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ORIGINAL ARTICLE

New record of metacercariae of the North American *Posthodiplostomum Centrarchi* (digenea, diplostomidae) in pumpkinseed (*Lepomis Gibbosus*) in Hungary

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ABSTRACT

Two species of the genus *Posthodiplostomum* (Digenea: Diplostomatidae) (*Posthodiplostomum brevicaudatum* Nordmann, 1832 and *Posthodiplostomum cuticola* Nordmann, 1832) are known as parasites of Hungarian native fishes. Metacercariae of *P. cuticola* are widespread in Europe and cause black spot disease. Several species of *Posthodiplostomum* were described also from North America but none of them has been isolated in Hungary up to now. *Posthodiplostomum centrarchi* Hoffman, 1958 has been detected recently in pumpkinseeds (*Lepomis gibbosus* L., 1758) in several European countries. *Posthodiplostomum centrarchi* was isolated for the first time in Hungary from pumpkinseeds caught in the Maconka water reservoir in 2015. Thereafter, several natural waters (e.g. the river Danube, Lake Balaton and the Sió channel) were sampled in order to determine its presence and distribution. Only the native species *P. cuticola* was detected in Lake Balaton on cyprinids but a relatively high infection rate was observed in the Sió channel close to the lake. Pathological changes were absent, and metacercariae were mostly attached to the surface of the liver, kidney and heart. The phylogenetic analysis of the ITS and COI sequences of *P. centrarchi* and *P. cuticola* clustered into two distinct branches, which was in agreement with the morphological results.

KEYWORDS

Lepomis gibbosus, *Posthodiplostomum*, non-native species, first observation in Hungary, histology

INTRODUCTION

Posthodiplostomum Dubois, 1936 (Digenea: Diplostomidae) species are distributed worldwide (López-Hernández et al., 2018; Niewiadomska 2002; Ritossa et al. 2013) and well known as fish and water-bird parasites (Lumsden and Zischke 1963; Miller 1954). The metacercariae of these species, especially *P. cuticola* (von Nordmann, 1832), cause black spot disease or *Posthodiplostomum* infection that occurs on the body surface, fins, scales, or in the musculature and body cavity of fishes (Horák et al. 2014; Ondračková et al. 2004; Tobler and Schlupp 2008; Zrnčić et al. 2009). These encysted stages often induce developmental disorders of the fish skeleton that may be lethal in the case of fingerlings (Lucký 1970). The most common signs are

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weight loss due to appetite decrease, lesions of the liver and kidney, and digestive dysfunction (Iqbal et al. 2014; Lane and Morris 2000; López-Hernández et al. 2018; Rolbiecki 2004; Schäperclaus 1990).

More than 25 species of *Posthodiplostomum* have been described in the world (López-Hernández et al. 2018; Ritossa et al. 2013) but in Europe only four species of this genus have been reported: *Posthodiplostomum cuticola* (von Nordmann, 1832) and *P. brevicaudatum* (von Nordmann, 1832) as native species, as well as *P. minimum* (McCallum, 1921) and *P. centrarchi* Hoffman (1958), both originating from North America. The occurrence of *P. minimum* in Europe was based on only three metacercariae (Grabda-Kazubska et al. 1987) and no further confirmations of this record were published. *Posthodiplostomum centrachi* had not been reported in the European pumpkinseed population before 2017 (Bykhovskaya-Pavlovskaya and Kulakovskaya 1985; Molnár 1969; Moravec 2001; Moshu 2012, 2014; Roman-Chiriac 1960). In Hungary, the native species *P. cuticola* and *P. brevicaudatum* were documented by Jaczó (1941) and Molnár (1968), while Székely and Molnár (1996) reported only *P. cuticola*. Recently, the presence of *P. centrarchi* has been reported published from several European countries: Bulgaria, Slovakia, the Czech Republic, Portugal, Ukraine and Germany (Kvach et al. 2017, 2018a; Ondračková et al., 2019; Stoyanov et al. 2017a).

The nomenclature and the fish intermediate host specificity of *P. centrarchi* are still under debate. Hoffman (1958, 1999) considered *P. centrarchi* a subspecies of *P. minimum* and named it *P. minimum centrarchi* because he proved that it could infect only centrarchid fishes while *P. minimum minimum* was able to infect only cyprinids. The strict host specificity of the *P. minimum* subspecies was supported by Locke et al. (2010a,b), Lane et al. (2015), Stoyanov et al. (2017a) and Boone et al. (2018). Accordingly, Stoyanov et al. (2017a) elevated *P. minimum centrarchi* to species rank as *P. centrarchi* and this novel name has become general since then (Kvach et al. 2018a; Ondračková et al. 2019).

Metacercariae of an unknown *Posthodiplostomum* species, non-indigenous in Hungary, were first recorded in 2015 when pumpkinseeds were sent to the National Food Chain Safety Office (NFCSO) from a North Hungarian water reservoir for a routine survey. These metacercariae were tentatively identified as *P. centrarchi*, a typical parasite of North American centrarchid fishes. To investigate the extent of this infection, we started a survey on the distribution, development and possible occurrence of these metacercariae in the Hungarian pumpkinseed population and on their pathogenic role in native fishes.

In this study, we report the first finding of *P. centrarchi* metacercariae in pumpkinseeds from a small water reservoir in Hungary. We also describe a recent occurrence of this non-native trematode parasite in the Sió channel close to Lake Balaton. In order to obtain adult stages and to study the histological changes induced in the host, we successfully infected chicks with metacercariae under laboratory conditions. Both metacercariae and adult stages were studied by morphological and molecular methods.

MATERIALS AND METHODS

Sample collection

Up to 2015, pumpkinseed specimens had been collected randomly from different areas of Hungary and studied for parasitic infections. In 2015, 6 out of 8 pumpkinseeds were found to be parasitised by *P. centrarchi* metacercariae during a general veterinary survey in the Maconka water reservoir. After the detection of the first metacercariae in pumpkinseed, our research group started to study the parasite fauna of the pumpkinseeds in Lake Balaton and in its outflow, the Sió channel (Table 1). In the littoral zone of Lake Balaton, the pumpkinseed is the most abundant non-native fish species (Czeglédi et al. 2019). Eighteen pumpkinseed specimens derived from the River Danube at Szentendre and a single specimen from the River Ipoly at Ipolytölgyes was examined (Table 2). Besides 142 pumpkinseeds, 16 specimens of roach (*Rutilus rutilus*) and 11 white breams (*Blicca bjoerkna*) were collected from Lake Balaton and preserved to check for *Posthodiplostomum* infection in cyprinid fishes. Moreover, 10 pumpkinseed individuals were caught from the Sió channel. Most of the fish were gathered using an electrofishing gear but several individuals were obtained by a 15-metre-long seine net. The fish were carried to the laboratory alive in oxygenated plastic bags, kept in aerated water tanks and subjected to complete parasitological dissection within 3 days.

Dissection, artificial digestion and microscopy

Before extermination, the pumpkinseeds were sedated with a drop of clove oil added to their water, then killed by a cervical cut. Each fish was investigated under a Zeiss dissecting microscope and the left side of the abdominal wall was cut down. Metacercariae were collected from the surface of the organs, excysted using a fine needle and placed in 0.9% saline solution for approximately 10 min. About 300 live metacercariae were kept alive for experimental infections. The rest of the parasites were counted and fixed in 80% ethanol for molecular examinations. The infected tissue samples were fixed in Bouin's solution, embedded in paraffin wax, cut to 4–5 μm thick sections and stained with haematoxylin and eosin. Live metacercariae and histological sections were studied with an Olympus BH2 compound microscope equipped with Nomarski differential interference contrast optics, then photographed with an Olympus DP 20 digital camera.

Experimental infection

Two experimental infections were carried out to obtain adult stages of *Posthodiplostomum* spp. for detailed morphological analysis and description. In the first case (09.02.2016–22.02.2016), each of two one-day-old unfed domestic chicks (*Gallus gallus domesticus* L., 1758) ('A' and 'B' individuals) was force-fed with 50 freshly collected *P. centrarchi* metacercariae from pumpkinseeds in the Maconka water reservoir. After the infection, the chicks were marked with green

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Table 1. Collection sites and dates of pumpkinseed (*Lepomis gibbosus*) in the Lake Balaton region

Collection site	Collection date	Number/fish	Size of fish (mean ± SE)	Infection with *P. centrarchi*
Balatonmáriafürdő 46° 42′ 34.3″ N, 17° 22′ 54.8″ E	08.02.2017	8	7.9 (±3.4)	No
Balatonszemes 46° 48′ 36.8″ N, 17° 45′ 55″ E	09.04.2008	1	9	No
	06.04.2012	6	9.6 (± 1)	No
	06.26.2018	5	8.8 (± 1.3)	No
Balatonvilágos 46° 58′ 0.5″ N, 18° 9′ 27.8″ E	07.01.2010	4	7.8 (± 3.9)	No
Keszthely 46° 45′ 22.1″ N, 17° 14′ 59.6″ E	07.27.2009	22	9.9 (± 2.3)	No
	05.04.2012	2	9 (± 1.4)	No
	04.11.2013	7	9.6 (± 1.3)	No
	06.20.2016	12	9.9 (± 1.7)	No
	05.17.2017	1	10	No
Siófok 46° 54′ 43.3″ N, 18° 2′ 32.5″ E	02.29.2015	1	10	No
	06.26–07.18.2018	40	9.8 (± 3.1)	No
Sió channel 46° 54′ 26.8″ N, 18° 2′ 48.5″ E	07.02.2018	10	12 (± 1.9)	**4**
Tihany 46° 54′ 51.6″ N, 17° 53′ 36.5″ E	07.30.2008	3	11 (± 4.6)	No
	05.07.2011	2	10.5 (± 2.1)	No
	07.19.2012	3	8.3 (± 1.2)	No
	06.26–07.02.2018	20	8.6 (± 3.2)	No
Zánka 46° 52′ 26.2″ N, 17° 42′ 17″ E	05.22.2017	5	8.8 (± 2.2)	No

Table 2. Collection sites and dates of pumpkinseed (*Lepomis gibbosus*)

Collection site	Collection date	Number/fish	Size of fish (cm)	Infection with *P. centrarchi*
Szentendre (Danube) 47° 39′ 50.3″ N, 19° 4′ 53.5″ E	04.30–05.04.2009	5	7.8 (± 1.8)	No
	07.20–08.06.2013	12	7.6 (± 2.1)	No
	09.07.2015	1	14	No
Maconka water-reservoir 48° 1′ 45.9″ N, 19° 49′ 8.9″ E	12.18.2015	8	10.6 (± 0.7)	**6**
Ipoly, Ipolytölgyes 47° 55′ 51.2″ N, 18° 46′ 15.3″ E	05.12.2017	1	11	No

colour on their head and wings. Two more individuals were preserved as negative control, which were left unmarked. Chick ‘A’ was killed by a cervical cut on 17.02.2016, while specimen ‘B’ and the negative controls were killed on 22.02.2016.

During the second experiment (03.10.2017–11.10.2017), 3 one-day-old unfed domestic chicks (‘C’, ‘D’, ‘E’) were infected per os with 50 *P. cuticola* metacercariae collected from white breams, and these birds were marked with red colour on their head. The two negative controls were unmarked. Chick ‘C’ was decapitated on 10.10.2017, then chicks ‘D’, ‘E’ and the negative controls were decapitated on 11.10.2017. All chicks had been purchased from a commercial supplier (Hegyhát BR Kft., Szentgotthárd-Rábafüzes, Hungary). Formal ethical approval was given by the Government Office of Pest County (permit PEI/001/1004-4/2015).

After the dissection, the gut system of each bird was isolated, then divided into three regions, namely duodenum plus jejunum, ileum and colon. These parts of the intestine were cut up and immersed in three different sedimentation cones containing 0.9% saline solution. After stirring for 10 min, they were allowed to subside for 30 min, then the pieces of gut and the intestinal content in the sediment were studied for trematodes under a Zeiss stereo microscope. All detected worms were removed and stored in 80% ethanol.

Molecular methods

Samples preserved in 80% ethanol were centrifuged at 8,000 *g* for 5 min to remove the ethanol. The DNA was extracted using a QIAGEN DNeasyTM tissue kit (animal tissue protocol; Qiagen, Hilden, Germany) and eluted in 100 μL AE buffer. The ITS region (part of 18S rDNA, ITS1, 5.8S rDNA, ITS2 and part of 28S rDNA) was amplified by a nested PCR as described by Sándor et al. (2017). The amplification of COI was performed using the primers (Plat-diploCOX1F and Plat-diploCOX1R) and protocol described by Moszczynska et al. (2009). PCR products were electrophoresed in 1.0% agarose gels in Tris-Acetate-EDTA (TAE) buffer gel, stained with 1% ethidium bromide. Purification was carried out with EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc., Markham, Canada). Purified PCR products of the ITS region and COI were sequenced with the PCR primers and with two additional inner primers 5.8Sr (5′-TGTCGATGAAGAGCGCAGC-3′) and 5.8S2 (5′-TAAGCCGACCCTCGGACAGG-3′) (Tkach et al. 2000) in the case of the ITS region. ABI BigDye Terminator v3.1 Cycle Sequencing Kit was used for sequencing and the sequences read using an ABI 3100 Genetic Analyser.

Phylogenetic analysis

Assembly of the sequenced fragments was done by MEGA version X (Kumar et al. 2018) and ambiguous bases clarified using corresponding ABI chromatograms. Alignments of the genes ITS and COI were done with the software CLUSTAL W (Thompson et al. 1994). The two alignments (ITS region and COI) were corrected manually using the alignment editor of the software MEGA version X. Sequences were deposited in the GenBank under the accession numbers (MN080274−92, MN179280−90, Table 3). DNA pairwise distances were calculated with the MEGA X software using the p-distance substitution model. Maximum likelihood (ML) analysis was performed for both alignments. The analysed samples are listed in Table 3. *Bolbophorus damnificus* and *Clinostomum marginatum* (AF470583 and MK426663) were chosen as the outgroup for ITS and COI genes. The dataset was tested using MEGA X for the nucleotide substitution model of best fit, and the model, shown by the Akaike Information Criterion (AIC) as the best-fitting one, was chosen for each partition. ML analyses were performed in MEGA X under the HKY + G model for the ITS region and GTR + G for the COI. Bootstrap values based on 1,000 resampled datasets were generated. The phylogenetic trees were visualised using the tree explorer of MEGA X.

RESULTS

The first *Posthodiplostomum centrarchi* infection of 6 pumpkinseed individuals was accidentally recorded from the Maconka water reservoir in 2015. Each fish was parasitised by 35–150 metacercariae located on the surface of internal organs and the mesentery; furthermore, some individuals were found in excysted condition. The excysted specimens clearly showed the morphological features of the genus *Posthodiplostomum* and corresponded to *P. centrarchi* (Figs 1 and 2) as characterised and depicted by Hoffman (1999) and Kvach et al. (2018a). In 2016 and 2017, large numbers of pumpkinseeds were examined from different regions of Lake Balaton and from the rivers Danube and Ipoly but such infection proved to be absent from these samples. Finally, our research isolated metacercariae of *P. centrarchi* in pumpkinseed specimens from the Sió channel in 2018, at the outflow of the water-gate. No *P. centrarchi* infection was found in roach and white bream dissected as controls; nevertheless, cysts of *P. cuticola* were recorded in 3 white bream specimens. In contrast, 4 out of the 10 pumpkinseed specimens collected from the Sió channel showed infection with 20–150 metacercariae.

These two *Posthodiplostomum* species could be identified and distinguished easily under compound and dissecting microscope based their morphology and predilection site. All specimens of *P. centrarchi* were found in the abdominal cavity contrary to metacercariae of *P. cuticola,* which were located in the skin, muscles and fins. In histological sections, the *P. centrarchi* cysts were mostly found in the mesenteries and inside the interstitium of the kidney and liver (Fig. 3). Less frequently they were located in the muscle of the heart's atrium (Fig. 4). They were never found in the skeletal muscle or in the muscle of the ventricle but they readily adhered to this latter. A special observation is that 3 out of the 4 *P. centrarchi* infected specimens from the Sió channel had a heavy co-infection with the nematode *Schulmanela petrushewskii* (Shulman 1948) Ivashkin 1964 in the liver (Fig. 5).

In the first experimental infection, two chicks were fed with *P. centrarchi* metacercariae per os. After 8 days, 5 and 4 adult trematodes, respectively, were found in their colon at dissection (Fig. 6). During the second experiment, when three chickens were infected with *P. cuticola* metacercariae, only 2 adult flukes were found in the bursa of Fabricius of a single chick after incubation for 8 days.

Nineteen *Posthodiplostomum* samples were analysed for the ITS region and COI genes, including metacercarial and adult developmental stages (Table 3). The amplified ITS region of the samples was more than 1,200 bps, with the alignment being 1,067 bps long after removing poorly aligned positions and divergent regions, and containing 807 conservative and 216 variable (116 of them parsimony-informative) sites. The COI fragments exceeded 460 bps and the alignment consisted of 458 bps, including 230 conservative and 228 variable (158 of them parsimony-informative) sites. The sequences of the ITS region and COI genes of the samples were in agreement with the results from the morphological and experimental studies. The analysed samples were similarly positioned on both phylogenetic trees (Figs 7A and B), one major group constituted by the samples

Table 3. List of the sequenced metacercariae and adult samples of *Posthodiplostomum* spp.

	Sample	Morphological identification	Host	Developmental stage	Date of collection	Site of collection	ITS sequenc	COI sequence
1	PD1	*Posthodiplostomum centrarchi*	Chick (first infection)	adult	17 February 2017	Maconka water reservoir	MN080274	MN179280
2	PD2	*Posthodiplostomum dentrarchi*	Chick (first infection)	adult	17 February 2017	Maconka water reservoir	MN080275	MN179281
3	PD3	*Posthodiplostomum centrarchi*	Chick (first infection)	adult	17 February 2017	Maconka water reservoir	MN080276	MN179282
4	PD4	*Posthodiplostomum* sp.	Pumpkinseed (*Lepomis gibbosus*)	metacercaria	27 November 2015	Maconka water reservoir	MN080277	MN179283
5	PD5	*Posthodiplostomum* sp.	Pumpkinseed (*Lepomis gibbosus*)		27 November 2015	Maconka water reservoir	MN080278	–
6	PD6	*Posthodiplostomum* sp.	Pumpkinseed (*Lepomis gibbosus*)		27 November 2015	Maconka water reservoir	MN080279	–
7	PD7	*Posthodiplostomum* sp.	Pumpkinseed (*Lepomis gibbosus*)		27 November 2015	Maconka water reservoir	MN080280	MN179284
8	PD8	*Posthodiplostomum* sp.	Pumpkinseed (*Lepomis gibbosus*)		27 November 2015	Maconka water reservoir	MN080281	MN179285
9	PD9	*Posthodiplostomum* sp.	Pumpkinseed (*Lepomis gibbosus*)		27 November 2015	Maconka water reservoir	MN080282	–
10	PD10	*Posthodiplostomum* sp.	Pumpkinseed (*Lepomis gibbosus*)		27 November 2015	Maconka water reservoir	MN080283	MN179286
11	PD11	*Posthodiplostomum* sp.	Pumpkinseed (*Lepomis gibbosus*)		27 November 2015	Maconka water reservoir	MN080284	MN179287
12	PD12	*Posthodiplostomum* sp.	Common bream (*Abramis brama*)		6 April 2016	Lake Balaton (Siófok)	MN080285	MN179288
13	PD15	*Posthodiplostomum* sp.	Common bream (*Abramis brama*)		19 May 2016	Lake Balaton (Balatonszemes)	MN080286	MN179289
14	PD16	*Posthodiplostomum* sp.	Common bream (*Abramis brama*)		19 May 2016	Lake Balaton (Balatonszemes)	MN080287	–
15	PD23	*Posthodiplostomum* sp.	Pumpkinseed (*Lepomis gibbosus*)		12 December 2015	Maconka water reservoir	MN080288	–
16	PD24	*Posthodiplostomum* sp.	Roach (*Rutilus rutilus*)		25 October 2016	River Danube (Szentendre)	MN080289	–
17	PD25	*Posthodiplostomum* sp.	White bream (*Blicca bjoerkna*)		25 February 2016	Lake Balaton (Siófok)	MN080290	–
18	PD26	*Posthodiplostomum* sp.	Rudd (*Scardinius erythrophthalmus*)		22 May 2017	Lake Balaton (Szigliget)	MN080291	MN179290
19	PD27	*Posthodiplostomum* sp.	Rudd (*Scardinius erythrophthalmus*)		22 May 2017	Lake Balaton (Szigliget)	MN080292	–

Figure 1. Encysted metacercaria of *Posthodiplostomum centrarchi* from the abdominal cavity of a pumpkinseed. Scale bar = 100 μm

Figure 2. Excysted metacercaria of *Posthodiplostomum centrarchi* from the abdominal cavity of a pumpkinseed. Scale bar = 100 μm

belonging to *P. centrarchi*, and apart from them another one consisting of *P. cuticola* samples, both groups supported by high bootstrap values (above 97). Between them, several other *Posthodiplostomum* and *Ornithodiplostomum* species were placed, most of them described and discussed by Locke et al. (2010a,b).

DISCUSSION

In this study, the first finding of *Posthodiplostomum centrarchi* metacercariae was reported from pumpkinseeds in a small Hungarian water reservoir (Maconka) in 2015. In 2018, additional data were presented about the current presence of this non-native trematode parasite from Sió channel, which is the outflow of Lake Balaton. This was the first record of *P. centrarchi* being in close relation with the waters of Lake Balaton. All samples were examined by morphological, histological and molecular methods; furthermore, 5 one-day-old chicks were infected with metacercariae in order to obtain adult stages of *P. centrarchi*.

The parasite fauna of pumpkinseed in Europe is relatively well studied: for a long time only three monogenean species, *Gyrodactylus avalonia* Hanek and Threlfall, 1969, *Onchocleidus similis* Müller, 1936 and *O. dispar* Müller, 1936 were known from Hungary and the neighbouring countries (Gussev et al. 1985; Kvach et al. 2018b; Moravec 2001; Ondračková et al. 2011; Roman 1953; Roman-Chiriac 1960; Stoyanov et al. 2017b; Vojtek 1958), representing the original American trematode parasite fauna of pumpkinseeds. However, several other protozoan and helminth species common in Europe were also recorded

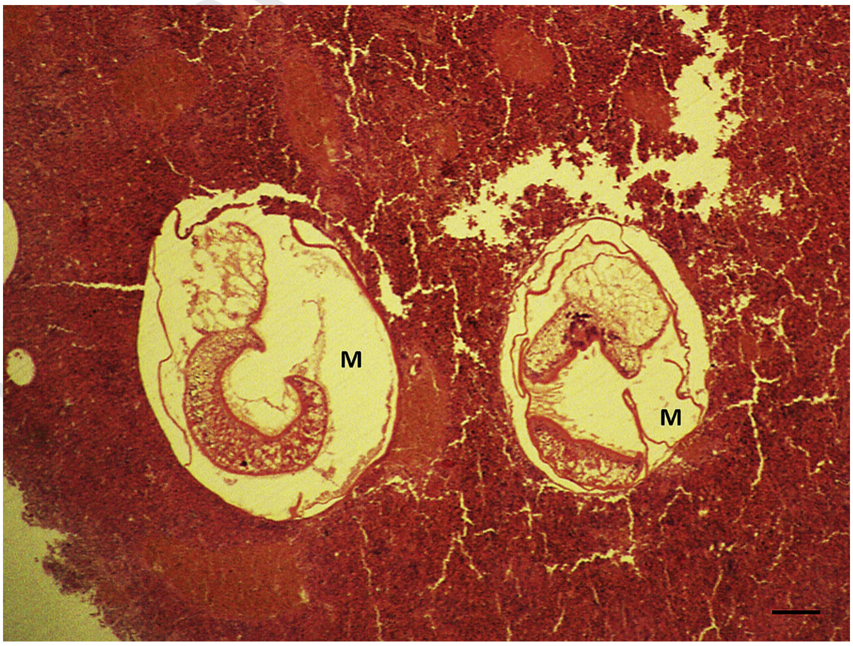

Figure 3. Histological section of the liver of a pumpkinseed infected with two metacercariae of *Posthodiplostomum centrarchi*. M: metacercaria. Haematoxylin and eosin (HE) staining. Scale bar = 100 μm

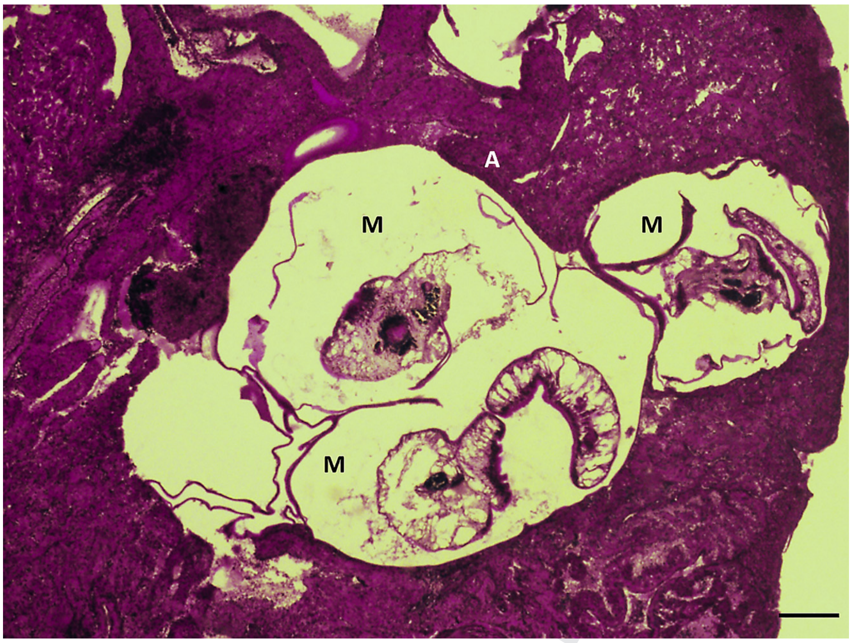

Figure 4. Histological section of the atrium of the heart of a pumpkinseed infected with metacercariae of *Posthodiplostomum centrarchi.* M: metacercaria. HE staining. Scale bar = 100 μm

in this fish (Moshu 2012, 2014; Roman-Chiriac 1960). The pumpkinseed has been present in Hungary since 1905 and it became widespread all over the country during the second half of the last century (Takács et al. 2017). Molnár (1963, 1968) was the first to study the parasite fauna of the pumpkinseed in Hungary and he reported also *O. similis* and *O. dispar*. Recently, Stoyanov et al. (2017a) has proved that *P. centrarchi* occurs in several European countries and even in Štúrovo (47° 49′ 34″ N, 18° 38′ 38″ E), Slovakia, next to the Hungarian border. Kvach et al. (2018a) stated that the infection of the European pumpkinseed population with *P. centrarchi* might be caused by the repeated imports of largemouth bass (*Micropterus salmoides*) to Europe. However, the results of Boone et al. (2018) seem to contradict this by emphasising that there are differences in the host specificity of *Postodiplostomum* species towards the fish species belonging to the genera *Micropterus* and *Lepomis*.

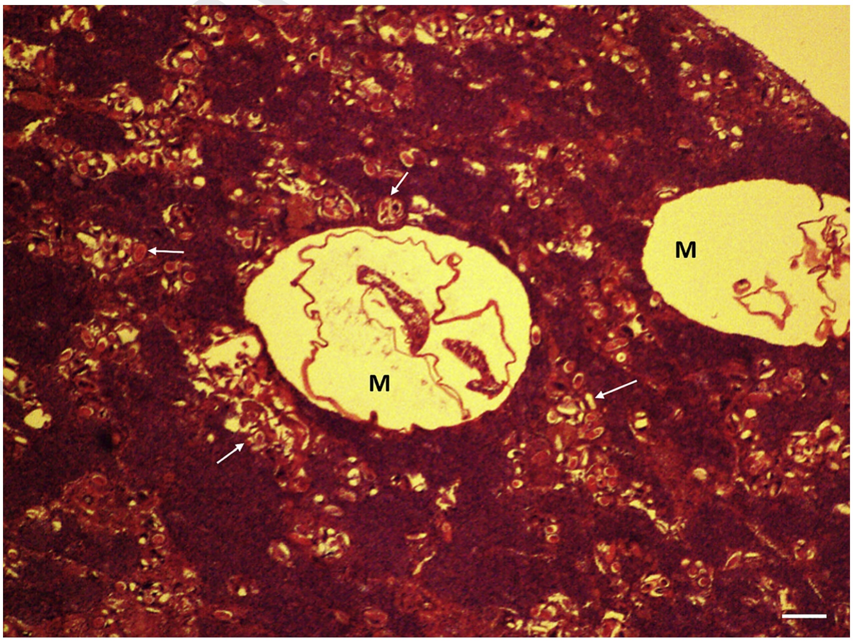

Figure 5. Histological section of the liver of a pumpkinseed containing two metacercariae of *Posthodiplostomum centrarchi* and several specimens of *Schulmanela petruschewskii* nematodes (arrows). M: metacercaria. HE staining. Scale bar = 100 μm

Figure 6. Micrographs of adult of *Posthodiplostomum centrarchi* collected post mortem from the guts of experimentally infected chicks. Scale bar = 100 μm

The occurrence of *P. centrarchi* in Hungary showed a unique pattern. After finding the parasite in a small water reservoir, several efforts were made to recover it from other areas in Hungary where pumpkinseed are abundant. Lake Balaton is the largest natural water in Hungary and the adjacent countries, where the most abundant population of pumpkinseeds is present. Despite dissecting pumpkinseeds of different size from distinct parts of Lake Balaton, no infected specimens were detected. Surprisingly, a relatively heavy infection was recorded in the Sió channel, only a few metres away from Lake Balaton. In contrast, the native *P. cuticola* was detectable in cyprinid fishes all over Lake Balaton as well as in the river Danube and its tributaries.

The spread of trematodes by their possible final host is understudied due to the difficulties of following water-birds and their parasites across countries. Up to now, Stoyanov et al. (2017a) has reported an adult *Posthodiplostomum centrarchi* from the small intestine of a grey heron (*Ardea cinerea* L.) at a bird recovery centre in Catalonia (Spain), originating from the Lagoon Bassa de les Olles, Ebro Delta, Spain. Our research group tried to get additional data on the host range of this trematode but wild water-birds were not available for research owing to the strict protection of wildlife. However, the infection experiments were successful, although the adult stages in chicks do not necessarily prove the wide host range of this trematode because these freshly hatched birds can be infected by several unspecific parasites. Nevertheless, the adult life stages obtained in infection experiments might serve as a basis for further research.

Despite heavy infections with *P. centrarchi* metacercariae, clinical changes were not found in the dissected pumpkinseed specimens. Remarkable histological changes with a general host reaction were not diagnosed either in the case when metacercariae were located inside internal organs or in the case when dozens of metacercariae covered the

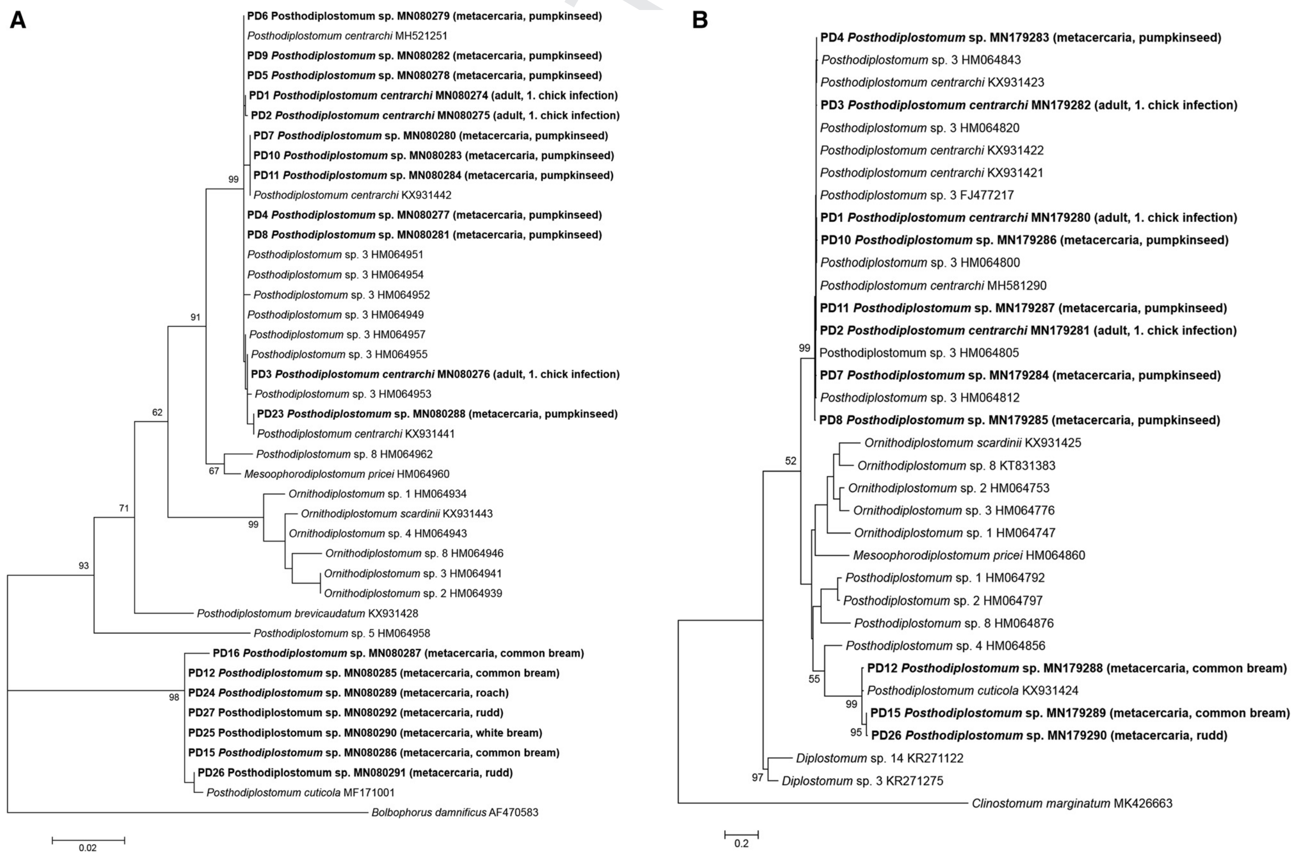

Figure 7. Maximum likelihood tree of the samples of *Posthodiplostomum* spp. from the present study (A: ITS region, B: COI) in relation to other diplostomid sequences deposited in GenBank. Bootstrap values are given at the nodes. Samples from the present study are in bold. The scale bar indicates the expected number of substitutions per site

serous membranes in the abdominal cavity. Infection of the liver of pumpkinseed with *Schulmanela petruschewskii* nematodes also seems to be common in the former USSR as Moravec (1994) reported the results of Shulman (1948) who had recorded heavy infections similar to our cases. Stoyanov et al. (2017b) also found this parasite frequently in pumpkinseed in Bulgaria. Nevertheless, changes in the liver due to *P. centrarchi* infection, especially in specimens also infected with *Schulmanela* nematodes, indicate the potential pathogenicity of *P. centrarchi*.

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