

Research Article

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The taxonomic identity and phylogenetic relationships of *Cercaria pugnax* and *C. helvetica* XII (Digenea: Lecithodendriidae) based on morphological and molecular data

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Abstract: The present study analysed the taxonomic status and phylogenetic relationships of two species of xiphidiocercariae of the ‘microcotylae’ group, *Cercaria pugnax* La Valette St. George, 1855, from *Viviparus viviparus* (Linnaeus) in the Ukraine and *Cercaria helvetica* XII Dubois, 1928 from *Bithynia tentaculata* (Linnaeus) in Lithuania. Molecular phylogenetic analyses based on sequences of the ITS2 region and partial 28S gene of the nuclear rDNA revealed that both these xiphidiocercariae belong to the Lecithodendriidae Lühe, 1901 and represent larval stages of lecithodendriids parasitic in bats. *Cercaria helvetica* XII clustered with the typical representatives of the genus *Lecithodendrium* Looss, 1896, being very close, but not identical, to *Lecithodendrium linstowi* Dollfus, 1931. Sequences of *C. pugnax* matched exactly the sequences of adult *Paralecithodendrium chilostomum* (Mehlis, 1831). Morphological descriptions of the cercariae are included; these represent the first report of non-irrigulate xiphidiocercariae belonging to the family Lecithodendriidae. Until now, the presence of glandular virgula organ in the region of the oral sucker was considered a robust synapomorphy for the Lecithodendriidae and several closely related families. Our results have shown that the relative importance of this character is in need of a re-assessment.

Keywords: *Paralecithodendrium*, *Lecithodendrium*, xiphidiocercaria, morphology, phylogeny, rDNA sequences

The taxonomic position of many digenean cercariae often cannot be established based on their morphology alone due to the scarcity of reliable taxonomic distinguishing characteristics at this stage of digenean development. Frequently, cercariae can be identified to the family or superfamily level only. The laboratory experiments on life cycles with the use of natural definitive hosts are not always feasible. Some of the difficulties in understanding trematode life cycles and identification of their larval stages may be overcome through the use of molecular techniques (Bartoli et al. 2000, Brant et al. 2006, Pina et al. 2007, Jensen and Bullard 2010, Locke et al. 2011).

As part of the study of digenean biodiversity in aquatic snails in the Ukraine and Lithuania, we have found two species of xiphidiocercariae previously described as *Cercaria pugnax* La Valette St. George, 1855 and *Cercaria helvetica* XII Dubois, 1928. *Cercaria pugnax* was initially described by La Valette St. George (1855) from the prosobranch *Viviparus viviparus* (Linnaeus) collected near Berlin, Germany (Prussia at the time). Since then, *C. pugnax*

has been reported from several other countries in Europe (Ginetsinskaya 1968, Cichy et al. 2011). Dubois (1928) described *C. helvetica* XII as a new species of xiphidiocercaria from *Bithynia tentaculata* (Linnaeus) collected in Neuchâtel, Switzerland. Based on their general morphology (size of the body, sucker ratio, position of the ventral sucker, number and position of the penetration gland-cells, absence of a fin-fold along the margins of the tail), both cercariae belong to the ‘microcotylae’ group of xiphidiocercariae. Due to the simple morphology and very limited development of most organs, proper taxonomic allocation of these cercariae based on morphology alone was problematic. Therefore, species affiliation and potential definitive hosts of *C. pugnax* and *C. helvetica* XII remained unknown.

The goal of the present study was to reveal identity of these cercariae and their phylogenetic affinities by using DNA sequences from the cercarial stages and sequences of adult digeneans currently available in GenBank and our data. For this purpose we used sequences of the nuclear ribosomal ITS2 region and partial 28S rRNA gene.

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MATERIALS AND METHODS

Snails were collected in the Ukraine and Lithuania. In May 2012, 31 *Viviparus viviparus* were collected from the Dnieper River at a site on the Truchaniv Island, Kyiv, Ukraine (50°27'19"N; 30°32'42"E). In June 2013, 121 *Bithynia tentaculata* were collected from the Curonian Bay near the village of Juodkrante, Lithuania (55°35'38"N; 21°7'57"E).

In the laboratory, snails were rinsed with water and placed individually in glass containers with aged tap water. The containers were examined daily under a stereo microscope for the presence of emerged cercariae. Morphology of the cercariae was studied on live specimens, with or without vital staining (neutral red and/or Nile blue). Afterwards, molluscs were dissected and their tissues were compressed between two Petri dishes of different diameters and examined under microscope in order to establish the localisation of the digenean larvae. Live larvae were rinsed in water and fixed in hot 4% formaldehyde solution for morphological study and in 96% ethanol for molecular study. Drawings of the cercarial morphology were made from a combination of live and fixed cercariae using a compound microscope equipped with a drawing tube. Measurements are presented in micrometres as the range followed by the mean in parentheses. Cercariae were identified by comparison with the descriptions in La Valette St. George (1855), Dubois (1928), Ginetsinskaya (1968), and Bychovskaya-Pavlovskaya and Kulakova (1971). Nomenclature of mollusc species follows Glöer (2002).

According to the most recent revision of the Lecithodendriidae Lühe, 1901 by Lotz and Font (2008), the genus *Prosthodendrium*

Dollfus, 1931 is considered a synonym of *Paralecithodendrium* Travassos, 1921. In order to be consistent with the current classification, in the present work we use the generic name *Paralecithodendrium* for all previously sequenced species of *Prosthodendrium*.

Total genomic DNA for molecular analysis was isolated from specimens of *C. pugnax* and *C. helvetica* XII according to the protocol by Stunžėnas et al. (2011) with a slight modification described in Petkevičiūtė et al. (2014). DNA fragments spanning the 3' end of 5.8S rRNA gene, complete internal transcribed spacer 2 region (ITS2) and a few nucleotides at the 5' end of the 28S gene were amplified using forward primer 3S (5'-CGG TGG ATC ACT CGG CTC GTG-3') and reverse primer 28S (5'-CCT GGT TAG TTT CTT TTC CTC CGC-3') (Bowles et al. 1995). An approximately 1300 bp long fragment at the 5' end of the 28S rRNA gene was amplified using forward primer Dig12 (5'-AAG CAT ATC ACT AAG CGG-3') and reverse primer L0 (5'-GCT ATC CTG AG (AG) GAA ACT TCG-3') (Tkach et al. 1999). The amplification protocols are described in Petkevičiūtė et al. (2014). PCR products were purified and sequenced in both directions at BaseClear B.V. (Leiden, The Netherlands) using PCR primers. We also obtained new ITS2 sequences of six adult specimens of lecithodendriid digeneans (Table 1), namely *Lecithodendrium linstowi* Dollfus, 1931, *Paralecithodendrium chilostomum* (Mehlis, 1831), *Paralecithodendrium hurkovaiae* Dubois, 1960, *Paralecithodendrium longiforme* (Bhalerao, 1926), *Pycnoporos heteroporus* (Dujardin, 1845) and *Pycnoporos megacotyle* (Ogata, 1939). We utilised the same DNA extracts that were used

Table 1. Digenean species used in the present study, their hosts, geographical origin of material and GenBank accession numbers for corresponding sequences. Taxa marked with asterisk were used as outgroups.

Species	Host	Geographical origin	GenBank No.		Reference
			ITS2	28S	
<i>Lecithodendrium linstowi</i> Dollfus, 1931	<i>Nyctalus noctula</i> (Schreber)	Ukraine	KJ934792	AF151919	present study, Tkach et al. 2000
<i>Lecithodendrium linstowi</i>	<i>Pipistrellus pipistrellus</i> (Schreber)	UK	JF784190	JF784191	Lord et al. 2012
<i>Lecithodendrium spathulatum</i> (Ozaki, 1929)	<i>Pipistrellus pipistrellus</i>	UK	JF784192	JF784193	Lord et al. 2012
<i>Lecithodendrium</i> sp. (= <i>Cercaria helvetica</i> XII)	<i>Bithynia tentaculata</i> (Linnaeus)	Lithuania	KJ126724	KJ126726	present study
<i>Paralecithodendrium chilostomum</i> (Mehlis, 1831)	<i>Nyctalus noctula</i>	Ukraine	KJ920281	AF151920	present study, Tkach et al. 2000
<i>Paralecithodendrium chilostomum</i> (= <i>Cercaria pugnax</i>)	<i>Viviparus viviparus</i> (Linnaeus)	Ukraine	KJ126723	KJ126725	present study
<i>Paralecithodendrium hurkovaiae</i> (Dubois, 1960)	<i>Myotis daubentoni</i> (Kuhl)	Ukraine	KJ920282	AF151922	present study, Tkach et al. 2000
<i>Paralecithodendrium longiforme</i> (Bhalerao, 1926)	<i>Myotis daubentoni</i>	Ukraine	KJ920283	AF151921	present study, Tkach et al. 2000
<i>Paralecithodendrium parvouterus</i> (Bhalerao, 1926)	<i>Miniopterus schreibersi</i> (Kuhl)	Spain	-	AY220617	Tkach et al. 2003
<i>Paralecithodendrium</i> sp.	<i>Pipistrellus pipistrellus</i>	UK	JF784195	JF784196	Lord et al. 2012
<i>Pycnoporos heteroporus</i> (Dujardin, 1845)	<i>Pipistrellus kuhlii</i> (Kuhl)	Ukraine	KJ920284	AF151918	present study, Tkach et al. 2000
<i>Pycnoporos megacotyle</i> (Ogata, 1939)	<i>Pipistrellus kuhlii</i>	Ukraine	KJ920285	AF151917	present study, Tkach et al. 2000
<i>Maritrema arenaria</i> Hadley et Castle, 1940*	<i>Semibalanus balanoides</i> (Linnaeus)	Northern Ireland	HM584171	-	Galaktionov et al. 2012
<i>Maritrema neomi</i> Tkach, 1998*	<i>Neomys anomalus</i> Cabrera	Ukraine	-	AF151927	Tkach et al. 2000
<i>Microphallus similis</i> (Jagerskiold, 1900)*	<i>Carcinus maenas</i> (Linnaeus)	Northern Ireland	HM584179	-	Galaktionov et al. 2012
<i>Microphallus fusiformis</i> Reimer, 1963*	Lab. host chicken egg	UK	-	AY220633	Tkach et al. 2003
<i>Collyriclum faba</i> (Bremser in Schmalz, 1831)*	<i>Saxicola rubetra</i> (Linnaeus)	Czech Republic	JQ231122	JQ231122	Heneberg and Literák 2013

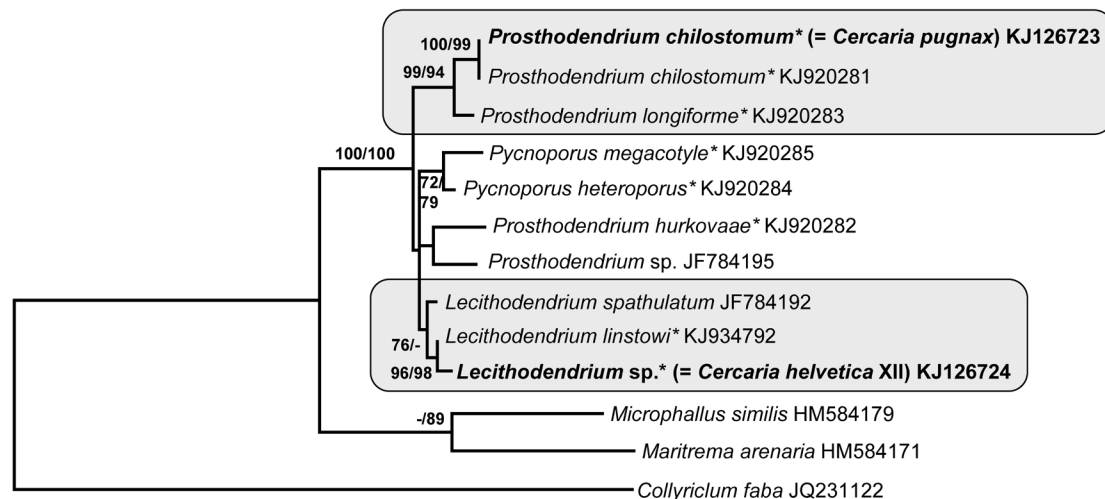


Fig. 1. Phylogenetic tree based on analysis of sequences of the ITS2 region of nuclear rDNA. Bootstrap support given for maximum likelihood/maximum parsimony analysis. Bootstrap support values lower than 70% are not shown. Sequences obtained in the present study indicated by asterisk. Shaded rectangles indicate clades containing *Cercaria helvetica* XII and *Cercaria pugnax*.

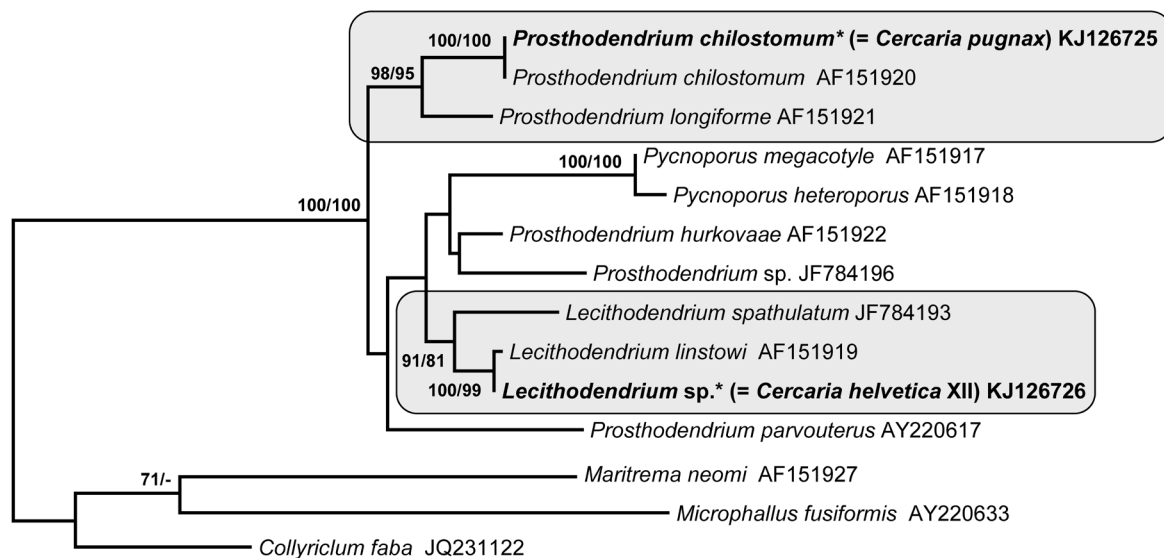


Fig. 2. Phylogenetic tree based on analysis of partial sequences of the 28S gene of nuclear rDNA. Bootstrap support given for maximum likelihood/maximum parsimony analysis. Bootstrap support values lower than 70% are not shown. Sequences obtained in the present study indicated by asterisk. Shaded rectangles indicate clades containing *Cercaria helvetica* XII and *Cercaria pugnax*.

by Tkach et al. (2000) to obtain 28S sequences of these species deposited in GenBank (Table 1). In this case, forward primer d58f (5'-GCGGTGGATCACTCGGCTCGTG-3') localised in the 5.8 gene and reverse primer digl2r (5'-CCGCTTAGTGATATGCTT-3') localised at the 5' end of the 28S gene were used for the PCR amplification and sequencing. The PCR reactions were performed following the protocols described by Tkach et al. (2003).

Contiguous sequences were assembled using Sequencher 4.7 software (Gene Codes Corporation). The new sequences were deposited in GenBank; accession numbers for both rDNA regions sequenced in this study are shown in Table 1.

Additional sequences of the Lecithodendriidae and outgroup taxa were downloaded from GenBank and included in the phylogenetic analysis and/or pairwise sequence comparisons (Table 1). For phylogenetic analyses, the sequences were aligned using ClustalW (Thompson et al. 1994) with an open gap penalty of 15

and gap extension penalty of 6.66. The best-fit model of sequence evolution for phylogenetic analysis was estimated using jModeltest v. 0.1.1 software (Posada 2008). Ambiguously aligned positions have been excluded from phylogenetic analysis. Maximum likelihood (ML) and maximum parsimony (MP) phylogenetic trees were obtained and analysed using MEGA v5 (Tamura et al. 2011). Branch support was estimated by bootstrap analyses with 1 000 replicates. The maximum likelihood trees were obtained using general time reversible model with a gamma distribution of rates and a proportion of invariant sites (GTR + G + I) for both the ITS2 and the 28S gene datasets. Gamma shape and number of invariant sites were estimated from the data. Parsimony analysis based on subtree pruning and regrafting (SPR) was used with default parsimony settings. Estimates of mean evolutionary divergence over sequence pairs within and between groups were calculated using MEGA v5 program.

RESULTS

Molecular analysis

In *Cercaria helvetica* XII the amplified and sequenced fragment of the 5.8S-ITS2-28S rDNA was 454 bp long and the partial sequence of 28S gene was 1113 bp. In *Cercaria pugnax* the 5.8S-ITS2-28S rDNA fragment was 472 bp and the partial sequence of 28S gene was 1212 bp long. The GenBank accession numbers are presented in Table 1. BLAST searches (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) and pairwise comparisons of ITS2 and 28S sequences with the newly obtained ITS2 sequences from adult lecitodendriids demonstrated that *C. helvetica* XII was closest to *Lecithodendrium linstowi* obtained from two different species of bats in the United Kingdom and Ukraine (Table 1). The sequences of *C. helvetica* XII differed from identical sequences of two *L. linstowi* by 5 out of 454 bp (1.1%) in the ITS2 region and by 7 out of 1010 bp (0.7%) in the sequenced portion of the 28S gene. Both ITS2 and 28S sequences of *C. pugnax* were identical to those of *Paralecithodendrium chilostomum*.

Two different sets of rDNA sequences were used in the phylogenetic analyses, namely the ITS2 region and partial 28S gene. Both (ML and MP) analyses of these datasets produced identical tree topologies (Figs. 1, 2). Each of the cercariae studied in this work was found in a distinct, strongly supported clade. *Paralecithodendrium chilostomum* was represented in the trees by the adult form obtained from bats and the larval stage *C. pugnax*. They clustered together with *P. longiforme* in a 94–99% supported clade (Figs. 1, 2). *Cercaria helvetica* XII clustered in a 96–100% supported clade with *L. linstowi*. In turn, these two species clustered in both trees with *Lecithodendrium spathulatum* (Ozaki, 1929). Among the remaining lecitodendriid taxa on the trees, two species of *Pycnoporos* Looss, 1899 formed a well-supported monophyletic clade, whereas the remaining topologies were weakly supported, among them the clade uniting *Paralecithodendrium* sp. and *P. hurkovaee* (Figs. 1, 2) and a separate branch formed by *Paralecithodendrium parvouterus* (Bhalerao, 1926) only.

Morphological examination

Cercaria pugnax and *Cercaria helvetica* XII belong to the ‘microcotylae’ group of xiphidiocercariae, which includes usually very small cercariae with body less than 200 µm long, with a postequatorial ventral sucker, which is smaller in size than the oral sucker and is situated behind the mid-body, and a tail without a fin-fold and not very different in length from the body.

Paralecithodendrium chilostomum (Mehlis, 1831) – cercaria Fig. 3

Syn. *Cercaria pugnax* La Valette St. George, 1855

Description (measurements based on 30 specimens). Cercariae develop in small oval sporocysts, 245–374 × 178–246 (323 × 210). Cercaria body oval, widest at its midpoint 90–180 × 70–114 (130 × 90). Tegument thin, uniformly covered with tiny spines. Oral sucker rounded, subterminal, 25–45 × 30–53 (35 × 38), much larger than

ventral sucker. Ventral sucker subspherical, 15–25 × 15–28 (19 × 21), slightly postequatorial. Oral/ventral sucker width ratio 1 : 0.30–1 : 0.73 (1 : 0.53). Oral sucker armed with large stylet, 28–30 (30) long, 5–6 (5) wide at base, with rounded, distinctly enlarged base and sharp distal end with conspicuous lateral thickenings (Fig. 3B). Two pairs of large penetration gland-cells with large nuclei; first pair anterior to ventral sucker, with secretory material in form of large granules; second pair at level of ventral sucker, with secretory material in form of small granules; gland ducts extend around oral sucker and open on either side of stylet. Prepharynx very short, pharynx small, 5–13 × 8–15 (8 × 11). Oesophagus very short; almost indistinct. Caeca not observed. Excretory vesicle thin-walled, V-shaped. Flame-cell formula: 2 [(2 + 2 + 2) + (2 + 2 + 2)] = 24. Excretory pore at tip of tail. Tail simple, 85–163 (127) long, 18–28 (21) wide at base, slightly shorter than body.

Host: *Viviparus viviparus* (Linnaeus) (Mollusca: Viviparidae).

Prevalence of infection: 20% (n = 31).

Site of infection: Hepatopancreas.

Locality: Dnieper River, Kyiv, Ukraine (50°27'19"N; 30°32'42"E).

GenBank Acc. Nos.: KJ126723, KJ126725.

Remarks. The morphological characters and measurements of the cercaria of *P. chilostomum* correspond well to the cercaria of *Lecithodendriidae* gen. sp. 3 previously described by Stenko et al. (2005) from naturally infected *V. viviparus* in water bodies of the Crimea (Ukraine).

Paralecithodendrium chilostomum is a common parasite of bats (see Sharpilo and Iskova 1989), but little is known of the spectrum of its intermediate hosts. Metacercariae occur in larvae, pupae and imagoes of dragonflies *Phryganea grandis* (Linnaeus) and *Phryganea* sp. (see Brown 1933, Shevchenko 1966). Lubarskaya and Galeeva (1980) found adults of *P. chilostomum* after feeding laboratory mice with infected dragonflies *Libellula quadrimaculata* (Linnaeus).

The first description of the cercaria of *P. chilostomum* was presented by Brown (1933) who found in *P. grandis* yet unencysted migrating cercariae possessing a stylet and remnants of penetration gland-cells (which he called salivary glands).

The comparison of the cercaria observed in the present work with the description given by Brown (1933) revealed differences in the morphology of cercariae. The cercaria of *P. chilostomum* has a shorter stylet (30 µm vs 37 µm) and fewer penetration gland-cells (two pairs vs three or four pairs). These differences indicate that these cercariae belong to two different digenean species, although the metacercariae described by Brown (1933) bear general morphological similarity with adult *P. chilostomum*. It should be mentioned, however, that *Paralecithodendrium* is a large genus with a number of morphologically similar species that are difficult to distinguish even at adult stage let alone metacercariae.

Lecithodendrium sp. – cercaria Fig. 3

Syn. *Cercaria helvetica* XII Dubois, 1928

Description (measurements based on 27 specimens). Cercariae develop in small elongate sporocysts. Body

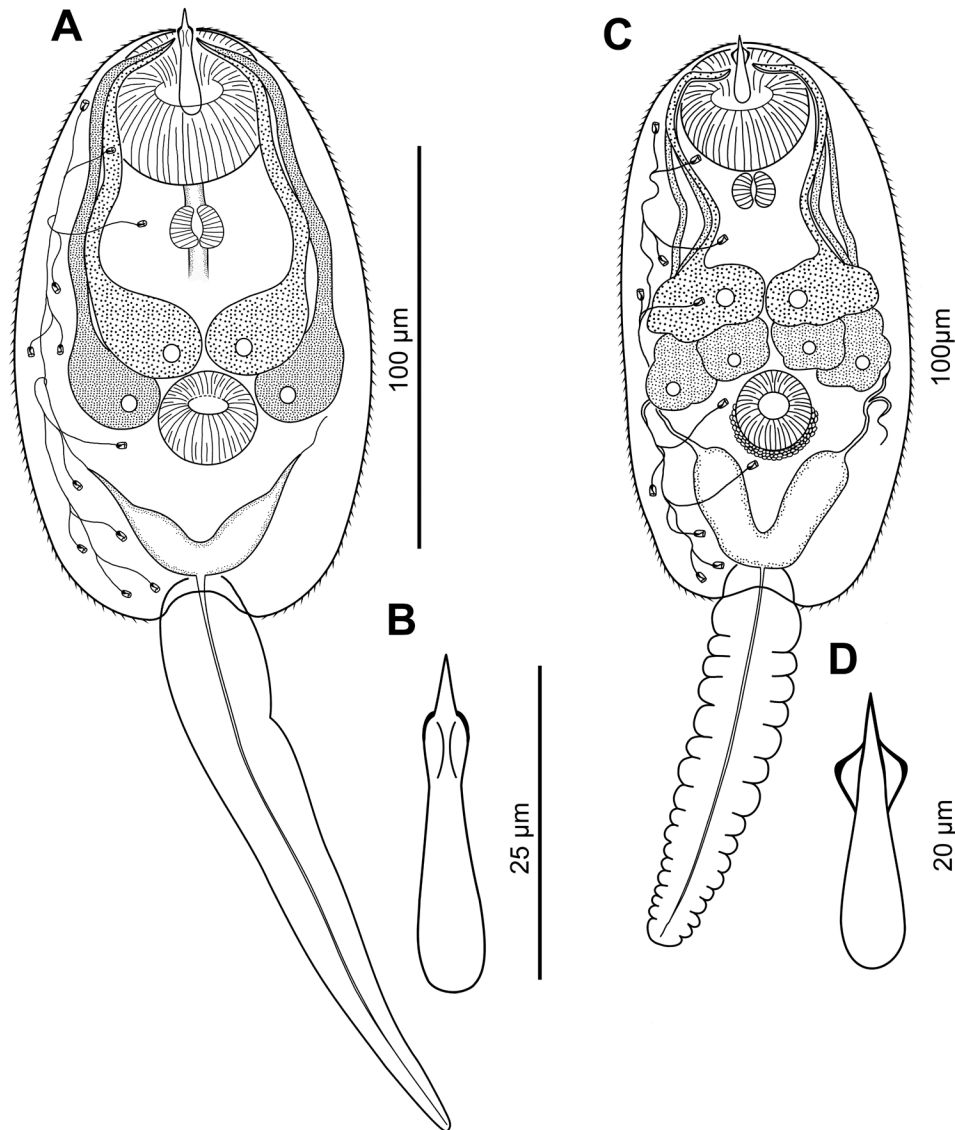


Fig. 3. *Paralecithodendrium chilostomum* (Mehlis, 1831) from *Viviparus viviparus* (A, B) and *Lecithodendrium* sp. from *Bithynia tentaculata* (C, D). A, C – whole cercaria; B, D – stylet.

small, oval, 125–158 × 50–95 (138 × 62). Tegument thin, uniformly covered with fine spines. Oral sucker rounded, subterminal, 20–33 × 23–35 (26 × 28), larger than ventral sucker. Ventral sucker 20–23 (21) in diameter, located at mid-body length. Oral/ventral sucker width ratio 1 : 0.57–1 : 0.90 (1 : 0.75). Stylet 16–19 (18) long, 4 wide at base, with strongly enlarged rounded base and sharp distal end with conspicuous lateral thickenings (Fig. 3D). Three pairs of penetration gland-cells with irregular shape located anterior to ventral sucker. One pair of large gland-cells with secretory material in form of large granules located at mid-distance between pharynx and ventral sucker. Two smaller pairs with secretory material in form of small granules located posterior to first pair. Gland ducts open on either side of stylet. Prepharynx almost indistinct; muscular pharynx small, spherical, 10–13 × 10–13 (11 × 11). Oesophagus almost indistinct. Genital primordium represented by a compact group of cells, posterodorsal to ventral sucker. Excretory vesicle thin-walled, V-shaped. Flame-cell formula:

2[(2 + 2 + 2) + (2 + 2 + 2)] = 24. Excretory pore subterminal. Tail simple, 100–137 (126) long, 15–23 (19) wide at base, somewhat shorter than body, with indented margins.

Host: *Bithynia tentaculata* (Linnaeus) (Mollusca: Bithyniidae).

Prevalence of infection: 6% (n = 121).

Site of infection: Hepatopancreas.

Locality: Curonian Bay, Juodkrante, Lithuania (55°35'38"N; 21°7'57"E).

GenBank Acc. Nos.: KJ126724, KJ126726.

Remarks. *Cercaria helvetica* XII was first described by Dubois (1928) from *B. tentaculata*. Since then, this species was reported by Bychovskaya-Pavlovskaya and Kulakova (1971) from the Curonian Bay and the Neman River in Lithuania. These authors suggested that this cercaria may belong to the genus *Prosthogonimus* Lühe, 1899, but their light microscopy data were insufficient to elucidate the taxonomic position of this cercaria. Our phylogenetic analyses have convincingly demonstrated that *C. helvetica*

XII is most closely related to typical representatives of *Lecithodendrium* Looss, 1896.

DISCUSSION

The molecular phylogenetic analyses have placed the non-irrigulate cercariae described herein among the lecithodendriids parasitic in bats, namely species of *Lecithodendrium* and *Paralecithodendrium*. The tree topology resulting from the analysis of both datasets left no doubt that *C. helvetica* XII belongs to the genus *Lecithodendrium* (Figs. 1, 2) and is closely related to *L. linstowi*. However, the two species are not identical because the sequence divergence between *C. helvetica* XII and *L. linstowi* (1.1% in ITS2 and 0.7% in 28S) is at the level usually observed among closely related congeneric species. It is noteworthy that both 28S and ITS2 sequences of *L. linstowi* obtained from two different bat species in the United Kingdom and Ukraine (Tkach et al. 2000, Lord et al. 2012) were completely identical.

The exact match of both the ITS2 sequences and 28S sequences of *C. pugnax* with those of adult *P. chilostomum* provides a convincing evidence that *C. pugnax* is the larval stage of *P. chilostomum*. Tkach et al. (2003) suggested that neither *P. parvouterus* nor *P. hurkovaee* belong to the genus *Paralecithodendrium*. Our phylogenetic analysis further corroborates this assumption, especially with respect to *P. hurkovaee*, which does not show a close affinity to *Paralecithodendrium* in both ITS2 and 28S trees.

The trematodes of the family Lecithodendriidae are characterised by a three-host life cycle (Lord et al. 2012) and may be parasitic as adults in birds and mammals, most prominently bats. The cercariae develop within daughter sporocysts in prosobranch molluscs. Encystment of the metacercariae occurs within aquatic insect larvae (Brown 1933, Azim 1936, Knight and Pratt 1955, Etges 1960, Besprozvannykh 1990), which may then be ingested, upon emergence of the adult insect, by a foraging bat (Lord et al. 2012). However, the complete life cycles have been elucidated in only a handful of lecithodendriid species, e.g. *Acanthatrium anaplocami* Etges, 1960, *Acanthatrium oregonense* Macy, 1939, *Acanthatrium ovatum* Yamaguti, 1939, *Paralecithodendrium dollfusi* Besprozvannykh, 1990 and *Paralecithodendrium pyramidum* (= *Prosthodendrium parvouterus* (Bhalerao, 1926) (see Azim 1936, Knight and Pratt 1955, Etges 1960, Burns 1961a,b, Besprozvannykh 1990).

All these studies reported irrigulate xiphidiocercariae. We also assume that the cercaria of *P. chilostomum* de-

scribed by Brown (1933) could be irrigulate. He found cercariae that recently penetrated the second intermediate host and the secretions of the irrigula glands may have been already used up at that point. At the beginning of penetration of the second intermediate host, the irrigula organ releases material that forms a pseudocyst around the cercarial body, securing its position while it penetrates the arthropod cuticle (Lotz and Font 2008). It should be noted that a large number of irrigulate cercariae described by several authors were also identified as representatives of the family Lecithodendriidae (e.g. Seitner 1945, Burns 1961a, Ginetsinskaya 1968, Stenko et al. 2005).

According to Lotz and Font (2008), the irrigula organ is an important characteristic that identifies 'lecithodendriid-like' species as a natural group, the 'irrigulate digeneans'. The five families thought to belong to this group are the Pleurogenidae Looss, 1899, Lecithodendriidae, Gyrobascidae Macy, 1935, Phaneropsolidae Mehra, 1935 and Leyogonimidae Dollfus, 1951. All these families contain at least one member for which the cercarial stage has been described. All of them have been found to have irrigulate xiphidiocercariae. Only *Ganeo* Klein, 1905, traditionally included in the Pleurogenidae, is known to have a non-irrigulate xiphidiocercaria. Brooks et al. (1985, 1989) considered the digeneans with irrigulate cercariae a monophyletic group, the Lecithodendriidae (*sensu lato*). Thus, the irrigula organ was used as a synapomorphy for the 'lecithodendriid-like' digenean lineages.

However, the results of the present study have demonstrated the presence of non-irrigulate xiphidiocercariae among the Lecithodendriidae. This implies that the irrigula organ is not an absolute synapomorphy in this group and the use of this character for broader phylogenetic and systematic assumptions is limited. Life cycles of a greater number of taxa need to be studied to adequately address the question of the evolution of the irrigula organ. In the present study, DNA sequencing has once again proved to be an extremely convenient, efficient and precise way of advancing our knowledge of digenean life cycles. Further molecular studies of 'microcotylae' group of xiphidiocercariae can provide opportunities to reveal identity of numerous previously reported cercariae, elucidate their life cycles and help in a better understanding of the evolution of the Lecithodendriidae and related digenean families.

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