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No strict host specificity: Brain metacercariae *Diplostomum petromyzifluviatilis* Müller (Diesing, 1850) are conspecific with *Diplostomum* sp. Lineage 4 of Blasco-Costa et al. (2014)

D.I. Lebedeva [a,*], I.Y. Popov [b], G.A. Yakovleva [a], D.O. Zaicev [c], S.V. Bugmyrin [a], A.A. Makhrov [b,d]

[a] Institute of Biology of Karelian Research Centre, RAS, Petrozavodsk, Russia
[b] Saint Petersburg State University, Saint Petersburg, Russia
[c] Petrozavodsk State University, Petrozavodsk, Russia
[d] Severtsov Institute of Ecology and Evolution, RAS, Moscow, Russia

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ABSTRACT

Metacercariae of *Diplostomum petromyzifluviatilis* (Digenea, Diplostomidae) from the brain of European river lamprey *Lampetra fluviatilis* from the Baltic Sea basin and Arctic lamprey *Lethenteron camtschaticum* from the White Sea basin were studied with the use of genetic and morphological methods. Phylogenetic analysis based on *cox*1 marker showed that the parasites of both lamprey species were conspecific with *Diplostomum* sp. Lineage 4 of Blasco-Costa et al. (2014). The name *Diplostomum petromyzifluviatilis* Müller (Diesing, 1850) has historical precedence as a species described from the brain of lampreys and should be used in genus nomenclature. There were no morphological qualitative differences between the metacercariae from the two lamprey species but those from *L. fluviatilis* were larger than those from L. *camtschaticum*. We expanded the data on the second intermediate hosts and the localization of *D. petromyzifluviatilis*, showing that its metacercariae occur not only in the brain of lampreys but also in the brain and the retina of three-spined stickleback *Gasterosteus aculeatus* and the vitreous humour of the perch *Perca fluviatilis* across the European part of the Palearctic.

1. Introduction

Diplostomum von Nordmann, 1832 is a large genus of widely distributed trematodes with complex life cycles. Their life cycles involve freshwater snails as the first intermediate hosts, various fish species and frogs as the second intermediate hosts, and fish-eating birds and mammals as definitive hosts Adult worms parasitize in the intestine of the birds or mammals. The eggs are released with the host faeces into water, where free-swimming miracidia hatch and infect freshwater snails. After asexual reproduction in the snail the larvae of the parasite, cercariae, leave the molluscan host and infect the second intermediate host, the fish or frogs. They actively penetrate the host and migrate into the eye or the brain of fish or frogs and develop into metacercariae [1–3].

Morphological identification of *Diplostomum* spp., is problematic due to their high morphological variability and phenotypic plasticity, also the morphological similarity of different species, especially their larval stages [2,4,5].

However, diplostomids can be identified on the basis of both mitochondrial *cox1* sequences and ribosomal ITS1-5.8S-ITS2 region. The first molecular studies of *Diplostomum* spp. employed ribosomal markers ITS1 and ITS1-5.8S-ITS2 [6,7]. Then it was found that the mitochondrial cytochrome *c* oxidase subunit 1 was best suited for the study of different development stages of this genus. This genetic marker has been successfully used to identify many *Diplostomum* isolates/species/lineages around the world and to study their life cycles [8–21]. Subsequent studies of adults from natural definitive hosts and the accumulation of numerous sequences have made it possible to arrange the data on the taxonomy, hosts and distribution of *Diplostomum* spp. and to identify different developmental stages of one and the same species [3,5,22–28].

Diplostomum metacercariae are major helminth pathogens of wild and reared fish [29]. It is unsurprising that these numerous and widely distributed life-cycle stages have received most scientific attention, not only in taxonomic respect but also in studies of genome, communities and fauna of *Diplostomum* spp. [30–33]. However, most of the data were obtained on metacercariae parasitizing in the lens ('lens' forms) and tissues under the retina ('non-lens' forms) in the eyes of fish. Information

* Corresponding author.
E-mail address: daryal78@gmail.com (D.I. Lebedeva).

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about metacercariae of *Diplostomum* spp. from the fish brain is limited.

To date five *Diplostomum* spp. whose metacercariae are located in the fish brain were currently known in the world [1–3,34]. The metacercariae of species *Diplostomum* VVT1 were found in brain of central mud minnow *Umbra limi* (Kirtland, 1840) in Minnesota, USA [3]. Metacercariae *Diplostomum phoxini* (Faust, 1989) Arvy et Buttner, 1954 were revealed from the brain of minnows *Phoxinus phoxinus* (Linnaeus, 1758) in Europe, whereas metacercariae in the brains of minnows in East Asia belong to another species, *Diplostomum* sp. MT [21,27]. One more unidentified *Diplostomum* sp. Lineage 4 was found in the brain and in the eyes of three-spined stickleback *Gasterosteus aculeatus* Linnaeus, 1758 in Iceland and Norway [10,13,35]. Finally, *Diplostomum petromyzifluviatilis* Müller (Diesing, 1850) was the only species known from the brain of lampreys [1,36].

It was traditionally believed that *D. petromyzifluviatilis* was the only species parasitizing the brain of different lamprey species of the European part of the Palearctic, including the basins of rivers flowing into the North, Baltic, Black, and Azov Seas [1,2]. Its metacercariae were observed in lampreys *Lampetra fluviatilis* Linnaeus, 1758 of Daugava and Neva Rivers, in other rivers flowing into the Gulf of Riga, and in the Curonian Gulf at the coast of Lithuania [37–39]. Evseeva [40] noted this parasite in *L. fluviatilis* from Lake Onega. Sobecka and colleagues [41] reported *D. petromyzifluviatilis* from river lamprey *L. fluviatilis* caught in Lake Dąbie, connected with the Odra river estuary in Poland. Zehnov [42] found *D. petromyzifluviatilis* in the brain of both *Eudontomyzon mariae* (Berg, 1931) from the upper Dnieper and *Lampetra planeri* (Bloch, 1784) from Daugava River and its tributaries. Gintovt [43] noted *D. petromyzifluviatilis* metacercariae parasitizing in the brain of *L. planeri* from the Lasosna River (a tributary of the Neman River near Grodno, Belarus). Sweeting [36] found metacercariae of *D. petromyzifluviatilis* in the brain of *L. fluviatilis* in the North Sea basin (River Ure, Yorkshire, UK). Another species of lamprey, Arctic lamprey *Lethenteron camtschaticum* (Tilesius, 1811), which is known to occur in Eurasia [44], was also considered as a potential host of *D. petromyzifluviatilis*. But no infection with *Diplostomum* spp. metacercariae has been found previously in the brain of *L. camtschaticum* in different parts of the range (the White Sea basin and rivers of the Far East) [37,45,46].

Life cycle of *D. petromyzifluviatilis* was independently investigated in the laboratory by Sweeting [36] and Shigin [1], who the described the morphology of all developmental stages. Sweeting [36] collected *D. petromyzifluviatilis* metacercariae from brain of *L. fluviatilis* and used them for invasion of different birds – ducks (*Anas platyrhynchos*), chickens (*Gallus gallus* (Linnaeus, 1758)) and herring gulls *Larus argentatus* Pontoppidan, 1763. As result he grew adults of *D. petromyzifluviatilis* in *A. platyrhynchos* only. Then miracidia obtained from eggs in the duck's faeces were used successfully in the infection of *Bithynia tentaculata* (Linnaeus, 1758).

Shigin [2] collected metacercaria *D. petromyzifluviatilis* from brain of *L. fluviatilis* from Sozh river (Sozh River, Republic of Belarus), successfully grew adults in laboratory mice (*Mus musculus*) and chickens (*Gallus gallus*). Then miracidia obtained from eggs in the mice's faeces were used successfully in the infection of *Ampullaceana balthica* (Linnaeus, 1758).

Though the findings of metacercariae *D. petromyzifluviatilis* were fairly common, its measurements or drawings were given only in a few studies involving one lamprey species, the river lamprey *Lampetra fluviatilis* [37,43]. No molecular or integrative approach studies of *Diplostomum* spp. from the brain of different lamprey species have been made.

In this study we described metacercariae of *Diplostomum petromyzifluviatilis* from the brain of *Lampetra fluviatilis* from the Baltic Sea basin and *Lethenteron camtschaticum* from the White Sea basin on the basis of molecular and morphological data. Our results could help to clarify the taxonomy, distribution and host range of *D. petromyzifluviatilis* Müller (Diesing, 1850), filling the gap in the knowledge of the 'brain' forms of larval *Diplostomum* spp. Our study stresses the importance of using fixed animal samples from collections [47,48].

Table 1
Investigated lampreys and sampling sites.

Lamprey species	Form	Sampling locality	Number of dissected lampreys
Lethenteron camtschaticum	resident	Umba River (White Sea Basin), (66°42′11.8″N 34°18′31.1″E)	1
	resident	Abakan River (Kara Sea Basin), (53°24′58.5″N 91°03′23.9″E)	2
	anadromous	Chernaya River (Baltic Sea Basin), 59°56′47.6″N 29°34′50.3″E	16
Lampetra fluviatilis	resident	Okhta River (Baltic Sea Basin), 60°10′49.1″N 30°17′55.1″E	11
	resident	Ptichya River (Baltic Sea Basin), 60°17′53.5″N 29°47′55.2″E)	4
Total number			34

2. Materials and methods

2.1. Sample collection

European river lampreys (*Lampetra fluviatilis*) and Arctic lampreys (*Lethenteron camtschaticum*) were collected by electrofishing and dip nets in the rivers of the basins of the Baltic Sea, the White Sea and the Kara Sea in 2013–2016. Some of the material was used for ichthyological and genetic studies [44,49] and the surveys of protected areas [50]. The remaining material was fixed in 96% ethanol and stored in the laboratory collection in fridge at temperature + 4. In this study we used 31 adult specimens of L. *fluviatilis* and three adult specimens of *L. camtschaticum* from this collection (Table 1).

We accept the taxonomy of lampreys suggested in Makhrov and Popov [49] and Artamonova et al. [44]: *Lampetra planeri* is considered as a resident form of *L. fluviatilis,* while *Lethenteron reissneri* (Dybowski, 1869) and *L. kessleri* (Anikin, 1905) are considered as resident forms of *L. camtschaticum.*

The brains of lampreys were examined for the presence of metacercariae of *Diplostomum* spp. The parasites were removed under a preparation stereomicroscope, counted and processed for morphological and molecular analyses.

Ecological terms characterizing fish infection were calculated according to Bush et al. [51]

Taxonomy and nomenclature of *Diplostomum* spp. followed the latest studies by Achatz et al. [3], Schwelm et al. [27] and Faltýnková et al. [28].

2.2. Morphological examination

Photomicrographs of metacercariae were made with a Levenhuk C1400 NG digital camera attached to Olympus CX41 microscope using LevenhukToupView image analysis software (V 3.5). Then some worms were stained with acetic acid carmine, dehydrated in ethanol, cleared in clove oil, and mounted in Canadian balsam [1].

The morphology of 40 metacercariae from lamprey brain was investigated (Table 2). Thirteen morphological characteristics [1] were scored (in μm): body length (BL), body width (BW), oral sucker length (OSL), oral sucker width (OSW), ventral sucker length (VSL), ventral sucker width (VSW), holdfast organ length (HL), holdfast organ width (HW), pseudosucker length (PSL), pharynx length (PHL), pharynx width (PHW), distance from centre of ventral sucker to anterior end of body (O), and number of excretory bodies. Seven indices of the relative values of these parameters were used [1]: BW × BL/HW × HL, BW × BL/VSW × VSL, OSW × OSL/VSW × VSL, HW × HL/ VSW × VSL, OSW × OSL/ PHW × PHL, BW/BL (%), O/BL (%).

Table 2
Morphometry of newly studied metacercariae of *D. petromyzifluviatilis* Diezing, 1850 from the brain of lampreys in comparison with previously published information. Figures are range with means in brackets.

Parasite species	*Diplostomum petromyzifluviatilis*					
Source	Present study	Present study	Shigin, 1986	Sweeting, 1976	Shulman, 1957	Gintovt, 1969
Host species	*L. fluviatilis*	*L. camtschaticum*	*L. fluviatilis*	*L. fluviatilis*	*L. fluviatilis*	*L. fluviatilis*
Origin	Chernaya River (Baltic Sea basin), Russia	Umba River (White Sea basin), Russia	Sozh River, Republic of Belarus	Ure River, England, UK	Daugava River (Western Dvina), Latvia	Lasosna River, Republic of Belarus
BL (μm)	299–459(404 ± 8.3)	245–379 (309 ± 7.2)	410–530 (458)	342–409 (360)	510–600 (555)	360–600 (470)
BW (μm)	224–315 (275 ± 5.8)	177–251 (220 ± 4.4)	213–265 (237)	262–312 (280)	320–325 (323)	200–380 (300)
OSL (μm)	50–68 (58 ± 1.3)	39–60 (50 ± 1)	55–62 (58)	51–55 (53)	60	45–58 (50)
OSW (μm)	42–65 (50 ± 1.3)	30–53 (38 ± 1.1)	40–47 (44)	45–54 (50)	61	39–60 (48)
VSL (μm)	44–72 (57 ± 1.4)	31–49 (43 ± 0.9)	45–51 (49)	42–50 (46)	69	33–54 (46)
VSW (μm)	49–71 (61 ± 1.1)	36–53 (46 ± 0.9)	50–56 (54)	51–65 (58)	62	42–60 (51)
HL (μm)	82–127 (91 ± 2.5)	76–117 (92 ± 2.2)	95–115 (106)	64–94 (80)	187	70–100 (80)
HW (μm)	94–134 (117 ± 2.3)	74–106 (90 ± 2.1)	100–115 (104)	93–117 (100)	182	80–120 (100)
PHL (μm)	28–52 (44 ± 1.4)	24–39 (33 ± 1)	38–45 (41)	32–44 (37)	34	30–54 (34)
PHW (μm)	40–52 (44 ± 0.8)	14–26 (18 ± 0.6)	25–30 (27)	22–34 (26)	54–61	26–36 (31)
PSL (μm)	39–52 (44 ± 0.6)	42–61 (51 ± 0.9)	–	–	–	
O (μm)	165–257 (214 ± 4.5)	136–214 (167 ± 4.2)	255	153	–	193
Number of excretory bodies	636–743 (669 ± 9.8)	612–768 (678 ± 5.5)	643–782 (724)	610–820 (690)	–	643–782 (724)
BW × BL/HW × HL	7–13 (9)	6–11 (8)	8.95–11.16 (9.81)	–	–	11.10–20.20
BW × BL/VSW × VSL	28–32 (31)	25–51 (35)	35.7–46.3 (41.4)	–	–	51.9–70.37
OSW × OSL/VSW × VSL	0.54–1.36 (0.84)	1–2 (1)	0.92–1.08 (0.98)	–	–	0.88–1.24
HW × HL/VSW × VSL	2.58–5.16 (3.51)	3–7 (4)	3.60–4.73 (4.22)	–	–	2.8–4.4
OSW × OSL/PHW × PHL	1.85–3.79 (2.65)	2–5 (3)	1.96–2.68 (2.27)	–	–	1.79–2.25
BW/BL (%)	56–80 (68)	54–93 (72)	45.4–60.8 (51.7)	–	–	50–73.2
O/BL (%)	50–57 (53)	49–74 (54)	52.8–57.6 (54.7)	–	–	50–63.1
Number of trematodes studied	15	20	20	10	11	26

Note: Body length (BL), body width (BW), oral sucker length (OSL), oral sucker width (OSW), ventral sucker length (VSL), ventral sucker width (VSW), holdfast organ length (HL), holdfast organ width (HW), pseudosucker length (PSL), pharynx length (PHL), pharynx width (PHW), distance from centre of ventral sucker to anterior end of body (O).

Morphological characteristics of the parasites were assessed with the use of discriminant analysis in PAST v. 4.05 [52]. Four groups with dimensions of the metacercariae examined in this study and the metacercariae from the brain of *P. phoxinus* examined by Lebedeva and co-authors [21] were generated for advanced comparison: (1) from *Leptenteron camtschaticum* in the White Sea basin (20 specimens); (2) from *Lampetra fluviatilis* in the Baltic Sea basin (20 specimens); (3) from *P. phoxinus* in Mongolia (24 specimens); (4) from three populations of *P. phoxinus* in Fennoscandia (75 specimens) (Supplementary material S1).

Values of ten morphometric parameters of different species of *Diplostomum* spp. metacercariae were chosen for discriminant analysis (BL, BW, OSL, OSW, PHL, PHW, VSL, VSW, HOL, HOW). The choice was based on the availability of published data and their relevance for our study [1, 13, 36, 37, 43, 53; Supplementary material S2). Average measurements for these species were used to determine their ordination in the canonical axes.

Voucher slides (DM/L1-DM/L3) of the parasites are deposited in the Helminthological Collections of Karelian Research Centre, Russian Academy of Sciences (Petrozavodsk, Karelia, Russia).

2.3. Molecular analysis

2.3.1. DNA extraction, PCR amplification, sequencing and phylogenetic analyses

Genomic DNA was isolated from ethanol-fixed single specimens using the DNA-Extran kits (Synthol, Moscow). Complete ITS1-5.8S-ITS2 cluster of the rRNA gene was amplified using primers designed by Galazzo et al. [6]: D1F (5′-AGGAA TTCCT GGTAA GTGCA AG-3′) and D2R (5′-CGTTA CTGAG GGAAT CCTGG-3′). Fragments of cytochrome *c* oxidase I gene were generated by the primers of Moszczynska et al. [19]: Plat-diploCOX1F (5′-CGTTT RAATT ATACG GATCC-3′) and Plat-diploCOX1R (5′-GCATA GTAAT MGCAG CAGC-3′). Cycling parameters of PCR amplification followed those of Moszczynska et al. [19] for *cox*1, and Gallazo et al. [6] for ITS1-5.8S-ITS2.

The amplified products were purified with Omnix Purification Kits (“Omnix”, St. Petersburg, Russia) following the manufacturer’s instructions, sequenced using the same primers of PCR reactions with MegaBACE 1000 DNA Analysis System (“Beagle”, St. Petersburg, Russia). Consensus sequences were assembled within MEGA v. 10 [54] and deposited in GenBank with accession numbers OM398933 – OM398938 for *cox1* and OM405144 – OM405149 for ITS1-5.8S-ITS2 cluster.

2.4. Phylogenetic analyses

Identity of newly-generated sequences was checked with the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nih.gov/BLAST/). The novel sequences were aligned with the representative sequences of *Diplostomum* spp. previously reported from different places (Supplementary material S3) with MUSCLE algorithms implemented in MEGA v. 10 [54] and edited manually. As a result, two datasets were prepared for the phylogenetic analyses (*cox*1 and ITS-5.8S-ITS2), consisting of 12 sequences obtained in the present study and 155 sequences for *Diplostomum* spp. available in GenBank.

The *cox*1 alignment (396 nt) comprised six novel sequences and 81 sequences of *Diplostomum* spp. from GenBank. The ITS-5.8S-ITS alignment (979 nt) included six novel sequences and 74 sequences of

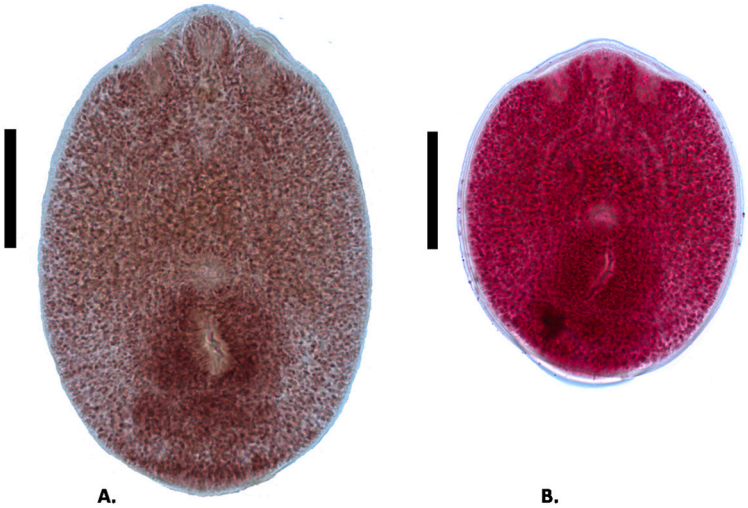


Fig. 1. Metacercariae of *Diplostomum petromyzifluviatilis* Diesing, 1850: **A.** from L. *fluviatilis*; **B.** from *Lethenteron camtschaticum* (scale bar 100 μm).

Diplostomum spp. from GenBank. Sequences of *Tylodelphys clavata* (von Nordmann, 1832) Diesing, 1850 were used as an outgroup in both alignments: JQ665459 for ITS-5.8S-ITS2 and JX986908 for *cox*1 (Supplementary material S3).

The best-fitting nucleotide substitution model for ML analysis was selected within the SMS algorithm [55] as the general time-reversible model incorporating invariant sites and gamma distributed among-site rate variations (GTR + I + G) for both alignments. ML analysis was conducted using PhyML version 3.0 [56] run on the ATGC bioinformatics platform (http://www.atgc-montpellier.fr/). Nodal support was estimated by performing 1000 bootstrap pseudoreplicates. BI analyses were conducted using MrBayes software (ver. 3.2.3) [57] with GTR + I + G model assigned in jModelTest 2.1.2 [58]. Markov chain Monte Carlo (MCMC) chains 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 trees by setting the 'burn in' parameter at 7500. FigTree ver. 1.4 software [59] was used to visualize the trees.

Distance matrices (*p*-distance) were calculated with MEGA v. 10 [54]. The unique *cox*1 haplotypes of *Diplostomum* spp. collected in the present and previous studies in different countries, 49 in total, were identified with DnaSP v. 6 [60]. Haplotype network was reconstructed using the Median-Joining method in PopART software v 1.7 (Population Analysis with Reticulate Trees, http://popart.otago.ac.nz).

3. Results

3.1. Prevalence of Diplostomum petromyzifluviatilis in the lamprey hosts

Lampreys from the Chernaya and the Umba Rivers were heavily infected with metacercariae of *D. petromyzifluviatilis* (Fig. 1) located in the brain. For *L. fluviatilis* from the Chernaya River the prevalence was 44%, mean abundance was 8.7, and the intensity varied from one to 66 specimens. The single specimen of *L. camtschaticum* had 193 metacercariae in the brain.

Lampreys *L. fluviatilis* from the Okhta and the Ptichya Rivers and the two specimens of *L. camtschaticum* from the Abakan River were uninfected with *D. petromyzifluviatilis* metacercariae.

3.2. Molecular investigation of Diplostomum petromyzifluviatilis

A total of six *cox*1 and six ITS1-5.8S-ITS2 sequences were obtained from eight isolates in the present study.

Newly generated sequences of *D. petromyzifluviatilis* strongly clustered together with the sequences representing isolates of *Diplostomum* sp. Lineage 4 from *Gasterosteus aculeatus* in Iceland, Norway, Scotland, Romania, and Italy and from *Perca fluviatilis* in Germany. The sequences of cercariae from *Ampullaceana balthica* in Norway, Denmark and Iceland also belonged to this clade [9,10,18,31,35,61]. The sequence divergence within this clade was 0–1.9%, which corresponds to the intraspecific level for *Diplostomum* spp. The *p*-distance between parasites from lampreys of the Chernaya and the Umba Rivers was 1%. Thus, molecular evidence indicates that the parasites of both lamprey species are conspecific with *Diplostomum* sp. Lineage 4 of Blasco-Costa et al. [10].

The 49 *cox*1 sequences (353 nt) of *Diplostomum petromyzifluviatilis* (*Diplostomum* sp. Lineage 4) from the present study and previous studies were represented by 31 haplotypes (Fig. 3, Supplementary Material S4). Twenty-five haplotypes were unique. One unique haplotype Hap4 was found in two metacercariae collected from *L. camtschaticum* in the Umba River (OM398937 and OM398938). Isolate OM398935 from the Chernaya River was also characterized by a single haplotype Hap2. Hap3 of isolate OM398936 from the same river was shared with the specimen from vitreous humour of *P. fluviatilis* in Germany (JQ639190; [9]).

Three haplotypes were the most frequent ones: Hap1, Hap6 and Hap9 (Fig. 3, Supplementary Material S4). Each of them was represented by six samples. Hap1 was represented by two specimens (OM398933, OM398934) from the brain of *L. fluviatilis* in the Chernaya River, two specimens from the vitreous humour of *P. fluviatilis* in Germany (JQ639180; JQ639184; [9]), one specimen from the brain of *G. aculeatus* in Iceland (KJ726480; [10]) and one specimen from the eye of *G. aculeatus* in Scotland (KX037877; [61]). Hap6 was represented by two specimens from the vitreous humour of *P. fluviatilis* in Germany (JQ639182; JQ639194; [9]), three specimens from the retina of *G. aculeatus* and one cercaria from *A. balthica* in Norway (KM212030, KM212032, KM212033, KY513192; [31,61]). Hap9 was found in one specimen from the vitreous humour of *P. fluviatilis* in Germany (JQ639186; [9]), in three specimens from *G. aculeatus* and in one cercariae from *A. balthica* in Norway (KJ726474, KJ726475, KJ726476, KJ726477, [10]), and one specimen from the eye of *G. aculeatus* in Scotland (KX037874; [61]).

A phylogram resulting from BI and ML analyses of *Diplostomum* spp. based on ITS1-5.8S-ITS2 sequence data is shown in Fig. 4. Six novel sequences clustered together with the sequences representing different isolates of *Diplostomum* sp. Lineage 4 of Blasco-Costa et al., 2014. They were metacercariae from the eyes of *Gasterosteus aculeatus* in Iceland and *Perca fluviatilis* in Germany and cercariae from *Ampullaceana balthica* in Iceland, Norway and Denmark [9,10,14,62]. This clade also included sequences of *Diplostomum* sp. Lineage 3 of Blasco-Costa et al. (2014)

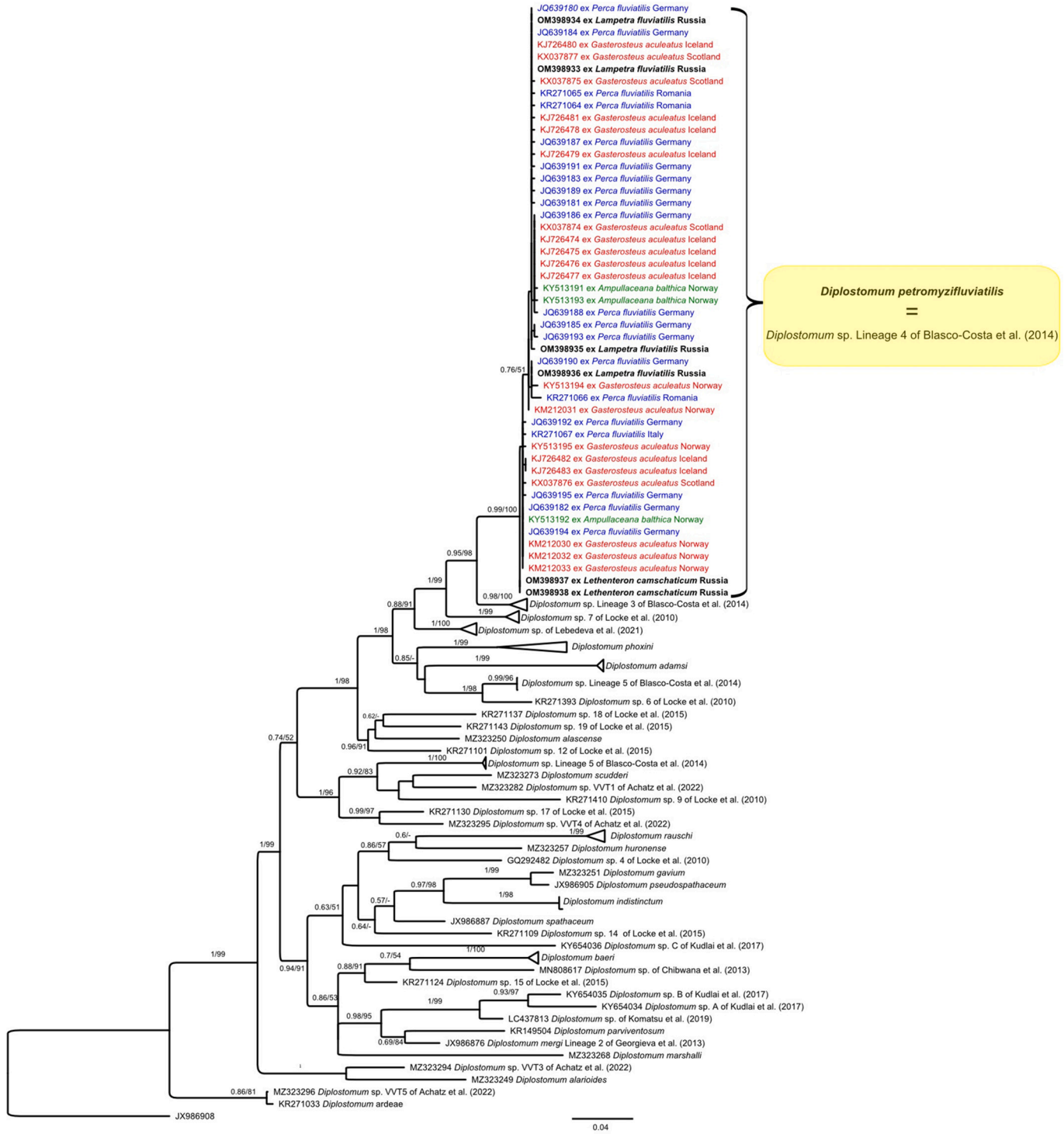
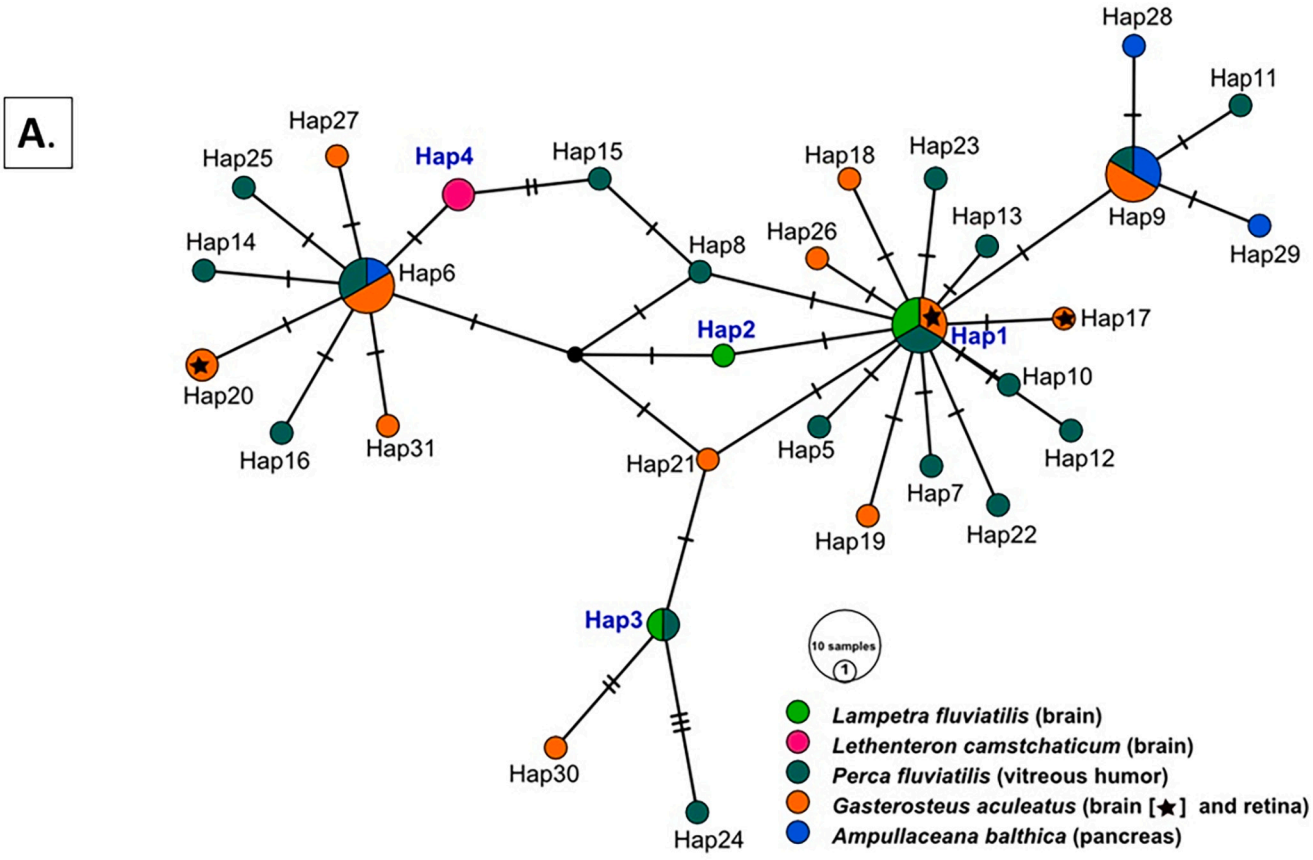


Fig. 2. Bayesian inference (BI) and maximum likelihood (ML) analyses tree for *Diplostomum* spp. based on the partial *cox*1 mtDNA sequences. Nodal supports from both analyses are indicated as BI/ML. The scale bar indicates the expected number of substitutions per site. Newly generated sequences are highlighted in black bold.

from different hosts and localities: *Gobio gobio* (Linnaeus, 1758) and *Oncorhynchus mykiss* (Walbaum, 1792) in Germany [35,63], *Salvelinus alpinus* (Linnaeus, 1758) and *Salmo trutta (Linnaeus, 1758)* from Norway and Iceland [10,35], *Thymallus brevirostris (Kessler, 1879)* from Mongolia [64] as well as *Diplostomum* sp. C57 from *Ladislavella elodes* (Say, 1821) in Canada [65]. There was no sequence divergence within the clade (*p*-distance was 0).

3.3. Morphological analysis of *Diplostomum petromyzifluviatilis*

Thirteen morphological characteristics of 40 newly found metacercariae of *D. petromyzifluviatilis* (20 specimens from each lamprey species) and seven indices of their relative values were obtained (Table 2). The parasites from the two host species did not differ in the qualitative morphological characters and had the same number of excretory granules. However, they differed in size (Fig. 5). The dimensions of parasites from the brain of *L. fluviatilis* were greater than



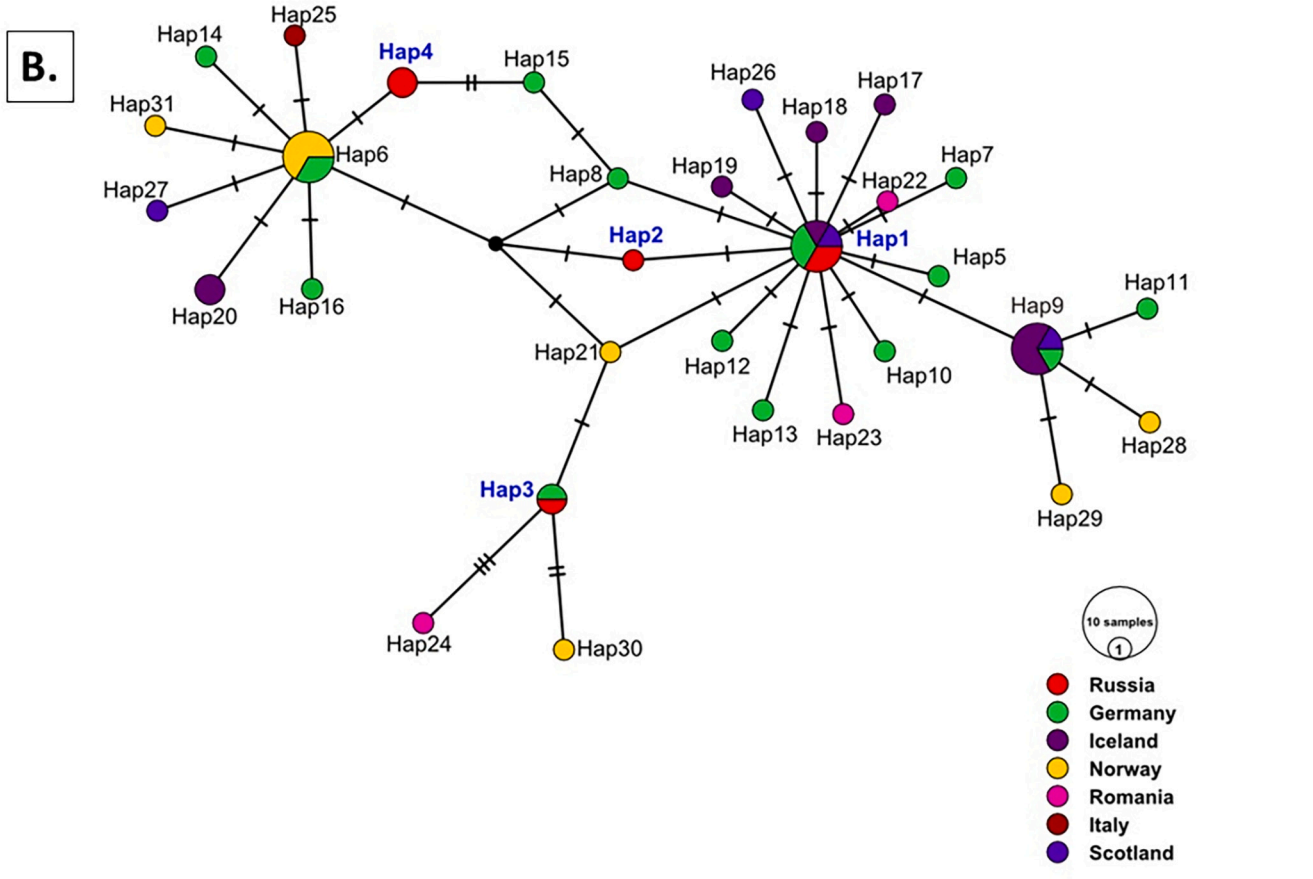


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Fig. 3. Haplotype network for *Diplostomum petromyzifluviatilis* based on published and novel *cox1* sequences (Supplementary Material S4): **A.** Host and localization; **B.** Locality. Haplotypes found in this study are marked in blue bold. Unsampled intermediate haplotype is represented by a short intersecting line; each branch corresponds to a single mutational difference and connective lines represent one mutational step. Circle size is proportional to the number of isolates sharing a haplotype; black circles indicate transitive haplotypes that are still not found. Haplotypes of parasites from the brain of *G. aculeatus* are marked with asterisks. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

those from the brain of *L. camtschaticum* ($p < 0.05$), except the pseudosucker length (PSL) and the distance from centre of ventral sucker to anterior end of body (O) (Table 2). The means of the three indices (BW/BL, O/BL, and BW × BL/VSW × VSL) of metacercariae from *L. fluviatilis* and *L. camtschaticum* did not differ ($p > 0.05$). Between-group differences of means of the other indices were significant ($p < 0.05$).

Discriminant analysis of the morphometric dataset on ten dimensions of brain larvae was conducted (Supplementary Material S1). The metacercariae of *D. petromyzifluviatilis* from lamprey brain in the Umba River and the Chernaya River on the one hand and the metacercariae of two species (*D. phoxini* and *Diplostomum* sp. MT) from minnow brains on the other hand belonged to two different groups (Fig. 5). The overall success of the discriminant function classification was estimated at 98%. The first canonical axis carried the highest functional load, 76%, while the second axis determined about 20% of the total variability. The most significant contribution to the divergence of the groups came from the length and the width of the metacercariae body (BL and BW) on both axes. Length and width of holdfast organ (HL and HW) were most significant on the first axis, while OSW and PHW were most significant on the second canonical axis. However, confidence zones overlapped for two groups of metacercariae from lamprey (Umba River and Chernaya River), in contrast with *D. phoxini* and *Diplostomum* sp. MT from minnows.

Analysis of the newly obtained and previously published data on the mean dimensions of *Diplostomum* spp. metacercariae parasitizing in the brain of lampreys and three-spined sticklebacks also showed high variation (Supplementary Material S2; Fig. 5).

Metacercariae of *D. petromyzifluviatilis* from the brain of the lamprey *L. fluviatilis* of the Chernaya River (Baltic Sea basin) were most similar in mean dimensions to the larvae found in the brain of *L. fluviatilis* in a tributary of the Neman River (Baltic Sea basin) by Shigin [1] and in Ure River, Yorkshire (North Sea basin) by Sweeting [36]. Metacercariae of *D. petromyzifluviatilis* from the brain of lamprey *L. camtschaticum* of the Umba River (White Sea basin) were the closest to those of *Diplostomum* sp. Lineage 4 of Blasco-Costa et al. (2014) from the brain and the retina of Icelandic three-spined stickleback [13] and those of *D. gasterostei* Williams, 1966 from the retina of three-spined stickleback in Scotland [53].

Metacercariae of *D. petromyzifluviatilis* from the brain of *L. fluviatilis* in the Daugava River [37] and from the brain of *L. planeri* in the Lasosna River [43] were larger than the metacercariae of other trematodes involved in the analysis and clustered separately from them (Fig. 5 and Table 2). Both these rivers belong to the Baltic Sea basin.

4. Discussion

In this study we provided a fairly complete characterization of *D. petromyzifluviatilis* on the basis of newly obtained combined morphological and molecular data on its metacercariae from the brains of lampreys *Lampetra fluviatilis* from the Baltic basin and *Lethenteron camtschaticum* from the White Sea basin.

The molecular analysis of *cox*1 showed that the sequences of metacercariae of *D. petromyzifluviatilis* from the brain of both lamprey species coincided with those of metacercariae of *Diplostomum* sp. Lineage 4 of Blasco-Costa et al. (2014) from different fish species (Fig. 2). The analysis of the haplotype network by *cox1* showed a high diversity of haplotypes both by hosts and by sampling localities (Fig. 3).

Our analysis of ITS1-5.8-ITS2 cluster of *D. petromyzifluviatilis* and *Diplostomum* sp. Lineage 3 showed that they were similar, apparently indicating a recent evolutionary divergence of these two species. This finding agrees with Faltýnková et al. [13], who showed that this marker, as well as ITS1, is of little use for separating *Diplostomum* species/lineages.

We also showed that morphological data on the metacercariae of *D. petromyzifluviatilis* are controversial. At this stage, morphological data alone cannot be used for the identification of this species, and molecular data are indispensable (Fig. 2). Morphometrically, the metacercariae of *D. petromyzifluviatilis* from the two lamprey species from this study were quite clearly divided into two groups, with an overlapping area between them (Fig. 2). Both newly and previously studied metacercariae were different in morphometric characteristics, forming heterogeneous size groups based on the results of discriminant analysis. These data support and expand the other investigations showed that morphology of *Diplostomum* metacercariae developing in different hosts varied [e.g. 22, 24, 26, [66–71],].

Our results extend the knowledge about the life cycle and distribution of *D. petromyzifluviatilis* and prompt a reconsideration of some previous ideas. The first intermediate host of *D. petromyzifluviatilis* is the snail *Ampullaceana balthica*. The sequence ITS1-5.8-ITS2 of the cercaria (MH108198; [65]) from the mollusc *Ladislavella elodes* from Canada belongs to a clade including *Diplostomum* sp. Lineage 3 and *D. petromyzifluviatilis* (Fig. 4). Moreover, Vinarski et al. [72] found the snail *Ampullaceana balthica* in June 2019 in a small lake in Québec, Canada. Future detailed parasitological studies of these two snail species in Canada, especially with the use of *cox*1 marker, are needed to clarify the distribution of *D. petromyzifluviatilis* and to check the possibility that the snail *L. elodes* may also serve as its first intermediate host.

We showed that metacercariae of *D. petromyzifluviatilis* have no strict specificity to the second intermediate host and the microhabitat within it. They have been recorded in *Lampetra fluviatilis* (brain), *Lethenteron camtschaticum* (brain), *Gasterosteus aculeatus* (brain and retina) and *Perca fluviatilis* (vitreous humour) in the European Palearctic from Iceland to the Black Sea coast of Romania. To date the distribution range of *D. petromyzifluviatilis* is coincides with are area of with that of its hosts, the lamprey as indicated by Shigin [1].

It has been thought the microhabitat within the fish utilised by the metacercariae is an important characteristic of *Diplostomum* spp. [1]. In particular, until recently *D. petromyzifluviatilis* has been considered to belong to the so-called "*D. baeri* species complex" [27]. This complex includes all “non-lens” species/lineages, that is, those recovered from the vitreous humour and the retina of the eye as well as from the brain of the fish hosts. However, *D. baeri* has been recently re-described and the concept of “*D. baeri* species complex” has been shown to be invalid [28]. These findings indicate that the importance of microhabitat and host species for diplostomid systematics should be reconsidered. On the other hand, a strict host specificity and a strict localization within the host proposed by Shigin [1] has been confirmed e.g. for *D. phoxini* in Europe and *D. adamsi*, and several *Diplostomum* spp. in Canada [18,21,27,73]. Parasitism in different fish species may be explained by habitat similarities and overlapping of their dispersal routes. The two lampreys, the stickleback and the perch inhabit freshwater rivers and lakes with low temperatures and stony and sandy substrates [74,75]. There is some evidence that shared habitats may lead to a parallel evolution of host-parasite relationships. Shulman [37] reports that out of ten parasites of river lamprey, four species (*Echinorynchus salmonis* (Müller, 1784), *Camalanus truttae* (Fabricius 1794), *Proteocephalus longicollis* ([Zeder, 1800] Benedict, 1900), *Raphidascaris acus* (Bloch, 1779) are specific or typical parasites of salmonid fish. The similarity of the parasitic fauna of

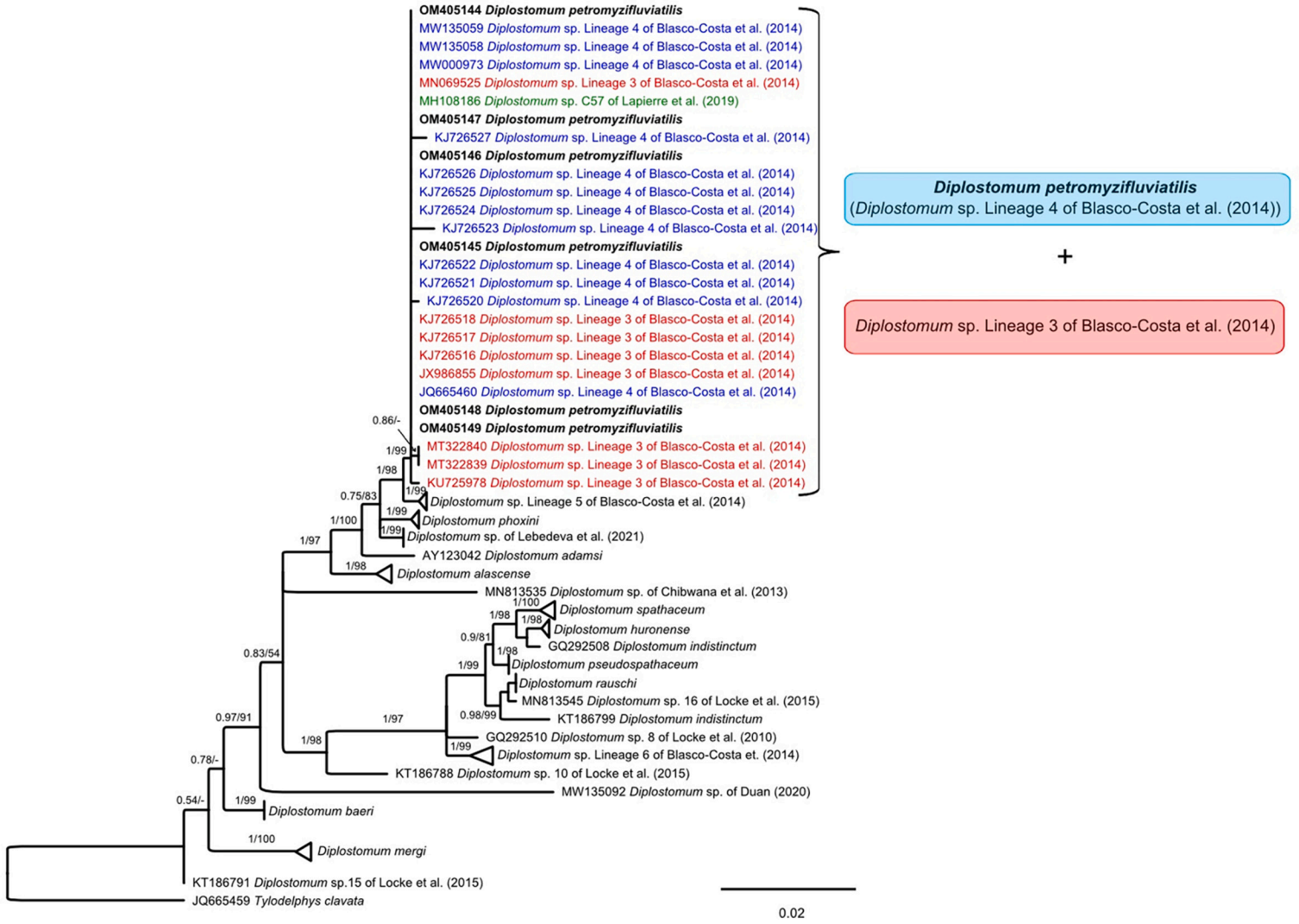

Fig. 4. Bayesian inference (BI) and maximum likelihood (ML) analyses tree for *Diplostomum* spp. based on the partial ITS1-5.8S-ITS2 sequences. Nodal supports from both analyses are indicated as BI/ML. The scale bar indicates the expected number of substitutions per site. The newly generated sequences are highlighted in black bold.

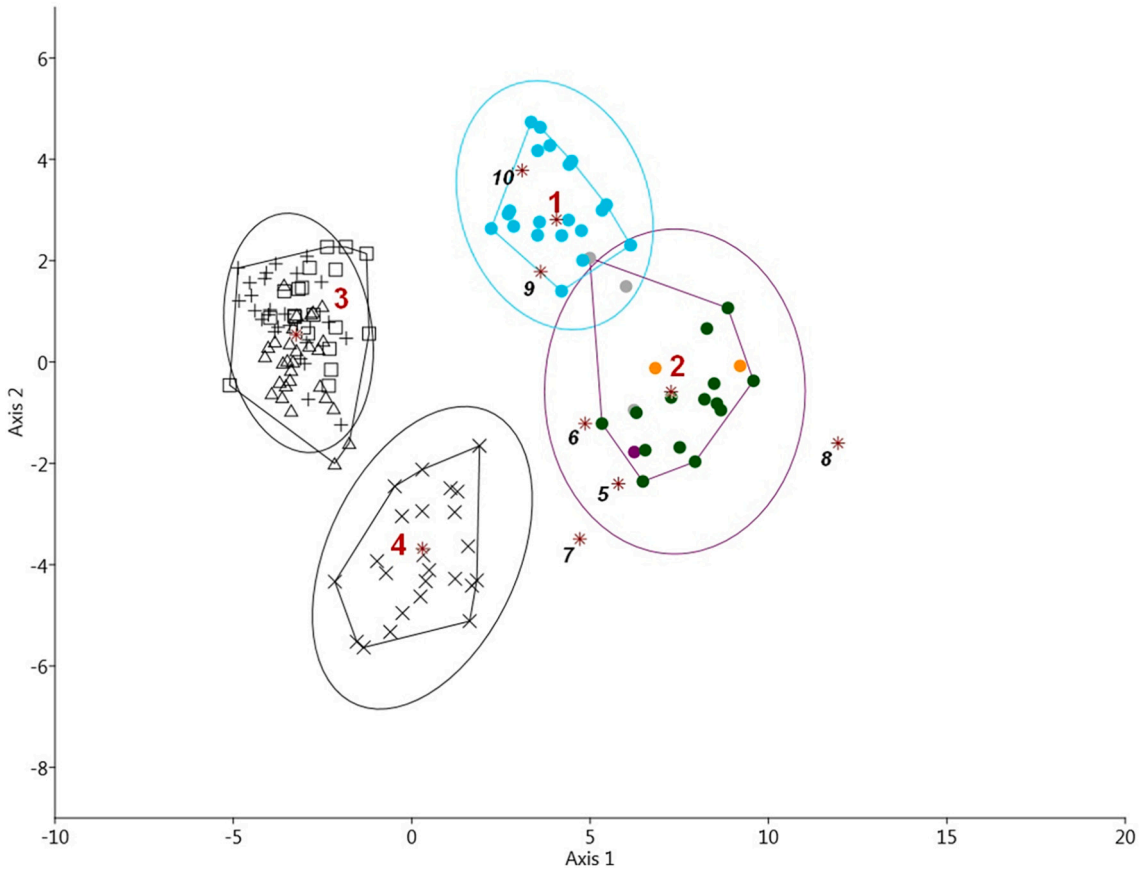

Fig. 5. Ordination of traits of *Diplostomum* spp. metacercariae from brain and retina by discriminant analysis. The means of 10 traits, including morphometry, from the present study and the literature were involved (Supplementary Material S2).
Data set for discriminant analysis. 1 – blue color: *Diplostomum petromyzifluviatilis* (*L. camtschaticum*; Umba River, White Sea Basin); 2 – purple color: *Diplostomum petromyzifluviatilis* (*L. fluviatilis*; Chernaya River, Baltic Sea Basin). The coloured points (green, orange, purple and gray) are specimens taken from different individuals of the host; 3 (including squares, triangles and crosses) – *Diplostomum phoxini* (Lebedeva et al., 2021); 4 (slanting crosses) – *Diplostomum* sp. MT (Lebedeva et al., 2021). Mean dimensions from the literature: 5. *D. petromyzifluviatilis* (Shigin, 1986); 6. *D. petromyzifluviatilis* (Sweeting, 1976); 7. *Diplostomum petromyzifluviatilis* (Gintovt, 1969); 8. *D. petromyzifluviatilis* (Shulman, 1957); 9. *Diplostomum* sp. Lineage 4 (Faltynkova et al., 2014); 10. *D. gasterostei* (Williams, 1966). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

these distantly related fish species is due to ecological parallelism between salmonids and lampreys [76]. Evseeva [40] observed a specific "stickleback" parasite, *Trichodina tenuidens* Fauré-Fremiet, 1943, in lamprey *L. fluviatilis* from the Lososinka River (Lake Onega basin), which probably resulted from close contact of lamprey and spawning three-spined stickleback in the river mouth. Several helminth species normally restricted to salmonids (*Crepidostomum* spp., *Eubothrium* spp., *Cystidicola farionis* (Fischer, 1798)) were found among the parasite communities of introduced three-spined stickleback [31]. It can be assumed that the dispersal of *D. petromyzifluviatilis* as well as some of its hosts (*Lampetra fluviatilis* and *Gasterosteus aculeatus*) proceeded from the Black Sea basin towards the northern Europe. Three-spined stickleback, in addition, crossed the Atlantic Ocean and reached Iceland and North America [77], also becoming a suitable host for *D. petromyzifluviatilis*. On the Kola Peninsula, the three-spined stickleback encountered the Arctic lamprey, which had been dispersing from the Pacific Ocean and advanced to northern Europe [44]. The snail *Ampullaceana balthica*, which is the only proven first intermediate host of *D. petromyzifluviatilis*, is common in Europe from Iceland to Spain [78,79]. Its range overlaps with that of all the fish species serving as second intermediate hosts, providing excellent transmission opportunities.

Natural definitive hosts of *D. petromyzifluviatilis* are still unknown. However, there are two morphological descriptions of *D. petromyzifluviatilis* adults experimentally reared from metacercariae isolated from the brain of the lamprey *L. fluviatilis*. Sweeting [36] grew adults of *D. petromyzifluviatilis* in ducks (*Anas platyrhynchos*). Shigin [2] grew *D. petromyzifluviatilis* in laboratory mice (*Mus musculus*) and chickens (*Gallus gallus*) and showed that morphology of adults from these two hosts differed significantly. Adults of *D. petromyzifluviatilis* grown in ducks were almost indistinguishable morphologically from *D. gasterostei* Williams, 1966. The coefficient of variation between these species did not reach the species level for 34 characters and reached the lower level of subspecies significance only in one character of little taxonomic importance [2].

The life cycle of *D. gasterostei* was elucidated by Williams (1966) in Scotland. He found its cercariae in the mollusc *Lymnaea peregra* (*A. balthica* according to the novel data of Vinarski et al. [79]) and used them to infect three-spined stickleback. Metacercariae that developed in its retina were used to infect ducklings (*Anas platyrhynchos*) and adults were successfully grown. As natural definitive hosts of *D. gasterostei* were identified smew *Mergellus albellus* (Linnaeus, 1758), goosander *Mergus merganser* (Linnaeus, 1758), red-breasted merganser *M. serrator* (Linnaeus, 1758), green sand piper *Tringa ochropus* (Linnaeus, 1758) [2].

Considering the morphological identity of adults of *D. gasterostei* and *D. petromyzifluviatilis* [2], the similarity of their distribution and second intermediate hosts (stickleback and perch) as well as the molecular identity in *cox*1 of metacercariae from lampreys and *G. aculeatus* in Scotland (Fig. 2) and their morphological similarity (Fig. 5), it could be suggest that they are in fact one species, *D. petromyzifluviatilis* Müller (Diesing, 1850). *Diplostomum gasterostei* (Williams, 1966) should be

considered as a junior synonym of *D. petromyzifluviatilis* Müller (Diesing, 1850) especially if barcoding will be used for this aim.

5. Conclusion

In this study we clarified the taxonomic status of *D. petromyzifluviatilis* and *Diplostomum* sp. Lineage 4 of Blasco-Costa et al. (2014). We also expanded the knowledge on the second intermediate hosts, localization and distribution of *D. petromyzifluviatilis*.

A careful examination of a broad range of waterfowl birds is needed to identify natural host species and to provide morphological and molecular description of adult *D. petromyzifluviatilis.* Assuming the conspecifity of *D. petromyzifluviatilis* and *D. gasterostei* we can suggest that its natural definitive hosts are different species of fish-eating birds including mentioned above. Also we need to pay attention to some other fish-eating bird species – common gull *Larus canus* (Linnaeus, 1758), European herring gull *L. argentatus* (Linnaeus, 1758), Lesser black-backed gull *L. fuscus* (Linnaeus, 1758), and terns *Sterna* spp. In a study carried out on the Ricklea River and in the nearby coastal area of the Gulf of Bothnia in northern Sweden, these birds have been shown to be especially active during the spawning season of the three-eared stickleback and lamprey, mostly feeding by these fishes [80,81]. All of birds listed are fairly common throughout the area where metacercariae of *D. petromyzifluviatilis* have been recorded [82]. We hope that morphological and molecular data on adult parasites of these two species will be described soon. However, the name *Diplostomum petromyzifluviatilis* Müller (Diesing, 1850) has historical precedence as a species described from the brain of lampreys and should be used in genus nomenclature.

The taxonomy of diplostomids remains incomplete. More data on morphology, molecular characteristic and biological features of *Diplostomum* parasites from various hosts (snails, fishes, birds) are required to elucidate the evolution of these important agents of fish diseases, and their interactions with the hosts and the environment.

Data availability statement

Data are available on request from the authors and in Supplementary material. Newly generated sequences were deposited in GenBank with the accession numbers listed in the manuscript.

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Author contributions

Concept and design of the study: DL, SB. Writing: DL, SB and AM. Review and editing: DL, SB, AM. Morphological analysis: GY, SB, DL. Molecular analyses: DL, DZ. Field work and data processing: AM, IP, DZ, DL. All authors approved the final version of the manuscript before submission.

Compliance with ethical standards

All procedures followed were in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123). All necessary permits for sampling and observational field studies were obtained by the authors from the Ministry of natural resources and environment of RF. Our study did not involve any endangered species.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2022.102654.

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