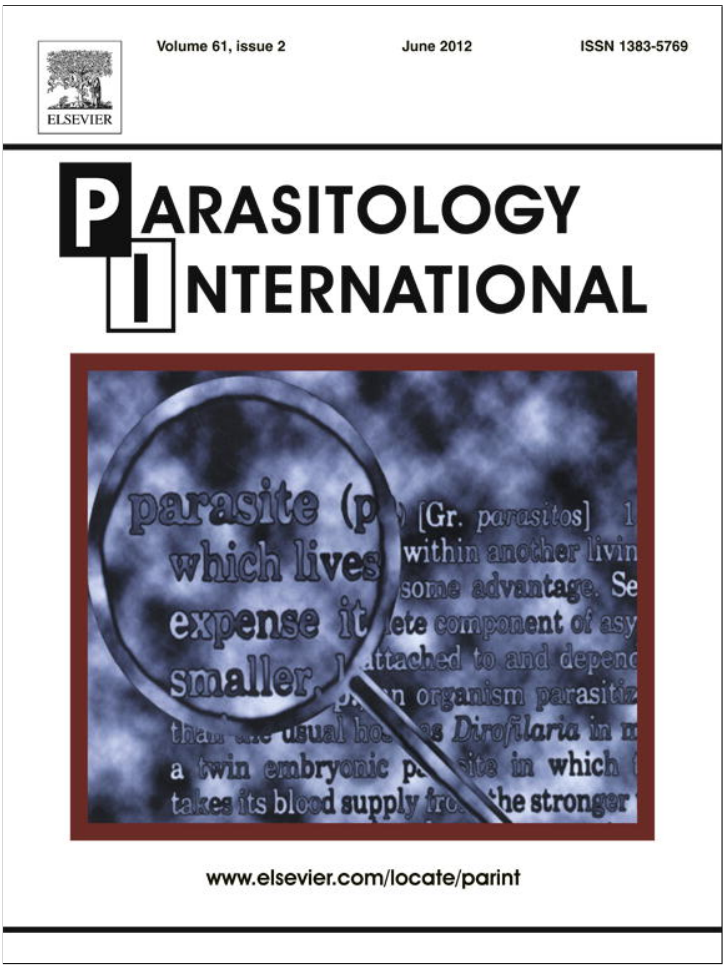

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The life cycle of *Asymphylodora perccotti* sp. n. (Trematoda: Lissorchiidae) in the Russian Southern Far East

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ABSTRACT

Specimens of *Asymphylodora perccotti* sp. n. (Trematoda: Lissorchidae) were found in the esophagus of the freshwater fish *Perccottus glenii* (Odobantidae) taken from the Bolshaya Ussurka River Basin (Primorsky Region, Russian Southern Far East). The first intermediate host of this trematode is a gastropod, *Parafossarulus manchouricus*, and the secondary hosts are the same mollusk and *Boreoelona ussuriensis*. Specimens of the new species are similar to *A. amnicolae* identified by Stunkard in 1959, but the mature worms have larger suckers and shorter ceca. The cercariae of these species are distinguished by body, suckers and pharynx size. These organs in *A. perccotti* sp. n. are more than one-third larger than what is observed in *A. amnicolae*. In addition, the new species lacks the capacity for progenesis. Finally, the new species is unusual in that it resides in the fish esophagus instead of the intestine, as is common for most *Asymphylodora* species.
Partial ribosomal DNA sequences and phylogenetic reconstruction sequence data indicate that these worms represent a new digenean species.

1. Introduction

In the Russian Southern Far East, three species from the Lissorchiidae have been discovered: *Asymphylotrema macrocetabulum* (Belous, 1953) Dvorjadkin et Besprozvannykh, 1985, *Asymphylodora japonica* Yamaguti, 1938 (*Parasymphylodora japonica*) and *Asymphylodora markewitschi* Kulakowskaja, 1947 (*P. markewitschi*) [1]. A fourth species from this family, *A. perccotti* sp. n., was discovered during a parasitological investigation of fish collected from the Ussuri river system in the summer of 2008. We dissected nine *Perccottus glenii* specimens and found the new *A. perccotti* species in seven of the fish. Other investigated fish species (ten species from three families, 100 specimens in total) did not carry this novel parasite.

2. Material and methods

2.1. Description of general morphology and life-cycle

During parasitological investigations of fish in the Bolshaya Ussurka River Basin, we found mature trematodes (10–20 per fish) in the esophagus of seven of ten 30–70-mm-long *Perccottus glenii* Dybowski (Chinese sleeper, family Odobantidae). The trematodes belonged to a new species in the *Asymphylodora* genus. In an attempt to identify first intermediate host in the same reservoir, we collected snails from the Bithyniidae family, as these snails are known to be first host of others parasites in this genus. In total, we collected 500 specimens of *Parafossarulus manchouricus* (Biurguignat) and *Boreoelona ussuriensis* (Ehrmann), and we dissected 150 snails of each species. Parthenitae and cercariae of two *Asymphylodora* species, which differed in morphology, were found only in the *Parafossarulus manchouricus* specimens. One of the *Asymphylodora* species, *Asymphylodora japonica* Yamaguti, 1938, is a common species in the Primorsky region [2]. To verify the existence of another species, we selected two snails that had emergent cercariae. We placed the snails in a small aquarium and infected the juvenile snails with laboratory specimens of *Boreoelona ussuriensis* (15 specimens) and *Anisus centrifugops* Prosorova and Starobogatov (15 specimens). These trematodes were isolated from various laboratory hosts, including juvenile *Perccottus glenii* (4), caddis *Semblis atrata* Fabricius larvae (5) and an amphipod *Gammarus* sp. (5) isolated from natural reservoirs. Thirty days after infection, we found *Asymphylodora* metacercariae in *Boreoelona ussuriensis,* while the other experimental animals were not infected. The metacercaria-infected snails were fed to two 7-cm long Chinese sleepers, and 25 days post-feeding, we observed mature worms with morphological similarity to *A. perccotti*. These worms were similar to those isolated from worms taken from the esophagus of naturally infected *Perccottus glenii* (3 and 7 specimens).

Studies examining the behavior of the cercariae and their life-span were carried out using larvae immediately after their emergence from snails. The maximum length of cercarial life was defined as the time elapsed from emergence to the death of all specimens. All experiments

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were carried out in laboratory conditions in water temperatures of 18–22 °C.

All measurements of the parthenitae, cercariae and metacercariae were made on specimens fixed with hot 4% formalin. Mature worms were washed in distilled water, killed in boiling distilled water and preserved in 70% ethanol. Whole-mounts were made by staining specimens with alum carmine, dehydrating the worms in a graded ethanol series and clearing in xylene. The xylene treatment was followed by mounting the specimens in Canada balsam under a coverslip on a glass slide. All measurements are given in millimeters (mm).

All photographs were taken of living specimens.

2.2. DNA extraction, amplification and sequencing

Adult *A. perccotti* specimens ($n = 20$) were obtained during experimental work and fixed in 96% ethanol for genetic analysis. DNA was extracted from whole worms using a "hot shot" technique [3].

Nuclear 28S rDNA was amplified using polymerase chain reaction with the following primers: DIG12 (5′-AAG CAT ATC ACT AAG CGG-3′) and 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) [4]. The initial PCR reaction was carried out in a total volume of 20 μl containing 0.25 mM of each primer pair, 1 μl DNA in water, 1× Taq buffer, 1.25 mM dNTP, 1.5 mM magnesium and 1 unit of Taq polymerase. The amplification of a 1200-bp fragment of 28S rDNA was performed in a GeneAmp 9700 (Applied Biosystems) with a 3-min denaturation hold at 94 °C; 40 cycles of 30 s at 94 °C, 30 s at 52 °C and 2 min at 72 °C; and a 7-min extension hold at 72 °C. Negative and positive controls, using both primers were used. The PCR products were directly sequenced using an ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit, as recommended by manufacturer, with the internal sequencing primers 300F, ECD2, 900F and 1200R (Tkach et al. 2003). The PCR products were analyzed using an ABI 3130 genetic analyzer at the Institute of Biology and Soil Sciences FEB RAS. The sequences have been submitted to GenBank with the following accession numbers: FR822715–FR822731.

2.3. Alignments and phylogenetic analysis

The ribosomal DNA sequences were assembled with SeqScape v.2.6 software and aligned using the MEGA 5.0 [5] alignment explorer with default options. The regions that could not be unambiguously aligned were excluded from the analyses. A number of variable, parsimony-informative sites, nucleotide composition and substitution analyses were performed using MEGA 5.0. Genetic divergence was estimated using genetic distance (d) values, which were calculated including all substitution types. The distance matrix was constructed using the general time reversible model [6]. This model showed the best fit to the data using Akaike's information criterion [7] in Modeltest v. 3.07 software [8]. Phylogenetic analysis of the nucleotide sequences was undertaken, using all accessible methods, including distance (neighbor-joining), parsimony (maximum parsimony and maximum likelihood) and Bayesian methods. All phylogenetic trees were reconstructed with PAUP v. 4b10 [9] and MrBayes v.3.1.2 software [10]. The resulting networks were rooted with the outgroup taxa. Neighbor-joining, maximum parsimony and maximum likelihood analyses were performed using a heuristic search strategy (10,000 rearrangements and 100 search replicates) with random addition of sequences. Bayesian inference was used with the following nucleotide substitution parameters: lset nst = 6, rates = equal, ncat = 4 and basefreq = empirical. The values corresponded to a general time reversible model, including estimates of equal distributed among-site rate variation. The phylogenetic relationship significance was estimated using a bootstrap analysis [11] with 100 replications.

The phylogenetic relationships of *Asymphylodora perccotti* were inferred from our data and the nucleotide sequences of 28S rDNA of other trematode specimens [12–17] obtained from the NCBI GenBank database. These other sequences represented different superfamilies (Table 2).

3. Results

3.1. Description

Asymphylodora perccotti Besprozvannykh, Ermolenko et Atopkin, sp. n.

Definitive host: *Perccottus glenii* (Odobantidae).

Site: Esophagus.

Habitat type: Bolshaya Ussurka River Basin (a tributary of the Ussuri River), Primorsky Region, Russian Southern Far East of Russia, 45°57′N, 133°53′E

Material examined: Rediae: 10 specimens; Cercariae: 10 specimens; Metacercariae: 10 specimens; Adults: 10 specimens.

Type-deposition: Holotype No 40-Tr, paratype No 41-50-Tr.

This material is held in the parasitological collection of the Zoological Museum (Institute of Biology and Soil Sciences, Far East Branch of the Russian Academy of Sciences, Vladivostok, Russia); e-mail: petrova@ibss.dvo.ru. Deposited: 2009.23.10.

Etymology: The specific name refers to the genus of the definitive host.

3.1.1. Adult worm (Table 1, Figs. 1a, 2a–b)

The oval body, from the anterior to posterior end, is covered by large needle-like spines. Fewer spines cover the suckers. The oral sucker is large and subterminal. The prepharynx is short and only observed in living worms (Fig. 2a, b) present in the esophagus. The worms exhibit bifurcation before the ventral sucker or underneath the anterior margin. The ceca terminated at the level of the anterior margin of the testis. The ventral sucker is larger than the oral sucker. The ratio of sucker length is 1:1.14–1.36, and the sucker width ratio is 1:1.14–1.60. The worms have a single testis, oval or irregularly shaped, which lies in the posterior third of the body, on the midline. There are pairs of seminal ducts that join near the cirrus sac. The ovary is large, bilaciniate and right from the midline of body, adjoining the anterior margin of the testis or covered by the testis. The seminal receptacle tubule is anterior to the ovary. The cirrus sac lies left of the midline, adjoining the left margin of the ventral sucker (Fig. 2c) or (on whole mounts) is covered by the suckers (Fig. 1a). The seminal vesicle is bipartite in the distal part of the cirrus sac. The vent ducts are surrounded by a small number of prostatic cells. The cirrus is covered with spines. The metraterm is short, with muscle walls that are covered with spines. The vitellaria lateral lie in intervals from the posterior margin of the ventral sucker up to the posterior half of the testis. They each consist of eight or nine follicles. Transversal vitelline ducts are joined at the midline, forming a vitelline reservoir at the level of the middle of the ovary. The uterus extends between the organs from the posterior margin of the testis up to the anterior border of the ventral sucker. The eggs are operculated and light yellow. The excretory bladder is tubular.

3.1.2. Redia (Fig. 1b)

The body is sac shaped and 0.5–1.5×0.2–0.4 mm. The pharynx is 0.05–0.06×0.05–0.067 mm. The cecum is short. A birth pore is located at the cecum level. Rediae contain cercariae at various stages of development.

3.1.3. Cercaria (Figs. 1c, 2d)

The body is oval and 0.4–0.5×0.18–0.54 mm. The maximum width of the worm occurs at the primordium ovary level. The posterior end is sharpened and has a pit-like deepening. The tegument is covered with spines and has sensible papillae (0.011–0.017 mm) from the anterior end up to the primordium ovary level. The oral

Table 1
Measurements (mm) of some adult digeneans from genus *Asymphylodora*.

	A .perccotti sp.n.			*A. japonica* in Yamaguti, 1938	*A. japonica* in Besprozvannykh, 2005	*A. demeli* in Markowski, 1935	*A. progenetica* in Serkova & Bychovsky, 1940	*A. progenetica* in Kulakowa, 1982	*A. amnicolae* in Stunkard, 1959
	holotype	limits	mean						
Body length	0.535	0.508–0.608	0.547	0.285–0.900	0.600–1.300	1.050–1.170	0.480–0.810	0.700–1.200	0.280–0.710
Body width	0.200	0.189–0.250	0.220	0.180–0.450	0.250–0.450	0.300–0.360	0.210–0.380	0,300–.0.500	0.140–0.250
Length of tegumental spines	-	0.0077–0.0115	-	-	-	-	0.003–0.006	0.003–0.014	0.006–0.008
Length of oral sucker	0.096	0.096–0.123	0.109	0.068–0.136	0.100–0.190	0.160–0.170	0.080–0.120	0.110–0.170	0.060–0.095
Width of oral sucker	0.100	0.096–0.139	0.107	0.068–0.136	0.100–0.190	0.160–0.170	0.080–0.130	0.100–0.190	0.060–0.095
Length of ventral sucker	0.127	0.123–0.158	0.137	0.082–0.177	0.150–0.240	0.160–0.200	0.100–0.170	0.160–0.230	0.090–0.130
Width of ventral sucker	0.142	0.131–0.166	0.149	0.082–0.190	0.150–0.240	0.160–0.200	0.110–0.180	0.160–0.250	0.090–0.130
Length of pharynx	0.042	0.042–0.054	0.047	0.041–0.090	0.070–0/090	0.056–0.078	0.030–0.050	0.060–0.080	0.036–0.044
Width of pharynx	0.046	0.046–0.058	0.049	0.027–0.090	0.054–0.070	0.062–0.084	0.040–0.050	0.050–0.110	0.036–0.044
Length of oesophagus	0.039	0.027–0.058	0.040	-	0.060–0.150	0.100–0.150	0.057	-	-
Length of testis	0.104	0.104–0.139	0.114	0.041–0.163	0.100–0.200	0.150–0.220	0.040–0.100	0.190–0.300	0.080–0.130
Width of testis	0.073	0.073–0.112	0.098	0.035–0.122	0.060–0.130	0.100–0.120	0.040–0.090	0.100–0.220	0.060–0.100
Length of ovary	0.077	0.073–0.108	0.090	0.022–0.125	0.060–0.180	0.084–0.300	-	0.100–0.190	0.060–0.110
Width of ovary	0.069	0.054–0.077	0.066	0.016–0.122	0.035–0.110	0.073–0.230	-	0.070–0.120	0.050–0.090
Length of cirrus sac	0.085	0.085–0.123	0.093	0.082–0.136	0.150–0.250	0.240–0.310	-	0.110–0.190	-
Width of cirrus sac	0.026	0.026–0.035	0.032	0.027–0.049	0.050–0.095	0.067–0.078	-	0.070–0.120	-
Length of metraterm	0.054	0.054–0.058	0.055	0.054–0.082	0.050–0.075	0.150	-	-	-
Width of metraterm	0.015	0.015–0.019	0.017	0.027–0.046	0.025–0.048	-	-	-	-
Length of eggs	-	0.022–0.027	-	0.022–0.027	0.030–0.045	0.031–0.034	0.020–0.033	0.018–0.024	0.025–0.029
Width of eggs	-	0.012–0.016	-	0.011–0.014	0.016–0.021	0.017–0.020	0.013–0.018	0.011–0.013	0.013–0.016

suckers are subterminal and 0.073–0.095 × 0.084–0.110 mm, with nine papillae located in a single circle on its internal margin. The prepharynx is short, the pharynx is 0.039–0.050 × 0.034–0.056 mm and the esophagus is 0.050–0.061 mm long and bifurcated immediately anterior to the ventral sucker. The ceca do not reach the far anterior margin of the testis primordium. The ventral sucker, 0.078–0.112 × 0.095–0.123 mm, lies 0.15–0.21 mm anterior from the end of body. The internal sucker cover has papillae located in three circles, containing six, six and eight papillae, respectively, beginning from the opening of the sucker. The cercariae have glands in four groups of cells left and right from the midline of body. Two of the glands each contain 30 cells and lie in the interval between the pharynx and the middle of the ventral sucker. The ducts from these cells open into the anterior end of the body. The two posterior groups, each containing 15–20 cells, are located in the interval between the middle of the ventral sucker and the anterior border of testis primordium. These ducts open around the ventral sucker. The testis and ovary primordia lie in the posterior third of the body. The testis primordium are oval or irregularly shaped, 0.084–0.134 × 0.064–0.112 mm. The ovary primordium (0.028–0.034 × 0.039–0.042 mm) adjoins the anterior border of the testis primordium. The cirrus sac primordium and metraterm, 0.056–0.061 × 0.019–0.021 mm, have spines, lie left of the midline of the body and adjoin the ventral body. The excretory bladder is tubular and anteriorly reaches to the anterior margin of the testis primordium. The flame cell formula is $2[(4+4+4+4)+(4+4+4+4)]=64$.

Table 2
List of the taxa incorporated in sequence analysis.

Species	n	Author	GenBank reg. number
Monorchioidea			
Asymphylodora perccotti	17	Original data	FR822715–FR822731
Lissorchis kritskyi	2	Curran et al., 2006; Olson et al., 2003	EF032689, AY222250
Lasiotocus (syn. *Ancylocoelium*) *typicum*	1	Olson et al., 2003	AY222254
Diplomonorchis leiostomi	1	Olson et al., 2003	AY222252
Monorchis monorchis	1	Tkach et al., 2001	AF184257
Provitellus turrum	1	Olson et al., 2003	AY222253
Allocreadioidea			
Allocreadium lobatum	1	Curran et al., 2006	EF032693
Gorgoderoidea			
Dicroroelum hospes	1	Dittmar, 2003	AY251233
Plagiorchioidea			
Plagiorchis vespertilionis	1	Tkach et al., 2000	AF151931
Gymnophalloidea			
Prosthenhystera obesa	1	Curran et al., 2006	EF032690
Haploporoidea			
Hapladena nasonis	1	Olson et al., 2003	AY222265
Echinosomatoidea			
Psilochasmus oxyurus		Tkach et al., 2000	AF151940
Fasciola hepatica	1	Olson et al., 2003	AY222244
Schistosomatoidea			
Schistosoma mansoni	1	Lockyer et al., 2003	AY157173

3.1.4. Metacercaria (Figs. 1d, 2e–f)

The cyst is thin–walled, transparent and 0.31–0.35 mm in diameter. As the overall metacercaria morphology is identical to cercaria morphology, only the sizes are provided. The body is 0.52–0.59 × 0.24–0.26, the oral suckers are 0.110–0.123 × 0.134–0.137 mm, the ventral suckers are 0.110–0.134 × 0.145–0.160 mm, the pharynx is 0.046–0.050 × 0.049–0.058 mm, the testis primordium is 0.072–0.10 × 0.067–0.089 mm and the ovary primordium is 0.028–0.039 × 0.034–0.042 mm. The cirrus sac and metraterm primordia are 0.056–0.061 × 0.019–0.021 mm.

3.2. Life-cycle

The extent of the natural infection of the first intermediate host (the freshwater snail *Parafossarulus manchouricus*) by *A. perccotti* parthenitae and cercariae was 1.25%. In laboratory conditions, a cercaria emission peak is absent. The trematodes live in the snail until they are fully mature. Typically, in a single day, a small number of cercariae (up to 40 specimens) emerged. When the worms emerged from the snail, they settled on the bottom of the aquarium as the host moved. Most of the cercariae remained along the host's movement path, but some were displaced 1–2 cm from the path. In the initial period of free living (up to 60 h), the cercariae stayed active. They used their ventral sucker and posterior body end for support, with the anterior end remaining free. Often, the worms moved their anterior end with a circular motion. During the second half of their life, the cercariae spent their time lying on the bottom of the aquarium, rarely elevating and rarely moving out their anterior ends. After contact with the second intermediate host (in the current experiments, the snail *Boreoelona ussuriensis*, family Bithyniidae Gray, 1857), the cercariae attached to the snail with the assistance of their oral sucker,

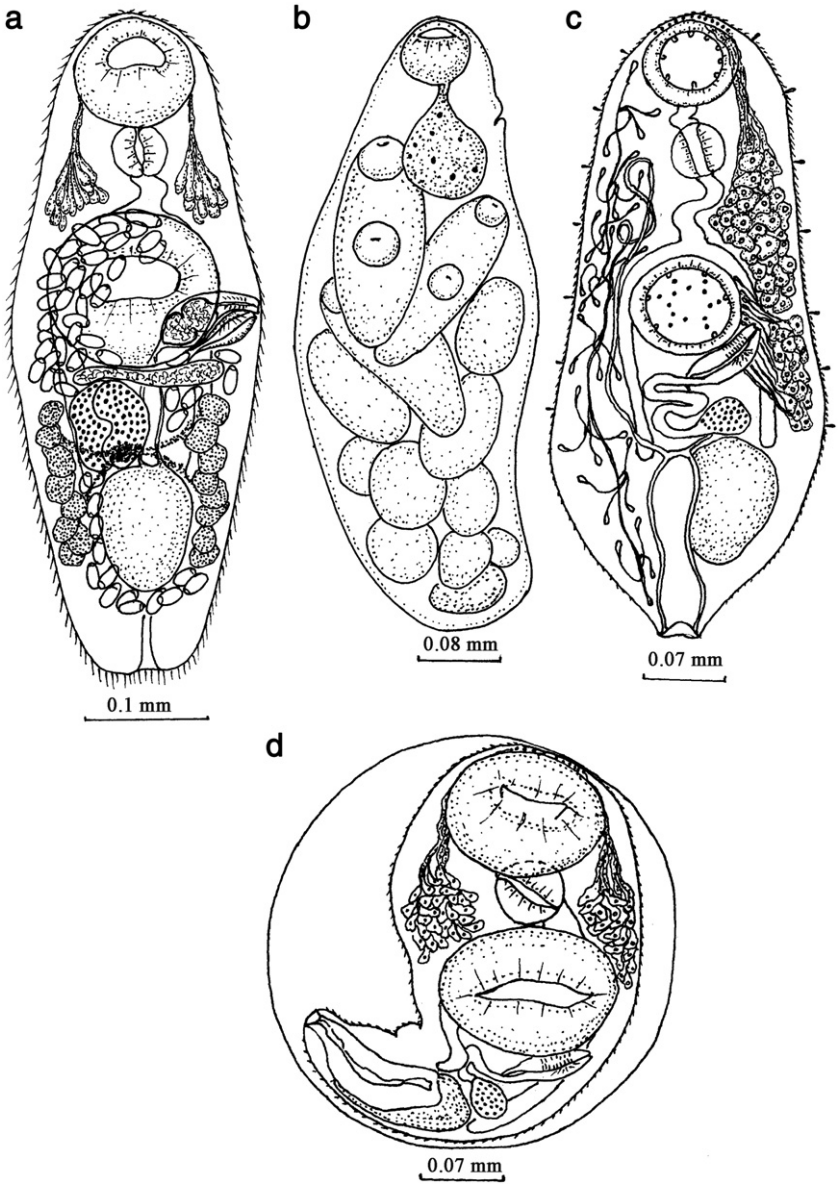

Fig. 1. *Asymphylodora perccotti* sp. n.: a — mature worm, b — redia, c — cercaria, d — metacercaria.

migrated under the host shell and formed cysts in mantle tissue. In natural conditions, the *A. perccotti* metacercariae, were found in *Boreoelona ussuriensis* and *Parafossarulus manchouricus* Dybowski. The definitive host (the fish *Perccottus glenii*) becomes infected following consumption of infected second intermediate *A. perccotti* hosts.

3.3. Alignments and phylogenetic analysis

To the best of our knowledge, we are presenting the first molecular data on *A. perccotti*. PCR amplification of the 28S rDNA produced a 1300-bp fragment for all *A. perccotti* specimens. After assembly and alignment procedures, the resulting 28S rDNA sequences from *A. perccotti* were 1153 bp (Fig. 3). All of the partial 28S rDNA *A. perccotti* sequences demonstrated complete (100%) identity with each other. The nucleotide composition of the obtained sequences was 22.5% A, 25.2% T, 31.6% G and 20.6% C. This composition indicates a high similarity with other digenetic suckers [18,19].

The genetic divergence was estimated between the *A. perccotti* 28S rDNA sequences and sequences from members of different genera of the superfamily Monorchioidea (Table 3). The lowest divergence values were obtained between *A. perccotti* and species of the genus *Lissorchis* ($d = 8.5\%$). While the lack of molecular data on Lissorchiidae species did not allow us to examine the range of divergence values between different genera, the divergence value between *A. perccotti* and *Lissorchis kruitsky* lies within the intergeneric divergence values of the family Monorchiidae ($d = 8.3$–15.8%).

All of the phylogenetic trees demonstrated close relationships between *A. perccotti* and *L. kruitsky*, a member of the family Lissorchiidae. *A. perccotti* and *L. kruitsky,* in turn, appear as sister groups to the Monorchiidae family (Fig. 3). Both families form a separate cluster with a high bootstrap support for the main nodes. Members of the other superfamilies, which were used in the analysis, formed a second distinct cluster. The maximum parsimony analysis yielded a single 1319-step tree with a CI = 0.45, RI = 0.64, RC = 0.29 and HI = 0.55. The calculated tree topology also indicated two different clusters, which correspond with Monorchioidea and other

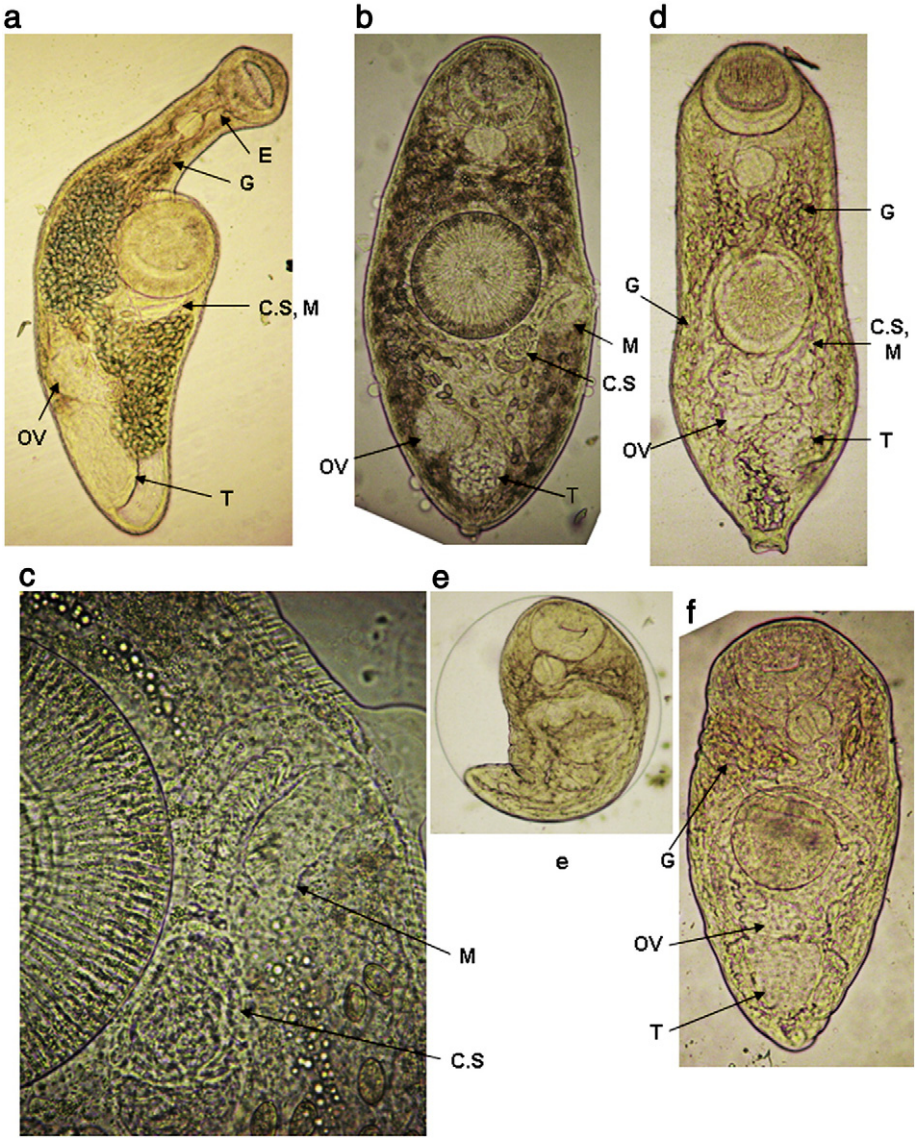

Fig. 2. *Asymphylodora perccotti* sp. n.: a, b, c — mature worm, d — cercaria, e — metacercaria in cyst, f — metacercaria without cyst (E — prepharynx, G — glands, C,S — cirrus sac, M — metraterm, OV — ovary, T — testis).

superfamilies. The maximum likelihood and the Bayesian inference analyses revealed an identical tree topology both with each other and with the neighbor-joining and maximum parsimony phylogenies.

4. Discussion

Species from the genus *Asymphylodora* are widely-distributed. They are found in fresh and brackish water fish throughout Asia, Europe and North America. In the Russian Southern Far East, two species of these worms, *A. japonica* Yamaguti, 1938 and *A. markewitschi* Kulakowskaja, 1947, in their mature stages were discovered in the small intestine of *Carassius gibelio* and *Cyprinus carpio haematopterus* (Cyprinidae), respectively [2]. A third species, *A. perccotti,* was isolated from the esophagus of *Perccottus glenii* (Odobantidae). In this study, we demonstrate that *A. perccotti*, similar to *A. japonica,* infects snails from the genus *Parafossarulus* as first intermediate hosts; however, *A. perccotti* sp. n. differs in size from *A. japonica* in its mature stage (Table 1) and other stages such as the cercaria (based on 2005 Besprozvannykh data, cercariae of *A. japonica* have a body 0.78–0.92 × 0.29–0.16 mm, an oral sucker 0.089–0.14 × 0.13–0.15 mm, a pharynx 0.056–0.078 × 0.067–0.078 mm, a ventral sucker 0.15–0.160 × 0.16–0.18 mm and a cirrus sac 0.093–0.10 × 0.037 mm). Furthermore, mature *A. perccotti* differ from most other worms in the genus *Asymphylodora*. *A. perccotti* have a smaller body and organs than what is observed with *A. atherinopsidis* Annereaux, 1947, *A. kubanicum* (Issaitschikoff, 1923), *A. carpiae* Szidat, 1943, *A. markewitschi* Kulakowskaja, 1947, *A. kafirnigan* Osmanov, 1965, *A. exspinosa* (Hausmann, 1897) and *A. ferruginosa* (Linstow, 1877). Compared with *A. tincae* (Modeer, 1790), *A. perccotti* has a smaller body, testis, ovary and cirrus sac. *A. perccotti* has a smaller body and suckers compared with *A. imitans* (Muhling, 1898) and shorter ceca than *A. macrostoma* Ozaki, 1925 and *A. kedarai* Srivastava, 1951. *A. perccotti* has a smaller pharynx, testis and cirrus sac than *A. kedarai,* but their eggs are larger (the eggs of *A. kedarai* are 0.014–0.015 × 0.009–0.010 mm). Compared with *A. indica* Srivastava, 1936, *A. perccotti* has a smaller body, pharynx and cirrus sac. Moreover, the vitellaria of *A. indica* lie between the middle of the esophagus and the anterior margin of the ovary. Compared with *A. pontica* (Tschernyschenko, 1949), *A. perccotti* has a smaller body, testis and eggs (the eggs of *A. pontica* are 0.036–0.041 × 0.016 mm). Furthermore, the position of the vitellaria differs between the two species. In *A. pontica,* the vitellaria extend from the posterior margin of the metraterm to the anterior margin of testis. Compared with *A. fishelsoni*

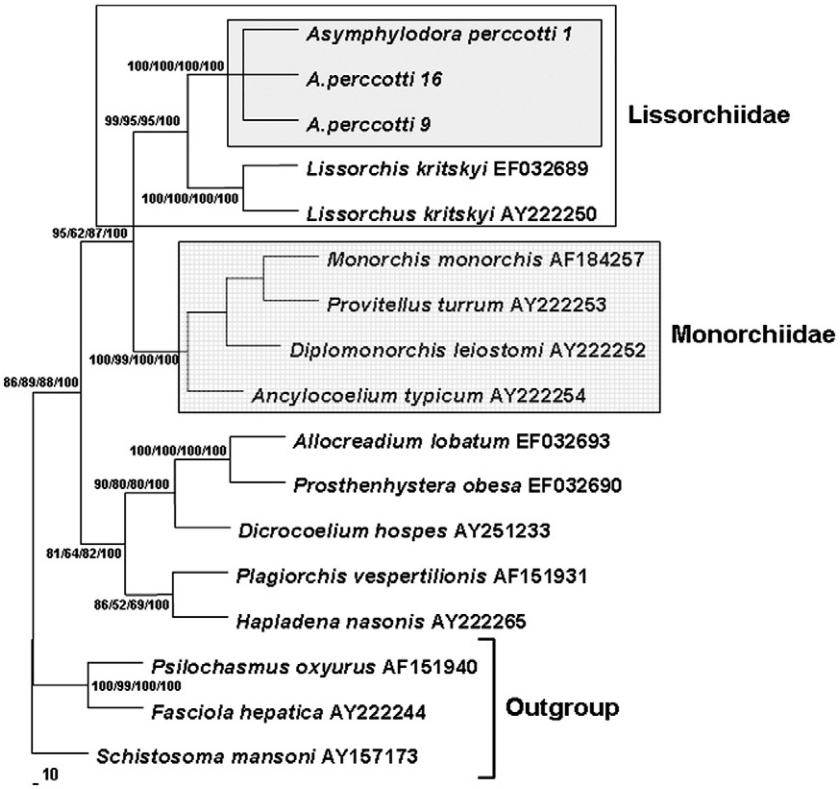

Fig. 3. Phylogenetic tree based on analysis of partial sequences of 28S rDNA of the members of some superfamilies using neighbor-joining algorithm. Nodal numbers is a bootstrap statistic values (%) for NJ/MP/ML/BI analyses.

Fischthal, 1979, *A. perccotti* has a smaller body and cirrus sac and a lower sucker ratio (the sucker length ratio in *A. fishelsoni* to *A. perccotti* is 1:1.00–1.09, and the width ratio is 1:1.04–1.19). Compared with *A. stenothyrae* Tang, 1980, *A. perccotti* has a smaller body and smaller eggs (the *A. stenothyrae* egg is 0.034–0.043 × 0.015–0.017 mm) [20–31].

Adult *A. perccotti* do not have noticeable size differences compared with *A. demeli* Markowski, 1935, *A. progenetica* Serkova et Bychovsky, 1940 (accordingly to [32]) or *A. amnicolae* Stunkard, 1959 [32–34]. An adult *A. demeli* is covered by spines only on the anterior end of the body, and while its vitellaria do not end at the testis, they do extend almost to the posterior end of the body. The definitive host of *A. demeli* is the marine fish *Gobius minutus*. Adult *A. perccotti* are similar in size to *A. progenetica* as described in the work of Serkova and Bychovsky (1940) but differ in size from specimens of the same species, as described by Kulakowa (1982) [35] (Table 1). The cercariae of *A. progenetica* have a smaller body (0.20–0.28 × 0.080–0.16 mm) and oral suckers (0.050–0.060 × 0.050–0.080 mm) than *A. perccotti*. The tegument of *A. perccotti* does not have spines [32].

Adult *A. perccotti* are most similar to *A. amnicolae*. These species have non-significant differences in the size of their ventral suckers (Table 1) and longer ceca. In *A. amnicolae*, they end at the level of the posterior margin of the testis. *A. perccotti* cercariae differ from those seen in *A. amnicolae* by a larger body, larger suckers and a larger pharynx (cercariae of *A. amnicolae*: body, 0.16–0.32 × 0.60–0.12 mm; oral sucker 0.050–0.060 mm; ventral sucker 0.060–0.070 mm; diameter of pharynx, 0.033–0.035 mm). The metacercariae of *A. perccotti* have a greater cyst diameter than *A. amnicolae* (0.19–0.22 mm) [34].

A. perccotti differ from *A. progenetica*, *A. stenothyrae* and *A. amnicolae* by the absence of progenesis. We did not observed any progenetic stages of *A. perccotti* in infected snails.

We believe that these differences are sufficient to establish *A. perccotti* as a new species. Moreover, *A. perccotti* is a single worm from the genus *Asymphylodora*. In the adult stage, they live in the esophagus. Until recently, this site has been recorded only for two digenean species: *Azygia hwangtsiytii* Tsin, 1933 and *Halipegus japonicus* Yamaguti, 1936 [36,37].

In the present study, the phylogenetic relationships of *A. perccotti* with related taxa were compared using partial sequences of the 28S ribosomal RNA gene. The results indicate that *A. perccotti* is a member of the family Lissorchiidae and is closely related to *Lissorchis kruitsky*, a representative member of that family. Moreover, the divergence level between these two species, which represent different genera, corresponds with a range of intergeneric divergence values of the related family Monorchiidae. This divergence level also supports that *A. perccotti* is a new species.

Table 3
Genetic divergence (%) between different genera of Monorchioidea by 28S rDNA partial sequence data.

	A.perccotti	*Lissorchis*	*Lasiotocus* (syn. *Ancylocoelium*)	*Diplomonorchis*	*Monorchis*	*Provitellus*
A.perccotti	-					
Lissorchis	8.5	-				
Ancylocoelium	16.1	14.9	-			
Diplomonorchis	13.3	11.6	11.9	-		
Monorchis	17.3	15.6	15.8	11.4	-	
Provitellus	13.1	10.7	11.9	8.3	10.9	-

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