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

J. Schwelm[1], O. Kudlai[2,3], N.J. Smit[3], C. Selbach[1,3,4,*] and B. Sures[1]

[1]Aquatic Ecology and Centre for Water and Environmental Research, University of Duisburg-Essen, Universitätsstraße 5, D-45141 Essen, Germany; [2]Institute of Ecology, Nature Research Centre, Akademijos 2, 08412 Vilnius, Lithuania; [3]Water Research Group, Unit for Environmental Sciences and Management, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa and [4]Department of Zoology, University of Otago, P.O. Box 56, Dunedin 9054, New Zealand

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 planorbids, 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.

Research Paper

*Current address: Department of Bioscience, Aquatic Biology, Aarhus University, 8000 Aarhus C, Denmark

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Author for correspondence:
J. Schwelm, E-mail: jessica.schwelm@uni-due.de

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 studied in a wide range of ecological contexts. For example, trematodes have been shown to make up a large proportion of an ecosystem's biomass (Kuris *et al.*, 2008; Preston *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; Huspeni & 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; Horák *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.*,

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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 asses 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; Faltýnková *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 Faltýnková *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 migrating 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′44.1″N, 8°10′23.9″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.*

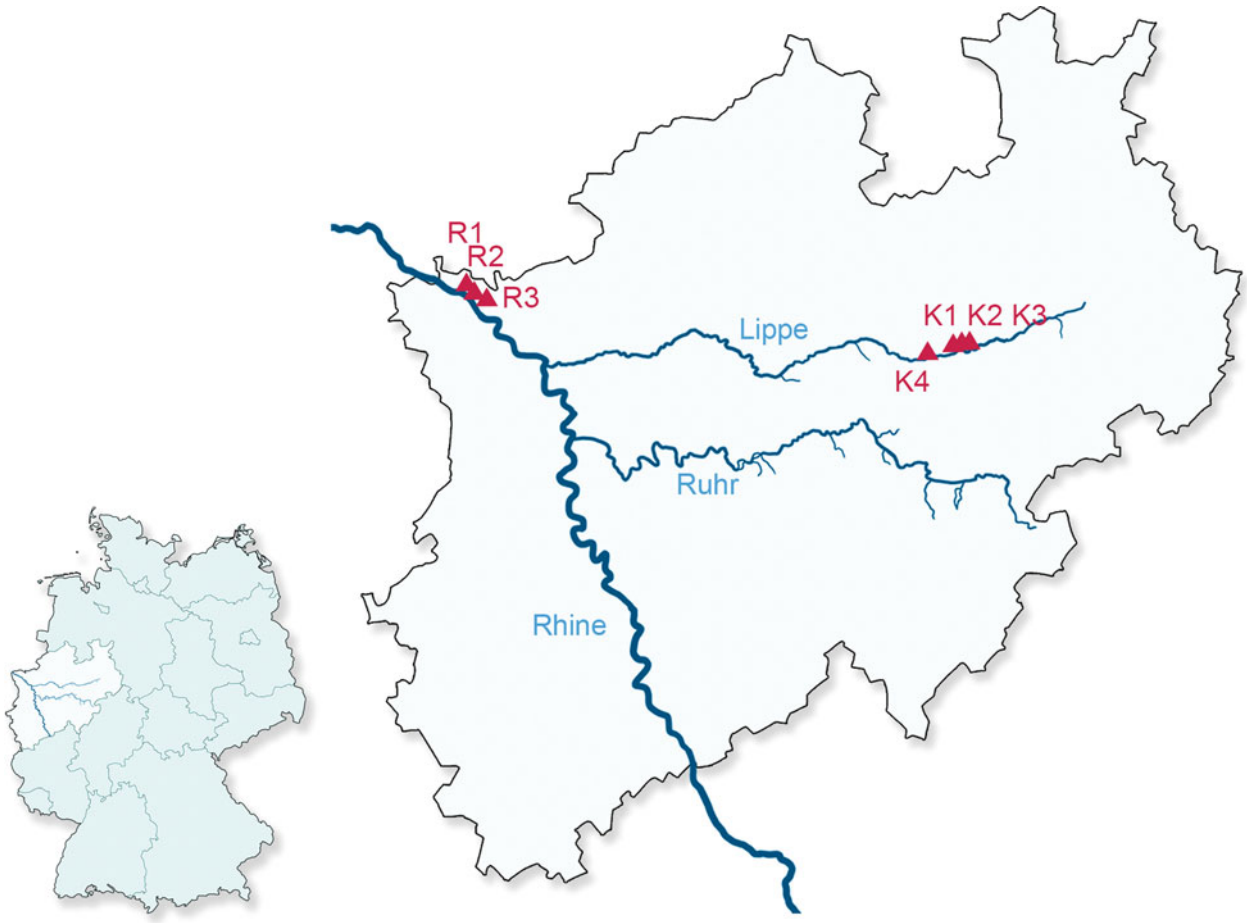

Fig. 1. Map of Germany and the federal state of North Rhine-Westphalia indicating sampling sites along the rivers Lippe and Rhine. Sampling sites are marked with red triangles.

(2015). 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:

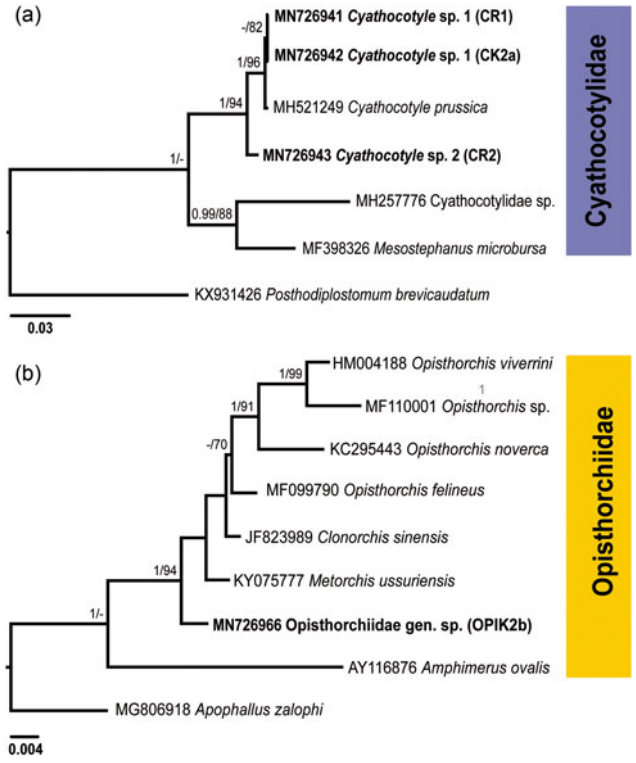

Fig. 2. Phylogenetic trees for Cyathocotylidae (a) and Opisthorchiidae (b) based on the partial sequences of the 28S rRNA gene. Numbers above branches indicate nodal support as posterior probabilities from the Bayesian inference (BI), followed by bootstrap values from the maximum likelihood (ML) analysis. Support values lower than 0.90 (BI) and 70 (ML) are not shown. The scale bar indicates the expected number of substitutions per site. The newly generated sequences are highlighted in bold.

Echinochasmidae and Psilostomidae (28S), Psilostomidae (28S), Notocotylidae (28S), Pleurogenidae and Prosthogonimidae (28S and internal transcribed spacer 2 (ITS2)), and Opecoelidae (28S); and (GTR + I) for two alignments: Cyathocotylidae (28S) and Opisthorchiidae (28S). BI analyses were performed using MrBayes v3.2.6 (Ronquist *et al.*, 2012). Markov chain Monte Carlo chains were run for ten million generations, log-likelihood scores were plotted and only the final 75% of trees were used to produce the consensus trees by setting the 'burnin' parameter at 2500 for six alignments: Echinochasmidae and Psilostomidae (28S), Psilostomidae (28S), Notocotylidae (28S) and Pleurogenidae and Prosthogonimidae (28S and ITS2). Markov chain Monte Carlo chains were run for three million generations, log-likelihood scores were plotted and only the final 75% of trees were used to produce the consensus trees by setting the 'burnin' parameter at 750 for two alignments: Cyathocotylidae (28S) and Opisthorchiidae (28S). ML analyses were performed using PhyML version 3.0 (Guindon *et al.*, 2010) with a non-parametric bootstrap validation based on 100 replicates. Trees were visualized using the FigTree ver. 1.4 software (http://tree.bio.ed.ac.uk/software/figtree/). One *cox*1 sequence alignment was analysed for the snails. The genetic divergence among taxa was estimated using uncorrected *p*-distances with the program MEGA version 6 (Tamura *et al.*, 2013).

Results

General observations

A total of 12.9% of all *B. tentaculata* from Germany showed larval trematode infections. Snails collected in Lithuania showed an overall prevalence of 14%. Phylogenetic and BLAST analyses based on 55 novel sequences for 36 isolates recovered from *B. tentaculata* collected in Germany and Lithuania (table 2) demonstrated high diversity of digeneans, including 20 species belonging to ten families: Cyathocotylidae, Echinochasmidae, Lecithodendriidae, Lissorchiidae, Notocotylidae, Opecoelidae, Opisthorchiidae, Pleurogenidae, Prosthogonimidae and Psilostomidae.

Six partial *cox*1 sequences were generated from isolates of *B. tentaculata* sampled in all German localities (MN720141–MN720146). The sequence difference between the isolates was 0–0.2% (1 nucleotide (nt) difference), thus confirming their conspecificity. Molecular identification of the snail isolates was achieved by comparing our sequences with sequences for *B. tentaculata* in 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 & Vojtkova, 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 Muhling, 1896

Cyathocotyle *sp. 1*

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

Table 1. PCR primers for gene-fragments used in the study.

Gene fragment	Primer name	Nucleotide sequence	Source
28S	Digl2	5′-GCTATCCTGAGGGAAACTTCG-3′	Tkach *et al.* (2003)
	1500R	5′-GCTATCCTGAGGGAAACTTCG-3′	Tkach *et al.* (2003)
	ECD2[a]	5′-CTTGGTCCGTGTTTCAAGACGGG-3′	Tkach *et al.* (2003)
	300F[a]	5′-CAAGTACCGTGAGGGAAAGTTG-3′	Tkach *et al.* (2003)
ITS1-5.8S-ITS2	D1F	5′-AGGAATTCCTGGTAAGTGCAA-3′	Galazzo *et al.* (2002)
	D2R	5′-CGTTACTGAGGGAATCCTGGT-3′	Galazzo *et al.* (2002)
ITS2	3S	5′-GGTACCGGTGGATCACGTGGCTAGTG-3′	Morgan & Blair (1995)
	ITS.2	5′-CCTGGTTAGTTTCTTTTCCTCCGC-3′	Cribb *et al.* (1998)
*cox*1	LCO1490	5′-GGTCAACAAATCATAAAGATATTGG-3′	Folmer *et al.* (1994)
	HCO2198	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′	Folmer *et al.* (1994)
*nad*1	NDJ11	5′-AGATTCGTAAGGGGCCTAATA-3′	Kostadinova *et al.* (2003)
	NDJ2a	5′-CTTCAGCCTCAGCATAAT-3′	Kostadinova *et al.* (2003)

[a]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 cyathocotylid cercariae according to Ginetsinskaya & Dobrovolskij (1968) and Niewiadomska (1980). To date, cyathocotylid 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* Opravilova, 1968, *H. curonensis* (Szidat, 1933), *H. dubinini* Vojtek & Vojtkova, 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

Table 2. Summary data for isolates collected from *Bithynia tentaculata* and used for generation of novel sequences.

		GenBank accession number			
Species	Isolate	28S	ITS1-5.8S-ITS2	ITS2	*nad*1
Family Cyathocotylidae Mühling, 1898					
Cyathocotyle sp. 1	CR1	MN726941	MN723852		
	CK2a	MN726942	MN723853		
Cyathocotyle sp. 2	CR2	MN726943	MN723854		
Family Echinochasmidae Odhner, 1910					
Echinochasmus coaxatus Dietz, 1909	ECR1	MN726944			MN720147
Echinochasmus bursicola (Creplin, 1837)	EBR1	MN726945			
Echinochasmus sp. 1	E2R1	MN726946			
	E1CB	MN726947			
Echinochasmus sp. 2	E1K2a	MN726948			MN720148
Family Psilostomidae Looss, 1900					
Sphaeridiotrema sp.	SR2	MN726949			MN720149
Psilostomidae gen. sp. 1	PS1R1	MN726950			
	PS1R2	MN726951			
	PS1K2a	MN726952			
	PSCB	MN726953			
Psilostomidae gen. sp. 2	PS2R2	MN726954			
Family Lissorchiidae Poche, 1926					
Asymphylodora sp.	ATK2b	MN726955			
Family Notocotylidae Lühe, 1909					
Notocotylus sp.	N11K0	MN726956			
	N12K0	MN726957			
Notocotylidae gen. sp.	N2K0	MN726958			
	N2K2b	MN726959			
Family Opecoelidae Ozaki, 1925					
Sphaerostoma sp.	O11K2a	MN726960		MN726988	
	O1K1	MN726961		MN726989	
	O1K2b	MN726962		MN726990	
	O12K2a			MN726991	
	O13K2a	MN726963			
Opecoelidae gen. sp.	O2K2a	MN726964		MN726992	
Family Lecithodendriidae Lühe, 1901					
Lecithodendrium linstowi (Dollfus, 1931)	LLK2a	MN726965		MN726993	
Family Opisthorchiidae Looss, 1899					
Opisthorchiidae gen. sp.	OPIK2b	MN726966			
Family Pleurogenidae Looss, 1899					
Parabascus duboisi (Hurkova, 1961)	PBK2b	MN726967		MN726994	
Pleurogenidae gen. sp. 1	PL11K2a	MN726968		MN726995	
	PL12K2a	MN726969		MN726996	
Pleurogenidae gen. sp. 2	PL2R1	MN726970		MN726997	
Family Prosthogonimidae Lühe, 1909					

(*Continued*)

Table 2. (*Continued.*)

Species	Isolate	GenBank accession number			
		28S	ITS1-5.8S-ITS2	ITS2	*nad*1
Prosthogonimus ovatus Rudolphi, 1803	PO1K2b	MN726971		MN726998	
	PO2K2b	MN726972			
	PO1R1	MN726973		MN726999	
	PO2R1	MN726974		MN727000	
	POK2a	MN726975		MN727001	

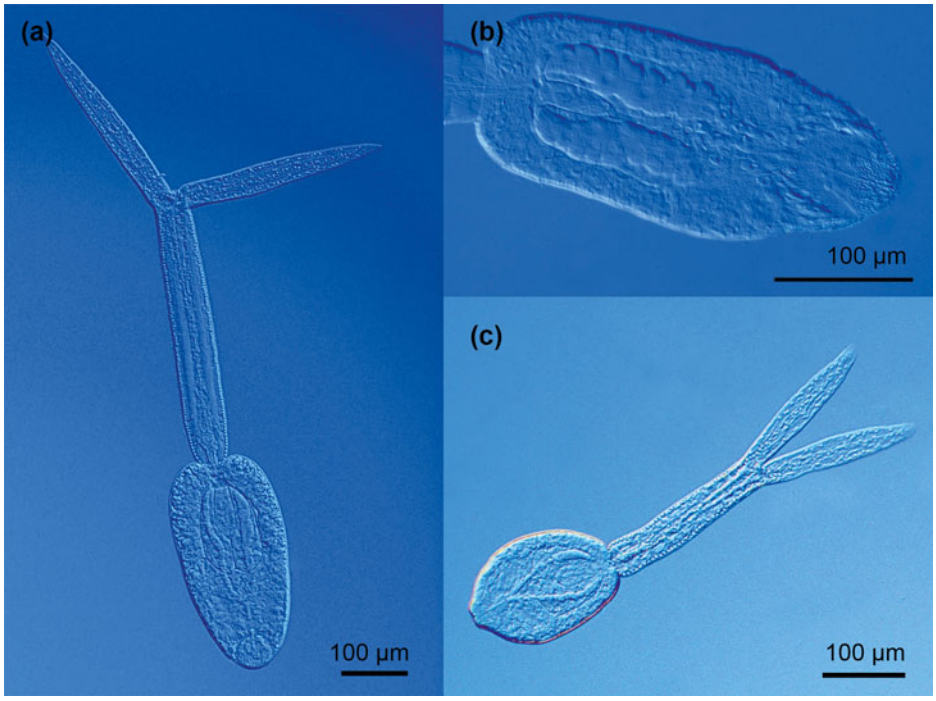

Fig. 3. Photomicrographs of live cercariae of the trematode family Cyathocotylidae. (a) *Cyathocotyle* sp.1; (b) *Cyathocotyle* sp.1, details of body spines and penetration gland-cells; (c) *Cyathocotyle* sp. 2.

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 *C. bithyniae*, *C. prussica* and *C. bushiensis* described by Niewiadomska (1980), Kanev (1984) and Khan (1962), respectively. The 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. 136–169; width: 116–147 (130) vs. 71–105], higher limits for the length and width of the anterior organ [length: 36–49 (40) vs. 32–45; width: 29–36 (32) vs. 26–32], smaller pharynx [9–13 (11) × 9–10 (9) vs. 14 × 14], shorter and narrower tail [length: 267–300 (288) vs. 400–500; width: 45–61 (54) vs. 56–65] and higher low limits for the length and width of furcae [length: 229–255 (245) vs. 208–234; width: 31–39 (35) vs. 26–39].

The cercariae of *Cyathocotyle* sp. 1 differ from the cercariae of *C. bithyniae* by larger body [length: 192–226 (205) vs. 161–187; width: 116–147 (130) vs. 102–119], longer tail [267–300 (288) vs. 192–204], shorter and wider anterior organ [length: 29–36 (32) vs. 37–44; width: 36–49 (40) vs. 30–39] and by the absence of the small triangular fins at the ends of the tip of furca. In addition, cercariae of *Cyathocotyle* sp. 1 bear a small elongate-oval pharynx, whereas the pharynx of *C. bithyniae* was described as fairly large and muscular [9–13 × 9–10 (11 × 9) vs. 11–17 × 10–15]. The differences between the cercariae of *Cyathocotyle* sp. 1 and *C. bushiensis* as described by Khan (1962) include: body 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 × 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

[28–41 (35) vs. 20–34] and shorter anterior organ [20–30 (32) vs. 37–44]. Additionally, the cercariae of *Cyathocotyle* sp. 2 possess a smaller pharynx than the cercariae of *C. bithyniae* [9–13 (11) × 6–10 (9) vs. 11–17 × 10–15].

Cercariae of *Cyathocotyle* sp. 2 differ from *C. prussica* as described by Kanev (1984) by larger body [length: 151–176 (165) vs. 136–169; width: 93–120 (107) vs. 71–105], lower low limits for length and width of the anterior organ [length: 29–36 (32) vs. 32–45; width: 20–30 (24) vs. 26–32], smaller pharynx [length: 9–13 (11) vs. 14; width: 6–10 (9) vs. 14], shorter and narrower tail [length: 164–207 (182) vs. 400–500; width: 39–53 (45) vs. 56–65] and shorter furcae [135–163 (150) vs. 208–234].

The cercariae of *Cyathocotyle* sp. 2 differ from cercariae of *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
Echinochasmidae Odhner, 1910 and Psilostomidae Looss, 1900

Molecular results

Infection with the cercariae of the families Echinochasmidae 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: Echinochasmidae: 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 echinoschasmids ($n = 15$) 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, Echinochasmidae 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 *Echinochasmus coaxatus* Dietz, 1909 (KT956928) ex *Podiceps nigricollis* and *E. bursicola* (Creplin, 1837) (KT956938) ex *Ardea alba* from Ukraine (Tkach *et al.*, 2016), respectively (fig. 4; supplementary table S1). The three remaining isolates within the Echinochasmidae represent two unidentified species of *Echinochasmus*. *Echinochasmus* sp. 1 (E2R1 and E1CB) clustered with the sequence for *Echinochasmus milvi* Yamaguti, 1939 (KT873319) from Russia, and *Echinochasmus* sp. 2 (E1K2a) with *E. beleocephalus* (Linstow, 1873) (KT956929) ex *A. alba* from Ukraine and *E. japonicus* Tanabe, 1926 (JQ890579) ex *Gallus gallus* from Vietnam. The interspecific divergence between *Echinochasmus* sp. 1 and *E. milvi* was 1.5% (58 nt), and between *Echinochasmus* 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

Echinochasmidae Odhner, 1910
Echinochasmus Dietz, 1909

Echinochasmus coaxatus *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). Tegument 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, subspherical, 23–32 × 28–36 (29 × 31). Ventral sucker subspherical, 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). Caeca indistinct. Penetration gland-cells 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).

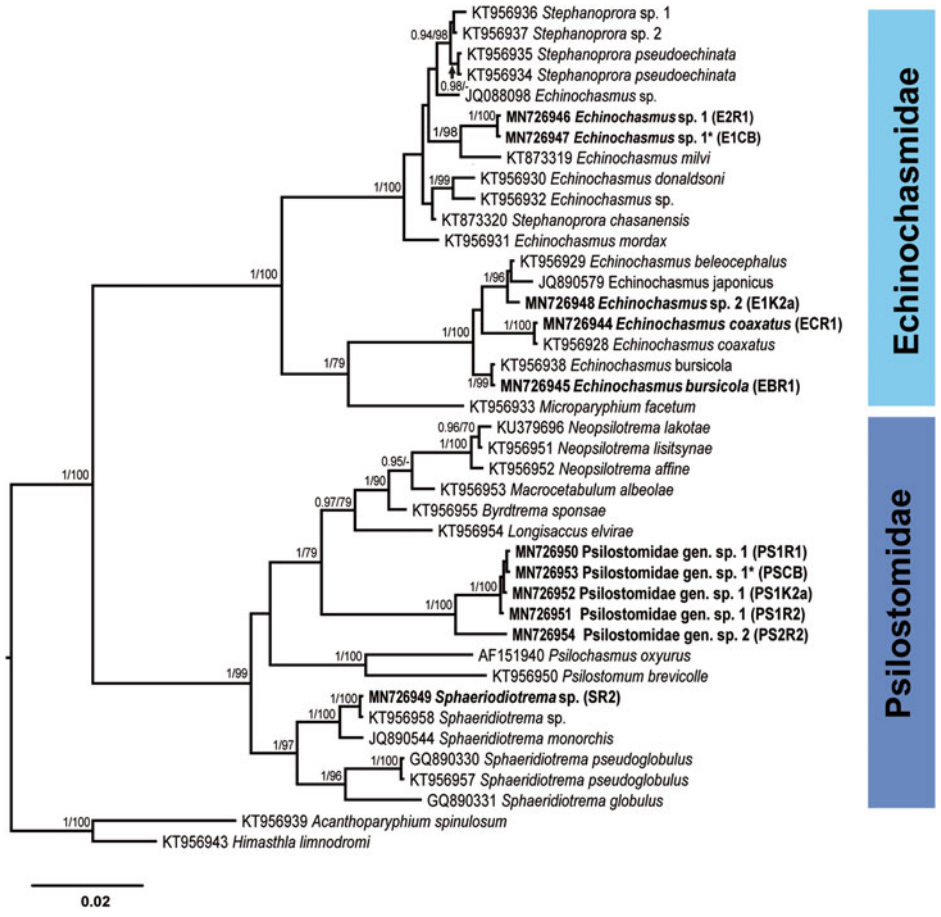

Fig. 4. Phylogenetic tree for Echinochasmidae and Psilostomidae based on the partial sequences of the 28S rRNA gene. Numbers above branches indicate nodal support as posterior probabilities from the Bayesian inference (BI), followed by bootstrap values from the maximum likelihood (ML) analysis. Support values lower than 0.90 (BI) and 70 (ML) are not shown. The scale bar indicates the expected number of substitutions per site. The newly generated sequences are highlighted in bold. Sequences obtained in Curonian Lagoon indicated by asterisk.

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).

Description

(Measurements from ten fixed specimens.) Gymnocephalous cercariae (fig. 5b). Body colourless, oval, with maximum width slightly anterior to ventral sucker, 110–132 × 71–105 (120 × 88). Tegument thick, tegumental spines absent. Collar well developed. Collar spines absent. Tail simple, muscular, contractile, 83–101 (93) long, with maximum width at base 31–39 (36), shorter than body [tail/body length ratio 1:1.18–1.42 (1:1.29)]. Oral sucker subterminal, muscular, transversely oval, 25–37 × 31–40 (30 × 35). Ventral sucker subspherical, just postequatorial, 27–38 × 25–37 (33 × 31). Oral/ventral sucker width ratio 1:0.90–1.27 (1:1.10). Prepharynx long, distinct, pharynx subspherical, muscular, 7–10 × 6–9 (9 × 7). Penetration gland-cells 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 narrow, containing large dark refractile excretory granules of different size (seven on each side).

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 unincisive. 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, subspherical 28–40 × 28–35 (32 × 31). Ventral sucker subspherical, 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

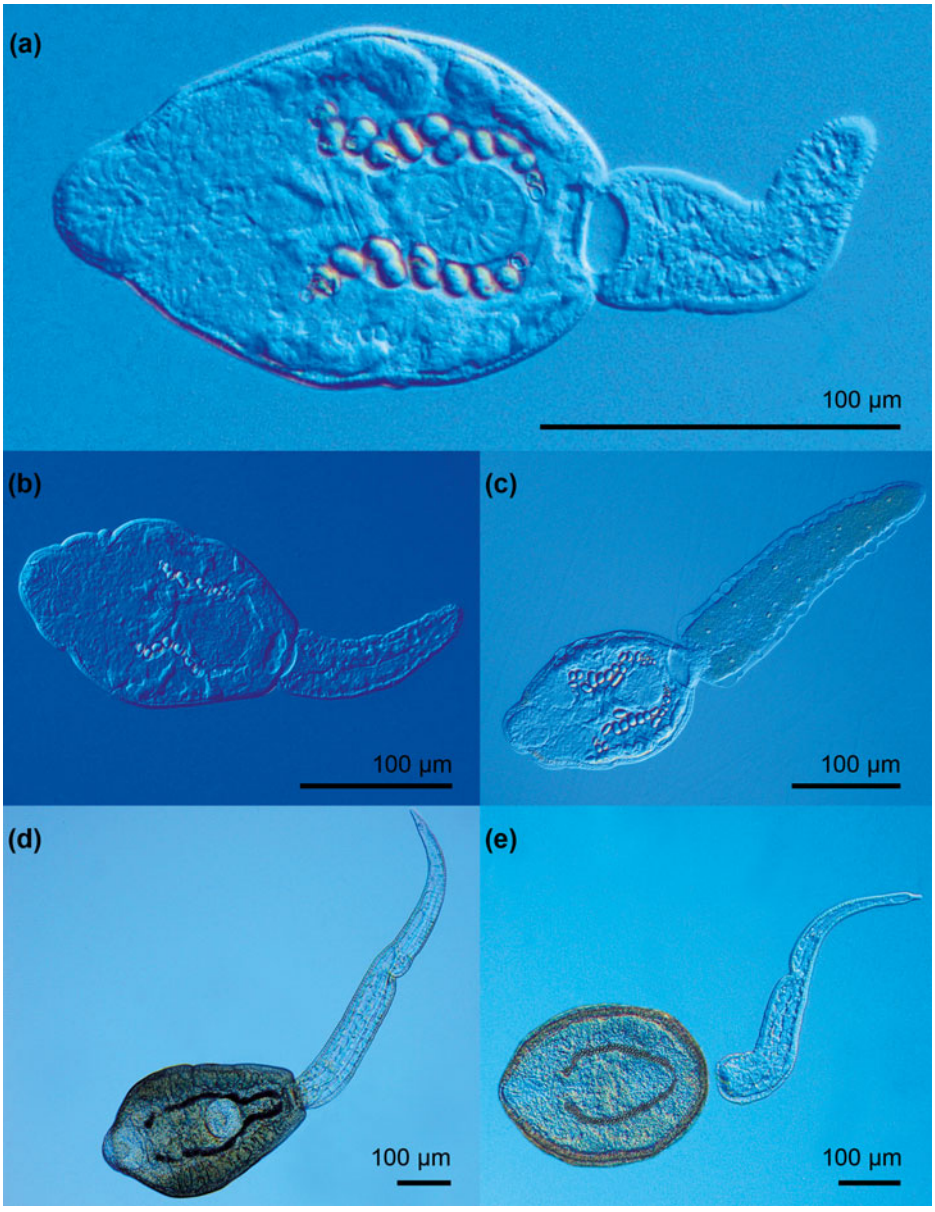

Fig. 5. Photomicrographs of live trematode cercariae of the families Echinochasmidae and Psilostomidae. (a) *Echinochasmus coaxatus*; (b) *Echinochasmus bursicola*; (c) *Echinochasmus* sp. 1; (d) Psilostomidae gen. sp. 1; (e) Psilostomidae gen. sp. 2.

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).

Echinochasmus *sp. 2*

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

Locality. River Lippe (K2), Germany.

Representative DNA sequences. 28S rDNA, one replicate (MN726948); *nad*1, one replicate (MN720148).

Description (no photomicrograph available)

(Measurements from seven fixed specimens.) Gymnocephalous cercariae. Body colourless, oval, with maximum width at level of ventral sucker, 98–133 × 66–84 (117 × 75). Tegument thick, tegumental spines absent. Collar poorly developed. Collar spines absent. Tail simple, muscular, contractile, 83–123 (102) long, with maximum width at base 23–31 (28), slightly shorter than body [tail/body length ratio 1:0.96–1.30 (1:1.15)]. Oral sucker subterminal, subspherical 27–37 × 26–36 (31 × 33). Ventral sucker spherical, muscular, 22–31 × 22–31 (28 × 28). Oral/ventral sucker width ratio 1:0.71–1.03 (1:0.90). Prepharynx indistinct, pharynx elongate-oval, muscular, 7–10 × 5–8 (9 × 7). 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 transversely oval at junction of body and tail; main collecting ducts wide, containing large, dark refractile excretory granules of different size (19 on each side).

Remarks

Cercariae of both identified species of *Echinochasmus* – *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. coaxatus* were also reported from *Radix auricularia* in the lake Gołdapiwo, Poland, by Wiśniewski (1957). General morphology of cercariae found in our study corresponded well to the description for cercariae of *E. coaxatus* 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 *Echinochasmus* sp. 1 and *Echinochasmus* sp. 2 differ from cercariae of *Echinochasmus* sp. reported from *Lithoglyphus naticoides* (Pfeiffer, 1928), 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 *Echinochasmus* sp. 1 and *Echinochasmus* 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 (MN726949); *nad*1, one replicate (MN720149).

Description

Cercariae of *Sphaeridiotrema* sp. were found in one snail at Locality R2 in the River Rhine. The species of the cercariae was 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).

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

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

Description

(Measurements from 11 fixed specimens.) Gymnocephalous cercariae (fig. 5d). Body dark, elongate-oval, with maximum width at level of posterior margin of pharynx, 243–314 × 218–267 (273 × 239). Tegument thick, tegumental spines not observed. Collar and collar spines absent. Tail simple, muscular, contractile, 454–495 (476) long, with maximum width at base 72–82 (76), longer than body [tail/body length ratio 1:0.51–0.66 (1:0.57)]. Oral sucker subterminal, subspherical, muscular, 56–75 × 52–71 (65 × 63). Ventral sucker subspherical, just postequatorial, 58–82 × 68–82 (71 × 72). Oral/ventral sucker width ratio 1:0.89–1.26 (1:1.09). Prepharynx indistinct, pharynx subspherical, muscular, 17–22 × 14–22 (19 × 17). Caeca indistinct. Penetration gland-cells numerous, on both sides posterior to oral sucker. Cystogenous gland-cells numerous on both sides posterior to oral sucker. Main collecting ducts narrow, containing numerous small dark refractile excretory granules of similar size. Main collecting ducts forming small lobes with an accumulation of slightly larger excretory granules posterior to oral sucker. Excretory vesicle transversely oval, at posterior margin of body.

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. simillimum* (Mühling, 1898) in Bulgaria (Samnaliev, 1981) and *P. spiculigerum* (Mühling, 1898) in Bulgaria, Russia, Ukraine and the UK (Zdun, 1961; Bykhovskaya-Pavlovskaya & Kulakova, 1971; Frolova, 1975; Samnaliev *et al.*, 1977; Morley & Lewis, 2006); and (iii) *Sphaeridiotrema*: *Sphaeridiotrema globulus* (Rudolphi, 1814) in Bulgaria, Finland, Russia and the UK (Szidat, 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. simillimum* described by Samnaliev (1981) and *P. spiculigerum* described by Bykhovskaya-Pavlovskaya & Kulakova (1971) and Samnaliev *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

[454–495 (476) vs. 261–461 (370)] and smaller pharynx [length: 17–22 (19) vs. 30–45 (39); width: 14–22 (17) vs. 22–39 (29)]; and from cercariae of *P. simillimum* by larger body [length: 243–314 (273) vs. 150–208 (178); width: 218–267 (239) vs. 92–135 (109)], larger oral [length: 56–75 (65) vs. 30–48 (38); width: 52–71(63) vs. 30–45 (37)] and ventral suckers [length: 68–82 (72) vs. 25–40 (32); width: 58–82 (71) vs. 28–40 (31)] and larger tail [length: 454–495 (476) vs. 288–350 (333); width: 72–82 (76) vs. 38–55 (46)]. Cercariae of Psilostomidae gen. sp. 1 differ from *P. spiculigerum* described by Bykhovskaya-Pavlovskaya & Kulakova (1971) in having larger low limits for body width (218–267 vs. 165–249), tail length (454–495 vs. 388–499), tail width (72–82 vs. 52–78), smaller low and high limits for oral sucker width (52–71 vs. 65–78) and smaller pharynx [length: 17–22 vs. 44–49; width: 14–22 vs. 26–39]. It also differs from *P. spiculigerum* described by Samnaliev *et al.* (1977) by larger body [length: 243–314 (273) vs. 204–242 (221); width: 218–267 (239) vs. 130–155 (138)], larger oral [length: 56–75 (65) vs. 50–62 (53); width: 52–71 (63) vs. 56–62 (56)] and ventral suckers [length: 68–82 (72) vs. 34–50 (41); width: 58–82 (71) vs. 37–56 (45)], smaller pharynx [length: 17–22 (19) vs. 19–31 (23); width: 14–22 (17) vs. 25–31 (28)] and larger tail [length: 454–495 (476) vs. 324–342 (336); width: 72–82 (76) vs. 43–56 (49)]. The above comparisons demonstrate that Psilostomidae gen. sp. 1 may represent a species of the genus *Psilotrema*, but is not conspecific with *P. oligoon*, *P. simillimum* or *P. spiculigerum*. Further identification of Psilostomidae gen. sp. 1 and Psilostomidae gen. sp. 2 to the species level requires the sequences of the adults from the definitive hosts, which are mainly birds and mammals (Kostadinova, 2005).

Systematics

Superfamily: Monorchioidea Odhner, 1911
Lissorchiidae Magath, 1917
Asymphylodora Looss, 1899

Asymphylodora *sp.*

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 *Perccottus glenii* Dybowski, 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, Rhabdiopoeidae and Labicolidae were used as the outgroup based on the topologies in the phylogenetic tree of the Digenea 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 notocotylid 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 *Catatropis* 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 notocotylid 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.

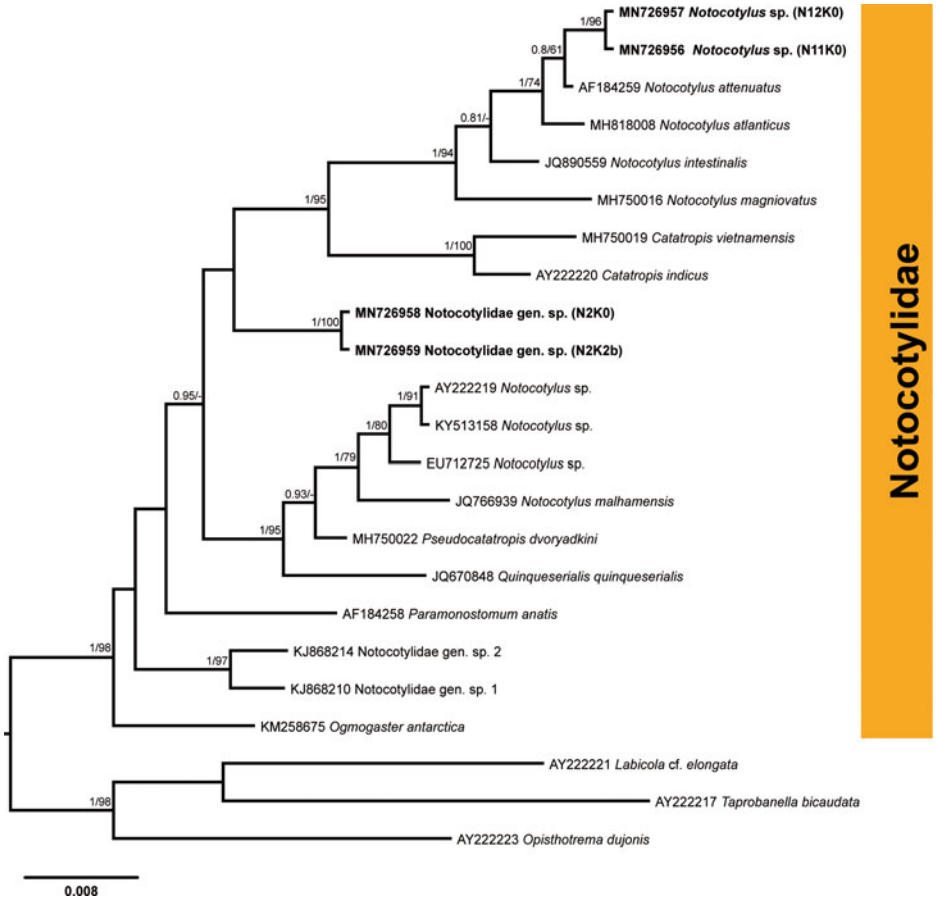

Fig. 6. Phylogenetic tree for Notocotylidae based on the partial sequences of the 28S rRNA gene. Numbers above branches indicate nodal support as posterior probabilities from the Bayesian inference (BI), followed by bootstrap values from the maximum likelihood (ML) analysis. Support values lower than 0.90 (BI) and 70 (ML) are not shown. The scale bar indicates the expected number of substitutions per site. The newly generated sequences are highlighted in bold.

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 *Sphaerostoma* sp. 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.

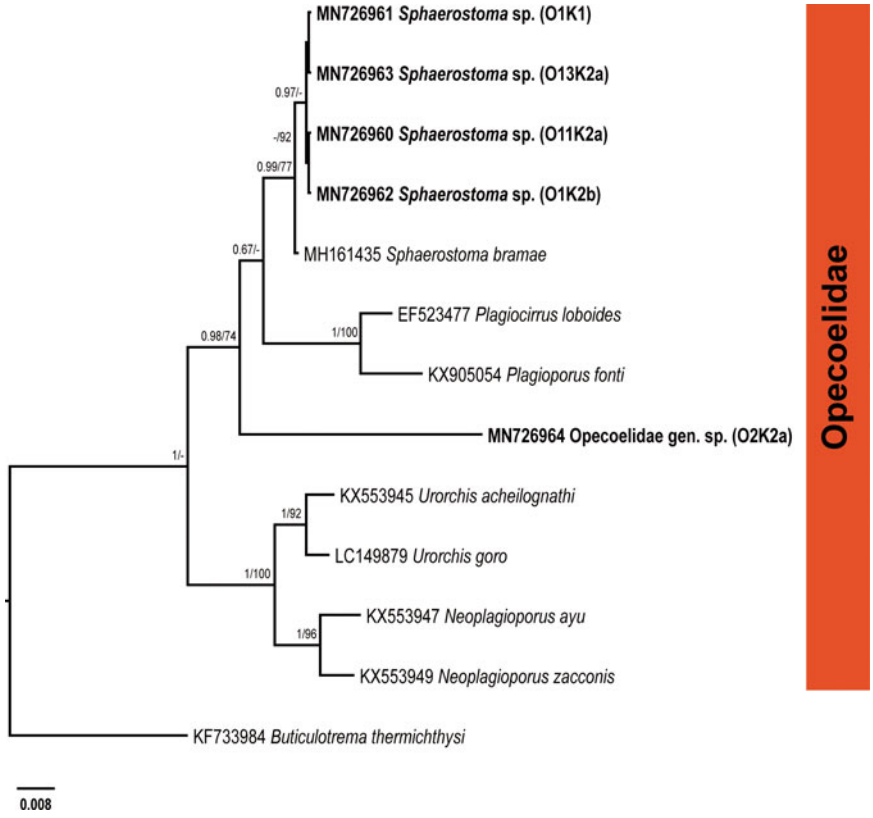

Fig. 7. Phylogenetic tree for Opecoelidae based on the partial sequences of the 28S rRNA gene. Numbers above branches indicate nodal support as posterior probabilities from the Bayesian inference (BI), followed by bootstrap values from the maximum likelihood (ML) analysis. Support values lower than 0.90 (BI) and 70 (ML) are not shown. The scale bar indicates the expected number of substitutions per site. The newly generated sequences are highlighted in bold.

Representative DNA sequences. 28S rDNA, four replicates (MN726960–MN726963); ITS2, four replicates (MN726988–MN726991).

Description

(Measurements from ten fixed specimens.) Microcercous cercariae (fig. 8a, b). Body colourless, elongate-oval, 207–285 × 110–132 (246 × 118), with maximum width at level just anterior to ventral sucker. Tegument thick, smooth. Tail reduced to small stump (cotylocercous), 22–42 (28) long with maximum width at base, 35–42 (40). Posterior half of the tail comprising glandular structures. Oral sucker large, subterminal, muscular, subspherical, 39–53 × 42–60 (49 × 50) armed with a small, simple stylet, 6–12 × 2–4 (8 × 3) dorsal to mouth opening. Ventral sucker subspherical, equatorial, 53–79 × 60–69 (68 × 64), larger than oral sucker, opening surrounded by one row of minute spines. Oral/ventral sucker width ratio 1:1.08–1.61 (1:1.39). Prepharynx long, pharynx distinct, elongate-oval, 20–25 × 15–19 (22 × 17). Caeca indistinct. Cystogenous gland-cells numerous, widespread throughout body. Four pairs of small penetration gland-cells, at level of prepharynx. Excretory vesicle broader anteriorly, heart-shaped.

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

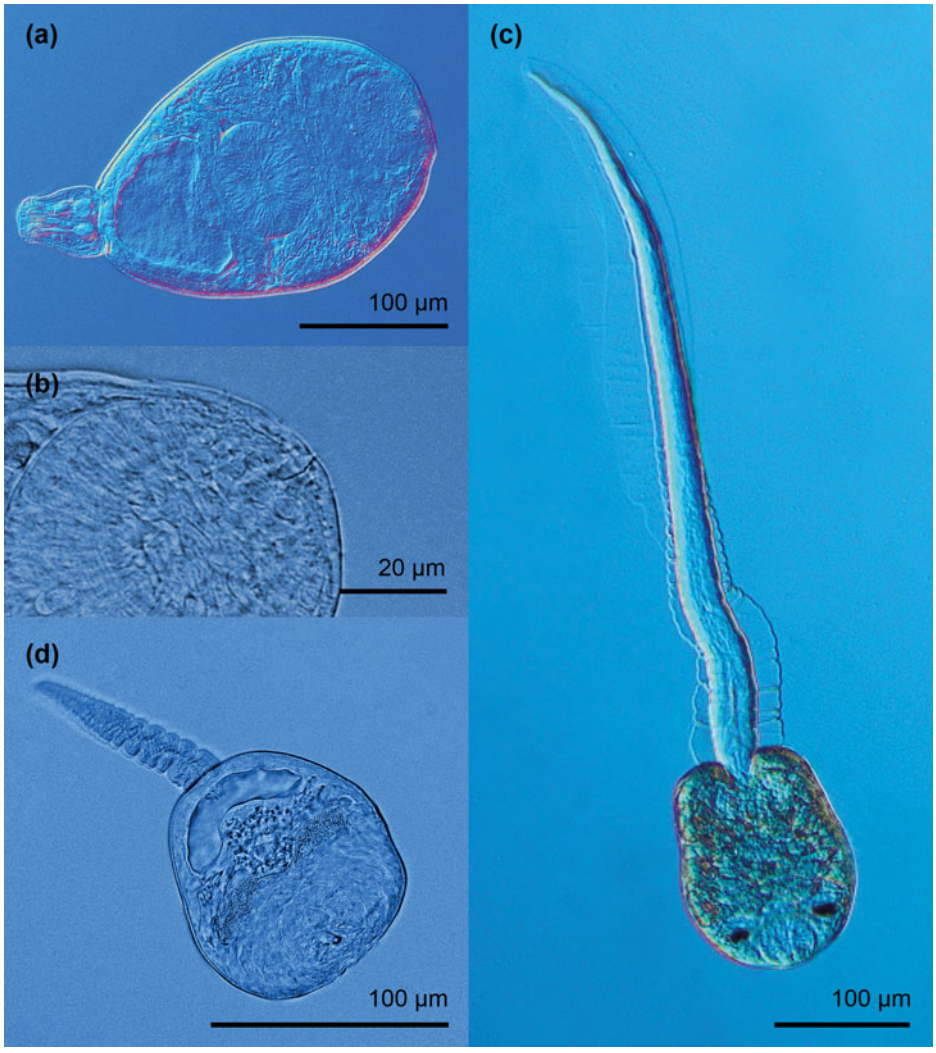

Fig. 8. Photomicrographs of live cercariae of the trematode families Opecoelidae, Opisthorchiidae and Lecithodendriidae. (a) *Sphaerostoma* sp.; (b) *Sphaerostoma* sp., stylet; (c) Opisthorchiidae gen. sp.; (d) *Lecithodendrium linstowi*.

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
Lecithodendriidae Lühe, 1901
Lecithodendrium 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 from *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

(Measurements from 11 fixed specimens.) Xiphidiocercariae (fig. 8d). Body colourless, elongate-oval, 118–148 × 67–85 (132 × 75). Tegument thin, covered with minute spines. Tail simple, 55–63 (64) long, with maximum width at base 16–23 (21), shorter than body. Tail/body length ratio 1:1.84–2.31 (1:2.06). Oral sucker subspherical, subterminal, muscular, 29–37 × 28–35 (33 × 30) armed with large stylet, 15–17 (16) long with maximum width at base 5–7 (6). Stylet with dilatation 1–2 (2). Virgula absent. Ventral sucker spherical, equatorial, 14–24 × 14–24 (19 × 18), smaller than oral sucker. Oral/ventral sucker width ratio 1:0.42–0.73 (1:0.58). Prepharynx indistinct, pharynx small, elongate-oval, 8–12 × 5–9 (10 × 7). Caeca indistinct. Penetration gland-cells three pairs, anterolateral to ventral sucker, filled with dark, granular secretory material. Excretory vesicle thin-walled, V-shaped.

Remarks

Digenean trematodes of the family Lecithodendriidae 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 cercariea 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

Molecular results

Infection with cercariae belonging to the families Pleurogenidae and Prosthogonomidae was detected in nine snails from three localities (prevalence: Pleurogenidae: River Lippe, 0.5%; River Rhine, 1.3%; Prosthogonomidae: 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 Microphallidae 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 Prosthogonomidae. The sequence of the isolate (PBK2b) 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 polyoon* (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 (PL11K2a and PL12K2a) 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 POK2a) 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 POK2a) clustered with the sequence of *P. ovatus* (KP192722) from *A. ferina* collected in the Czech Republic (Heneberg *et al.*, 2015). The four remaining isolates (PBK2b, PL2R1, PL11K2a and PL12K2a) identified as the members of

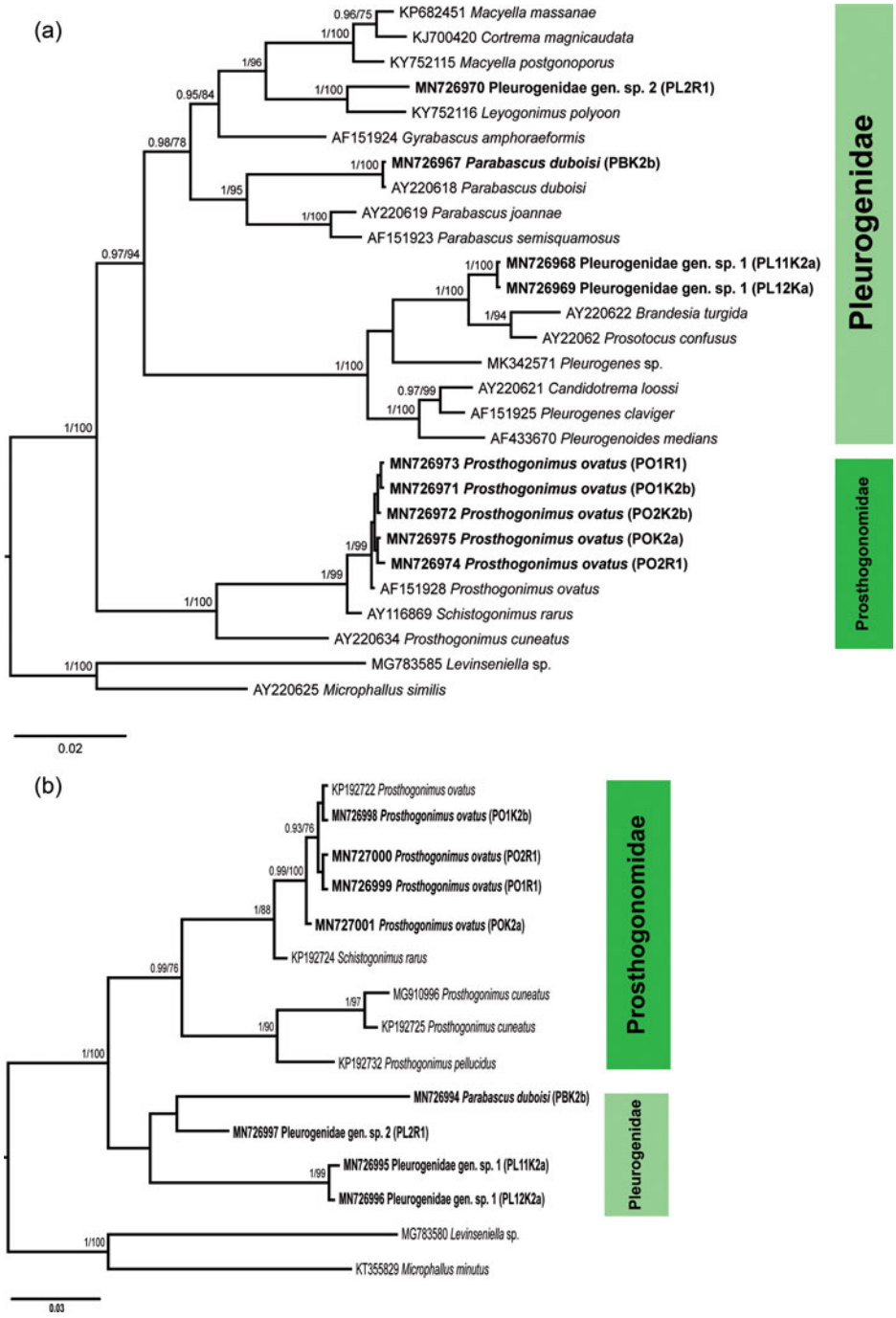

Fig. 9. Phylogenetic tree for Pleurogenidae and Prosthogonimidae based on the partial sequences of the 28S rRNA gene (a) and the internal transcribed spacer 2 (ITS2) region (b). Numbers above branches indicate nodal support as posterior probabilities from the Bayesian inference (BI), followed by bootstrap values from the maximum likelihood (ML) analysis. Support values lower than 0.90 (BI) and 70 (ML) are not shown. The scale bar indicates the expected number of substitutions per site. The newly generated sequences are highlighted in bold.

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).

Description

(Measurements from ten fixed specimens.) Virgulate xiphidiocercariae (fig. 10a, b). Body colourless, oval, 82–115 × 69–94 (92 × 82). Tail simple, 41–48 (45) long with maximum width at base 14–20 (17), longer than body. Tail/body length ratio 1:1.82–2.56 (1:2.04). Oral sucker subterminal, elongate-oval, 32–40 × 21–28 (37 × 25), armed with small stylet 14–20 (16) long with maximum width 3–5 (4). Stylet in anterior part of oral sucker, with anterior dilatation of blade. Small pyriform virgula organ in posterior part of oral sucker. Ventral sucker subspherical, 14–19 × 11–16 (16 × 14), smaller than oral sucker. Oral/ventral sucker width ratio 1:0.38–0.51 (1:0.43). Prepharynx long, pharynx spherical, 8–11 × 7–10 (9 × 8). Caeca indistinct. Few medium-sized fat inclusions in body parenchyma. Cystogenous gland-cells numerous, widespread throughout body. Four pairs of penetration gland-cells, anterolateral to ventral sucker. Excretory vesicle thin-walled, Y-shaped.

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).

Description

(Measurements from seven fixed specimens.) Virgulate xiphidiocercariae (fig. 10c, d). Body colourless, elongate oval, 120–154 × 77–109 (135 × 90). Tail simple, contractile, 77–150 (105) long with maximum width 21–27 (25), slightly longer than body. Body/tail length ratio 1:1.14–1.47 (1:1.10). Oral sucker subspherical, subterminal, 31–39 × 30–36 (35 × 33), armed with small stylet, 13–19 (16) long with maximum width 4–6 (5). Stylet in anterior part of oral sucker, with anterior dilatation of blade. Virgula organ large, bilobed, in posterior part of oral sucker. Ventral sucker subspherical, equatorial, 21–25 × 16–25 (23 × 21), smaller than oral sucker. Oral/ventral sucker width ratio 1:0.60–0.71 (1:0.66). Prepharynx long, pharynx distinct, close to virgula organ, oval, 10–12 × 12–16 (11 × 14). Caeca indistinct. Numerous small fat inclusions in body parenchyma. Penetration gland-cells four pairs, posterolateral to ventral sucker. Excretory vesicle thin-walled, Y-shaped.

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 (Ždárská, 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

Prosthogonimidae Lühe, 1909
Prosthogonimus Lühe, 1899

Prosthogonimus ovatus *Rudolphi, 1803*

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

Localities. River Lippe (K2, K3), River Rhine (R1), Germany.

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

Description

(Measurements from 11 fixed specimens.) Xiphidio cercariae (fig. 10e, f). Body colourless, elongate-oval, 104–126 × 60–79 (115 × 69). Tail simple, 57–86 (72) long with maximum width 19–26 (23), shorter than body. Body/tail length ratio 1:1.44–1.75 (1:1.60). Oral sucker subspherical, subterminal, 23–35 × 20–33 (30 × 29) armed with small stylet, 14–20 (18) long with maximum width 4–6 (5). Stylet in anterior part of oral sucker with thickening and incomplete wall at basis and anterior dilatation of blade. Ventral sucker subspherical, equatorial, 18–32 × 17–30 (22 × 20), smaller than oral sucker. Oral/ventral sucker width ratio 1:0.60–1.07 (1:0.73). Prepharynx indistinct. Pharynx spherical, muscular, 7–11 × 6–10 (9 × 8). Caeca indistinct. Four pairs of large, overlapping conspicuous penetration gland-cells anterior to ventral sucker. Excretory vesicle thin-walled, Y-shaped.

Remarks

Three species of *Prosthogonimus* – namely, *P. cuneatus*, *P. ovatus* and *Prosthogonimus* sp. – have been reported to parasitize *B. tentaculata* in Europe: in the Netherlands (Boddeke, 1960), Germany (Palm, 1966), Lithuania (Bykhovskaya-Pavlovskaya & Kulakova, 1971), Russia (Ginetsinskaya & Dobrovolskij, 1968; Sharpilo & Iskova, 1989; 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

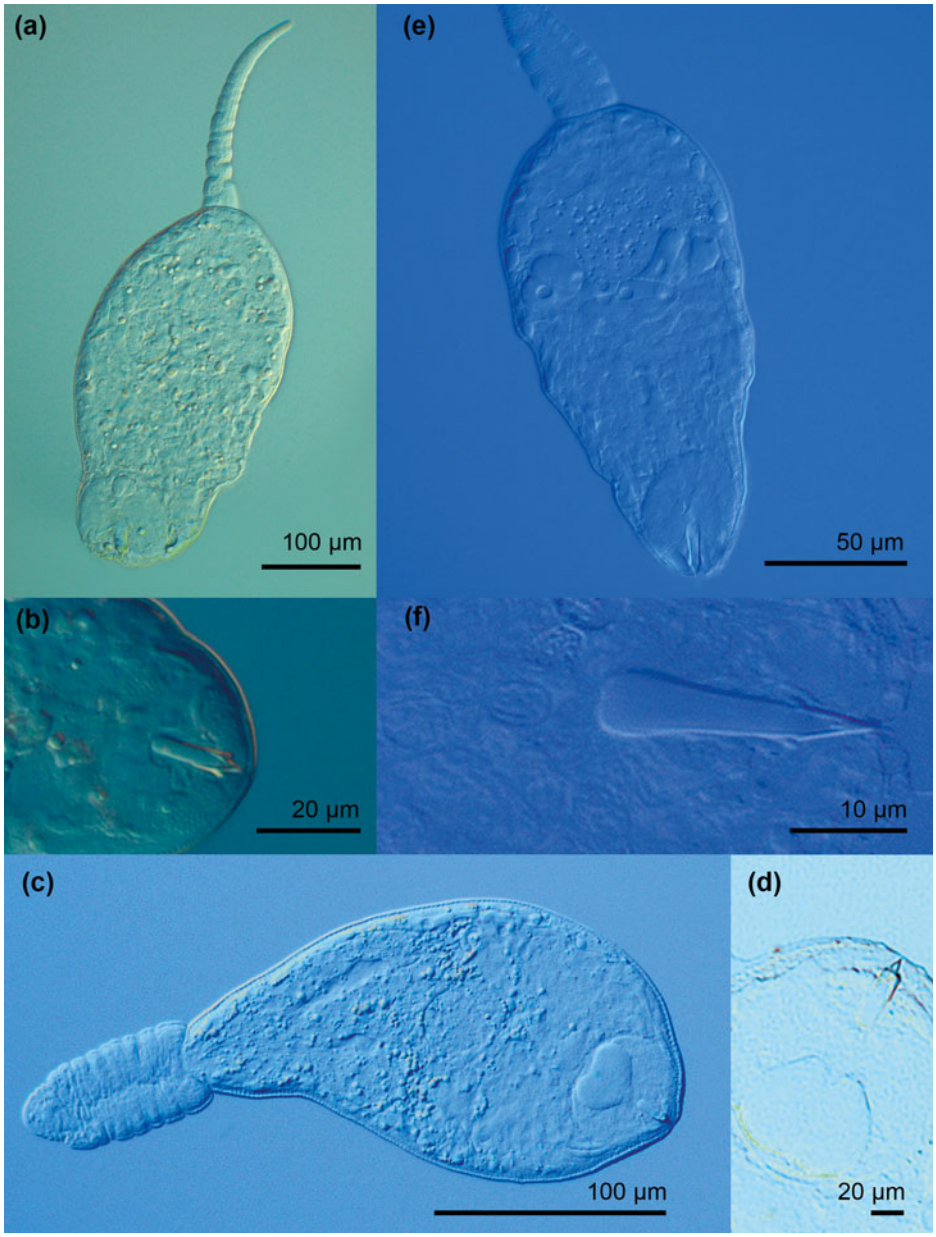

Fig. 10. Photomicrographs of live cercariae of the trematode families Pleurogenidae and Prosthogonomidae. (a) Pleurogenidae gen. sp. 1; (b) Pleurogenidae gen. sp. 1, stylet; (c) Pleurogenidae gen. sp. 2; (d) Pleurogenidae gen. sp. 2, virgula organ and stylet; (e) *Prosthogonimus ovatus*; (f) *P. ovatus*, stylet.

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

(Frolova, 1975; Karmanova, 1975), so our records constitute the first record for Central Europe.

Echinochasmus sp. 1 and Psilostomidae gen. sp. 1 are the only species that were both detected in Germany and Lithuania. The definitive hosts of echinochasmids and psilostimids 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 fulfils vital and central ecological functions, ranging from contributing to ecosystem diversity, structuring food webs or serving as ecosystem engineers (Thomas *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 (Sharpilo & 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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