High parasite diversity in a neglected host: larval trematodes of *Bithynia tentaculata* in Central Europe

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**Abstract**

Bithynids snails are a widespread group of molluscs in European freshwater systems. However, not much information is available on trematode communities from molluscs of this family. Here, we investigate the trematode diversity of *Bithynia tentaculata*, based on molecular and morphological data. A total of 682 snails from the rivers Lippe and Rhine in North Rhine-Westphalia, Germany, and 121 *B. tentaculata* from Curonian Lagoon, Lithuania were screened for infections with digeneans. In total, *B. tentaculata* showed a trematode prevalence of 12.9% and 14%, respectively. The phylogenetic analyses based on 55 novel sequences for 36 isolates demonstrated a high diversity of digeneans. Analyses of the molecular and morphological data revealed a species-rich trematode fauna, comprising 20 species, belonging to ten families. Interestingly, the larval trematode community of *B. tentaculata* shows little overlap with the well-studied trematode fauna of lymnaeids and planorbid, and some of the detected species (*Echinochasmus beleocephalus* and *E. coaxatus*) constitute first records for *B. tentaculata* in Central Europe. Our study revealed an abundant, diverse and distinct trematode fauna in *B. tentaculata*, which highlights the need for further research on this so far understudied host-parasite system. Therefore, we might currently be underestimating the ecological roles of several parasite communities of non-pulmonate snail host families in European fresh waters.

**Introduction**

With about 25,000 species and a cosmopolitan distribution, digenetic trematodes constitute one of the most diverse and ubiquitous groups of parasites on the planet (Esch *et al.*, 2002). Despite their complex life history with a wide variety of vertebrate definitive hosts, including fish, amphibians, reptiles, birds and mammals, this group shares a unifying character: practically all species require molluscs (usually gastropods) as first intermediate hosts. Due to their complex interaction with their hosts and their wide distribution and abundance, trematodes have been shown to make up a large proportion of an ecosystem’s biomass (Kuris *et al.*, 2008; Presten *et al.*, 2013; Soldánová *et al.*, 2016), contribute significantly to the energy flow within ecosystem (Thieltges *et al.*, 2008), function as structuring forces in food webs (Lafferty *et al.*, 2008; Thieltges *et al.*, 2013) and can affect host populations by influencing host mortality, fecundity, growth and behaviour (Mouritsen & Jensen, 1994; Marcogliese, 2004; Lagrue & Poulin, 2008; Rosenkranz *et al.*, 2018). Moreover, they can serve as useful bioindicators to assess environmental conditions and changes due to their intricate life cycles (e.g. Lafferty, 1997; Hupshi & Lafferty, 2004; Vidal-Martínez *et al.*, 2010; Shea *et al.*, 2012; Nachev & Sures, 2016). Altogether, there is increasing awareness that trematodes are important ecosystem components that require our attention in order to fully understand the complex interactions and dynamics in ecosystems.

In freshwater systems, snails of the families Lymnaeidae and Planorbidae play a key role in the life cycle of trematodes. In Europe, members of both families serve as important first intermediate hosts to a wide variety of digenean trematodes, with 87 and 92 described species, respectively, which accounts for more than 85% of the described trematode species from gastropod hosts from this region (Faltýnková *et al.*, 2016; Schwelm *et al.*, 2018). Both groups are well-studied model host-parasite systems in terms of their diversity, ecological function and their role as infectious agents (e.g. Faltýnková & Haas, 2006; Faltýnková *et al.*, 2007, 2008, 2016; Soldánová *et al.*, 2010, 2013, 2017; Novobilský *et al.*, 2014; Horařík *et al.*, 2015; Selbach *et al.*, 2015a, b). Consequently, detailed identification keys (Faltýnková *et al.*, 2007, 2008; Selbach *et al.*, 2014) as well as accessible molecular vouchers (e.g. Georgieva *et al.*,...
2013, 2014; Selbach et al., 2014, 2015b; Zikmundová et al., 2014; Soldánová et al., 2017) are available for these parasite taxa, which enable studies to accurately identify trematodes and assess their ecological role. Moreover, the life cycles of many trematodes are known in detail (Brown et al., 2011 and references therein), which allows conclusions to be drawn about the presence or absence of free-living organisms in ecosystems (Byers et al., 2010).

In contrast to the detailed knowledge about lymnaeid and planorbid host-trematode systems, the role of bithyniid snails and other non-pulmonate snails, such as Hydrobiidae, Melanopsidae, Neritidae, Valvatidae and Viviparidae, as first intermediate hosts for trematodes in Central Europe remains largely unexplored. Although snails of these families are known to host digenean trematodes and these data were included in some faunistic surveys (e.g. Cichy et al., 2011; Faltyňková et al., 2016), these studies were mostly focused on lymnaeid and planorbid hosts. The faucet snail, Bithynia tentaculata, which is common and widespread throughout Europe, has established itself as a non-indigenous species in North America (Mills et al., 1993; Duggan et al., 2003; Bachtel et al., 2019). Bithynia tentaculata is highly tolerant towards salinity and temporal droughts and occurs in most waterbodies throughout Europe (Glöer, 2002; Welter-Schultes, 2012). With 32 trematode species according to Cichy et al. (2011) and 14 species according to Faltyňková et al. (2016) known from B. tentaculata, it represents the most species-rich snail–parasite assemblage among the group of non-pulmonate freshwater snails (formerly known as ‘Prosobranchia’) in Europe. However, due to the lack of focussed faunistic studies on trematode communities in this host species, with the exception of individual studies investigating selected parasite groups (e.g. Serbina, 2005; Kudlai et al., 2015, 2017), the number of trematode species known from B. tentaculata may well grossly underestimate the true diversity of this host–parasite system.

A serious obstacle for most freshwater ecologists and parasitologists encountering trematodes of the snail families Bithynidae, Hydrobiidae, Melanopsidae, Neritidae, Valvatidae and Viviparidae is the lack of morphological and molecular information for species identification. Unlike for planorbid and lymnaeid snails, there are no keys for larval trematodes parasitizing snails from these families, and morphological descriptions are often restricted to adult stages (e.g. Gibson et al., 2002; Tkach et al., 2003; Jones et al., 2005; Bray et al., 2008; Besprozvannykh et al., 2017; Kudlai et al., 2017). Moreover, existing literature is often not available in English (e.g. Našincová, 1992; Ataev et al., 2002; Serbina, 2005; Besprozvannykh, 2009), which also exacerbates the investigation of this parasite–host system. These obstacles lead to a further bias towards well-studied species, such as Lymnaea stagnalis, Radix spp. and Planorbarius corneus, while other snail species continue to remain overlooked and avoided in the assessments of the ecological role of trematodes. It is, therefore, important to characterize the trematode fauna in B. tentaculata, and thus facilitate further studies on the ecological role of this host–parasite system, as is possible for lymnaeid and planorbid snails. Moreover, some of the trematodes utilizing Bithynia spp. are important pathogens of wildlife that can have serious impacts on migratory birds (e.g. Herrmann & Sorensen, 2009; Roy & St-Louis, 2017; Bachtel et al., 2019), which further highlights the need for a better understanding of this host and its parasite fauna.

Here, we assess the diversity of the larval trematodes of B. tentaculata in Central Europe and provide molecular and morphological reference material to fill this gap in our knowledge. With this study, we also hope to draw more attention to this essential and largely overlooked parasite–host system and promote further studies on this group.

**Material and methods**

**Sample collection**

In total, 682 snails of the species B. tentaculata were collected and examined for trematode infections during monthly collections in 2016 and 2017. Snails were collected at four sampling sites at the River Lippe in summer and autumn in 2016 and 2017 (K4: 51°39′44″N, 8°10′23″E; K1: 51°39′42.3″N, 8°13′49.3″E; K2: 51°39′41.5″N, 8°14′18.1″E; K3: 51°39′41.8″N, 8°14′19.2″E) and at two sampling sites at the lower River Rhine (R1: 51°47′59.2″N, 6°21′46.3″E; R3: 51°48′37.1″N, 6°21′23.4″E) and one pond of its adjacent floodplain (R2: 51°49′07.0″N, 6°20′26.8″E) in spring, summer and autumn in 2017 in North Rhine-Westphalia, Germany (fig. 1). Additionally, 121 B. tentaculata were collected from the Curonian Lagoon near the village of Juodkrante, Lithuania (55°35′38″N, 21°7′57″E) in June 2013. Snails from Germany were identified using the identification keys of Glöer (2002) and Welter-Schultes (2012).

All snails were collected with strainers or hand-picked from sediments, stones, macrophytes and floating vegetation from the riverside or along the littoral zone of the pond. In the laboratory, snails were placed in individual cups with filtered river water at 20°C and exposed to a light source to induce the emergence of cercariae. Each cup was screened for the presence of cercariae three times over three consecutive days after sampling under a stereomicroscope. Snails that did not shed cercariae during this time period were dissected and examined for prepatent infections (rediae/sporocysts). To obtain isolates for molecular analyses, cercariae, rediae and sporocysts were pooled from one single infected snail host and fixed in molecular-grade ethanol. Additionally, cercariae were fixed in 4% formaldehyde solution for measurements of fixed material. For documentation and measurements of the snail hosts, photomicrographs of the snail shell were taken with a Keyence VHX5000 microscope (Osaka, Japan). Foot tissue from infected snails was fixed in molecular-grade ethanol for molecular analysis and identification.

**Morphological analyses**

Larval stages were preliminarily identified under an Olympus BX51 microscope (Tokyo, Japan) using morphological descriptions of Našincová (1992) and Bykhovskaya-Pavlovskaya & Kulakova (1971) and other relevant publications (e.g. Heinemann, 1937; Zdun, 1961; Našincová & Scholz, 1994; Kudlai et al., 2015). Preliminary identification was made to the family or genus level. Morphology of cercariae was studied on live and fixed specimens. Series of photomicrographs were taken for collected isolates with an Olympus UC30 digital camera (Tokyo, Japan) for measurements and further identification. Measurements were taken from the digital images using cellSens 1.16 Life Science image software (https://www.olympus-lifescience.com/en/software/cellsens). Measurements are in micrometres (μm) and are presented as a range, followed by a mean in parentheses.

**Molecular sequencing**

DNA isolation was performed following a modified salt precipitation protocol after Sunnucks & Hales (1996) and Grabner et al.
To each sample, 600 µl TNES Buffer and 10 µl proteinase K solution were added. Trematode samples were incubated at 50°C for two to three hours depending on the quantity of the sample material. Snail tissue samples were incubated overnight at 35°C. In order to precipitate the proteins, 170 µl of 5 M sodium chloride was added, followed by vortexing and centrifuging for 5 min at 20,000×g at room temperature. The supernatant was transferred into a new reaction tube and centrifuging was repeated. The supernatant was again transferred into a new reaction tube, the pellet was discarded and 800 µl of 99% ice-cold ethanol was added to the supernatant and mixed by repeated inverting. The solution was centrifuged at 20,000×g for 15 min at 4°C. In order to purify the sample, 180 µl of 70% ethanol was added after the supernatant was discarded. The sample was centrifuged for 15 min at 20,000×g at 4°C, the ethanol was discarded and the pellet air-dried. The DNA pellet was dissolved in 100 µl TE buffer.

Target gene fragments were chosen based on preliminary identification of cercariae to the family level and were amplified via polymerase chain reaction (PCR) (table 1) following the corresponding protocols (Folmer et al., 1994; Cribb et al., 1998; Galazzo et al., 2002; Kostadinova et al., 2003; Tkach et al., 2003). Tissue of snails was also used for DNA extraction and PCR amplification using the primers and protocols by Folmer et al. (1994). PCR products were purified using purification kits (GATC Biotech, Constance, Germany). The original PCR primers and the internal primers for 28S were used for sequencing (table 1). Contiguous sequences were assembled and edited in Geneious ver. 11 (https://www.geneious.com). All sequences were submitted to GenBank under accession numbers MN720141–MN720149; MN723852–MN723854; MN726941–MN726975; and MN726988–MN727001. For species identification, each sequence was compared with sequences available in GenBank by using the Basic Local Alignment Tool (BLAST).

**Phylogenetic analyses**

The newly generated sequences were aligned with sequences available in GenBank according to the trematode family and gene amplified (supplementary table S1). Sequences were aligned with MUSCLE (Edgar, 2004) implemented in Geneious ver. 11. A total of eight alignments for nine families were analysed. Outgroup selection was based upon the molecular phylogenies of Olson et al. (2003), Tkach et al. (2016), Kanarek et al. (2017) and Hernández-Orts et al. (2019). Phylogenetic trees for each dataset were constructed with Bayesian inference (BI) and maximum likelihood (ML) analyses on the CIPRES portal (Miller et al., 2010) and the ATGC bioinformatics platform, respectively. The Akaike Information Criterion implemented in jModelTest 2.1.1 (Guindon and Gascuel, 2003; Darriba et al., 2012) was used to determine the best-fit nucleotide substitution model for each dataset. These were the general time reversible model, with estimates of invariant sites and gamma distributed among-site rate variation (GTR + I + G) for six alignments:
Echinochasmusidae and Psilostomidae (28S), Psilostomidae (28S), Notocotylidae (28S), Pleurogenidae and Prosthogonimidae (28S and internal transcribed spacer 2 (ITS2)), and Opecoelidae (28S); Notocotylidae (28S), Pleurogenidae and Prosthogonimidae (28S, Echinochasmidae and Psilostomidae (28S), Psilostomidae (28S), and ITS1-5.8S-ITS2 data revealed two closely related sequences of the rDNA alignment included novel sequences and those retrieved from GenBank. A BLAST search analysis indicated a 86% coverage and 98% of similarity with two isolates of *B. tentaculata* from Germany (AF445334) (Hausdorf *et al.*, 2003) and North America (JX970605) (Wilke *et al.*, 2013); and a 89% coverage and 92% of similarity with one isolate from Croatia (AF367643) (Wilke *et al.*, 2001). Snails from the Lithuanian system were identified morphologically.

### Systematics

**Superfamily:** Diplostomoidea Poirier, 1886  
**Cyathocotylidae Mühling, 1898**

### Molecular results

In total, four snails from three localities were infected with cercariae belonging to the family Cyathocotylidae (prevalence: River Lippe: 0.2%; River Rhine: 4%). Sequences for the partial 28S rRNA gene and entire ITS1-5.8S-ITS2 gene cluster were generated for one isolate per locality.

Both BI and ML analyses of the Cyathocotylidae based on 28S rDNA alignment included novel sequences and those retrieved from GenBank (fig. 2a; supplementary table S1), and resulted in trees with similar topologies. Sequences for the isolates CR1 and CK2a clustered with the sequence for *Cyathocotyle prussica* Mühling, 1896 with a strong support. A single isolate (CR2) formed a branch basal in the clade of *Cyathocotyle* spp.

The two identical 28S rDNA sequences (isolates CR1 and CK2a) differed from the sequence for *C. prussica* (MH521249) by 0.2% (2 nt) and from the sequence for the isolate CR2 by 1.4% (17 nt). The ITS1-5.8S-ITS2 sequences for the isolates CR1 and CK2a were identical and differed from the sequence for the isolate CR2 by 3.7% (48 nt). A BLAST analysis based on ITS1-5.8S-ITS2 data revealed two closely related sequences of the Cyathocotylidae available in GenBank. These were *Holostephanus dubinini* Vojtek & Vojtěchova, 1968 (AY245707) and *C. prussica* (MH521249) (supplementary table S1). However, the isolates CR1 and CK2a differed from *C. prussica* by 0.2% (3 nt) and from *H. dubinini* by 3.8% (49 nt), whereas the isolate CR2 differed from the same species by 3.6% (47 nt) and 3.5% (45 nt), respectively. Based on the results of molecular analyses, the isolates CR1 and CK2a were identified as *Cyathocotyle* sp. 1 and isolate CR2 as *Cyathocotyle* sp. 2.

### Systematics

**Cyathocotyle Mühling, 1896**

**Cyathocotyle sp. 1**

*First intermediate host.* *Bithynia tentaculata* (L.) (Gastropoda: Bithyniidae).
Table 1. PCR primers for gene-fragments used in the study.

<table>
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<tr>
<th>Gene fragment</th>
<th>Primer name</th>
<th>Nucleotide sequence</th>
<th>Source</th>
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<td>28S</td>
<td>DigII</td>
<td>5′-GCTATCTGAGGAAAAGTTCG-3′</td>
<td>Tkach et al. (2003)</td>
</tr>
<tr>
<td>1500R</td>
<td></td>
<td>5′-GCTATCTCAGGAAAACGG-3′</td>
<td>Tkach et al. (2003)</td>
</tr>
<tr>
<td>ECD2*</td>
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<td>5′-CTTGGTCCTGGTTCTCAAGACGG-3′</td>
<td>Tkach et al. (2003)</td>
</tr>
<tr>
<td>300R*</td>
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<td>5′-CAAGTCCTGAGGAAAAGTTCG-3′</td>
<td>Tkach et al. (2003)</td>
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<td>ITS1-5.8S-ITS2</td>
<td>DIF</td>
<td>5′-AGGAATCTCTGTAATGCCAA-3′</td>
<td>Galazzo et al. (2002)</td>
</tr>
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<td></td>
<td>D2R</td>
<td>5′-CGTACTGAGGAAATCTCTGCGT-3′</td>
<td>Galazzo et al. (2002)</td>
</tr>
<tr>
<td>ITS2</td>
<td>3S</td>
<td>5′-GGTACCGGTGGATACGTTGCTAGTG-3′</td>
<td>Morgan &amp; Blair (1995)</td>
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<td>ITS.2</td>
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<td>cox1</td>
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<td>Folmer et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>HCO2198</td>
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<td>NDJ2a</td>
<td>5′-CTTCCAGCTTCAGATATA-3′</td>
<td>Kostadinova et al. (2003)</td>
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</table>

*Internal primers. PCR conditions were followed as described in the source papers.

Localities. River Rhine (R1), River Lippe (K2), Germany.

Representative DNA sequences. 28S rDNA, two replicates (MN726941, MN726942); ITS1-5.8S-ITS2, two replicates (MN723852, MN723853).

Description

(Measurements from eight fixed specimens.) Furcocercous cercariae (fig. 3a, b) with colourless, opaque, elongate-oval body, 192–226 × 116–147 (205 × 130). Entire body surface covered with minute spines. Tail with furcae; tail stem 267–300 (288) long with maximum width 45–61 (54). Tail stem longer than body [tail stem/body length ratio 1:0.67–0.78 (1:0.71)]. Furcae 229–255 (245) long with maximum width 31–39 (35). Furcae without finfold. Tail stem/furcal length ratio 1:0.80–0.78 (1:0.85). Tips of furcae form contractile processes. Anterior organ terminal, elongate-oval, 36–49 × 29–36 (40 × 32) with several rows of spines (10–12). Prepharynx distinct, pharynx small, elongate-oval, 9–13 × 9–10 (11 × 9); oesophagus short, bifurcating in first third of body, intestinal caeca well developed, conspicuous and wide with lobate walls, terminating blindly at posterior extremity of body. Ventral sucker absent. Penetration gland-cells numerous, small, pear-shaped, posterior to anterior organ and lateral to prepharynx and pharynx, with narrow ducts opening subterminally at the anterior end of anterior organ. Excretory commissures forming two conspicuous loop-like structures. Genital primordium in one group of compact small cells anterior to excretory vesicle. Excretory vesicle thin-walled, transversely oval.

Cyathocotyle sp. 2

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Locality. River Rhine (R2), Germany.

Representative DNA sequences. 28S rDNA, one replicate (MN726943); ITS1-5.8S-ITS2, one replicate (MN723854).

Description

(Measurements from ten fixed specimens.) Furcocercous cercariae (fig. 3c) with colourless, opaque, elongate-oval body, 151–176 × 93–120 (165 × 107). Entire body surface covered with minute spines. Several rows (7–8) of small postoral spines at anterior body part. Tail with furcae; tail stem 164–207 (182) long with maximum width at base 39–53 (45). Tail stem longer than body [tail stem/body length ratio 1:0.93–0.97 (0.91)]. Furcae lance-shaped, 135–163 (150) long, maximum width 28–41 (35), with small triangular fins, forming contractile processes. Tail stem/furcal length ratio 1:0.74–0.90 (1:0.82). Anterior organ terminal, elongate-oval, 29–36 × 20–30 (32 × 24). Prepharynx distinct; pharynx small, elongate-oval, muscular, 9–13 × 6–10 (11 × 9), conspicuous; oesophagus very short, indistinct, bifurcation just postpharyngeal. Intestinal caeca well developed, with slightly lobate walls, terminating blindly at posterior extremity of body. Ventral sucker absent. Penetration gland-cells numerous, small, pear-shaped, posterior to anterior organ. Main collection ducts without refractile excretory granules. Excretory commissures forming two loop-like structures. Genital primordium in one group of compact medium-sized cells anterior to excretory vesicle. Excretory vesicle thin-walled, transversely oval.

Remarks

The morphological features of cercariae of both species are consistent with the morphology of cyathocotyloid cercariae according to Ginetsinskaya & Dobrovolskij (1968) and Niewiadomska (1980). To date, cyathocotyloid cercariae of nine species have been reported from B. tentaculata in Europe: Cyathocotyle bithyniae Sudarikov, 1974, C. bushiensis (Khan, 1962), C. prussica Muhling, 1896; Holostephanus cobitidis Oprivilova, 1968, H. curonensis (Szidat, 1933), H. dubini Vojtek & Vojtěch, 1968, H. luehei Szidat, 1936, H. volgensis (Sudarikov, 1962) and Prohemistomum vivax (Sonsino, 1892).

The cercariae of Cyathocotyle sp. 1 and Cyathocotyle sp. 2 show several distinctive features that allow the separation of the two species. Cercariae of Cyathocotyle sp. 1 differ by body size [length: 192–226 (205) vs. 151–176 (165); width: 116–147 (130) vs. 93–120 (107)], number of rows of postoral spines (10–12 vs. 7–8), shape of furcae (more slender with a sharp tip vs. more compact with small triangular fins), size of furcae [length: 229–255 (245) vs. 135–163 (150); width: 31–39 (45) vs. 39–53 (35)] and tail shape of furcae (more slender with a sharp tip vs. more compact with small triangular fins).
Table 2. Summary data for isolates collected from *Bithynia tentaculata* and used for generation of novel sequences.

<table>
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<th>Species</th>
<th>Isolate</th>
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<tr>
<td></td>
<td></td>
<td>28S</td>
</tr>
<tr>
<td><strong>Family Cyathocotylidae Mühling, 1898</strong></td>
<td></td>
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<tr>
<td>Cyathocotyle sp. 1</td>
<td>CR1</td>
<td>MN726941</td>
</tr>
<tr>
<td></td>
<td>CK2a</td>
<td>MN726942</td>
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<tr>
<td>Cyathocotyle sp. 2</td>
<td>CR2</td>
<td>MN726943</td>
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<tr>
<td><strong>Family Echinocasmiidae Odhner, 1910</strong></td>
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<td><em>Echinocausmus coaxatus</em> Dietz, 1909</td>
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<td><strong>Family Notocotylidae Lühe, 1909</strong></td>
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(Continued)
stem/furcal length ratio [1:0.80–0.89 (1:0.85) vs. 1:0.74–0.90 (1:0.82)]. The oesophagus of the cercariae of Cyathocotyle sp. 2 is much shorter than in Cyathocotyle sp. 1 and it bifurcates just behind the pharynx (fig. 3a, b). The intestinal caeca are well developed in both species, but the cercariae of Cyathocotyle sp. 1 show more conspicuous ceaca with wide and lobate walls, whereas the ceaca of the cercariae of Cyathocotyle sp. 2 exhibit narrower and straighter walls.

General morphology of cercariae of Cyathocotyle sp. 1 and Cyathocotyle sp. 2 corresponds well to cercariae of Cyathocotyle sp. 1 resemble cercariae of C. prussica as described by Kanev (1984) in the absence of the small triangular fins on the tip of furca. However, cercariae of Cyathocotyle sp. 1 have larger body [length: 192–226 (205) vs. 161–187; width: 116–147 (130) vs. 90–93 (92), respectively], size of the furca [229–255 (245) × 31–39 (35) vs. 200–216 (209) × 13–20 (17)], anterior organ [36–49 (40) × 29–36 (32) vs. 43–46 (44) × 33–36 (38)] and pharynx [9–13 (11) × 9–10 (9) vs. 16–17 × 10–15. The differences between the cercariae of Cyathocotyle sp. 1 and C. bushiensis as described by Khan (1962) include: body length [116–147 (130) vs. 90–93 (92), respectively], size of the furca [229–255 (245) × 31–39 (35) vs. 200–216 (209) × 13–20 (17)], anterior organ [36–49 (40) × 29–36 (32) vs. 43–46 (44) × 33–36 (38)] and pharynx [9–13 (11) × 9–10 (9) vs. 16–17 × 16].

The present cercariae identified as Cyathocotyle sp. 2 appeared most similar to the cercariae of C. bithyniae and C. bushiensis based on the presence of the small triangular fins at the ends of the tail furcae, but differ from cercariae of C. bithyniae as described by Niewiadomska (1980) by the lower limits for body length [151–176 (165) vs. 161–187], lower low limits for body width [93–120 (107) vs. 102–119], lower low limits for tail length [164–207 (182) vs. 192–204], higher limits for the width of furca

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**Table 2.** (Continued.)

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Echinochasmidae Odhner, 1910 and Psilostomidae Looss, 1900

Cercariae of *Cyathocotyle* sp. 2 differ from *C. bushiensis* as described by Khan (1962) by smaller body dimensions except the width of body [93–120 (107) vs. 90–93 (92), respectively] and furca [28–41 (35) vs. 13–20 (17)].

**Systematics**

Superfamily: Echinostomatoidea Looss, 1899

Echinococidae Odhner, 1910 and Psilostomidae Looss, 1900

**Molecular results**

Infection with the cercariae of the families Echinococidae and Psilostomidae was detected in nine snails from two localities in the River Rhine and one locality at the River Lippe, and in two snails collected in the Curonian Lagoon (prevalence: Echinococidae: River Lippe, 0.2%; River Rhine, 4%; Curonian Lagoon, 0.8%; Psilostomidae: River Lippe, 0.2%; River Rhine, 6.7%; Curonian Lagoon, 13.2%). Partial 28S rDNA sequences were generated for all collected isolates (*n* = 11); *nad*1 sequences were successfully generated for three isolates (fig. 4; supplementary fig. S1; table 2).

Sequences of partial 28S rDNA obtained in this study were aligned with the available sequences for echinococids (*n* = 15) and psilostomids (*n* = 13) from GenBank (supplementary table S1). Two species of the Himasthlidae, *Acanthoparyphium spinulosum* Johnston, 1917 and *Himasthla limnodromi* Didyk & Burt, 1997 were used as the outgroup based on the topologies in the phylogenetic tree of the Echinostomatoidea published by Tkach et al. (2016). Both BI and ML analyses yielded a similar topology, with two main clades representing the two families, Echinococidae and Psilostomidae. The newly generated sequences fell into two distinct and strongly supported clades within each family. Two isolates, ECR1 and EBR1, collected from the River Rhine were identical with the sequences for *Echinococoma coxatus* Dietz, 1909 (KT956928) ex *Podiceps nigricollis* and *E. busricola* (Creplin, 1837) (KT956938) ex *Ardea alba* from Ukraine (Tkach et al., 2016), respectively (fig. 4; supplementary table S1). The three remaining isolates within the Echinococidae represent two unidentified species of *Echinococoma*. *Echinococoma* sp. 1 (E2R1 and E1CB) clustered with the sequence for *Echinococoma mivii* Yamaguti, 1939 (KT873319) from Russia, and *Echinococoma* sp. 2 (E1K2a) with *E. beleocephalus* (Linstow, 1873) (KT956929) ex *A. alba* from Ukraine and *E. japonicus* Tanabe, 1926 (JQ890579) ex *Gallus gallus* from Russia. The interspecific divergence between *Echinococoma* sp. 1 and *E. mivii* was 1.5% (58 nt), and between *Echinococoma* sp. 2 and *E. beleocephalus* and *E. japonicus* was 0.2% (2 nt) and 0.6 (7 nt), respectively.

Six isolates (SR2, PS1R1, PS1R2, PS1K2a, PSCB and PS2R2) represented by three species fell within the clade for the Psilostomidae. One isolate (SR2) collected from the River Rhine was identical to the isolate for *Sphaeridiotrema* sp. ex *B. tentaculata* (KT956958) from Lithuania (fig. 5; supplementary table S1). Five remaining isolates formed a strongly supported clade with four of them (PS1R1, PS1R2, PS1K2a and PSCB) representing the same species, whereas the fifth isolate (PS2R2) was distinct. The sequences for these five isolates did not show affiliation to any of the psilostomid genera included in the analyses. The interspecific divergence between the two species was 1.8% (20 nt). Based on these results, both species were identified to the family level as Psilostomidae gen. sp. 1 and Psilostomidae gen. sp. 2.

Additional analyses were conducted for the Psilostomidae in order to include sequences of the three species of the genus *Psilotrema* Odhner, 1913 available in GenBank (supplementary table S1). The sequences for these species were not included in the main analyses due to their short length (759 nt). In these analyses, the isolates for Psilostomidae gen. sp. 1 and Psilostomidae gen. sp. 2 clustered in a clade with representatives of the genus *Psilotrema*, *P. oschmarini*, *P. simillium* and *P. acutirostris*, while Psilostomidae gen. sp. 1 appeared to be conspecific with *P. oschmarini* (see supplementary fig. S1). Based on this result, both species – Psilostomidae gen. sp. 1 and Psilostomidae gen. sp. 2 – may belong to the genus *Psilotrema*. However, at this stage, we refrain from identifying cercariae in our material as *P. oschmarini* due to the results being based on a short dataset and the lack of morphological vouchers for the sequences in GenBank.

**Systematics**

Echinococidae Odhner, 1910

*Echinococoma* Dietz, 1909

*Echinococoma coxatus* Dietz, 1909

First intermediate host. *Bithynia tentaculata* (L.) (Gastropoda: Bithyniidae).

Locality. River Rhine (R1), Germany.

Representative DNA sequences. 28S rDNA, one replicate (MN726944); *nad*1, one replicate (MN720147).

**Description**

(Measurements from 11 fixed specimens.) Gymnocephalous cercariae (fig. 5a). Body colourless, oval, with maximum width slightly anterior to ventral sucker, 103–124 × 68–102 (115 × 86). Tegmentum thick, tegumental spines absent. Collar poorly developed. Collar spines absent. Tail simple, muscular, contractile, 72–115 (92) long, with maximum width at base 22–33 (29), slightly shorter than body [tail/body length ratio 1:1.12–1.35 (1:1.25)]. Oral sucker subterminal, muscular, subcircular, 23–32 × 28–36 (29 × 31). Ventral sucker subcircular, just postequatorial, 26–33 × 23–31 (30 × 27). Oral/ventral sucker width ratio 1:0.90–1.14 (1:1.03). Prepharynx distinct, pharynx spherical, muscular, 6–10 × 5–10 (8 × 7). Ceca indistinct. Penetration glands numerous, on both sides posterior to oral sucker. Cystogenous gland-cells few, rounded, with rhabditiform contents. Excretory vesicle bipartite, anterior part transversely oval, at level of posterior margin of body, posterior part transversely oval, at junction of body and tail; main collecting ducts wide, dilated between mid-level of pharynx and level of posterior margin of ventral sucker, containing large dark refractile excretory granules of different size (12 on each side).
Echinochasmus bursicola (Creplin, 1837)

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Locality. River Rhine (R1), Germany.

Representative DNA sequences. 28S rDNA, one replicate (MN726945).


Echinochasmus sp. 1

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Locality. River Rhine (R1), Germany; Curonian Lagoon (CB), Lithuania.

Representative DNA sequences. 28S rDNA, two replicates (MN726946, MN726947).

Description (Measurements from seven fixed specimens.) Gymnocephalous cercariae (fig. 5c). Body colourless, oval, with maximum width at level of ventral sucker, 105–136 × 74–125 (123 × 95). Tegument thick, brownish, tegumental spines absent. Collar uncinose. Collar spines absent. Tail simple, leaf-like, brownish, muscular, contractile, with few randomly arranged subspherical concretions, 115–170 (141) long with maximum width at base 27–82 (53), longer than body [tail/body length ratio 1:0.74–0.96 (1:0.87)]. Oral sucker subterminal, subterminal, 28–40 × 28–35 (32 × 31). Ventral sucker subterminal, 25–34 × 26–33 (30 × 29). Oral/ventral sucker width ratio 1:0.78–1.06 (1:0.94). Prepharynx indistinct, pharynx elongate-oval, muscular, 12–16 × 6–10 (13 × 8). Caeca indistinct. Penetration gland-cells numerous, on both sides posterior to oral sucker. Cystogenous gland-cells numerous, with rhabditiform contents, on both sides anterior to ventral sucker. Excretory vesicle bipartite; anterior part smaller, transversely oval, at level of posterior margin of body, posterior part larger, transversely oval, at junction of body and tail; main collecting ducts wide, dilated between level of pharynx and anterior...
margin of ventral sucker, narrowing posteriorly and anteriorly, containing dark refractile excretory granules of different size (13–15 large ones on each side plus several small ones at posterior and anterior ends).

**Echinococmus sp. 2**

*First intermediate host.* Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

*Locality.* River Lippe (K2), Germany.

*Representative DNA sequences.* 28S rDNA, one replicate (MN726948); nad1, one replicate (MN720148).

**Description (no photomicrograph available)**


**Remarks**

Cercariae of both identified species of *Echinococmus – E. coaxatus* and *E. bursicola* – have been previously reported from *B. tentaculata* (Karmanova, 1973, 1974). The life cycle of *E. coaxatus*...
was described by Karmanova (1974) in the Astrakhan Nature Reserve, Russia. Cercariae were found in *B. tentaculata* and metacercariae were found in the freshwater fishes of the families Cyprinidae and Percidae, collected in the River Volga. Cercariae of *E. coxatus* were also reported from *Radix auricularia* in the lake Goldapiwo, Poland, by Wiśniewski (1957). General morphology of cercariae found in our study corresponded well to the description for cercariae of *E. coxatus* by Karmanova (1974), except in the number of the refractile excretory granules per collecting duct (12 vs. 13–14, respectively). The differences in the metrical data for body [114–184 vs. 103–124 (115)] and tail lengths [123–140 vs. 72–115 (92)] may be due to the different fixation methods. Karmanova (1974) did not indicate whether the measurements were taken from live or fixed cercariae.

Cercariae of *E. bursicola* were previously described by Karmanova (1973) from *B. tentaculata* collected in the Lower Volga, Russia. Although the method of fixation was not specified, the present cercariae differ from cercariae described by Karmanova (1973) by a shorter [110–132 (120) vs. 129–340, respectively] and narrower body [71–105 (88) vs. 104–110], shorter tail [83–101 (93) vs. 114–300] and the number of the refractile excretory granules per collecting duct (seven vs. six).

Cercariae of both *Echinocochasmus* sp. 1 and *Echinocochasmus* sp. 2 differ from cercariae of *Echinocochasmus* sp. reported from *Lithoglyphus naticoides* (Pfeiffer, 1828), as described by Stanevičiūtė et al. (2008) in Lithuania, by smaller dimensions for all morphological characters, in particular by smaller body (105–136 × 74–125 vs. 98–133 × 66–84 vs. 240–280 × 136–144) and much smaller tail (115–170 × 27–82 vs. 83–123 × 23–31 vs. 1120–1360 × 132), respectively.

Further identification of *Echinocochasmus* sp. 1 and *Echinocochasmus* sp. 2 to the species level requires the sequences of the adults from the definitive hosts, which are typically fish-eating birds and rarely mammals (Tkach et al., 2016).

**Systematics**

Psilostomidae Looss, 1900

*Sphaeridiotrema* Odhner, 1913

*Sphaeridiotrema* sp.

**First intermediate host.** *Bithynia tentaculata* (L.) (Gastropoda: Bithyniidae).

**Locality.** River Rhine (R2), Germany.

**Representative DNA sequences.** 28S rDNA, one replicate (MN726954).

**Description**

Cercariae of *Sphaeridiotrema* sp. were found in one snail at Locality R2 in the River Rhine. Cercariae were identified based on the results of molecular analyses. No morphological data were obtained for cercariae of this species.

Psilostomidae gen. sp. 1

**First intermediate host.** *Bithynia tentaculata* (L.) (Gastropoda: Bithyniidae).

**Locality.** River Lippe (K2), River Rhine (R1, R2), Germany; Curonian Lagoon (CB), Lithuania.

**Representative DNA sequences.** 28S rDNA, four replicates (MN726950–MN726953).

Description


Psilostomidae gen. sp. 2

**First intermediate host.** *Bithynia tentaculata* (L.) (Gastropoda: Bithyniidae).

**Locality.** River Rhine (R2), Germany.

**Representative DNA sequences.** 28S rDNA, one replicate (MN726954).

**Description**

Cercariae of Psilostomidae gen. sp. 2 (fig. 5e) were found in one snail at Locality R2 in the River Rhine. Cercariae were identified based on the results of molecular analyses. No morphological data were obtained for cercariae of this species.

**Remarks**

To date, *B. tentaculata* was reported as the first intermediate host for six species of psilostomids from three genera: (i) *Psilochasmus: Psilochasmus oxyurus* (Creplin, 1825) in Poland (Wiśniewski, 1958); (ii) *Psilotrema: Psilotrema oligoon* (Linstow, 1887) in the UK (Pike, 1968), *P. similimum* (Mühling, 1898) in Bulgaria (Samnaliiev, 1981) and *P. spiculigerum* (Mühling, 1898) in Bulgaria, Russia, Ukraine and the UK (Zdun, 1961; Bykhovskaya-Pavlovskaya & Kulakova, 1971; Frolova, 1975; Samnaliiev et al., 1977; Morley & Lewis, 2006); and (iii) *Sphaeridiotrema: Sphaeridiotrema globulus* (Rudolph, 1814) in Bulgaria, Finland, Russia and the UK (Sziadat, 1937; Selinheimo, 1956; Bykhovskaya-Pavlovskaya & Kulakova, 1971; Kanev & Vasilev, 1984; Morley & Lewis, 2006) and *Sphaeridiotrema* sp. in Lithuania (Tkach et al., 2016).

*Sphaeridiotrema* sp. in our material appeared to be conspecific to the species that was previously reported from Lithuania (Tkach et al., 2016). Both isolates were identified only to the genus level and require sequences of adults from the definitive hosts (water birds), to complete identification to the species level.

General morphology of cercariae of *Psilostomidae* gen. sp. 1 resemble morphology of cercariae of *P. oligoon* described by Pike (1968), *P. similimum* described by Samnaliiev (1981) and *P. spiculigerum* described by Bykhovskaya-Pavlovskaya & Kulakova (1971) and Samnaliiev et al. (1977). However, cercariae of *Psilostomidae* gen. sp. 1 differ from cercariae of *P. oligoon* by wider body [218–267 (239) vs. 165–235 (196)], longer tail...
Lissorchiidae Magath, 1917

P or Cercariae of Molecular results

Superfamily: Monorchioidea Odhner, 1911

Asymphylodora sp. (L.) (Gastropoda: Bithyniidae).

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Locality. River Lippe (K3), Germany.

Representative DNA sequences. 28S rDNA, one replicate (MN726955).

Molecular results

Cercariae of Asymphylodora sp. were found in one snail in the River Lippe (prevalence: 0.2%). Partial 28S rDNA sequences were generated from one isolate (table 2). The partial 28S rDNA sequence for Asymphylodora sp. obtained in the present study was compared to the sequences of Asymphylodora perccotti Besprozvannykh, Ermolenko & Atopkin, 2012 (FR822715–FR822731) ex Percottus gleni Dybowsky, 1877 from Russia (Besprozvannykh et al., 2012), the only sequences for this genus currently available in GenBank. The sequence divergence between our isolate and 17 isolates for A. perccotti ranged between 2.7% and 2.8% (31–32 nt).

Description

The species of the cercariae was identified based on the results of molecular data. No morphological data were obtained for cercariae of this isolate.

Systematics

Superfamily: Pronocephaloidea Looss, 1899
Notocotylidae Lühe, 1909

Molecular results

Infection with the cercariae of the family Notocotylidae was detected in nine snails from four localities in the River Lippe (prevalence: 1.5%). Partial 28S rDNA sequences were generated for four isolates (fig. 6; table 2) and aligned with 16 sequences for species of the Notocotylidae available in GenBank (supplementary table S1). Members of the families Opisthotrematidae, Rhabdioptidae and Labicolidae were used as the outgroup based on the topologies in the phylogenetic tree of the Digesta published by Olson et al. (2003). The results of phylogenetic analyses demonstrated that two isolates preliminarily identified as Notocotylus sp. (N11K0 and N12K0) clustered within a clade comprising Notocotylus spp., demonstrating the close affinity to the isolate of Notocotylus attenuatus (Rudolphi, 1809) (AF184259), the type species of the genus Notocotylus, collected from Aythya ferina in Ukraine (Tkach et al., 2001). Sequences for two isolates of Notocotylus sp. from our study were identical and differed from N. attenuatus by 0.4% (3 nt). The sequences for two other notocotyloid isolates (N2K0 and N2K2b) from B. tentaculata collected in the River Lippe were identical and formed a basal branch to the clade consisting of Notocotylus spp. and Catastrapis spp., albeit without support (fig. 6). The taxonomic identity of these two isolates was not justified based on the phylogenetic analyses and we, thus, provide the identification for this species only to the family level, as Notocotylidae gen. sp. The sequence divergence between two notocotyloid species recorded in our study was 2.9% (23 nt).

Systematics

Notocotylus Diesing, 1839

Notocotylus sp.

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Locality. River Lippe (K4), Germany.

Representative DNA sequences. 28S rDNA, two replicates (MN726956, MN726957).

Description

No morphological data were obtained for cercariae of these isolates since the infections were prepatent.

Notocotylidae gen. sp.

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Localities. River Lippe (K3, K4), Germany.

Representative DNA sequences. 28S rDNA, two replicates (MN726958, MN726959).

Description

No morphological data were obtained for cercariae of these isolates since the infections were prepatent.
Remarks

Members of the family Notocotylidae are reported to utilize lymnaeids, planorbids and a variety of other snail families in their life cycles (Filimonova, 1985). To date, six species – namely, *N. attenuatus*, *N. ponticus*, *N. parviovatus*, *N. imbricatus*, *Notocotylus* sp. and *Catatropis verrucosa* – were reported to develop in *B. tentaculata* in Europe (Bock, 1982; Filimonova, 1985 and references therein; Morley et al., 2004). Further identification of the two species collected from *B. tentaculata* in the River Lippe to the species level requires the sequences of adult worms from the definitive hosts, which are mammals and birds (Filimonova, 1985).

Systematics

Superfamily: Allocreadioidea Looss, 1902
Opecoelidae Ozaki, 1925

Molecular results

Infection with cercariae of the family Opecoelidae was detected in 45 snails from four localities in the River Lippe (prevalence: 7.4%). Sequences for the partial 28S rDNA and ITS2 region were generated for six isolates (fig. 7; table 2). Comparative sequence analyses of 28S and ITS2 datasets revealed the presence of two species of the family Opecoelidae in our material. Five sequences of partial 28S rRNA gene (table 2) were aligned with seven GenBank sequences for species of the Opecoelidae known to parasitize freshwater fish (supplementary table S1). A species of the Opecoelidae, *Buticulotrema thermichthysi* Bray, Waeschenbach, Dyal, Littlewood & Morand, 2014, was used as the outgroup based on the topologies in the phylogenetic tree of the Opecoelidae published by Martin et al. (2019). The results of the phylogenetic analyses demonstrated a close affinity of the four isolates (O11K2a, O1K1, O1K2b and O13K2a) with *Sphaerostoma bramae* (Müller, 1776) (MH161435) collected from *Abramis brama* in Russia (Sokolov et al., 2019). The sequences for our isolates were identical and differed from the sequence of *S. bramae* by 0.2% (3 nt), which is considered as interspecific variation and, thus, this species was identified as *Sphaerostoma* sp. The intraspecific divergence between the isolates of *Sphaerostoma* sp. (O11K2a, O1K1, O1K2b and O12K2a) within the ITS2 dataset was 0.2% (1 nt).

The remaining isolate (O2K2a) collected in the River Lippe, representing a second opecoelid species in our material, formed a branch basal to a clade consisted of *Sphaerostoma* spp. and *Plagiocirrus* spp. The 28S rDNA sequence of this species differed from the sequence of *S. bramae* by 5.9% (71 nt), from *S. bramae* by 5.6% (68 nt) and from *Plagiocirrus* spp. by 7.1–7.2% (85–87 nt), whereas the ITS2 sequence differed from the sequence of *Sphaerostoma* sp. by 4.7–4.9% (21–22 nt). Based on the results, we identified this species only to the family level as Opecoelidae gen. sp.

*Sphaerostoma* sp.

First intermediate host. *Bithynia tentaculata* (L.) (Gastropoda: Bithyniidae).

Localities. River Lippe (K1, K2, K3), Germany.
Representative DNA sequences. 28S rDNA, four replicates (MN726960–MN726963); ITS2, four replicates (MN726988–MN726991).


Opecoelidae gen. sp.

First intermediate host. Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

Locality. River Lippe (K2), Germany.

Representative DNA sequences. 28S rDNA, one replicate (MN726964); ITS2, one replicate (MN726992).

Description (no photomicrograph available)

No morphological data were obtained for cercariae of this isolate since the infections were prepatent.

Remarks

To date, one species of the Opecoelidae, S. bramae, has been reported in B. tentaculata in Europe: in Denmark (as Cercaria micrura; Wesenberg-Lund, 1934), Finland (Wikgren, 1956), Lithuania (Petkevičiūtė et al., 1995), the Netherlands (as C. micrura; Keulen, 1981), Russia (as C. micrura; Bykhovskaya-Pavlovskaya & Kulakova, 1971), Ukraine (as C. micrura; Zdun, 1961) and the UK (Pike, 1967). Comparative sequence analysis suggested Sphaerostoma sp. of the present study to be close but not conspecific with S. bramae. Morphology of cercariae of Sphaerostoma sp. corresponds well to cercariae of S. bramae described by Wikgren (1956), Wesenberg-Lund (1934), Pike (1967) and Bykhovskaya-Pavlovskaya & Kulakova (1971). However, our cercariae differ from cercariae of S. bramae as described by Wesenberg-Lund (1934) by lower maximum of the body length (207–285 vs. 255–450), body width (110–132 vs. 85–135) and shorter tail [22–42 (28) vs. 45–50]; and from cercariae as described by Pike (1967) by shorter body [207–285 (246) vs. 261–418 (299)] and smaller oral [39–53 (49) × 42–60 (50) vs. 54–61 (57) × 50–59 (55)] and ventral suckers [53–79 (68) × 60–69 (64) vs. 63–81 (68) × 68–84 (73)]. Further identification of Sphaerostoma sp. and Opecoelidae gen. sp. to the species level requires the sequences of the adults from the definitive hosts, freshwater fish.

Systematics

Superfamily: Opisthorchioidea Looss, 1899
Opisthorchiidae Looss, 1899

Molecular results

Cercariae of the family Opisthorchiidae were found in one snail in the River Lippe (prevalence: 0.2%). A partial 28S rDNA sequence...
was generated for one isolate (fig. 2b; table 2) and aligned with sequences of seven species belonging to the family Opisthorchiidae available in GenBank (supplementary table S1). *Apophallus zalophi* Price, 1932 (Heterophyidae) was used as the outgroup based on the topologies in the phylogenetic tree of the Opisthorchioidea published by Hernández-Orts et al. (2019). In phylogenetic analyses of the Opisthorchiidae (fig. 2b), the novel sequence formed a branch at a basal position in the low supported clade consisting of *Opisthorchis* spp., *Clonorchis sinensis* (Cobbold, 1875) and unidentified species of *Metorchis*. Within this clade, our isolate demonstrated the lowest level of sequence divergence relative to the isolate of *Metorchis ussuriensis* (KY075777) (0.8%, 9 nt) and the highest level of divergence to the isolate of *Opisthorchis* sp. (MF110001) (2.3%, 25 nt).

Based on the results of molecular analyses, cercariae were identified to the family level as Opisthorchiidae gen. sp.

**Opisthorchiidae gen. sp.**

*First intermediate host.* Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

*Locality.* River Lippe (K3), Germany.

*Representative DNA sequences.* 28S rDNA, one replicate (MN726966).

*Description (fig. 8c)*

(Measurements from 11 fixed specimens.) Pleurolophocercariae (fig. 8c) with elongate-oval body 143–191 × 89–118 (169 × 101), with brown pigment. Tegument thick with minute spines in anterior part. Tail simple, longer than body, 437–494 (459) × 24–40 (33) with dilatation in anterior part and conspicuous finfold present in the posterior two thirds of tail, with maximum width 11–22 (16). Tail/body length ratio 1:0.31–0.42 (1:0.37). Crescent-shaped eye-spots two, with black pigment, large, 13–18 (15) long and maximum width 7–10 (9), mediolateral at level of prepharynx. Oral sucker subterminal, subspherical, 29–39 × 28–36 (34 × 33). Ventral sucker inconspicuous, rudimentary, subspherical 13–25 × 11–23 (19 × 17), smaller than oral sucker. Oral/ventral sucker width ratio 1:0.38–0.74 (1:0.56). Cystogenous gland-cells large, nucleated, posterior to eye-spots. Preacetabular penetration gland-cells six pairs, with long ducts, dilated anteriorly, opening at the anterior margin of oral sucker. Excretory vesicle thick-walled, transversely oval.

*Remarks*

Four species of the family Opisthorchiidae – *Metorchis bilis* (Braun, 1890), *M. intermedius* Heinemann, 1937, *M. xanthosomus* (Creplin, 1846) and *Metorchis* sp. – have been reported from *B. tentaculata* in Europe (Zdun, 1961; Bykhovskaya-
Pavlovskaya & Kulakova, 1971; Cichy et al., 2011). Based on sequence data analyses, the present cercariae may belong to the genus *Metorchis*. Morphologically, cercariae collected from *B. tentaculata* in the River Lippe resemble cercariae of *Metorchis intermedius* Heinemann, 1937 reported from the same snail host in the Curonian Lagoon by Bykhovskaya-Pavlovskaya & Kulakova (1971). However, cercariae in our material differ by having a larger body [143–191 (169) × 89–118 (101) vs. 135–170 × 78–81] and longer tail [437–494 (459) × 24–40 (33) vs. 350–390 × 26]. The present cercariae differ from *Metorchis* sp. as described by Zdun (1961) by smaller body [143–191 × 89–118 (169 × 101) vs. 200–320 × 32–70] and oral sucker [29–39 × 28–36 (34 × 33) vs. 48 × 48].

**Systematics**

Superfamily: Microphalloidea Ward, 1901

*Lechiodendriidae* Lühe, 1901

*Lechiodendrium* Looss, 1896

**Lecithodendrium linstowi** (Dollfus, 1931)

**Molecular results**

Cercariae of *L. linstowi* were found in one snail in the River Lippe (prevalence: 0.2%). Sequences for the partial 28S rDNA and ITS2 region were generated for one isolate (table 2). Newly generated 28S rDNA sequence appeared identical to sequence of *L. linstowi* (AF151919) obtained from an adult collected by *Nyctalus noctula* in Ukraine (Tkach et al., 2000) and sequence of *L. linstowi* (MF498821) obtained from cercariae from *Radix balthica* in the UK (Enabulele et al., 2018). The sequence for ITS2 region from the present study showed 99% similarity to those of *L. linstowi* (JF784190 and KJ934792) from *Pipistrellus pipistrellus* in England (Lord et al., 2012) and *N. noctula* from Ukraine (Kudlai et al., 2015), and 94% similarity with the sequence of *L. linstowi* (MF498820) from *R. balthica* (Enabulele et al., 2018).

*First intermediate host.* *Bithynia tentaculata* (L.) (Gastropoda: Bithyniidae).

*Locality.* River Lippe (K2), Germany.

*Representative DNA sequences.* 28S rDNA, one replicate (MN726965); ITS2, one replicate (MN726993).

**Description**


*Remarks*

Digenean trematodes of the family *Lechiodendriidae* infect insectivorous vertebrates (most prominently bats, occasionally birds), using aquatic insect larvae as second intermediate hosts and usually snails of the group formerly known as ‘prosobranchia’ as first intermediate hosts (Enabulele et al., 2018). However, cercariae of *L. linstowi* were also reported from *R. auricularia* collected in the Queen’s River, England (Enabulele et al., 2018).

Adults were found in a wide range of bat species, e.g. *Myotis daubentonii* (Kuhl) in Germany (Gottschalk, 1970), in *N. noctula* (Schreber) in Ukraine (Tkach et al., 2000; Kudlai et al., 2015) and in *P. pipistrellus* (Schreber) in Spain and the UK (Esteban et al., 2001; Lord et al., 2012). Morphology of cercariae found in our study corresponded well to the description of the cercaria of *L. linstowi* by Enabulele et al. (2018). However, the present cercariae differ in having a shorter body [78–116 (88) vs. 118–148 (132)] and smaller oral [19–14 (11) × 9–15 (11) vs. 29–37 (33) × 28–35 (30)] and ventral suckers [10–13 (11) × 8–14 (9) vs. 14–24 (19) × 14–24 (18)].

**Systematics**

Pleurogenidae Looss, 1899 and Prosthogonimidae Lühe, 1909

Infection with cercariae belonging to the families Pleurogenidae and Prosthogonimidae was detected in nine snails from three localities (prevalence: Pleurogenidae: River Lippe, 0.5%; River Rhine, 1.3%; Prosthogonimidae: River Lippe, 0.7%; River Rhine, 1.3%). Sequences for the partial 28S rDNA (n = 9) and ITS2 region (n = 8) were generated for the isolates from all localities (fig. 9; table 2). The 28S rDNA sequences were aligned with the sequences for pleurogenids (n = 13) and prosthogonomids (n = 5) available in GenBank. Two species of the family *Microphalloidea* were used as the outgroup based on the topologies in the phylogenetic tree of the *Microphalloidea* published by Kanarek et al. (2017) (fig. 9; supplementary table S1). Both BI and ML analyses yielded similar topology with two main clades corresponding to the Pleurogenidae and Prosthogonimidae. The sequence of the isolate (PKB2b) collected from the River Lippe (K3) appeared to be identical to the sequence of *Parabascus duboisi* ex *M. daubentonii* from Ukraine (AY220618) (Tkach et al., 2003). The isolate (PL2R1) collected from *B. tentaculata* in the River Rhine clustered with *Leyogonimus polynoeum* (KY752116) from *Fulica atra* collected in Poland (Kanarek et al., 2017). The sequence divergence between two species was 2% (23 nt). This isolate was identified to the family level as Pleurogenidae gen. sp. 2. The two isolates (PL1K2a and PL1K2a) collected from the River Lippe (K2) clustered with pleurogenid species from the genera *Brandesia*, *Candidotrema*, *Pleurogenes*, *Pleurogenoides* and *Prosotocus*, and identified only to the family level as Pleurogenidae gen. sp. 1.

The 28S rDNA sequences of the remaining five isolates (PO1K2b, PO2K2b, PO1R1, PO2R1 and PO1K2a) collected from the River Rhine (R1) and from the River Lippe (K2 and K3) were identical with the sequence of *Prosthogonimus ovatus* from *Pica pica* collected in Ukraine (AF151928) (Tkach et al., 2000) (fig. 9b; supplementary table S1).

Sequences of the ITS2 region for pleurogenids and prosthogonomids obtained in this study were aligned with sequences of prosthogonomids available in GenBank (supplementary table S1). Sequences of the four isolates (PO1K2b, PO1R1, PO2R1 and PO2K2a) clustered with the sequence of *P. ovatus* (KF192722) from *A. ferina* collected in the Czech Republic (Heneberg et al., 2015). The four remaining isolates (PKB2b, PL2R1, PL1K2a and PL1K2a) identified as the members of
the family Pleurogenidae based on 28S rDNA analyses clustered within a nearly supported clade (fig. 9a).

**Systematics**

Pleurogenidae Looss, 1899
Parabascus Looss, 1907

**Parabascus duboisi (Hurkova, 1961)**

*First intermediate host.* Bithynia tentaculata (L.) (Gastropoda: Bithyniidae).

*Locality.* River Lippe (K3), Germany.

Representative DNA sequences. 28S rDNA, one replicate (MN726967); ITS2, one replicate (MN726994).

**Description**

No morphological data were obtained for cercariae of this isolate since the infection was prepatent.

**Remarks**

The life cycle of *P. duboisi* is unknown and our finding is the first to report *B. tentaculata* serving as the first intermediate host for this species. *Parabascus duboisi* is known to parasitize, among other bats, those of the genera *Eptesicus*, *Miniopterus*, *Myotis*, *Pipistrellus* and *Rhinolophus* (Sharpilo & Iskova, 1989).
**Pleurogenidae gen. sp. 1**

*First intermediate host. Bithynia tentaculata* (L.) (Gastropoda: Bithyniidae).

*Locality. River Lippe (K2), Germany.*

*Representative DNA sequences. 28S rDNA, two replicates (MN726968, MN726969); ITS2, two replicates (MN726995, MN726996).*


**Pleurogenidae gen. sp. 2**

*First intermediate host. Bithynia tentaculata* (L.) (Gastropoda: Bithyniidae).

*Locality. River Rhine (R1), Germany.*

*Representative DNA sequences. 28S rDNA, one replicate (MN726970); ITS2, one replicate (MN726997).*


**Remarks**

*Bithynia tentaculata* is known to be the intermediate hosts for three species of the Pleurogenidae – *Pleurogenes claviger* (Rudolphi, 1819), *Pleurogenoides medians* (Olsson, 1876) and *Pleurogenoides* sp. – in the Czech Republic (Zdarská, 1963), Germany (Palm, 1966), Lithuania (Bykhovskaya-Pavlovska & Kulakova, 1971), Poland (Grabda-Kazubska, 1971), Russia (Frolova, 1975) and Ukraine (Zdun, 1961).

The cercariae of Pleurogenidae gen. sp. 1 and Pleurogenidae gen. sp. 2 show some distinctive features that distinguish the two species. Differences between cercariae of Pleurogenidae gen. sp. 1 and Pleurogenidae gen. sp. 2 comprise the size of body [body length: 82–115 (92) vs. 120–154 (135); width: 69–94 (82) vs. 77–109 (90), respectively], the size and shape of virgula (small pyriform vs. large bilobed), position of the penetration gland-cells (anterolateral to the ventral sucker vs. posterolateral to the ventral sucker), the length of tail [41–48 (45) vs. 77–150 (105)] and the width of tail [14–20 (17) vs. 21–27 (25)]. Both species possess distinct fat inclusions in the body parenchyma, which are medium-sized and low in numbers in Pleurogenidae gen. sp. 1 and small and numerous in Pleurogenidae gen. sp. 2.

The cercariae of both species in the present study differ from cercariae of *P. claviger* as described by Grabda-Kazubska (1971), *P. medians* as described by Chernogorenko (1983) and *Pleurogenoides* sp. as described by Palm (1966) in having much smaller dimensions for all morphological characters.

**Systematics**

Prothogonimidae Lühe, 1909

Prothogonimus Lühe, 1899

**Prothogonimus ovatus Rudolphi, 1803**

*First intermediate host. Bithynia tentaculata* (L.) (Gastropoda: Bithyniidae).

*Locality. River Lippe (K2, K3), River Rhine (R1), Germany.*

*Representative DNA sequences. 28S rDNA, five replicates (MN726971–MN726975); ITS2, four replicates (MN726998–MN727001).*


**Remarks**

Three species of *Prothogonimus* – namely, *P. cuneatus*, *P. ovatus* and *Prothogonimus* sp. – have been reported to parasitize *B. tentaculata* in Europe; in the Netherlands (Boddeke, 1960), Germany (Palm, 1966), Lithuania (Bykhovskaya-Pavlovska & Kulakova, 1971), Russia (Ginetsinskaya & Dobrovolskij, 1968; Sharpilo & Iskova, 1963; Grabda-Kazubska, 1963), Poland (Serbina, 2005), Ukraine (Sergienko, 1972; Sharpilo & Iskova, 1989) and the UK (Probert, 1965; Morley & Lewis, 2006). The morphology of cercariae found in our study corresponded well to the description of the cercariae of *P. ovatus* by Boddeke (1960). The only feature that differs in the present cercariae is the number of penetration gland-cell pairs (four vs. three). However, this difference might be due to a miscount in
the previous description, as penetration gland-cells can be overlapping and hard to count.

**Discussion**

This study examined the parasite diversity in the faucet snail *B. tentaculata* in Central European fresh waters. To the best of our knowledge, this is the first broad faunistic survey investigating the trematode fauna in *B. tentaculata* in Central Europe and providing a combined morphological and molecular dataset. The study reveals a high trematode diversity of 20 species belonging to ten families and a high overall prevalence of infection of 12.9% in this snail host.

Not surprisingly, the larval trematode community of *B. tentaculata* shows little overlap with that of lymnaeids and planorbids, highlighting the distinctive host–parasite associations of pulmonate and non-pulmonate snails. Only one (*L. linstowi*) out of 20 species has also been recorded from lymnaeid and planorbid snail hosts (Enabulele *et al.*, 2018). Bithynids are the typical hosts of *L. linstowi*, and the single finding of this species in *Radix* sp. most probably represents an accidental infection. The species has only been reported in a single lymnaeid host, so it is reasonable to consider it as an accidental infection. Looking at the faunistic overlap of trematodes at the family level, we find a slightly different picture. Out of ten recorded families, six (Lecithodendriidae, Lissorchidae, Notocotylidae, Opecoelidae, Pleurogenidae, Psilostomidae) are also known to occur in lymnaeids and planorbids (Faltýnková *et al.*, 2007, 2008, 2016; Cichy *et al.*, 2011; Enabulele *et al.*, 2018). On the other hand, species of the families Cyathocotylidae, Echinochasmidae, Prosthogonomidae and Opisthorchiidae seem to be strictly host-specific to non-pulmonate freshwater molluscs as first intermediate hosts. Some species, such as *E. beleocephalus* and *E. coaxatus*, have been recorded from *B. tentaculata* only in Russia.
Echinocochasmus sp. 1 and Pisolostomidae gen. sp. 1 are the only species that were both detected in Germany and Lithuania. The definitive hosts of echinocochsmds and pisolostimds are typically birds and mammals (Kostadinova, 2005; Tkach et al., 2016). Birds are especially mobile, so the occurrence of the two species in both countries can be easily explained by seasonal migration. However, since the sampling effort in Germany was much higher (682 vs. 121 B. tentaculata), we would expect more trematode species to be present in Curonian Lagoon, which our limited survey did not detect.

Overall, this diverse and distinctive trematode community of B. tentaculata, and the high prevalence of infection, reveal the important role of this snail species as a first intermediate host for trematodes in European freshwater ecosystems. Similar to other well-studied host–parasite systems, B. tentaculata supports a parasite community that presumably fulfills vital and central ecological functions, ranging from contributing to ecosystem diversity, structuring food webs or serving as ecosystem engineers (Thomason et al., 1999; Mouritsen & Poulin, 2002; Lafferty et al., 2008; Hatcher et al., 2012; Dunne et al., 2013). Moreover, since parasites can also serve as indicators of the local diversity and trophic interactions of free-living organisms (Hechinger et al., 2007; Byers et al., 2010; Shea et al., 2012), the distinct trematode communities of B. tentaculata offer valuable insights into local habitat conditions.

One interesting example of the indication of local diversity and trophic interaction using digenean trematodes might be the detection of P. duboisi and L. linstowi. On the basis of our findings, we can infer the presence of bats in the studied habitat, as both are known to parasitize bats, e.g. the Daubenton’s bat M. daubentonii (Gottschalk, 1970; Esteban et al., 2001; Tkach et al., 2003). The Daubenton’s bat feeds on aquatic insects and insects with aquatic larvae, such as Lepidoptera, Diptera and Trichoptera, and it is, therefore, highly dependent on water sources. It hunts over standing or slow-moving water bodies and takes its prey from the water surface (Krapp, 2011 and references therein). Based on the finding of P. duboisi and L. linstowi, we are able to make inferences about the presence of bats at the studied habitat and the trophic relations between aquatic insects, which most probably serve as second intermediate hosts for the detected parasite species (Sharpil & Iskova, 1989; Kudlai et al., 2015; Enabulele et al., 2018) and its final bat host.

The current study was limited by the lack of relevant sequences for many trematode families in GenBank. Consequently, a major proportion of our isolates could only be identified to the genus or family level. Moreover, the complete life cycle of many trematodes parasitizing B. tentaculata have not yet been elucidated (see Kudlai et al., 2015) and remain unclear. Such obstacles impede and exacerbate extensive studies on the diversity, the ecological role and the influence of digeneans on food webs. Therefore, it is important to extend and compile morphological data of cercariae also from non-pulmonate snails, obtain more molecular isolates of adult specimens to facilitate molecular identification and clarify the still unknown life cycles of many trematode species. The present study can be seen as an important step in compiling morphological and molecular data on the digenean parasite fauna of bithyniids. Among the 20 digenean species, we are able to present the characteristics (measurements and/or photomicrographs) for 14 taxa. With the present host–parasite list we hope to foster parasitological research on parasites of understudied snail families.

Taken together, our findings and the limitations we encountered demonstrate unambiguously that our knowledge of the studied parasite–host system remains limited and large-scale studies focussing on non-pulmonate freshwater snails are lacking. This fits the overall trend of a currently highly patchy research effort on parasite diversity, which not only prevents a full inventory of parasite biodiversity but also impedes predictions of their role in ecosystems (Jorge & Poulin, 2018). Our study revealed an abundant and diverse trematode fauna in B. tentaculata, which highlights the need for further research on this host–parasite system. Therefore, we might currently be underestimating the ecological roles and impacts of parasite communities of non-pulmonate freshwater snails in European fresh waters. In order to fully comprehend the numerous and often central roles these parasites play in aquatic ecosystems, we need to better understand such understudied host–parasite systems.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/S0022149X19001093

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Conflicts of interest. None.


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