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Description of a new species, *Cryptocotyle lata* sp. nov., and discussion of the phylogenetic relationships in Opisthorchioidea

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ABSTRACT

Adult *Cryptocotyle lata* sp. nov. worms were obtained from experimental studies. In the Russian southern Far East, the life cycle of this parasite is carried out using freshwater snails (*Boreoelona ussuriensis*), freshwater fish, and birds as the first intermediate, second intermediate, and definitive hosts, respectively. The morphological indices of *C. lata* sp. nov. are closest to *Cryptocotyle concava*; however, these two species differ in terms of their sizes of body, oral and ventral suckers, eggs, and the shape of their testes and ovaries. Analysis of the life cycles of the *Cryptocotyle* representatives suggested that *C. concava* were at least two cryptic species, one of which circulates using brackish water *Hydrobia* snails, and the other using freshwater *Amnicola* snails as the first intermediate hosts. Molecular data (i.e., the 28S gene and the second internal transcribed spacer (ITS2) of rDNA) were used to analyze the phylogenetic relationships of *C. lata* sp. nov. and other representatives of Opisthorchioidea. The long repeats and secondary structure of the ITS1 region were studied. Representatives of the Opisthorchiidae and several species from Heterophyidae (including the genus *Cryptocotyle*) were found to have molecular features that suggested that these species belonged to Opisthorchiidae. At the same time, the genetic relatedness of worms, which are united in common clusters on phylogenetic trees, is consistent with the use of the first intermediate hosts from different taxonomic groups in their life cycles; namely, snails of the Truncatelloidea are hosts of trematodes from a cluster with Opisthorchiidae and a number species of the family Heterophyidae, while snails of the Cerithioidea are hosts of worms from a cluster that includes only the Heterophyidae. In addition, the results of genetic studies indicate that *Clonorchis sinensis*, *Metorchis ussuriensis, Metorchis bilis*, *Metorchis xanthosomus*, and *Metorchis orientalis* should be included in the genus *Opisthorchis*.

1. Introduction

Representatives of the genus *Cryptocotyle* Lühe, 1899 are parasites in the intestines of birds and mammals. The genus includes eight valid species, of which *Cryptocotyle lingua* (Creplin, 1825), *Cryptocotyle jejuna* (Nicoll, 1907), *Cryptocotyle badamshini* (Kurochkin, 1958), and *Cryptocotyle delamurei* (Jurachno, 1987) circulate using the inhabitants of marine or brackish water environments [1–4]. *Cryptocotyle thapari* uses *Lutra longicaudis* Olfers, which is an animal associated with the freshwater environment [5]. There is currently insufficient data to confirm the involvement of the inhabitants of marine or freshwater environments in the life cycles of *Cryptocotyle quinqueangularis* (Skrijabin, 1923) and *Cryptocotyle cryptocotyloides* (Issaitschikoff, 1923). Only two species, *C. lingua* and *Cryptocotyle concava* (Creplin, 1825), have had their life cycles studied in addition to analysis regarding the morphology of their developmental stages [1,6]. *C. lingua* is a cosmopolitan species that uses snails of the family Hydrobiidae Stimpson and various euryhaline fish as the first and second intermediate hosts, respectively. The definitive hosts for this species include piscivorous birds. In Europe, these same hosts are involved in the circulation of *C. concava* [7–9]. In North America, *C. concava* worms were discovered in freshwater snails of the family Amnicolidae Tryon and freshwater fish, which were reported as the first and second intermediate hosts, respectively [6,10]. Adult worms of this species were also found in *Felis catus* in Korea [11].

Genetic data are only available for one representative of the genus, *C. lingua* [12–17]. GenBank contains nucleotide sequences of the 28S and 18S genes and the ITS region of nuclear rDNA, as well as the *cox1* gene of mitochondrial DNA.

Cercariae belonging to the Pleurolophocerca group from *Boreoelona ussuriensis* Ehrmann snails were collected in the dead stream branch of the Bolshaya Ussurka River in the southern part of the Russian Far East. Further experimental studies on the life cycle and genetic data of this trematode showed that it belonged to a new species, *Cryptocotyle lata*

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sp. nov. The results of these investigations and discussion of the phylogenetic relationships of *C. lata* sp. nov. with other representatives of Opisthorchioidea Loos, 1899 are presented below.

2. Materials and methods

In this study, 10 adult worms, 10 rediae, 10 cercariae, and 10 metacercariae of *C. lata* sp. nov. were used for morphological analysis. Holotype No 128–Tr, paratypes No 129–137–Tr are held in the parasitological collection of the Zoological Museum (Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia); e-mail: petrova@ibss.dvo.ru. Deposited 2017.11.20. Two individuals were analyzed using nucleotide sequences of ITS1-5.8S-ITS2 (*ITS*) and 28S rRNA gene (*28S*). The data were not published earlier.

2.1. Life-cycle and morphology of worms

The freshwater snail, *B. ussuriensis*, was collected in the dead stream branch in the middle reaches of the Bolshaya Ussurka River (Primorsky Region, Russia). It was naturally infected by rediae and emitted cercariae of the Pleurolophocerca group. To establish the second intermediate hosts, experimental infection with cercariae were conducted using *B. ussuriensis* snails (ten specimens), tadpoles of *Rana dybowskii* (Guenther) (ten specimens), and *Rhynchocypris percnurus mantschuricus* (Berg) fish (eight specimens). *B. ussuriensis* snails were grown in laboratory conditions from clutches, while the tadpoles and young fish were caught in a pond that was free from metacercariae (the presence of metacercariae was examined in 30 specimens of fish and tadpoles). The animals were placed separately in 0.5 (snails) or 1.5 (tadpoles and fish) L vessels for the infection period. Water from Petri dish containing a *Boreoelona* snail that was emitting Pleurolophocerca cercariae was poured into the animal-containing vessels for 3 days. Presence of cercariae in the Petri dish was checked under a binocular microscope. After the infection period, animals were kept in separate aquaria at a water temperature of 18–22 °C. At autopsy of the experimental animals, it was established which of them were infected with metacercariae. To obtain adult worms, the infected animals were fed to two ducklings that were hatched from eggs under laboratory conditions.

The ducklings were dissected on day eight post-infection, and adult flukes were detected in their intestines. After the completion of the life-cycle experiment, the snail that emitted the cercariae was crushed for the study of rediae. Euthanasia of laboratory animals was carried out in accordance with the Committee on the Ethics of Animal Experiments of Federal Scientific Center of the East Asia Terrestrial Biodiversity, Russia (Permit Number: 3 of 02.06.2011).

Rediae and metacercariae were measured on live specimens, while cercariae were fixed in hot 4% formalin before measurement. The detection of sensillae on the cercariae body was performed using the method of Ginetsinskaya and Dobrovolskij [18], while the position was described according to Richard [19]. Adult flukes were fixed in 70% ethanol and stored in 96% ethanol. Whole mounts of adult flukes were prepared by staining with alum carmine, dehydrating in a graded ethanol series, clearing in clove oil and mounting in Canada balsam. All measurements were given in millimeters (mm).

2.2. Genetic analysis

The complete sequences of the ITS1-5.8S-ITS2 rDNA and partial sequences of the 28S rRNA gene from two samples of adult *C. lata* sp. nov. were used to genetically identify the new species. Nucleotide sequences used in this study are listed in Table 1. Information about the DNA extraction, the primers for amplification and sequencing, the composition of the reaction mixture, and the PCR cycling conditions are reported in Shumenko et al. [20]. The nucleotide sequences were assembled manually in MEGA version 5.03 [21]. The nucleotide composition of the obtained sequences, the amount and type of nucleotide substitutions, and the *p*-distances between species were also analyzed using the same program. Phylogenetic reconstructions used aligned sequences of 283 and 1160 bp from ITS2 and 28S, respectively.

Table 1
List of analyzed sequences. *n* – number of sequences; accession numbers in bold are newly determined sequences; underlined species were attributed to Opisthorchiidae according to the results of this study.

Family/species	GenBank accession number		
	ITS1-5.8S*/ITS1	ITS2	28S
Opisthorchiidae			
Cryptocotyle lata sp. nov.	**MH025622***	**MH025622**	**MH025622**
	MH025623*	**MH025623**	**MH025623**
Cryptocotyle lingua	KJ641518	KJ641518	AY222228
	KJ641521*	KJ641521	
	KJ641523*	KJ641523	
Clonorchis sinensis	MF319617	KJ137228	JF823989
Metorchis bilis	EU038154	KT740976	–
Metorchis albidus	–	JF710316	–
Metorchis xanthosomus	JQ716400	KT740977	–
Metorchis orientalis	HM347228	KX832894	–
Opisthorchis viverrini	EU038152	AY584735	HM004188
Opisthorchis felineus	EU038139	EF688141	MF099790
Apophallus muehlingi	MF438049	MF438049	–
Apophallus donicus	MF438056	MF438056	–
Euryhelmis costaricensis	AB521798	AB521800	AB521800
Euryhelmis zelleri	–	KM594133	–
Euamphimerus pancreaticus	–	KT740984	–
Amphimerus sp.	–	AB678442	–
Amphimerus ovalis	–	–	AY116876
Heterophyidae			
Phagicola longa	AY245703	AY245703	–
Pygidiopsis genata	AY245710	AY245710	–
Centrocestus sp.	AY245699	AY245699	–
Centrocestus formosanus		–	HQ874609
Haplorchis taichui	KX815126	KX815126	HM004181
Haplorchis yokogawai		HM004158	HM004177
Haplorchis pumilio	KX815125	KX815125	HM004173
Procerovum cheni		HM004164	HM004193
Procerovum varium		HM004169	HM004182
Metagonimus katsuradai		KM061400	KM061391
Metagonimus otsurui		KM061403	KM061394
Metagonimus suifunensis	KX387461	KX387461	KX387456
Metagonimus yokogawai	KJ631740	HQ832621	HQ832639
Metagonimus takahashii		HQ832620	HQ832636
Metagonimus hakubaensis		KM061397	KM061388
Metagonimus miyatai		HQ832615	HQ832633
Metagonimoides oregonensis		–	JQ995473
Galactosomum lacteum		–	AY222227
Stellantchasmus falcatus		KJ630833	HM004176
Acanthotrema tridactyla	KF447592	KF447593	
Cryptogonimidae			
Acanthostomum burminis	–	–	KC489791
Gynichthys diakidnus	FJ907332	FJ907332	–
Siphoderina subuterus	EU571252	EU571252	–
Siphoderina grunnitus	EU571257	EU571257	–
Siphoderina jactus	EU571253	EU571253	–
Siphoderina virga	EU571258	EU571258	–
Caulanus thomasi	EF428141	EF428141	–
Beluesca longicolla	EF566871	EF566871	–
Retrovarium valdeparvum	EF116627	EF116639	–
Retrovarium formosum	EF116625	EF116637	–
Varialvus lacertus	HM187780	HM187780	–
Siphomutabilus gurukun	KF417628	KF417628	–
Mitotrema anthostomatum	–	–	AY222229
Adlardia novaecaledoniae	–	–	FJ788496
Siphodera vinaledwardsii	–	–	AY222230
Caecincola parvulus	–	–	AY222231
Outgroup			
Paragonimus paishuihoensis	–	AB679285	–
Paragonimus kellicotii	–	–	HQ900670
Homalometron armatum	–	–	KC710975

The Bayesian inference (BI) method in MrBayes version 3.1.2 [22] was used to reconst interspecific phylogenetic trees. According to the Akaike criteria in Modeltest version 3.7 [23], TVM + G and TVM + I + G were the optimal models for determining genetic distances for the ITS2 region and 28S rDNA sequences, respectively. The BI analysis was performed using 1,100,000 and 200,000 generations of Markov chain Monte Carlo (MCMC) for the ITS2 region and 28S rRNA gene, respectively. This number of generations was sufficient as the SD value was < 0.01. A total of 25% samples were excluded to construct the consensus trees. The chain was sampled every 100th generation. *Predicted s*econdary structures of the ITS1 transcripts of *C. lata* sp. nov. and *C. lingua* were created using Mfold version 3.0 (http://mfold.rit.albany.edu, [24]), and long repeats were identified in UGENE version 1.10 (http://ugene.unipro.ru).

3. Results

3.1. Morphological description

Cryptocotyle lata sp. nov.

Definitive host: *Anas platyrhynchos* dom. (experimentally).

Site: small intestine.

Intensity of infection: 25 and 57 individuals.

First intermediate host: *Boreoelona ussuriensis*.

Second intermediate host: *Rhynchocypris percnurus mantschuricus, Rana dybowskii* (experimentally).

Type-locality: the Bolshaya Ussurka River, Primorsky Region, the Russian southern Far East; 45°57′N, 133°53′E.

Type-deposition: Holotype No. 128–Tr, paratypes No. 129–137–Tr. This material is held in the parasitological collection of the Zoological Museum (Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia); e-mail: petrova@ibss.dvo.ru. Deposited 2017.11.20.

Etymology: The species is named due to its large body width. The width of some specimens exceeds their length.

3.1.1. Adult worm (material examined: 10 specimens) (Fig. 1A, B; Fig. 2A–C; Table 2)

Body trapezoidal, length greater or equal to or less than width. Maximum body width at level of testes. Posterior end of body at median line, concave. Tegument covered with spines to level of testes. Anterior part of body with numerous gland-cells. Ducts of these gland-cells open on ventral surface of body. Oral sucker subterminal, spherical or transversaly oval. Prepharynx very short, pharynx spherical, esophagus slender, bifurcating at border between anterior and middle thirds of body. Caeca slender, laterally relative testes, at the level of the posterior edge of testes turn into the median line. At this point intestinal branches follow curvature of body, but never come in contact with excretory vesicle. Ventral sucker rudimentary, at level of anterior edge of ventrogenital sac, covered by tegument. Two testes opposite, transversely oval, with 3–4 large lobes, closely located posterior end of body, separated from each other by excretory bladder. Ventrogenital sac in middle third of body, surrounded by glandular cells. Genital pore opened ventrally, at level of anterior edge of ventrogenital sac. Seminal vesicle elongated, S-shaped. Ovary triangular, transversely elongated, consists of 3–4 large lobes, adjacent to anterior edge of right testis. Uterus short, located between ovary and ventrogenital sac. Eggs at various stages of embryonic development, elliptical, slightly pointed at opercular end, with small knob at opposite end. Surface of eggs with

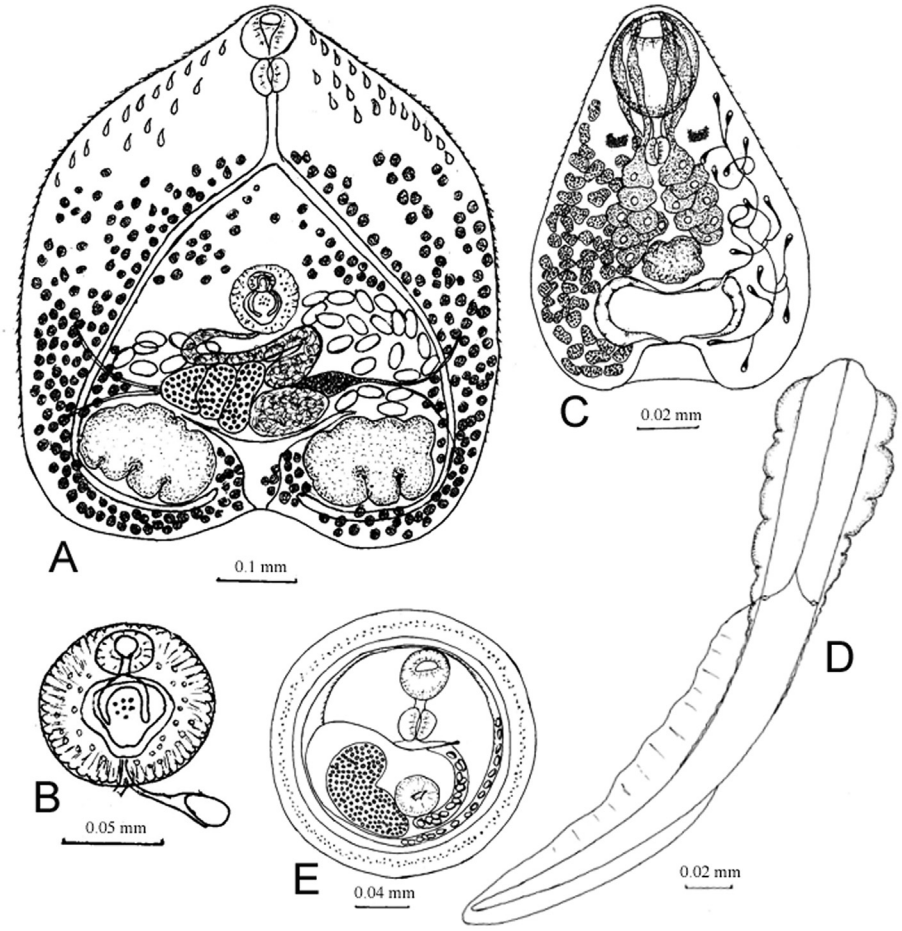


Fig. 1. *Cryptocotyle lata* sp. nov.: (A) adult worm; (B) ventrogenital sac; (C) body of cercaria; (D) tail of cercaria; (E) metacercaria.

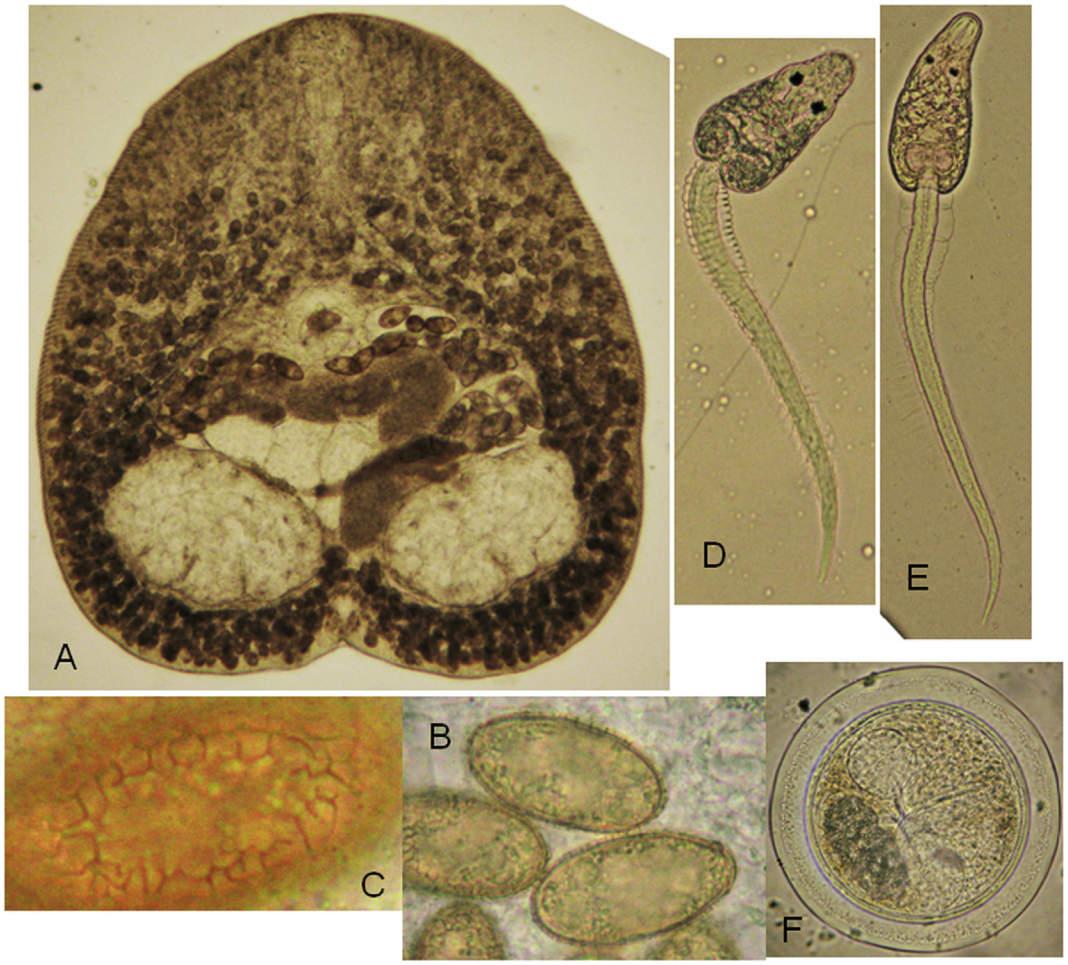


Fig. 2. *Cryptocotyle lata* sp. nov.: (A) adult worm; (B) eggs; (C) surface of egg with numerous folds; (D) cercaria immediately after emission from the snails; (E) cercaria with "Cobra-like" hood on the tail; (F) metacercaria.

numerous folds. Vitelline follicles laterally from level of caecal bifurcation to posterior end of body, almost contiguous in post-testicular region. Vitelline reservoir, seminal receptacle and Mehlis' gland between ovary and left testis. Excretory vesicle Y-shaped.

3.1.2. Redia (based on 10 specimens)

Body elongated, 0.70–1.20 × 0.10–0.20. Pharynx 0.025–0.036 in diameter. Caecum short, sac-shaped.

3.1.3. Cercaria (based on 10 specimens) (Fig. 1C, D; Fig. 2D, E; Fig. 3)

Body 0.123–0.139 × 0.092–0.100, elongated, triangle, covered with fine spines from anterior end to middle part. Two pigment eye-spots on both sides of body, on dorsal side, at short distance from posterior border of oral sucker. Oral sucker 0.027–0.031 × 0.031–0.039 subterminal, prepharynx short, pharynx 0.0077–0.0116 spherical, at level of eye-spots, Esophagus and intestine absent. Ventral sucker not developed. Primordium of genital system in posterior third of body. Penetrating glands 14, 7 on each side of median line of body, pre-primordium of genital system. Ducts of these glands open anterior to oral sucker according formula 3 + 4 + 4 + 3. Cystogenous glands on both sides of body, from level of pigment eye-spots to posterior end of body. Excretory vesicle Y-shaped with thick walls. Flame cell formula: 2[(3 + 3) + (3 + 3 + 3)] = 30. Tail 0.331–0.362 × 0.027–0.031, inserted deeply in tail socket, provided with dorsoventral finfold. Finfold begins on dorsal side of middle part of tail, extending around its end, on ventral side of tail reaches 1/3 of its length. Tegument of tail at anterior 1/3–1/2 length can form "Cobra-like" hood. "Cobra-like" hood forms after a certain amount of time after leaving the mollusk (Fig. 2, D, E). Formula of sensory structures: CI = 4 V_1; CII = 1 V_1, 1 V_2, 1 V_3, 6D; CIII = 1 V_1, 1 V_2, 1 V_3, 2 V_4, 1D, 2 L; CIV = 3 V_1, 1 V_2, 2D, 5 L; AI = 3 V, 4D, 5 L; AII = 2 V, 1D, 4 L; AIII = 2 V, 2D, 3 L; M = 2D, 4 L; *P* = 4PIL, 2PIIL, 2PIIIV, 2PIIID, 2PIIIL; U = 3 L.

3.1.4. Metacercaria (based on 10 specimens) (Fig. 1E; Fig. 2F)

Cyst 0.181–0.231 in diameter. Cyst shell multi-layer, 0.015–0.031. Body covered with fine spines. Oral sucker 0.031–0.039 × 0.039–0.042, pharynx spherical, 0.027–0.031 in diameter. Caecal cavity filled with disc-shaped granules. Same granules are in cavity of cyst. Primordium of ventrogenital sac 0.027–0.031 in diameter. Excretory bladder Y-shaped, filled with granules.

3.1.5. Life cycle

Experimental studies showed that fish and frogs are second intermediate hosts of *C. lata* sp. nov.; however, the metacercariae were not obtained from snails. All experimental animals were infected (100%) by *C. lata* sp. nov. metacercariae with an intensity of 10–25 metacercariae per fish and 20–39 metacercariae per tadpole. Trematodes were located in the muscles of fish and in the muscle and surface tissues of internal organs in tadpoles. It was observed that after emission from the snails cercariae actively moved within 20–30 min in a water column without a resting phase. During this period, if cercariae encountered a second intermediate host, they did not show infective activity. After this period, the active movements of cercariae alternated with phases of rest

Table 2
Measurements (mm) of adult *Cryptocotyle* worms.

	Cryptocotyle lata sp. nov. (present study)			*C. concava* (Ransom, 1920 cited in [5])	*C. concava* (Issaitschikoff, 1925 cited in [2])	*C. concava* [11]	*C. concava* [10]	*C. quinqueangularis* [2]	*C. cryptocotyloides* [25]
	Holotype	Range (n = 10)	Mean						
Body L	0.693	0.554–0.739	0.656	**1.00**	**0.769–1.00**	0.500–**1.170 (0.778)**	**0.418–0.585**	**0.85**	**1.039–1.092**
Body W	0.616	0.570–0.770	0.644	**0.850**	0.678–0.791	0.468–0.732 (0.576)	**0.240–0.313**	0.74	0.625–0.689
Forebody	0.347	0.231–0.347	0.282	–	–	–	–	–	–
Body L/forebody ratio	1:1.125	1:0.900–1.216	1:1.023	–	–	–	–	1:1.149	–
Oral sucker L	0.065	0.050–0.073	0.062	0.060–0.087	0.064–0.079	0.050–0.084 **(0.075)**	**0.034–0.039**	0.060	0.064–0.074
Oral sucker W	0.073	0.058–0.085	0.069	0.060–0.087	0.074–0.084	0.070–0.105 **(0.089)**	**0.034–0.039**	0.060	0.074–0.085
Ventral sucker L	0.027	0.023–0.039	0.028	–	**0.054**	–	–	–	–
Ventral sucker W	0.031	0.027–0.039	0.031	–	**0.067**	–	–	–	–
Suckers L ratio	1:2.41	1:1.77–2.56	1:2.24	–	–	–	–	–	–
Suckers W ratio	1:2.35	1:1.97–2.43	1:2.21	–	–	–	–	–	–
Pharynx L	0.050	0.039–0.062	0.048	0.060	–	**0.028**–0.049 **(0.036)**	**0.024–0.036**	0.046	0.047
Pharynx W	0.050	0.039–0.062	0.049	0.050	–	**0.025**–0.055 **(0.043)**	**0.024–0.031**	0.046	0.047
Esophagus L	0.077	0.035–0.096	0.059	0.095	–	0.063–**0.230 (0.114)**	0.036–0.061		0.065–0.095
Testis left L	0.119	0.096–0.154	0.136	**0.300**	0.095–0.102	0.110–**0.307 (0.169)**	**0.078**–0.115	0.090–0.100	0.105–**0.187**
Testis left W	0.173	0.154–0.243	0.186	**0.300**	0.191–0.254	**0.050**–0.197 **(0.089)**	**0.036–0.073**	**0.130–0.150**	0.207–0.212
Testis right L	0.123	0.108–0.177	0.138	–	–	0.108–**0.332 (0.182)**	–	–	–
Testis right W	0.177	0.146–0.250	0.199	–	–	0.050–**0.161 (0.085)**	–	–	–
Ventrogenital sac L	0.080	0.069–0.085	0.077	**0.150–0.300**	–	**0.035**–0.088 **(0.052)**	**0.012–0.022**	**0.060**	**0.127–0.150**
Ventrogenital sac W	0.085	0.081–0.096	0.085	**0.150–0.300**	–	**0.048**–0.088 **(0.065)**	**0.012–0.022**	**0.060**	**0.127–0.150**
Ovary L	0.104	0.092–0.154	0.109	–	**0.063–0.074**	**0.038**–0.133 **(0.070)**	**0.049–0.054**	**0.040**	**0.053–0.085**
Ovary W	0.123	0.112–0.173	0.139	–	0.169–**0.233**	**0.050**–0.120 **(0.076)**	**0.049–0.054**	**0.080**	0.159
Eggs L	0.035–0.039	0.035–0.039	–	0.034–0.038	**0.027**–0.036	**0.0230–0.0281**	**0.032**	0.038	**0.040**
Eggs W	0.019–0.023	0.019–0.023	–	**0.016–0.020**	**0.018**	**0.0110–0.0166**	**0.018**	**0.015**	0.020

L – length; W – width; n – number of specimens. The significant differences in measurements between *C. lata* sp. nov. and other species are in bold.

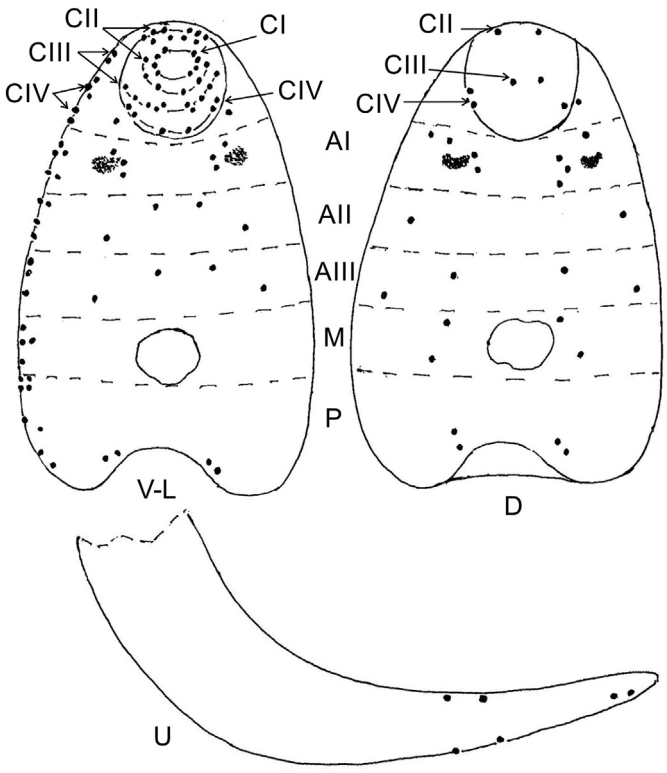


Fig. 3. Sensory structures of *Cryptocotyle lata* sp. nov. cercaria: V-L – ventrolaterally; D – dorsally; U – tail.

that were interrupted by a display of rheotaxis. If cercariae encountered the host in this period, they attached using an oral sucker and invaded into the internal tissues of the host. Cercariae penetrated fish within 20–25 min and tadpoles within 10 min. The metacercariae were well-developed larvae at 25 days after the infection of the second intermediate host. Adult worms were obtained in ducklings by feeding them the tadpoles of *R. dybowskii* and *R. percnurus mantschuricus* fish that were infected by 25-days-old metacercariae.

3.2. Genetic data

1401 bps of the 28S gene were identical between both specimens of *C. lata* sp. nov. The length of the ITS1–5.8S-ITS2 rDNA region was 1331 bp: ITS1was 891 bp in length and contained one A ↔ G transition at position 139, ITS2 was 280 bp in length and contained one A ↔ T transversion at position 180, and the 5.8S rRNA gene was 160 bp in length and did not contain any substitutions between the *C. lata* sp. nov. samples. Between the aligned ITS1–5.8S-ITS2-28S sequences (2787 bp) of *C. lata* sp. nov. and *C. lingua*, 119 variable sites were detected, of which 115 were parsimony informative (Fig. 4). In addition, seven 1-bp, three 2-bp, one 4-bp and one 89-bp indels were obtained between these species.

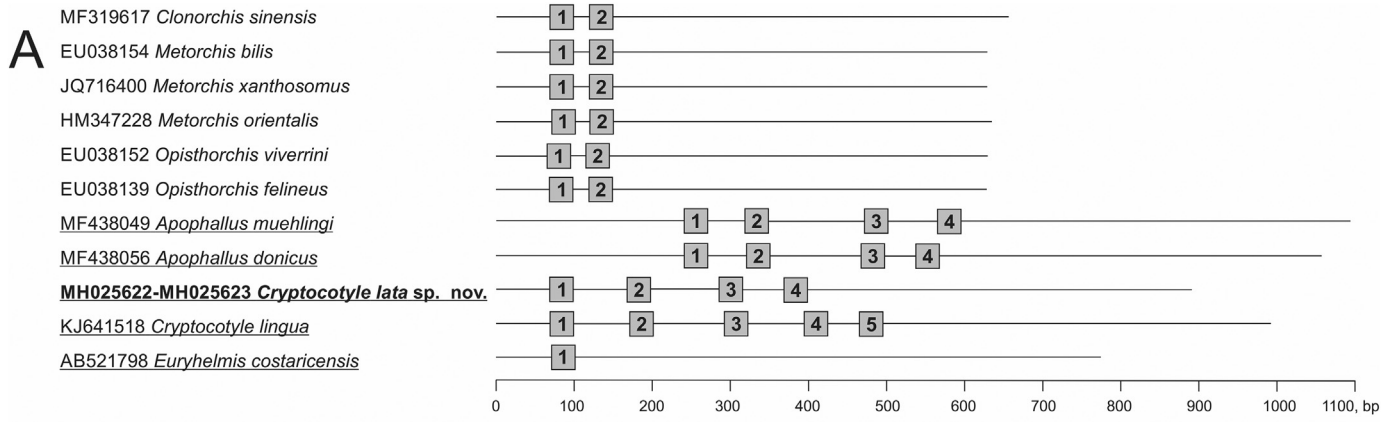
Four and five long (30 bp) repeats were detected in the ITS1 region of *C. lata* sp. nov. and *C. lingua*, respectively (Fig. 5). We have tried to relate the long repeats of the secondary structure of the ITS1 region. An additional long repeat in the ITS1 region of *C. lingua* was located in an 89-bp insertion within a region on top of helix 2; however, this did not affect the secondary structure of the ITS1 transcript, as well as the other four repeats (Fig. 6). The predicted secondary structure of the ITS1 region demonstrated a high similarity within the genus *Cryptocotyle.* The putative model includes three helices around a core structure; all of them have similar size.

Trees based on the nucleotide sequences of the 28S gene and ITS2 region showed that *C. lata* sp. nov. was clustered with *C. lingua* with high values of posterior probabilities (Fig. 7; Fig. 8). The *p*-distances between these species were 0.017 ± 0.004 and 0.020 ± 0.009 for the 28S gene and ITS2 region, respectively. Thus *C. lata* sp. nov. differs from *C. lingua* by 2% according to the data from both markers (i.e., the 28S gene and the ITS2 region).

4. Discussion

The adult worms presented in this study correspond to the genus *Cryptocotyle* in terms of their morphology and genetic characterization. Of the known species in this genus, *C. lata* sp. nov. shows the greatest morphometric similarities with *C. quinqueangularis*, *C. cryptocotyloides*, and *C. concava*. At the same time, *C. lata* sp. nov. differs from *C. quinqueangularis* as it has larger dimensions of the ventrogenital sac and ovary (Table 2) and another shape of the ovary, testes, and eggs (*C. quinqueangularis* has entire ovary, entire testes, and curved eggs). It differs from *C. cryptocotyloides* due to its smaller body length, smaller

Fig. 4. Variable sites of ITS1-5.8S-ITS2-28S rDNA region for *Cryptocotyle* species. * – KJ641521/KJ641523 and AY222228 sequences of *C. lingua* were used for ITS1-5.8S-ITS2 region and the 28S rRNA gene, respectively; the numbers at the top of figure are the nucleotide positions of substitutions; *N* – unknown nucleic acid residue; dashes mean indels; arrows show the 5′- and 3′-ends; grey boxes indicate differences within species.



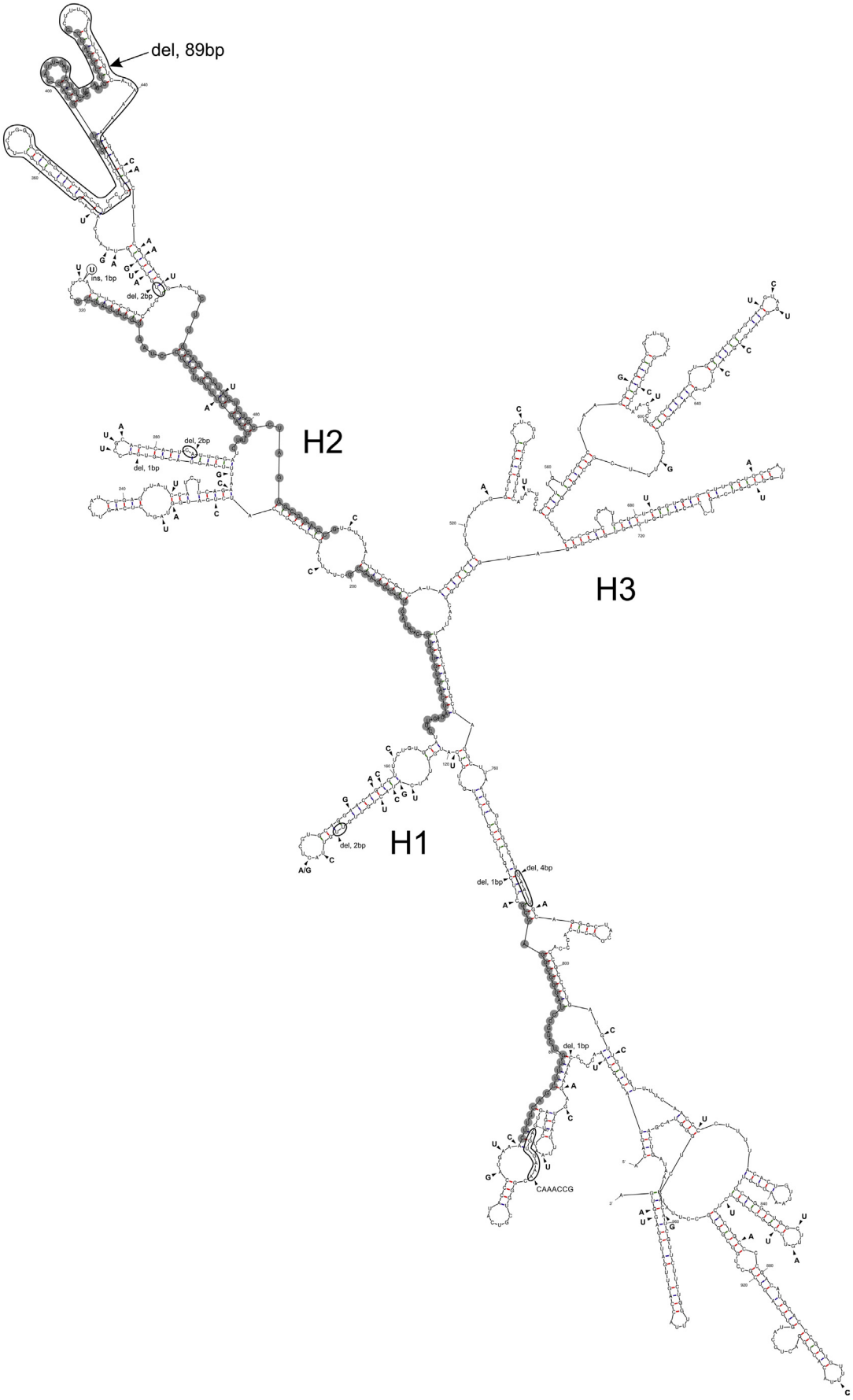
B

Species	Repeat		Position, bp	Length of ITS1 region, bp
MF319617 *Clonorchis sinensis*	1	CTTGCAGC***C***-TTG***C***CTGCCTAGGGCGGAGCG	68-97	656
	2	CTTGCAGC***A***-TTG***T***CTGCCTAGGGCGGAGCG	118-147	
EU038154 *Metorchis bilis*	1	CTTGCAGC***C***-TTG***A***CTGCCTAGGGCGGAGCG	68-97	629
	2	CTTGCAGC***A***-TTG***T***CTGCCTAGGGCGGAGCG	118-147	
JQ716400 *Metorchis xanthosomus*	1	CTTGCAGC***C***-TTG***A***CTGCCTAGGGCGGAGCG	68-97	629
	2	CTTGCAGC***A***-TTG***T***CTGCCTAGGGCGGAGCG	118-147	
HM347228 *Metorchis orientalis*	1	CTTGCAGC***C***-TTG***C***CTGCCTAGGGCGGAGCG	71-100	634
	2	CTTGCAGC***AA***TTG***T***CTGCCTAGGGCGGAGCG	120-150	
EU038152 *Opisthorchis viverrini*	1	CTTGCAGC***A***-TTG***T***CTGCCTAGGGCGGAGCG	65-94	630
	2	CTTGCAGC***A***-TTG***T***CTGCCTAGGGCGGAGCG	115-144	
EU038139 *Opisthorchis felineus*	1	CTTGCAGC***C***-TTG***T***CTGCCTAGGGCGGAGCG	68-97	629
	2	CTTGCAGC***A***-TTG***T***CTGCCTAGGGCGGAGCG	118-147	
MF438049 *Apophallus muehlingi*	1	CTTGCAG***AC***-TTG***T***CTGCCTAGGGCGGAGCG	241-270	1094
	2	CTTGCAGC***A***-TTG***A***CTGCCTAGGGCGGAGCG	318-347	
	3	CTTGCAGC***T***-TTG***T***CTGCCTAGGGCGGAGCG	471-500	
	4	CTTGCAGC***A***-TTG***T***CTGCCTAGGGCGGAGCG	565-594	
MF438056 *Apophallus donicus*	1	CTTGCAG***AC***-TTG***T***CTGCCTAGGGCGGAGCG	241-270	1057
	2	CTTGCAGC***A***-TTG***A***CTGCCTAGGGCGGAGCG	320-349	
	3	CTTGCAGC***T***-TTG***T***CTGCCTAGGGCGGAGCG	467-496	
	4	CTTGCAGC***A***-TTG***T***CTGCCTAGGGCGGAGCG	537-566	
MH025622-MH025623 *Cryptocotyle lata* sp. nov.	1	CTTGCAGC***T***-TTG***T***CTGCCTAGGGCGGAGCG	68-97	891
	2	CTTGCAGC***A***-TTG***T***CTGCCTAGGGCGGAGCG	167-196	
	3	CTTGCAGC***A***-TTG***T***CTGCCTAGGGCGGAGCG	285-314	
	4	CTTGCAGC***T***-TTG***T***CTGCCTAGGGCGGAGCG	368-397	
KJ641518 *Cryptocotyle lingua*	1	CTTGCAGC***T***-TTG***T***CTGCCTAGGGCGGAGCG	69-98	992
	2	CTTGCAGC***A***-TTG***T***CTGCCTAGGGCGGAGCG	171-200	
	3	CTTGCAGC***T***-TTG***T***CTGCCTAGGGCGGAGCG	292-321	
	4	CTTGCAGC***A***-TTG***T***CTGCCTAGGGCGGAGCG	394-423	
	5	CTTGCAGC***T***-TA***G***TCTGCCTAGGGCGGAGCG	465-494	
AB521798 *Euryhelmis costaricensis*	1	CTTGCAGC***T***-TTG***T***CTGCCTAGGGCGGAGCG	71-100	775

Fig. 5. The number of 30 bp repeats, their location in the ITS1 region (A) and variants of repeats within each species (B) from the family Opisthorchiidae. Grey rectangles indicate the localization of repeats within the ITS1 region; substitutions between repeats are in italic; accession numbers in bold are newly determined sequences; underlined species were attributed to Opisthorchiidae according to the results of this study.

ventrogenital sac dimensions (Table 2), and another shape of the ovary and eggs (*C. cryptocotyloides* has transversally elongated ovary and curved eggs). Unlike *C. quinqueangularis* and *C. cryptocotyloides*, for which morphometric data are only available in publications from authors who identified these species [2,25], much more data are available for *C. concava*. Morphometric data were published for specimens of this species from Europe, North America, and Korea (Issaitschikoff, 1925 cited in [2] [6,10,11],), and worms from these regions differ in many metric indices (Table 2). In comparison with specimens presented in the papers of Issaitschikoff (1925 cited in [2]), Hoffman [10], and Chai et al. [11], adult *C. lata* sp. nov. worms differ in terms of sizes of body, oral and ventral suckers, and eggs (Table 2). Moreover, *C. lata* sp. nov. differs from *C. concava* in terms of the shape of the testes and ovary (entire testes and ovary) according to Issaitschikoff (1925 cited in [2]), in terms of the shape of the testes and ovary (oval and round) according to Hoffman [10], and in terms of the shapes of the body, ovary, and seminal vesicle (oval or spatulate body, spherical ovary, and saccate seminal vesicle) according to Chai et al. [11].

Of the *Cryptocotyle* worms with a wider body, only the life cycle of *C. concava* has been studied. European studies indicated that brackish water *Hydrobia* (Hydrobiidae) snails and euryhaline fish were involved in the life cycle of *C. concava* as the first and second intermediate hosts, respectively [7–9,26]; however, no description of cercariae from *Hydrobia* snails has been made and it has not been experimentally confirmed that they belong to the *C. concava* species. All adult *C. concava* in Europe were obtained from naturally-infected definitive hosts or by feeding the definitive hosts with metacercariae from naturally-infected fish. Identifying cercariae from *Hydrobia* is usually based on a comparison with *C. concava* cercariae, which was described previously by Wootton [6]. The later author experimentally established that worms belonging to *C. concava*, in his opinion, were able to circulate in North America using freshwater *Amnicola* (Amnicolidae) snails and freshwater fish as the first and second intermediate hosts, respectively. The worms discovered in the Russian southern Far East, as well as those studied by Wootton [6], circulate using freshwater snails and freshwater fish; however, in contrast to the *C. concava* described by Wootton [6], *C. lata* sp. nov. uses *Boreoelona* (Bythiniidae Gray) snails in their life cycle. In addition, the mature *C. lata* sp. nov. have smaller oral suckers, larger egg sizes, and differently shaped ovaries compared to the *C. concava* described by Wootton [6] (the oral sucker has a diameter of 0.09–0.10 mm, the eggs are 0.027–0.031 × 0.016–0.017 mm, and the ovary is irregularly ovoid or slightly lobed). Therefore data on the life cycles of *C. concava* and *C. lata* sp. nov. and the differences in the morphometry of the adult worms confirm that they belong to different



(caption on next page)

Fig. 6. Predicted secondary structure of the *C. lingua* ITS1 transcript created using the program Mfold (Zuker, 2003, http://mfold.rit.albany.edu). Differences from *C. lata* sp. nov. are indicated by arrows. The values of free energy (*dG*) were −339.14 and −317.57 for *C. lingua and C. lata* sp. nov., respectively. *H* – helices; grey circles show location of 30 bp repeats.

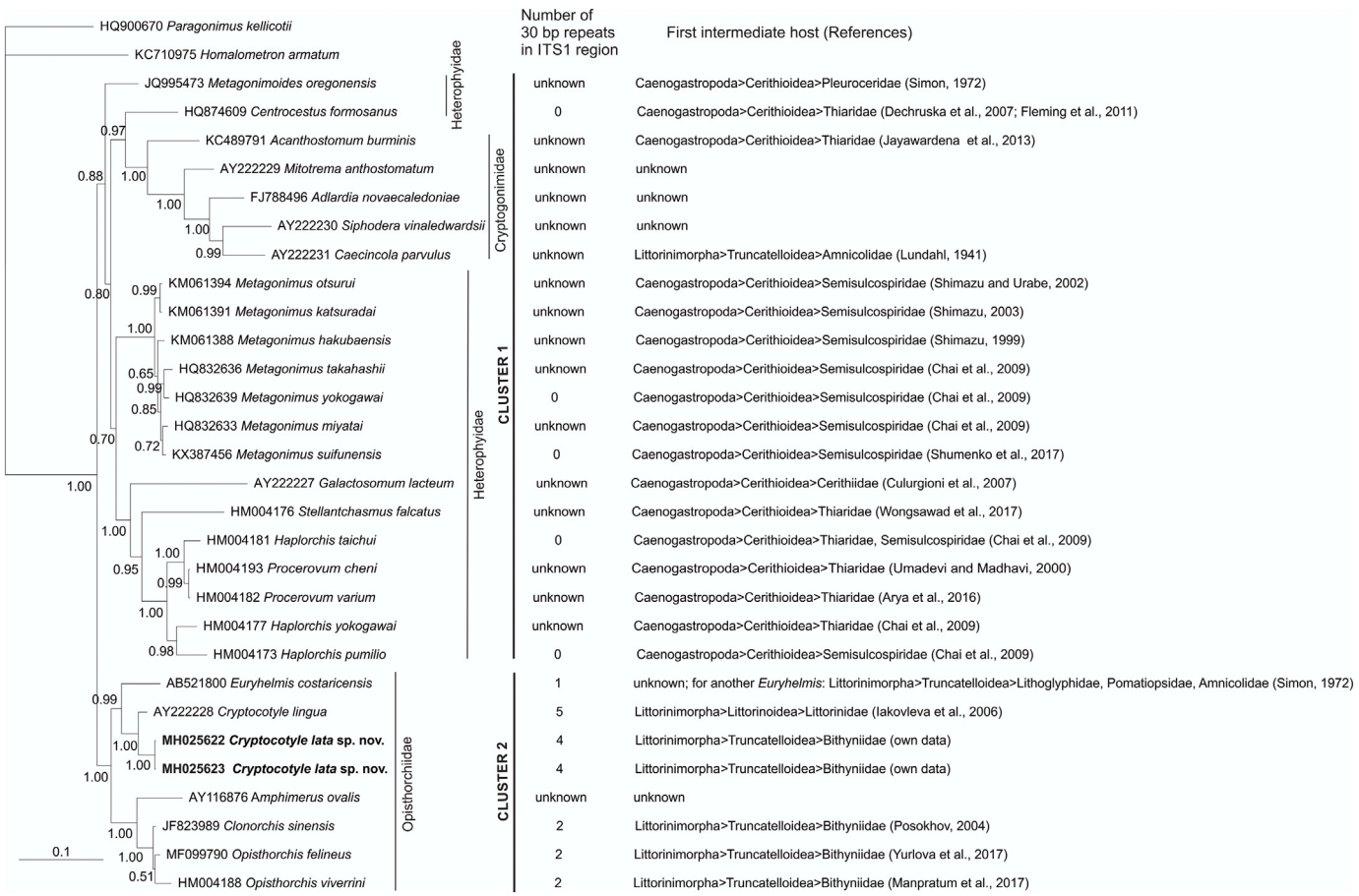


Fig. 7. Phylogeny based on 28S rRNA gene sequences using the Bayesian inference (BI) method. Bayesian posterior probabilities of ≥0.50 are shown. Accession numbers in bold are newly determined sequences; underlined species were attributed to Opisthorchiidae according to the results of this study.

species. With regard to *C. concava*, it is likely that there are at least two cryptic species with this name, one of which is circulating using *Hydrobia* snails while the other uses *Amnicola* snails as the first intermediate host.

The genetic distances between *C. lata* sp. nov. and *C. lingua* correspond to the distances between species of different genera of Opisthorchioidea [27]. The phylogenetic relationships between *C. lata* sp. nov. and other representative of the genus and Opisthorchioidea as a whole were examined using nucleotide sequences of the ITS2 region and the 28S gene of nuclear rDNA. The results showed that some species of the Heterophyidae (including *Cryptocotyle* spp.) formed a common group with representatives of the Opisthorchiidae on both trees (Fig. 7; Fig. 8). Based on the currently available genetic data, the species composition of this cluster was more numerous according to the ITS2 region and less numerous according to the 28S gene due to limited data on the second marker. At the same time, most branches of the tree based on the ITS2 region had lower support (posterior probability) that the tree based on the 28S gene. Together with the data from the literature [28], these results indicate that the 28S gene is more suitable for analyzing phylogenetic relationships both within Opisthorchioidea and in other groups of parasitic worms [13,29]. A close relationship between some heterophyid and opisthorchiid representatives was previously noted in phylogenetic analysis using the 18S and 28S genes [13,28,30,31]. Based on these data, it was suggested that the Heterophyidae was paraphyletic with respect to the Opisthorchiidae [30]. The impossibility of separating these families is associated with the fact that representatives of both families have common definitive hosts (fish-eating birds and mammals including humans) and second intermediate hosts (freshwater fish). In addition, Thaenkham et al. [30] reported that representatives of these families did not share first intermediate hosts; however, the authors did not analyze this issue in more depth.

In our opinion, the group of species from cluster two, which includes representatives of both families, is well-divided on the tree based on the 28S rRNA gene sequences (Fig. 7) and can, therefore, be regarded as a monophyletic group. In addition, all species were analyzed for the presence of long (30 bp) repeats in the ITS1 region. Representatives of Cryptogonimidae and Heterophyidae, which were included in the cluster one of tree based on the 28S rRNA gene sequences, did not have any repeats; however, representatives of Heterophyidae and Opisthorchiidae, which were included in the well-supported cluster two, contained these repeats (Fig. 5; Fig. 7). Despite the differences in the length of the ITS1 sequences, one to five long repeats were detected. All repeats had over 87% identity for all species from cluster two. This is interesting as the ITS1 region is usually so variable that it is often impossible to align sequences even within the same genus. It is noteworthy that the number of repeats and their location in the ITS1 region was conservative within the genus (Fig. 5). Based on this fact, *Opisthorchis viverrini*, *Opisthorchis felineus*, *Clonorchis sinensis*, *Metorchis ussuriensis, Metorchis bilis*, *Metorchis xanthosomus*, and *Metorchis orientalis* should be combined into one genus, which according to the seniority of

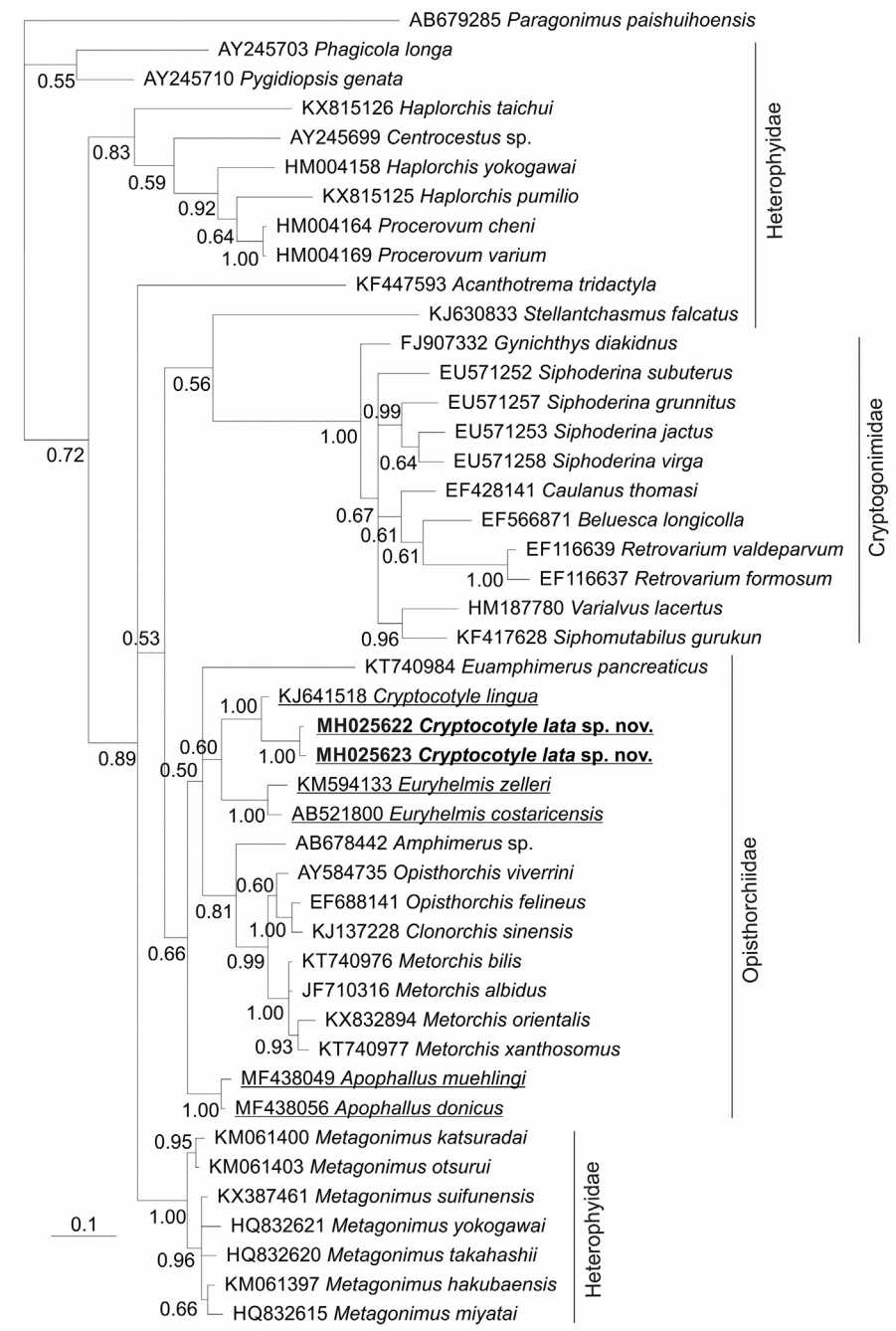


Fig. 8. Phylogeny based on ITS2 rDNA sequences using the Bayesian inference (BI) method. Bayesian posterior probabilities of ≥0.50 are shown. Accession numbers in bold are newly determined sequences; underlined species were attributed to Opisthorchiidae according to the results of this study.

the names of genera (*Opisthorchis* Blanchard, 1895, *Metorchis* Looss, 1899 and *Clonorchis* Looss, 1907) is necessary to name *Opisthorchis*. This consumption was also supported by previously stated hypotheses that the representatives of these genera may belong to one genus, which were based on the data of mitochondrial and nuclear DNA [27,32].

At the same time, the conservative repeats in different species included in cluster two of tree based on the 28S rRNA gene sequences may indicate the presence of a common ancestor and confirmed the monophyletic nature of this trematode group. The monophyliticity of this group was also confirmed by data on the circulation of heterophyids and opisthorchiids. The history of trematodes is inextricably linked with mollusks, which were the first intermediate hosts of these worms throughout the evolutionary path of the parasite–host system [33–35]. As noted above, the first intermediate hosts for Opisthorchiidae and Heterophyidae differ [30]. However, the Heterophyidae from cluster one used snails of the Cerithioidea as their first intermediate hosts, whereas the Heterophyidae from cluster two parasitized on Truncatelloidea snails, as well as representatives of the Opisthorchiidae. The study of trematodes for which freshwater snails of the Cerithioidea and Truncatelloidea are used as first intermediate hosts, on the one hand shows a significant similarity of worm faunas at the family level with less similarity at the genus level. On the other hand, each obtained cluster of trematodes has no genus and species that uses representatives of both superfamilies of snails. The association of trematodes from the first and second clusters with snails from the Cerithioidea and Truncatelloidea, respectively, indicates that the fauna of trematodes from each group has a common origin. In the period of life cycle formation, possibly the end of the Devonian–Carbonian period

[36], the fauna of trematodes was likely formed as a general fauna for snails of the Truncatelloidea and Cerithioidea. The further fate of the trematode faunas of these mollusks was predetermined by the different times of mollusk colonization in continental reservoirs. Snails of the Truncatelloidea (paleolimnic) occupied freshwater stagnant reservoirs, while snails of the Cerithioidea (mezolimnic) inhabited flowing water bodies (i.e., rivers) [37]. In conditions of temporary and ecological isolation, it is possible to preserve some generality of faunas for trematodes circulating with snails of the Truncatelloidea and Cerithioidea at the generic and higher taxonomic level, but worm faunas will differ at the species level. Isolation and divergence processes predetermined the formation of monophyletic trematode groups circulating using snails of the Truncatelloidea or Cerithioidea. This was reflected in the composition of trematodes using mollusks of these families as first intermediate hosts. The separation of the two groups identified in this study by genetic data (i.e., phylogenetic relationships based on the 28S rRNA gene and the presence or absence of long repeats), as well as the composition of the first intermediate hosts, indicates that all worms in cluster two are affiliated to Opisthorchiidae. Thus it is expedient to unite *Cryptocotyle* Lühe, 1899, *Euryhelmis* Poche, 1926, and *Apophallus* Lühe, 1909 into Opisthorchiidae.

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