*diversity*

MDPI

*Article*

# Resurrection of *Diplostomum numericum* Niewiadomska, 1988 (Digenea, Diplostomatoidea: Diplostomidae) Based on Novel Molecular Data from the Type-Host

**Sergey G. Sokolov [1], Evgeny P. Ieshko [2] and Daria I. Lebedeva [2,*]**

[1] Alexey Nikolaevich Severtsov Institute of Ecology and Evolution, RAS, 119071 Moscow, Russia; sokolovsg@mail.ru
[2] Institute of Biology, Karelian Research Centre, RAS, 185000 Petrozavodsk, Russia; ieshkoep@gmail.com
* Correspondence: daryal78@gmail.com

**Abstract:** *Diplostomum* is a taxonomically problematic genus of trematodes, with many members harmful at the metacercarial stage to fish in aquaculture. We found metacercariae in the eye vitreous humor of the rudd, *Scardinius erythrophthalmus*; the bleak, *Alburnus alburnus*; and the roach, *Rutilus rutilus*, in Karelia (Russia) and identified them as *Diplostomum numericum* based on a combination of morphological and ecological data. Previously, *D. numericum* was thought to be conspecific with *Diplostomum gavium*. However, our phylogenetic analysis based on the sequences of the *cox*1 mitochondrial DNA gene did not support this hypothesis. We demonstrated the conspecificity of *D. numericum* with the larval trematode *Diplostomum* sp. Lineage 3 of Blasco-Costa et al. (2014) from the eye vitreous humor of European salmonid and cyprinid fishes. Therefore, we resurrected the species *D. numericum* and identified *Diplostomum* sp. Lineage 3 of Blasco-Costa et al. (2014) as *D. numericum*. Finally, we hypothesized that *D. numericum* was, in fact, conspecific with *Diplostomum colymbi* and that the latter was distinct from *D. gavium sensu stricto*.

**Keywords:** *Diplostomum gavium*; *Alburnus alburnus*; *Rutilus rutilus*; *Scardinius erythrophthalmus*; eye vitreous humor; cox1 mitochondrial DNA gene; molecular phylogeny

check for updates

**Citation:** Sokolov, S.G.; Ieshko, E.P.; Lebedeva, D.I. Resurrection of *Diplostomum numericum* Niewiadomska, 1988 (Digenea, Diplostomatoidea: Diplostomidae) Based on Novel Molecular Data from the Type-Host. *Diversity* **2023**, *15*, 840. https://doi.org/10.3390/d15070840

Academic Editors: José Luis Luque, Philippe Vieira Alves and Simon Blanchet

Received: 25 May 2023
Revised: 27 June 2023
Accepted: 7 July 2023
Published: 9 July 2023

**Copyright:** © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

## 1. Introduction

After the 1832 proposal of the genus *Diplostomum* Nordmann by the Russian naturalist Alexander von Nordmann [1], numerous papers have been published on this taxon, covering a wide range of issues from the taxonomy of these parasites to their modulating influence on host behavior and their ecological role in aquatic ecosystems (e.g., [2–7]). Metacercariae of many *Diplostomum* spp. cause eye disease in fish (e.g., [8,9]). Intensive molecular phylogenetic studies of *Diplostomum* spp. in the last two decades have exposed the extensive species richness of these parasites (e.g., [10–25]). Unfortunately, all modern researchers, with few exceptions [15], in their taxonomic conclusions on Palearctic *Diplostomum* spp. adhere to the synonymy of species proposed by Niewiadomska [26] and lack a balanced approach to this author's criticism of Shigin's taxonomic ideas [4,5].

*Diplostomum numericum* Niewiadomska, 1988, described metacercariae from the eye vitreous humor and the eye fluid of the rudd, *Scardinius erythrophthalmus* (Linnaeus, 1758), the European perch, *Perca fluviatilis* Linnaeus, 1758, and the ruffe, *Gymnocephalus cernua* (Linnaeus, 1758), from Poland [27]. The type host of this species is the rudd; the type site of infection is the eye vitreous humor [27,28]. Later, Shigin and Stanislavetz [29] found *Diplostomum* metacercariae in the eye vitreous humor of the roach, *Rutilus rutilus* (Linnaeus, 1758), the bleak, *Alburnus alburnus* (Linnaeus, 1758), and the vendace, *Coregonus albula* (Linnaeus, 1758), from Lake Oster (Karelia, Russia). Being unaware of the study by Niewiadomska [27], Shigin and Stanislavetz [29] described these metacercariae as belonging to a new species, *Diplostomum vitreophilum* Shigin and Stanislavetz, 1989.

Soon after that discovery, Shigin [5] proposed to consider *D. numericum* and *D. vitreophilum* as junior synonyms of *Diplostomum gavium* (Guberlet, 1922) described based on adult specimens found in the common loon, *Gavia immer* (Brunnich, 1764), in Oklahoma, the USA [30]. Metacercariae of *D. gavium* were unknown at that time. The reasons for the synonymization proposed by Shigin [5] were the similarity of the metacercariae of *D. numericum* and *D. vitreophilum* in morphology and localization in the host body and the similarity of the adults experimentally reared in ducklings infected with *D. vitreophilum* metacercariae with the adults of *D. gavium*. Recently, Achatz et al. [24] determined the *cox*1 gene sequences of *D. gavium* adults from the type-host captured in North Dakota (USA). However, the relationship between *D. gavium* and *D. numericum* has not been tested by genetic methods because of the lack of molecular data for the latter species.

After Niewiadomska [27] and Shigin and Stanislavetz [29], *Diplostomum* spp. metacercariae have also been recorded in the eye vitreous humor of European fishes by Brady [31], Valtonen and Gibson [32], Behrmann-Godel [33], Georgieva et al. [15], Blasco-Costa et al. [17], Faltýnková et al. [25], Soldánová et al. [19], and Landeryou et al. [34]. In all cases, however, the authors identified these metacercariae as either *Diplostomum baeri* Dubois, 1937, *Diplostomum gasterostei* Williams, 1966; or *Diplostomum* sp. The phylogenetic analyses have shown that the metacercariae found by Behrmann-Godel [33], Georgieva et al. [15], Blasco-Costa et al. [17], Faltýnková et al. [25], Soldánová et al. [19], and Landeryou et al. [34] are distributed across two species-level lineages, *Diplostomum* sp. Lineage 3 of Blasco-Costa et al. (2014) and *Diplostomum* sp. Lineage 4 of Blasco-Costa et al. (2014), with most of them belonging to the former lineage [17,22,35]. The metacercariae of *Diplostomum* sp. Lineage 4 of Blasco-Costa et al., 2014 have been shown to be conspecific with *Diplostomum petromyzifluviatilis* (Diesing, 1850) [22]. As for the metacercariae of *Diplostomum* sp., Lineage 3 of Blasco-Costa et al. (2014), no nomenclatural proposals concerning them have been made so far.

The aim of this study was to elucidate the taxonomic status of *D. numericum* on the basis of morphological and molecular data.

## 2. Materials and Methods

### *2.1. Sample Collection and Morphological Study*

*Diplostomum* spp. metacercariae were isolated from the eye vitreous humor of the rudd, *S. erythrophthalmus*, the bleak, *A. alburnus*, and the roach, *R. rutilus*, from various water bodies in Karelia, Russia. Twelve specimens of the rudd were caught in Lake Vanchozero (64°04′ N, 32°49′ E) in June 2022. One specimen of the bleak was caught in Lake Koivas (64°48′ N, 31°00′ E) in June 2012 and one in Lake Kostomukshskoye (64°47′ N, 30°21′ E) in June 2018. Ten, four, one, and one specimens of the roach originated from Lake Gimoilampi (62°17′ N, 33°53′ E) in April 2023, Lake Onega near Kondopoga (62°16′ N, 34°31′ E) in June 2022, and Lake Koivas and Lake Kostomukshskoye in June 2012, respectively. The eyes of fish were dissected and examined under a stereomicroscope for the presence of *Diplostomum* spp. metacercariae. The parasites were counted and processed for morphological and/or phylogenetic analyses.

Metacercariae from the rudd and the roach intended for morphometric study were processed as suggested by Shigin [4]. Live metacercariae were transferred to acetocarmine for fixation and simultaneous staining, then dehydrated in the graded ethanol series, cleared in dimethyl phthalate, and mounted in Canada balsam. According to Sudarikov and Shigin [36], fixation of *Diplostomum* metacercariae with acetocarmine reduces the dispersion of morphometric features, which makes it preferable to other fixation options (using ethanol or formalin). Drawings were made with the aid of a camera lucida.

A few live metacercariae were fixed in 96% ethanol and later used for counts of the excretory bodies (only specimens from the rudd) and/or for the molecular study (specimens from all three host species). In order to count the number of excretory bodies, the three ethanol-fixed metacercariae from the rudd were placed in distilled water for 1 min and then transferred into a 0.5% silver nitrate solution for 2 min. They were then sequentially

transferred into a saline, 3% sodium thiosulfate solution, dehydrated in the graded ethanol series, cleared in dimethyl phthalate, and mounted in Canada balsam [36]. The excretory body count from metacercariae isolated from the roach (two trematode specimens from Lake Gimoilampi) was carried out on live specimens.

In the course of fixing metacercariae from the rudd with both acetocarmine and ethanol, we paid attention to the diversity of the metacercariae body shapes, which ranged from oval to elongate-oval. Both oval and elongate-oval trematode specimens from this host were used for all morphometric and molecular procedures. Metacercariae from the remaining fish species were relatively homogeneous in shape.

### *2.2. Molecular Data and Phylogenetic Analysis*

Genomic DNA was isolated from individual ethanol-fixed metacercariae using DNA-Extran kits (Synthol, Moscow, Russia). A total of fifteen specimens, six from the rudd (three oval and three elongate-oval metacercariae), two from the bleak, and seven from the roach (three trematode specimens from Lake Gimoilampi, 2 from Lake Onega, and 2 from Lake Kostomukshskoye and Lake Koivas), were studied molecularly. For each of these larvae, we amplified a fragment of the mtDNA *cox1* gene using the primers Cox1_schist_5′ (5′-TCT TTR GAT CAT AAG CG-3′) and Cox1_schist_3′ (5′-TAA TGC ATM GGA AAA AAA CA-3′) of Lockyer et al. (2003) [37].

The PCR assay was carried out in 20 µL of reaction mixture containing ready 5X ScreenMix (Evrogen, Moscow, Russia), 1.5 pmol of each primer, and 2 µL of DNA; an annealing temperature of 50 °C was used for amplification [37].

PCR products were purified using the Cleanup Standard Extraction Kit (Evrogen, Moscow, Russia) following the manufacturer's instructions and then sequenced directly with the automatic sequencing system ABI PRISM 3100-Avant (Applied Biosystems Inc., Foster City, CA, USA). Consensus sequences (1170 bp length) were assembled in MEGA v. 10 [38] and deposited in GenBank with accession numbers OQ214204–OQ214209 (metacercariae ex S. *erythrophthalmus*), OQ257342, OR044094 (metacercariae ex *A. alburnus*), and OR044091–OR044093, OR044095–OR044097 (metacercariae ex *R. rutilus*).

The identity of the newly generated sequences was checked with the Basic Local Alignment Search Tool (BLASTn) (www.ncbi.nih.gov/BLAST/, accessed on 15 January 2023 ). The novel sequences were aligned with those of 172 representatives of the genus *Diplostomum* (Table S1; [10,12,15,17–24,33–35,39–48]) in MEGA v. 10 [38] and trimmed to the shortest length, 384 nt. The nomenclature of *Diplostomum* spp. followed the latest studies [22–24,35]. The *cox1* sequence for *Tylodelphys clavata* (Nordmann, 1832) (JX986909) was used as an outgroup (Table S1; [15]).

To assess the phylogenetic relationships of the newly found *Diplostomum* metacercariae, we applied Bayesian inference analysis to the dataset. Prior to the analyses, the best-fitting GTR + G+ I model was identified with jModelTest v2.1.2 [49]. Bayesian inference analyses were conducted using MrBayes (v3.2.3) [50]. Markov chain Monte Carlo (MCMC) simulations were run for 3,000,000 generations, log-likelihood scores were plotted, and only the final 75% of trees were used to produce the consensus tree. Posterior probability was calculated to estimate nodal support. FigTree v1.4 [51] was used to visualize the tree. The genetic divergence (as pairwise distances) between the taxa was estimated with the bootstrap method (1000 replicates) and with a uniform nucleotide substitution (transition + transversion) rate using MEGA v. 10 [38].

## 3. Results

Taxonomy
Family Diplostomidae Poirier, 1886
Genus *Diplostomum* Nordmann, 1832
*Diplostomum numericum* Niewiadomska, 1988, metacercaria (Figures 1 and 2)

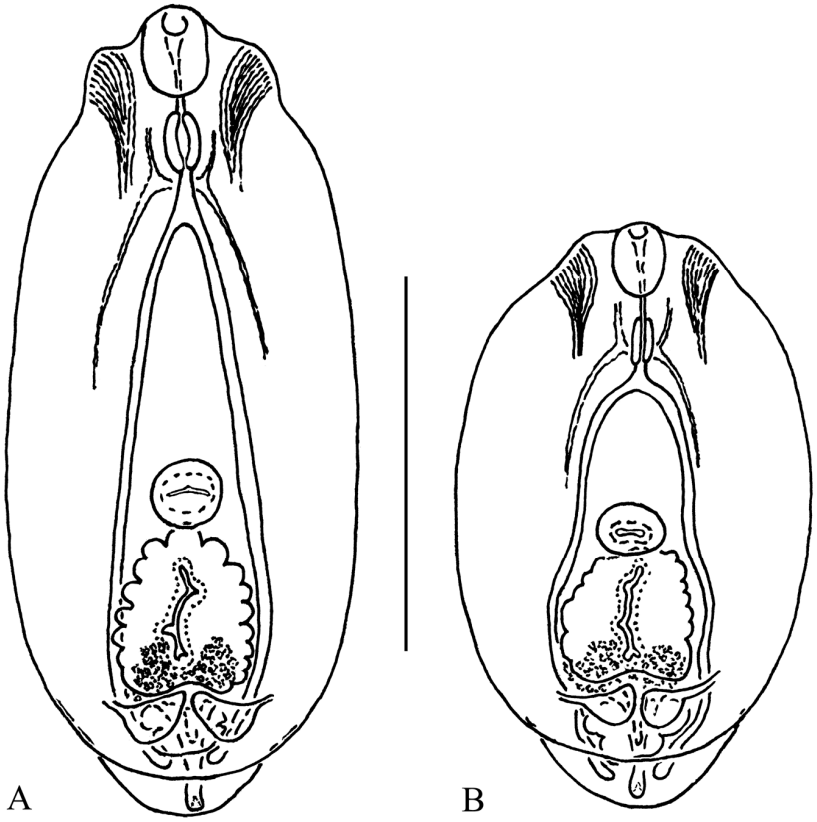


**Figure 1.** Metacercariae of *Diplostomum numericum* ex *Scardinius erythrophthalmus* from Lake Vanchozero, Karelia, Russia, with elongate-oval (***A***) and oval (***B***) body shapes at the same scale. Scale bar = 300 μm.

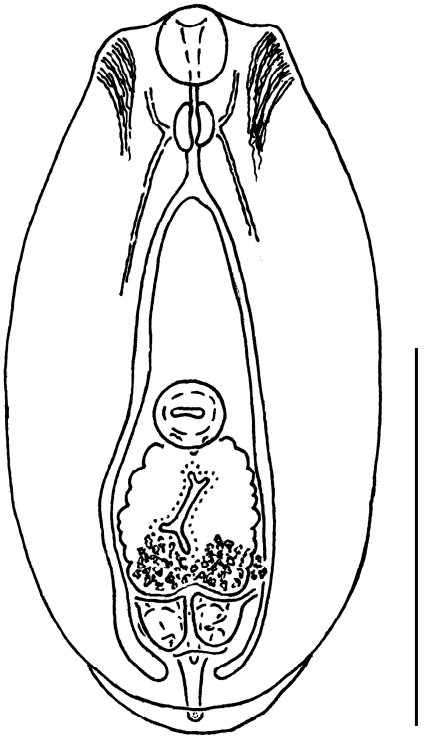

**Figure 2.** Metacercaria of *Diplostomum numericum* ex *Rutilus rutilus* from Lake Gimoilampi, Karelia, Russia. Scale bar = 300 μm.

**Synonyms:***Diplostomum* sp. Lineage 3 of Blasco-Costa et al., 2014; *Diplostomum baeri* 1 of Georgieva et al., 2013; *Diplostomum baeri* of Landeryou et al., 2020; *Diplostomum gavium* of Shigin, 1993, *part.*; *Diplostomum vitreophilum* Shigin & Stanislavetz, 1989.

**Type-host:** *Scardinius erythrophthalmus* (Linnaeus, 1758) (Actinopterygii, Cyprinidae).

**Other hosts:** metacercariae—*Alburnus alburnus* (Linnaeus, 1758), *Rutilus rutilus* (Linnaeus, 1758), *Gobio gobio* (Linnaeus, 1758) (Actinopterygii, Cyprinidae), *Salnelinus alpinus* (Linnaeus, 1758), *Salmo trutta* (Linnaeus, 1758), and *Coregonus albula* (Linnaeus, 1758) (Actinopterygii, Salmonidae); adults (only in experiment)—*Anas platyrhynchos domesticus* Linnaeus, 1758 (Aves, Anatidae).

Type-site of infection: Eye vitreous humor.

**Type-locality:** Lake Warniak, Poland.

**Other localities:** Lake Vanchozero (64°04′ N, 32°49′ E), Karelia, Russia (*S. erythrophthalmus*); Lake Gimoilampi (62°17′ N, 33°53′ E); Lake Koivas (64°48′ N, 31°00′ E); Lake Kostomukshskoye (64°47′ N, 30°21′ E); and Lake Onega (62°16′ N, 34°31′ E) in Karelia, Russia (*R. rutilus*); Lakes Koivas and Kostomukshskoye in Karelia, Russia (*A. alburnus*); as well as Iceland [17,25], Germany [15], Norway [19], Scotland [34], and other water bodies in Karelia, Russia [29].

**Specimens deposited:** Three slides with whole-mounted metacercariae from *S. erythrophthalmus* (IPEE RAS 14332–14334), paragenophores.

**Sequences deposited:** Six *cox*1 sequences, GenBank NCBI, as OQ214204–OQ214209 (ex *S. erythrophthalmus* from Lake Vanchozero); two as OQ257342 and OR044094 (ex *A. alburnus* from Lake Koivas and Lake Kostomukshskoye, respectively); three as OR044095–OR044097 (ex *R. rutilus* from Lake Gimoilampi); two as OR044093 and OR044096 (ex *R. rutilus* from Lake Koivas and Lake Kostomukshskoye, respectively); and two as OR044091 and OR044092 (ex *R. rutilus* from Lake Onega).

**Description** (based on 15 acetocarmine-stained paragenophores from *S. erythrophthalmus* and the same number from *R. rutilus*; measurements in Table 1): Body is oval to elongate-oval, distinctly bipartite, with a relatively large, concave prosoma and a small, conical opisthosoma. Oral sucker is muscular, longer than wide, and mouth subterminal. Pseudosuckers are two, well developed, lateral, on either side of the oral sucker, completely protruded or partly introverted. Ventral sucker muscles are muscular, transversely oval or rounded, and slightly post-equatorial. Holdfast organs are suboval or rounded, with a longitudinal fissure just posterior to the ventral sucker. Prepharynx distinct. Pharynx elongate-oval, muscular; oesophagus distinct, shorter than pharynx. Cerebral ganglia with suprapharyngeal commissure, posterior longitudinal ventral nerve cords, and anterior nerves clearly visible. Caeca long, terminating in opisthosoma. Proteolytic gland bilobed, dorsal to posterior end of holdfast organ. Gonad primordium posterior to the holdfast organ. The excretory vesicle is V-shaped and reaches to the holdfast organ.

**Table 1.** Measurements of *Diplostomum numericum* metacercariae.

| **Characters** | **Our Material; Range (Mean)** | | **Niewiadomska [27]; Range (Mean)** | **Shigin and Stanislavetz [29] [as *D. vitreophilum*]; Range (Mean)** |
|---|---|---|---|---|
| Host | *Scardinius erythrophthalmus* | *Rutilus rutilus* | *Scardinius erythrophthalmus* | *Rutilus rutilus*, **Coregonus albula*, **Alburnus alburnus* * |
| Total body length (BL), µm | 440–651 (545) | 543–644 (578) | 451–547 (512) | 465–587 (526) |
| Maximum body width (BW), µm | 242–336 (289) | 256–298 (280) | 244–288 (270) | 210–265 (239) |
| Oral sucker length (OL), µm | 52–69 (64) | 58–72 (64) | 51–68 (62) | 48–62 (56) |
| Oral sucker width (OW), µm | 45–55 (50) | 48–58 (52) | 40–54 (47) | 45–58 (51) |
| Pharynx length (PL), µm | 31–48 (40) | 34–52 (42) | 30–42 (37) | 28–40 (33) |
| Pharynx width (PB), µm | 22–31 (27) | 26–34 (32) | 17–30 (24) | 25–35 (29) |
| Ventral sucker length (VL), µm | 41–58 (49) | 50–58 (53) | 40–57 (47) | 43–51 (47) |
| Ventral sucker width (VW), µm | 52–62 (56) | 52–58 (56) | 40–64 (51) | 46–55 (51) |
| Distance from anterior margin of body to center of ventral sucker (O), µm | 242–402 (304) | 298–367 (322) | 234–306 (275) | – |
| Holdfast organ length (HL), µm | 107–144 (124) | 110–138 (120) | 81–119 (98) | 100–130 (112) |
| Holdfast organ width (HW), µm | 103–120 (112) | 103–120 (113) | 74–108 (87) | 92–135 (107) |
| Proteolytic gland length, µm | 28–55 (44) | 41–69 (54) | 23–40 (30) | – |
| Proteolytic gland width, µm | 76–110 (94) | 79–110 (97) | 68–102 (81) | – |
| Number of excretory bodies | 638, 659, 813 | 957, 1088 | ~ 700 | 760–1150 (965) |
| BW/BL, % | 42.62–61.54 (53.32) | 43.09–53.75 (48.52) | 40.67–58.42 (52.39) | 41.70–50.50 (45.50) |
| O/BL, % | 50.63–61.70 (55.61) | 52.49–58.33 (55.62) | 50.38–55.94 (53.74) | 51.30–56.60 (54.50) |
| [BL × BW]/[VL × VW] | 43.43–77.17 (57.69) | 45.05–63.27 (54.52) | 41.89–78.64 (58.40) | 43.20–58.90 (53.10) |
| [BL × BW]/[HL × HW] | 9.87–14.22 (11.38) | 10.54–13.21 (11.99) | 11.35–20.46 (16.51) | 8.37–12.89 (10.58) |
| [OL × OW]/[VL × VW] | 0.94–1.34 (1.15) | 0.96–1.31 (1.13) | 0.94–1.84 (1.22) | 1.06–1.39 (1.21) |
| [HL × HW]/[VL × VW] | 4.13–6.25 (5.10) | 3.76–5.47 (4.56) | 2.63–5.04 (3.63) | 3.89–7.02 (5.02) |
| [OL × OW]/[PL × PW] | 2.08–3.72 (2.94) | 1.90–3.36 (2.57) | 2.13–4.21 (3.22) | 2.29–3.79 (3.02) |

Note: * It is unclear whether Shigin and Stanislavetz (1989) measured metacercariae from the bleak and the vendace.

*Phylogenetic Analysis*

All 15 newly obtained sequences were grouped together with the sequences of the metacercariae identified previously as *Diplostomum* sp. Lineage 3 of Blasco-Costa et al. (2014) (=*Diplostomum baeri* 1 of Georgieva et al., 2013 and *D. baeri* of Landeryou et al., 2020) is a strongly supported clade (Figure 3). The p-distance between members of the clade was 0–1% for a sequence length of 388 nt. For the full length (1170 bp), p-distance ranged from 0 to 2%. Hereafter, we refer to this clade as *D. numericum*.

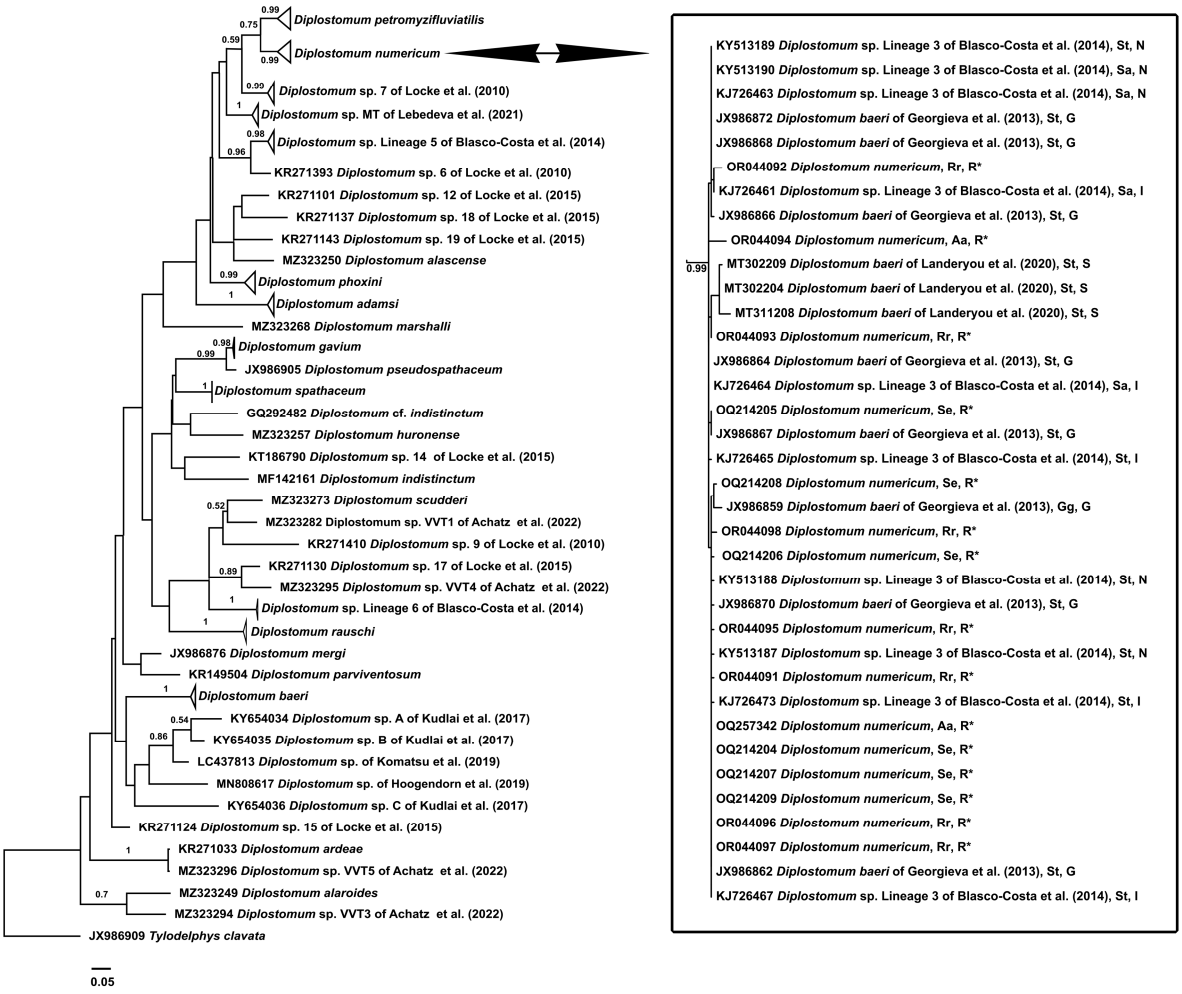


**Figure 3.** Phylogenetic tree of *Diplostomum* spp. based on partial *cox*1 (388 bp) inferred using Bayesian inference analysis. Posterior probability values lower than 0.5 are not shown. Newly obtained sequences are marked with an asterisk (*). Abbreviations: **Aa**—*Alburnus alburnus*; **Rr**—*Rutilus rutilus*; **Sa**—*Salvelinus alpinus*; **Se**—*Scardinius erythrophthalmus*; **St**—*Salmo trutta*; **Gg**—*Gobio gobio*; **I**—Iceland; **G**—Germany; **N**—Norway; **R**—Russia; **S**—Scotland.

*Diplostomum numericum* appeared as a poorly supported sister taxon to *D. petromyzifluviatilis*. The p-distance values between these species were 6%. The *D. numericum* + *D. petromyzifluviatilis* clade occupied a terminal position in the large group of *Diplostomum* spp. with brain or non-lens eye localization, which also contained *Diplostomum* sp. 7 of Locke et al., 2010, *Diplostomum* sp. of Lebedeva et al., 2021, *Diplostomum* sp. Lineage 5 of Blasco-Costa et al., 2014, *Diplostomum* sp. 6 of Locke et al., 2010, *Diplostomum* sp. 12 of Locke et al., 2015, *Diplostomum* sp. 18 of Locke et al., 2015, *Diplostomum* sp. 19 of Locke et al., 2015, *Diplostomum alascense* Dubois, 1969, *Diplostomum phoxini* (Faust, 1919), *Diplostomum adamsi* Lester & Huizinga, 1977 and *Diplostomum marshalli* Chandler, 1954 (Figure 3).

*Diplostomum gavium* occupied a well-supported sister position to *Diplostomum pseudospathaceum* Niewiadomska, 1984. The *D. gavium* + *D. pseudospathaceum* groups had no direct phylogenetic connections with the aforementioned large clade of *Diplostomum* spp. The p-distance values between *D. numericum* and *D. gavium* were 15%.

## 4. Discussion

The metacercariae from the rudd and the roach (Lake Gimoilampi) examined in our study are similar to each other in morphological and ecological (site of infection, cyprinid host) features, as well as molecularly. In turn, they are close to those of *D. numericum* and

*D. vitreophilum* in a number of the key characters, namely, the site of infection, the number of excretory bodies, and the mean and/or range values of the indexes describing the body proportions or organ ratios. (Table 1). However, the specimens in our study were slightly larger (in mean and maximum values) than the metacercariae described by Niewiadomska [27] and Shigin and Stanislavetz [29]. These authors fixed metacercariae using different techniques, specifically fixation in 75% ethanol of metacercariae killed by hot water [27] vs. fixation of live metacercariae in acetocarmine [29]. Different methods of fixation are known to affect the morphometric characteristics of *Diplostomum* metacercariae [31,36]. However, the differences in size indicated above do occur despite the identity of the techniques used by us and by Shigin and Stanislavetz [29].

Shigin [5] has suggested that *D. vitreophilum* is conspecific with *D. numericum* because they are very similar in general morphology and have an identical localization. In turn, as mentioned above, both *D. vitreophilum* and *D. numericum* were considered by this author to be synonymous with *D. gavium.* Niewiadomska [28] agreed with these conclusions. Our morphological and phylogenetic data confirm only the conspecificity of *D. vitreophilum* to *D. numericum and* allow us to identify all the specimens we examined, including those from the bleak and the roach (Lakes Koivas, Onega, and Kostomukshskoye), as *D. numericum.*

The molecular results of our study allow us to conclude with certainty that *D. numericum* is conspecific with the metacercariae of *Diplostomum* sp., Lineage 3 of Blasco-Costa et al. (2014) from the eye vitreous humor of European salmonid and cyprinid fishes. Therefore, we identify this lineage as *D. numericum*. Morphometric data on the metacercariae of this lineage from the brown trout, *Salmo trutta* Linnaeus, 1758, and the Arctic char, *Salvelinus alpinus* (Linnaeus, 1758) from Iceland were provided by Faltýnková et al. [25]. However, the measurements in that study were performed on live larvae and specimens fixed with 96% ethanol without pre-treatment by the standard staining, cleaning, and mounting techniques.Therefore, we cannot compare the morphometrics of specimens from Faltýnková et al. [25] with those examined in our study. It would be correct to compare them only in terms of the number of excretory bodies. Metacercariae of *Diplostomum* sp. Lineage 3 of Blasco-Costa et al. (2014) studied by Faltýnková et al. [25] had fewer excretory bodies than the specimens of *D. numericum* examined by us and by Shigin and Stanislavetz [29]: 450–600 vs. 638–1150. Combined with data published by Faltýnková et al. [25] and Lebedeva et al. [22] on metacercariae of *D. petromyzifluviatilis* (=*Diplostomum* sp. Lineage 4 of Blasco-Costa et al., 2014), these findings indicate considerable intraspecific variability in the number of excretory bodies in *Diplostomum* metacercariae and contradict Shigin's [4] notion of the high taxonomic importance of this trait for species identification in these parasites.

Niewiadomska [27] noted the great morphological similarity between the metacercariae of *D. numericum* and those of *Diplostomum baeri* of Niewiadomska, 1988 (=*Diplostomum volvens* of Shigin, 1986) from the retina, choroid, and eye fluid of percid, lotid, gasterosteid, salmonid, and, less frequently, cyprinid fishes. Shigin and Stanislavetz [29] report great similarities between *D. vitreophilum* (=*D. numericum*), *D. volvens* of Shigin, 1986, and *D. petromyzifluviatilis.* It is worth noting that *D. petromyzifluviatilis* is probably conspecific to *D. volvens* of Shigin, 1986. This hypothesis is supported by the similarities between metacercariae of *D. volvens* of Shigin, 1986, and *Diplostomum* sp. Lineage 4 of Blasco-Costa et al. (2014), in terms of localization and hosts, and recent data on the conspecificity of *D. petromyzifluviatilis* with *Diplostomum* sp. Lineage 4 of Blasco-Costa et al., 2014 [22]. At this point, however, we regard *D. volvens* of Shigin, 1986 and *D. petromyzifluviatilis* as separate species. Morphological differences between metacercariae of *D. numericum*, on the one hand, and metacercariae of *D. volvens* of Shigin, 1986, and *D. petromyzifluviatilis*, on the other hand, are the size of the ventral sucker compared to that of the body and the oral sucker: mean values of the [BL × BW]/[VL × VW] and [OL × OW]/[VL × VW] indexes are 53.10–58.40 vs. 37.20–45.26 and 1.13–1.22 vs. 0.98–1.05, respectively ([4,27,29], present data).

Of the metacercariae found in the eye vitreous humor of European fishes, morphological data are also available for *Diplostomum* sp. type 2 of Brady [31]. These larvae have

been described from the farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), from Scotland and, despite the perfunctory similarity to *D. numericum*, differ significantly from it in body size (302–397 × 155–262 µm vs. 440–651 × 210–336 µm). Brady [31] suggested that the metacercariae she described probably belonged to *Diplostomum mergi* Dubois, 1932. However, given the known facts on the confinement of *D. mergi* metacercariae to the lens [4], this taxonomic conclusion seems doubtful. The taxonomic status of *Diplostomum* sp. type 2 of Brady [31] remains unclear.

Niewiadomska ([27], Figure 3) noted the variability of the body shape of *D. numericum* metacercariae. Both elongate-oval and oval specimens were represented in her material. Our data, supported by the results of phylogenetic analysis, also demonstrate the variability of body shape in metacercariae of this species we isolated from the rudd (Figure 1). This is probably because both the previous author and we were dealing with a mixture of specimens at different stages of development (early and fully formed metacercariae). It is known that the morphology of *Diplostomum* metacercariae varies notably across development stages [4].

We can now reject the hypothesis that *D. numericum* is conspecific with *D. gavium* [5]. Our molecular genetic data showed that the p-distance values between *D. numericum* and *D. gavium* were 15%, which corresponds to a level of difference between species. In addition, *D. numericum* and *D. gavium sensu stricto* seem to be different ecologically at the main site within the host where their metacercariae reside. According to the molecular data of Achatz et al. [24], adults of *D. gavium sensu stricto* are conspecific with the metacercariae of *Diplostomum* sp. 3 of Moszczynska et al. (2009) from the eyes of various North American fishes and amphibians and the metacercariae of *Diplostomum* specimens from the northern hog sucker, *Hypentelium nigricans* (Lesueur, 1817), also from North America ([10–12,24,37,52]; Achatz, personal communication). In fish, a majority of metacercariae molecularly conspecific with *D. gavium sensu stricto* were found in the lens (for amphibians, the specific localization of these metacercariae within the eye was not indicated). Only two specimens of metacercariae of *D. gavium sensu stricto* have ever been found outside the lens in fish: one in an unspecified site in the brown bullhead *Ameurus nebulosus* (Lesueur, 1819) (D.J. Marcogliese and S. Locke, personal communications) and one in the eye vitreous humor in the northern hog sucker (T. Achatz, personal communication). Marcogliese and Locke [42] also identified metacercariae of *D. gavium sensu stricto* in the lens of the invasive round goby *Neogobius melanostomus* (Pallas, 1814) in North America.

However, it is noteworthy that the adult specimen experimentally reared by Shigin [5] from metacercaria of *D. vitreophilum* (=*D. numericum*) was similar in morphological form to *D. gavium* formed under conditions of high infection intensity in the final host (492 specimens per bird). Shigin [5] adhered to the hypothesis of Dubois and Rausch [53] about the conspecificity of *D. gavium sensu stricto* with *Diplostomum colymbi* (Dubois, 1928), described based on adult specimens from *Gavia arctica* (Linnaeus, 1758) in Switzerland. It cannot be ruled out that *D. numericum* is, in fact, conspecific with *D. colymbi* and that the latter is distinct from *D. gavium sensu stricto*. If so, it is likely that *D. gavium sensu stricto* is widespread in North America, while *D. colymbi* inhabits Eurasia. Molecular evidence is necessary to test this hypothesis. Nevertheless, at this point, the species *D. numericum* must be resurrected.

To date, metacercariae molecularly identified as *D. numericum* (=*Diplostomum* sp., Lineage 3 of Blasco-Costa et al., 2014) have been found only in the eye vitreous humor of European salmonids and cyprinids. Niewiadomska [27] reported the localization of some specimens of *D. numericum* metacercaria in the "eyeball outside eyelens" of European percid fishes. However, these data need molecular verification because it has already been proven that *Diplostomum* metacercariae localized in the eye vitreous humor of European percids are conspecific with *D. petromyzifluviatilis* [22,33].

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15070840/s1, Table S1: Summary data for cox1 sequences of *Diplostomum* spp. retrieved from GenBank.

**Author Contributions:** Conceptualization and morphological study—S.G.S.; writing—original draft preparation, S.G.S. and D.I.L.; sample collection, S.G.S. and E.P.I.; molecular and phylogenetic analyses, D.I.L.; writing—editing, S.G.S., D.I.L. and I E.P.I. All authors have read and agreed to the published version of the manuscript.

**Funding:** The study was funded by the Russian Ministry of Science and Higher Education: FFER-2021-0005 (SS) and FMEN-2022-0005 (DL, EI).

**Institutional Review Board Statement:** No applicable.

**Data Availability Statement:** The data presented in this study are available on request.

**Acknowledgments:** Special thanks to Olga Novokhatskaya (Unicaf University, Cyprus) for help with proofreading the text.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Nordmann, A.v. *Mikrographische Beiträge zur Naturgeschichte der Wirbellosen Thiere*; Zweites Heft. Mit zehn Kupfertafeln, G. Reimer: Berlin, Germany, 1832; pp. 1–150.
2. Dubois, G. Synopsis des Strigeidae et des Diplostomatidae (Trematoda). *Douxième Partie. Mem. Soc. Neuchl. Sci. Nat.* **1970**, *10*, 259–727.
3. Shigin, A.A. Trematodes of the genus *Diplostomum* in the biocenosis of the trout farm "Skhodnia". *Trudy Gel'mintologicheskoi Lab. Akad. Nauk SSSR* **1980**, *30*, 140–202.
4. Shigin, A.A. *Trematodes in the Fauna of the USSR: The Genus Diplostomum. Metacercariae*; Nauka: Moscow, Russia, 1986; p. 252.
5. Shigin, A.A. *Trematodes of the Fauna of Russia and Neighbouring Regions. Genus Diplostomum. Adults*; Nauka: Moscow, Russia, 1993; p. 207.
6. Mikheev, V.N.; Pasternak, A.F.; Taskinen, J.; Valtonen, E.T. Parasite-induced aggression and impaired contest ability in a fish host. *Parasites Vectors* **2010**, *3*, 17. [CrossRef]
7. Karvonen, A.; Marcogliese, D.J. Diplostomiasis (*Diplostomum spathaceum* and related species). In *Climate Change and Infectious Fish Diseases*; Woo, P.T.K., Leong, J.-A., Buchmann, K., Eds.; CAB International: Wallingford, UK, 2020; pp. 434–456.
8. Stables, J.N.; Chappell, L.H. The epidemiology of diplostomiasis in farmed rainbow trout from north-east Scotland. *Parasitology* **1986**, *92*, 699–710. [CrossRef] [PubMed]
9. Avsever, M.L.; Selver, M.M.; Yazicioğlu, Ö.; Tokşen, E.; Tay, S.; Erdal, G.; Günen, M.Z. The first report of diplostomiasis from cultured rainbow trout (*Oncorhynchus mykiss*) in Turkey. *Ank. Üniv. Vet. Fak. Derg.* **2016**, *63*, 377–381.
10. Locke, S.A.; McLaughlin, J.D.; Dayanandan, S.; Marcogliese, D.J. Diversity and specificity in Diplostomum spp. metacercariae in freshwater fishes revealed by cytochrome c oxidase I and internal transcribed spacer sequences. *Int. J. Parasitol.* **2010**, *40*, 333–343. [CrossRef]
11. Locke, S.A.; McLaughlin, J.D.; Marcogliese, D.J. DNA barcodes show cryptic diversity and a potential physiological basis for host specificity among Diplostomoidea (Platyhelminthes: Digenea) parasitizing freshwater fishes in the St. Lawrence River, Canada. *Mol. Ecol.* **2010**, *19*, 2813–2827. [CrossRef]
12. Locke, S.A.; Al-Nasiri, F.S.; Caffara, M.; Drago, F.; Kalbe, M.; Lapierre, A.R.; McLaughlin, J.D.; Nie, P.; Overstreet, R.M.; Souza, G.T.R.; et al. Diversity, specificity and speciation in larval Diplostomidae (Platyhelminthes: Digenea) in the eyes of freshwater fish, as revealed by DNA barcodes. *Int. J. Parasitol.* **2015**, *45*, 841–855. [CrossRef]
13. Locke, S.A.; Van Dam, A.; Caffara, M.; Pinto, H.A.; Blanard, C.A. Validity of the Diplostomoidea and Diplostomida (Digenea, Platyhelminthes) upheld in phylogenomic analysis. *Int. J. Parasitol.* **2018**, *48*, 1043–1059. [CrossRef]
14. Locke, S.A.; Drago, F.B.; Núñez, V.; Souza, G.T.R.E.; Takemoto, R.M. Phylogenetic position of *Diplostomum* spp. from New World herons based on complete mitogenomes, rDNA operons, and DNA barcodes, including a new species with partially elucidated life cycle. *Parasitol. Res.* **2020**, *119*, 2129–2137. [CrossRef]
15. Georgieva, S.; Soldánová, M.; Pérez-del-Olmo, A.; Dangel, R.D.; Sitko, J.; Sures, B.; Kostadinova, A. Molecular prospecting for European *Diplostomum* (Digenea: Diplostomidae) reveals cryptic diversity. *Int. J. Parasitol.* **2013**, *43*, 57–72. [CrossRef]
16. Chibwana, F.D.; Blasco-Costa, I.; Georgieva, S.; Hosea, K.M.; Nkwengulila, G.; Scholz, T.; Kostadinova, A. A first insight into the barcodes for African diplostomids (Digenea: Diplostomidae): Brain parasites in *Clarias gariepinus* (Siluriformes: Clariidae). *Infect. Genet. Evol.* **2013**, *17*, 62–70. [CrossRef] [PubMed]
17. Blasco-Costa, I.; Faltýnková, A.; Georgieva, S.; Skírnisson, K.; Scholz, T.; Kostadinova, A. Fish pathogens near the Arctic Circle: Molecular, morphological and ecological evidence for unexpected diversity of *Diplostomum* (Digenea: Diplostomidae) in Iceland. *Int. J. Parasitol.* **2014**, *44*, 703–715. [CrossRef] [PubMed]
18. Pérez-del-Olmo, A.; Georgieva, S.; Pula, H.J.; Kostadinova, A. Molecular and morphological evidence for three species of *Diplostomum* (Digenea: Diplostomidae), parasites of fishes and fish-eating birds in Spain. *Parasites Vectors* **2014**, *7*, 502. [CrossRef] [PubMed]

19. Soldánová, M.; Georgieva, S.; Rohacova, J.; Knudsen, R.; Kuhn, J.A.; Henriksen, E.H.; Siwertsson, A.; Shaw, J.C.; Kuris, A.M.; Amundsen, P.A.; et al. Molecular analyses reveal high species diversity of trematodes in a sub-Arctic lake. *Int. J. Parasitol.* **2017**, *47*, 327–345. [CrossRef]
20. Hoogendoorn, C.; Smit, N.J.; Kudlai, O. Resolution of the identity of three species of *Diplostomum* (Digenea: Diplostomidae) parasitising freshwater fishes in South Africa, combining molecular and morphological evidence. *Int. J. Parasitol. Parasites Wildl.* **2020**, *11*, 50–61. [CrossRef]
21. Lebedeva, D.I.; Chrisanfova, G.G.; Ieshko, E.P.; Guliaev, A.S.; Yakovleva, G.A.; Mendsaikhan, B.; Semyenova, S.K. Morphological and molecular differentiation of *Diplostomum* spp. metacercariae from brain of minnows (*Phoxinus phoxinus* L.) in four populations of northern Europe and East Asia. *Infect. Genet. Evol.* **2021**, *92*, 104911. [CrossRef]
22. Lebedeva, D.I.; Popov, I.Y.; Yakovleva, G.A.; Zaicev, D.O.; Bugmyrin, S.V.; Makhrov, A.A. No strict host specificity: Brain metacercariae *Diplostomum petromyzifluviatilis* Müller (Diesing, 1850) are conspecific with *Diplostomum* sp. Lineage 4 of Blasco-Costa et al. (2014). *Parasitol. Int.* **2022**, *91*, 102654. [CrossRef]
23. Schwelm, J.; Georgieva, S.; Grabner, D.; Kostadinova, A.; Sures, B. Molecular and morphological characterisation of *Diplostomum phoxini* (Faust, 1918) with a revised classification and an updated nomenclature of the species-level lineages of *Diplostomum* (Digenea: Diplostomidae) sequenced worldwide. *Parasitology* **2021**, *148*, 1648–1664. [CrossRef]
24. Achatz, T.J.; Martens, J.R.; Kostadinova, A.; Pulis, E.E.; Orlofske, S.A.; Bell, J.A.; Fecchio, A.; Oyarzún-Ruiz, P.; Syrota, Y.Y.; Tkach, V.V. Molecular phylogeny of *Diplostomum*, *Tylodelphys*, *Austrodiplostomum* and *Paralaria* (Digenea: Diplostomidae) necessitates systematic changes and reveals a history of evolutionary host switching events. *Int. J. Parasitol.* **2022**, *52*, 47–63. [CrossRef]
25. Faltýnková, A.; Georgieva, S.; Kostadinova, A.; Blasco-Costa, I.; Scholz, T.; Skírnisson, K. *Diplostomum* von Nordmann, 1832 (Digenea: Diplostomidae) in the sub-Arctic: Descriptions of the larval stages of six species discovered recently in Iceland. *Syst. Parasitol.* **2014**, *89*, 195–213. [CrossRef] [PubMed]
26. Niewiadomska, K. The genus *Diplostomum*—Taxonomy, morphology and biology. *Acta Parasitol.* **1996**, *41*, 55–66.
27. Niewiadomska, K. *Diplostomum* metacercariae (Digenea) in fish of the Dgal Wielki and Warniak lakes: *D. numericum* sp. n. and *D. baeri* Dubois, 1937, with comments on the synonymy of this species. *Acta Parasitol.* **1988**, *33*, 7–24.
28. Niewiadomska, K. Fauna słodkowodna Polski. 34A. In *Przywry (Trematoda).Część ogólna; Część systematyczna—Aspidogastrea, Digenea: Strigeida*; Wydawnictwo Uniwersytetu Łódzkiego: Łódź, Poland, 2010; p. 388.
29. Shigin, A.A.; Stanislavetz, A.N. On the species validity of metacercariae of the genus *Diplostomum* from the vitreous body of the eyes of freshwater fish. *Mater. Nauchnoi Conf. Vsesoyuznogo Obschestva Gel'mintolgov* **1989**, *38*, 253–259.
30. Guberlet, J.E. Three new species of Holostomidae. *J. Parasitol.* **1922**, *9*, 6–14. [CrossRef]
31. Brady, A. Studies on the taxonomy and biology of *Diplostomum* species (Digenea). Ph.D. Thesis, University of Stirling, Stirling, UK, 1989; p. 277.
32. Valtonen, E.T.; Gibson, D.I. Aspects of the Biology of Diplostomid Metacercarial (Digenea) Populations Occurring in Fishes in Different Localities of Northern Finland. *Ann. Zool. Fenn.* **1997**, *34*, 47–59. Available online: http://www.jstor.org/stable/23735430 (accessed on 1 June 2023).
33. Behrmann-Godel, J. Parasite identification, succession and infection pathways in perch fry (*Perca fluviatilis*): New insights through a combined morphological and genetic approach. *Parasitology* **2013**, *140*, 509–520. [CrossRef]
34. Landeryou, T.; Kett, S.M.; Ropiquet, A.; Wildeboer, D.; Lawton, S.P. Characterization of the complete mitochondrial genome of *Diplostomum baeri*. *Parasitol. Int.* **2020**, *79*, 102166. [CrossRef]
35. Faltýnková, A.; Kudlai, O.; Pantoja, C.; Yakovleva, G.; Lebedeva, D. Another plea for 'best practice' in molecular approaches to trematode systematics: *Diplostomum* sp. clade Q identified as *Diplostomum baeri* Dubois, 1937 in Europe. *Parasitology* **2022**, *149*, 503–518. [CrossRef]
36. Sudarikov, V.E.; Shigin, A.A. On the methods for working with metacercariae of the Strigeidida. *Trudy Gel'mintologicheskoi Lab. Akad. Nauk SSSR* **1965**, *15*, 158–166.
37. Lockyer, A.E.; Olson, P.D.; Stergaard, P.; Rollinson, D.; Johnston, D.A.; Attwood, S.W.; Southgate, V.R.; Horak, P.; Snyder, S.D.; Le, T.H.; et al. The phylogeny of the Schistosomatidae based on three genes with emphasis on the interrelationships of *Schistosoma* Weinland, 1858. *Parasitology* **2003**, *126*, 203–224. [CrossRef]
38. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef]
39. Ubels, J.L.; DeJong, R.J.; Hoolsema, B.; Wurzberger, A.; Nguyen, T.T.; Blankespoor, H.D.; Blankespoor, C.L. Impairment of retinal function in yellow perch (*Perca flavescens*) by *Diplostomum baeri* metacercariae. *Int. J. Parasitol.* **2018**, *7*, 171–179. [CrossRef] [PubMed]
40. Gordy, M.A.; Kish, L.; Tarrabain, M.; Hanington, P.C. A comprehensive survey of larval digenean trematodes and their snail hosts in central Alberta, Canada. *Parasitol. Res.* **2016**, *115*, 3867–3880. [CrossRef]
41. Gordy, M.A.; Hanington, P.C. A fine-scale phylogenetic assessment of digenean trematodes in central Alberta reveals we have yet to uncover their total diversity. *Ecol. Evol.* **2019**, *9*, 3153–3238. [CrossRef] [PubMed]
42. Marcogliese, D.J.; Locke, S.A. Infection of *Diplostomum* spp. in invasive round gobies in the St Lawrence River, Canada. *J. Helminthol.* **2021**, *95*, e64. [CrossRef] [PubMed]

43. Selbach, C.; Soldánová, M.; Georgieva, S.; Kostadinova, A.; Sures, B. Integrative taxonomic approach to the cryptic diversity of *Diplostomum* spp. in lymnaeid snails from Europe with a focus on the '*Diplostomum mergi*' species complex. *Parasites Vectors* **2015**, *8*, 300. [CrossRef]
44. Rahn, A.K.; Krassmann, J.; Tsobanidis, K.; MacColl, A.D.C.; Bakker, T.C.M. Strong neutral genetic differentiation in a host, but not in its parasite. *Infect. Gen. Evol.* **2016**, *44*, 261–271. [CrossRef]
45. Kuhn, J.A.; Kristoffersen, R.; Knudsen, R.; Jakobsen, J.; Marcogliese, D.J.; Locke, S.A.; Primicerio, R.; Amundsen, P.-A. Parasite communities of two three-spined stickleback populations in subarctic Norway—Effects of a small spatial-scale host introduction. *Parasitol. Res.* **2015**, *114*, 1327–1339. [CrossRef]
46. Brabec, J.; Kostadinova, A.; Scholz, T.D.; Littlewood, T.J. Complete mitochondrial genomes and nuclear ribosomal RNA operons of two species of *Diplostomum* (Platyhelminthes: Trematoda): A molecular resource for taxonomy and molecular epidemiology of important fish pathogens. *Parasites Vectors* **2015**, *8*, 336. [CrossRef]
47. Komatsu, N.; Itoh, N.; Ogawa, K. Worm Cataract of Hatchery-Reared Japanese Dace *Tribolodon hakonensis* Caused by *Diplostomum* sp. (Digenea: Diplostomidae). *Fish Pathol.* **2019**, *54*, 1–11. [CrossRef]
48. Kudlai, O.; Oros, M.; Kostadinova, A.; Georgieva, S. Exploring the diversity of *Diplostomum* (Digenea: Diplostomidae) in fishes from the river Danube using DNA mitochondrial barcodes. *Parasites Vectors* **2017**, *10*, 592. [CrossRef] [PubMed]
49. Darriba, D.; Taboada, G.L.; Doallo, R.; Posada, D. jModelTest 2: More models, new heuristics and parallel computing. *Nat. Methods* **2012**, *9*, 772. [CrossRef] [PubMed]
50. Ronquist, F.; Teslenko, M.; Van Der Mark, P.; Ayres, D.L.; Darling, A.; Höhna, S.; Huelsenbeck, J.P. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* **2012**, *61*, 539–542. [CrossRef]
51. Rambaut, A. FigTree v1. 4. Molecular Evolution, Phylogenetics and Epidemiology. 2012. Available online: http://tree.bio.ed.ac.uk/software/figtree/ (accessed on 1 January 2023).
52. Moszczynska, A.; Locke, S.A.; McLaughlin, J.D.; Marcogliese, D.J.; Crease, T.J. Development of primers for the mitochondrial cytochrome c oxidase I gene in digenetic trematodes (Platyhelminthes) illustrates the challenge of barcoding parasitic helminths. *Mol. Ecol. Resour.* **2009**, *9*, 75–82. [CrossRef]
53. Dubois, G.; Raush, R. Les Strigeata (Trematoda) des Gaviides nord-americans. *Rev. Suisse Zool.* **1967**, *74*, 399–407. [CrossRef]


**Disclaimer/Publisher's Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.