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The first modern morphological description of *Cercaria pennata* and molecular evidence of its synonymy with *Pronoprymna ventricosa* in the Black Sea

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Research Paper

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Abstract

During the parasitological examination of molluscs *Abra segmentum* obtained from the Black Sea basin, parthenitae belonging to the family Faustulidae were found. The cercariae were obtained by natural emergence and were studied using differential interference contrast microscopy and scanning electron microscopy. Specimens resemble *Cercaria pennata* ex *Tapes rugatus* which was described from the Sevastopol area, in the shape and length of the body, tail length, location and shape of internal organs, suckers, pharynx, testicular rudiments, and the number and position of longitudinal lamellae on the tail finlets. To date, there are only limited descriptions of the parthenitae of *C. pennata* without detailed measurements, thus the taxonomic position of the individuals studied needs thorough revision and molecular verification. According to the molecular analyses, *C. pennata* was identical to that of published sequences of *Pronoprymna ventricosa*.

Introduction

To date, a large number of helminthological studies have focused on the descriptions of trematode parthenitae. However, this is not enough to correlate the taxonomic position of trematode larvae with the systematic status of the maritae with only morphology. Identification of parthenitae has previously been on the basis of morphological characteristics, but it is very difficult to compare the morphological characteristics of trematode larval stages with that of adults. With the advent of current molecular studies, however, it has become possible to reconstruct the life cycles of trematodes through the comparison of larval and adult stages using genetic markers.

Abra segmentum (Recluz, 1843) is a common and abundant mollusc in the Black Sea (Kiseleva *et al.*, 1996) and Mediterranean Sea (Denis, 1981) coastal lagoons, sometimes playing a dominant role in these habitats and is an important food for a variety of marine species (Cilenti *et al.*, 2009). Sinitzin in his work (1911) described two closely related species, which have subsequently been considered to represent the family Faustulidae – *Cercaria pennata* in *Polititapes aureus* (Gmelin, 1791) (=*Tapes rugatus*) and *Cercaria plumosa* in *Abra alba* (Wood, 1802). The species differ in several characters (size of the body, the length of the tail and finlets and in the number of longitudinal lamellae) (Sinitzin, 1911).

In this study, first complex data, including morphological descriptions and molecular characteristics of parthenitae of *Cercaria pennata* from bivalve molluscs *A. segmentum* in the Black Sea are presented. The molecular phylogeny was reconstructed based on the nucleotide sequences of the 28S rRNA gene fragment.

Material and methods

Sample collection

A total of 1053 *A. segmentum* specimens were collected in two different biotopes – the estuary of the Chernaya River (44°27′49″ N, 33°51′37″ E) and in Kazachya Bay (44°36′29″ N, 33°35′54″ E) (Sevastopol, Black Sea) monthly during 2011–2013. All snails were examined for helminthic infections using standard methods (Bykhovskaya-Pavlovskaya, 1969). Parthenitae of trematodes were studied alive and stained using an Olympus CX41 microscope equipped with a CX50 camera with software Infinity Analyze. Trematodes were fixed under a cover glass with slight pressure and stained with acetocarmine. The degree of colour was differentiated by 'iron water' (water + iron(III) oxide) and acidified alcohol (70% ethanol + 3% hydrochloric acid). After dehydration in ethanol of increased concentrations (70, 80, 90 and 100%) and clarification in clove oil, trematodes were mounted in Canada balsam (Roskin & Levinson, 1957).

Morphological data

All measurements were made on stained parasites. The abbreviations for the metrical features are as follows: BL, body length; BW, body width; OSL, oral sucker length; OSW, oral sucker width; PL, prepharynx length; PHL, pharynx length; PHW, pharynx width; OL, oesophagus length; VSL, ventral sucker length; VSW, ventral sucker width;TL,testis length; TW, testis width; OVL, ovary length; OVW, ovary width; CEND, post-caecal field length; TEND, post-testicular field length (Blasco-Costa *et al.*, 2009). The excretory system of the larvae was researched on living individuals when the larvae were stained with neutral red, as a result of which the flickering of the flame cells was observed. Drawings were made using a drawing software Inkscape 0.48.2.-1 (Scalable Vector Graphics, 2011).

Scanning electron microscopy (SEM)

Live sporocysts and spontaneously emitting cercariae were fixed in 2.5% (v/v) glutaraldehyde buffered with 0.1 M Sorensen phosphate for 24 h at 5°C, after which samples were dehydrated through an ethanol series (70–96°C) and dried in a Leica EM CPD 300 critical point dryer using liquid carbon dioxide as a transitional medium. After drying, they were mounted on aluminium stubs and coated with gold in an ion-sputtering apparatus Leica EM ACE 200.

Molecular data

One cercaria was fixed in 96% ethanol. Total DNA was extracted using innuPREP Mini Kit (Analytik Jena, Germany). The specimen was incubated in 100 µl of lysis buffer with 5 µl of Proteinase K at 56°C for one hour with following mix by vortex for 20 s. DNA extraction was carried out according to the manufacturer's protocol. The elution volume was 20 µl. The DNA was stored at −20°C.

The ribosomal 28S RNA gene fragment was amplified using the 28S-A forward primer (5′ GCA CCC GCT GAA YTT AAG 3′) (Matejusova & Cunningham, 2004) and 1500R (5′ GCT ATC CTG AGG GAA ACT TCG 3′) (Tkach *et al.*, 1999, 2000). An initial polymerase chain reaction (PCR) was performed in a total volume of 25 µl containing 0.25 mM of each primer pair, 25 ng of total DNA in water and 12.5 µl of Promega GoTaq Green Master mix (Madison, Wisconsin, USA). Amplification of a 1200-base pairs (bp) fragment of the 28S rRNA gene was performed in a GeneAmp 9700, Applied Biosystems, with a 5-min denaturation at 96°C, 35 cycles of 1 min at 96°C, 20 s at 55°C and 2 min 30 s at 72°C, and a 10-min extension at 72°C. Negative and positive controls were made with the use of both primers.

The PCR product was directly sequenced using an ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, USA), as recommended by the manufacturer, with the internal sequencing primers, described by Tkach *et al.* (2003) for 28S rDNA. The PCR product was analysed using an ABI 3500 genetic analyser at the Federal Scientific Center of the East Asia Terrestrial Biodiversity, Far Eastern Branch of the Russian Academy of Sciences. The sequence was submitted to the United States National Center for Biotechnology Information (NCBI) database with accession number: (will be available after manuscript acceptance).

Alignment and phylogenetic analysis

Ribosomal DNA sequences were assembled with SeqScape v.2.6 software. Alignments and estimation of the number of variable sites and sequence differences were performed using the MEGA 7.1 (Kumar *et al.*, 2016).

Phylogenetic relationships were inferred from our data and the nucleotide sequences of 28S rDNA from other trematode specimens from the families Faustulidae, Zoogonidae, Tandanicolidae and Gymnophalloidea *incertae sedis* (*Pseudobacciger cheneyae*) obtained from the NCBI GenBank database (table 1).

Phylogenetic analysis of nucleotide sequences was undertaken using maximum likelihood (ML) and Bayesian (BI) methods. Prior to analysis, the nucleotide substitution model was estimated using Akaike's information criterion for ML (Akaike, 1974) and Bayesian information criterion for BI (Huelsenbeck *et al.*, 2001) using jModeltest v.3.07 software (Darriba *et al.*, 2012). The model TVM + I + G (Posada, 2003) was estimated as those best fitting the 28S rDNA sequence data of the dataset used for both ML and BI analyses. Phylogenetic trees were reconstructed with PhyML 3.1 (Guindon & Gascuel, 2003) and MrBayes v.3.1.2 software (Huelsenbeck *et al.*, 2001). A Bayesian algorithm was performed using the Markov chain Monte Carlo option with ngen = 10,000,000, nruns = 2, nchains = 4, temp = 0.5 and samplefreq = 100. Burning values for 'sump' and 'sumt' options composed 25% of number of generations (ngen). Phylogenetic relationship significance was estimated using approximate likelihood-ratio test using eBayes support (Anisimova & Gascuel, 2006) for ML and posterior probabilities for BI analyses (Huelsenbeck *et al.*, 2001).

Statistical methods

After the parasites were detected, we assessed the infection indexes in the *Abra*, including invasion intensiveness (prevalence) (IE), invasion intensity (II), and abundance index (AI). For each morphological parameter, the arithmetic mean with standard error (mean ± SE) was calculated. To calculate the statistical parameters, the Statistica 6 software package for Windows (Statsoft) was used.

Results

Cercaria pennata Sinitzin, 1911

Host: *Abra segmentum* (Recluz, 1843)

Locality: Black Sea, near Sevastopol (44°27′49″ N, 33°51′37″ E); (44°36′29″ N, 33°35′54″ E)

Site: Hepatopancreatic gland

IE = 2%: II = 1–30

Description

Sporocysts long, saccular, 2082–5110 (3852) × 437–645 (552) µm, and fill hepatopancreatic gland of their host.

Cercariae (based on 15 specimens, table 2, figs 1 and 2).

All measurements and body proportions are listed in table 2.

Body oval. Tegument spined: spines small. Oral sucker subterminal, almost the same size as ventral sucker. Ventral sucker in middle of body. Oral to ventral sucker distance slightly less than ventral sucker to end of body distance. Prepharynx absent. Pharynx muscular, well-defined. Oesophagus narrow. Intestinal bifurcation slightly anterior to ventral sucker. Caeca reach posterior margin of testes. Testes two, arranged symmetrically, laterally, posterior to ventral sucker. Ovary oval slightly posterior to testes. Tail length 0.8 mm, almost twice as long as body length, with row of feathers on each side perpendicular to tail surface, 20 pairs in number. Number of longitudinal lamellae on feathers ranged

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Table 1. List of taxa incorporated in the molecular analysis of the superfamily Microphalloidea with the number of 28S rDNA sequences given in parentheses.

Species	Author	Accession numbers
Microphalloidea		
Zoogonidae		
Cephaloporinae		
Zoogonoides viviparus (*n* = 1)	Olson *et al.* (2003)	AY222271
Plectognathotrema kamegaii (*n* = 1)	Cutmore *et al.* (2014)	KM505035
Lecithostaphylinae		
Deretrema nahaense (*n* = 1)	Olson *et al.* (2003)	AY222273
Lecithostaphylus brayi (*n* = 3)	Cabañas-Granillo *et al.* (2020)	MT704137- MT704139
Lecithostaphylus halongi n. sp. (*n* = 2)	present study	n/a
Proctophantastes gillissi (*n* = 2)	Sokolov *et al.* (2016)	KU163452- KU163453
Lepidophyllinae		
Lepidophyllum cameroni (*n* = 2)	Sogrina *et al.* (2019) (unpublished)	MN217107- MN217108
Lepidophyllum steenstrupi (*n* = 1)	Lockyer *et al.* (2003)	AY157175
Faustulidae		
Antorchis pomacanthi (*n* = 2)	Olson *et al.* (2003) and Cribb *et al.* (2015)	AY222268, KR149729
Bacciger lesteri(*n* = 1)	Olson *et al.* (2003)	AY222269
Trigonocryptus conus (*n* = 1)	Olson *et al.* (2003)	AY222270
Eucotylidae		
Paratanaisia bragai (*n* = 2)	Unwin *et al.* (2013)	JX231098- JX231099
Tamerlania zarudnyi (*n* = 2)	Tkach *et al.* (2001) and Suleman Muhammad *et al.* (2021)	AF184248, MW131090
Tanaisia fedtschenkoi (*n* = 1)	Olson *et al.* (2003)	AY116870
Tanaisia valida (*n* = 3)	Soares *et al.* (2016) (unpublished)	KX913712- KX913714
Gymnophalloidea		

from eight to 12 in number. Excretory bladder V-shaped, both branches approach level of ventral sucker. Formula of excretory system is 2 [(3 + 3) + (3 + 3)] = 24.

Molecular data

Partial 28S rRNA gene sequence of a single specimen of *Cercaria pennata* of 1182 bp in the length (GenBank accession numbers will be provided after acceptance) was generated and aligned with all available ribosomal large subunit sequences of Faustulidae from gymnophalloid and microphalloid clades and also representatives of Gymnophalloidea and Microphalloidea and trimmed to the most optimal alignment length (1114 bp) for the available dataset.

Based on these data, ML and Bayesian algorithms generated phylogenetic trees with identical topologies (fig. 3). Results of phylogenetic analysis indicate that *C. pennata* ex *A. segmentum* from the Black Sea from our material clustered with *Pronoprymna ventricosa* (Rudolphi, 1819) Poche, 192 with high statistical support within gymnophalloid clade of Faustulidae as nucleotide sequences of 28S rDNA fragment of *C. pennata* and *P. ventricosa* (Rudolphi, 1819) Poche, 1926 were identical. *Pronoprymna petrowi* (Layman, 1930) Bray & Gibson, 1980 appears as sister species to [*C. pennata* + *P. ventricosa*] subclade. *Cercaria pennata and P. ventricosa* differed from that of *P. petrowi* on 3.25 ± 0.55%. Genetic *p*-distance values between *C. pennata* and other species within gymnophalloid clade ranged from 12.2 ± 1.04% to 13.9 ± 1.2%.

Discussion

The family Faustulidae is a member of the superfamily Microphalloidea. On the basis of molecular, morphological and life cycle data, Hall *et al.* (1999) removed the subfamily Baccigerinae from the Fellodistomiae. The Baccigerinae Yamaguti, 1959 was considered as the junior synonym of the Faustulidae, Poche, 1926. Members of family Faustulidae are known to present gymnocephalous cercariae.

Daughter sporocysts of *C. pennata* were found in the hepatopancreatic gland of *A. segmentum* hemipopulations. This type of parthenitae with similar characteristics were firstly detected by Sinitzin (1911), who described them as *C. plumosa* and *C. pennata*. The author registered these two morphologically similar faustulid cercariae in the bivalve molluscs *Abra alba* and *Polititapes aureus* (Gmelin, 1791) (=*Tapes rugatus)* in the water off Sevastopol. The trematode fauna of molluscs in the Black

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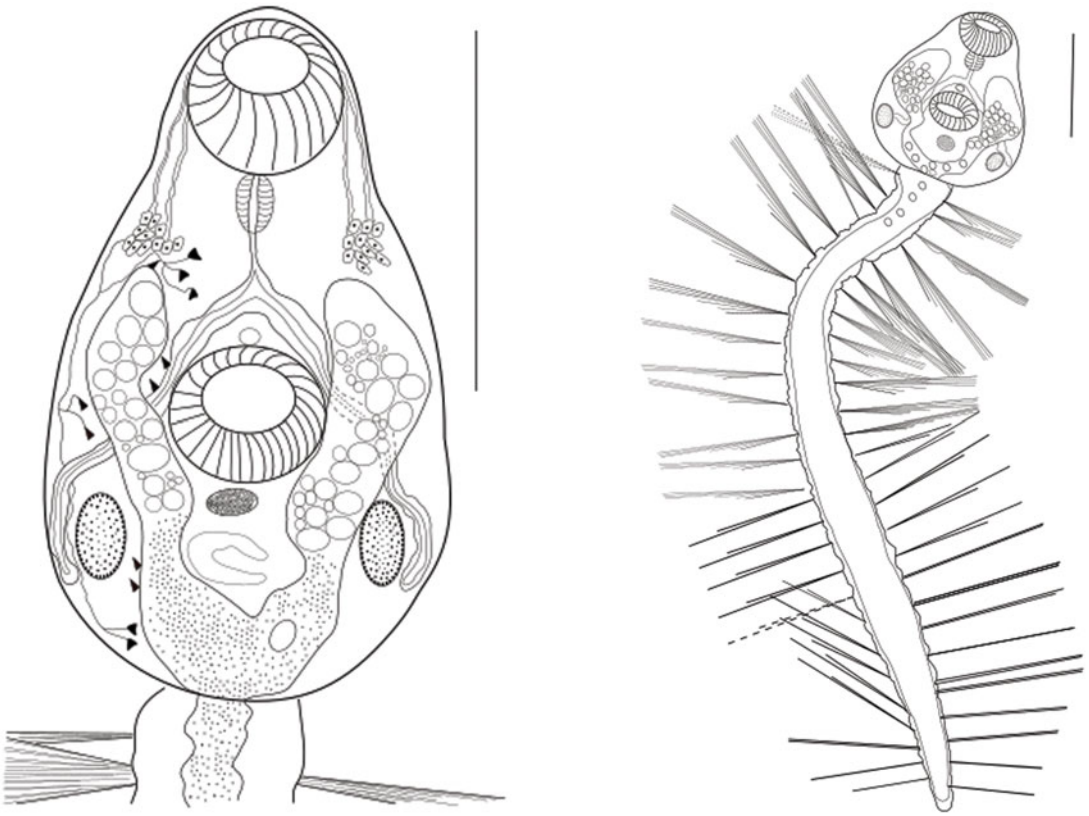

Fig. 1. Live *Cercaria pennata* from the mollusc *Abra segmentum*: (A) morphology of the cercaria body; (B) position of cercaria tail finlets. Scale: 200 μm.

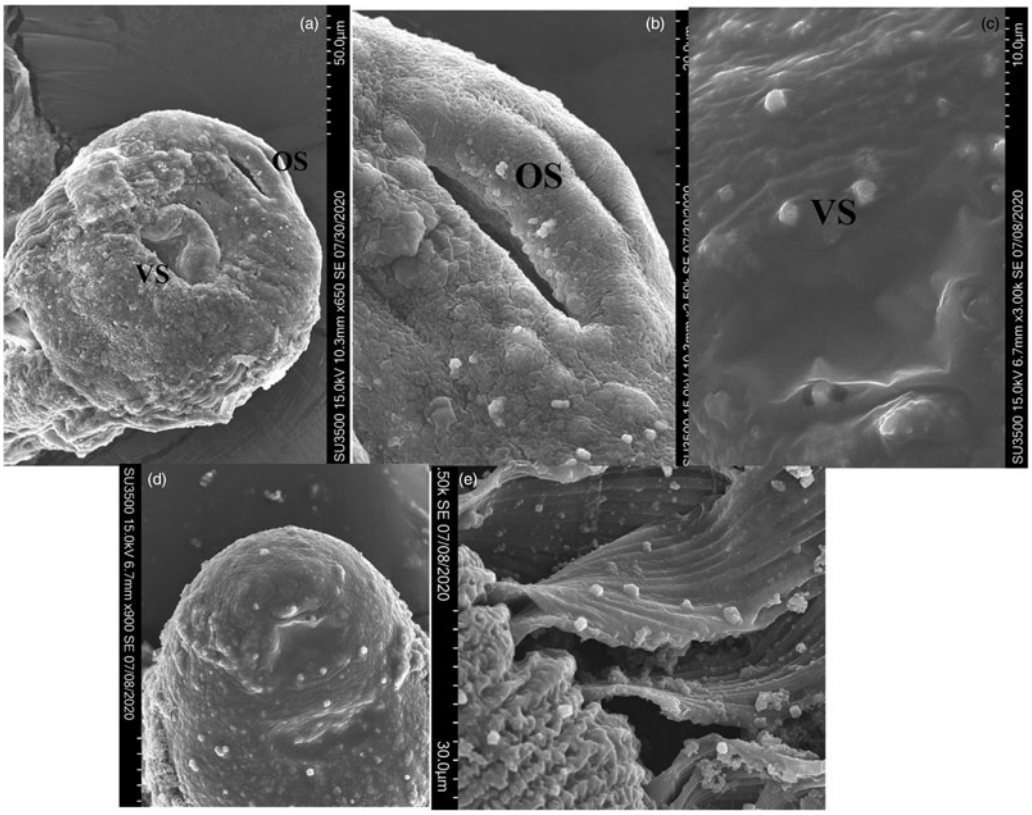

Fig. 2. Scanning electron microscopy photomicrographs of *Cercaria pennata* from mollusc *Abra segmentum*: (A, B) views of suckers: OS – oral sucker; VS – ventral sucker; (C, D) body surface structure;and (E) tail feathers and longitudinal lamellae.

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Table 2. Comparing morphological features of faustulid cercariae in the Black Sea.

Features	Present study, μm	Sinitsin, 1911 *Cercaria pennata*, mm	Sinitsin, 1911 *Cercaria plumosa*, mm
BL	330–530 (450)	0.45	0.24
BW	100–200 (165)		
OSL	50–100 (80)		
OSW	60–100 (90)		
VSL	70–100 (80)		
VSW	60–95 (80)		
PL	–		
PHL	25–35 (27)		
PHW	20–25 (24)		
OL	25–55 (37)		
OVL	20–35 (27)		
OVW	15–35 (22)		
TL	25–65 (40)		
TW	25–35 (28)		
Tail length	800–900 (840)	0. 6	0.36
Finlets length	0.19	0.21	0.072
Numerous of tail finlets	20		
Number of longitudinal lamellas	8–12	8–12	18–22
FO	140–220		
TEND	70–150		
CEND	0.125–180		
OSL/BL	0.125–0.2		
VSL/BL	0.14–0.2		
PL/BL	–		
PHL/BL	0.05–0.08		
OL/BL	0.06–0.14		
OVL/BL	0.04–0.08		
TL/BL	0.05–0.15		
FO/BL	0.35–0.5		
TEND/BL	0.2–0.3		
CEND/BL	0.25–0.3		
0SW/BW	0.2–0.6		
VSW/BW	0.4–0.6		
PHW/BW	0.1–0.2		
OVW/BW	0.1–0.2		
TW/BW	0.1–0.3		
OSL/VSL	0.7–1.4		

Sea was widely studied by Dolgikh (1965). In this work there were no mentions on *C. plumosa* and *C. pennata* in any of the studied areas.

Based on morphological data, our samples belong to family Faustulidae. Most of the morphological characteristics of the specimens investigated agree with those of *C. pennata* and *C. plumosa*, reported by Sinitzin (1911). Nevertheless, some morphological differences are present. According to Sinitzin (1911), *C. pennata* differs from *C. plumosa* in the size of the body, the length of the tail and finlets, and in the number of longitudinal lamellae. Our specimens differ from *C. plumosa* in the numbers of finlets on the tail (20 pairs vs. 25–28 pairs for *C. plumosa*), the number of longitudinal lamellae (8–12 vs. 18–22 for *C. plumosa*), and body and tail length (table 1). Samples from the present study are most similar to *C. pennata* by shape and length of the body, tail length, arrangement and shape of internal organs, shape and measurements of both suckers, pharynx, testicular rudiments, and the number and position of the longitudinal lamellae on the tail finlets (table 2). Thus, morphological data support the identification of faustulid cercaria from mollusc *A. segmentum* in the Black Sea as *C. pennata*.

The body surface structure, number and precise arrangement of feathers and longitudinal lamellae on the tail described in the present SEM study were not reported for *P. ventricosa* trematode larvae by other authors. Thus, the new characters are described here. The entire surface of the cercaria was armed with sharp, single-pointed spines arranged in regular rows in the tegumentary papillae. Number and precise arrangement of feathers and longitudinal lamellae of the cercariae of *P. ventricosa* appears well established by SEM. SEM observations of the cercarial tail feathers confirm its composite character, the rib-like supports acting as a skeleton.

Based on molecular data, *C. pennata* from our study is a synonym of *P. ventricosa* based on their identical partial 28S rDNA sequences. At present, there are no morphological or molecular studies on cercariae of *P. ventricosa* from bivalve molluscs (Bray & Gibson, 1980). *Pronoprymna ventricosa* is the type-species of the genus. These trematodes have been recorded from the intestine of various species of marine shads in the Black Sea (Chulkova, 1939; Nikolaeva, 1963; Popjuk, 2009; Ozer *et al.*, 2013), Azov Sea (Solonchenko, 1982), Mediterranean Sea (Bray, 2008), Pontic and Caspian Seas (Kurochkin, 1964; Kornijchuk & Barzegar, 2005; Youssefi *et al.*, 2011), north-eastern Atlantic Ocean (Bray, 2008), Dnieper River (Komarova, 1964), and Severn and Rhine Rivers (Bray & Gibson, 1980). An adult specimen of *P. ventricosa* ex *Alosa volgensis* (Berg, 1913), used in phylogenetic analysis, was described from the delta of the Volga River and genotyped by Sokolov *et al.* (2021).

In the Black Sea, mature worms of *P. ventricosa* described from different definitive hosts, including *Alosa tanaica* (Grim, 1901), *Alosa immaculata* Bennett, 1835, *Atherina boyeri* Risso, 1810, *Alosa fallax* (Lacepède, 1803) (Cetindag, 1993), *Gobius niger* Linnaeus, 1758, *Neogobius fluviatilis* (Palas, 1814), *Neogobius melanostomus* (Palas, 1814), *Proterorhinus marmoratus* (Palas, 1814), *Zosterisessor ophiocephalus* (Palas, 1814), *Symphodus roissali* (Risso, 1810), and *Sciaena umbra* Linnaeus, 1794 (Gaevskaya & Kornijchuk, 2003). These records indicate that *P. ventricosa* has a wide spread of distribution and range of definitive hosts. In the Black Sea, *A. segmentum* is found to be a first intermediate host for *P. ventricosa*. However, this mollusc is widespread from the coasts of England through the Atlantic Ocean, Mediterranean Sea (Denis, 1981) and up to the Caspian Sea (Romanova, 1977; Latypov, 2004). *Abra* spp. natural habitats cover all northern, western and southern littoral areas of the Caspian Sea (Romanova, 1977). Based on our molecular results we propose a role of this bivalve species as first intermediate host of *P. ventricosa* larvae.

Faustulid trematodes can use different species of bivalves from different orders as first intermediate hosts: *Polititapes aureus*,

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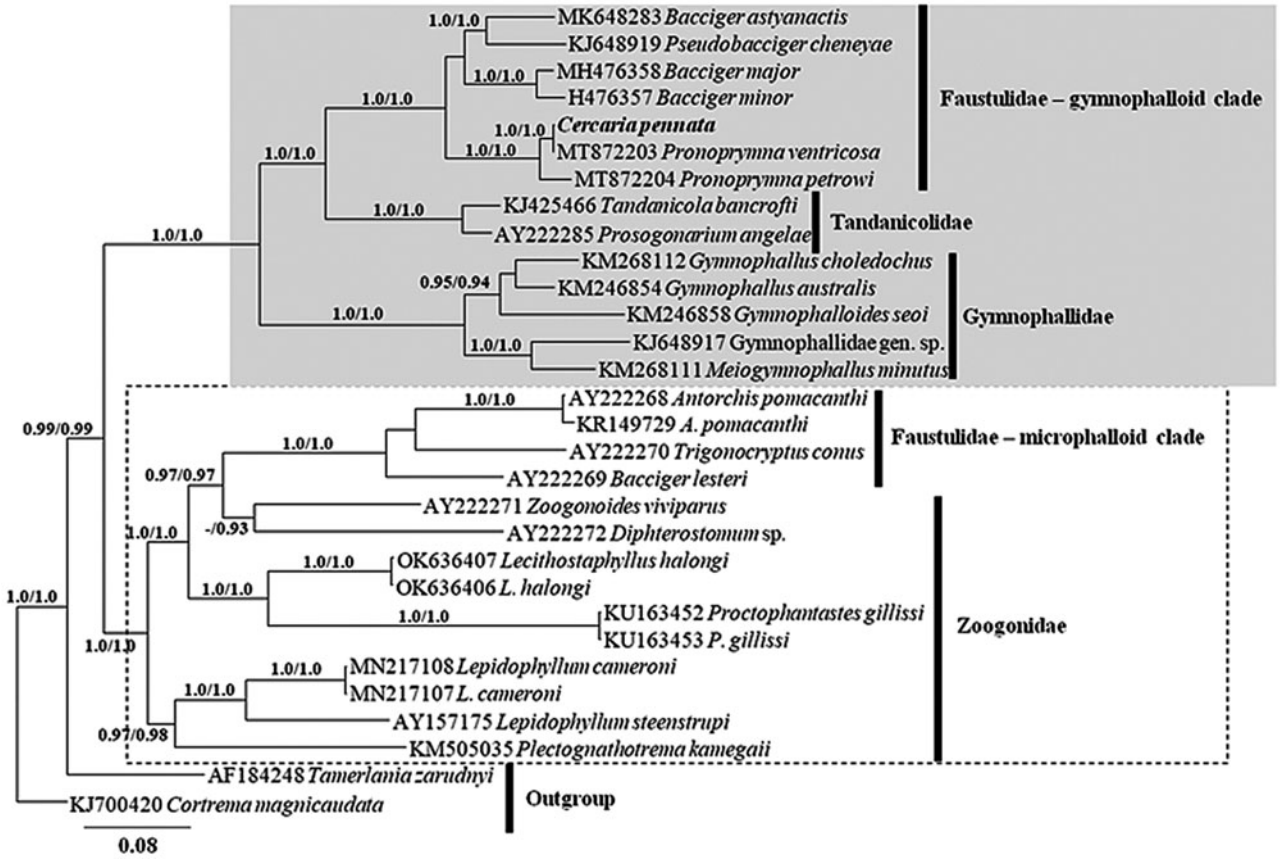

Fig. 3. Phylogenetic tree showing the relationships of various members of the families Faustulidae, Zoogonidae, Tandanicolidae and Gymnophalloidea based on 28S rDNA.

Chamelea gallina (Linnaeus, 1758) (Venerida), *Donax vittatus* (da Costa, 1778) (Cardiida), *Barnea candida* (Linnaneus, 1758) (Myida) (Palombi, 1934a, 1940; Dolgikh, 1968). In the present study we registered the *A. segmentum* mollusc as a first intermediate host from the order Cardida for *P. ventricosa*.

In the life cycle of some faustulid trematodes, marine clams act as first intermediate host, and crustaceans as second intermediate hosts. For example, these hosts are known to be used by *Cercaria lata* (Faustulidae) (Gargouri *et al.*, 2008). The life cycle of *C. lata* was described as cercariae after escaping from sporocysts parasitizing the first intermediate host, *Ruditapes decussatus* (Linnaeus, 1758) (=*Tapes decussata*), penetrate and encyst as metacercariae in the Amphipoda *Erichthonius difformis*, and develop into adults in the alimentary tract of *Atherina* spp. (Palombi, 1934b). In the Black Sea Grintsov & Sezgin (2011) registered the Amphipoda *Erichthonius difformis* in Sevastopol Bay.

Thus, we can expect that the *P. ventricosa* trematode completes its life cycle in the Black Sea, using molluscs *A. segmentum* as first intermediate host, the Amphipoda *Erichthonius difformis* can be used as second intermediate host and the definitive host are shad fishes. However, to accurately prove this fact, additional studies on the experimental equipment of the life cycle are required to recover this life cycle completely.

Conclusion

Morphological characteristics of cercariae emerging from *A. segmentum* from the Black Sea, correspond to *C. pennata*. Analysis of partial 28S rDNA sequences indicates that these cercariae are identical to *P. ventricosa* mature worms. Accepting that *P. ventricosa* is a widespread trematode species in the Black Sea, we suppose that the life cycle of this species involves the bivalve molluscs *A. segmentum* in this region.

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Conflicts of interest. None.

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

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