Parasitology

cambridge.org/par

Another plea for 'best practice' in molecular approaches to trematode systematics: *Diplostomum* sp. clade Q identified as *Diplostomum baeri* Dubois, 1937 in Europe

Anna Faltýnková[1,2], Olena Kudlai[3], Camila Pantoja[3], Galina Yakovleva[4] and Daria Lebedeva[4]

[1]Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, Branišovská 31, 370 05 České Budějovice, Czech Republic; [2]Department of Forest Ecology, Faculty of Forestry and Wood Technology, Mendel University in Brno, Zemědělská 3, Brno 613 00, Czech Republic; [3]Institute of Ecology, Nature Research Centre, Akademijos 2, 08412 Vilnius, Lithuania and [4]Institute of Biology, Karelian Research Center, Russian Academy of Sciences, Pushkinskaya St. 11, 185910 Petrozavodsk, Russia

Research Article

Cite this article: Faltýnková A, Kudlai O, Pantoja C, Yakovleva G, Lebedeva D (2022). Another plea for 'best practice' in molecular approaches to trematode systematics: *Diplostomum* sp. clade Q identified as *Diplostomum baeri* Dubois, 1937 in Europe. *Parasitology* **149**, 503–518. https://doi.org/10.1017/S0031182021002092

Received: 21 September 2021
Revised: 8 November 2021
Accepted: 2 December 2021
First published online: 7 January 2022

Keywords:
Ireland; Laridae; Lymnaeidae; nuclear and mitochondrial DNA; Russia; trematoda

Author for correspondence:
Olena Kudlai, E-mail: olena.kudlai@gmail.com

Abstract

DNA sequence data became an integral part of species characterization and identification. Still, specimens associated with a particular DNA sequence must be identified by the use of traditional morphology-based analysis and correct linking of sequence and identification must be ensured. Only a small part of DNA sequences of the genus *Diplostomum* (Diplostomidae) is based on adult isolates which are essential for accurate identification. In this study, we provide species identification with an aid of morphological and molecular (*cox*1, ITS-5.8S-ITS2 and 28S) characterization of adults of *Diplostomum baeri* Dubois, 1937 from naturally infected *Larus canus* Linnaeus in Karelia, Russia. Furthermore, we reveal that the DNA sequences of our isolates of *D. baeri* are identical with those of the lineage *Diplostomum* sp. clade Q , while other sequences labelled as the '*D. baeri*' complex do not represent lineages of *D. baeri*. Our new material of cercariae from *Radix balthica* (Linnaeus) in Ireland is also linked to *Diplostomum* sp. clade Q. We reveal that *D. baeri* is widely distributed in Europe; as first intermediate hosts lymnaeid snails (*Radix auricularia* (Linnaeus), *R. balthica*) are used; metacercariae occur in eye lens of cyprinid fishes. In light of the convoluted taxonomy of *D. baeri* and other *Diplostomum* spp., we extend the recommendations of Blasco-Costa *et al.* (2016, Systematic Parasitology **93**, 295–306) for the 'best practice' in molecular approaches to trematode systematics. The current study is another step in elucidating the species spectrum of *Diplostomum* based on integrative taxonomy with well-described morphology of adults linked to sequences.

Introduction

Species identification is often emphasized as a basic prerequisite for the understanding of diversity, ecology and evolution of the living world (Hey *et al.*, 2003; Olson and Tkach, 2005; Shaffer *et al.*, 2019). For the last three decades, DNA sequence data including DNA barcoding became an integral part of species characterization and identification. Nevertheless, the actual specimen associated with a particular DNA sequence must still be identified by the use of traditional morphology-based analysis to ensure that the sequence and identification are linked correctly (Blasco-Costa *et al.*, 2016; Schwelm *et al.*, 2021). Trematode species identification has been and continues to be based on morphological data collected from adult specimens since the larval stages often lack reliable distinguishing morphological characters. Thereafter, specimens of either adults or larval stages can be accurately identified if they match the sequence of the known species. DNA sequence databases rely on sequences that are derived from taxonomically correctly identified isolates. Although the DNA sequences became a primary source for assessment and measure of biodiversity, there is a growing problem of taxonomic misidentification in public DNA databases (Bridge *et al.*, 2003; Tautz *et al.*, 2003; Vilgalys, 2003; Valkiūnas *et al.*, 2008; Locke *et al.*, 2015; Achatz *et al.*, 2021; Bensch *et al.*, 2021; Pantoja *et al.*, 2021). The problems in each dataset are different. The sequences may be incorrectly labelled, of poor quality, incomplete for reliable comparison or without voucher specimens (Bridge *et al.*, 2003; Locke *et al.*, 2015; Blasco-Costa *et al.*, 2016).

One of the trematode groups that has largely benefited from application of the molecular genetic methods is the genus *Diplostomum* von Nordmann, 1832 – a species-rich genus with complex taxonomy distributed worldwide in freshwater ecosystems (Niewiadomska, 2002). The members of *Diplostomum* are important fish pathogens, with a three-host life-cycle encompassing lymnaeid snails, fish and fish-eating bird hosts; the most pathogenic stage are metacercariae infecting fish eyes or brain, which can impair vision and lead to cataract

formation in wild and farmed fish (Shigin, 1986; Karvonen *et al.*, 2004; Karvonen and Marcogliese, 2020).

The development of suitable molecular markers, particularly the barcode region of the cytochrome *c* oxidase subunit (*cox*1), allowed a wealth of studies to prospect for *Diplostomum* with an aid of molecular genetic methods; thus, in North America, Europe, Africa and Asia an unexpectedly wide spectrum of lineages and complexes of cryptic species were revealed (Galazzo *et al.*, 2002; Moszczynska *et al.*, 2009; Locke *et al.*, 2010*a*, 2015; Georgieva *et al.*, 2013; Hoogendoorn *et al.*, 2020).

The recent intensive studies resulted in generating sequence libraries, presenting a platform for further molecular delineation and linking larval stages with adults (Kudlai *et al.*, 2017; Achatz *et al.*, 2021; Schwelm *et al.*, 2021). To date, libraries contain more than 40 species and lineages from Africa, Asia, Europe and North America (Hoogendoorn *et al.*, 2020). However, still a small part of these sequences is based on adult isolates (18 species in total), of which 15 represent identified species, i.e. *Diplostomum adamsi* Lester & Huizinga, 1977, *Diplostomum alarioides* Dubois, 1937, *Diplostomum alascense* Dubois, 1969, *Diplostomum ardeae* Dubois, 1969, *Diplostomum gavium* (Guberlet, 1922), *Diplostomum huronense* (La Rue, 1927), *Diplostomum indistinctum* (Guberlet, 1923), *Diplostomum marshalli* Chandler, 1954 and *Diplostomum scudderi* Olivier, 1941 in North America (Galazzo *et al.*, 2002; Locke *et al.*, 2015; Achatz *et al.*, 2021), *Diplostomum lunaschiae* Locke, Drago, Núñez, Rangel e Souza & Takemoto, 2020 in South America (Locke *et al.*, 2020) and *Diplostomum mergi* Dubois, 1932, *Diplostomum spathaceum* (Rudolphi, 1819), *Diplostomum pseudospathaceum* Niewiadomska, 1984 and *Diplostomum rauschi* Shigin, 1993 in Europe (Pérez-del-Olmo *et al.*, 2014; Selbach *et al.*, 2015; Achatz *et al.*, 2021; Schwelm *et al.*, 2021). However, this number is most likely to be changed as Achatz *et al.* (2021) questioned the identification of *D. ardeae sensu* Locke *et al.* (2015). Thanks to recent studies (particularly Achatz *et al.*, 2021) out of these 15 species, specimens of 13 species (*D. alarioides*, *D. alascense*, *D. ardeae*, *D. gavium*, *D. huronense*, *D. indistinctum*, *D. lunaschiae*, *D. marshalli*, *D. mergi*, *D. pseudospathaceum*, *D. rauschi*, *D. scudderi* and *D. spathaceum*) were obtained from naturally infected bird hosts and connected to the original description (Pérez-del-Olmo *et al.*, 2014; Locke *et al.*, 2015; Heneberg *et al.*, 2020; Achatz *et al.*, 2021) or described as new species (Locke *et al.*, 2020). Still, most of the sequences available in GenBank are based on metacercariae, a stage with least distinguishing characters, and only a small portion of them is linked to voucher material, thus there is no unequivocal identification which would warrant assignment to valid species for many of isolates (Selbach *et al.*, 2015; Hoogendoorn *et al.*, 2020). Another basic issue concerns the consistency and uniformity in naming unidentified species and lineages by different authors that often follow different nomenclature. This creates misidentifications and misinterpretations in later studies when authors solely rely on identifications provided for sequences in GenBank.

In this study, we provide species identification for the 'questionable' lineage of *Diplostomum* of Georgieva *et al.* (2013) in Europe and we extend the recommendation of Blasco-Costa *et al.* (2016) for the 'best practice' in molecular approaches to trematode systematics. We characterize morphologically and molecularly (*cox*1, ITS-5.8S-ITS2 and 28S) adults of *Diplostomum baeri* based on new material collected from naturally infected *Larus canus* Linnaeus in Karelia, Russia; thus, we link the original description by Dubois (1937, 1938, 1970) with our morphological and DNA sequence data. Furthermore, *via* molecular tools we reveal that sequences of our isolates of *D. baeri* are identical with those of the lineage *Diplostomum* sp. clade Q of Georgieva *et al.* (2013). Also, we review the morphology of cercariae reported in the literature as *D. baeri* or *Diplostomum volvens* Nordmann, 1832 (based on views of Shigin, 1993 and Niewiadomska, 2010), and we accompany our data with new material of cercariae from Ireland, with DNA sequences and morphology corresponding to *Diplostomum* sp. clade Q of Georgieva *et al.* (2013) and Selbach *et al.* (2015), respectively. The current study is another step in elucidating the species spectrum of *Diplostomum* based on integrative taxonomy with a well-described morphology of adults, linked to sequences.

Materials and methods

Sample collection

Samples of adults were collected from a single specimen of the common gull *L. canus* found dead on the shore of the Kostomukshskoye Lake (64°39′34″N, 30°48′10″E), Karelia, northwest Russia, in June 2010. The bird was transported on ice to the laboratory and immediately dissected following the protocol of Dubinina (1971). A total of 154 worms of the genus *Diplostomum* were found in the duodenum and small intestine. Collected digeneans were preserved in 96% ethanol for both morphological investigation (without additional pressure) and DNA extraction; a total of five adult worms were used for molecular and morphological analyses. Samples of cercariae were obtained from snails *Radix balthica* (Linnaeus) [*Ampullaceana balthica* (Linnaeus) being considered senior synonym by Aksenova *et al.*, 2018] (two snails infected out of a total of 573) collected in the Lake Lough Corrib (53° 20′24.3″N, 9°05′28.6″W), Ireland in July 2019. In the laboratory, snails were placed individually in plastic cups filled with dechlorinated tap water and left for 24 h to detect emergence of cercariae. Emerged cercariae were studied alive, fixed in 96% ethanol for DNA isolation and in 4% formalin for measurements.

Morphological examination

Specimens recovered from the bird were identified as members of the genus *Diplostomum*, based on the generic diagnosis provided by Dubois (1970), Shigin (1993) and Niewiadomska (2002). Specimens of adults ($n = 5$) selected for molecular analysis were vouchered following the concept of Pleijel *et al.* (2008) and the recommendation of Blasco-Costa *et al.* (2016) and series of photomicrographs of vouchers were taken with a digital camera of an Olympus CX41 and BX51 microscope. Thereafter, a small piece of worm body was excised and used for DNA extraction. The remaining voucher (hologenophore) was stained in iron acetocarmine, dehydrated in ethanol, cleared in clove oil, mounted in Canada balsam and used for detailed morphological analyses. Measurements were taken from the digital photomicrographs and total mounts in Canada balsam with the use of the Levenhuk C1400 NG, Levenhuk ToupView image analysis software, V.3.5 and the QuickPHOTO CAMERA 2.3 image analysis software. All measurements in the descriptions and tables are in μm. For the description of morphological characters, we followed the terminology of Niewiadomska (2002); for anterior and posterior parts of body, we used the terms 'prosoma' and 'opisthosoma' proposed by Achatz *et al.* (2021). Morphometric variables were used as in Dubois (1970) and Shigin (1993). The voucher specimens were deposited in the Helminthological Collection of Karelian Research Centre RAS, Petrozavodsk, Russia (nos. DB1LC26 and DB2LC26) and in the Helminthological Collection of the Institute of Parasitology (IPCAS, D-829, three hologenophores; D-845, two vials with cercariae), Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic. The morphology of cercariae was studied on live specimens under a light microscope and series of photomicrographs (of three specimens) were taken with a digital camera

https://doi.org/10.1017/S0031182021002092 Published online by Cambridge University Press

Table 1. Summary data for the sequences of *Diplostomum baeri* generated in the current study and used for morphological analyses

Host	Stage	Country	Voucher no.	Isolate	GenBank ID		
					*cox*1	ITS	28S
Larus canus	Adult	Russia	DB1LC26	D224	OK632471	OK631872	–
L. canus	Adult	Russia	DB2LC26	D411	OK632472	–	–
L. canus	Adult	Russia	IPCAS D-829	AF465	OK632473	–	–
L. canus	Adult	Russia	IPCAS D-829	AF466	OK632474	OK631873	OK631869
L. canus	Adult	Russia	IPCAS D-829	AF467	OK632475	–	OK631870
R. balthica	Cercariae	Ireland	IPCAS D-845	AF290	OK632476	OK631874	–
R. balthica	Cercariae	Ireland	IPCAS D-845	AF291	OK632477	OK631875	OK631871

on Olympus BX51 to obtain measurements with the aid of QuickPHOTO CAMERA 2.3 image analysis software. Cercariae were identified following description and DNA sequences of Selbach *et al.* (2015). For abbreviations and explanations of characters measured, see Table 2.

DNA amplification and sequencing

Genomic DNA was isolated from an excised part of adult worms and 20–25 ethanol-fixed cercariae following the protocol described by Antar *et al.* (2015) or using DNA-Extran kits (Synthol, Moscow). As suggested in Blasco-Costa *et al.* (2016), we used ribosomal and mitochondrial molecular genetic markers in the current study.

The *cox*1 region of the mtDNA was amplified using the primers Dice1F (forward: 5′-ATTAACCCTCACTAAATTWCNTTRGATCATAAG-3′) and Dice14R (reverse: 5′-TAATACGACTCACTATACCHACMRTAAACATATGATG-3′) (van Steenkiste *et al.*, 2015), or Plat-diploCOX1F (5′-CGTTTRAATTATACGGATCC-3′) and Plat-diploCOX1R (5′-GCATAGTAATMGCAGCAGC-3′) (Moszczynska *et al.*, 2009).

The 28S region of the rDNA was amplified using the primers digl2 (5′-AAGCATATCACTAAGCGG-3′) and 1500R (5′-GCTATCCTGAGGGAAACTTCG-3′) (Snyder and Tkach, 2001); additional internal primers ECD2 (5′-CCTTGGTCCGTGTTTCAAGACGGG-3′) (Littlewood *et al.*, 1997) and 300F (5′-CAAGTACCGTGAGGGAAAGTTG-3′) (Littlewood *et al.*, 2000) were used for sequencing. The ITS1-5.8S-ITS2 region of the rDNA was amplified using the primers D1 (F) (5′-AGGAATTCCTGGTAAGTGCAAG-3′) and D2 (R) (5′-CGTTACTGAGGGAATCCTGGT-3′) (Galazzo *et al.*, 2002).

Polymerase chain reactions (PCRs) (25 μL) included 12.5 μL of MyFi™ mix, 1.25 μL of each oligonucleotide primer (10 mM), 8 μL of H_2O and 1.5 μL of genomic DNA. Cycling parameters of PCR amplification were the same as in van Steenkiste *et al.* (2015) and Moszczynska *et al.* (2009) for *cox*1, Tkach *et al.* (2003) for 28S and Galazzo *et al.* (2002) for ITS1-5.8S-ITS2. The amplified products were purified with the Exo-SAP-IT Kit™ Express Reagent (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) following the manufacturer's instructions, sequenced using the same primers of PCRs and the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems-Perkin Elmer, Waltham, Massachusetts) in a MegaBACE sequencer (GE Healthcare Life Sciences). Contiguous sequences were assembled using Geneious v. 11 (Biomatters, Auckland, New Zealand) and deposited in GenBank.

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 (Table 1) (*cox*1, 473 and 836 nucleotides (nt); ITS1-5.8S-ITS1, 1250 nt; 28S, 1300 nt) were aligned with the representative sequences of *Diplostomum* spp. ($n = 38$ species/lineages) previously reported from Europe (Supplementary Table S1) with MUSCLE (Edgar, 2004) implemented in Geneious v.11. The ITS1-5.8S-ITS2 sequence of a single species, *D. adamsi* (syn. *D. baeri*; AY123042; Galazzo *et al.*, 2002) reported from North America was included in analyses due to its relevance to the current study. Two datasets (*cox*1 and ITS-5.8S-ITS2) were prepared. The *cox*1 alignment (356 nt) comprised of seven novel sequences and 35 sequences of the representatives of *Diplostomum* from GenBank. The ITS-5.8S-ITS alignment (960 nt) included four novel sequences and 24 sequences of *Diplostomum* spp. from GenBank. Sequences of *Tylodelphys clavata* (von Nordmann, 1832) (JX986908, *cox*1; JQ665459, ITS1-5.8S-ITS2) (Digenea: Diplostomidae) were used as the outgroup based on the results of the phylogenetic analyses of Georgieva *et al.* (2013).

To assess the phylogenetic relationships of *Diplostomum* spp., we used Bayesian inference (BI) and maximum likelihood (ML) analyses for both datasets. Prior to analyses, the best-fitting model was estimated with jModelTest 2.1.2 (Darriba *et al.*, 2012). This was the general time-reversible model incorporating invariant sites and gamma distributed among-site rate variations (GTR + I + G) for both alignments. BI analyses were conducted using MrBayes software (ver. 3.2.3) (Ronquist *et al.*, 2012). Markov chain Monte Carlo 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 tree. ML analyses were conducted using PhyML version 3.0 (Guindon *et al.*, 2010) run on the ATGC bioinformatics platform (http://www.atgc-montpellier.fr/). Nodal support was estimated by performing 100 bootstrap pseudoreplicates. FigTree ver. 1.4 software (Rambaut, 2012) was used to visualize the trees. Genetic distances (uncorrected *P*-distance) were calculated in MEGA ver. 6. The unique *cox*1 haplotypes collected in Ireland and Russia in the current study and in Germany and Spain in the previous studies were identified with DnaSP (Rozas *et al.*, 2003). A haplotype network was reconstructed using the median-joining method in PopART software (Population Analysis with Reticulate Trees, http://popart.otago.ac.nz).

Results

Description of the molecular voucher material

Diplostomidae Poirier, 1886
Diplostomum Nordmann, 1832
Diplostomum baeri Dubois, 1937
Synonym: *Diplostomum* sp. Clade Q of Georgieva *et al.* (2013).

https://doi.org/10.1017/S0031182021002092 Published online by Cambridge University Press

Table 2. Metrical data of adults of *Diplostomum* spp.

Species	*D. baeri*	*D. baeri*	*D. baeri*	*D. baeri*	*Diplostomum volvens*	*D. volvens*
Source	Our material	Dubois (1937, 1938, 1970)	Niewiadomska and Kiseliene (1990)	Galazzo *et al.* (2002)	Shigin (1977, 1993)	Shigin (1977, 1993)
Locality	Lake Kostomukshskoye, Karelia, Russia	Lac Léman, Switzerland	Lithuania	Canada	Rybinsk reservoir, Russia	Rybinsk reservoir, Russia
Host	*L. canus*	*Stercorarius parasiticus*, *S. longicaudus*	Exp. chicken	*Larus delawarensis* (exp.)	*Larus ridibundus* (exp., 9 days p.i.)	*L. ridibundus* (exp., 5 days p.i.)
No.	*n* = 5		*n* = 1	*n* = 10	*n* = 21	*n* = 100
Total body length	1592–1955 (1758)	830–1780	1536	1660–2110 (1870)	1782–2376 (2016)	1617–1980 (1866)
Prosoma length	954–1076 (1008)	500–930	851	810–1020 (930)	875–1888 (988)	924–1221 (1089)
Prosoma width	487–590 (560)	260–600	355	370–420 (400)	425–575 (491)	500–625 (551)
Opisthosoma length	638–904 (767)	310–850	740	810–1100 (950)	908–1188 (1019)	677–891 (777)
Opisthosoma width	424–526 (464)	300–520	318	320–430 (380)	394–487 (432)	450–588 (501)
Pseudosucker length	79–182 (108)	75–95	68	–	90–125 (104)	–
Pseudosucker width	49–123 (68)	–	–	–	50–70 (57)	–
Oral sucker length	57–99 (85)	50–85	81	48–84 (77)	60–95 (78)	70–90 (80)
Oral sucker width	69–90 (80)	72–100	74	84–108 (93)	70–90 (82)	80–95 (89)
Pharynx length	56–79 (68)	60–96	61	55–72 (62)	65–85 (77)	65–85 (76)
Pharynx width	46–54 (50)	40–67	51	57–72 (62)	55–70 (62)	55–67 (61)
Ventral sucker length	76–104 (90)	60–103	88	84–104 (93)	80–100 (88)	70–90 (81)
Ventral sucker width	74–127 (107)	63–108	–	84–105 (94)	80–100 (92)	75–100 (88)
Holdfast organ length	193–253 (222)	145–270	162	192–300 (240)	200–312 (243)	263–388 (332)
Holdfast organ width	217–294 (251)	120–225	170	144–211 (173)	238–325 (266)	288–365 (327)
Anterior testis length	154–302 (223)	110–235	185	168–365 (234)	250–388 (310)	163–275 (219)
Anterior testis width	235–380 (316)	250–360	244	288–360 (286)	275–363 (325)	163–313 (234)
Posterior testis length	118–272 (205)	115–250	221	192–444 (294)	275–400 (343)	250–338 (280)
Posterior testis width	251–427 (325)	280–435	310	240–444 (353)	363–463 (411)	365–550 (436)
Ovary length	84–311 (166)	90–105	88	96–144 (118)	100–225 (146)	113–213 (153)
Ovary width	97–188 (152)	105–155	96	96–144 (131)	75–150 (103)	88–138 (112)
Egg length	99–109 (105)	96–113	No eggs	–	95–110 (104)	115–125 (118)
Egg width	63–77 (69)	60–77	–	60–67 (63)	60–75 (67)	52–60 (56)
Ratios						
PR/OP length	1.16–1.50 (1.33)	–	1.15	–	0.9–1.13 (0.98)	1.22–1.51 (1.41)

OP/PR length	0.67–0.86 (0.76)	0.53–1.00	–	0.80–1.09 (1.01)	–	–
PR/OP width	1.12–1.26 (1.21)	–	1.12	–	1.05–1.21 (1.13)	1.05–1.19 (1.10)
VS/OS length	0.90–1.33 (1.04)	–	1.09	–	–	–
VS/OS width	0.91–1.52 (1.29)	–	1.19	–	–	–
OS/PH length	1.02–1.42 (1.24)	0.96–1.27	–	–	OSL < PHL	–
PR/HO length	4–5 (5)	2–3	–	–	–	–
Distances						
PTR length	93–306 (218)	–	–	–	263–463 (352)	213–350 (266)
VS-DIST	499–654 (575)	–	–	–	400–550 (464)	413–563 (488)
HO-DIST	570–746 (655)	–	–	–	450–695 (566)	516–600 (566)
VIT-DIST	356–559 (478)	–	–	–	275–431 (346)	338–450 (389)
AT-DIST	497–531 (514)	–	–	–	896–1226 (1038)	677–903 (792)
OV-DIST	25–102	–	–	–	–	–
VS-HO-DIST	0–114	10–63	–	–	45	12–100 (39)
GP-DIST	63–163 (110)	50–90	–	–	–	–
Proportions of TB length						
PR/TB length %	54–60 (57)	–	55	–	–	–
PTR length %	13–42 (29)	60–75/100	–	–	25.7–41.2 (34.4)	28.1–44.2 (34.1)
VS-DIST %	47–62 (57)	47–59/100	61	38–56 (46)	47.5	–
HO-DIST%	60–77 (65)	55–76/100	–	50–68 (59)	–	–
VIT-DIST %	34–54 (48)	33–55/100	–	–	38.4	–
OV-DIST %	3–13 (9)	0–8/100	–	2–16 (7)	–	–

PR, prosoma; OP, opisthosoma; VS, ventral sucker; OS, oral sucker; PH, pharynx; HO, holdfast organ; TB, total body. PTR, post-testicular region; VS-DIST, distance of centre of ventral sucker from anterior margin of prosoma; HO-DIST, distance of anterior margin of holdfast organ from anterior margin of prosoma; VIT-DIST, distance between front level of vitelline follicles and anterior margin of prosoma; AT-DIST, distance of anterior margin of anterior testis from posterior margin of opisthosoma; OV-DIST, distance of ovary from anterior margin of opisthosoma, VS-HO-DIST, distance between ventral sucker and holdfast organ; GP-DIST, distance of genital pore from posterior margin of opisthosoma.

Host: *Larus canus canus* Linnaeus.
First intermediate host: *Radix balthica* (Linnaeus).
Locality: Kostomukshskoye Lake, Karelia, northwest Russia (64°39′34″N, 30°48′10″E) (adults); Lough Corrib, Ireland (53° 20′24.3″N, 9°05′28.6″W) (cercariae).
Site in host: small intestine and duodenum in bird (adult stage); hepatopancreas in snail (larval stage).
Infection rates: prevalence, 1 of 1 bird; 2 of 573 snails (0.35%); intensity, 5 specimens per bird.
Material: two voucher specimens (DB1LC26, DB2LC26), three hologenophores (IPCAS D-829).
Representative DNA sequences: 28S, three sequences (OK631869–OK631871); ITS1-5.8S-ITS2, four sequences (OK631872–OK631875), *cox*1, seven sequences (OK632471–OK632477).

Adult *(Fig. 1, Table 2)*

[Description based on five specimens.] Body distinctly bipartite, partly retroflexed, i.e. prosoma and opisthosoma usually forming dorsally a sharp angle (Fig. 1B). Prosoma elongate-oval, dorsoventrally flattened, anterior extremity tapered and trilobed, with maximum width at the level of holdfast organ, longer than opisthosoma, posterior rim of prosoma elevated ventrally, slightly forming a cup. Opisthosoma cylindrical, with stout, rounded posterior extremity; slightly narrower anteriorly, maximum width at its mid-level. Tegument smooth.

Oral sucker small, weakly muscular, ventro-subterminal, subspherical. Pseudosuckers well developed, posterolateral to oral sucker, reaching back to the level of pharynx. Prepharynx very short. Pharynx well-developed, small. Oesophagus shorter than pharynx. Intestinal bifurcation in first quarter of prosoma. Caeca long, narrow, terminate blindly close to posterior extremity of opisthosoma. Ventral sucker weakly muscular, transversely oval to oval, positioned in its third quarter (Fig. 1A) or at the mid-level of prosoma (Fig. 1B); slightly larger than oral sucker (Table 2). Holdfast organ sub-globular, with median slit, posterior to ventral sucker and contiguous or separated (no further than diameter of ventral sucker).

Testes 2, large, entire, tandem, contiguous or overlapping, in mid-part of opisthosoma. Anterior testis asymmetrical, with one developed lappet. Posterior testis symmetrical, with two lappets turned ventrally. Seminal vesicle coiled, posttesticular, median, contiguous with posterior testis. Ovary subspherical to transversely oval, entire, sub-median, pretesticular, contiguous with anterior testis; close to anterior extremity of opisthosoma or in its first quarter. Vitellarium follicular, vitelline follicles numerous, small; in prosoma most dense in its posterior part at the level of holdfast organ, anteriorly protruding in three or four branches on each side of ventral sucker and extending in front of it. In opisthosoma, vitelline follicles most dense in its anterior and posterior extremity; confluent in front of testes, forming a ventral field at the level of both testes and being also confluent in posttesticular region. Uterus short, with few (1–7), large eggs. Copulatory bursa small, hermaphroditic duct short, opening dorsosubterminally (Fig. 1B). Excretory vesicle not observed.

Remarks

The present material agrees well with the diagnosis of the genus *Diplostomum* of Niewiadomska (2002) in the presence of a distinctly bipartite body, a trilobate anterior extremity with pseudosuckers, vitelline follicles distributed in prosoma and opisthosoma, tandem testes with the anterior one being asymmetrical, a non-protrusible copulatory bursa and ovary being pretesticular. *Diplostomum baeri* was originally described by Dubois (1937) ex *Stercorarius longicaudus* Vieillot and *Stercorarius parasiticus* (Linnaeus) from Lac Léman in Switzerland; the description of Dubois (1937) was very brief, thus the species was redescribed by Dubois (1938, 1970) and provided with drawings. The morphology of the present material of adults agrees well with the description of *D. baeri* of Dubois (1938, 1970) in the ratio of the opisthosoma to the prosoma length (OPL/PRL = 0.67–0.86 *vs* 0.53–1.00), i.e. the prosoma is always longer than the opisthosoma as stated in the description by Dubois (1970), although the range of the ratio given by him indicates that the body segments can be up to the same length. We infer that the typical character for this species is that the prosoma is longer than the opisthosoma or can be nearly equal in length. Furthermore, our material agrees in the vitelline follicles extending in front of the ventral sucker, in the elongate-oval shape of the prosoma being trilobed anteriorly and exhibiting the maximum width at the level of holdfast organ, in the position of the pseudosuckers (posterolateral to oral sucker) and in the ovary being close to the anterior extremity of the opisthosoma. The body dimensions in our material and in *D. baeri* Dubois, 1937 are very similar and overlap, including the large, not too numerous eggs; only the minima for total body length and length and width of both prosoma and opisthosoma in our material exhibit higher values, while the prosoma in our material is longer (954–1076 *vs* 500–930 μm) than that in *D. baeri* of Dubois (1970) (Table 2). Because of the correspondence in morphology, dimensions of body and internal organs and ratios of dimensions we consider our material of adults identical with *D. baeri* Dubois, 1937.

In Canada, adults under the name *D. baeri* were obtained experimentally *ex Larus delawarensis* Ord by Galazzo *et al.* (2002). However, Schwelm *et al.* (2021) re-classified this material as *D. adamsi* based on morphology of adults and the microhabitat of metacercariae (located in the peripheral retina). We agree with this concept, because the sequences (ITS1-5.8S-ITS2) of the adult worms obtained by Galazzo *et al.* (2002) do not match ours (see below). Our material of *D. baeri* resembles that of Galazzo *et al.* (2002) in dimensions, of which almost all overlap (Table 2). However, our adult worms differ in the OPL/PRL ratio [0.67–0.86 (0.76) *vs* 0.80–1.09 (1.01)], and although there is a slight overlap, still it indicates that the worms of Galazzo *et al.* (2002) have a prosoma shorter than opisthosoma, which is never the case in our material, neither it is in the American subspecies, *D. baeri bucculentum* Dubois & Rausch, 1948 described from Michigan, USA (Dubois, 1970). Moreover, the worms of *D. adamsi* from Canada differ in their biology, as the adults were obtained from metacercariae recovered from the vitreous humour of eyes of *Perca flavescens* (Mitchill) by Galazzo *et al.* (2002) or retina (see Schwelm *et al.*, 2021), while the European *D. baeri* occurs in the eye lens of cyprinid fishes (see below).

The other most similar species to our material, and also to *D. baeri* of Dubois (1970), is *D. volvens*. This species was characterized by Shigin (1977), who at first recognized it under the name *D. yogenum* (Cort and Brackett, 1937), based on the description by Cort and Brackett (1937) of *Cercaria yogena ex Stagnicola emarginata* (Say) and *Stagnicola palustris elodes* (Say) from Michigan, USA. Later Shigin (1993) considered *D. yogenum* a synonym of *D. volvens*. The present material resembles *D. volvens* of Shigin (1977, 1993) in the shape of the whole body and prosoma, and in dimensions which are very similar (Table 2). However, the main difference is in the prosoma being longer than opisthosoma in our material *vs* prosoma being shorter or of similar length as opisthosoma in *D. volvens* [PRL/OPL ratios: 1.16–1.50 (1.33) *vs* 0.9–1.13 (0.98)/1.22–1.51 (1.41)]. Another similar worm is the one presented as *D. baeri* by Niewiadomska and Kiseliene (1990), who obtained one adult experimentally from chicken, the material originating from cercariae in Lithuania. Our material and *D. volvens* of Shigin (1977, 1993)

https://doi.org/10.1017/S0031182021002092 Published online by Cambridge University Press

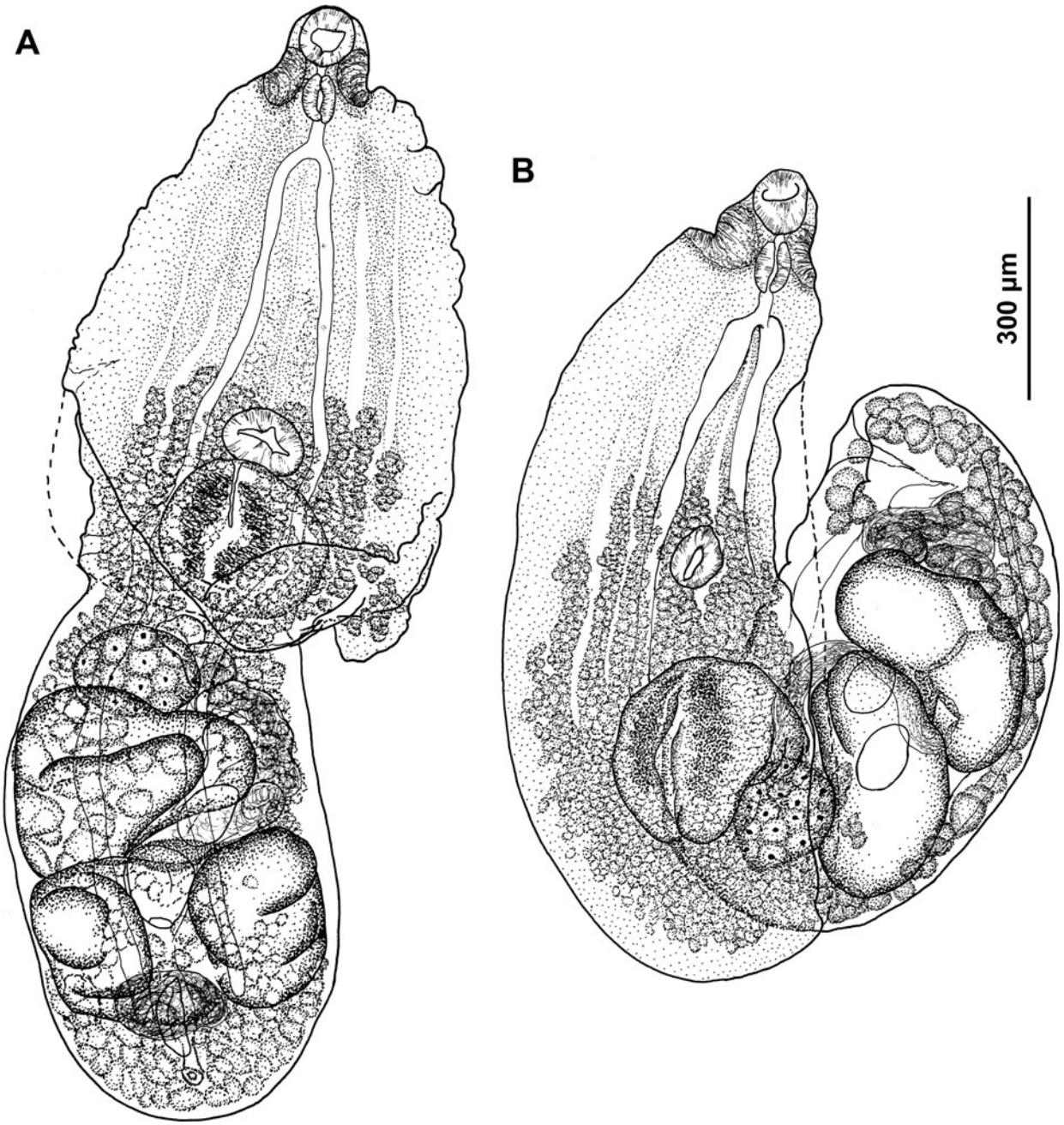

Fig. 1. Adult *Diplostomum baeri* ex *Larus canus* (IPCAS D-829): (A) ventral view and (B) partly retroflexed specimen, ventral view of prosoma, lateral view of opisthosoma.

resemble to the specimen of Niewiadomska and Kiseliene (1990) in shape of prosoma and opisthosoma, in the vitelline follicles reaching in front of ventral sucker, in the prosoma being longer than the opisthosoma, and in similar dimensions (Table 2). However, the cercariae and metacercariae (found outside the eye lens) described and linked to that adult by Niewiadomska and Kiseliene (1990) are clearly different from our material of cercariae and from metacercariae characterized by Pérez-del-Olmo *et al.* (2014) as *Diplostomum* sp. clade Q (see below for details). Another material collected in Ireland and identified as *D. volvens* is that of McKeown and Irwin (1995), who did not provide comparable measurements, however, from their figure it is clear that the prosoma is shorter than the opisthosoma.

From *D. mergi* Dubois, 1932 which is similar in body shape, our material differs in the prosoma to opisthosoma length ratio (PRL/OPL), which is lower than in *D. mergi* [1.16–1.50 *vs* 1.06–2.43 of Dubois (1970), 1.83–2.52 of Shigin (1993)] and in showing higher minima for body size and internal organs. From *Diplostomum nordmanni* Shigin & Shapirov, 1986, which has a similar PRL/OPL ratio (1.09–1.46) and was found in Karelia and is typically occurring in larids (Shigin, 1993), our material differs in body shape (stout *vs* slender) and in being smaller (mean: 1758 *vs* 2443 µm); moreover, *D. nordmanni* has a smaller holdfast organ (193–253 *vs* 120–175).

Cercariae (Fig. 2)

Remarks

Cercariae of our material from Ireland are genetically identical (see below) and agree well with the morphology of *Diplostomum* sp. clade Q described by Selbach *et al.* (2015), in the presence of the same pattern of body spination, i.e. number of pre-oral spines (Fig. 2C), shape and number of rows of post-oral spines, number of

https://doi.org/10.1017/S0031182021002092 Published online by Cambridge University Press

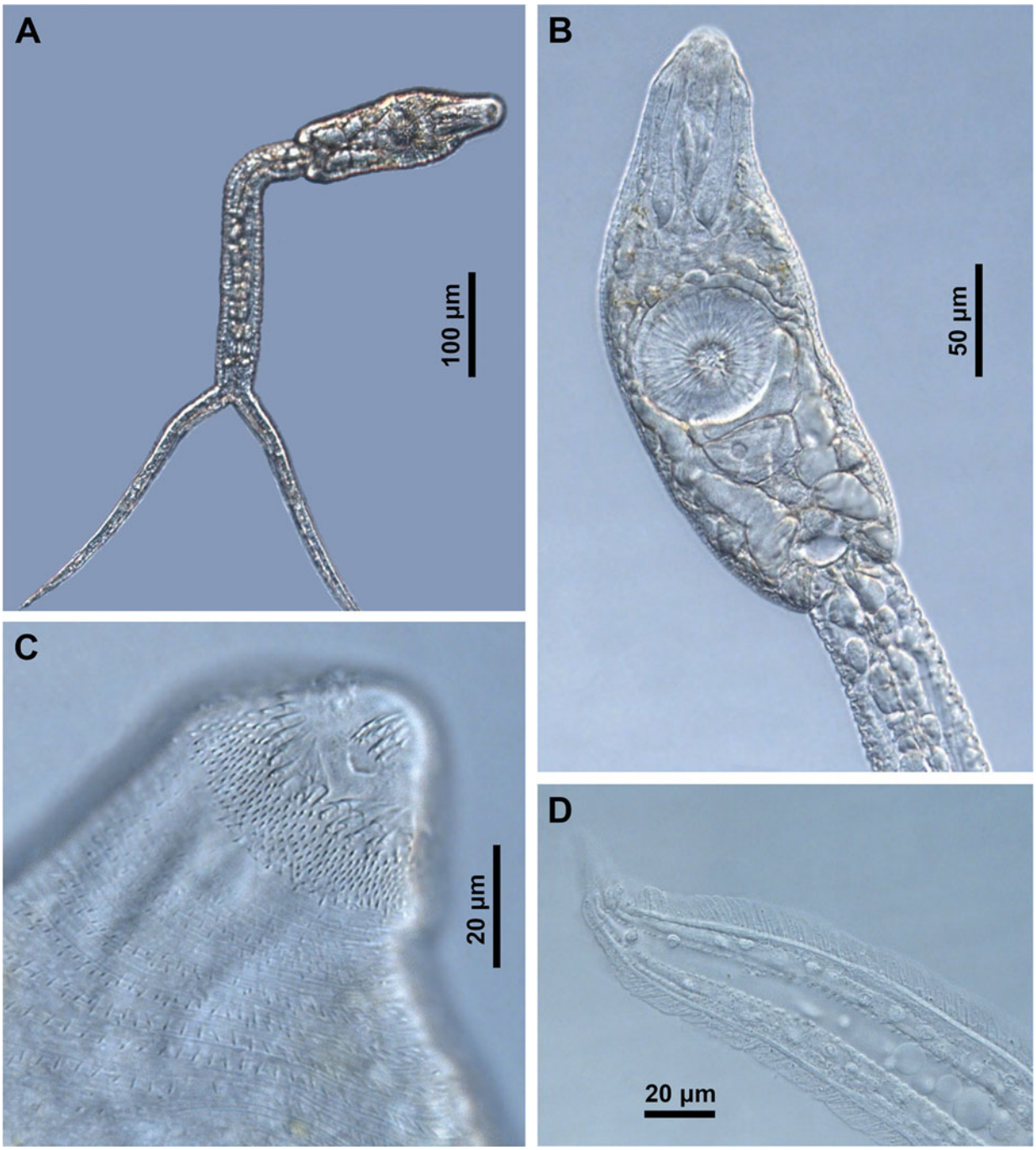

Fig. 2. Cercaria of *Diplostomum baeri ex Radix balthica* (IPCAS D-845): (A) total view with resting position; (B) ventral view of body; (C) anterior extremity with pre-oral and post-oral tegumental spines, ventral view and (D) detail of fish-fin like fin-fold on furca.

transverse rows on body, non-converging lateral spined fields posterior to ventral sucker, the number of rows and spines on ventral sucker, the spination on tail stem and furca and the fish-fin like fin-fold on furca (Fig. 2D). We newly add the information on the resting position of the cercariae, which is characteristic with a bent tail stem and widespread furcae (Table 3, Fig. 2A). As stated in Selbach *et al.* (2015), their cercariae of *Diplostomum* sp. clade Q agree in part with the description of *D. spathaceum* of Niewiadomska and Kiseliene (1994), however, they differ in tail stem and furca spination (spined *vs* devoid of spines) and in presence of a fin-fold on furcae, with which we agree.

The current cercariae clearly differ in morphology from those assigned to *D. volvens*, i.e. *C. yogena* of Cort and Brackett (1937), *D. yogenum* of Shigin (1977) and *D. volvens* of McKeown and Irwin (1995), and to *D. baeri* as presented by Niewiadomska and Kiseliene (1990); the most striking difference is the resting position (Fig. 2A, tail stem bent in our material *vs* tail stem straight in all four descriptions), furcae with a clearly visible fin-fold (Fig. 2D) *vs* no fin-fold, a tail stem with caudal bodies with incised contours *vs* tail stem with smooth caudal bodies; also, the arrangement of tegumental spines on body differs (Table 3).

Phylogenetic results

Fourteen novel sequences including seven *cox*1 (473 and 836 nt), four ITS1-5.8S-ITS2 (1250 nt) and three 28S (1300 nt) were obtained from seven isolates (Table 1). The 28S sequences were identical.

A phylogram resulted from BI and ML analyses based on the *cox*1 sequences generated in the current study (Table 1) and 35

https://doi.org/10.1017/S0031182021002092 Published online by Cambridge University Press

Table 3. Distinctive characters of cercariae of *D. baeri* and cercariae originally associated with it

Parasite species	*D. baeri*	*Diplostomum* sp. clade Q	*D. baeri*	*Cercaria yogena*	*Diplostomum yogenum* (syn. of *D. volvens*)	*D. volvens*
Source	Current study	Selbach *et al*. (2015)	Niewiadomska and Kiseliene (1990, 1994)	Cort and Brackett (1937)	Shigin (1977)	McKeown and Irwin (1995)
Locality	Lough Corrib, Ireland	Germany	Lithuania	North America, Michigan	Russia, Rybinsk reservoir	Ireland (obtained experimentally)
Host species	*R. balthica*	*Radix auricularia*	*Radix ovata*	*Stagnicola emarginata*, *Stagnicola palustris elodes*	*R. auricularia*	*L. stagnalis*, *R. peregra* (exp.)
Yellow pigment in body	Present	Present	–	present	present	present
Relation BL-TSL-FL	Live: BL < TSL = FL	Live: BL < TSL = FL	BL < TSL = FL	BL < TSL = FL	BL < TSL > FL	BL < TSL > FL
Relation VSW-AOW	Live: VSW > AOW	Live: VSW > AOW	VSW = AOW	VSW = AOW	VSW = AOW	–
No. of pre-oral spines (median group)	9–10 in 3 rows	9 in 3 rows	7–11 in 3 rows	12	9–11 in 3 rows	–
No. of pre-oral spines in lateral groups	Absent	Absent	–	–	–	–
No. of rows of post-oral spines	12	12	7–9	7	6–7	10
Incomplete rows of post-oral spines	Row 1 with median interruption	Row 1 with median interruption, rows 11–12 interrupted laterally	–	–	–	–
Size of post-oral spines	Spines in row 1 largest; spines in rows 1–4 larger than in other rows	First 5 spines in row 1 on both sides of median interruption largest; spines in rows 1–4 distinctly larger than in other rows	Size diminishing posteriorly	–	First rows with larger spines, size diminishing in posterior rows	–
Zone of dispersed post-oral spines	Present (wide)	Present (wide)	Present (wide)	–	–	–
Spineless area posterior to dispersed spines	Present (narrow)	Present (narrow)	–	Present (narrow)	–	–
No. of transverse rows of spines on body	10	10	10	9	10	10
Double transverse rows	Row 1	Row 1	Row 1	–	–	–
Incomplete transverse rows	Rows 5–10 discontinuous ventrally and dorsally	Rows 5–10 discontinuous ventrally and dorsally	Rows 8–10 discontinuous ventrally and dorsally	Row 9	Rows 7–10 discontinuous ventrally and dorsally	–
Transverse rows with additional spines laterally	Rows 2–5	Rows 2–3	Rows 2–8	–	–	–
Zone of dispersed spines in hind body	2 lateral non-converging fields posterior to VS	2 lateral non-converging fields posterior to VS	2 lateral fields converging posterior to VS and at posterior body extremity	2 lateral fields posterior to VS	2 lateral non-converging fields posterior to VS	–
No. of spine rows on ventral sucker	2	2	3	c. 3	3	–

(*Continued*)

Table 3. (*Continued.*)

Parasite species	*D. baeri*	*Diplostomum* sp. clade Q	*D. baeri*	*Cercaria yogena*	*Diplostomum yogenum* (syn. of *D. volvens*)	*D. volvens*
No. of spines on ventral sucker (mean)	113–117	112–116 (114)	90–130 (112)	–	105–120	–
Penetration gland-cells	Large, do not cover ends of caeca	Large, do not cover ends of caeca	Larger, cover ends of caeca	Large, do not extend laterally beyond ceca	Do not cover ends of caeca	–
Spines on tail stem	Present (2 ventral and 2 dorsal bands)	Present (2 ventral and 2 dorsal bands)	Absent	Absent	Absent	–
Spines on furcae	Present	Present	Absent	Absent	Absent	–
Fin-folds on furcae	Present (fish-fin like fin-fold)	Present (fish-fin like fin-fold)	Absent	Absent	Absent	–
No. of caudal bodies	10 pairs	10 pairs	6–7 pairs	6 pairs	5–7 (6) pairs	13 caudal bodies
Shape of caudal bodies	With incised contours	With incised contours	Entire, rounded	Entire, rounded	Entire, rounded	–
Resting position	Tail stem bent (90°), furcae forming angle 45–90°	–	Tail stem straight, furcae forming an angle of 180°	Tail stem straight, furcae forming an angle of 90°	Tail stem straight, furcae forming an angle of 180°	Tail stem straight, furcae widespread

BL, body length; BW, maximum body width; AOW, anterior organ width; VS, ventral sucker; VSW, ventral sucker width; TSL, tail stem length; FL, furca length.

sequences of *Diplostomum* spp. retrieved from GenBank (Supplementary Table S1) is presented in Fig. 3. Seven novel sequences clustered in a strongly supported clade with the sequences of the isolates previously identified as *Diplostomum* sp. clade Q collected from their first intermediate hosts, *Radix auricularia* (Linnaeus) and *R.* cf. *peregra* in Germany and second intermediate hosts, *Rutilus rutilus* (Linnaeus) and *Cyprinus carpio* Linnaeus in Germany and Spain, respectively. The sequence divergence within this clade was 0–1.4% (0–5 nt) which corresponds to the intraspecific level for members of *Diplostomum*. Sequences of the isolates that were identified to belong to the '*D. baeri*' complex *sensu* Georgieva *et al.* (2013), including *Diplostomum* sp. lineage 3 of Blasco-Costa *et al.* (2014) ('*D. baeri* Lineage 1' of Georgieva *et al.*, 2013) and *Diplostomum* sp. lineage 4 of Blasco-Costa *et al.* (2014) ('*D. baeri* Lineage 2' Georgieva *et al.*, 2013) and recently published complete mitochondrial genome sequences of metacercarial isolates from *Salmo trutta* Linnaeus identified as *D. baeri* (see Landeryou *et al.*, 2020) clustered in a distant clade. Importantly, the metacercarial isolates used for generation of mitochondrial genome were identified based solely on DNA sequence data. Within this clade, sequences of *D. baeri* of Landeryou *et al.* (2020) clustered with sequences of *Diplostomum* sp. lineage 3 of Blasco-Costa *et al.* (2014) ('*D. baeri* Lineage 1' Georgieva *et al.*, 2013). The sequence divergence between these species was 1.3–2% (6–7 nt) suggesting that they belong to the same species.

Diplostomum sp. clade Q was delineated by Georgieva *et al.* (2013) in Europe as consisting of eight sequences, five identical ITS1 sequences: two of cercarial isolates identified originally as *D. spathaceum* (AF419275 and AF419276) and one identified as *D. parviventosum* (AF419278) ex *Radix ovata* in Poland by Niewiadomska and Laskowski (2002); another cercarial isolate submitted to GenBank under the name *D. mergi* (JQ665458) but designated as *D. spathaceum* ex *R. auricularia* in Germany by Behrmann-Godel (2013); one of a metacercarial isolate submitted to GenBank as *D.* cf. *parviventosum*/*spathaceum* (JF775727) ex *R. rutilus* in Finland by Rellstab *et al.* (2011). And the three *cox*1 sequences were from *R. auricularia* (JQ639179) and from *R. rutilus* (JQ639177 and JQ639178) added by Behrmann-Godel (2013). Pérez-del-Olmo *et al.* (2014) obtained two more *cox*1 and ITS1-5.8S-ITS2 sequences (KP025770 and KP025788) for a metacercaria from the eye lens ex *C. carpio* in Spain, which also clustered with *Diplostomum* sp. clade Q; they were the first to combine their genetic data with a morphological description of the metacercaria, as the previously obtained sequences were not linked to any voucher material. Pérez-del-Olmo *et al.* (2014) assumed the questionable clade, which was most close to the '*D. mergi*' complex, could represent *D. parviventosum*. This assumption was disproved by Selbach *et al.* (2015) who by integrative taxonomy characterized cercariae of both *Diplostomum* sp. clade Q and *D. parviventosum* and proved that they differed genetically and morphologically. While cercariae of *D. parviventosum* corresponded to those described by Niewiadomska and Kiseliene (1994), cercariae of *Diplostomum* sp. clade Q did not correspond to any of their descriptions.

The 14 *cox*1 sequences (359 nt) of *D. baeri* generated in the present ($n = 7$) and previous ($n = 7$; identified as *Diplostomum* sp. clade Q) studies were collapsed into eight haplotypes (Fig. 3) including seven unique haplotypes and one haplotype shared by seven isolates collected from *L. canus* in Russia (AF465 and AF467), *R.* cf. *peregra* (KR271470 and KR271471; Locke *et al.*, 2015), *R. auricularia* (KR149554; Selbach *et al.*, 2015) and *R. rutilus* (JQ639178; Behrmann-Godel, 2013) in Germany, and from *C. carpio* in Spain (KP025770; Pérez-del-Olmo *et al.*, 2014).

Figure 4 presents the phylogram resulting from BI and ML analyses based on the ITS1-5.8S-ITS2 dataset. Four novel sequences obtained from *L. canus* in Russia and *R. balthica* in

https://doi.org/10.1017/S0031182021002092 Published online by Cambridge University Press

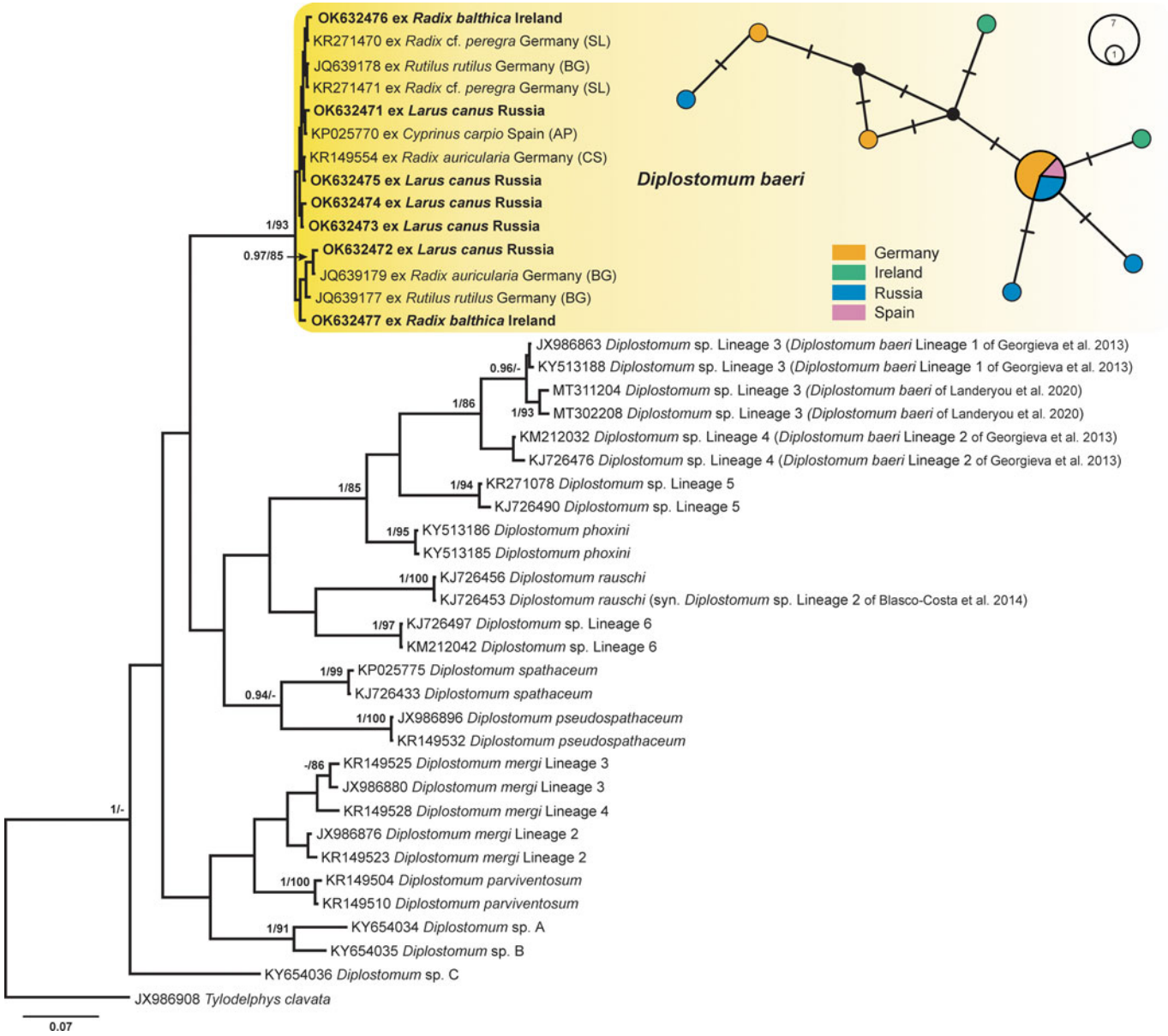

Fig. 3. BI tree for *Diplostomum* spp. based on the partial *cox*1 mtDNA sequences. Nodal support from BI and ML analyses is indicated as BI/ML; values <0.90 (BI) and <70 (ML) are not shown. The scale bar indicates the expected number of substitutions per site. The newly generated sequences are highlighted in bold. Yellow rectangle indicates the clade with published and novel sequences of *D. baeri*. Sequence names followed by abbreviations: AP, Pérez-del-Olmo *et al.* (2014); BG, Behrmann-Godel (2013); CS, Selbach *et al.* (2015); SL, Locke *et al.* (2015). Haplotype network for *D. baeri* based on published and novel *cox*1 sequences. Unsampled intermediate haplotype is represented by 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; haplotype frequency is indicated by colourless circles.

Ireland clustered with a sequence of the isolate collected from *R. auricularia* in Germany (Supplementary Table S1). Sequences in this clade were identical. Importantly, a sequence of the adult isolate identified as *D. adamsi* and reported from North America (Galazzo *et al.*, 2002) clustered in a distant clade with representatives previously considered to belong to the '*D. baeri*' complex and recently published sequences of *D. baeri* obtained from the metacercarial isolates by Landeryou *et al.* (2020). Our sequences of *D. baeri* differed from the sequence of Galazzo *et al.* (2002) by 2.7% (26 nt) suggesting that these species are not conspecific. Similar to the *cox*1 analyses, sequences of *D. baeri* by Landeryou *et al.* (2020) clustered with sequences of *Diplostomum* sp. lineage 3 (*D. baeri* lineage 1) and *Diplostomum* sp. lineage 4 (*D. baeri* lineage 2). The comparative analysis of our sequences and sequences originally assigned to the clade of *Diplostomum* sp. clade Q (Fig. 5 in Georgieva *et al.*, 2013) restricted to the ITS1 region showed no nucleotide difference.

Based on the results of the phylogenetic analyses, we conclude that (i) isolates previously reported as *Diplostomum* sp. clade Q belong to the species of *D. baeri*, (ii) species of *D. baeri* lineage 1 and *D. baeri* lineage 2 do not belong to the '*D. baeri*' complex and should be referred to as *Diplostomum* sp. lineage 3 and *Diplostomum* sp. lineage 4 as proposed by Blasco-Costa *et al.* (2014), (iii) sequence of complete mitochondrial genome and the rest of sequences obtained from isolates identified as *D. baeri* in the study of Landeryou *et al.* (2020) belong to *Diplostomum* sp. lineage 3 of Blasco-Costa *et al.* (2014) and (iv) the isolate of *D. adamsi* [originally identified as *D. baeri* by Galazzo *et al.* (2002)] represent a distinct species different from our material.

Discussion

The present new material of adult *D. baeri* corresponds well with the description of *D. baeri* Dubois, 1937 by Dubois (1937, 1938,

https://doi.org/10.1017/S0031182021002092 Published online by Cambridge University Press

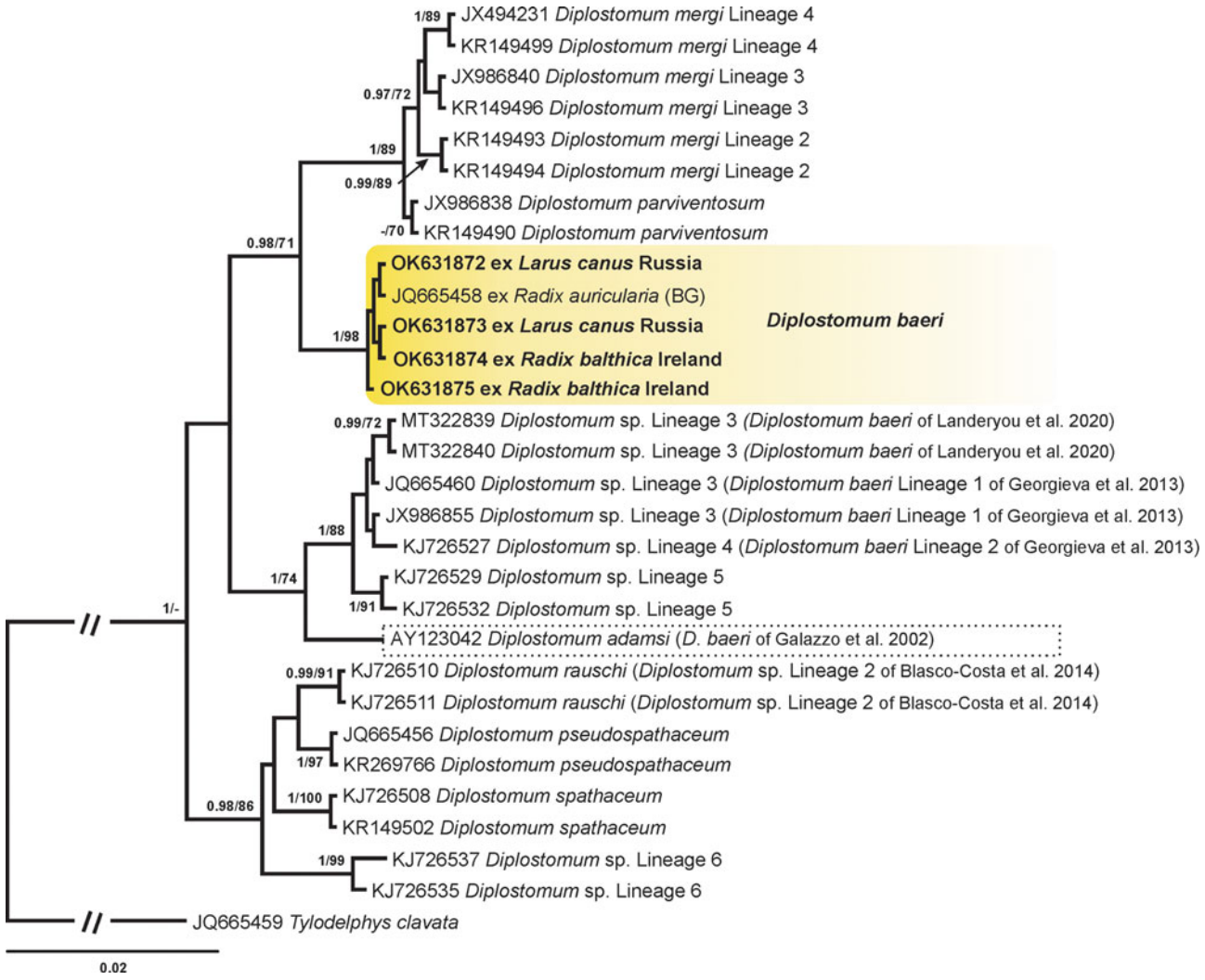

Fig. 4. BI tree for *Diplostomum* spp. based on ITS1-5.8S-ITS2 sequences. Nodal support from BI and ML analyses is indicated as BI/ML; values <0.90 (BI) and <70 (ML) are not shown. The scale bar indicates the expected number of substitutions per site. The newly generated sequences are highlighted in bold. Yellow rectangle indicates the clade with published and novel sequences of *D. baeri*. Dotted rectangle indicates the sequence of *Diplostomum adamsi* identified as *D. baeri* by Galazzo *et al*. (2002) in North America. Sequence name followed by abbreviation: BG, Behrmann-Godel (2013).

1970). Therefore, we consider *D. baeri* as a species occurring in Europe in birds *L. canus*, *S. longicaudus* and *S. parasiticus* with metacercariae using cyprinid fishes with location in the eye lens and using snails *R. auricularia* and *R. balthica* (syn. *R. ovata*) as first intermediate hosts. With molecular genetic analyses we proved that this species is identical to *Diplostomum* sp. clade Q. Also, our new material of cercariae is identical in morphology and in DNA sequences with *Diplostomum* sp. clade Q characterized by Selbach *et al.* (2015). As the most typical characters in adults of *D. baeri* we view the ratio of prosoma to opisthosoma length, which means that the prosoma is always longer than the opisthosoma or almost of the same size, but never shorter than opisthosoma; the other features are the extent of vitelline follicles not far in front of the ventral sucker and a relatively stout body. For morphological investigations always a set of more specimens is needed, as the PRL/OPL ratio can be dependent on age of the worms.

We reveal that our material of *D. baeri* cannot be assigned to *D. volvens* recognized by Shigin (1977, 1993), although both species are highly similar and their PRL/OPL ratio is overlapping, i.e. also in *D. volvens* the prosoma can be of almost the same length as the opisthosoma, however, it can be shorter, which is never true for *D. baeri*. Neither can our material be assigned to *D. baeri* of Niewiadomska and Kiseliene (1990, 1994), which has a similar PRL/OPL ratio, because the corresponding cercariae are different in morphology (resting position, presence/absence of fin-folds on furca, spination of body and tail). It is possible that both authors (Niewiadomska and Kiseliene, 1990 and Shigin, 1977, 1993) might have had the same species, or they eventually had a mixture of species, because for experimental infections, they used whole fish eyes of *Ctenopharyngodon idella* (Valenciennes), *Oncorhynchus mykiss* (Walbaum) and *Perca fluviatilis* Linnaeus (potentially there could have been simultaneous infections with specimens from different locations in eyes). This overlap in characters (PRL/OPL) signals that there is no feature reliable enough to distinguish unambiguously between *D. baeri* of Dubois (1937, 1970) and *D. baeri* of Niewiadomska and Kiseliene (1990) and *D. volvens* of Shigin (1977, 1993), and the high morphological similarity documents the difficulties in identification and proves that it is possible to reliably distinguish the species only with both molecular analyses and detailed morphological examination.

Another typical feature of *D. baeri* is the host specificity of metacercariae, which were so far found in the eye lens in cyprinid fishes (*C. carpio*, *R. rutilus*) by Rellstab *et al.* (2011), Behrmann-Godel (2013) and Pérez-del-Olmo *et al.* (2014). However, this contradicts the previous data on the life-cycle of *D. volvens* (syn. *D. baeri*), because metacercariae of *D. baeri* of Shigin (1968), *D. volvens* and *D. yogenum* were consistently

https://doi.org/10.1017/S0031182021002092 Published online by Cambridge University Press

reported from the Percidae or Lottidae and never from eye lens, i.e. from retina or between sclera (see Shigin, 1977, 1993; McKeown and Irwin, 1995). Metacercariae of *D. baeri* of Niewiadomska and Kiseliene (1990) were reported from *C. idella*, however they were obtained experimentally, and they were located outside the lens. The metacercariae differ also morphologically, those of Shigin (1968) and Niewiadomska and Kiseliene (1990) are much larger than *Diplostomum* sp. clade Q of Pérez-del-Olmo *et al.* (2014) (body size: $405 \times 205\,\mu$m and $518 \times 244\,\mu$m, respectively *vs* $229 \times 180\,\mu$m). In summary, the present results contradict those of the previous studies reporting that percids (and *P. fluviatilis* in particular) serve as the main fish hosts for *D. baeri* [*D. volvens* in view of Shigin (1986, 1993) and McKeown and Irwin (1995)] and that the metacercariae are located between the sclera and retina or even deeper in the eye, under the retina (Shigin, 1993), it indicates that the authors (Shigin, 1977, 1993; Niewiadomska and Kiseliene, 1990, Niewiadomska and Laskowski, 2002) were dealing with a species different from *D. baeri*.

The first material characterized molecularly under the name *D. baeri* was a metacercaria recovered from *P. fluviatilis* by Niewiadomska and Laskowski (2002); the morphological identification was based on the concept of Niewiadomska and Kiseliene (1990). This identification was followed by authors subsequently providing the corresponding sequences of metacercariae from outside lens of percid fishes (e.g. Rellstab *et al.*, 2011; Behrmann-Godel, 2013; Georgieva *et al.*, 2013; Landeryou *et al.*, 2020), and Schwelm *et al.* (2021) claimed that metacercariae of all species/lineages of the '*D. baeri*' complex represent non-lens-dwelling forms. However, these metacercariae identified as belonging to the '*D. baeri*' complex are genetically distant from *Diplostomum* sp. clade Q. Therefore, the sequences labelled as the '*D. baeri*' complex *sensu* Georgieva *et al.* (2013) ('*D. baeri* Lineage 1' and '*D. baeri* Lineage 2'); *sensu* Blasco-Costa *et al.* (2014) [*Diplostomum* sp. lineages 3–5 of Blasco-Costa *et al.* (2014)], *D. adamsi* and *Diplostomum* sp. lineages 2, 5–7 of Locke *et al.* (2010*a*, 2010*b*); and *sensu* Schwelm *et al.* (2021) (*D. adamsi*, *D. phoxini*, *Diplostomum* sp. lineages 3–5 of Blasco-Costa *et al.* (2014), *Diplostomum* spp. lineages 5–7 of Locke *et al.* (2015), *Diplostomum* sp. of Lebedeva *et al.* (2021) do not represent lineages of *D. baeri*.

Another important outcome of the molecular genetic analyses is that the recently published complete mitochondrial genome of *D. baeri* and the rest of sequences obtained from isolates identified as *D. baeri* in the study of Landeryou *et al.* (2020) do not correspond to our material of *D. baeri* and in fact belong to an unknown species of *Diplostomum*, *Diplostomum* sp. lineage 3 of Blasco-Costa *et al.* (2014) (see also Schwelm *et al.*, 2021). A metacercarial isolate used for construction of the mitochondrial genome was identified as *D. baeri* based on a comparison with the published *cox*1 and ITS1-5.8S-ITS2 sequences of the '*D. baeri*' complex obtained in Germany and Iceland (Georgieva *et al.*, 2013; Blasco-Costa *et al.*, 2014; Unger and Palm, 2017), however without specifying to which of the lineages it belongs.

Based on the results of ITS1-5.8S-ITS2 sequence analyses we showed that the isolate identified as *D. baeri* in Canada by Galazzo *et al.* (2002) does not cluster with the 'true' *D. baeri* (Fig. 4) and represents a different species, which is in accordance with Achatz *et al.* (2021) and Schwelm *et al.* (2021), who re-classified it as *D. adamsi*; the metacercariae were obtained from the vitreous humour of eyes of *P. flavescens* and the experimentally obtained adults differ morphologically from our material in the PRL/OPL ratio. Consequently, sequences from the North American isolates identified to belong to the '*D. baeri*' complex [*Diplostomum* sp. lineages 2, 5–7 of Locke *et al.* (2010*a*, 2010*b*)] by Blasco-Costa *et al.* (2014) represent an unknown species.

Diplostomum baeri is widely distributed in Europe, as the known range is from north/east Europe (Finland and Northwest Russia) *via* Poland, Germany and Switzerland up to most west Europe (Ireland and Spain). This was also supported by the results of the haplotype analysis. Out of eight haplotypes of *D. baeri* identified, one was shared between isolates from all three hosts of species life-cycle distributed in Germany, Russia and Spain (Fig. 3). However, despite numerous previous studies on metacercariae of *Diplostomum* from various fish species, including cyprinids, these parasites were found sporadically (Rellstab *et al.*, 2011; Behrman-Godel, 2013; Pérez-del-Olmo *et al.*, 2014). Potential previous records of metacercariae from eye lens of fishes could be masked by putting all findings under the collective name *D. spathaceum* or *Diplostomum* sp.

DNA sequence data for *Diplostomum* spp. have accumulated from around the world (Locke *et al.*, 2015), however, the majority of these data is represented by unidentified species of larval isolates, thus the identification and interpretation are not satisfactory yet and keeping vouchers is still nearly non-existent. This indicates that there is still a long way to comprehend the importance of vouchering samples including host species, and yet again, the holistic approach (which considers the parasite morphology, biology and ecology) in interpreting and evaluating data should be advocated as done by Blasco-Costa *et al.* (2016). Consequently, the taxonomy of *Diplostomum* will remain unclear until molecular data from adults are available for accurate species identification. However, at present it is crucial to collect standardized data that can be later integrated into analyses to answer questions related to systematics, biology, ecology and evolution of *Diplostomum* spp. Therefore, we extend the recommendations by Blasco-Costa *et al.* (2016) and provide a checklist including key aspects that need to be considered when studying *Diplostomum* spp. at any life-cycle stage in a molecular-taxonomic work.

(i) **What is the host species of *Diplostomum* spp.?** The accurate identification of the host organism is crucial and reliable information will aid identification of *Diplostomum* spp. Moreover, recording host species spectrum data will aid studies of ecology, host–pathogen coevolution and epidemiological aspects of *Diplostomum* spp. (see Thompson *et al.*, 2021). Patterns of host–parasite associations revealed by recent molecular genetic studies indicated higher host specificity in metacercariae than reported by previous morphology or experiment-based studies (Blasco-Costa and Locke, 2017).

(ii) **What is the microhabitat of *Diplostomum* spp. in the host?** Cercariae and adults of *Diplostomum* spp. occur in the same discrete microhabitats in their snail and bird hosts – hepatopancreas and digestive tract, respectively. Metacercariae of *Diplostomum* spp. occur in either eyes or brain of their fish host. Recent molecular genetic surveys demonstrated that metacercariae of different species in fish eye may restrict their distributions to precise locations such as retina, vitreous humour or lens (Locke *et al.*, 2010*b*; Blasco-Costa *et al.*, 2014; Schwelm *et al.*, 2021).

(iii) **What type of voucher is required for the sample?** Morphological vouchers are crucial for taxonomic identification of genetic lineages especially when multiple infections occur. The best practice in vouchering of material is described by Pleijel *et al.* (2008). For adults we suggest excising a small piece of tissue from the lateral side of forebody or hindbody without removing informative morphological structures. For larval stages which are substantially smaller than adults, electronic vouchers (E-vouchers), i.e. photomicrographs of each individual showing details of morphology should be applied. E-voucher individuals

https://doi.org/10.1017/S0031182021002092 Published online by Cambridge University Press

are used for generation of DNA sequences. Live cercariae and metacercariae are preferable for E-vouchers as some details are not visible on fixed individuals. Morphological vouchers need to be deposited in recognized parasitological collections and E-vouchers are available in Supplementary materials.

(iv) **Which morphological traits should be considered and of which life-cycle stage?** Morphology of any of the life-cycle stages of *Diplostomum* spp. is valuable and will contribute to completing the picture of the particular species. Characters in adults and cercariae are the most informative. In adults (which are most decisive for species description), the body shape and proportions (prosoma to opisthosoma ratio and sucker ratio), the extent and development of vitelline follicles, and the position of gonads are important. In cercariae, particularly the arrangement and numbers of tegumental spines are important and should be best studied with aid of scanning electron microscopy to obtain reliable counts; furthermore, proportions of body to tail stem and furca, sucker ratios and prominence of penetration gland cells should be considered as well. The metacercariae are the stages with least characters (size of body, suckers, and holdfast organ, number of excretory granules), however, together with other traits (host species, microhabitat and geographical distribution), they are an important counterpart to genetic data. Photos of live material (see above for E-vouchers) can be used for descriptions and measurements. When possible, a wider lot of specimens of one species should be examined to catch the variability.

(v) **Which molecular markers can aid identification?** The results of molecular genetic studies of *Diplostomum* depend on the use of standardized molecular markers and obtaining DNA sequences that are compatible with those available in molecular library. Thus far, the mitochondrial *cox*1 gene and the ribosomal gene cluster ITS1-5.8S-ITS2 are the most employed markers and therefore, their corresponding sequences are available for the majority of *Diplostomum* spp. in GenBank for analysis. DNA-based identification of *Diplostomum* spp. relies on phylogenetic analyses and estimation of genetic divergence (*P*-distance).

(vi) **Has the species been reported previously?** Linking morphological descriptions of *Diplostomum* spp. to the existing descriptions as well as linking novel sequences to those available in molecular library helps to identify material. The name used for the new material should correspond to the names (or provisional names) used in previous studies. If an unidentified isolate of a larval stage is novel it requires a unique name, and we recommend the numbering system as suggested by Locke *et al.* (2015).

(vii) **Does the species require morphological description?** Morphological descriptions with illustrations need to be provided for adults representing a new species of *Diplostomum* and unidentified isolates of larval stages of *Diplostomum* reported for the first time. Also, sequences of *Diplostomum* spp. published for the first time should be supplemented with a description or illustration of morphological vouchers.

(viii) **Do the names of the species and metadata in GenBank correspond to data in publication?** Publication is the main source for the identification. Currently, there are discrepancies between data in the publications and GenBank annotations (Locke *et al.*, 2015) and therefore, identification should not be based solely on the comparison of DNA sequences in GenBank but should always be checked with the original publication and recently published papers reporting on any changes in taxonomy. The most updated revision of classification and nomenclature of the species and species-level lineages of *Diplostomum* until 2021 are presented in Schwelm *et al.* (2021).

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182021002092.

Data. Data are available on request from the authors and in the Supplementary material. Newly generated sequences were deposited in GenBank with the following accession numbers: OK631869–OK631875 and OK632471–OK632477.

Acknowledgements. The authors are grateful to Dr Alexandr Artem'ev (Laboratory of Zoology, IB, KRC RAS) for help with bird identification. We thank Katie O'Dwyer (Marine and Freshwater Research Centre, Galway-Mayo Institute of Technology) for help with sampling and processing trematode material in Ireland.

Author contributions. Concept and design of the study: AF, DL, OK. Writing: AF, DL, OK. Review and editing: CP, GY. Morphological analysis: AF. Molecular analyses: CP, OK, DL. Fieldwork and data processing: AF, CP, GY, DL. All authors approved the final version of the manuscript before submission.

Financial support. The current study was funded by the Russian Ministry of Science and Education (state order 0218-2019-0075) and the Czech Grant Agency (project No. 18-18597S).

Conflict of interest. None.

References

Achatz TJ, Martens JR, Kostadinova A, Pulis EE, Orlofske SA, Bell JA, Fecchio A, Oyarzún-Ruiz P, Syrota YY and Tkach VV (2021) Molecular phylogeny of *Diplostomum*, *Tylodelphys*, *Austrodiplostomum* and *Paralaria* (Digenea: Diplostomidae) necessitates systematic changes and reveals a history of evolutionary host switching events. *International Journal for Parasitology* (in press). doi: 10.1016/j.ijpara.2021.06.002

Aksenova OV, Bolotov IN, Gofarov M, Kondakov AV, Vinarski MV, Bespalaya Y, Kolosova Y, Palatov DM, Sokolova SE, Spitsyn VM, Tomilova AA, Travina OV and Vikhrev IV (2018) Species richness, molecular taxonomy and biogeography of the radicine pond snails (Gastropoda: Lymnaeidae) in the Old World. *Scientific Reports* **8**, 11199.

Antar R, Georgieva S, Gargouri L and Kostadinova A (2015) Molecular evidence for the existence of species complexes within *Macvicaria* Gibson & Bray, 1982 (Digenea: Opecoelidae) in the western Mediterranean, with descriptions of two new species. *Systematic Parasitology* **91**, 211–229.

Behrmann-Godel J (2013) Parasite identification, succession and infection pathways in perch fry (*Perca fluviatilis*): new insights through a combined morphological and genetic approach. *Parasitology* **140**, 509–520.

Bensch S, Inumaru M, Sato Y, Lee Cruz L, Cunningham AA, Goodman SJ, Levin II, Parker PG, Casanueva P, Hernández MA, Moreno-Rueda G and Rojo MA (2021) Contaminations contaminate common databases. *Molecular Ecology Resources* **21**, 355–362.

Blasco-Costa I and Locke SA (2017) Life history, systematics and evolution of the Diplostomoidea Poirier, 1886: progress, promises and challenges emerging from molecular studies. In Rollinson D and Stothard JR (eds), *Advances in Parasitology*, Vol. **98**. UK: London: Academic Press, pp. 167–225.

Blasco-Costa I, Faltýnková A, Georgieva S, Skírnisson K, Scholz T and Kostadinova A (2014) Fish pathogens near the Arctic circle: molecular, morphological and ecological evidence for unexpected diversity of *Diplostomum* (Digenea: Diplostomidae) in Iceland. *International Journal for Parasitology* **44**, 703–715.

Blasco-Costa I, Cutmore SC, Miller TL and Nolan MJ (2016) Molecular approaches to trematode systematics: 'best practice' and implications for future study. *Systematic Parasitology* **93**, 295–306.

Bridge PD, Roberts PJ, Spooner BM and Panchal G (2003) On the unreliability of published DNA sequences. *New Phytologist* **160**, 43–48.

Cort WW and Brackett S (1937) A new strigeid cercaria which produces a bloat disease in tadpoles. *Journal of Parasitology* **23**(Suppl.), S563–S564.

Darriba D, Taboada GL, Doallo R and Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods* **9**, 772–772.

Dubinina MN (1971) *Parasitological Study of Birds*. Leningrad: Nauka (in Russian).

Dubois G (1937) Sur quelques strigéidés. Notes préliminaires. *Revue Suisse de Zoologie* **44**, 391–396.

Dubois G (1938) Monographie des Strigeida (Trematoda). *Mémoires de la Société des Sciences Naturelles de Cherbourg* **6**, 1–535.

Dubois G (1970) Synopsis des Strigeidae et des Diplostomatidae (Trematoda). *Mémoires de la Société des Sciences Naturelles de Cherbourg* **10**, 259–727.

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**, 1792–1797.

Galazzo DE, Dayanandan S, Marcogliese DJ and McLaughlin JD (2002) Molecular systematics of some North American species of *Diplostomum* (Digenea) based on rDNA-sequence data and comparisons with European congeners. *Canadian Journal of Zoology* **80**, 2207–2217.

Georgieva S, Soldánová M, Pérez-del-Olmo A, Dangel RD, Sitko J, Sures B and Kostadinova A (2013) Molecular prospecting for European *Diplostomum* (Digenea: Diplostomidae) reveals cryptic diversity. *International Journal for Parasitology* **43**, 57–72.

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W and Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology* **59**, 307–321.

Heneberg P, Sitko J and Těšínský M (2020) Paraphyly of *Conodiplostomum* Dubois, 1937. *Parasitology International* **76**, 102033.

Hey J, Waples RS, Arnold ML, Butlin RK and Harrison RG (2003) Understanding and confronting species uncertainty in biology and conservation. *Trends in Ecology and Evolution* **18**, 97–603.

Hoogendoorn C, Smit NJ and Kudlai O (2020) Resolution of the identity of three species of *Diplostomum* (Digenea: Diplostomidae) parasitising freshwater fishes in South Africa, combining molecular and morphological evidence. *International Journal for Parasitology: Parasites and Wildlife* **11**, 50–61.

Karvonen A and Marcogliese DJ (2020) Diplostomiasis (*Diplostomum spathaceum* and related species). In Woo PTK, Leong J-A and Buchmann K (eds), *Climate Change and Infectious Fish Diseases*. Wallingford: CABI, pp. 434–456.

Karvonen A, Seppälä O and Valtonen ET (2004) Eye-fluke induced cataract formation in fish: quantitative analysis using and ophthalmological microscope. *Parasitology* **129**, 473–478.

Kudlai O, Oros M, Kostadinova A and Georgieva S (2017) Exploring the diversity of *Diplostomum* (Digenea: Diplostomidae) in fishes from the river Danube using DNA mitochondrial barcodes. *Parasites & Vectors* **10**, 592.

Landeryou T, Ropiquet A, Kett SM, Wildeboer D and Lawton SP (2020) Characterization of the complete mitochondrial genome of *Diplostomum baeri*. *Parasitology International* **79**, 102166.

Lebedeva DI, Chrisanfova GG, Ieshko EP, Guliaev AS, Yakovleva GA, Mendsaikhan B and Semyenova SK (2021) Morphological and molecular differentiation of *Diplostomum* spp. metacercariae from brain of minnows (*Phoxinus phoxinus* L.) in four populations of northern Europe and East Asia. *Infection, Genetics and Evolution* **92**, 104911.

Littlewood DT, Rohde K and Clough KA (1997) Parasite speciation within or between host species? – Phylogenetic evidence from site-specific polystome monogeneans. *International Journal for Parasitology* **27**, 1289–1297.

Littlewood DT, Curini-Galletti M and Herniou EA (2000) The interrelationships of Proseriata (Platyhelminthes: Seriata) tested with molecules and morphology. *Molecular Phylogenetics and Evolution* **16**, 449–466.

Locke SA, McLaughlin JD and Marcogliese DJ (2010a) 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. *Molecular Ecology* **19**, 2813–2827.

Locke SA, McLaughlin JD, Dayanandan S and Marcogliese DJ (2010b) Diversity, specificity and evidence of hybridization in *Diplostomum* spp. metacercariae in freshwater fishes is revealed by DNA barcodes and ITS sequences. *International Journal for Parasitology* **40**, 333–343.

Locke SA, Al-Nasiri FS, Caffara M, Drago F, Kalbe M, Lapierre AR, McLaughlin JD, Nie P, Overstreet RM, Souza GTR, Takemoto RM and Marcogliese DJ (2015) Diversity, specificity and speciation in larval Diplostomidae (Platyhelminthes: Digenea) in the eyes of freshwater fish, as revealed by DNA barcodes. *International Journal for Parasitology* **45**, 841–855.

Locke SA, Drago FB, Núñez V, Souza GTRE and Takemoto RM (2020) 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. *Parasitology Research* **119**, 2129–2137.

McKeown CA and Irwin SWB (1995) The life cycle stages of three *Diplostomum* species maintained in the laboratory. *International Journal for Parasitology* **25**, 897–906.

Moszczynska A, Locke SA, McLaughlin JD, Marcogliese DJ and Crease TJ (2009) Development of primers for the mitochondrial cytochrome *c* oxidase I gene in digenetic trematodes (Platyhelminthes) illustrates the challenge of barcoding parasitic helminths. *Molecular Ecology Resources* **9**, 75–82.

Niewiadomska K (2002) Family Diplostomidae Poirier 1886. In Gibson DI, Jones A and Bray RA (eds), *Keys to the Trematoda*. London, Wallingford: CABI, Natural History Museum, pp. 167–196.

Niewiadomska K (2010) *Fauna słodkowodna Polski. 34A. Przywry (Trematoda). Część ogólna; Część systematyczna – Aspidogastrea, Digenea: Strigeida*. Łódź: Wydawnictwo Uniwersytetu Łódzkiego (in Polish).

Niewiadomska K and Kiseliene V (1990) *Diplostomum baeri* Dubois, 1937 (Digenea, Diplostomidae) from Lithuania. *Acta Parasitologica Polonica* **35**, 277–283.

Niewiadomska K and Kiseliene V (1994) *Diplostomum* cercariae (Digenea) in snails from Lithuania. II. Survey of species. *Acta Parasitologica* **39**, 179–186.

Niewiadomska K and Laskowski Z (2002) Systematic relationships among six species of *Diplostomum* Nordmann, 1832 (Digenea) based on morphological and molecular data. *Acta Parasitologica* **47**, 20–28.

Olson PD and Tkach VV (2005) Advances and trends in the molecular systematics of the parasitic Platyhelminthes. *Advances in Parasitology* **60**, 165–243.

Pantoja C, Faltýnková A, O'Dwyer K, Jouet D, Skírnisson K and Kudlai O (2021) Diversity of echinostomes (Digenea: Echinostomatidae) in their snail hosts in high latitudes. *Parasite* **28**, 59.

Pérez-del-Olmo A, Georgieva S, Pula HJ and Kostadinova A (2014) Molecular and morphological evidence for three species of *Diplostomum* (Digenea: Diplostomidae), parasites of fishes and fish-eating birds in Spain. *Parasites & Vectors* **7**, 502.

Pleijel F, Jondelius U, Norlinder E, Nygren A, Oxelman B, Schander C, Sundberg P and Thollesson M (2008) Phylogenies without roots? A plea for the use of vouchers in molecular phylogenetic studies. *Molecular Phylogenetics and Evolution* **48**, 369–371.

Rambaut A (2012) *FigTree v*1. 4. *Molecular evolution, phylogenetics and epidemiology*. Available at http://tree.bio.ed.ac.uk/software/figtree/ (Accessed 1 February 2021).

Rellstab C, Louhi K-R, Karvonen A and Jokela J (2011) Analysis of trematode parasite communities in fish eye lenses by pyrosequencing of naturally pooled DNA. *Infection, Genetics and Evolution* **11**, 1276–1286.

Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S and Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**, 539–542.

Rozas J, Sánchez-DelBarrio JC, Messeguer X and Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics (Oxford, England)* **12**, 2496–2497.

Schwelm J, Georgieva S, Grabner D, Kostadinova A and Sures B (2021) 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* **148**, 1648–1664. doi: 10.1017/S0031182021001372

Selbach C, Soldánová M, Georgieva S, Kostadinova A and Sures B (2015) 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* **8**, 300.

Shaffer MR, Davy SK and Bell JJ (2019) Hidden diversity in the genus *Tethya*: comparing molecular and morphological techniques for species identification. *Heredity* **122**, 354–369.

Shigin AA (1968) On the study of life cycle and morphology of the cercaria *Diplostomum indistinctum* (Trematoda, Diplostomatidae). *Trudy GELAN SSSR* **19**, 208–217 (in Russian).

Shigin AA (1977) Morphology, biology and taxonomy of *Diplostomum* from Palearctic Laridae. *Trudy GELAN SSSR* **27**, 5–64 (in Russian).

Shigin AA (1986) *Trematodes in the fauna of the USSSR: the genus Diplostomum. Metacercariae*. Moscow: Nauka (in Russian).

Shigin AA (1993) *Trematodes of the fauna of Russia and neighbouring regions. Genus Diplostomum. Adults*. Moscow: Nauka (in Russian).

Snyder SD and Tkach VV (2001) Phylogenetic and biogeographical relationships among some Holarctic frog lung flukes (Digenea: Haematoloechidae). *The Journal of Parasitology* **87**, 1433–1440.

https://doi.org/10.1017/S0031182021002092 Published online by Cambridge University Press

Tautz D, Arctander P, Minelli A, Thomas RH and Vogler AP (2003) A plea for DNA taxonomy. *Trends in Ecology & Evolution* **18**, 70–74.

Thompson CW, Phelps KL, Allard MW, Cook JA, Dunnum JL, Ferguson AW, Gelang M, Khan FAA, Paul DL, Reeder DM, Simmons NB, Vanhove MPM, Webala PW, Weksler M and Kilpatrick CW (2021) Preserve a voucher specimen! The critical need for integrating natural history collections in infectious disease studies. *Host-Microbe Biology* **12**, e02698–20.

Tkach VV, Littlewood DT, Olson PD, Kinsella JM and Swiderski Z (2003) Molecular phylogenetic analysis of the Microphalloidea Ward, 1901 (Trematoda: Digenea). *Systematic Parasitology* **56**, 1–15.

Unger P and Palm HW (2017) Parasite risk of maricultured rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) in the Western Baltic Sea, Germany. *Aquaculture International* **25**, 975–989.

Valkiūnas G, Atkinson CT, Bensch S, Sehgal RN and Ricklefs RE (2008) Parasite misidentifications in GenBank: how to minimize their number? *Trends in Parasitology* **24**, 247–248.

van Steenkiste N, Locke SA, Castelin M, Marcogliese DJ and Abbott CL (2015) New primers for DNA barcoding of digeneans and cestodes (Platyhelminthes). *Molecular Ecology Resources* **15**, 945–952.

Vilgalys R (2003) Taxonomic misidentification in public DNA databases. *New Phytologist* **160**, 4–5.

https://doi.org/10.1017/S0031182021002092 Published online by Cambridge University Press