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First Modern Morphological and Molecular Description of *Saccocoelium Cephali* Larvae Stages (Digenea: Haploporidae) from the Black Sea

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Abstract

Purpose Knowledge of haploporid trematode larvae is very poor. Until recently, only scattered reports from the Black and Medditeranean Seas were known. The present research is the first report of haploporid cercariae *S. cephali* from the gastropod *Hydrobia acuta* inhabiting the Black Sea. Thus, the present study aimed to investigate the larvae of one species from the Haploporidae family.

Methods *Hydrobia acuta* molluscs were collected in the estuary of the Chernaya River (the Black Sea) during 2011–2013 and examined for trematode larvae. Found parthenitae were drawn and preserved for DNA extractions. Morphological features were compared to nominal species, and molecular comparisons were made of the subunit ribosomal DNA with other Haploporidae species.

Results The haploporid parthenitae were found in the gonads of *Hydrobia acuta* molluscs. Most of the morphological characteristics of the specimens investigated agree with those of *Saccocоcoelium* sp. and are closest to those of *S. cephali* by shape and length of the body, ratio of suckers, ratio of tail length to body, shape, and size of the pharynx. Phylogenetic analysis from our study generated with Bayesian algorithm showed that studied cercariae specimens from the Black Sea were within the hapolporine clade and closely related to *S. cephali* from Spain.

Conclusion Morphological characteristics of cercariae emerging from *Hydrobia acuta* from the Black Sea and the analysis of partial 28S rDNA sequences support the conspecificity of the parasite with *S. cephali* mature worms from the Mediterranean Sea.

Keywords Trematoda · Larva · *Saccocoelium* · *Hydrobia acuta* · Black Sea

Introduction

Haploporidae is a family of the suborder Haploporata Pérez-Ponce de León & Hernández Mena, 2019 (Digenea), which includes eight subfamilies of marine or brackish-water mullet fishes of the Atlantic, Indo-West Pacific, and Mediterranean Regions. Of these, only adult worms of four genera of Haploporinae Nicoll, 1914, including members of *Saccocoelium*, *Haploporus, Dicrogaster,* and *Lecithobotrys,* were registered from the Black Sea [25, 35, 44], as well as in the Mediterranean Sea [17, 18]. On this background, fauna haploporine trematodes and their life cycles are well studied in the Mediterranean Sea. Particularly, the species *Sacccocoelium obesum* Looss, 1902, *S. tensum* Looss, 1902, and *Haploporus benedeni* (Stossich, 1887) Looss, 1902 have been shown to realise their life cycles through gastropods from either the family Rissoidae Gray, 1847 or Hydrobiidae W. Stimpson, 1865 as first intermediate hosts, and Mugilidae Jarocki, 1822 fish species as definitive hosts [27]. In the Black Sea, the life cycles of haploporid trematodes as well as species diversity are still poorly studied. The only report on two morphologically similar haploporid cercariae from gastropods *Iravadia quadrasi* (O. Boettger, 1893) (=*Rissoa venusta)* and *Ecrobia ventrosa* (Montagu, 1803) is available

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[43]. This is largely due to the fact that most of the previous studies have been focused on the parasites of marine fishes [28, 35]. In a whole, the life cycles described for few species of Haploporidae at present [27, 41, 42]. Moreover, some haploporid species from the Mediterranean Sea, for instance, members of the genus *Saccocoelium*, were revised based on morphological data [17]. This creates difficulties in making an accurate species identification and studies of their life cycles.

In this study, the first complex data, including morphological descriptions and molecular characteristics of larvae of *Saccocoelim cephali* Blasco-Costa, Montero, Gibson, Balbuena, Raga & Kostadinova, 2009 from gastropod molluscs *Hydrobia acuta* (Draparnaud, 1805) in the Black Sea are presented.

Materials and Methods

Sample Collection

A total of 5188 specimens of *H. acuta* were collected in the estuary of the Chernaya River (44°27′49″ N, 33°51′37″ E) (Sevastopol City, the Black Sea) during 2011–2013. All snails were examined for helminthic infections using standard methods [21]. Parthenitae of trematodes were studied as alive asstained using an Olympus CX41 microscope equipped with an CX50 camera with software Infinity Analyze 1 (Canada). Trematodes were fixed under a cover glass with slight pressure, stained with acetocarmine. The colour grade was differentiated by "iron water" ($H_2O + Fe_2O_3$) and acidified alcohol (70% ethanol + 3% HCl). After dehydration in ethanol of increased concentrations (70, 80, 90, and 100%) and clarification in clove oil, trematodes were mounted in Canada balsam [40].

Morphological Data

All measurements were made on stained parasites. The abbreviations of metric features included in Table 1 are according to [17]. The excretory system of the larvae was investigated 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 the drawing software Inkscape 0.48.2.-1 (Scalable Vector Graphics, 2011).

Scanning Electron Microscopy

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 samples were dehydrated through an ethanol series (70–96 °C). Dried in 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).

Statistical Methods

After the parasites were identified, we assessed the infection indexes in the *Hydrobia acuta*, including invasion intensiveness (prevalence) (IE), invasion intensity (II), and abundance index (AI) [20]. For each morphological parameter, the mean with standard error (mean ± SE) was calculated. Statistical parameters were calculated with the Statistica 6 software package for Windows (Statsoft).

DNA Extraction, Amplification and Sequencing

Two haploporid cercariae specimens fixed by 96% ethanol were used for molecular analysis (Table S1). Total DNA was extracted using QIAamp DNA Micro Kit (Qiagen, Germany). A worm was incubated in 180 µl of Genomic Digestion buffer with 20 µl of Proteinase K at 55 °C for one hour with the following mix by vortex for 20 s. DNA extraction was carried out according to the manufacturer's protocol. The elution volume was 25 µl. The DNA was stored at − 20 °C. Fragment of 28S ribosomal DNA (rDNA) 1200 base pairs (bp) in length was amplified by a polymerase chain reaction (PCR) method using the 2 × GoTaq Green Master mix (Promega, Madison, Wisconsin, USA) and the primers 28S-A (5′-TCG ATT CGA GCG TGA WTA CCC GC-3′) [34] and 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′) [45] with an annealing temperature of 55 °C. Negative and positive controls using both primer pairs were included. PCR parameters began with a 1 min denaturation at 98 ºC, followed by 35 cycles of 10 s at 98 ºC, 5 s at 55 ºC and 2 min 30 s at 72 ºC, and concluded with a 7 min extension at 72 ºC.

PCR products were directly sequenced using an ABI Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, Massachusetts, USA) as recommended by the manufacturer. The internal sequencing primers for 28S rDNA are described in [45]. PCR product sequences were analysed using an ABI 3500 genetic analyser at the FSC of Biodiversity FEB RAS. Sequences were submitted to the GenBank database under accession numbers PQ131205-PQ131206.

Alignments and Phylogenetic Analysis

Ribosomal DNA sequences were assembled using the SeqScape v. 2.6 software provided by Applied Biosystems Company. Alignments, estimations of the number of variable sites, and sequence differences through p-distance

Table 1 Comparing morphological features of haploporid cercariae in the Black Sea and Mediterranean Seas (in µm)

Features	*Saccocoelium cephali*, Present study	*Saccocoelium tensum*, Fares, Maillard, 1974	*Saccocoelium obesum*, Fares, Maillard, 1974	*Haploporus benedeni*, Fares, Maillard, 1974
BL	315–514 (404 ± 41)	210	500	350
BW	162–249 (205 ± 21)	120	220	240
OSL	51–69 (60 ± 3)	53	90	100
OSW	84–94 (88 ± 2)			90
VSL	57–68 (63 ± 3)	50	80	
VSW	63–83 (73 ± 6)			
PL	12–69 (37 ± 10)			30
PHL	35–48 (42 ± 2)	29	70	50
PHW	38–57 (48 ± 4)	25	60	60
OL	74–131 (106 ± 17)			
Tail length	139–260 (200 ± 61)	330		380
FO	186 – 370 (244 ± 32)			
CEND	109–174 (132 ± 15)			
OSL/BL	0,2	0,25		
VSL/BL	0,2	0,2		
PL/BL	0,1			
PHL/BL	0,1	0,1		
OL/BL	0,3			
FO/BL	0,6			
TEND/BL				
CEND/BL	0,3			
0SW/BW	0,4			
VSW/BW	0,4			
PHW/BW	0,2	0,2		
TW/BW				
OSL/VSL	1.05	1.06		1

calculation were performed using the MEGA 7.1 software [33].

Phylogenetic analysis was performed with Bayesian Inference (BI) using the MrBayes 3.2.6 program [39] for subfamily Haploporinae and for overall Haploporidae. The best nucleotide substitution model, the TVM + I + G [37], was estimated with jModeltest v. 2.1.5 software [24] using the BIC criterion [39] for both datasets.

The Monte Carlo Markov chain algorithm was performed with 10,000,000 generations during two independent runs, sampling each 1000 generation and burnin the first 25% of all generations. The significance of phylogenetic relationships was estimated with posterior probabilities; positive values ranged from 0.9 to 1.0 [29]. The average standard deviation of split frequencies after MCMC was 0.001660, which was enough to stop the algorithm and summarise the sampled parameter values and trees [29].

The sequences of 28S rDNA of all members of Haploporidae, available in GenBank, were incorporated into phylogenetic analysis [1–16, 18, 19, 22, 23, 30, 37, 38] (Table S1). The 28S rDNA sequences of *Brachycladium goliath* (van Beneden, 1858) Fraija-Fernández, Aznar, Raga, Gibson and Fernández, 2014 and *Hurleytrematoides chaetodonti* (Manter, 1942) Yamaguti, 1954 from GenBank were used as the outgroup [6, 19] (Table S1).

Results

Saccocoelium cephali Blasco-Costa, Montero, Gibson, Balbuena, Raga & Kostadinova, 2009.

Host:Hydrobia acuta (Draparnaud, 1805).

Locality: the Black Sea, near Sevastopol City (44°27′49″ N, 33°51′37″ E).

Site: gonads.

Intensity of infection IE = 1%, II = 1–30.

Description

Sporocyst long, 238–356 (302 ± 24) × 73 – 133 (95 ± 11) µm, fill gonads of their host.

Table 2 Interspecific genetic p-distance values (%, below diagonal) and standard errors (above diagonal) for Haploporinae

		1	2	3	4	5	6	7	8	9	10
1	Cercaria ex *H. acuta* #1		0	0.093	0.621	0.632	0.508	0.828	0.832	0.884	0.791
2	Cercaria ex *H. acuta* #2	0		0.093	0.621	0.632	0.508	0.828	0.832	0.884	0.791
3	*Saccocoeliumcephali*	0.098	0.098		0.61	0.62	0.495	0.817	0.823	0.872	0.783
4	*Saccocoeliumobesum*	4.617	4.617	4.519		0.293	0.558	0.764	0.798	0.878	0.777
5	*Saccocoeliumbrayi*	4.813	4.813	4.715	0.884		0.593	0.777	0.84	0.88	0.799
6	*Saccocoeliumtensum*	2.75	2.75	2.652	3.733	4.126		0.72	0.797	0.829	0.761
7	*Dicrogastercontracta*	6.883	6.883	6.785	6.293	6.686	5.801		0.606	0.809	0.823
8	*Dicrogasterperpusilla*	8.358	8.358	8.26	8.063	8.456	7.473	4.523		0.881	0.863
9	*Haploporusbenedeni*	8.644	8.644	8.546	8.35	8.743	7.859	6.785	8.063		0.844
10	*Litosaccusbrisbanensis*	7.389	7.389	7.291	7.685	8.374	6.7	7.594	9.172	8.473	

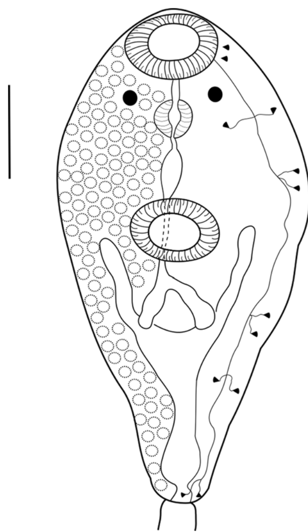

Fig. 1 Alive *Saccocoelium cephali* cercaria from the mollusc *Hydrobia acuta*: A. The morphology of the cercaria body. B. Alive *Saccocoelium cephali* adolescaria Scale: 200 µm

Cercariae (Based on 10 specimens, Table 1, Figs. 1 and 2).

Body oval. Tegument without spines. Oral sucker subterminal, almost the same size as ventral sucker. Ventral sucker in middle of body. Two global eye spots behind oral sucker. Oral to ventral sucker distance (493 ± 54) slightly less than ventral sucker to end of body distance (651 ± 114). Prepharynx present. Pharynx round, muscular, well defined. Oesophagus long. Intestinal bifurcation slightly posterior to ventral sucker in two sacciform branches. Two digestive branches reach the posterior 1/3 of body. Excretory bladder Y-shaped, both branches approach level of ventral sucker. Stenostome excretory system. Formula of excretory system is $2 [(2+2+2)+(2+2+2)]=24$.Tail slender, unadorned, almost twice as short as body length.

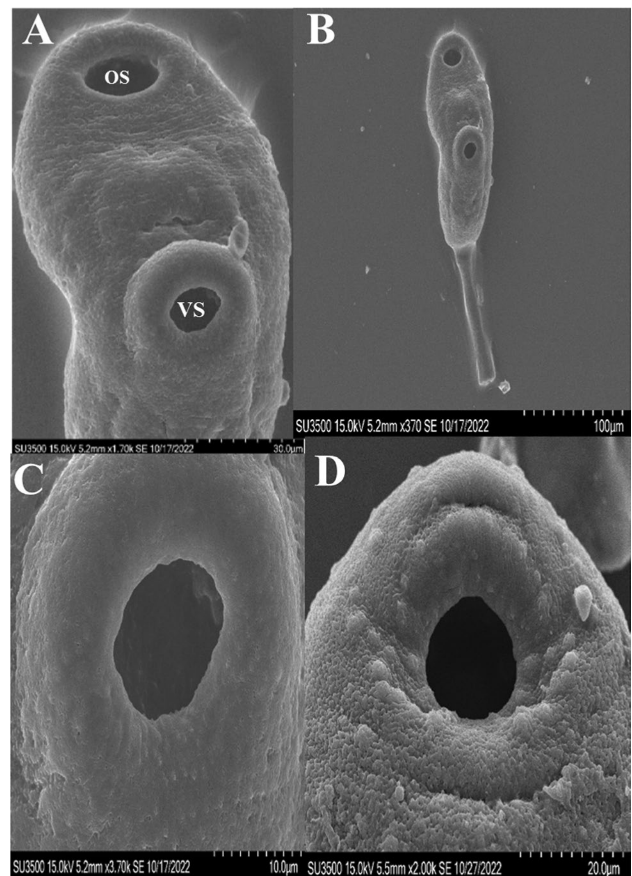

Fig. 2 SEM photomicrographs of *Saccocoelium cephali* cercariae from mollusc *Hydrobia acuta*: A- body surface structure, ratio of suckers, *OS* oral sucker, *VS* ventral sucker. B—view of body C –ventral sucker surface structure. D–oral sucker surface structure

Adolescaria

The process of encystation of 1 cercaria in same mollusc was observed (adolescaria). Cyst oval, elongate, 180 × 116 µm (Figure S1).

Molecular Data

The alignment consisted of 1022 bp for the 28S rRNA gene of two investigated haploporid cercariae specimens collected from *H. acuta* from the Black Sea basin. The nucleotide composition of the 28S rDNA fragment was 25.6% for T(U), 21.9% for C, 21.0% for A, and 31.5% for G bases, which was typical for *Saccocoelium* species. Two 1022 bp 28S rDNA sequences from this study were highly similar to those for *S. cephali* ex *Mugil cephalus* from Spain (FJ211233). These sequences differ from each other with a single T/C transition. However, we cannot identify if this mutation is fixed because a single sequence of adult *S. cephalic* is available. Genetic p-distances between our specimens and *S. cephali* were 0.098 ± 0.093%, whereas between our samples and other *Saccocoelium* species ranged from 2.75 ± 0.5 (*S. tensum*) to 4.8 ± 0.6% (*S. brayi*), demonstrating typical interspecific differentiation level for Haploporidae by 28S rDNA sequence data (Table 2).

Phylogenetic analysis from our study generated with Bayesian algorithm showed that studied cercariae specimens ex *H. acuta* from the Black Sea, were within the haploporine clade and closely related to *S. cephali* from Spain on both only Haploporinae and overall Haploporidae phylogenetic trees (Fig. S2).

Discussion

Adult worms of *S. cephali* were first described by Isabel Blasco-Costa with co-authors in 2009 [17] from the Mediterranean Sea (Spain). The Black Sea cercariae and metacercariae of *S. cephali* trematodes have not been described before.

In the Black Sea, parthenithae of haploporid trematodes were first noted by Sinitzin [43]. The author reported two morphologically similar haploporid cercariae: *Cercaria metentera* (Sinitzin, 1911) from gastropod *Iravadia quadrasi* (O. Boettger, 1893) (=*Rissoa venusta)* and *C. mesentera* (Sinitzin, 1911) from mollusc *Ecrobia ventrosa* (Montagu, 1803) (=*Hydrobia ventrosa*) in the water off Sevastopol City. According to Sinitzin [43], *C. mesentera* differs from *C. metentera* in the sizes of the body, oesophagus, caeca, excretory bladder form, and absence of the prepharynx. Afterwards, the trematode fauna of molluscs in the Black Sea was widely studied by Dolgikh [26]. The author reported one type of haploporid parthenitae, *C. metentera,* from the mollusc *Rissoa splendida* (Eichwald, 1830), and discussed their biology and the identity of the adult form, suggesting that it is a species of *Saccocoelium tensum* Looss, 1902.

Based on morphological data, our samples belong to the family Haploporidae. Most of the morphological characteristics of the specimens investigated agree with those of *Saccococoelium* sp. and closest to *S. cephali* by shape and length of the body, ratio of suckers, ratio of tail length to body, shape and size of pharynx. The samples of *S. cephali* we found differ from all other species of *Saccocoelium* in their distinctly bigger size (size of body and other organs see in Table 1). Thus, morphological data supports the identification of haploporid cercariae from mollusc *H. acuta* in the Black Sea as *S. cephali.*

The body surface, suckers, and tail structure are described in present SEM research. But they were not reported for *Saccocoelium* trematode larvae until now. Thus, the new characters are provided in this article. The entire surface of the cercaria was not armed with spines. On the figures, some authors illustrated the armed protein coagulation on the body surface of *Saccocoelium* cercaria from the Mediterranean [27] and the Azov Seas [31]. Our SEM study reflects the absence of the formations on the body surface (Fig. 2).

Based on the molecular data, cercariae ex *H. acuta* from our study belong to the species *S. cephali* unambiguously, despite the single substitution between the 28S rDNA sequences of our specimens and the individual of *S. cephali* from the Ebro Delta, Spain (FJ211233). We do not exclude that this substitution will be characterised as the fixed mutation after additional 28S rDNA sequence data of adult *S. cephali* will be available. In any case, our taxonomical conclusion about cercariae we found in the Black Sea will stand firm. Besides, fixed mutations in 28S rDNA sequences within the same species are known for Haploporidae [7, 14].

In the life cycle of haploporid trematodes, marine gastropods of the families Hydrobiidae and Rissoidae serve as the first intermediate hosts [27] and mullet fish as definitive hosts [25, 35].

Thus, we can expect that *S. cephali* trematode realises the life cycle in the Black Sea, using the mollusc *H. acuta* as the first intermediate host and the mullets as the most probably definitive host.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11686-024-00934-8.

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Author Contributions Y.V.B. provided support for the fieldwork portion of the study, including transport, supplies, and catching of mollusks. Y.V.B. carried out dissections and parasitological examinations. When parasites were encountered, they were photographed by Y.V.B. Figure 1, Fig. 2 and Figure S1 was made by Y.V.B. All molecular biology work and analysis was carried out by D.M.A. Figure S2 were created by A.D.M. Table 1 was created by Y.V.B and Table S1 was

made by D.M.A. All authors reviewed the manuscript and provided edits on the final version.

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Data Availability DNA sequence data was deposited in GenBank.

Code Availability Not applicable.

Declarations

Conflict of Interest The authors declare no conflicts of interest.

Ethical Approval All applicable institutional, national and international guidelines for the care and use of animals were followed, protocol No 2(7)/24 of March 28, 2024.

Human and Animal Rights All applicable institutional, national and international guidelines for the care and use of animals were followed.

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