

# Morphology and molecules reveal the alien *Posthodiplostomum centrarchi* Hoffman, 1958 as the third species of *Posthodiplostomum* Dubois, 1936 (Digenea: Diplostomidae) in Europe

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**Abstract** Metacercariae of two species of *Posthodiplostomum* Dubois, 1936 (Digenea: Diplostomidae) were subjected to morphological and molecular studies: *P. brevicaudatum* (von Nordmann, 1832) from *Gasterosteus aculeatus* (L.) (Gasterosteiformes: Gasterosteidae), Bulgaria (morphology, *cox1* and ITS1-5.8S-ITS2) and *Perca fluviatilis* L. (Perciformes: Percidae), Czech Republic (morphology, *cox1*, ITS1-5.8S-ITS2 and 28S); and *P. centrarchi* Hoffman, 1958 from *Lepomis gibbosus* (L.) (Perciformes: Centrarchidae), Bulgaria (morphology, *cox1* and ITS1-5.8S-ITS2) and Slovakia (*cox1* and ITS1-5.8S-ITS2). In addition, cercariae of *P. cuticola* (von Nordmann, 1832) from *Planorbis planorbis* (L.) (Mollusca:

Planorbidae), Lithuania (morphology and *cox1*) and metacercariae of *Ornithodiplostomum scardinii* (Schulman in Dubinin, 1952) from *Scardinius erythrophthalmus* (L.) (Cypriniformes: Cyprinidae), Czech Republic, were examined (morphology, *cox1*, ITS1-5.8S-ITS2 and 28S). These represent the first molecular data for species of *Posthodiplostomum* and *Ornithodiplostomum* Dubois, 1936 from the Palaearctic. Phylogenetic analyses based on *cox1* and ITS1-5.8S-ITS2, using *O. scardinii* as the outgroup and including the three newly-sequenced *Posthodiplostomum* spp. from Europe and eight published unidentified (presumably species-level) lineages of *Posthodiplostomum* from Canada confirmed the distinct status of the three European species (contrary to the generally accepted opinion that only *P. brevicaudatum* and *P. cuticola* occur in the Palaearctic). The subspecies *Posthodiplostomum minimum centrarchi* Hoffmann, 1958, originally described from North America, is elevated to the species level as *Posthodiplostomum centrarchi* Hoffman, 1958. The undescribed “*Posthodiplostomum* sp. 3” of Locke et al. (2010) from centrarchid fishes in Canada has identical sequences with the European isolates of *P. centrarchi* and is recognised as belonging to the same species. The latter parasite, occurring in the alien pumpkinseed sunfish *Lepomis gibbosus* in Europe, is also supposed to be alien for this continent. It is speculated that it colonised Europe long ago and is currently widespread (recorded in Bulgaria, Slovakia and Spain); based on the *cox1* sequence of an adult

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digenean isolate from the Ebro Delta, Spain, only the grey heron (*Ardea cinerea* L.) (Ciconiiformes: Ardeidae) is known to be its definitive host in Europe.

## Introduction

Species of *Posthodiplostomum* Dubois, 1936 are widespread around the world (Dubois, 1938, 1970; Niewiadomska, 2002). This genus includes digeneans with a three-host life-cycle, with snails acting as first intermediate hosts, fishes as second intermediate hosts and different fish-eating birds as definitive hosts (Wisniewski, 1958; Dönges, 1964, 1965; Dubois, 1970; Niewiadomska, 2003). In Europe, two species of this genus, *Posthodiplostomum cuticola* (von Nordmann, 1832) Dubois, 1936 (type-species) and *P. brevicaudatum* (von Nordmann, 1832) Dubois, 1936, have been reported from various fish species and localities (Dubois, 1970; Sudarikov et al., 2002; Niewiadomska, 2003). Grabda-Kazubska et al. (1987) reported a third species in Europe, *P. minimum* (McCallum, 1921) Dubois, 1936, a widespread North-American parasite occurring in a wide range of fish species (Sudarikov, 1971; Gibson, 1996). This record was based on three metacercariae, one from the common bream *Abramis brama* (L.), one from the common roach *Rutilus rutilus* (L.) and one from the rudd *Scardinius erythrophthalmus* (L.), from two lakes in Poland. However, these identifications were considered uncertain due to the lack of detailed descriptions or deposited specimens (Niewiadomska, 2003). Therefore, only the occurrence of *P. cuticola* and *P. brevicaudatum* can be considered as currently generally recognised in European fishes.

During the course of a helminthological survey of fishes from Lake Atanasovsko and its adjacent wetlands, on the Bulgarian Black Sea coast, we found metacercariae of two morphologically distinct species of *Posthodiplostomum*, the first isolated from the three-spined stickleback *Gasterosteus aculeatus* (L.) and the second from the alien pumpkinseed sunfish *Lepomis gibbosus* (L.). In order to aid identification, representative isolates of the two species were characterised molecularly and included in phylogenetic analyses with additional novel sequences of isolates of *Posthodiplostomum* spp. and *Ornithodiplostomum scardinii* (Schulman in Dubinin, 1952) sampled in central and northern Europe. This paper aims to

increase our knowledge of the species of *Posthodiplostomum* and the related genus *Ornithodiplostomum* Dubois, 1936 by providing the first molecular data from the Palaearctic, which are also accompanied by morphological characterisations of the larval stages of the diplostomid species sequenced.

## Materials and methods

In 2012 and 2013, we surveyed the helminth parasites of fishes in the northern part (declared as a managed natural reserve and a Ramsar site) of Lake Atanasovsko, a coastal wetland situated close to the city of Burgas on the Bulgarian Black Sea coast. The core of the wetland represents a shallow lake (mean depth of 0.3 m) divided by dykes into ponds of various salinity (from brackish to hyperhaline), the majority being used in the cycle of salt production; in addition, this wetland includes several canals, freshwater ponds and marshes surrounding the lake. The wetland is used by numerous and diverse birds (317 species) for nesting or roosting during migrations and over-wintering (Vassilev et al., 2013).

Fishes were sampled three times per year (May, July and September). We examined for helminth parasites 134 individuals of *Gasterosteus aculeatus* (92 from freshwater parts and 42 from brackish water parts of the wetland), as well as 107 individuals of *Lepomis gibbosus*, entirely from freshwater habitats. Each fish was dissected under a stereomicroscope. The majority of the isolated metacercariae were excysted using a fine needle and placed in saline for approximately 10 min to relax, then fixed in hot saline and transferred to 70% ethanol. Some metacercariae were not excysted and also fixed in hot saline and preserved in 70% ethanol. In the laboratory, most of them (excysted and unexcysted) were stained in iron acetocarmine, dehydrated in an ascending ethanol series, cleared in dimethyl phthalate and mounted in Canada balsam.

Comparative materials from Europe comprised metacercariae of *Posthodiplostomum brevicaudatum* from the vitreous humour of the eyes of *Perca fluviatilis* L. and *Ornithodiplostomum scardinii* from the optic lobes of the brain of *Scardinius erythrophthalmus* sampled in Lake Mácha, Czech Republic; metacercariae identified by us as *Posthodiplostomum centrarchi* Hoffman, 1958 from the mesenteries of *Lepomis gibbosus* in a blind river arm of the Danube

near Štúrovo, Slovakia; an adult isolate of *Posthodiplostomum centrarchi* from the small intestine of *Ardea cinerea* L. at a bird recovery centre in Catalonia (Spain) originating from the Lagoon Bassa de les Olles, Ebro Delta, Spain; and a single cercarial isolate of *Posthodiplostomum cuticola* from the hepatopancreas of *Planorbis planorbis* (L.) in the Curonian Bay near Juodkrantė, Lithuania. Prior to sequencing, the larval stages were examined live, and series of photomicrographs were made with a digital camera on an Olympus BX51 microscope. Measurements were taken from the digital images with the aid of Quick Photo Camera 2.3 image analysis software.

The following measurements were taken from the specimens studied (Tables 1, 2): CL, cyst length; CW, cyst width; BTL, body total length; FL, forebody length; FW, forebody width; HL, hindbody length; HW, hindbody width; OSL, oral sucker length; OSW, oral sucker width; VSL, ventral sucker length; VSW, ventral sucker width; PL, prepharynx length; PHL, pharynx length; PHW, pharynx width; OL, oesophagus length; OW, oesophagus width; HOL, holdfast organ length; HOW, holdfast organ width; HGL, holdfast gland length; HGW, holdfast gland width; OVL, ovary length; OVW, ovary width; ATL, anterior testis length; ATW, anterior testis width; PTL, posterior testis length; PTW, posterior testis width; CBL, copulatory bursa length; CBW, copulatory bursa width. The following distances were measured: VSDAB, distance from ventral sucker to anterior extremity of body; VSDPFB, distance from ventral sucker to posterior margin of forebody; HODVS, distance from holdfast organ to ventral sucker. The metrical data are presented as the range followed by the mean and the number of the measurements taken (n) in parentheses. All measurements are given in micrometres.

After morphological identification and the recognition of distinct morphological groups of the metacercariae studied, several individuals of each group were selected for sequencing and preserved in molecular grade ethanol. Total genomic DNA was extracted from single ethanol-fixed specimens or from a pool of 10–15 cercariae, as described by Georgieva et al. (2013). Partial fragments of the cytochrome *c* oxidase subunit 1 (*cox1*) and the entire ITS1-5.8S-ITS2 gene cluster were amplified using the polymerase chain reaction (PCR) protocols of Moszczynska et al. (2009)

and Galazzo et al. (2002). Partial 28S rDNA sequences were also amplified for a subset of samples under the conditions described by Georgieva et al. (2014).

Two sets of primers were used for the *cox1* amplification: (i) the degenerate primers for the barcode region MplatCOX1dF (forward; 5'-TGT AAA ACG ACG GCC AGT TTW CIT TRG ATC ATA AG-3') and MplatCOX1dR (reverse; 5'-CAG GAA ACA GCT ATG ACT GAA AYA AYA IIG GAT CIC CAC C-3'); and (ii) the diplostomid-specific primers Plat-diploCOX1F (forward; 5'-CGT TTR AAT TAT ACG GAT CC-3') and Plat-diploCOX1R (reverse; 5'-AGC ATA GTA ATM GCA GCA GC-3') (Moszczynska et al., 2009).

ITS1-5.8S-ITS2 gene cluster of the rRNA gene was amplified using the primers D1 (forward; 5'-AGG AAT TCC TGG TAA GTG CAA G-3') and D2 (reverse; 5'-CGT TAC TGA GGG AAT CCT GGT-3') (Galazzo et al., 2002). Partial 28S rDNA sequences (domains D1–D3; c.1,200 nt) were amplified using primer combination ZX-1 (5'-ACC CGC TGA ATT TAA GCA TAT-3') (Bray et al., 2009) and 1500R (5'-GCT ATC CTG AGG GAA ACT TCG-3') (Tkach et al., 2003). PCR amplicons were purified with a QIAquick PCR purification kit (Qiagen Ltd, UK), following the manufacturer's protocol and sequenced directly on both strands using the PCR primers (*cox1* and 28S) and the primers of Luton et al. (1992) for ITS1-5.8S-ITS2: BD1 (forward; 5'-GTC GTA ACA AGG TTT CCG TA-3') and BD2 (reverse; 5'-TAT GCT TAA ATT CAG CGG GT-3'). Sequencing was performed on an Applied Biosystems 3730xl DNA Analyser, using Big Dye v.1.1 chemistry (ABI Perkin-Elmer) according to the manufacturer's protocol. Contiguous sequences were assembled and edited using MEGA v.6 (Tamura et al., 2013) and submitted to GenBank.

Two alignments were constructed using MUSCLE (Edgar, 2004) implemented in MEGA v.6. The *cox1* alignment (352 nt) comprised seven newly-generated sequences for the species of *Posthodiplostomum* from Europe and nine sequences for *Posthodiplostomum* spp. from Canada retrieved from GenBank (representative sequences with the longest length were included in the alignment). Sequences were aligned with reference to the amino acid translation, using the echinoderm and flatworm mitochondrial code (Telford et al., 2000). The ITS1-5.8S-ITS2 alignment (1,021 nt) initially included 16 newly-

generated sequences for *Posthodiplostomum* spp. and six sequences retrieved from GenBank representing four species from Canada; the number of the novel sequences was subsequently reduced to five. Three of the North American species represented in the *cox1* dataset did not have matching sequences for the ITS1-5.8S-ITS2 in the GenBank database. Pairwise genetic distances were calculated in MEGA v. 6. All sequences retrieved from GenBank represented unidentified isolates of *Posthodiplostomum*. The newly-generated sequences for *O. scardinii* were used as the outgroups in all analyses. Partial 28S rDNA sequences of *P. brevicaudatum* and *O. scardinii* are provided for further studies.

Phylogenetic analyses were carried out using both Bayesian inference (BI) and maximum likelihood (ML) methods. The best-fitting models of nucleotide substitution for each data set were estimated prior to the analyses based on Akaike Information Criteria with a correction for small sample sizes (AICc) (Sugiura, 1978; Hurvich & Tsai, 1989) using jModelTest 2.1.4 (Guindon & Gascuel, 2003; Darriba et al., 2012). These were the HKY+I+ $\Gamma$  (*cox1* dataset) and GTR+I (ITS1-5.8S-ITS2 dataset). ML analyses were performed with PhyML version 3.0 (Guindon et al., 2010) run on the ATGC bioinformatics platform (<http://www.atgc-montpellier.fr/phyml/>); nodal support was estimated from 1,000 bootstrap pseudoreplicates. BI analyses were carried out with MrBayes v.3.2.6 (Ronquist et al., 2012) run on the CIPRES Science Gateway v. 3.3 ([http://www.phylo.org/sub\\_sections/portal/](http://www.phylo.org/sub_sections/portal/)) (Miller et al., 2010) using Markov chain Monte Carlo (MCMC) searches on two simultaneous runs of four chains for 10,000,000 generations and sampling every 1,000th generation. The 'burn-in' period was set for the first 25% of the sampled trees. Consensus topology and nodal support were estimated from the rest of the sampled trees. Tracer v. 1.5 (Rambaut & Drummond, 2009) was used for data convergence and the stationary distribution evaluation of the runs. FigTree v.1.4.2 was used for tree visualisation (Rambaut, 2012).

Voucher specimens (paragenophores, as defined by Pleijel et al., 2008) were deposited in the Natural History Museum of Geneva, Switzerland (MHNG) and the Helminthological Collection of the Institute of Parasitology (HCIP; paragenophores and isogenophores), Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic.

**Family Diplostomidae Poirier, 1886**  
**Subfamily Crassiphialinae Sudarikov, 1960**  
**Genus *Posthodiplostomum* Dubois, 1936**

***Posthodiplostomum brevicaudatum* (von Nordmann, 1832) Dubois, 1936**

*Second intermediate hosts*: Three-spined stickleback *Gasterosteus aculeatus* L. (Gasterosteiformes: Gasterosteidae); European perch *Perca fluviatilis* (L.) (Perciformes: Percidae).

*Site in host*: Vitreous humour of the eye.

*Localities*: Ex *G. aculeatus*: freshwater canal at Lake Atanasovsko, Bulgaria (42.5783N, 27.4932E); ex *P. fluviatilis*: Lake Mácha, Czech Republic (50.5834N, 14.6481E).

*Voucher material*: MHNG-PLAT-95107 (Morph A, paragenophore) and MHNG-PLAT-95108 (Morph B, paragenophore) (ex *G. aculeatus*, Bulgaria); HCIP D-730 (paragenophore ex *P. fluviatilis*, Czech Republic); a hologenophore ex *P. fluviatilis*, Czech Republic, is shown in Fig. 3A.

*Representative DNA sequences*: GenBank: *cox1*: KX931418 and KX931419 (ex *P. fluviatilis*), KX931420 (ex *G. aculeatus*); ITS1-5.8S-ITS2: KX931428 (ex *P. fluviatilis*), KX931429–KX931440 (ex *G. aculeatus*); 28S: KX931426 (ex *P. fluviatilis*).

Description (Figs. 1A–D, 2A, B, 3A)

*Metacercaria* [Based on material ex *G. aculeatus* from Lake Atanasovsko: 20 excysted fixed specimens and 19 fixed specimens in cysts; Figs. 1A–D, 2A, B; metrical data in Table 1.] Cyst oval to elongate-oval, colourless, transparent, with thin walls (Fig. 1D). Body distinctly bipartite. Forebody 63.9–75.3 (68.2)% of total body length, larger than hindbody, elongate, lancet-shaped (Fig. 1A) or leaf-shaped (Fig. 1B), with ventral concavity, covered with numerous tiny spines (Fig. 2A, B). Hindbody typically bulb-shaped, with forebody and hindbody almost equal in width (Fig. 1A); in some specimens hindbody narrower than forebody, with hindbody subcylindrical (Fig. 1B); FW/HW ratio 1:0.77–1.83 (1.29).

Oral sucker terminal or slightly subterminal, elongate-oval, similar in size to or slightly larger than ventral sucker; sucker width ratio 1:0.89–1.25 (1:1.10). Pseudosuckers absent. Ventral sucker elongate-oval; situated at approximately mid-length of forebody

**Table 1** Metrical data for metacercariae of *Posthodiplostomum brevicaudatum* (von Nordmann, 1858) from fishes in Lake Atanasovsko, Bulgaria, and comparative data

| Source              | Dubois (1970)  |      | Sudarikov et al. (2002) | Present study (Morph A)       |       |    | Present study (Morph B)       |       |    |
|---------------------|----------------|------|-------------------------|-------------------------------|-------|----|-------------------------------|-------|----|
| Host                | Various fishes |      | Various fishes          | <i>Gasterosteus aculeatus</i> |       |    | <i>Gasterosteus aculeatus</i> |       |    |
| Locality            | Europe         |      | Russia, European part   | Lake Atanasovsko, Bulgaria    |       |    | Lake Atanasovsko, Bulgaria    |       |    |
| Character           | Range          | Mean | Range                   | Range                         | Mean  | n  | Range                         | Mean  | n  |
| <i>Measurements</i> |                |      |                         |                               |       |    |                               |       |    |
| CL                  | 1,200–1,720    | –    | 1,524                   | 525–775                       | 688   | 16 | 680–805                       | 737   | 3  |
| CW                  | 450–640        | –    | 437                     | 200–350                       | 266   | 16 | 325–375                       | 350   | 3  |
| BTL                 | 750–1,500      | –    | 843–1,169               | 900–1,160                     | 1,040 | 10 | 940–1,250                     | 1,120 | 10 |
| FL                  | 430–1,070      | –    | 653–843                 | 640–855                       | 739   | 10 | 640–825                       | 758   | 10 |
| FW                  | 195–540        | –    | 205–275                 | 170–225                       | 199   | 10 | 290–350                       | 324   | 10 |
| HL                  | 185–480        | –    | 274–410                 | 245–410                       | 330   | 10 | 290–440                       | 367   | 10 |
| HW                  | 137–450        | –    | 137–275                 | 130–248                       | 190   | 10 | 180–290                       | 220   | 10 |
| OSL                 | 24–29          | 27   | 22–38                   | 25–33                         | 30    | 10 | 28–43                         | 37    | 10 |
| OSW                 | 21–26          | 25   | 22–38                   | 20–28                         | 23    | 10 | 25–30                         | 28    | 10 |
| VSL                 | 24–29          | 25   | 19–32                   | 25–35                         | 29    | 10 | 23–30                         | 26    | 10 |
| VSW                 | –              | –    | 19–32                   | 20–28                         | 25    | 10 | 23–30                         | 27    | 10 |
| PL                  | –              | –    | –                       | 5–8                           | 6     | 8  | 0                             | 0     | 10 |
| PHL                 | 24–30          | 27   | 22–28                   | 23–33                         | 29    | 10 | 18–28                         | 22    | 10 |
| PHW                 | 18–24          | 20   | 13–26                   | 18–25                         | 21    | 10 | 18–23                         | 21    | 10 |
| OL                  | 25–60          | –    | –                       | 75–118                        | 104   | 8  | 93–128                        | 107   | 9  |
| OW                  | –              | –    | –                       | 5–13                          | 9     | 8  | 5–10                          | 8     | 9  |
| HOL                 | 60–91          | 74   | 96–128                  | 70–95                         | 80    | 10 | 70–88                         | 80    | 10 |
| HOW                 | 57–84          | 72   | 96–141                  | 68–85                         | 73    | 10 | 75–103                        | 84    | 10 |
| HGL                 | –              | –    | –                       | 23–38                         | 27    | 10 | 25–53                         | 34    | 8  |
| HGW                 | –              | –    | –                       | 45–73                         | 58    | 10 | 50–75                         | 65    | 8  |
| OVL                 | –              | –    | –                       | 35–60                         | 51    | 5  | 30–50                         | 40    | 10 |
| OVW                 | –              | –    | –                       | 25–35                         | 29    | 5  | 25–38                         | 30    | 10 |
| ATL                 | –              | –    | –                       | 50–75                         | 61    | 8  | 23–45                         | 34    | 10 |
| ATW                 | –              | –    | –                       | 53–95                         | 72    | 8  | 25–48                         | 38    | 10 |
| PTL                 | –              | –    | –                       | 43–93                         | 63    | 8  | 25–50                         | 39    | 10 |
| PTW                 | –              | –    | –                       | 60–113                        | 79    | 8  | 38–75                         | 53    | 10 |
| CBL                 | –              | –    | –                       | 47–75                         | 60    | 9  | 33–75                         | 49    | 5  |
| CBW                 | –              | –    | –                       | 40–55                         | 49    | 9  | 38–83                         | 71    | 5  |
| <i>Distances</i>    |                |      |                         |                               |       |    |                               |       |    |
| VSDAB               | –              | –    | –                       | 303–438                       | 383   | 10 | 320–450                       | 399   | 10 |
| VSDPFB              | –              | –    | –                       | 278–388                       | 318   | 10 | 280–380                       | 325   | 10 |
| HODVS               | –              | –    | –                       | 143–223                       | 174   | 10 | 158–208                       | 178   | 10 |

[distance between anterior body extremity to ventral sucker 49.1–55.9 (52.6)% (n = 20) of forebody length]. Prepharynx very short or apparently absent. Pharynx elongate-oval, muscular. Oesophagus long, narrow; its anterior part (adjacent to pharynx) often

with thicker walls than remainder. Intestinal bifurcation in anterior quarter of forebody. Caeca long, narrow, almost straight, terminating blindly at level of posterior testis; terminal portions of caeca slightly expanded in some specimens.



Holdfast organ elongate-oval, opens via longitudinal slit or circular aperture, lobulate, located near to posterior margin of forebody. Proteolytic gland typically with bipartite appearance, situated at posterior margin of holdfast organ, sometimes slightly overlapping it dorsally. Primordial testes 2, tandem, transversely-oval; posterior testis somewhat larger. Primordial ovary intertesticular, elongate-oval. Primordial copulatory bursa globular; genital atrium deep; genital pore terminal.

**Additional data** [Based on 4 live specimens ex *P. fluviatilis* from Lake Mácha; Fig. 3A.] Excretory system of ‘*neascus*’ type, composed of ramified median and 3 lateral canals on each side forming net in forebody; most lateral canals with numerous anastomoses; excretory concretions small, free in canals, significantly larger in hindbody. Measurements: Cyst 1,502–1,595  $\times$  677–746 (1,562  $\times$  709). Total body length 822–1,192 (969). Forebody 515–810  $\times$  373–482 (672  $\times$  399). Hindbody 331–466  $\times$  259–400 (411  $\times$  328). Oral sucker 73–78  $\times$  53–86 (76  $\times$  70). Ventral sucker 40–63  $\times$  43–60 (52  $\times$  51); distance from ventral sucker to anterior extremity of body 368–451 (406); distance from ventral sucker to posterior margin of forebody 129–222 (184). Pharynx 53–72  $\times$  42–45 (63  $\times$  44). Holdfast organ 119–141  $\times$  173–203 (130  $\times$  188).

## Remarks

Initially, specimens from Lake Atanasovsko were considered to represent two morphs indicated as Morph A (Fig. 1A) and Morph B (Fig. 1B) (see also Table 1), which differ mainly in the shape of the forebody (lanceolate in Morph A vs leaf-shaped in Morph B) and hindbody (bulb-shaped in A vs subcylindrical in B, see Fig. 1A, B); in the presence (vs apparent absence) of a prepharynx; and in the shape of the orifice of the holdfast organ (slit-shaped vs circular; see Fig. 1A, B). Metrical differences (Table 1) observed are few: the size of the forebody (wider in Morph B) and the size of the anterior testis (larger in Morph A). Comparative sequence analysis revealed that isolates assigned to Morph A and B represent a single haplotype (see below). Therefore, the morphological differences between Morph A and Morph B described above are recognised here as individual variations, probably reflecting the degree of development of the metacercariae. Fixation may also

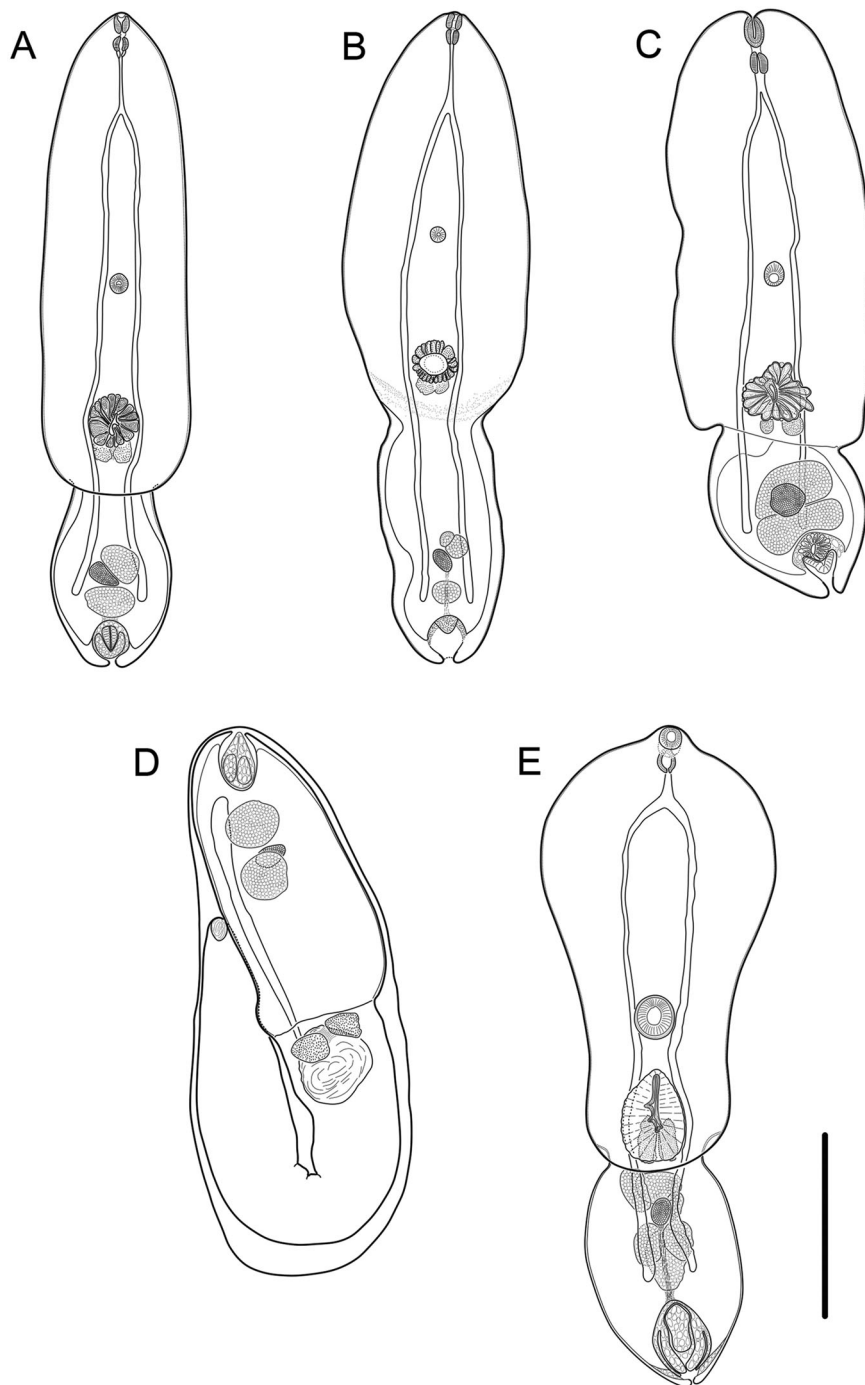
result in variations in the shape of the body, as demonstrated for slightly contracted specimens (Fig. 1C).

The present material corresponds well with previous descriptions of metacercariae of *Posthodiplostomum brevicaudatum* (see Dönges, 1965; Dubois, 1970; Sudarikov et al., 2002; Niewiadomska, 2003). The cysts described by Dönges (1965), based on material from experimental infections (c.5 years post-infection) from *Phoxinus phoxinus* (L.) in Germany, are larger: cyst size 1,280–1,600 vs up to 805  $\mu$ m in our specimens; however, the body parts of metacercariae described by Dönges (1965) are smaller than the body parts of our specimens: forebody size 430–635  $\times$  195–274 vs 640–855  $\times$  170–350  $\mu$ m; hindbody size 185–254  $\times$  137–185 vs 245–440  $\times$  130–290  $\mu$ m. In addition, the specimens described by Dönges (1965) have a shorter oesophagus (25–60 vs 75–128  $\mu$ m).

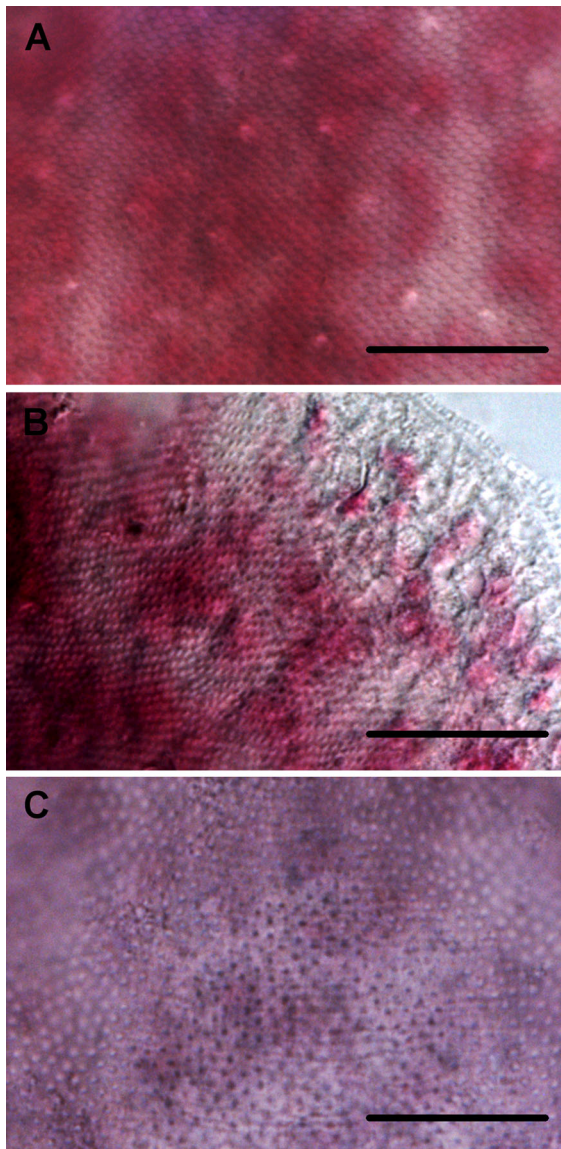
The metrical data from our specimens correspond well with those presented by Dubois (1970) and Sudarikov et al. (2002) originating from natural infections of various European freshwater fishes (Table 1). There is a substantial difference in the holdfast organ size, which is greater in specimens described by Sudarikov et al. (2002) than in those reported by Dubois (1970) and the present material (Table 1). Since there is good agreement with regard to the remaining morphological data (see also Table 1), we believe that the reported minor morphological differences indicate intraspecific variation.

Our specimens were identified as *P. brevicaudatum* based on the shape of the forebody and the hindbody, which are of comparable width [forebody to hindbody width ratio on average 1:1.29 in the present material; 1:1.33 in figure 15 of Dönges (1965) and 1:1.19 in figure 59 of Niewiadomska (2003)], the position of the ventral sucker at the mid-length of forebody and at some distance from the holdfast organ, the oval posterior testis and the colourless cyst. These characters, mentioned in previous descriptions of metacercariae of this species (Dönges, 1965; Dubois, 1970; Sudarikov et al., 2002; Niewiadomska, 2003), might be considered as having diagnostic value for *P. brevicaudatum*.

According to Sudarikov et al. (2002) and Niewiadomska (2003), in the second European species, *P. cuticola*, the cysts are pigmented (black), the forebody is significantly wider than the hindbody [forebody to hindbody width ratio 1:1.43 in figure 20 of Dönges (1964) and 1:1.70 in figure 61 of Niewiadomska (2003)], the pharynx is longer than the oesophagus (55–61 and



**Fig. 1** Metacercariae of *Posthodiplostomum* spp. from fishes in Lake Atanasovsko, Bulgaria. A–D, *P. brevicaudatum* (von Nordmann, 1832) ex *Gasterosteus aculeatus* L. A, Morph A, ventral view; B, Morph B, ventral view; C, Slightly contracted specimen, ventral view. D, Cyst, lateral view; E, *P. centrarchi*. Scale-bar: 250 μm



**Fig. 2** *Posthodiplostomum* spp. from fishes in Lake Atanasovsko, Bulgaria. Forebody, ventral view, spines. A, B, *P. brevicaudatum* (von Nordmann, 1832); C, *P. centrarchi*. Scale-bars: 20  $\mu$ m

22–55  $\mu$ m, respectively), the ventral sucker is located fairly close to the holdfast organ, and the posterior testis is V-shaped (the included metrical data after Dubois, 1970). Another description (Ciurea, 1930, cited after Sudarikov, 1971) reported Y-shaped posterior testis. The main metrical differences between *P. cuticola* and *P. brevicaudatum* include narrower cysts, narrower forebody and hindbody, smaller oral and ventral suckers and a smaller pharynx in *P. brevicaudatum* (Table 1).

The presence of a prepharynx in *P. brevicaudatum* has been mentioned only in the description by Sudarikov et al. (2002); it has not been explicitly mentioned in the other previous descriptions but can be observed in figures 4 and 5 of the description by Wisniewski (1958), figure 15 by Dönges (1965) and figure 59 by Niewiadomska (2003). In addition, the site of infection of metacercariae of *P. cuticola* is typically the skin, subcutaneous connective tissue and subcutaneous musculature, sometimes on gills and fins and buccal muscles and, rarely, in the eye cornea (Dubois, 1970; Sudarikov et al., 2002; Niewiadomska, 2003). In contrast, the most frequent site of infection of metacercariae of *P. brevicaudatum* is in the retina and the vitreous humour of the eyes (Dubois, 1970; Sudarikov et al., 2002; Niewiadomska, 2003) and, rarely, in the brain (Dubois, 1970; Sudarikov, 1971; Niewiadomska, 2003).

Sudarikov et al. (2002) considered the nature of the cysts as a distinguishing feature between *P. cuticola* and *P. brevicaudatum*, i.e. single cysts in the former and typically an aggregation of cysts in a common envelope (termed “syncysts”) in the latter. All the specimens of *P. brevicaudatum* recovered by us were in single cysts. Therefore, we can speculate that the presence of “syncysts” of this species depends on the intensity of infection and occurs when metacercariae are densely located in the host’s tissue.

#### ***Posthodiplostomum centrarchi* Hoffman, 1958**

Syns *Neascus vancleavei* (Agersborg, 1926) of Hughes (1928); *Posthodiplostomum minimum centrarchi* Hoffman, 1958; “*Posthodiplostomum* sp. 3” of Locke et al. (2010)

**Second intermediate host:** Pumpkinseed sunfish *Lepomis gibbosus* L. (Perciformes: Centrarchidae).

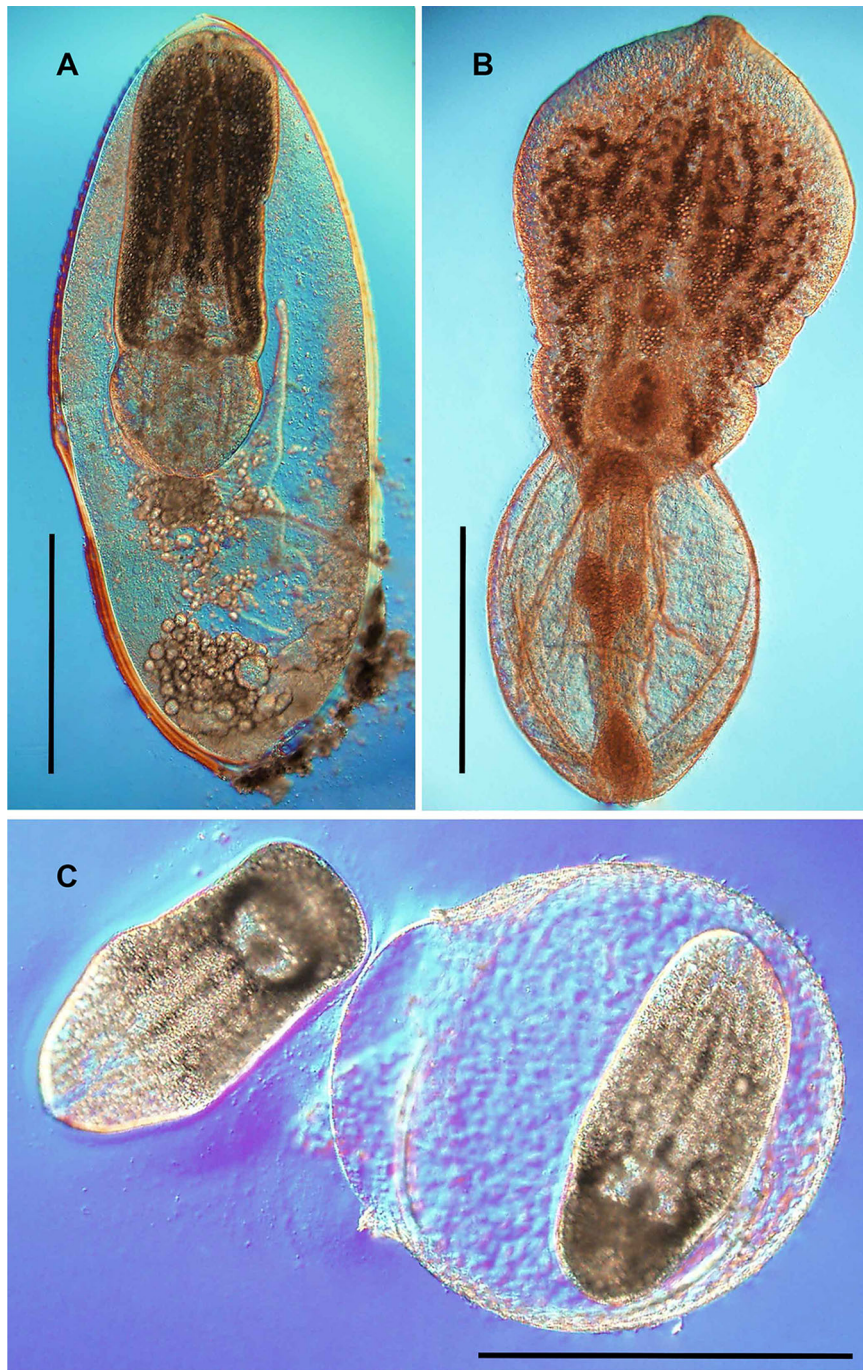
**Definitive host:** Grey heron *Ardea cinerea* L. (Ciconiiformes: Ardeidae).

**Site in host:** *L. gibbosus*: liver, body cavity and mesenteries; *A. cinerea*: small intestine.

**Localities:** Ex *L. gibbosus*: freshwater pond at Lake Atanasovsko, Bulgaria (42.5781N, 27.4944E); blind arm of River Danube near Šturovo, Slovakia (47.8197N, 18.7286E); ex *A. cinerea*: Lagoon Bassa de les Olles, Ebro Delta, Spain (40.7895N, 0.7047E).

**Voucher material:** MHNG-PLAT-95109 (paragenophores); a hologenophore ex *L. gibbosus*, Lake Atanasovsko, Bulgaria is shown in Fig. 3B.





**Fig. 3** Metacercariae of *Posthodiplostomum* spp. and *Ornithodiplostomum scardinii* (Schulman in Dubinin, 1952). A, Live encysted *P. brevicaudatum* (von Nordmann, 1832) from the vitreous humour of the eye of *Perca fluviatilis* L. in Lake Mácha, Czech Republic (hologenophore; GenBank KX931419 and KX931428); B, Excysted fixed specimen of *P. centrarchi* ex *Lepomis gibbosus* L., Lake Atanasovsko, Bulgaria (hologenophore; GenBank KX931421 and KX931441); C, Cyst containing two metacercariae of *O. scardinii* from the optic lobes of the brain of *Scardinius erythrophthalmus* (L.) in Lake Mácha, Czech Republic (one hologenophore; GenBank KX931425, KX931427 and KX931443). Scale-bars: 500  $\mu$ m

**Representative DNA sequences:** GenBank: *cox1*: KX931421 and KX931423 (ex *L. gibbosus*), KX931422 (ex *A. cinerea*); ITS1-5.8S-ITS2: KX931441 and KX931442 (ex *L. gibbosus*).

**Description** (Figs. 1E, 2C, 3B)

*Metacercaria* [Based on material ex *L. gibbosus*, Lake Atanasovsko: 10 fixed specimens; metrical data in Table 2.] Cyst oval to elongate-oval, colourless, transparent, thin-walled. Body distinctly bipartite. Forebody 60.5–75.8 (69.3)% of total body length, larger than hindbody, ovate, with ventral concavity, covered with numerous tiny spines (Fig. 2C). Hindbody bulb-shaped or elongate-oval, without spines; FW/HW ratio 1:1.10–1.75 (1.40).

Oral sucker elongate-oval, muscular, subterminal, distinctly smaller than ventral sucker; sucker width ratio 1:1.36–1.95 (1.61). Pseudosuckers absent. Ventral sucker subspherical, muscular, located between second and last third of forebody, fairly close to holdfast organ. Prepharynx apparently absent. Pharynx lemon-shaped to elongate-oval. Oesophagus short. Caeca long, narrow, straight, terminating blindly at level of posterior testis; posterior extremities slightly widened.

Holdfast organ elongate-oval, rounded posteriorly and gradually narrowing anteriorly, with longitudinal slit-like aperture, fairly close to posterior margin of forebody. Proteolytic gland consisting of 2 thick connected bodies, dorsal to posterior half of holdfast organ. Primordial testes 2, tandem, lobed, in anterior and mid-hindbody; anterior testis smaller than posterior, consisting of several lobes (typically 3), differing in size; posterior testis elongate-oval, typically tri-lobed. Primordial ovary elongate-oval, located between testes. Primordial copulatory bursa ovoid; genital atrium relatively shallow; genital pore terminal.

Excretory system of ‘*neascus*’ type, composed of ramified median and 3 lateral canals on each side forming net in forebody; most lateral canals with numerous anastomoses; excretory concretions smaller in forebody, free in canals (Fig. 3B).

**Remarks**

The present material differs morphologically from metacercariae of the two other European species. On the basis of the molecular sequences obtained during

the course of the present study (see below), it is conspecific with “*Posthodiplostomum* sp. 3” of Locke et al. (2010) from centrarchid fishes in Canada. The present specimens can be distinguished from *P. brevicaudatum* by their ovate forebody (vs lanceolate or leaf-shaped), ventral sucker located fairly close to the holdfast organ (vs at large distance from holdfast organ) and tri-lobed (vs smooth) posterior testis. The ventral sucker of the present specimens is much larger than the oral sucker (mean sucker width ratio 1:1.61), whereas, in *P. brevicaudatum*, the oral and ventral suckers are of almost the same size (mean sucker width ratio 1:1.10). The main metrical differences between these two species include the greater forebody width and size of the oral sucker, ventral sucker, pharynx, holdfast organ, primordial testes and primordial copulatory bursa in the present specimens (Tables 1, 2). In addition, the latter metacercariae are located in the liver and body cavity (vs eyes) of the fish intermediate host.

The present material differs from *P. cuticola* in its location within the fish host (liver and body cavity vs skin and superficial musculature) and in encysting within thin-walled, colourless cysts (vs thick-walled cysts with strong melanisation), ovate forebody (vs leaf-shaped), apparent absence (vs presence) of a prepharynx, and typically 3-lobed oval posterior testis (vs V or Y-shaped). In addition, although the metrical data show overlapping ranges for most characters, the present metacercariae from *Lepomis gibbosus* possess a smaller oral sucker (63–77 × 55–61 µm in *P. cuticola*, after Dubois, 1970) and, on average, a smaller forebody and hindbody (720–1,050 × 520–560 and 330–420 × 260–330 µm, respectively, in *P. cuticola*, see Dubois, 1970).

The above comparisons indicate that the metacercariae from *Lepomis gibbosus* in the wetlands of Lake Atanasovsko in Bulgaria (and also metacercariae from the River Danube in Slovakia and the adult from the Ebro Delta in Spain with identical or almost identical gene sequences, see below) represent an additional, third, species of *Posthodiplostomum* occurring in Europe.

Since the present specimens exhibit identical sequences with an unnamed Canadian species of *Posthodiplostomum*, i.e. “*Posthodiplostomum* sp. 3” of Locke et al. (2010), we have attempted to identify them with the congeneric North American species.

**Table 2** Metrical data for metacercariae of *Posthodiplostomum centrarchi* Hoffman, 1958 from fishes in Lake Atanasovsko, Bulgaria, and comparative data

| Species             | <i>P. minimum centrarchi</i> Hoffman, 1958 |      |    | <i>P. centrarchi</i> Hoffman, 1958 |       |    |
|---------------------|--|------|----|------------------------------------|-------|----|
| Host                | <i>Lepomis gibbosus</i>                    |      |    | <i>L. gibbosus</i>                 |       |    |
| Locality            | Huron River, Ann Arbor (USA)               |      |    | Lake Atanasovsko, Bulgaria         |       |    |
| Source              | Hughes (1928)                              |      |    | Present study                      |       |    |
| Character           | Range                                      | Mean | n  | Range                              | Mean  | n  |
| <i>Measurements</i> |  |      |    |                                    |       |    |
| BTL                 | 560–1,060                                  | 730  | 10 | 958–1,535                          | 1,171 | 10 |
| FL                  | 300–410                                    | 350  | 8  | 605–1,020                          | 811   | 10 |
| FW                  | 280–440                                    | 350  | 8  | 370–550                            | 459   | 10 |
| HL                  | 220–330                                    | 280  | 7  | 295–515                            | 386   | 10 |
| HW                  | 250–380                                    | 310  | 7  | 243–459                            | 340   | 10 |
| OSL                 | 18–28                                      | 20   | 5  | 51–63                              | 56    | 10 |
| OSW                 | –  | –    | –  | 34–50                              | 43    | 10 |
| VSL                 | 42–53                                      | 46   | 5  | 68–80                              | 74    | 10 |
| VSW                 | 60–70                                      | 64   | 5  | 55–80                              | 69    | 10 |
| PL                  | –  | –    | –  | 0                                  | 0     | 10 |
| PHL                 | 28–39                                      | 33   | 5  | 33–45                              | 40    | 10 |
| PHW                 | 18–28                                      | 21   | 5  | 20–38                              | 30    | 10 |
| OL                  | –  | –    | –  | 13–40                              | 31    | 9  |
| OW                  | –  | –    | –  | 5–15                               | 11    | 9  |
| HOL                 | 67–88                                      | 74   | 5  | 118–180                            | 144   | 10 |
| HOW                 | 81–105                                     | 95   | 5  | 90–147                             | 115   | 10 |
| HGL                 | –  | –    | –  | 20–80                              | 60    | 7  |
| HGW                 | –  | –    | –  | 55–108                             | 75    | 7  |
| OVL                 | –  | –    | –  | 40–58                              | 48    | 6  |
| OVW                 | –  | –    | –  | 28–33                              | 32    | 6  |
| ATL                 | –  | –    | –  | 78–170                             | 129   | 6  |
| ATW                 | –  | –    | –  | 63–153                             | 96    | 6  |
| PTL                 | –  | –    | –  | 120–235                            | 162   | 6  |
| PTW                 | –  | –    | –  | 68–105                             | 97    | 6  |
| CBL                 | 53–88                                      | 68   | 5  | 95–163                             | 121   | 7  |
| CBW                 | –  | –    | –  | 100–158                            | 119   | 7  |
| <i>Distances</i>    |  |      |    |                                    |       |    |
| VSDAB               | –  | –    | –  | 343–688                            | 512   | 10 |
| VSDPFB              | –  | –    | –  | 175–275                            | 227   | 10 |
| HODVS               | –  | –    | –  | 8–70                               | 49    | 10 |

The identification key presented by Hoffman (1998) leads to the subspecies *Posthodiplostomum minimum centrarchi* Hoffman, 1958. The latter taxon is believed to represent a specific parasite to the fishes of the family Centrarchidae (Hoffman, 1958, 1998; Gibson,

1996) and especially to those of the genus *Lepomis* (see Lane et al., 2015). The only detailed morphological study on *P. m. centrarchi* was presented by Hughes (1928) who designated it as *Neascus van-cleavei* (Agersborg, 1926), a larval form originally

described from a cyprinid host in Illinois. Hughes (1928) used metacercariae from several centrarchid hosts for morphological characterisation of *N. vancleavei* and provided a detailed description. Dubois (1938) recognised *Diplostomum vancleavei* Agersborg, 1926 (syn. *Neascus vancleavei*) as metacercariae of *Posthodiplostomum minimum* (MacCallum, 1921), a species described from the great blue heron (*Ardea herodias* L.) in the New York Zoo. On the basis of experimental examinations of the specificity of metacercariae of *P. minimum*, Hoffman (1958) proposed to subdivide this species into two subspecies, the nominotypical subspecies *P. minimum minimum* MacCallum, 1921, exhibiting a strong specificity to cyprinid second intermediate hosts, and *P. minimum centrarchi* Hoffman, 1958, with metacercariae specific to centrarchid species. The validity of these two subspecies was adopted in the subsequent compendia (Gibson, 1996; Hoffman, 1998).

Hoffman (1958) described in detail the cysts of the subspecies proposed by him but referred to the description presented by Hughes (1928) for the body morphology of metacercariae. Our specimens differ from the worms described by Hughes (1928) in the length of the forebody, the size of the oral sucker, the length of the ventral sucker, the length of the holdfast organ and the length of the copulatory bursa (Table 2). Although the metrical data presented by Hughes (1928) have been based on relatively small samples from a few geographical localities, his study remains the only detailed source for the morphometry of metacercariae of *P. minimum centrarchi*. However, Davis & Miller (1989) reported this subspecies from five centrarchid hosts in North Carolina and provided a detailed drawing of a metacercaria. The illustrated metacercaria is 1,540 µm long, with forebody 1,154 × 577 µm, hindbody 500 × 462 µm, oral sucker 58 × 77 µm, ventral sucker 77 × 77 µm and holdfast organ 116 × 77 µm. These metrical data expand the known ranges of variation of the main metrical characters of *P. minimum centrarchi* in North America and correspond well to the measurements of the specimens from *Lepomis gibbosus* in Bulgaria described here (Table 2).

As demonstrated by Hoffman (1958), *P. minimum minimum* and *P. minimum centrarchi* differ in their life-cycles (utilising different fish groups as second intermediate hosts); therefore, they should be considered as distinct ecological and evolutionary entities.

Based on this, we prefer to recognise them as distinct species and elevate the subspecies *P. minimum centrarchi* Hoffman, 1958 to the species level as *P. centrarchi* Hoffman, 1958.

The species diversity of the genus *Posthodiplostomum* in North American fish-eating birds includes six taxa (Dubois, 1970; Dronen & Chen, 2002) but the morphology of their metacercariae is unknown or poorly known (Gibson, 1996; Hoffman, 1998). Further studies may show that *P. centrarchi* can be conspecific with any of *Posthodiplostomum* spp. described on the basis of adult worms from North American fish-eating birds.

### ***Posthodiplostomum cuticola* (von Nordmann, 1832)**

*First intermediate host:* *Planorbis planorbis* (L.) (Mollusca: Planorbidae).

*Site in host:* Hepatopancreas.

*Locality:* Curonian Bay near Juodkrantė, Lithuania (55.5370N, 21.1267E).

*Voucher material:* HCIP D-731 (isogenophore sensu Pleijel et al., 2008).

*Representative DNA sequence:* GenBank: *cox1*: KX931424.

### **Description**

*Cercaria* [Measurements based on 5 live specimens examined under slight coverslip pressure.] Body elongate-oval, 179–227 × 43–69, shorter than tail stem (tail stem length/body length ratio 1:1.39–1.42), with 2 small, pigmented eye-spots, just post-equatorial. Anterior organ elongate-oval, 68–80 × 33–38, with posterior margin reaching second third of body. Ventral sucker rudimentary, between first and second pairs of penetration gland-cells. Mouth opening ventro-subterminal; prepharynx 10–11 long; pharynx small, subspherical to transversely-oval, 11–13 × 13–18; oesophagus short, bifurcates just posterior to pharynx; intestinal caeca very short, terminate blindly just anterior to first pair of penetration gland-cells. Penetration gland-cells 3 pairs, large, with fine granular content, in posterior half of body; ducts open antero-laterally to mouth. Anlagen of reproductive organs a compact, round mass between last pair of penetration gland-cells and excretory vesicle. Tail stem 249–290 long, 31–46 wide at base, equal in length to or slightly shorter than furcae (tail stem length to furcal length ratio 1:0.96–1.05). Furcae



without fin-folds, 259–276 long, 18–25 wide at base. Excretory vesicle Y-shaped; caudal excretory duct passes through tail stem; Cort's isle present at level of anterior extremity of tail stem.

**Body armature:** Post-oral spines arranged in more than 7 alternate rows, encircling anterior organ along its entire length. Transverse rows of spines 7, extending to mid-level of body or just posterior.

### Genus *Ornithodiplostomum* Dubois, 1936

#### *Ornithodiplostomum scardinii* (Schulman in Dubinin, 1952)

**Second intermediate host:** Common rudd *Scardinius erythrophthalmus* (L.) (Cypriniformes: Cyprinidae).

**Site in host:** Optical lobes of brain.

**Locality:** Lake Mácha, Czech Republic (50.5834N, 14.6481E).

**Voucher material:** One paragenophore (HCIP D-732) and one hologenophore; both shown in Fig. 3C.

**Representative DNA sequences:** GenBank: *cox1*: KX931425; ITS1-5.8S-ITS2: KX931443; 28S: KX931427.

#### Description (Fig. 3C)

**Metacercaria** [Based on 2 live, excysted specimens.] Metacercariae encysted in pairs (Fig. 3C). Cyst spherical, thin-walled, 654 × 596. Body linguiform, medium-sized, 503–520 long, with indistinct division between forebody and hindbody. Forebody with slight ventral concavity, 231–337 × 238–256. Hindbody short, semi-circular, not well differentiated from forebody, 124 × 214–222.

Oral sucker small, subspherical, terminal, 35–36 × 34–37. Pseudosuckers absent. Ventral sucker small, 34–36 × 34–36, similar in size to oral sucker, just posterior to mid-body, at 231–337 from anterior extremity of body and at 146–152 from posterior margin of forebody. Prepharynx absent; pharynx slightly elongate-oval, 34–35 × 23–25; oesophagus very short, bifurcates closely posterior to pharynx; caeca masked by excretory concretions. Holdfast organ oval, bipartite, 66–91 × 122–142.

Excretory system of 'neascus' type, composed of ramified median and 3 lateral canals on each side forming net in forebody and commissure close to posterior margin of forebody; excretory concretions

small in forebody; very large in hindbody and excretory bladder.

#### Remarks

The morphology of the material sequenced here agrees well with the description of the metacercaria of *O. scardinii* ex *Scardinius erythrophthalmus* given by Sudarikov et al. (2002). The present material exhibits slightly greater ranges for hindbody width (214–222 vs 137–182 µm), oral sucker (35–36 × 34–37 vs 25–31 × 20–25 µm), ventral sucker (34–36 × 34–36 vs 22 × 22–23 µm) and the width of the holdfast organ (122–142 vs 70–106 µm). These minor differences are likely due to the fact that the measurements of Sudarikov et al. (2002) were taken from fixed specimens.

#### Molecular data and phylogenetic analyses

Successful DNA amplification was achieved for 21 representative specimens: (i) 15 *P. brevicaudatum* [12 ex *G. aculeatus* (both loci sequenced for three specimens; ITS only sequenced for 14 specimens) and two ex *Perca fluviatilis* (both loci sequenced; 28S rDNA sequenced for one specimen)]; (ii) four individuals of *P. centrarchi*, three ex *Lepomis gibbosus* (both loci sequenced) and one ex *Ardea cinerea* (*cox1* sequence only); (iii) one *P. cuticola* (*cox1* sequence only); and (iv) one *O. scardinii* (all three loci sequenced) (see Table 3 for details). Partial *cox1* sequences were obtained with both, i.e. the degenerate *cox1* and the diplostomid-specific primers, with a similar, moderate success [*cox1* degenerated primers yielded sequences in 10 of 21 (47.6%) of the isolates and the diplostomid-specific primers yielded sequences in 9 of 21 (42.9%) of the cases]. Amplification of the ITS1-5.8S-ITS2 gene cluster was successfully achieved for most of the isolates (17 newly-generated sequences) (Table 3).

The alignment of the *cox1* dataset comprised seven newly-generated sequences for *Posthodiplostomum* spp. from fishes in Europe and eight unidentified (presumably species-level) lineages from fishes in Canada (Locke et al., 2010) retrieved from the GenBank database (Table 4). Both, ML and BI analyses of the set yielded similar phylogenetic hypotheses (Fig. 4). The newly-generated sequences

**Table 3** Summary data for the isolates of *Posthodiplostomum* spp. used for generation of the new *cox1*, ITS1–5.8S-ITS2 and 28S sequences

| Digenean species  | Isolate | Host species                       | Site in host <sup>a</sup> | Life-cycle stage <sup>b</sup> | Locality <sup>c</sup>                      | GenBank accession number |                                    |
|---|---------|------------------------------------|---------------------------|-------------------------------|--|--------------------------|------------------------------------|
|   |         |                                    |                           |                               |  | <i>cox1</i>              | ITS1–5.8S-ITS2/28S                 |
| <i>Posthodiplostomum brevicaudatum</i> (von Nordmann, 1832)               | PBPF1   | <i>Perca fluviatilis</i>           | Eye VH                    | M                             | Lake Mácha (CZ)                            | KX931418                 | KX931426 <sup>d</sup>              |
|   | PBPF2   | <i>Perca fluviatilis</i>           | Eye VH                    | M                             | Lake Mácha (CZ)                            | KX931419                 | KX931428                           |
|   | PBGA1   | <i>Gasterosteus aculeatus</i>      | Eye VH                    | M                             | Lake Atanasovsko (BG)                      |                          | KX931429                           |
|   | PBGA2   | <i>Gasterosteus aculeatus</i>      | Eye VH                    | M                             | Lake Atanasovsko (BG)                      |                          | KX931430                           |
|   | PBGA3   | <i>Gasterosteus aculeatus</i>      | Eye VH                    | M                             | Lake Atanasovsko (BG)                      |                          | KX931431                           |
|   | PBGA4   | <i>Gasterosteus aculeatus</i>      | Eye VH                    | M                             | Lake Atanasovsko (BG)                      | KX931420                 | KX931432                           |
|   | PBGA5   | <i>Gasterosteus aculeatus</i>      | Eye VH                    | M                             | Lake Atanasovsko (BG)                      |                          | KX931433                           |
|   | PBGA6   | <i>Gasterosteus aculeatus</i>      | Eye VH                    | M                             | Lake Atanasovsko (BG)                      |                          | KX931434                           |
|   | PBGA7   | <i>Gasterosteus aculeatus</i>      | Eye VH                    | M                             | Lake Atanasovsko (BG)                      |                          | KX931435                           |
|   | PBGA8   | <i>Gasterosteus aculeatus</i>      | Eye VH                    | M                             | Lake Atanasovsko (BG)                      |                          | KX931436                           |
|   | PBGA9   | <i>Gasterosteus aculeatus</i>      | Eye VH                    | M                             | Lake Atanasovsko (BG)                      |                          | KX931437                           |
|   | PBGA10  | <i>Gasterosteus aculeatus</i>      | Eye VH                    | M                             | Lake Atanasovsko (BG)                      |                          | KX931438                           |
| <i>Posthodiplostomum centrarchi</i> Hoffman, 1958                         | PBGA11  | <i>Gasterosteus aculeatus</i>      | Eye VH                    | M                             | Lake Atanasovsko (BG)                      |                          | KX931439                           |
|   | PBGA12  | <i>Gasterosteus aculeatus</i>      | Eye VH                    | M                             | Lake Atanasovsko (BG)                      |                          | KX931440                           |
|   | PCLG1   | <i>Lepomis gibbosus</i>            | Liver                     | M                             | Lake Atanasovsko (BG)                      | KX931421                 | KX931441                           |
|   | PCAC1   | <i>Ardea cinerea</i>               | Intestine                 | A                             | Lagoon Bassa de les Olles, Ebro Delta (SP) | KX931422                 |                                    |
| <i>Posthodiplostomum cuticola</i> (von Nordmann, 1832)                    | PCLG2   | <i>Lepomis gibbosus</i>            | Mesenteries               | M                             | Danube near Štúrovo (SK)                   | KX931423                 |                                    |
|   | PCLG3   | <i>Lepomis gibbosus</i>            | Mesenteries               | M                             | Danube near Štúrovo (SK)                   |                          | KX931442                           |
|   | PCPP1   | <i>Planorbis planorbis</i>         | Hepatopancreas            | C                             | Curonian Bay near Juodkrantė (LT)          | KX931424                 |                                    |
|   |         |                                    |                           |                               |  |                          |                                    |
| <i>Ornithodiplostomum scardinii</i> (Shulman in Dubinin, 1952) (outgroup) | OSSE1   | <i>Scardinius erythrophthalmus</i> | Brain                     | M                             | Lake Mácha (CZ)                            | KX931425                 | KX931443/<br>KX931427 <sup>d</sup> |

<sup>a</sup> Eye VH, eye vitreous humour<sup>b</sup> A, adult; M, metacercaria; C, cercaria<sup>c</sup> BG, Bulgaria; CZ, Czech Republic; LT, Lithuania; SK, Slovakia; SP, Spain<sup>d</sup> 28S rDNA sequences

for *P. brevicaudatum*, including two isolates ex *P. fluviatilis* from the Czech Republic and a single isolate ex *G. aculeatus* from Lake Atanasovsko in Bulgaria, formed a strongly supported monophyletic lineage resolved as the earliest divergent to the remaining representatives of the genus. The newly-sequenced metacercarial isolates of *P. centrarchi* ex *L. gibbosus* and the adult isolate ex *Ardea cinerea* clustered together with sequences for “*Posthodiplostomum* sp. 3” of Locke et al. (2010) ex *L. gibbosus* from Canada. The single cercarial isolate for *P. cuticola* branched apart from *P. brevicaudatum* and *P. centrarchi* [and the identical “*Posthodiplostomum* sp. 3” of Locke et al. (2010)], thus confirming the distinct status of the three newly-sequenced species of *Posthodiplostomum* from Europe.

The intraspecific divergence for *P. brevicaudatum* ranged between 0.3–1.7% (1–6 nt difference). The sequence divergence between the two haplotypes ex *P. fluviatilis* from the Czech Republic was 0.3% (1 nt difference), and the divergence between these and the haplotype ex *G. aculeatus* from Lake Atanasovsko was 1.4–1.7% (5–6 nt difference). Sequence divergence between the isolates of *P. centrarchi* from Europe and “*Posthodiplostomum* sp. 3” from Canada was between 0–0.3% (0–1 nt difference). Notably, the isolates from Europe and one isolate from Canada (HM064838) shared the same haplotype and the second Canadian haplotype (HM064801) exhibited a single nucleotide difference.

The interspecific divergence between species represented in the *cox1* dataset was distinctly higher: 15.3–16% (54–56 nt difference) between *P. brevicaudatum* and *P. centrarchi*; 21.6–21.9% (76–77 nt difference) between *P. brevicaudatum* and *P. cuticola*; and 18.5–18.8% (65–66 nt difference) between *P. cuticola* and *P. centrarchi*. The overall interspecific divergence in the *cox1* dataset ranged between 3.1% [11 nt difference; “*Posthodiplostomum* sp. 1” of Locke et al. (2010) vs “*Posthodiplostomum* sp. 2” of Locke et al. (2010)] and 21.9% (77 nt difference; *P. brevicaudatum* vs *P. cuticola*).

Phylogenetic hypotheses resulting from ML and BI analyses of the ITS1–5.8S–ITS2 rDNA dataset, with slightly different representation of the taxa, supported the results obtained from the mitochondrial marker. The newly-generated sequences clustered in two strongly supported monophyletic lineages (Fig. 5),

with *P. brevicaudatum* again at a basal position and isolates of *P. centrarchi* from Europe clustering together with “*Posthodiplostomum* sp. 3” of Locke et al. (2010) from Canada.

Intraspecific sequence divergence in the ITS1–5.8S–ITS2 dataset was detected only for the isolates of *P. centrarchi* (0–0.3%; 0–3 nt difference). The interspecific divergence between *P. brevicaudatum* and *P. centrarchi* was 4.0–4.1% (40–42 nt difference). The overall interspecific divergence in the ITS1–5.8S–ITS2 dataset ranged between 1.1% [11 nt difference; “*Posthodiplostomum* sp. 6” of Locke et al. (2010) vs “*Posthodiplostomum* sp. 8” of Locke et al. (2010)] and 5.7% [53 nt difference; “*Posthodiplostomum* sp. 5” of Locke et al. (2010) vs “*Posthodiplostomum* sp. 8” of Locke et al. (2010)].

## Discussion

To the best of our knowledge, this is the first study to generate molecular data for species of *Posthodiplostomum* and *Ornithodiplostomum* from the Palaearctic, and the first to provide detailed morphological description of the isolates sequenced (hologenophores, paragenophores and isogenophores sensu Pleijel et al., 2008). This integrative taxonomic characterisation of the four species will enable reliable identification of the larval stages in their intermediate snail and fish hosts in future studies. Locke et al. (2010) reported eight lineages of *Posthodiplostomum* from fishes, i.e. *Ambloplites rupestris* (Rafinesque), *Lepomis gibbosus*, *Micropterus salmoides* (Lacépède), *Morone americana* (J. F. Gmelin) and *Pimephales notatus* (Rafinesque), collected from the St. Lawrence River, Canada. However, the metacercariae recorded by Locke et al. (2010) have remained undescribed and a direct morphological comparison is impossible.

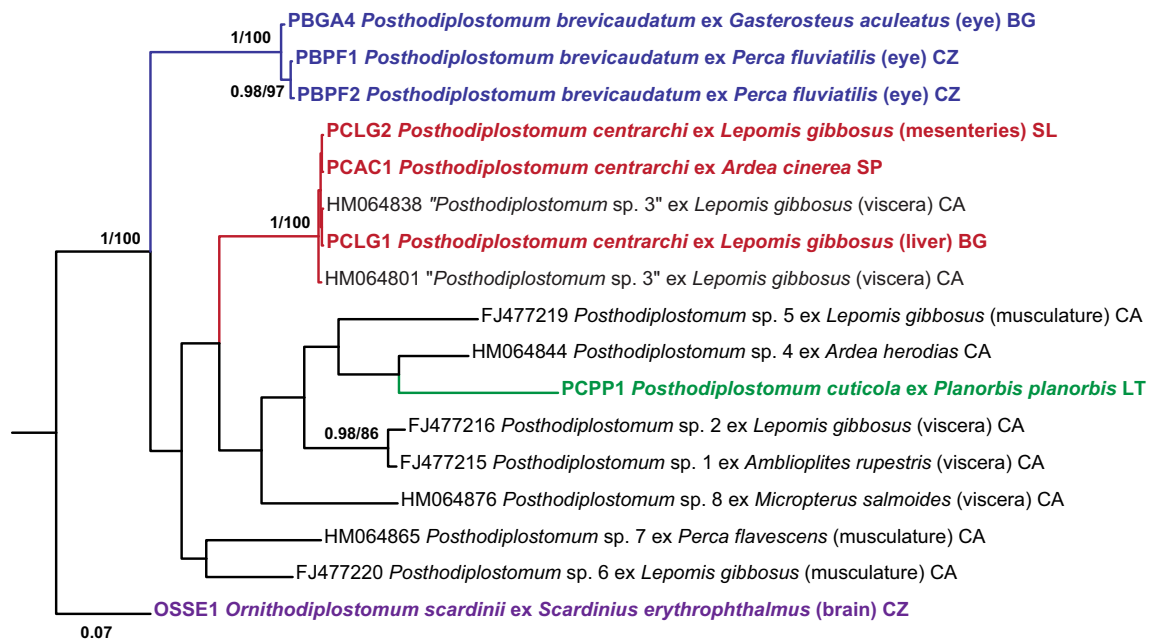
The metacercariae recorded from Lake Atanasovsko, Bulgaria, possess morphological characteristics corresponding to those of *Posthodiplostomum* (see Dubois, 1938, 1970; Gibson, 1996; Niewiadomska, 2003). The metacercariae collected from the eye vitreous humour of *G. aculeatus* were identified at the species level as *P. brevicaudatum* based on comparisons with previous descriptions (Dönges, 1965; Dubois, 1970; Sudarikov et al., 2002; Niewiadomska, 2003). Representative isolates of *P. brevicaudatum* ex *G. aculeatus* formed

**Table 4** Summary data for isolates of *Posthodiplostomum* spp. retrieved from GenBank

| Digenean species   | Isolate         | Host species                 | Site in host | Life-cycle stage <sup>a</sup> | Locality   | GenBank accession number |                    | Reference                 |
|--|-----------------|------------------------------|--------------|-------------------------------|--|--------------------------|--------------------|---------------------------|
|  |                 |                              |              |                               |  | <i>cox1</i>              | ITS1-5.8S-ITS2/28S |                           |
| " <i>Posthodiplostomum</i> sp. 1" of Locke et al. (2010) | P.LV.1.R.3.1    | <i>Ambloplites rupestris</i> | Viscera      | M                             | Lac Saint-François (Ontario, Canada)               | FJ477215                 |                    | Moszczyńska et al. (2009) |
| " <i>Posthodiplostomum</i> sp. 2" of Locke et al. (2010) | O.LV.O.S.22.1   | <i>Lepomis gibbosus</i>      | Viscera      | M                             | Lac Saint-Pierre, Île aux Ours (Québec, Canada)    | FJ477216                 |                    | Moszczyńska et al. (2009) |
| " <i>Posthodiplostomum</i> sp. 3" of Locke et al. (2010) | P.KY.B08.S.1F.3 | <i>Lepomis gibbosus</i>      | Viscera      | M                             | Lac Saint-Louis, Beauharnois (Québec, Canada)      | HM064801                 | HM064949           | Locke et al. (2010)       |
|  | P.HT.B07.S.1.3  | <i>Lepomis gibbosus</i>      | Viscera      | M                             | Lac Saint-Louis, Beauharnois (Québec, Canada)      | HM064838                 |                    | Locke et al. (2010)       |
|  | P.BC.B08.S.1F.5 | <i>Lepomis gibbosus</i>      | Viscera      | M                             | Lac Saint-Louis, Beauharnois (Québec, Canada)      |                          | HM064951           | Locke et al. (2010)       |
|  | P.BC.B08.S.1F.4 | <i>Lepomis gibbosus</i>      | Viscera      | M                             | Lac Saint-Louis, Beauharnois (Québec, Canada)      |                          | HM064952           | Locke et al. (2010)       |
| " <i>Posthodiplostomum</i> sp. 4" of Locke et al. (2010) | P.GRI.Ah.1F.3   | <i>Ardea herodias</i>        | Intestine    | A                             | Lac Saint-Pierre, Grand Île (Québec, Canada)       | HM064844                 |                    | Locke et al. (2010)       |
| " <i>Posthodiplostomum</i> sp. 5" of Locke et al. (2010) | P.LM.S.S.4.2    | <i>Lepomis gibbosus</i>      | Musculature  | M                             | Lac Saint-Pierre, Îles aux Sables (Québec, Canada) | FJ477219                 |                    | Moszczyńska et al. (2009) |
|  | P.LM.O.S.10.1   | <i>Lepomis gibbosus</i>      | Musculature  | M                             | Lac Saint-Pierre, Île aux Ours (Québec, Canada)    |                          | HM064958           | Locke et al. (2010)       |
| " <i>Posthodiplostomum</i> sp. 6" of Locke et al. (2010) | P.BC.1.S.9.2    | <i>Lepomis gibbosus</i>      | Musculature  | M                             | Lac Saint-François (Ontario, Canada)               | FJ477220                 |                    | Moszczyńska et al. (2009) |
|  | P.RM.B.Ma.1.1   | <i>Morone americana</i>      | Musculature  | M                             | Lac Saint-Louis, Beauharnois (Québec, Canada)      |                          | HM064960           | Locke et al. (2010)       |
| " <i>Posthodiplostomum</i> sp. 7" of Locke et al. (2010) | P.BCM.B07.P.f.1 | <i>Perca flavescens</i>      | Musculature  | M                             | Lac Saint-Louis, Beauharnois (Québec, Canada)      | HM064865                 |                    | Locke et al. (2010)       |
| " <i>Posthodiplostomum</i> sp. 8" of Locke et al. (2010) | P.LV.B.MS.1.1   | <i>Micropterus salmoides</i> | Viscera      | M                             | Lac Saint-Louis, Beauharnois (Québec, Canada)      | HM064876                 | HM064962           | Locke et al. (2010)       |

<sup>a</sup> A, adult; M, metacercaria





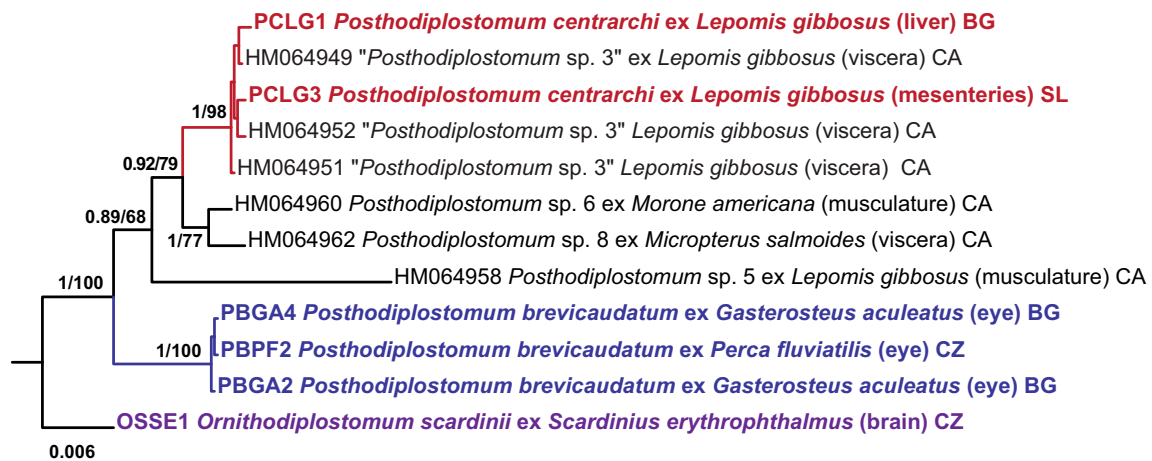
**Fig. 4** Bayesian inference (BI) tree for *Posthodiplostomum* spp. based on the mitochondrial gene *cox1*. Nodal support from BI and maximum likelihood (ML) analyses is indicated as BI/ML; values < 0.95 (BI) and < 70 (ML) are not shown. The scale-bar indicates the expected number of substitution per site. Sequence annotations are as in the GenBank database; host, site and country of origin are also indicated. *Abbreviations*: BG, Bulgaria; CA, Canada; CZ, Czech Republic; LT, Lithuania; SL, Slovakia; SP, Spain

strongly supported reciprocally monophyletic lineage for both loci together with specimens ex *Perca fluviatilis* from the Czech Republic, also identified, based on morphology, as *P. brevicaudatum*. Notably, the intraspecific divergence detected for the *cox1* gene sequences was much lower than the interspecific divergence between the three European species of *Posthodiplostomum* (0.3–1.7 vs 15.3–21.9%), thus allowing unequivocal molecular identification of these species in future barcoding projects.

The metacercariae of *P. brevicaudatum* are widespread parasites in many freshwater fishes in the Palearctic Region (Niewiadomska, 2003; Pugachev, 2003). In Bulgaria, this species was reported as adults from *Botaurus stellaris* (L.) (Ardeidae) along the Danube by Jančev et al. (1984) and from *Ixobrychus minutus* (L.) (Ardeidae) from the region of the present study (Chernomorets, near Burgas) by Kostadinova (1995). In her unpublished dissertation, Kostadinova (1993) reported metacercariae ex *Scardinius erythrophthalmus* from Lake Durankulak on northern part of the Bulgarian Black Sea coast. Therefore, the record of this species from the Burgas wetlands is not unexpected.

The second species of *Posthodiplostomum* in the present study, *P. centrarchi*, which was isolated from the pumpkinseed sunfish *Lepomis gibbosus* in the Lake Atanasovsko wetland of Bulgaria and River Danube in Slovakia, and from a heron in Ebro Delta, was identical with a *cox1* haplotype of "*Posthodiplostomum* sp. 3" of Locke et al. (2010) isolate collected from the same centrarchid fish host species in Lake Saint-Louis along the St. Lawrence River, Canada (GenBank HM064838), i.e. from the native geographical range of this fish species. Phylogenetic analyses based on both loci recovered the European and Canadian isolates in a strongly supported, reciprocally monophyletic species-level lineage, thus confirming their conspecificity.

"*Posthodiplostomum* sp. 3" of Locke et al. (2010) has been considered as a parasite specific to fish species of the family Centrarchidae, since it has not been recorded from several hundred specimens of the Percidae and Cyprinidae from the same habitats in Canada (Locke et al., 2010). The host species, *L. gibbosus*, is alien in Europe (Kottelat & Freyhof, 2007) and centrarchid fishes are not native to this continent. It seems likely that *P. centrarchi* (syn.



**Fig. 5** Bayesian inference (BI) tree for *Posthodiplostomum* spp. based on the ITS1-5.8S-ITS2 sequence alignment. Nodal support from BI and maximum likelihood (ML) analyses is indicated as BI/ML; values < 0.95 (BI) and < 70 (ML) are not shown. The scale-bar indicates the expected number of substitution per site. Sequence annotations are as in the GenBank database; host, site and country of origin are also indicated. *Abbreviations:* BG, Bulgaria; CA, Canada; CZ, Czech Republic; SL, Slovakia

"*Posthodiplostomum* sp. 3" of Locke et al., 2010) is also alien to Europe. The range of definitive hosts of this undescribed species is unknown in its native geographical range in North America but there are sequences for "*Posthodiplostomum* sp. 3" deposited in GenBank (HM064809, HM064810 and HM064815) linked to voucher specimens (BOLD:TREMA579-08.COI-5P; BOLD:TREMA570-08.COI-5P and BOLD:TREMA580-08.COI-5P) from the great blue heron (*Ardea herodias* L.) in Canada (no information can be found in the reference linked with these sequences, see Locke et al., 2010). However, we obtained a *cox1* sequence from a worm in the small intestine of the grey heron *Ardea cinerea* in the Ebro Delta, Spain, which was identical with a sequence from an isolate in Canada (HM064838), thus providing the first report of *P. centrarchi* from a definitive host in the Palaearctic. Unfortunately, the worm was too macerated to be used for a morphological description of the adult stage of this species. The novel data on the geographical range of *P. centrarchi* in Europe (i.e. Spain, Slovakia and Bulgaria) suggest that this species is widespread throughout the continent, thus indicating its long-lasting undetected presence in Europe. Furthermore, it can be expected that its distribution in Europe probably coincides with that of its centrarchid second intermediate host. Further studies are needed to understand the range of definitive and first intermediate hosts of this species as well as its impact on the native biodiversity.

The present study demonstrates that the combination of molecular and morphological methods may further contribute to better understanding of parasite diversity in European fishes.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All applicable institutional, national and international guidelines for the care and use of animals were followed.

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