follicles, extracaecal, located in posterior half of body. Vitellarium anteriorly reaches level of 8–9th uterine coils, posterior part lies at level of anterior margins of testes or slightly laterally to testes. Eggs oval, smooth, operculate, with two polar filaments. Excretory vesicle saccular with diverticulum.

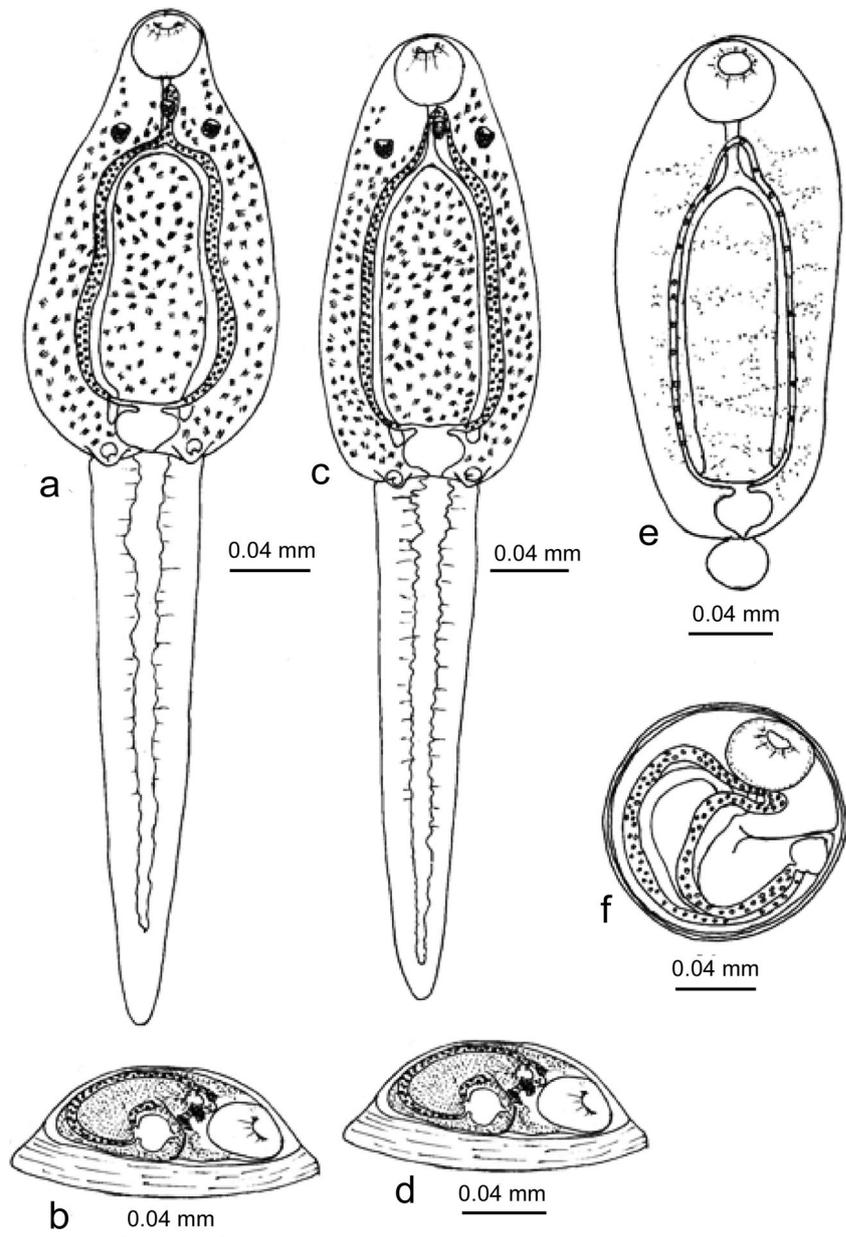
Redia (material examined: 5 specimens)

Body 1.155–1.232 × 0.277–0.354, elongate, yellow-brown, pharynx 0.046–0.069 × 0.046–0.050, caecum long, birth pore at level of pharynx.

Cercaria (material examined: 10 specimens) (Figs. 2c and 3e; Table 3)

Cercariae belonging to “Imbricata” group. Body 0.293–0.312 × 0.142–0.181, oval or triangular, brown pigmented, tri-oculate. Median eyespot less developed

Fig. 2 *Notocotylus magniovatus* Yamaguti, 1934: **a** Cercaria. **b** Metacercaria. *Catatropis vietnamensis* n. sp.: **c** Cercaria. **d** Metacercaria. *Pseudocatropis dvoryadkini* n. sp.: **e** Cercaria. **f** Metacercaria



than lateral ones. Posterior-lateral body parts bearing adhesive pockets. Body filled with numerous cystogenous glands. These glands absent around ocelli and oral sucker. Oral sucker 0.042–0.050 in diameter, subterminal, esophagus well developed, intestinal bifurcation posterior to median eyespot. Caeca reached level of excretory vesicle. Excretory vesicle sac-like, opening by excretory pore near posterior body end, between dorsal adhesive pockets. Main excretory ducts fused immediately after oral sucker. Main excretory ducts filled with excretory granules. Tail 0.304–0.454 × 0.069–0.077.

Metacercaria (material examined: 10 specimens) (Figs. 2d and 3f; Table 3)

Cyst dome-shaped, 0.146–0.177 in diameter. Width of wall 0.054–0.062, forming dome, which provides attachment of metacercariae to substrate.

Pseudocatropis dvoryadkini n. sp.

Host: *Anas platyrhynchos* dom. (experimental).

Site: Caeca of the intestine.

Intermediate host: *Helicorbis suffunensis*.

Site: Digestive gland.

Table 3 Measurements of larval stages of Notocotylidae

Features	<i>Notocotylus magniovatus</i>	<i>Catantropis vietnamensis</i> n. sp.	<i>Pseudocotatropis dvoryadkini</i> n. sp.	<i>Pseudocotatropis joyexi</i> (Joyeux, 1922 cit. from Skrjabin, 1953)	<i>Pseudocotatropis joyexi</i> (Odening, 1966)
Redia					
Body	0.93–1.20 × 0.34–0.40	1.155–1.232 × 0.277–0.354	1.232–1.309 × 0.200–0.308	0.10	0.771–1.798 × 0.264–0.367
Pharynx	0.10–0.11	0.046–0.069 × 0.046–0.050	0.062–0.073 × 0.058	–	0.041–0.069 × 0.052–0.072
Cercaria					
Body	0.220–0.260 × 0.140–0.150	0.293–0.312 × 0.142–0.181	0.231–0.258 × 0.104–0.116	0.20–0.35 × 0.10–0.13	0.311–0.499 × 0.162–0.250
Oral sucker	0.028–0.033 × 0.033–0.039	0.042–0.050	0.035–0.042 × 0.039–0.046	–	0.037–0.051 × 0.046–0.053
Tail	0.280–0.390 × 0.050–0.056	0.304–0.454 × 0.069–0.077	0.045–0.062 length	0.050–0.060 length	0.045–0.072 × 0.031–0.041
Metacercaria					
Cyst	0.156–0.169 diameter	0.146–0.177 diameter	0.119–0.135 diameter	0.110–0.120 diameter	0.138–0.139 × 0.147

Locality: Slavyanka channel, Soldatskoe lake (43°77'N, 131°94'E), Primorsky Region, Russia.

Etymology: The species was named in honor of V.A. Dvoryadkin (Dvoryadkin, 1989), who first discovered this trematode in the Russian southern Far East.

Adult worm (material examined: 10 specimens) (Figs. 1g–i and 3g, h; Table 2)

Body flat, elongate, ventrally concave, with tapered anterior and rounded posterior ends. Ventral surface of anterior half of body covered by scale-like spines. Ventral papillae in median row united in continuous ridge and arranged from level of internal seminal vesicle to level of posterior ovary end. Two lateral rows with 6–7 papillae in each. First pair of papillae in lateral rows located posterior to anterior border of ridge. Posterior papillae in lateral rows at level of anterior or posterior half of testes. Oral sucker subterminal, esophagus short, caeca extends laterally to uterine coils, between ovary and testes and ends blindly at level of posterior edges of testes. Testes at posterior end of body, symmetrical, lobed on external and internal margins. External seminal vesicle coiled, located at level of anterior uterine coils. Cirrus sac elongated, narrowed anteriorly and roundly expanded posteriorly, contains curved internal seminal vesicle, short prostatic and cirrus covered with papillae. Genital pore median, immediately posteriorly to intestinal bifurcation. Ovary intertesticular, consisting of three lobes. Mehli's gland preovarian. Uterus forms 15–17 coils. Metraterm reaches half of cirrus sac length. Vitellarium consisting of irregular follicles, extracaecal, located in posterior half of body. Vitellarium anteriorly reaches level of 12–13th uterine coils, posterior part lies slightly laterally to testes. Eggs oval, smooth, operculate, with two polar filaments. Excretory vesicle saccular with diverticulum.

Redia (material examined: 5 specimens)

Body 1.232–1.309 × 0.200–0.308, elongate, yellow-brown, pharynx 0.062–0.073 × 0.058, caecum long, birth pore at level pharynx.

Cercaria (material examined: 15 specimens) (Figs. 2e and 3i; Table 3)

Cercariae belonging to “Imbricata” group. Body 0.231–0.258 × 0.104–0.116, elongate, oval, with diffused gray pigment and without eyespots. Body filled with numerous cystogenous glands. Oral sucker 0.035–0.042 × 0.039–0.046, subterminal, esophagus well developed, caeca slightly does not reach level of excretory vesicle. Excretory vesicle sac-like. Main excretory ducts fused at level of esophagus. Main excretory ducts contain few excretory granules. Tail short, 0.02–0.03 in diameter.

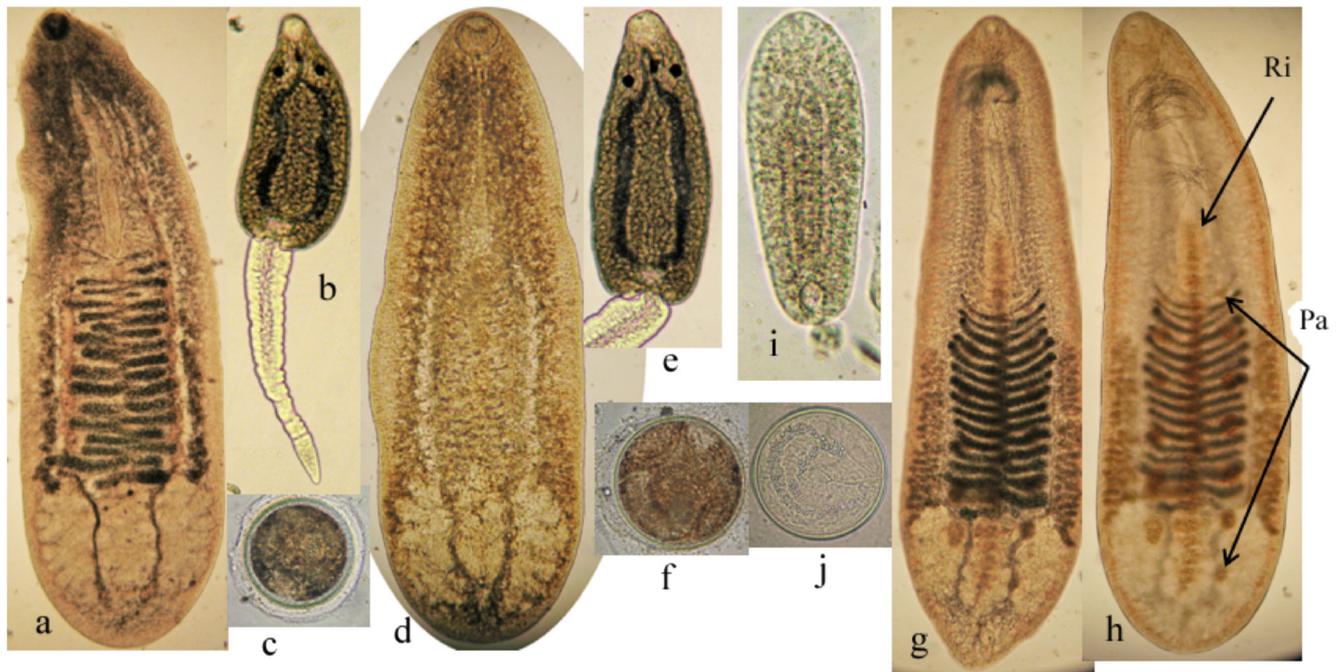


Fig. 3 *Notocotylus magniovatus* Yamaguti, 1934: **a** Adult worm. **b** Cercaria. **c** Metacercaria. *Catatropis vietnamensis* n. sp.: **d** Adult worm. **e** Cercaria. **f** Metacercaria. *Pseudocatatropis dvoryadkini* n. sp.: **g**, **h** Adult worms. **i** Cercaria. **j** Metacercaria (Ri, ridge, Pa, papillae)

Metacercaria (material examined: 10 specimens) (Figs. 2f and 3j; Table 3)

Cyst spherical, 0.119–0.135 in diameter, clear. Wall of cyst 0.008–0.019. Excretory ducts contain numerous excretory granules.

Genetic data

The length of the partial nucleotide sequences of 28S was 1277 bp for *N. intestinalis*, *C. vietnamensis*, and *P. dvoryadkini*, and 1276 bp for *N. magniovatus*. The length of the complete nucleotide sequences of ITS2 was 285 bp for *N. magniovatus* and 282 bp for other species. No nucleotide substitutions were observed within these species for both markers, with the exception of the sample MN750021 of *C. vietnamensis*, which had C/T double peak at position 278 bp of ITS2. The nucleotide composition of the analyzed sequences did not show any significant differences between species (Table 4).

The length of aligned sequences used for phylogenetic analysis was 1197 and 292 bp for 28S and ITS2, respectively. For 28S, the trees reconstructed using the Bayesian method, the maximum likelihood algorithm, and UPGMA (Fig. 4), as well as, for ITS2, phylogenetic trees reconstructed using Bayesian algorithms and maximum likelihood (Fig. 5), had differences in topology and were analyzed separately.

The reconstruction of phylogenetic relationships based on 28S sequences showed that the analyzed species are also

divided into three groups with high statistical support (Fig. 4a–c). Group 1 includes *N. attenuatus*, *N. intestinalis*, and *N. magniovatus*, while group 2 combines *C. indicus* and *C. vietnamensis* n. sp.; both groups form a common cluster. The remaining Notocotylidae representatives (*P. dvoryadkini* n. sp., *Notocotylus* sp. 1, *Notocotylus* sp. 2, *Notocotylus* sp. 3, and *N. malhamensis*) form group 3. The above-mentioned groups are represented in all phylogenetic trees. However, there are some differences between trees built using different methods. According to reconstruction made using the maximum likelihood algorithms (Fig. 4b) and UPGMA (Fig. 4c), the members of *Paramonostomum* Lühe, 1909 and *Ogmogaster* Jägerskiöld, 1891 are united in a common cluster

Table 4 Nucleotide composition of analyzed sequences

Species	Nucleotide frequency (%)			
	T(U)	C	A	G
28S gene, partial				
<i>Notocotylus intestinalis</i>	26.1	21.2	20.6	32.0
<i>Notocotylus magniovatus</i>	26.8	21.2	20.3	31.8
<i>Pseudocatatropis dvoryadkini</i> n. sp.	25.8	21.3	20.8	32.0
<i>Catatropis vietnamensis</i> n. sp.	26.5	21.1	20.8	31.6
ITS2 region, complete				
<i>Notocotylus intestinalis</i>	31.1	22.0	22.0	25.9
<i>Notocotylus magniovatus</i>	29.8	22.8	22.1	25.3
<i>Pseudocatatropis dvoryadkini</i> n. sp.	31.6	21.6	21.6	25.2
<i>Catatropis vietnamensis</i> n. sp.	31.6	19.9	22.3	26.2

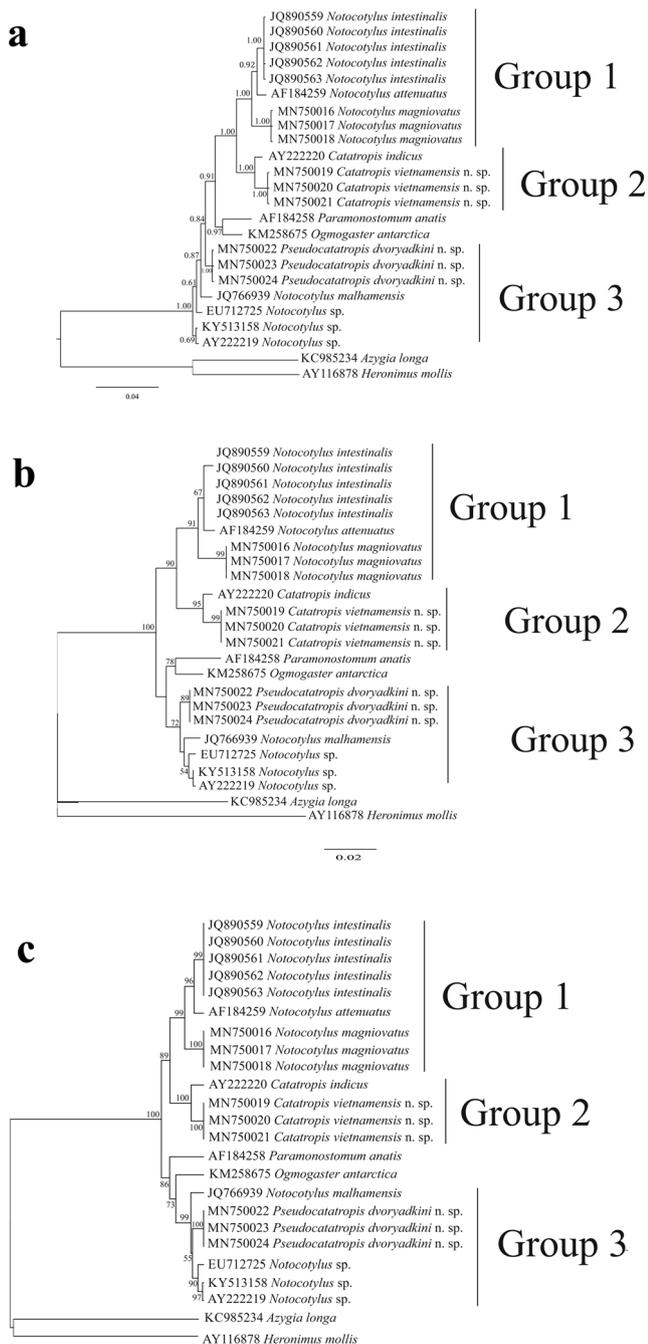


Fig. 4 The phylogeny based on the 28S rRNA gene sequences using. **a** Bayesian algorithm. **b** Maximum likelihood algorithm. **c** UPGMA. Posterior probability ≥ 50 (nodal support of ≥ 0.50) is shown in the nodes, respectively. The outgroup species are listed in Table 1

with group 3, whereas according to the Bayesian tree, they form a common cluster with groups 1 and 2 (Fig. 4a). Genetic distances between groups 1, 2, and 3 vary from 2.7 to 3.3%. The genetic distance between *Paramonostomum anatis* Garkawi, 1965 and *Ogmogaster antarctica* Johnston, 1931 is 2.6%. The genetic distances between representatives of *Paramonostomum* and *Ogmogaster* and the species of groups 1 and 2 (3.6–4.0%) are higher than the distances between

these genera and the species of group 3 (2.2–2.7%). Within groups 1, 2, and 3, the genetic distances do not exceed 0.8%. Genetic distances with outgroup species are 17.7–18.3% (Table 5).

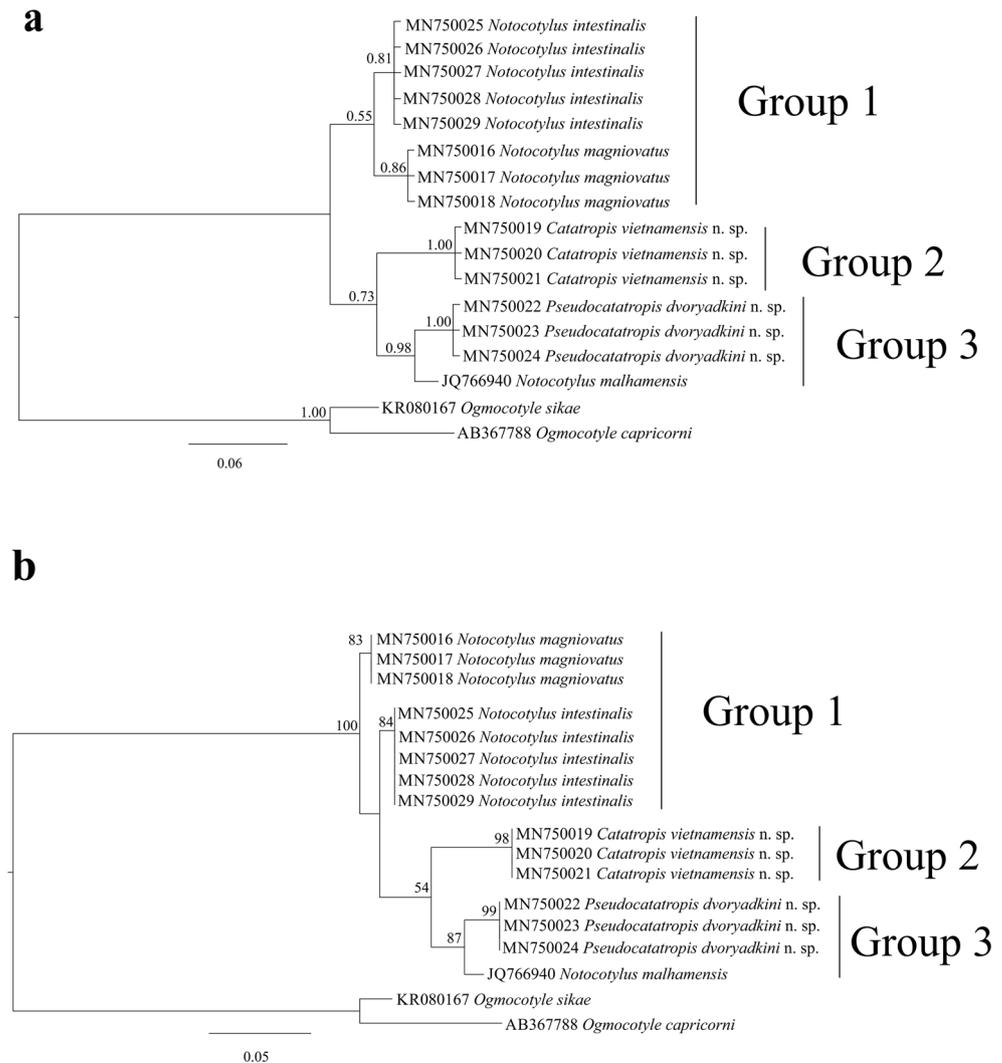
Phylogenetic reconstruction based on sequencing data of *ITS2* using the Bayesian method shows that *N. magniovatius* and *N. intestinalis* were grouped together (group 1), species *C. vietnamensis* n. sp. forms a separate cluster (Group 2), and representatives of *P. dvoryadkini* n. sp. and *N. malhamensis* are united in one cluster (group 3) (Fig. 5a). Genetic distances between groups varied from 5.7 to 6.8%. The distance between *N. magniovatius* and *N. intestinalis* was 1.0%, between *P. dvoryadkini* n. sp. and *N. malhamensis* was 1.1%. The genetic distances between all analyzed species and outgroup, including two species of *Ogmocotyle* Skrjabin and Schulz, 1933, were more than 23% (Table 6). Groups 2 and 3 were also presented on the tree based on the maximum likelihood algorithm. At the same time, sequences of *N. magniovatius* form an external group (Fig. 5b).

Discussion

Adult flukes obtained from cercariae from the snails *P. subtegulata* (Cerithioidea Fleming) are identical to *N. magniovatius* according to the morphometric indices of different developmental stages. In the Russian southern Far East, flukes of this species at different developmental stages were previously reported by Alekseev (1970), Dvoryadkin (1989), Besprozvannykh (2000), and others. The affiliation of *N. magniovatius* flukes is also confirmed by the utilization of *Parajuga* spp. snails as the first intermediate hosts in their life cycle (Dvoryadkin 1989; Besprozvannykh 2000). These snails are closely related to *Semisulcospira* spp. and *Melanoides obliquigranosa* Smith, 1878 (Cerithioidea), in which trematodes of this species being found in Japan, Korea, and the Philippines (Ito 1964). However, it should be noted that the status of the *Parajuga* genus is still being discussed and requires additional research (Strong, Köhler 2009; Köhler 2017).

C. vietnamensis n. sp. is the first trematode from this genus for which *Melanoides* Olivier, 1804 snails (Cerithioidea) serve as an intermediate host. Prior to this, as mentioned above, only one trematode from Notocotylidae, *N. magniovatius*, was known, in which Cerithioidea mollusks are involved in the life cycle. Other *Catatropis* species for which life cycles have been studied use as intermediate hosts branchial snails, Bithyniidae Gray, 1857 and Hydrobiidae Stimpson, 1865, as well as pulmonate snails of the families Planorbidae Rafinesque, 1815 and Chiliniidae Dall, 1870 (Flores and Brugni 2006). Among *Catatropis* species, only adult flukes of *C. indicus*, *Catatropis pricei* Harwood, 1939, *Catatropis harwoodi* Bullock, 1952, *Catatropis chinensis* Lai, Sha, Zang

Fig. 5 The phylogeny based on the ITS2 rDNA sequences using **a** Bayesian algorithm. **b** Maximum likelihood algorithm. Posterior probability ≥ 50 (nodal support of ≥ 0.50) is shown in the nodes, respectively. The outgroup species are listed in Table 1



et Yang, 1984, *Catatropis misrai* Gupta & Sing, 1984, *Catatropis poecylorhynchai* Gupta & Sing, 1984, and *Catatropis pakistanensis* Schuster and Wibbelt, 2012 (Bullock 1952; Lai et al. 1984; Gupta and Sing 1984;

Schuster and Wibbelt 2012), as in *C. vietnamensis* n. sp., have a genital pore situated anterior to the intestinal bifurcation. Judging by the number of papillae in each lateral row, *C. vietnamensis* n. sp. are close to *C. harwoodi* (7–9 papillae)

Table 5 Genetic distances based on partial nucleotide sequences of the 28S rRNA gene

Groups	Distances between species					Distances within species
	Group 1	Group 2	Group 3	<i>Paramonostomum anatis</i>	<i>Ogmogaster antarctica</i>	
Group 1	–	–	–	–	–	0.008
Group 2	0.027	–	–	–	–	0.005
Group 3	0.031	0.033	–	–	–	0.007
<i>Paramonostomum anatis</i>	0.038	0.040	0.027	–	–	n/c
<i>Ogmogaster antarctica</i>	0.036	0.037	0.022	0.026	–	n/c
Outgroup	0.183	0.182	0.177	0.182	0.179	0.176

Group 1: *Notocotylus intestinalis*, *Notocotylus magniovatus* and *Notocotylus attenuatus*. Group 2: *Catatropis vietnamensis* n. sp. and *Catatropis indicus*. Group 3: *Pseudocatatropis dvoryadkini* n. sp. *Notocotylus* sp. 1, *Notocotylus* sp. 2 and *Notocotylus* sp. 3. Outgroup: *Heronimus mollis*, *Azygia longa*. The presence of n/c in the results denotes cases in which it was not possible to estimate evolutionary distances

Table 6 Genetic distances based on complete nucleotide sequences of the ITS2 rDNA

Groups	Distances between species			Distances within species
	Group 1	Group 2	Group 3	
Group 1	–	–	–	0.012
Group 2	0.072	–	–	0.000
Group 3	0.061	0.062	–	0.000
Outgroup	0.230	0.232	0.230	0.072

Group 1: *Notocotylus intestinalis* and *Notocotylus magniovatus*. Group 2: *Catatropis vietnamensis* n. sp. Group 3: *Pseudocatropis dvoryadkini* n. sp. Outgroup: *Ogmocotyle sikae*, *Ogmocotyle capricorni*

and *C. pakistanensis* (9–10 papillae). However, in contrast to both of these species, *C. vietnamensis* n. sp. has the following features: ridge anteriorly reaches the level of the internal seminal vesicle vs. ridge just behind the cirrus sac; metraterm reaches 1/3 length of the cirrus sac vs. metraterm is longer or equal to cirrus sac; anterior pair of lateral papillae far anterior from vitellarium vs. this pair of papillae being located immediately posterior to the cirrus sac (*C. harwoodi*) or at the level of the vitellarium (*C. pakistanensis*). The results of studying the morphology of the developmental stages (Figs. 1d–f, 2c, d, and 3d–f) indicate that the notocotylids from Vietnam, which used *Melanoides tuberculata* as their intermediate hosts, belong to a new *Catatropis* species, *C. vietnamensis* n. sp. Genetic data also indicate that this species belongs to the genus *Catatropis*, because it is clustered with *C. indicus*.

Another notocotylid trematode, which was found in the territory of the Russian southern Far East and designated by us as *P. dvoryadkini* n. sp., is similar to *Pseudocatropis joyeuxi* Kanev, Vassilev, 1986, based on the features of the life cycle. *P. joyeuxi* was found in Europe, where its life cycle is carried out using snails, *Anisus leucostomus* (Millet, 1813), *Segmentina nitida* (Muller, 1774), and *Gyraulus albus* (Mueller, 1774) (Pullomonata, Planorbidae), as intermediate hosts (Odening 1966). In the life cycle of both trematode species, there are short-tailed, no-eyed, unpigmented cercariae, which do not leave the intermediate host and are encysted there. In the Russian southern Far East, notocotylids with this development type were first recorded by Dvoryadkin (1989) and named *Catatropis joyeuxi*. However, the description of developmental stages, including adult flukes, was not given. The presence of six to nine papillae in each of the lateral rows in specimens was only referred to for mature worms. The trematodes discovered by us, as well as those found by Dvoryadkin, are circulated with the involvement of *Helicorbis suifunensis* snails. Based on the common location of the finding, the identity of the life cycle, and a single intermediate host of these trematodes, we believe that the flukes in our study and in Dvoryadkin's material belong to the same species.

In addition to the similarity of life cycles, *P. dvoryadkini* n. sp. and *P. joyeuxi* have similar morphometric indices of cercariae and adult flukes. There are insignificant differences in the number of lateral papillae in each row for adult flukes: 6–9 (Dvoryadkin 1989) and 6–7 (our data) for *P. dvoryadkini* n. sp. and 7–10 for *P. joyeuxi* (Kanev et al. 1994). At the same time, the metric indicators of cercariae and metacercariae of *P. joyeuxi* are equal according to Joyeux (Joyeux 1922, citation to Skrjabin 1953), and greater according to Odening (1966) than those of *P. dvoryadkini* n. sp. (Table 3). In the presence of morphological similarity of flukes at different developmental stages, there is spatial isolation between the European and Asian populations of these trematodes, which is caused by the absence of interrelations between the animals participating in the life cycles of flukes in these territories. The isolation of snails is not in doubt, and ducks (the definitive hosts of the flukes) from these territories do not have common nesting locations and wintering grounds. Following on from this, two variants of the taxonomic status are possible for the examined trematodes from the European and East Asian populations. They can be either representatives of one or different species. The solution to this issue will remain open until genetic data for *P. joyeuxi* are obtained. In the meantime, taking into account the above, as well as the intermediate hosts of European and Asian populations of flukes belonging to different genera, *Anisus* Studer, 1820, *Segmentina* Fleming, 1818, *Gyraulus* Charpentier, 1837 for *P. joyeuxi* and *Helicorbis* Benson, 1855 for *P. dvoryadkini* n. sp., we believe it is advisable to consider the trematode found in the Russian southern Far East as a new species of genus *Pseudocatropis* Kanev, Vassilev, 1986. In 2005, Barton and Blair (2005) felt that data on the morphology of developmental stages and the life cycle of *P. joyeuxi* were not sufficient to isolate a new genus and reduced *Pseudocatropis* to a synonym for *Catatropis*. However, when analyzing the obtained genetic data, a stable tendency was noted in the division of *C. vietnamensis* n. sp. and *P. dvoryadkini* n. sp. into different clades, which is confirmed by trees, constructed using both 28S and ITS2 rDNA (Fig. 4). According to the tree based on 28S, the clade with *C. vietnamensis* n. sp. includes another *Catatropis* species, *C. indicus*. At the same time, genetic distances between species from groups 1, 2, and 3 based on the nucleotide sequences of 28S are higher than the distances between representatives of the *Ogmogaster* and *Paramonostomum* genera. Thereby, the distances between the obtained groups constitute a generic level. The results of genetic studies obtained in this research confirm the correctness of isolating notocotylids with shorter life cycles from *Catatropis* into a separate genus, *Pseudocatropis*, which was proposed previously by Kanev and Vassilev (1986).

28S, ITS, and 18S rDNA are the most commonly used markers to construct phylogenetic relationships of trematodes (Nolan and Cribb 2005). However, the 28S and ITS

rDNA sequences have a higher rate of change in the variable sites than the 18S rRNA gene, which allows systematic problems of the different taxa at the species and generic levels to be solved. The 18S marker is considered more conservative, and for this reason, it is more often used to separate species at the level of families and higher (Hillis and Dixon 1991). For this reason, less conservative markers, nucleotide sequences of 28S and ITS2 of ribosomal DNA, were used in this study for the analysis of phylogenetic relationships of Notocotylidae. Genetic data showed that species with three rows of papillae, *N. attenuatus*, *N. intestinalis*, and *N. magnioartus*, were combined in a single cluster; species with two rows of papillae and ridge, *C. indicus* and *C. vietnamensis* n. sp., were included into the other cluster. At the same time, the third cluster included flukes with the first and second types of these formations: *P. dvoryadkini* n. sp., *Notocotylus* sp. 1, *Notocotylus* sp. 2, *Notocotylus* sp. 3, and *N. malhamensis* (Figs. 4 and 5). This indicates that the main morphological criteria for separating the representatives of *Notocotylus* and *Catatropis* genera, namely, the presence of three rows of glandular papillae or a continuous median ridge and two rows of lateral papillae on the ventral side of the body, are not determinate in establishing the fluke affiliation to a particular genus. The data obtained indicate that revision of the Notocotylidae family is needed, but further morphological and molecular studies are required for this group of flukes.

Funding information This work was partially supported by the Program of fundamental research of the Far Eastern Branch of Russian academy of Sciences «Far East» 2018–2020, project number 18–4–039 and the Vietnam’s National Foundation for Science and Technology Development (NAFOSTED), grant number 106.05-2018.17.

Compliance with ethical standards

Euthanasia of laboratory animals was carried out in accordance with the Committee on the Ethics of Animal Experiments of the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Russia.

Conflict of interest The authors declare that they have no conflicts of interest.

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