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# Life-cycle and genetic characterization of *Astiotrema odhneri* Bhalerao, 1936 sensu Cho & Seo 1977 from the Primorsky Region (Russian Far East)

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ABSTRACT

Adult *Astiotrema odhneri* Bhalerao, 1936 sensu Cho & Seo 1977 were found in the intestine of a freshwater turtle, *Pelodiscus sinensis* (Wiegmann), from the Komissarovka River Basin, Primorsky Region, Russia. It was established that the first intermediate host of this parasite is a snail, *Anisus centrifugops*, and that the second intermediate hosts include the snails, *Helicorbis sujfunensis* and *A. centrifugops*, tadpoles of the frog *Rana dybowskii*, and the fish *Perccottus glenii*. The development of *A. odhneri* includes the formation of sporocyst and xiphidiocercariae, which is typical for species belonging to Plagiorchioidea. Phylogenetic analysis based on 28S rRNA gene sequences showed that *A. odhneri*, together with *Astiotrema monticellii*, form a monophyletic clade that was closer to Opisthorchioidea than to any other taxon represented in the tree. However, phylogenetic analysis without outgroup taxon indicated a high degree of differentiation of *Astiotrema* from both Plagiorchioidea and Opisthorchioidea.



## 1. Introduction

The genus *Astiotrema* Looss, 1900 includes intestinal parasites of fish and reptiles in the Palearctic, Indo-Malayan and Ethiopian Regions [1]. At present, data concerning the species composition of this genus and the validity of its representatives are contradictory. A total of 28 species of *Astiotrema* have been described [2–4]. However, as a result of numerous revisions, the number of valid species has repeatedly changed. Depending on the assessment and significance of various criteria for the delineation of *Astiotrema* species, many identified species of *Astiotrema* were reduced to synonyms [5–10 and others]. Yeh and Fotedar [9], as a result of their revision, radically changed the number of assigned *Astiotrema* species. They recognized as valid only: *Astiotrema reniferum* (Looss, 1898), *Astiotrema impletum* (Looss, 1898), *Astiotrema monticellii* Stossich, 1904 and *Astiotrema odhneri* from an original 21 species that were included in this genus. Later, Agrawal [2] described a new species, *Astiotrema lissemydis*, and decided that six species from an original 26 were now valid: *A. reniferum*, *A. impletum*, *A. monticellii*, *Astiotrema emydis* Ejsmont, 1930, *Astiotrema cyclemydis* [10] and *A. lissemydis* [2].

Thus, no consensus has been established for either the species composition of the genus or their relative positions within the phylogeny. At present time, *Astiotrema*, which belonged to the family Plagiorchiidae Lűhe, 1901, is assigned as genus *incertae sedis* within the system of Trematoda [1,11]. Reasons for the removal of *Astiotrema* from Plagiorchiidae include data on the morphology of adult worms, their life-cycle and the morphology of the parthenitae and cercariae of *A. monticellii* Stossich, 1904, as well as the results of molecular phylogenetic studies.

Trematodes of the genus *Astiotrema* were separated from the family Plagiorchiidae based on the following criteria (*Astiotrema* vs. Plagiorchiidae): (1) seminal vesicle sac-shaped vs. seminal vesicle straight bipartite or convoluted undivided tubular [1,11]; (2) first intermediate host of *A. monticellii* according to Schevtschenko & Vergun [12] is the prosobranch snail *Bithynia leachi* (Seppard) vs. first intermediate host of Plagiorchiidae are pulmonate molluscs; (3) *A. monticellii*, as Schevtschenko & Vergun [12] wrote, has rediae and cercariae from the Pleurolophocerca group, which is typical for the species of the superfamily Opisthorchioidea Looss, 1899 vs. species of Plagiorchiidae, which have sporocysts and cercariae from the Xiphidiocercaria group. In addition, molecular phylogenetic studies of *A. monticellii*, *A. reniferum* and *Astiotrema turneri* Bray, van Oosterhout, Blais & Cable, 2006 showed that they form a monophyletic clade close to Heterophyidae Odhner, 1914 [1]. On the basis of the morphology of various developmental stages, life-cycle and composition of intermediate hosts typical for Plagiorchiidae, a new genus termed *Neoastiotrema* [11] was included in this family, which was founded for *Astiotrema trituri* Grabda, 1959 [11].

The first data on parasite fauna of *Pelodiscus sinensis* (Wiegmann) (syn. *Amyda sinensis*) in the territory of the Russian Far East were obtained in 1950 and 1960 [13,14]. Belous [14] found six digenean species, two of which belonged to *Astiotrema*, in *P. sinensis* of the

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Khanka Lake Basin. These were identified as *Astiotrema amydae* Ogata, 1938 and *Astiotrema spinosa* Chatterji, 1933.

In 2003, during parasitological investigations of the Komissarovka River Basin (Khanka Lake Basin), we found 37 specimens of adult flukes belonging to *Astiotrema* in the small intestine of the turtle *P. sinensis*. Subsequent research has focused on identifying possible routes of circulation of these worms in the conditions of the Primorsky Region through study of the morphology and developmental stages and by obtaining DNA sequence data. According to this research, it was found that these worms belong to *A. odhneri* [5] sensu Cho & Seo [15].

## 2. Material and methods

### 2.1. Morphology and life-cycle

The material for research included 37 adult *A. odhneri* that were found in the intestine of *Pelodiscus sinensis* from the Komissarovka River Basin. After preliminary identification conducted on live parasites, 17 specimens were fixed in 70% alcohol. The remaining 20 worms were rinsed in distilled water for 8 h, after which the uteri of these flukes were dissected by needles to obtain their eggs. To identify the first intermediate hosts, young laboratory-reared snails *Cipangopaludina ussuriensis* (Gerstfeldt), *Boreoelona ussuriensis* (Ehrmann), *Anisus centrifugops* (Prozorova et Starobogatov), *Helicorbis sujfunensis* (Starobogatov), and *Lymnaea ussuriensis* (Kruglov et Starobogatov) (15 specimens of each species) were placed in Petri dishes with the eggs of flukes. The control group consisted of ten specimens of each species. After two days, snails of each species were placed in separate aquaria. On the 38th day after the beginning of the experiments, snails were placed individually in Petri dishes. On the 45th day, cercariae from the Xiphidiocercaria group were found in dishes with *Anisus centrifugops*. On the 47th day, experimental snails of the control group were dissected: no parthenithae of any digenean species were identified. Second intermediate hosts were also defined using two aquaria containing cercariae-releasing *A. centrifugops*, together with five specimens of laboratory-reared *C. ussuriensis*, *B. ussuriensis*, *A. centrifugops*, *H. ujfunensis*, *L. ussuriensis*, larvae of dragonflies *Cordulia* sp., tadpoles of *Rana dybowskii* Günther, and the fish *Perccottus glenii* Dybowski. Dragonflies, tadpoles and fish were captured in an artificial reservoir where they were free from infection by any metacercariae. Thirty specimens of each species of these animals were examined for the presence of metacercariae. After 48 h of exposure, dragonflies, tadpoles, and fish were transferred to separate aquaria, but snails were not transferred. On the second day of the experiment, one specimen of each species (dragonfly, tadpole and fish) was dissected. Other animals, such as first intermediate hosts, were dissected on the 18th day. Metacercariae were found in snails *A. centrifugops* and *H. sujfunensis*, tadpoles *R. dybowskii* and the fish *P. glenii*. All experiments to determine intermediate hosts were carried out in water at 18 °C to 22 °C. Measurements of parthenithae and metacercariae were made on living specimens. Cercariae used for measurements were fixed in hot 4% formalin. Whole-mounts for adult descriptions were made by staining the specimens with alum carmine, dehydrating the worms in a graded ethanol series and clearing in xylene, followed by mounting in Canada balsam under a coverslip on a slide.

### 2.2. DNA extraction, amplification and sequencing

Adult *A. odhneri* specimens ($n = 6$) were obtained during parasitological fieldwork and were fixed in 96% ethanol for genetic analysis. Total DNA was extracted from whole worms using the "hot shot" technique [16].

The nuclear 28S rDNA was amplified by polymerase chain reaction (PCR) using the following primers: DIG12 (5′-AAG CAT ATC ACT AAG CGG-3′) and 1500R (5′-GCT ATCCTGAGGGAA ACT TCG-3′) [17]. Nuclear 18S rDNA was amplified using the primers 18S-E and 18S-F [18]. The initial PCR reaction was carried out using Dream-*Taq*- polymerase (Thermo Scientific). Amplification of the 1200-bp fragment of the 28S rDNA was performed in a GeneAmp 9700 thermocycler (Applied Biosystems) with annealing temperatures of 55 °C for 28S rDNA and 58 °C for 18S rDNA. Negative and positive controls were used. The 28S rDNA PCR products were directly sequenced using an ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit, as recommended by the manufacturer, with the internal sequencing primers described in Tkach et al. [17]. Amplified 18S rDNA fragments were sequenced with the primers for amplification and additional internal primers, as described in Littlewood and Olson [18]. The PCR products were analysed using an ABI 3130 genetic analyser at the Institute of Biology and Soil Science. The sequences have been submitted to the GenBank database with the following accession numbers: LN589984–LN589989 for 18S rDNA and LN589990–LN589991 for 28S rDNA.

### 2.3. Alignments and the phylogenetic analysis

The ribosomal DNA sequences were assembled with SeqScape (v. 2.6) software and aligned using the MEGA (v. 5.22) [19] alignment explorer with default options. The regions that could not be unambiguously aligned were excluded from the analyses. Phylogenetic analysis was performed using Bayesian algorithms with MrBayes (v. 3.1.2) software [20]. Phylogenetic algorithms were used with the general time reversible model, including gamma-distributions and proportion of invariant sites (GTR + G + I) [21]. This model showed the best fit to the data using Bayesian information criterion (BIC) in Modeltest (v. 3.07) software [22]. Bayesian analysis was performed using 10,000,000 generations and with four independent runs. Burn-in values for "sump" and "sumt" commands were 1,000,000, which were established with the Tracer software [23]. The significance of the phylogenetic relationship was estimated by posterior probabilities [20].

The phylogenetic relationships of *A. odhneri* were inferred from our data and the 28S rDNA nucleotide sequences of other trematode specimens obtained from the NCBI GenBank [24–34] (Table 1).

## 3. Results

### 3.1. Description

*A. odhneri* [5] sensu [15].
*Definitive host*: *P. sinensis* (Wiegmann).
*Site*: small intestine.
First intermediate host: *A. centrifugops* Prozorova et Starobogatov (experimentally).
Second intermediate hosts: snails *H. sujfunensis* Starobogatov and *A. centrifugops*; tadpoles *R. dybowskii*; fish *P. glenii* (experimentally).
*Locality*: Komissarovka River Basin, Primorsky Region, southern Far East of Russia; 44°54′N, 133°57′E.

#### 3.1.1. Adult worms (material examined: 10 specimens) (Fig. 1a, Table 2)

Body elongated, tapered towards each end. Surface spined from anterior end to level of posterior margin of ventral sucker. Oral sucker subterminal. Prepharynx very short, pharynx small, round or transversely elongated. Oesophageal bifurcation anterior to ventral sucker. Caeca reach level of posterior testis, but do not extend beyond it. Ventral sucker in anterior third of body, equal to, or somewhat larger than, oral sucker. Testes round or transversely oval, one behind other, anterior testis slightly submedian left and second testis slightly submedian right. Anterior testis immediately post equatorial. Testes separated by uterine loops. Seminal ducts merge into vas deferens which enters cirrus sac. Cirrus sac large, preequatorial between oesophageal bifurcation and anterior testis, contains saccate seminal vesicle and muscular prostatic duct. Cirrus everted in most fixed specimens, forming globular structure

**Table 1**
List of taxa, incorporated into molecular analysis by 28S rRNA gene sequences.

| Species | n | Author | ENA accession number |
|---|---|---|---|
| *Astiotrema odhneri* | | This study | LN589990–LN589991 |
| *Astiotrema monticellii* | | Tkach et al., 2001 [24] | AF184253 |
| *Opisthorchioidea* | | | |
| *Heterophyidae* | | | |
| *Metagonimus yokogawai* | 1 | Thaenkham, Kino, Nawa, 2010, unpublished | HQ832641 |
| *Procerovum cheni* | 1 | Thaenkham et al., 2010 [26] | HM004193 |
| *Haplorchis yokogawai* | 1 | Thaenkham et al., 2010 [26] | HM004192 |
| Opisthorchiidae | | | |
| *Clonorchis sinensis* | 1 | Thaenkham et al., 2010a, unpublished [26] | JF823989 |
| *Opisthorchis viverrini* | 1 | Thaenkham et al., 2010a, unpublished [26] | JF823990 |
| *Cryptogonimidae* | | | |
| *Acanthostomum burminis* | 1 | Jayawardena et al., 2013 [34] | KC489791 |
| *Adlardia novaecaledoniae* | 1 | Bray et al., 2009 [33] | FJ788496 |
| *Caecincola parvulus* | 1 | Olson et al., 2003 [25] | AY222231 |
| *Mitotrema anthostomatum* | 1 | Olson et al., 2003 [25] | AY222229 |
| *Siphodera vinaledwardsii* | 1 | Olson et al., 2003 [25] | AY222230 |
| *Lepocreadioidea* | | | |
| *Brachycladiidae* | | | |
| *Zalophotrema hepaticum* | 1 | Olson et al., 2003 [25] | AY222255 |
| *Acanthocolpioidea* | | | |
| *Acanthocolpidae* | | | |
| *Stephanostomum cestillium* | 1 | Olson et al., 2003 [32] | DQ248226 |
| *Gorgoderoidea* | | | |
| *Paragonimidae* | | | |
| *Paragonimus westermani* | 1 | Devi et al., 2011, unpublished | JN656173 |
| *Plagiorchioidea* | | | |
| *Reniferidae* | | | |
| *Renifer aniarum* | 2 | Santoro et al., 2010, unpublished | HQ665459–HQ665460 |
| *Telorchiidae* | | | |
| *Telorchis bonnerensis* | 1 | Pulis, Tkach, Newman, 2011 [27] | JF820592 |
| *Telorchis assula* | 1 | Tkach, Pawlowski, Mariaux, 2000 [28] | AF151915 |
| *Haematoloechidae* | | | |
| *Haematoloechus varioplexus* | 1 | Snyder and Tkach, 2001 [29] | AF387798 |
| *Macroderoididae* | | | |
| *Macroderoides typicus* | 1 | Tkach, Snyder, Swiderski, 2002, unpublished [29], unpublished | AF433673 |
| *Paramacroderoides kinsellai* | 1 | Tkach, Pulis, Overstreet, 2010 [30] | HM137662 |
| *Alloglossidium kenti* | 1 | Tkach and Mills, 2011 [31] | JF440809 |
| *Plagiorchiidae* | | | |
| *Plagiorchis elegans* | 1 | Boyce et al., 2012, unpublished | JX522535 |
| *Echinostomatoidea* | | | |
| *Fasciolidae* | | | |
| *Fasciola hepatica* | 1 | Olson et al., 2003 [25] | AY222244 |

0.25–0.28 × 0.27–0.35 mm in size. Vitellarium in two lateral fields of small follicles, between level of ventral sucker and mid-level of second testis. Ovary spherical, midway between ventral sucker and anterior testis, right of base of cirrus sac. Large U-shaped seminal receptacle. Vitelline reservoir and Laurer's canal between ovary and anterior testis. Uterine loops in posterior part of body and between testes. Metraterm reaches half of length of cirrus sac. Genital pore directly in front of ventral sucker. Eggs operculated, in distal part of uterus contain miracidia. Excretory bladder Y-shaped.

*3.1.2. Sporocyst (material examined: 10 specimens) (Fig. 1b)*

Thin-walled oval body 0.350–0.550 × 0.062–0.10 mm. Birth pore terminal. Each sporocyst contains up to eight cercariae at various developmental stages.

*3.1.3. Cercaria (material examined: 15 specimens) (Fig. 1c, d)*

Body oval, 0.18–0.20 × 0.09–0.13 mm, covered by small spines. Large spines in folds of caudal pocket. Oral sucker oval, with stylet, 0.039–0.053 × 0.045–0.048 mm. Stylet with underdeveloped shoulders and small bulb, 0.019–0.020 × 0.0046 mm. Prepharynx short. Pharynx 0.014–0.019 mm in diameter. Oesophagus long. Oesophageal bifurcation in front of ventral sucker. Caeca dorsal to lateral margins of ventral sucker, not extending beyond it. Ventral sucker in post equatorial zone, 0.050–0.056 mm in diameter. Two lateral groups of penetration glands, each of six cells, close to anterior margin of ventral sucker. Ducts of these glands open near tip of stylet. Two groups of metacercarial glands, each consisting of four cells on level of pharynx. Their ducts open on anterior end of body. Medium-sized cystogenous cells extend from middle of oesophagus to posterior end of body. Curved primordium of genital system dorsal to ventral sucker. Excretory bladder Y-shaped. Flame cell formula: $2[(3 + 3 + 3) + (3 + 3 + 3)] = 36$. Tail simple, 0.150–0.160 × 0.022 mm. Base of tail immersed in caudal pocket.

*3.1.4. Metacercaria (material examined: 10 specimens) (Fig. 1e, f)*

Cyst round, 0.129–0.15 × 0.13–0.14 mm. Body of excysted metacercaria 0.29–0.30 × 0.16–0.17 mm. Anterior end of body spined to level of ventral sucker. Subterminal oral sucker 0.042–0.045 ×

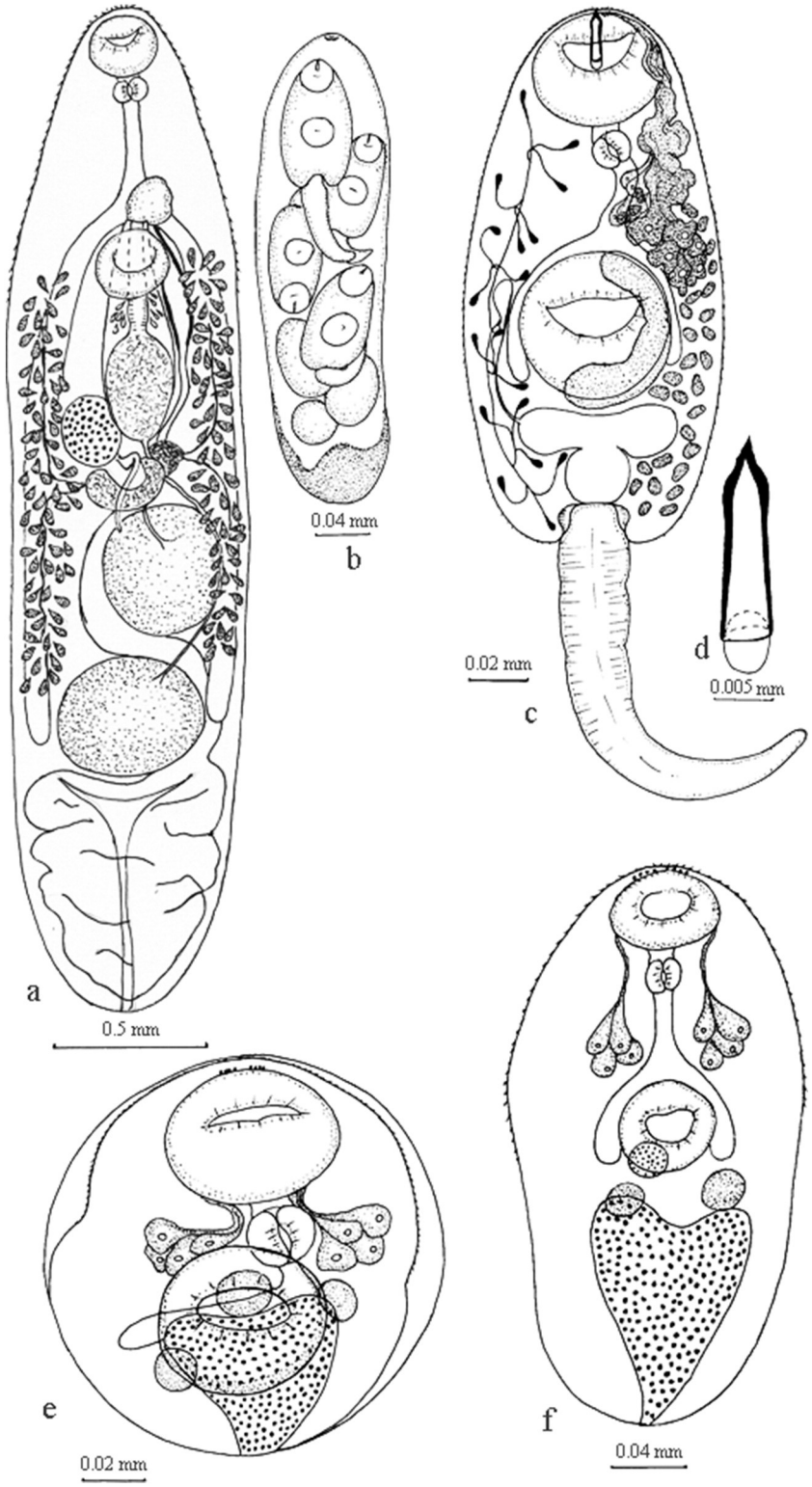


**Fig. 1.** *Astiotrema odhneri*: a — adult worms, b — sporocyst, c — ceracaria, d — stylet, e — metacercaria in cyst, f — metacercaria excysted.

0.056–0.059 mm. Prepharynx and oesophagus present. Pharynx 0.017–0.019 × 0.019–0.022 mm. Oesophageal bifurcation in front of ventral sucker. Caeca reach level of second half of ventral sucker. Ventral sucker 0.048–0.050 × 0.050–0.056 mm. Primordia of testes submedian, opposite, slightly posterior to ventral sucker, left and right from median line of body. Ovary primordium dorsal to ventral sucker. Two glands of four cells each, on both sides of oesophagus. Their ducts open on anterior end of body. Excretory bladder V-shaped, filled with granules.

### 3.2. Life-cycle

It was experimentally established that the snail, *A. centrifugops*, is a first intermediate host of *A. odhneri*. Other snails used in the

**Table 2**
Comparative metrical (mm) data for *Astiotrema odhneri*.

| Features | Present study | Yeh & Fotedar (1958) [9] | Belous (1958) (from Skrjabin & Antipin 1958 [35]) | Tang (1941) (from Skrjabin & Antipin [35]) | Gupta (1954) [6] | Cho & Seo (1977) [15] | |
|---|---|---|---|---|---|---|---|
| | Range | Mean | | | | | |
| Body length | 3.20–3.54 | 3.30 | 4.0–5.0 | 4.20 | 5.0 | 4,08–5,304 | 3.35 (2.75–3.79) |
| Body width | 0.678–0.79 | 0.74 | 1.0 | 1.10 | 1.21 | 0,85 | 0.76 (0.57–0.89) |
| Forebody | 0.660–0.755 | 0.724 | | | | 0.65 | |
| Oral sucker length | 0.20–0.216 | 0.212 | 0.25 | 0.215 | 0.249 | 0.210 | 0.17 |
| Oral sucker width | 0.20–0.231 | 0.211 | 0.30 | 0.215 | 0.209 | 0.225 | 0.17 |
| Ventral sucker length | 0.216–0.246 | 0.234 | 0.25 | 0.30 | – | 0.18 | 0.22 |
| Ventral sucker width | 0.216–0.246 | 0.226 | 0.30 | 0.30 | – | 0.18 | 0.20 |
| Sucker length ratio | 1:1.0–1.16 | 1:1.1 | – | – | – | – | 1:1.22 |
| Sucker width ratio | 1:1.0–1.16 | 1:1.08 | – | – | – | – | – |
| Pharynx length | 0.092–0.108 | 0.097 | – | 0.08 | 0.090 | 0.12 | 0.09 |
| Pharynx width | 0.108–0.139 | 0.122 | – | 0.08 | 0.104 | 0.15 | 0.07 |
| Oesophagus length | 0.26–0.40 | 0.32 | – | 0.260 | 0.498 | 0.510–0.525 | 0.17 |
| Anterior testis length | 0.35–0.416 | 0.381 | 0.45 | 0.450 | – | 0.465–0.495 | 0.38 |
| Anterior testis width | 0.339–0.493 | 0.399 | 0.65 | 0.450 | – | 0.345–0.375 | 0.31 |
| Posterior testis length | 0.370–0.416 | 0.387 | 0.45 | 0.450 | – | 0.51–0.525 | 0.39 |
| Posterior testis weight | 0.370–0.508 | 0.416 | 0.65 | 0.450 | – | 0.39–0.465 | 0.34 |
| Cirrus sac length | 0.74–0.86 | 0.78 | – | 1.320 | 1.054 | 0.51–0.54 | 0.76 (0.43–0.91) |
| Cirrus sac width | 0.169–0.231 | 0.22 | – | 0.240 | 0.207 | 0.225 | – |
| Ovary length | 0.216–0.277 | 0.241 | – | 0.280 | 0.282 | 0.25–0.30 | 0.18 |
| Ovary width | 0.169–0.262 | 0.203 | – | 0.280 | 0.282 | 0.21–0.255 | 0.17 |
| Metraterm length | 0.45–0.46 | – | – | 0.400 | – | – | – |
| Metraterm width | 0.123–0.154 | – | – | 0.160 | – | – | – |
| Distances | | | | | | | |
| Pre-ovarian field length | 1.26–1.39 | 1.31 | – | – | – | – | – |
| Pre-testicular field length | 1.54–1.76 | 1.64 | – | – | – | – | – |
| Pre-vitellarium field length | 0.601–0.862 | 0.814 | – | – | – | – | – |
| Post-vitellarium field length | 1.02–1.31 | 1.21 | – | – | – | – | – |
| Post-testicular field length | 0.755–0.97 | 0.862 | – | – | – | 0.969–1.394 | – |
| Eggs length (0.041–0.046) | 0.031–0.039 | – | 0.026–0.029 | 0.033 | 0.028–0.034 | 0.0238–0.034 | 0.044 |
| Eggs width | 0.015–0.019 | – | 0.011 | 0.016 | 0.012–0.016 | 0.0102–0.0136 | 0.013 (0.012–0.015) |

experiments (e.g. *C. ussuriensis*, *B. ussuriensis*, *H. sujfunensis* and *L. ussuriensis*) were not infected. The incidence of infection of *A. centrifugops* by parthenitae of *A. odhneri* was 53.3%. The time from infection of snails to emission of first cercariae was 45 days. From 2 to 4 h after leaving the snail, cercariae were actively swimming in the water column and did not show either positive or negative phototaxis. Later, movement of cercariae in the water column alternated with periods of settling to the bottom. Larvae settled at the bottom moved using their suckers. The second intermediate hosts of *A. odhneri* include tadpoles of *R. dybowskii*, the fish *P. glenii*, and the snails *H. sujfunensis* and *A. centrifugops*. The intensity of infection of tadpoles reached 30 metacercariae, whereas this value was 17 in fish and not more than five in snails. In our experiments, the larvae of dragonflies *Cordulia* sp. and the snails *B. ussuriensis* and *C. ussuriensis* were not infected. Observation showed that cercariae, during swimming or moving along the tank bottom, attached to their second intermediate hosts using suckers and actively penetrated into the body. The penetration time into tadpoles was around 20 to 30 min. Penetrated cercariae moved into the hosts' muscles and organ tissues and encysted. Granules filling the excretory bladder of metacercariae during the first two days of infection area characteristic feature of metacercariae development. On day 18, metacercariae were found in experimentally infected second intermediate hosts, which corresponded to the above description. A fairly wide range of second intermediate hosts for *A. odhneri* is probably a result of historical ties, as *P. sinensis* has a varied diet including snails, fishes and amphibians.

### 3.3. Alignments and phylogenetic analyses

Molecular data were used for two main goals: (1) species-level comparison among samples of *A. odhneri* and *A. montichellii*; and (2) for inference of the affinities and the proper systematic position of the genus *Astiotrema*. Species comparison was performed using only the 28S rRNA gene fragment, while phylogenetic analysis was performed using the 28S rRNA gene and 18S rRNA gene separately. This was because of different availability of nucleotide sequences in the GenBank database.

#### 3.3.1. 18S rDNA sequence data

Despite successful amplification of the complete 18S rRNA gene (about 1800 bp in length) for many trematode species, we were unable to obtain a large PCR product for the 18S rRNA gene for *A. odhneri* with the primers described above. There was a single PCR product approximately 300 bp in length exclusively for this species, which contained the 5′ end of the 18S rRNA gene. Nucleotide sequences of this fragment were identical for different specimens of *A. odhneri*. Phylogenetic tree based on short 18S rRNA gene sequences showed unresolved topology with low statistical support. For that reason we didn't made any claims and conclusions based on this phylogeny.

#### 3.3.2. 28S rDNA sequence data

The alignment of 28S rDNA of *A. odhneri* and *A. monticellii* used a 1135-bp long sequence. The alignment of these two species contained 17 variable sites, distributed across the whole of the compared fragment.

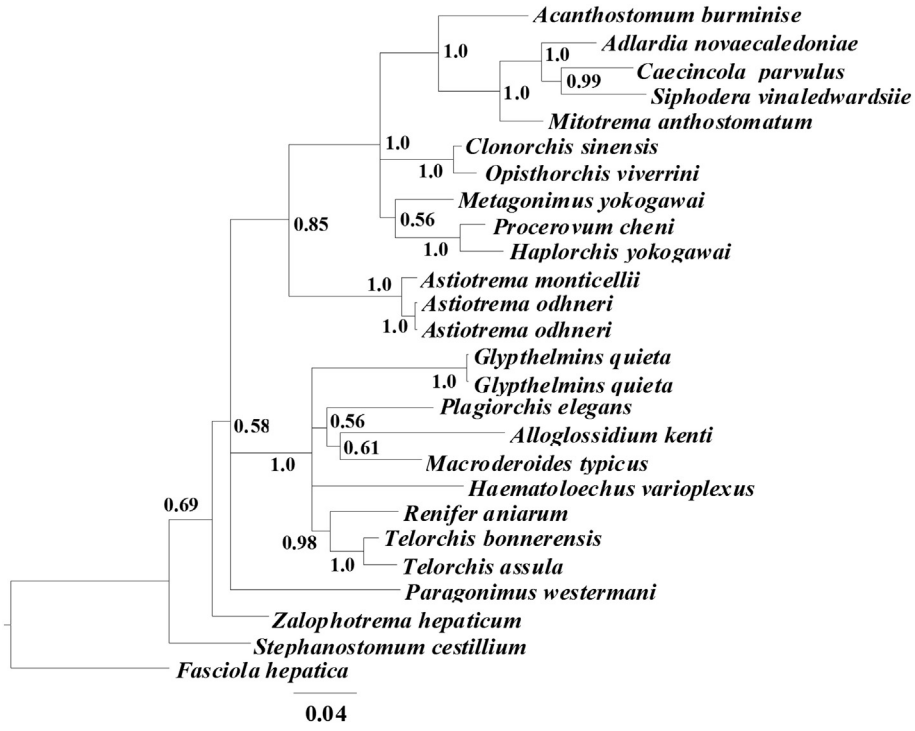


**Fig. 2.** Phylogenetic relationships of *Astiotrema odhneri*, reconstructed with the Bayesian analysis based on 28S rRNA gene partial sequences with outgroup.

Results of the Bayesian analysis yielded a tree topology with high support of posterior probability values at most nodes. *A. odhneri* and *A. monticellii* were closely related to each other. The genus *Astiotrema* was closer to the superfamily Opisthorchioidea on the phylogenetic tree with outgroup species, *F. hepatica* (Fig. 2). Alternatively we performed Bayesian algorithm without outgroup taxa. Obtained phylogenetic tree separates out *Astiotrema* as significantly differentiated from both the Plagiorchioidea and the Opisthorchioidea (Fig. 3).

## 4. Remarks

In our material, all worms had a similar morphology: an elongated body, non-lobed ovary and testes, vitellarium and caeca that did not extend beyond the posterior margin of the second testis. By features such as body size and organs, worms from our material are most similar to *A. odhneri* found by Cho and Seo [15] in *P. sinensis* from Korea (Table 2). We agree with the views of Pojmanska et al. [1], i.e. that a

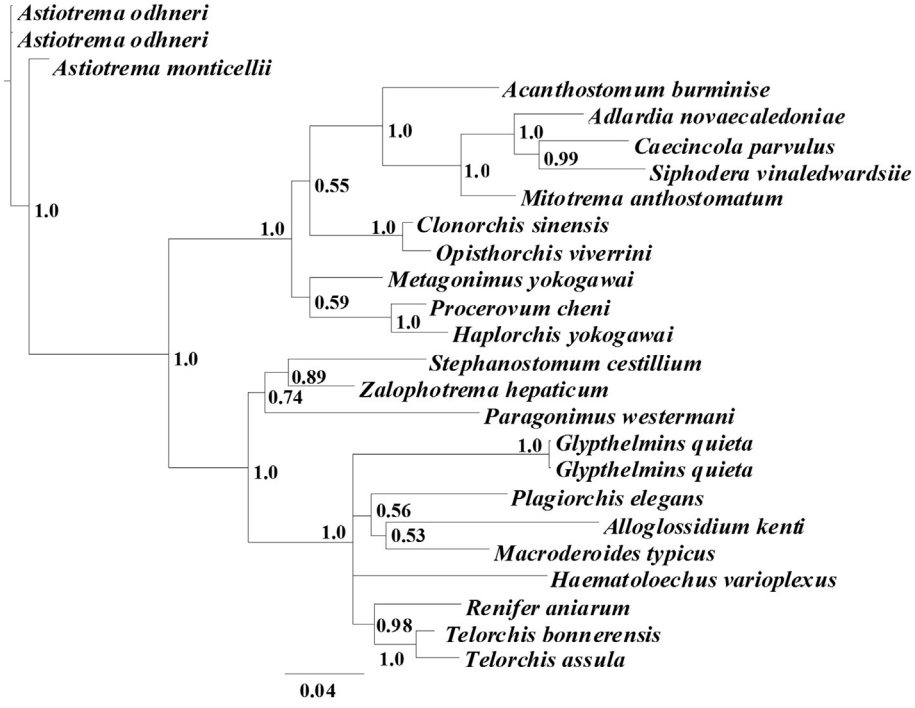


**Fig. 3.** Phylogenetic relationships of *Astiotrema odhneri*, reconstructed with the Bayesian analysis based on 28S rRNA gene partial sequences without outgroup.

thorough revision of *Astiotrema* is required. To avoid further complicating the species composition of this genus, we applied the name *A. odhneri* [5] sensu [15] to our detected specimens.

At present, *Astiotrema* can be removed from Plagiorchiidae and temporarily regarded as *incertae sedis* [1,11]. This conclusion was based on results of molecular phylogenetic studies, which demonstrated that *A. reniferum*, *A. monticellii* and *A. turneri* formed a monophyletic clade, which was closer to Opisthorchioidea [1]. Only a single species from *Astiotrema*, *A. trituri*, for which a new genus, *Neoastiotrema*, was established, remains in Plagiorchiidae [11]. This species is similar to Plagiorchiidae in terms of: (1) species specific relationships with snails of the genus *Planorbarius* — first intermediate host, (2) development stages, including sporocyst and xiphidiocercaria and (3) bipartite seminal vesicle of adult worms.

Further analysis of the results obtained by Schevtschenko & Vergun [12] shows the incorrectness of their interpretation of the *A. monticellii* life-cycle; the authors found adult worms in snakes from the middle reaches of Seversky Donets, as well as cercariae from pleurolophocerca in *Bithynia leachi* and metacercariae in naturally infected amphibians. These larvae were a priori classified as *A. monticellii*. The experimental component of this work included only infection of uninfected amphibians by cercariae from naturally infected *B. leachi*. As a result of this experiment, metacercariae morphologically identical to those in amphibians from Seversky Donets River were obtained. In experimental part of their work Schevtschenko & Vergun did not fulfil at least one of the two obligate conditions that are necessary during the study of the life cycle. First, experimental infection of sterile snails by miracidia of *A. monticellii* did not occur. Second, adult worms from metacercariae obtained in the experiment were not grown. Cercariae that were morphologically identical to those obtained by Schevtschenko & Vergun were found by us in *Boreoelona* (Bithyniidae) from the Russian Far East, and in experiments, these larvae infected amphibians. Later, from metacercariae, we were able to grow adult worms that belonged to *Metorchis* (Opisthorchiidae). Thus, we consider that Schevtschenko & Vergun [12] were not dealing with cercariae and metacercariae of *Astiotrema*, but instead most likely described larvae belonging to Opisthorchioidea.

Molecular studies of Tkach et al., 2001 [24] and Olson et al., 2003 [25] demonstrated that *A. monticellii* is not closely related to plagiorchioid digeneans. Later, Pojmanska et al. reported that a few *Astiotrema* species formed a monophyletic clade that was closest to heterophyids by molecular data [1]. The phylogenetic analyses reported here show that *A. odhneri*, together with *A. monticellii*, formed a monophyletic clade. Molecular data on the other species of the genus *Astiotrema* are not available in GenBank. Phylogenetic analysis performed using outgroup taxon, *F. hepatica*, showed that the genus *Astiotrema* is closer to Opisthorchioidea than to any other taxon represented in the tree (Fig. 2). Phylogenetic analysis, performed without outgroup taxa, indicate that *Astiotrema* belongs neither to the Opisthorchioidea nor the Plagiorchioidea (Fig. 3). At the same time, participation of the pulmonate snail in the life-cycle of *A. odhneri* and development with forming sporocysts and Xiphidiocercaria are evidence that it belongs to Plagiorchioidea.

In conclusion we have to say that taxonomical status of *Astiotrema* is still not clear. There are not enough molecular data for plagiorchioid trematodes to perform representative analysis and to discuss phylogenetic position of the genus *Astiotrema*.

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