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Discovery of adults of the gorgoderid trematode *Cercaria duplicata* with first morphological description, molecular identification and notes on host specificity

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Rhopalocercous *Cercaria duplicata* von Baer, 1827 develops in an intermediate host, the unionid bivalve *Anodonta anatina* (L.), but its adult form has been unknown. We examined eight fish species occurring in the presence of a highly infested population of *A. anatina* in the Zesławice reservoir (S Poland). Gravid *Phyllodistomum* specimens were obtained from the ureters of ide, *Leuciscus idus* (L.) and common rudd, *Scardinius erythrophthalmus* (L.). One of the rudd specimens was doubly infected, a trematode was also found in the urinary bladder. In addition, a gravid *Phyllodistomum* specimen was found in the ureter of a tench *Tinca tinca* (L.), caught in Lake Ilmėdas (Lithuania). In order to clarify the phylogenetic position of larval and adult gorgoderids and to establish their life cycle, ITS2 and 28S rDNA sequences were analysed. The analysis showed that adult *Phyllodistomum* specimens located in the ureters are conspecific with *C. duplicata*. The trematode found in the urinary bladder of *S. erythrophthalmus* was *P. folium* (Olfers, 1816). It is suggested that adult stages of *C. duplicata* should be referred to as *Phyllodistomum duplicatum* n. comb. The intercaecal position of the uterus and the deeply-lobed ovary are the main features distinguishing it from other *Phyllodistomum* species. Host specificity and ecology are discussed.

Keywords Fish parasite, Digenean trematode, *Phyllodistomum*, 28S rDNA, ITS2, Life cycle

Phyllodistomum Braun, 1899, a widespread genus of digeneans with a global distribution, comprises over 120 species, identified principally on the basis of morphological characters. New species are still being described using an integrative morphological and molecular approach[1–12]. GenBank (www.ncbi.nlm.nih.gov/genbank/) contains publicly available nucleotide sequences for somewhat more than 30 formally described *Phyllodistomum* species.

Considerable variation occurs in the life-cycles of *Phyllodistomum* species, which is related to the morphology and biology of their larval stages. One type of life-cycle known for *Phyllodistomum* spp. is that characterized by rhopalocercous cercariae (with a comparatively short, club-shaped tail but no stylet or pharynx) developing in sporocysts in the viscera of unionid bivalves. Rhopalocercous cercariae encyst soon after emergence from the bivalve host; the tail swells rapidly to form a spherical structure into which the cercarial body is quickly retracted, which, presumably, can then be directly eaten by the definitive host, a fish[13,14].

The only known species with such a life cycle in Europe is *Cercaria duplicata*, discovered and described by von Baer[15] probably somewhere in former Prussia (now Northeastern Poland, Kaliningrad Oblast and Western Lithuania). Molecular data based on 28S and ITS2 sequences and karyotype structure, 2n = 18, confirmed that *C. duplicata* was a member of Gorgoderinae[16]. Two genetic lineages were discovered in that study: 28S 1103

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bp length and 5.8S-ITS2-28S 425 bp length sequences of *C. duplicata* from Finland differed by 4 bp and 1 bp, respectively, from cercariae collected in Lithuania, Ukraine and Russia. The results of published experimental studies on the life cycle are contradictory, because different species of adult trematodes have been identified after experimental fish infections with *C. duplicata* under controlled conditions[17,18]. Moreover, molecular studies have revealed no match between *C. duplicata* and any species of *Phyllodistomum* investigated in comparative studies, including adults found in the experimental infections[9,19]. Ultimately, the correct affiliation of larval specimens of *C. duplicata* and recognition of its complete life cycle remain unresolved questions.

Cercaria duplicata has been found most frequently in the duck mussel, *Anodonta anatina* (L.) (Bivalvia: Unionidae), which acts as the first intermediate host. Some publications have reported it from *A. cygnea* (L.) in Italy[17], and Russia[20] and also from *A. cygnea*, *A. anatina* and *Unio* sp. from Ukraine (Fig. 1.)[21–23].

The prevalences of infection in previously studied populations of *A. anatina* were relatively low; for example, 5.4% in the River Slutch in Ukraine[24] and 7% in Lake Võrtsjärv in Estonia[25]. Our previous helminthological studies of bivalves[9,19] also revealed a low prevalence of *C. duplicata.* More than 100 individuals of *A. anatina* from each population were studied and the following data on the prevalence of infection were obtained (unpublished): 1.88% in Kaunas reservoir, Lithuania; 0.92 % in Lake Saraavesi, Finland; 0.76 % in the River Chesnava, Russia. In Poland, the prevalence of infection of different populations ranged from 0.2 to 8.6%[25–27].

Although *C. duplicata* has been recorded in *A. anatina* in various water bodies of Estonia, Finland, Lithuania, Poland, Ukraine and the European part of Russia (Fig.1)[9,19,24,25,27,28], its host *A. anatina* has a wider geographical distribution that includes almost all parts of Europe and parts of Asia[29,30]. Moreover, identification of definitive hosts is potentially important in explaining the geographic range of this trematode species; for example, the presence of *Phyllodistomum* cercariae in *A. anatina* from the Amu-Daria Delta[31] requires further detailed study, because it may be the first record of *C. duplicata* or of different rhopalocercous species of *Phyllodistomum* in Asia. Given the obvious knowledge gap and uncertainty surrounding this topic, there is a clear need to address these questions in order to aquire a better understanding of the species' ecology and patterns of host-parasite coevolution. These studies may also be useful in evaluating the impact of *C. duplicata* on intermediate host populations, because in some cases, parthenogenetically reproducing larvae of digeneans fill a gonad of a mussel, eventually causing its castration and, in consequence, decreasing the reproductive capacity of mussel populations[32,33].

Here, we present the results of a search for the adult stage of *C. duplicata* in natural definitive hosts in the Zesławice reservoir in Krakow, (S Poland), which is inhabited by a population of *A. anatina* heavily infected with *C. duplicata* (according to our unpublished data). This study also provides information on the definitive host from another location, Lake Ilmėdas in Lithuania.

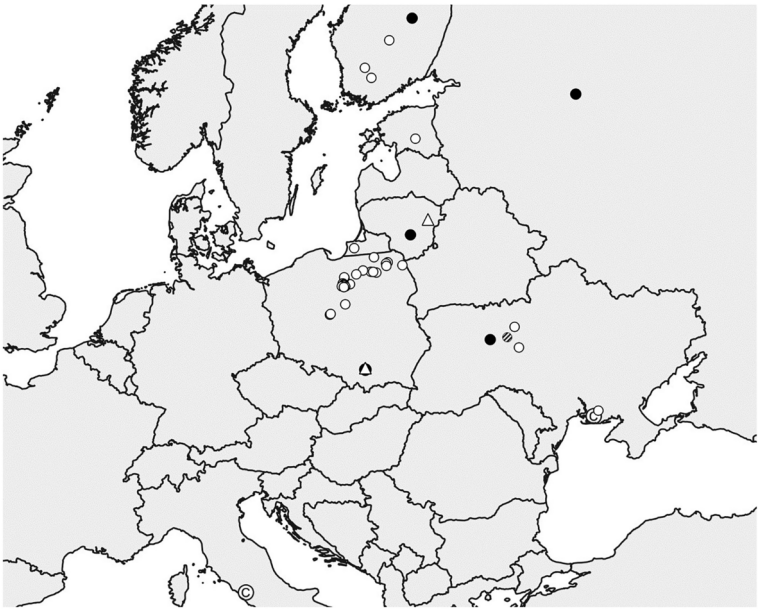

Fig. 1. Distribution map of *C. duplicata* and its adult stage locations: white circles – *C. duplicata* identified by morphology (found in *A. anatina*), black circles – *C. duplicata* identified by morphology and subjected to molecular studies (found in *A. anatina*), circle with a letter "C" inside – *C. duplicata* identified by morphology (found in *A. cygnea*), circle with lines inside – *C. duplicata* identified by morphology (found in *Unio* sp.), white triangles – adult stage distinguished with molecular characters (found in fish host). The base of the map: Lambert Azimuthal Equal Area projection; 1:14 000 000 map scale; based on a modified World Bank dataset licensed under CC-BY-SA 4.0

Results

Helminthological studies of the population of *A. anatina* living in the Zesławice reservoir, conducted in 2021 and 2022, revealed a varying prevalence of *C. duplicata* infection: in 2021, the prevalence was 6.52% (n=42), while in 2022 it was 11.90 % (n=46). Samples of *U. pictorum* (n=35), were completely free of gorgoderids.

Infected bivalves had their gonads filled with sporocysts and did not produce their own gametes (Fig. 2): the parasite led to the castration of the mussel host.

Nine mature *Phyllodistomum* sp. were obtained from the urinary system of four specimens of *S. erythrophthalmus* (prevalence 75%) and four worms were found in the ureters of two specimens of *L. idus* (prevalence 100%). All other fish collected at the same location on the same day were negative for gorgoderids. The partial sequences of 28S rDNA and ITS2 from adult and larval samples were analysed. One gravid specimen, identical to the genetic lineage of *C. duplicata* from Lithuania, Ukraine and Russia[19], was retrieved from the ureter of *T. tinca*, caught in Lake Ilmėdas, Lithuania. The sequences of the adult *Phyllodistomum* sp. from ureters of common rudd and ide were identical to the sequences of *C. duplicata* from that study; the 28S analysis revealed no difference between those larval samples and *C. duplicata* from Lithuania, Ukraine and Russia. The ITS2 of *C. duplicata* from Poland is identical to *C. duplicata* from Finland and differs by 1bp from the ITS2 rDNA of samples collected in Lithuania, Ukraine and Russia. In one case of double infection, a specimen recovered from the urinary bladder of one common rudd was genetically identical to *Phyllodistomum folium* specimens collected in Norway, Ukraine and Russia in our previous studies[19].

Phylogenetic analyses of ITS2 and 28S rDNA nucleotide sequences of Gorgoderinae were based on 368 and 797 positions in the final datasets, respectively. In the phylogenetic tree based on 28S rDNA, *P. duplicatum* formed a clade with the rhopalocercous cercaria from the unionid bivalves of North America, while *Phyllodistomum* species developing in Japanese unionids were phylogenetically more distant (Fig. 3). These results are consistent with the study by Gosho *et al.*[12]. The *P. duplicatum* clade appears to be sister to that of gorgoderids for which only cystocercous cercariae are known and the first intermediate hosts are exclusively species of Sphaeriidae.

Other gorgoderids were clustered into two distinct clades (Fig. 3). One of these comprises exclusivelly marine *Phyllodistomum* species from the Australasian region, including two sequences of larvae collected from venerid bivalves. The other, most diverse clade comprises *Pseudophyllodistomum* and marine *Xystretrum* species together with freshwater *Phyllodistomum* with different cercaria types and a wide range of bivalvia species as intermediate hosts, including Dreissenidae, Sphaeriidae, Unionidae and Corbiculidae. The branch of *P. cyprini* developing in Japanese unionids is the longest and, like a branch of *Phyllodistomum chauhani* from India, lacks bootstrap support with other groups in this clade. Also, besides the highly supported subclades of *Xystretrum* and *Pseudophyllodistomum,* there are two highly supported subclades of *Phyllodistomum* species. One of them comprises six species with unknown first intermediate hosts from the Neotropical and Paleotropical regions; the other consists of European species *P. pseudofolium* and *P. macrocotyle*, which have macrocercous cercariae developing in the sphaeriid clam *Pisidium amnicum* and microcercous cercariae developing in *Dreissena polymorpha*, respectively.

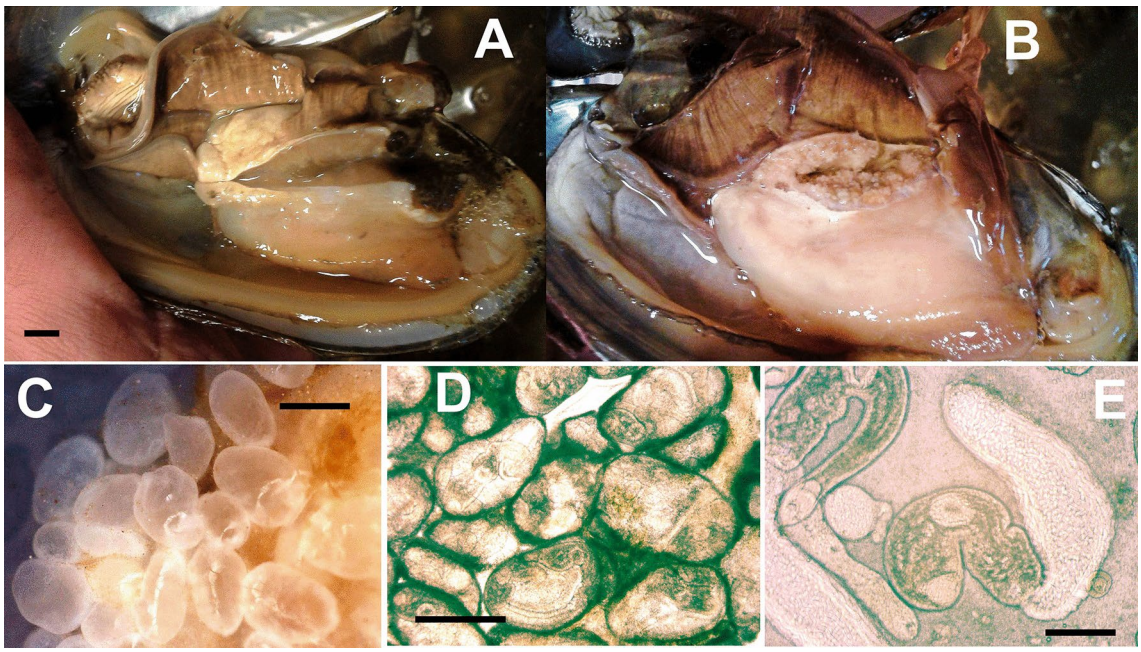

Fig. 2. Fresh specimens of *A. anatina* with: (**A**) dissected uninfected gonad, (**B**) dissected infected gonad full of sporocysts, scale bar 1 cm; (**C**) and (**D**) microphotographs of sporocysts, scale bar 500 µm, (**E**) *Cercaria duplicata*, scale bar 300 µm.

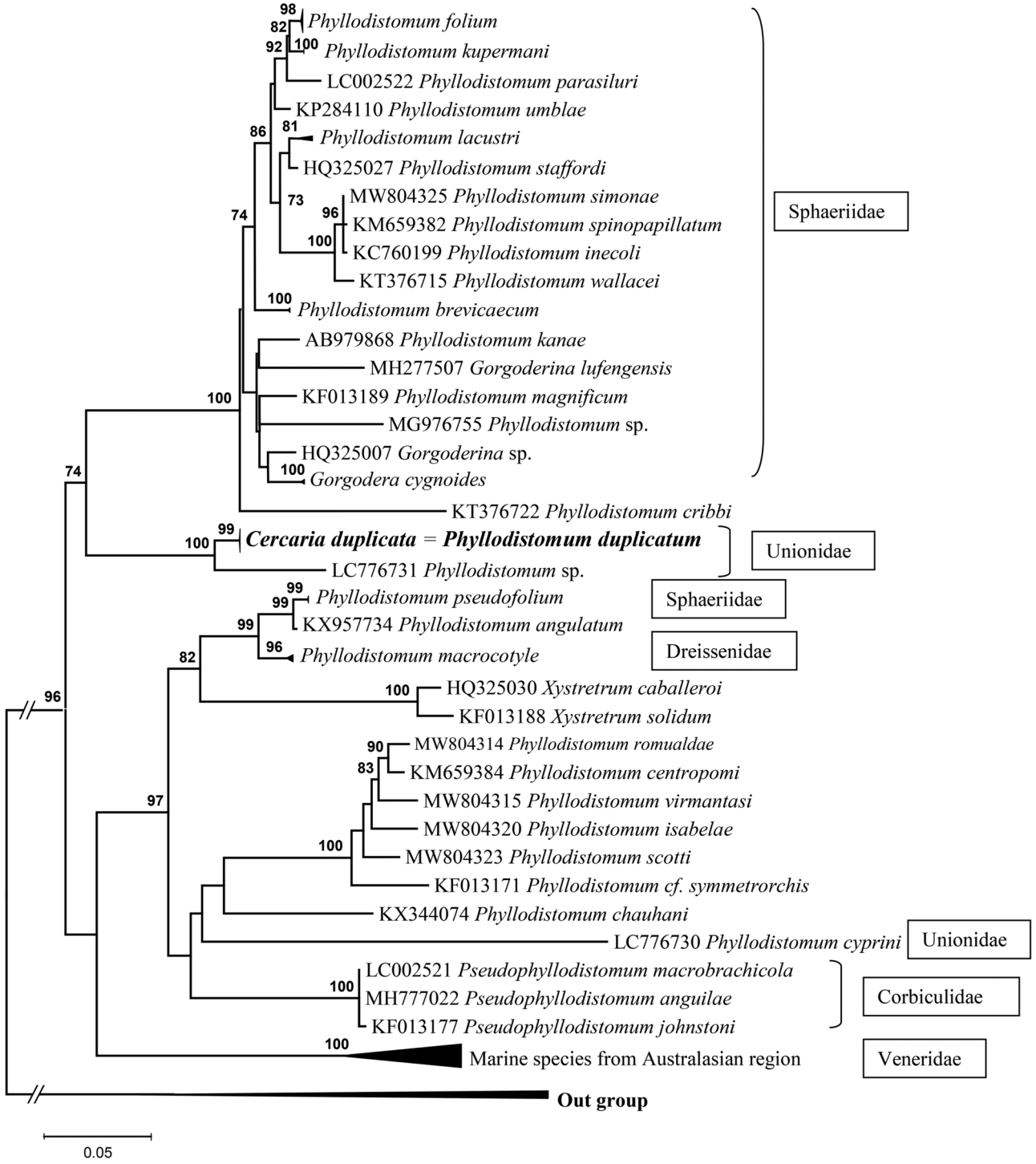

Fig. 3. Phylogenetic tree of gorgoderid species based on the Maximum Likelihood analysis of partial sequences of the 28S nuclear rDNA gene, with notes about first intermediate host taxa. Bootstrap support values lower than 70% are not shown. GenBank accession numbers of the collapsed clades are provided in Table 1.

Although there are significantly fewer data for ITS2 than for 28S, the topology of the ITS2 tree is identical to previously obtained molecular phylogenetic trees of gorgoderids, with the position of *P. duplicatum* being the same as in the 28S tree[9].

Morphological description

Family Gorgoderidae Looss, 1899

Genus *Phyllodistomum* Braun, 1899

Phyllodistomum duplicatum (von Baer, 1827) n. comb.

Syn.*Cercaria duplicata* von Baer 1827

Type-host: *Scardinius erythrophthalmus* (L.), (Cypriniformes: Cyprinidae).

Other hosts: *Leuciscus idus* (L.) (Cypriniformes: Cyprinidae), *Tinca tinca* (L.) (Cypriniformes: Tincidae).

Type-locality: Zesławice reservoir, Krakow, Poland.

Site in host: Ureters.

Voucher material: EKOI-252PL EKOI -253PL, EKOI-226LT are deposited in the Helminthological Collection of the Nature Research Centre, Lithuania.

Representative DNA sequences: 28S rDNA, PP061384 - PP061387, ITS1-ITS2 rDNA, PP082092 - PP082094, PP748525.

Description based on 4 gravid specimens; Fig. 4. Body elongate, lanceolate, 2400-3050 (2700) long, maximum width 690–920 (800) at level of anterior testis; lateral margins smooth. Forebody 900–1020 (950) long, representing 35% of total body length. Body length to width ratio 1:3.35. Oral sucker rounded, subterminal, 180–225 × 150–260 (200 × 210). Ventral sucker round or oval, approximately the same size as or slightly larger than oral sucker, 180–240 × 190–220 (210 × 220). Pharynx absent. Oesophagus straight, 190–220 (200) long. Intestinal bifurcation closer to oral than ventral sucker. Caeca simple, wide, blind, extending close to posterior end.

Gonads and vitelline masses located intercaecally, posterior to ventral sucker. Testes two, slightly lobed, oblique, in mid-body, not overlapping caeca; anterior testis 170–300 × 110–150 (230 × 130); posterior testis 160–260 × 125–180 (220 × 150). Seminal vesicle saccular (spherical), comparatively large 80–85 × 80–90 (84 × 82), dorsal to genital pore. Pars prostatica not observed. Genital pore median, close to anterior margin of ventral sucker.

Ovary irregular, lobed (3-4 lobes), dextral in all four specimens, slightly overlapping and ventral to vitellarium, 100–145 × 95–160 (120 × 135).

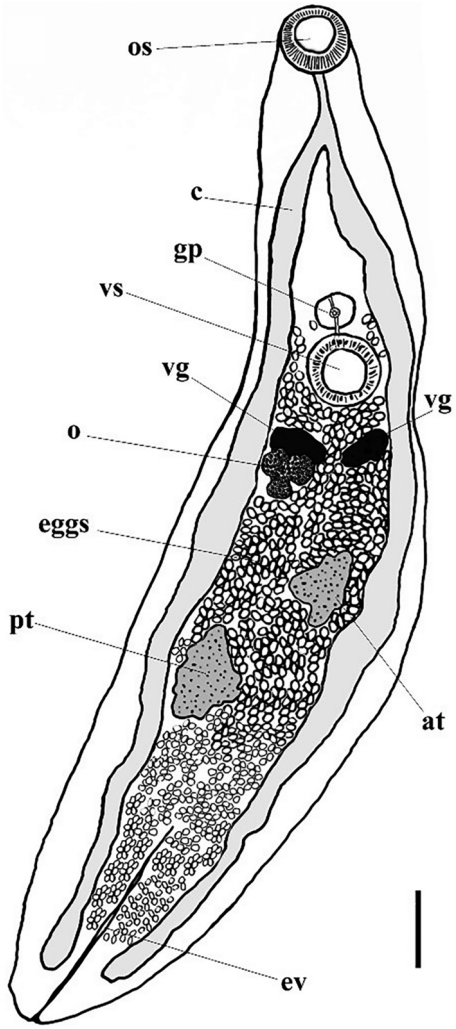

Fig. 4. *Phyllodistomum duplicatum* n. comb., ex. *Scardinius erythrophthalmus*: ventral view of adult specimen; os, oral sucker; c, caecum; gp, genital pore; vs, ventral sucker; vg, vitelline gland; o, ovary; at, anterior testis; pt, posterior testis; ev, excretory vesicle. Scale bar: 200 µm.

Vitelline masses two, strongly elongated in direction transverse to long axis of body, irregular, posterior to ventral sucker. Right vitelline mass 70–95 × 120–155 (85 × 145), left vitelline mass 60–75 × 120–160 (65 × 140).

Uterus extensively coiled, almost entirely intercaecal in hindbody. Eggs oval, 33–40 × 20–24 (35 × 21). Excretory vesicle tubular; anterior extent obscured by uterus in all specimens. Excretory pore terminal.

Remarks

Phyllodistomum duplicatum is only the second described species of the genus that has an intercaecal uterus. This condition is also characteristic of *P. cyprini* Feng et Wang, 1995, recovered from the ureters of *Cyprinus carpio* (L.) in Japan[12], but the ovary of *P. cyprini* is smooth and oval. Moreover, these species are phylogenetically distant. All other valid *Phyllodistomum* species are characterized by the distribution of the uterus in inter- and extracaecal space.

Morphologically, *Phyllodistomum duplicatum* closely resembles *P. macrocotyle* (Lühe, 1909) Odhner, 1911 (syn. *P. dogieli* Pigulevsky, 1953). Both parasitize the same fish host species, i.e. *S. erythrophthalmus* and *L. idus*, and have been isolated from the ureters rather than the urinary bladder, but the two species are not closely related in molecularly based phylogenies. *Phyllodistomum duplicatum* can be differentiated from *P. macrocotyle* by its intercaecal uterus, the relatively larger seminal vesicle, and the more elongated vitelline masses; the oral sucker to ventral sucker size ratio varies in the descriptions of *P. macrocotyle* by different authors. In the description according to Pigulevsky[34] and[35], the ventral sucker is twice as large as the oral sucker. In the recent redescription of *P. macrocotyle* the suckers were about the same size[9], as *P. duplicatum* suckers.

Phyllodistomum duplicatum has many morphological similarities to *P. elongatum* Nybelin, 1926: similar body shape, sucker sizes, genital pore near to ventral sucker margin, and lobed ovary. However, many reasonable doubts have arisen regarding the morphological descriptions of *P. elongatum* provided by different researchers. Specimens of different species were probably described under this name[34,36], but the position of the uterus in all descriptions is both intra- and inter-caecal. Reports incorporating molecular data have shown that *Phyllodistomum* specimens, recovered from ureters of fish hosts and morphologically resembling *P. elongatum*, may be representatives of multiple species[9,19]. Due to the morphological plasticity of *Phyllodistomum* specimens located in ureters, they tend to have an elongated body shape and resemble '*P. elongatum*'. In our previous studies, *P. elongatum*-like trematodes isolated from the ureters of European cyprinid fish (*Abramis bramae*, *S. erythrophthalmus*, *L. idus*, *R. rutilus*) were identified by DNA analysis as *P. macrocotyle* or *P. folium,* suggesting that location in the fish excretory system leads to a similar elongated body shape and morphology[9].

Discussion

Despite the increasing availability of molecular data, there is still confusion and difficulty in determining the taxonomy of the genus *Phyllodistomum*, particularly in relating morphological species to genetic lineages. Most species of trematodes are only known from their adult stage, while some well-known larval (asexual) trematodes have been described from their intermediate hosts and have never been associated with any adult. However, a species cannot be fully understood without knowledge of its life-cycle. Different approaches, including morphological matching and ecological evidence, experimental infections, and molecular matching, have been used in life cycle studies. Recent comparative molecular analysis has shown that inferences based on morphological similarities or ecological evidence are often incorrect, and that even experimental data can be contradictory[6,19,37,38].

The host specificity of *P. duplicatum* differs for its adult and larval stages. According to our molecular studies, the larval stage of *P. duplicatum* is strictly host-specific to *A. anatina* (i.e. oioxeny also known in *P. cyprini, P. macrocotyle* and *P. pseudofolium*), while larval stages of other *Phyllodistomum* species exhibit much broader host specificity, reaching the genus or family level[6]. In view of the fact that in North America at least six different rhopalocercous gorgoderid cercariae with strict host specificity to the first intermediate host have been described[13,14], the identity of *C. duplicata* reported from *A. cygnea* should be verified by molecular methods, because it may be another species of rhopalocercous *Phyllodistomum* in Europe.

In this study, three fish species were identified as definitive hosts of *P. duplicatum*, suggesting that the host range includes relatively closely-related species from the families Leuciscinae and Tincidae (stenoxeny). However, it is still unclear whether other fish species may be suitable definitive hosts. The host specificity patterns of *P. duplicatum* are identical to those of the morphologically similar *P. macrocotyle*.

Phenotypic plasticity poses challenges in species recognition as it can obscure species identity. Adult trematodes that grow in different hosts can exhibit intraspecific, host-induced variation in morphology[6,39,40]. The morphology of *Phyllodistomum* species depends not only on the fish host species but also on the site within the host, principally the ureters or urinary bladder[41]. Specimens obtained from the ureters of the fish host have an elongated form and thus are "*P. elongatum* – like".

Urabe *et al.*[2] described rhopalocercous gorgoderid cercaria found in the unionid mussel *Nodularia douglasiae* (now *N. nipponensis* (Martens, 1877)[42]) from the Yodo River in Japan, along with immature phyllodistomes collected from the ureters of the common carp, *C. carpio*, from the same water body. Comparative molecular analysis confirmed the conspecificity of the two developmental stages. Based on their general morphology, this species does not correspond to any other species previously described in freshwater fish from Japan. The morphology of the immature trematodes resembles that of *P. elongatum* recorded in Europe, but molecular and morphological studies of gravid trematodes from ureters of *C. carpio* have revealed that this species is *P. cyprini*[12].

Our previous studies based on comparative sequence analysis showed that specimens of *Phyllodistomum* species recovered from the ureters and urinary bladder of *S. erythrophthalmus* represent two species, *P. macrocotyle* and *P. folium*, while *Phyllodistomum* from the ureters of *L. idus* proved to be *P. macrocotyle*[9]. It is

likely that *Phyllodistomum* species typically have a location preference in the urinary system of their definitive hosts.

Our phylogenetic analysis does not fully support the suggestions that Gorgoderinae species associated with distinct intermediate host groups represent distinct main clades in the species tree[43] or that the morphology of cercariae may be a more accurate indicator of the phylogenetic distinctions than the taxonomy of their molluscan hosts[12]. The present phylogenetic analyses confirm our previous observations[6,9] that the morphological or biological features of adult forms, as well as cercariae, do not provide a reliable basis for recognizing distinct phylogenetic units in Gorgoderinae. Indeed, in the 28S tree at least two main clades of Gorgoderinae species characterized by a single type of cercariae are associated with distinct first intermediate host groups. The main clade including *P. folium* consists of species for which only cystocercous cercariae are known to develop only in sphaeriid bivalves. Another significant group consists of *P. duplicatum* and rhopalocercous gorgoderid cercaria from North America, which are associated exclusively with unionid mussels. But the most diverse major clade includes species with different cercaria morphologies and distinct pattern of first intermediate host identity (Fig. 3). Thus, the phylogenetically closely-related *P. pseudofolium* and *P. macrocotyle* have morphologically very different macrocercous and microcercous cercariae that develop in the phylogenetically distant bivalves *Pisidium amnicum* and *Dreissena polymorpha*. The rhopalocercous cercaria of *P. cyprini* developing in unionid bivalves from the Yodo River (Japan)[2] is phylogenetically distant from *P. duplicatum*. Incidentally, Gosho et al.[12] stated that Urabe et al.[2] incorrectly assigned this cercaria to the rhopalocercous morphological group and that the morphology of cercariae resembles that of macrocercous cercariae, but no arguments were provided to support this decision. The rhopalocercous cercariae are characterized as follows: they are produced by sporocysts in the viscera of unionid bivalves; they have no stylet and only a short tail, and they use cystogenous cells surrounding the excretory bladder to encyst within their own tail soon after emergence from the bivalve[13,14]. The cercaria of *P. cyprini* meets all these morphological characteristics, but data regarding the metacercarial stage and its formation are not provided. Therefore, the assignment of *P. cyprini* cercaria to a group other than rhopalocerca cannot be accepted without further observations.

It is obvious that some phylogenetic groups of Gorgoderinae species are associated with distinct intermediate host groups and the morphology of cercariae, but these are not regularities common to all groups of phylogenetically closely-related *Phyllodistomum* species. It is worth noting that the first intermediate hosts are unknown for most *Phylodistomum* species, especially those of marine species, and no intermediate host is known for species from the Neotropical and Paleotropical regions.

Numerous populations of the unionids *A. anatina* and *U. pictorum* inhabit the Zesławice reservoir. Mussels were found along the whole surveyed shoreline (about 300 m along the west bank of the Zesławice II reservoir, which is about 13.5 % of its total shoreline length). Locally, the densities of both mussel species, co-occuring in equal measure, reached 10 specimens per 1 m^2.

Dreissenid and sphaeriid bivalves were not recorded in this reservoir. The majority of *S. erythrophthalmus* and *L. idus* examined were infected with *P. duplicatum*, suggesting a high prevalence of infection in these definitive hosts in the wild (unfortunately, only a small number of these fish were studied). However, during more than ten years of molecular - helminthological studies of fish in Lithuania, *P. duplicatum* was detected only in *T. tinca*, while during this study not a single infected *T. tinca* was found in the Zesławice reservoir. The three fish species that act as definitive hosts, i.e. *T. tinca, S. erythrophthalmus* and *L. idus,* belong to the order Cypriniformes and have distinct characteristics in terms of biology and ecological preferences[44,45]. Given the different preferences for either benthic or benthopelagic activity, the definitive host range of *P. duplicatum* cannot be explained solely on the basis of fish feeding behaviour. Two of them, *T. tinca* and *S. erythrophthalmus,* prefer slow-moving or stagnant waters like ponds and lakes, whereas *L. idus* is typically found in flowing waters such as rivers and streams. Benthopelagic *L. idus* and *S. erythrophthalmus,* feed on plankton, various aquatic and terrestrial animals and plant material. *Tinca tinca*, a bottom feeder, preys mainly on molluscs and other benthic animals. There is also a noticeable difference in the type of waterbodies which held definite hosts inspected during the study: both *L. idus* and *S. erythrophthalmus* were caught in a small artificial water body that was very shallow and eutrophic, whereas *T. tinca* was caught in a large oligotrophic, natural, glacial lake. Both waterbodies are of the inflow-outflow type.

This study corroborates the strict specificity of *P. duplicatum* to its first intermediate host *A. anatina* and identifies three final host species from two different families of fish. Further research is needed to understand the range and host specificity, ecology, behaviour, and population dynamics of *P. duplicatum* in its intermediate and definitive hosts. Conducting research on larger fish populations with a focus on ecology could provide valuable insights into these matters.

Materials and methods

Research sites

The biological material was collected at two sites (Fig. 1). One of them, the Zesławice dam reservoir (50° 6' 31.475" N, 20° 1' 50.064" E), was created in 1987 by damming the valley of the River Dłubnia, ca 8 km from its confluence with the River Vistula; it is surrounded by the suburbs of Kraków in southern Poland. The research was conducted in the eastern part of the reservoir (area = 11.3 ha, water depth rarely > 2.0 m). The bottom substrate consists solely of a thick layer of silt, fine organic sediment and detritus, ca 15 to 30 cm deep in the littoral zone[46,47]. The water transparency varies during the year; it is mostly dark and murky, with significant amounts of suspended matter, so that visibility is limited; in September 2023, the Secchi disc visibility was no more than 30 cm, which suggests eutrophication.

The second site where research material was obtained was the natural, glacial Lake Ilmėdas in the Labanoras Regional Park, a protected area, in eastern Lithuania (55°15'39.7"N 25°33'44.8"E). The lake is 83.6 ha in area, with maximum and average depths of 17.5 and 6.6 m, respectively. The lake is situated in a glacial trough with a

very winding shoreline and small islets in the western and central parts. The shores are mostly high and covered with forest, while in some places they are flat and swampy. On the other shores, there are meadows and cultivated fields. Ilmėdas is an inflow-outflow lake with a 17% water exchange rate; it belongs to the catchment area of the River Žeimena.

Mussel collection and examination

Two species of unionid mussels, *Unio pictorum* (L.) (painter's mussel) and *A. anatina* (duck mussel) were identified in the Zesławice reservoir. To examine the presence of trematodes in the mussels' gonads, 46 specimens of *A. anatina* were collected in January 2021, and 42 specimens of *A. anatina* in October 2022, when 35 specimens of the co-occurring *U. pictorum* were also collected.

The mussels were sampled close to the reservoir shore using bottom-scrapers (rakes with nets) and manually (through tactile means). The mussels were carefully placed in multiple 10-litre containers filled with fresh water and transported to the laboratory at the Institute of Nature Conservation, Polish Academy of Sciences, Kraków.

In the laboratory, the mussels were stored in pails (the water was aerated) and examined for the presence of trematodes in their gonads. Syringe puncture and microscope examination were employed, following the method described by Brian and Aldridge[48], utilizing a Leica S8 APO stereo microscope and a Phenix BMC100 light microscope at magnifications ranging from 40× to 400×. Heavily infected specimens were selected and dissected in order to obtain intra-molluscan stages for subsequent analysis.

Fish collection and manipulation

In October 2022, fish were caught in the littoral zone along a 300 m stretch of the shoreline using electrofishing techniques from an inflatable dinghy. An engine-powered EL62IIGI (Hans-Grassl) device, was used for this purpose. A maximum of four individuals per species were captured for subsequent dissection. To euthanise the fish, anaesthetics (MS-222, Sigma Aldrich) were administered through immersion in a buffered solution: a dosage of 250 mg/L to sedate the specimens and then 500 mg/L to euthanize them. Mortality was confirmed after 30 minutes by the onset of rigor mortis. The following species were analyzed: Cypriniformes: *Abramis brama* (L.) (3 specimens), *Rutilus rutilus* (L.) (2), *Scardinius erythrophthalmus* (L.) (4), *Leuciscus idus* (L.) (2), *Blicca bjoerkna* (L.) (1), *Tinca tinca* (4); Perciformes: *Perca fluviatilis* (L.) (4), *Sander lucioperca* (L.) (1). After being euthanized, the fish were immediately transported to the laboratory at the Kraków University of Agriculture and stored in a cooler set at 3°C for 20 hours before dissection could take place on the following day. These storage conditions were sufficient to prevent the material from freezing and to maintain the viability of most of the trematodes present in the fish.

Live *T. tinca* were caught with a fishing rod in Lake Ilmėdas in August 2018 and were dissected the next day at the Nature Research Centre, Vilnius, Lithuania.

Adult stages of trematode collection

Adult gorgoderids were collected from naturally infected fish. The urinary bladder and ureters of the fish were isolated and placed in saline solution (0.65% NaCl). They were carefully examined under a stereomicroscope. Mature adult worms were recovered from the ureters and urinary bladder of common rudd *S. erythrophthalmus*, as well as from the ureters of ide *L. idus* and tench *T. tinca*. Specimens for molecular analysis were fixed in absolute ethanol and stored in a freezer.

Five gravid specimens of *Phyllodistomum* from the ureters of the fish were used for light microscopy examination and molecular analysis: one live specimen from *T. tinca* and two live and two fixed specimens from *S. erythrophthalmus.* One specimen of *Phyllodistomum* from *L. idus* was used for molecular analysis. To prepare permanent slides, the flukes were rinsed in saline solution, fixed by pipetting into near-boiling saline, and preserved in 70% ethanol. The specimens were stained with alum carmine, dehydrated in ascending concentrations of ethanol, cleared in dimethyl phthalate and mounted in Canada balsam. All measurements are in micrometres (μm) and given as a range followed by the mean in parentheses.

Samples for the molecular analysis of rhopalocercous cercariae were obtained from three specimens of *A. anatina* collected at the Zesławice reservoir, at roughly the same time two days later.

The voucher specimens are deposited in the helminthological collection of P.B. Šivickis Laboratory of Parasitology, Nature Research Centre, Vilnius, Lithuania.

Molecular sequencing and phylogenetic analysis

Genomic DNA of adult specimens and larval trematodes was extracted from ethanol-fixed material according to the protocol of Stunžėnas[16,49]. Amplification and sequencing of the two rDNA markers, the nuclear internal transcribed spacers (ITS1-5.8S-ITS2 rDNA) and the beginning of the large (28S) ribosomal subunit RNA coding regions, were performed following the protocol used in our previous studies[16]. A primer pair designed for Gorgoderidae species[9], the forward primer GoJe-F (5′-CTTGCAATTGTTCCCCGTGA-3′) and the reverse primer GoJe-R (5′-CTGTTCACTCGCCGTTACTG-3′), were used to amplify part of the internal transcribed spacer 1 (ITS1), the complete 5.8S rDNA and ITS2. A fragment at the 5′ end of the 28S rRNA gene was amplified using the forward primers Digl2 (5′-AAG CAT ATC ACT AAG CGG-3′) or ZX-1 (5′-ACC CGC TGA ATT TAA GCA TAT-3′)[50] and the reverse primers L0 (5′-GCT ATC CTG AG (AG) GAA ACT TCG-3′)[51] or 1500R (5′-GCT ATC CTG AGG GAA ACT TCG-3′)[52,53]. Polymerase chain reaction (PCR) products were purified and sequenced by the Sanger sequencing method in both directions at Macrogen Europe (Amsterdam, The Netherlands) using PCR primers. Contiguous sequences were assembled using Sequencher 4.10.1 software (Gene Codes Corporation, Ann Arbor, USA). Estimates of mean evolutionary divergence over sequence pairs within and between groups were calculated using the MEGA v.11.0.11 programme[54]. Newly generated 28S and ITS2 sequences from larval intramolluscan and adult stages were compared and identical, similar and related

sequences for phylogenetic analyses were found by the "Basic Local Alignment Search Tool" (BLAST)[55]. Both the ITS2 and 28S datasets were aligned independently using ClustalW[56] with an open gap penalty of 15 and a gap extension penalty of 6.66. The maximum likelihood (ML) trees were obtained using the general time reversible model with a gamma distribution rate (GTR + G) for both the ITS2 and the 28S gene datasets. The value for gamma and the number of invariant sites were estimated from the data. Parsimony analysis based on subtree pruning and regrafting (SPR) was used with default parsimony settings. Branch support was estimated by bootstrap analyses with 1000 pseudoreplicates. If two or more sequences belonged to one species, they were collapsed into one branch, except those newly obtained in this study. Furthermore, additional rDNA sequences of gorgoderid species and outgroup taxa were downloaded from GenBank and included in phylogenetic analysis (Table 1).

Ethical approval

The Polish Act relating to the Protection of Animals Used for Scientific and Educational Purposes does not apply to the euthanasia of animals when it is intended solely to obtain tissues or organs. An ethical review from the relevant Local Ethics Committee was therefore not required. The anaesthesia and euthanasia of the fish which provided tissues for examination was carried out in compliance with the American Veterinary Medical Association's (AVMA) Guidelines for the Euthanasia of Animals, and with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. This research was conducted in compliance with local regulations and legal requirements in the Republic of Poland and the Republic of Lithuania: for fishing with electric devices in the Zesławice reservoir, we obtained a permit from the Provincial Marshall's Office (RO-II.7143.1.7.2022), and also a letter with a permit from the Polish Angling Association (L.dz. GRW 400/1/2022). The fishing group was headed by a competent person (valid electrofishing certificate number 1825/14) and the capture caused no avoidable pain or distress to the fish. The tenches (*T. tinca*) from Lake Ilmėdas were caught by an angler possessing a valid license, and in accordance with the regulations and limits applicable in inland fisheries in the Republic of Lithuania. The study is reported in accordance with ARRIVE guidelines.

Species	Host	Locality	GenBank ID[a] [Reference]	
			28S	5.8S-ITS2-28S
Phyllodistomum angulatum	*Sander lucioperca*	River Chesnava, Russia		KJ740511, KJ740512[19]
P. angulatum	*S. lucioperca*	Rybinsk water reservoir on the Volga River, Russia	KX957734[19]	
Phyllodistomum brevicaecum	*Umbra limi*	Brokenhead, Manitoba, Canada	KC760207[57]	
Phyllodistomum brevicaecum	*U. limi*	Canada	HQ325008[58]	
Phyllodistomum centropomi	*Centropomus parallelus*	Tlacotalpan, Veracruz, Mexico	KM659384[4]	
Phyllodistomum chauhani	*Channa punctatus*	Uttar Pradesh, India	KX344074[59]	
Phyllodistomum cribbi	*Zoogoneticus quitzeoensis*	Mexico	KT376718[3]	
Phyllodistomum cyprini	*Cyprinus carpio*	Yodo River, Japan	LC776730[12]	
Phyllodistomum duplicatum n. comb. (= *C. duplicata*)	*Anodonta anatina*	Zesławice Reservoir, Poland	**PP061387**	**PP082092**
P. duplicatum	*Leuciscus idus*	Zesławice Reservoir, Poland	**PP061385**	**PP082094**
P. duplicatum	*Scardinius erythrophthalmus*	Zesławice Reservoir, Poland	**PP061386**	**PP082093**
P. duplicatum	*Tinca tinca*	Lake Ilmėdas, Lithuania	**PP061384**	**PP748525**
P. duplicatum (= *C. duplicata*)	*A. anatina*	Lake Saravesi, Finland	KJ729516[19]	KJ740490[19]
P. duplicatum (= *C. duplicata*)	*A. anatina*	Kaunas water Reservoir, Lithuania	KJ729515[19]	KJ740492[19]
P. duplicatum (= *C. duplicata*)	*A. anatina*	River Sluch, Ukraine	KJ729517[19]	KJ740489[19]
P. duplicatum (= *C. duplicata*)	*A. anatina*	River Chesnava, Russia	KJ729514[19]	KJ740491[19]
Phyllodistomum folium	*Esox lucius*	River Ild, Russia	KJ729542[19]	KJ740500[19]
P. folium	*Cottus gobio*	River Neris, Lithuania	KJ729550[19]	KJ740507[19]
P. folium	*Gasterosteus aculeatus*	River Vilnelė, Lithuania	AY277707[60]	AY277705[60]
P. folium	*Scardinius erythrophthalmus*	Zesławice Reservoir, Poland	**PP716808**	**PP718688**
P. folium	*Sphaerium corneum*	River Hegga, Norway	KJ729551[19]	KJ740495[19]
P. folium	*Pisidium supinum*	River Ūla, Lithuania	KJ729544[19]	KJ740496[19]
Phyllodistomum hoggettae	*Plectropomus leopardus*	Lizard Island, Queensland, Australia	KF013191[43]	KF013148[43]
Phyllodistomum hyporhamphi	*Hyporhamphus australis*	Australia	KF013190[43]	KF013150[43]
Phyllodistomum inecoli	*Heterandria bimaculata*	Agua Bendita, Xico, Veracruz, Mexico	KC760199[57]	
Phyllodistomum isabelae	*Maskaheros regansis*	Río Grande, Matías Romero, Oaxaca, Mexico	MW804320[10]	
Phyllodistomum kanae	*Hynobius retardatus*	Pippu, Hokkaido, Japan	AB979868[5]	
Phyllodistomum kupermani	*Perca fluviatilis*	Rybinsk water reservoir on the Volga River, Russia	MT875008, MT875009[9]	MT875012, MT875013[9]
Phyllodistomum lacustri	*Noturus flavus*	Canada	HQ325010[58]	
Phyllodistomum macrocotyle	*Dreissena polymorpha*	Lake Vilkokšnis, Lithuania	KJ729518[19]	KJ740518[19]
P. macrocotyle (= *P. folium* sensu Sinitsin, 1905)	*D. polymorpha*	Lake Lepelskoe, Belarus	AY288828[61]	AY288831[61]
P. macrocotyle (= *P. folium* sensu Sinitsin, 1905)	*D. polymorpha*	Lake Lukomskoe, Belarus	AY281127[61]	AF533015[61]
P. macrocotyle	*S. erythrophthalmus*	Rybinsk water reservoir on the Volga River, Russia	MT872664[9]	MT875011[9]
Phyllodistomum magnificum	*Tandanus tandanus*	Moggill Creek, Queensland, Australia	KF013189[43]	KF013153, KF013156[43]
Phyllodistomum pacificum	*Pantolabus radiatus*	Moreton Bay, Queensland, Australia	MG845599[7]	MG845601[7]
Phyllodistomum parasiluri	*Silurus asotus*	Japan	LC002522[5]	
Phyllodistomum pseudofolium	*Gymnocephalus cernuus*	River Chesnava, Russia	KX957732[6]	KY307875[6]
P. pseudofolium	*Pisidium amnicum*	River Chesnava, Russia		KJ740513[19]
P. pseudofolium (= *Phyllodistomum* sp. Ginetzinskaya (1959))	*P. amnicum*	Lithuania	AY281126[60]	
Phyllodistomum romualdae	*Archocentrus centrarchus*	Finca el Espavel, San Carlos, Nicaragua	MW804314[10]	
Phyllodistomum scotti	*Rhamdia nicaraguensis*	Finca El Espavel, San Carlos, Nicaragua	MW804323[10]	
Phyllodistomum simonae	*Profundulus labialis*	Río San Carlos, Chiapas, Mexico	MW804325[10]	
Phyllodistomum cf. *symmetrorchis*	*Clarias gariepinus*	Lake Victoria, Kenya	KF013171[43]	KF013162[43]
Phyllodistomum spinopapillatum	*Profundulus balsanus*	Rio PuebloViejo, Mexico	KM659382[4]	
Phyllodistomum staffordi	*Ameiurus melas*	Canada	HQ325027[58]	
Phyllodistomum wallacei	*Xenotaenia resolanae*	Mexico	KT376714[3]	
Phyllodistomum vaili	*Mulloidichthys flavolineatus*	Lizard Island, Queensland, Australia	KF013173[43]	KF013155[43]
Phyllodistomum virmantasi	*Gobiomorus dormitor*	Río La Palma, Veracruz, Mexico	W804315[10]	

Continued

Species	Host	Locality	GenBank ID[a] [Reference]	
			28S	5.8S-ITS2-28S
Phyllodistomum sp.	*Perca fluviatilis*	Rybinsk water reservoir on the Volga River, Russia	KY307869[6]	KY307886[6]
Phyllodistomum sp. 5	*Cephalopholis boenak*	Australia	KF013175[43]	
Phyllodistomum sp. 4	*Epibulus insidiator*	French Polynesia	KF013179[43]	
Phyllodistomum sp.	*Lampsilis siliquoidea*	USA	LC776731[12]	
Pseudophyllodistomum anguilae	*Siniperca scherzeri*	China	MH777022, Zhang,S. unpublished	
Pseudophyllodistomum johnstoni	*Macrobrachium australiense*	Warrill Creek, Queensland, Australia	KF013177[43]	KF013166[43]
Pseudophyllodistomum macrobrachicola	*Anguilla japonica*	Japan	LC002521[2]	
Gorgodera cygnoides	*Rana ridibunda*	Switzerland	AF151938[62]	
G. cygnoides	*R. ridibunda*	Kokaljane, near Sofia, Bulgaria	AY222264[52]	
Gorgodera amplicava	*Rana catesbeiana*	Nebraska, USA		FJ445743[63]
Gorgoderina attenuata	*Rana clamitans*	Nebraska, USA		FJ445741[63]
Gorgoderina simplex	*R. clamitans*	Nebraska, USA		FJ445742[63]
Gorgoderina lufengensis	*Nanorana yunnanensis*	China	MH277507 Ding,J. unpublished	MH257738, Ding,J. unpublished
Gorgoderidae larvae	*Lioconcha castrensis*	Australia Heron Island, Queensland, Australia	KF013172, KF013193[43]	KF013157, KF013169[43]
Xystretrum solidum	*Sphoeroides testudineus*	Conch Key, Florida, USA	KF013188[43]	KF013149[43]
Xystretrum caballeroi	*Balistes polylepis*	Mexico	HQ325030[58]	
Xystretrum sp.	*Sufflamen fraenatum*	Ningaloo, Western Australia, Australia		KF013160[43]
Outgroup				
Allocreadium crassum	*S. erythrophthalmus*	Lithuania: Lake Ilmėdas	OQ359133[38]	OQ359141[38]
Bucephalus polymorphus	*D. polymorpha*	Belarus	AY289248[61]	AY289239[61]
B. polymorphus	*P. fluviatilis*	Curonian Lagoon, Lithuania		JQ346725[16]
Nagmia floridensis	*Rhinoptera bonasus*	Gulf of Mexico, East Ship Island, Mississippi, USA	AY222262[52]	
Maritrema arenaria	*Semibalanus balanoides*	Belfast Lough, Northern Ireland		HM584171[64]

Table 1. Species subjected to molecular phylogenetic analysis with information of their host, locality and GenBank accession numbers. [a]Sequences generated in the present study are indicated in bold.

Data availability

All the authors declare that all the data supporting the results reported in our article have been included in this article.Dataset can be download at https://doi.org/https://doi.org/10.5281/zenodo.10335684. The datasets generated or analysed during this study are available in the GENE BANK repository, accession numbers: PP061384 - PP061387, PP082092 - PP082094, PP716808, PP718688, PP748525.

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Author contributions

K.Z., R.P., G.S. and V.S. put forward the idea of the project and its implementation plan; K.Z., E. Ł-T and J.D. organized and undertook the fieldwork to capture fish and unionid bivalves; K.Z. carried out the mussel dissections and took the microphotographs; R.P., G.S., and V.S. carried out the fish dissections, collected and fixed the helminths for further morphological and molecular analysis; R.P. and V.S. performed the morphological analysis of the adult helminths and prepared a morphological description and figure with the drawing of an adult; V.S. carried out the molecular phylogenetic analysis; R.P. and V.S. prepared the draft of the article; all the authors revised the manuscript critically for important intellectual content. All the authors read and approved the final manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

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