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MOLECULAR BIOLOGICAL STUDIES OF ADULT AND METACERCARIAL STAGES OF *PETASIGER EXAERETUS* DIETZ, 1909 (DIGENEA: ECHINOSTOMATIDAE)

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Molnár et al. (2015) reported two types of echinostomatid metacercariae in the lateral line organ of Hungarian fish species. Type 1 metacercariae possessed 27 collar spines and 16 uniform and three larger dorsal spines, whereas Type 2 metacercariae bore 27 collar spines and 19 equal-sized dorsal spines. In the recent work, molecular studies carried out on the ITS region and partial 28S rDNA sequences of two types of echinostomatid metacercariae and the sequences of adult stages of the species of *Petasiger* Dietz, 1909 collected from cormorants (*Phalacrocorax carbo* L.) showed that some of the Type 2 metacercariae corresponded to *Petasiger exaeretus* Dietz, 1909, whereas other morphologically similar metacercariae were identified as *Petasiger phalacrocoracis* (Yamaguti, 1939). The sequences of the Type 1 metacercariae with three larger dorsal spines could not be identified with any of the known sequences from echinostomatid trematodes.

Key words: Digenea, *Petasiger,* ITS, 28S rDNA, metacercaria

In a recent paper, Molnár et al. (2015) reported finding echinostomatid metacercariae in the lateral line organ of some Hungarian fish species. These metacercariae, characterised by 27 collar spines, could be related to *Paryphostomum* Dietz, 1909 and *Petasiger* Dietz, 1909 species infecting the intestine of fish-eating birds, especially cormorants. Two types of metacercariae were collected. One of them (Type 1) had 16 uniform and three larger dorsal spines, whereas the other (Type 2) had 19 similarly sized dorsal spines. Later, by dissecting the gut of cormorants [*Phalacrocorax carbo* (Linnaeus)], the same authors collected two echinostomatid species, *Petasiger phalacrocoracis* (Yamaguti, 1939) and *Paryphostomum radiatum* (Dujardin, 1845). By analysing the ITS sequences (including parts of 18S rDNA, ITS1, 5.8S rDNA, ITS2 and parts of 28S rDNA) of the two metacercarial types and adult specimens of *Paryphostomum radiatum* and *Petasiger phalacrocoracis,* the authors concluded that

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the sequences of five samples of Type 2 metacercariae corresponded to those of adult *P. phalacrocoracis* and to the sequence of *P. phalacrocoracis* AY245709 deposited in GenBank. The sequences of Type 1 metacercariae differed significantly from those of *P. phalacrocoracis* and any other genetically characterised species. A sixth sequence of one of the Type 2 metacercariae (PA3) gave a surprising result and, consequently, the authors postponed its study.

In recent years, two studies using molecular markers have been published on species of the genus *Petasiger*. Georgieva et al. (2012) described the life cycle of *Petasiger islandicus* Kostadinova et Skirnisson, 2007 applying 28S rDNA and nadI sequences, and Selbach et al. (2014) described four new cercariae of *Petasiger* spp. based on their morphology and 28S rDNA and nadI sequences. Recently, Tkach et al. (2016) have provided a comprehensive molecular phylogeny of the Echinostomatoidea Looss, 1899, using 28S rDNA sequences, which included *Petasiger* spp.

The present paper has a dual purpose. On the one hand, we report results showing that one sequence of a Type 2 metacercaria showed 100% identity with the sequences of adult *Petasiger exaeretus* Dietz, 1909. On the other hand, our studies were extended to analyse the 28S rDNA of *Petasiger* and *Paryphostomum* spp., proving that the sequences of the species studied by us differ from those of the *Petasiger* spp. published by both Georgieva et al. (2012) and Selbach et al. (2014).

Materials and methods

The collection of parasites, as well as the morphological and histological methods used basically corresponded to those described by Molnár et al. (2015). In that study, only specimens of *Petasiger phalacrocoracis* were recorded from the gut of cormorants. During a re-examination of the *Petasiger* collection, 14 specimens of *Pet. exaeretus* were also found in one cormorant among *Pet. phalacrocoracis* specimens. Two of these (KM2, KM4) were studied for their ITS (including part of the 18S rDNA, ITS1, 5.8S rDNA, ITS2 and part of the 28S rDNA) and partial 28S rDNA sequences. Similarly, the postponed sequencing of a Type 2 echinostomatid metacercaria (PA3) collected from a cyprinid fish, the roach [*Rutilus rutilus* (Linnaeus)], was carried out for both genes. Additionally, the 28S rDNA was partially sequenced for the *Petasiger* and *Paryphostomum* samples published by Molnár et al. (2015). Adult worms were identified according to the keys of Kostadinova (2005), Kostadinova et al. (2002) and Faltýnková et al. (2008).

Molecular methods

For DNA extraction, samples preserved in ethanol were centrifuged at 8,000 × *g* for 5 min, after which the ethanol was removed. The DNA was ex-

tracted using a QIAGEN DNeasy™ tissue kit (animal tissue protocol; Qiagen, Hilden, Germany) and eluted in 100 µl AE buffer. The ITS region (part of the 18S rDNA, ITS1, 5.8S rDNA, ITS2 and part of the 28S rDNA) was amplified and sequenced as described by Molnár et al. (2015). Partial 28S rDNA was amplified using primers ZX-1 (forward; 50-ACC CGC TGA ATT TAA GCA TAT-30) (Bray et al., 2009) and 1500R (reverse; 50-GCT ATC CTG AGG GAA ACT TCG-30) (Tkach et al., 2003) and following the thermocycling conditions described by Selbach et al. (2014). PCR fragments of 28S rDNA were sequenced directly for both strands using the PCR primers.

The sequence fragments were assembled using MEGA 6.06 (Tamura et al., 2013) and ambiguous bases clarified using corresponding ABI chromatograms. Nucleotide sequences of the ITS region and 28S rDNA were aligned with the software CLUSTAL W (Thompson et al., 1994). The alignments were corrected manually using the alignment editor of the software MEGA 6.06. DNA pairwise distances were calculated with the MEGA 6.06 software using the Tamura-Nei substitution model. Maximum likelihood (ML) and Bayesian inference (BI) analyses were performed. The liver fluke (*Fasciola hepatica* Linnaeus, 1758) was chosen as the outgroup. The dataset was tested using MEGA 6.06 for the nucleotide substitution model of best fit and the model shown by the Akaike Information Criterion (AIC) was chosen. ML analyses of the ITS region and 28S rDNA were performed under the GTR + G +I model. Bootstrap values based on 1000 resampled datasets were generated. BI was computed by Topali 2.5 (Milne et al., 2004). The likelihood parameters for BI were based on the GTR + G +I model. Posterior probabilities (PP) were estimated over 1,000,000 generations via two independent runs of four simultaneous MCMCMC chains with every 100th tree saved. The first 25% of the sampled trees were discarded as 'burn in'. The ML trees were visualised using the tree explorer of MEGA 6.06.

Results

The metacercariae were recovered from an extensive infection of roach, as described previously (Molnár et al., 2015). The infection mainly affected the scales of the lateral line organ. Two types of metacercariae were differentiated.

The single metacercaria sample (PA3), which gave a surprising molecular result, belonged to the second type of metacercariae described by Molnár et al. (2015). That type of metacercariae had a cyst size of 128–157 × 105–115 µm and bore 27 collar spines: four pairs of angle spines of 28–40 µm and 19 dorsal spines all of a similar size (17–23 µm) (Fig. 1). The excysted metacercaria of sample (PA3) had a size of 425 × 115 µm (Fig. 2). Morphologically it was completely identical with the metacercariae of *Petasiger phalacrocoracis* (PA1, PA2, PA4, CK1, CK2).

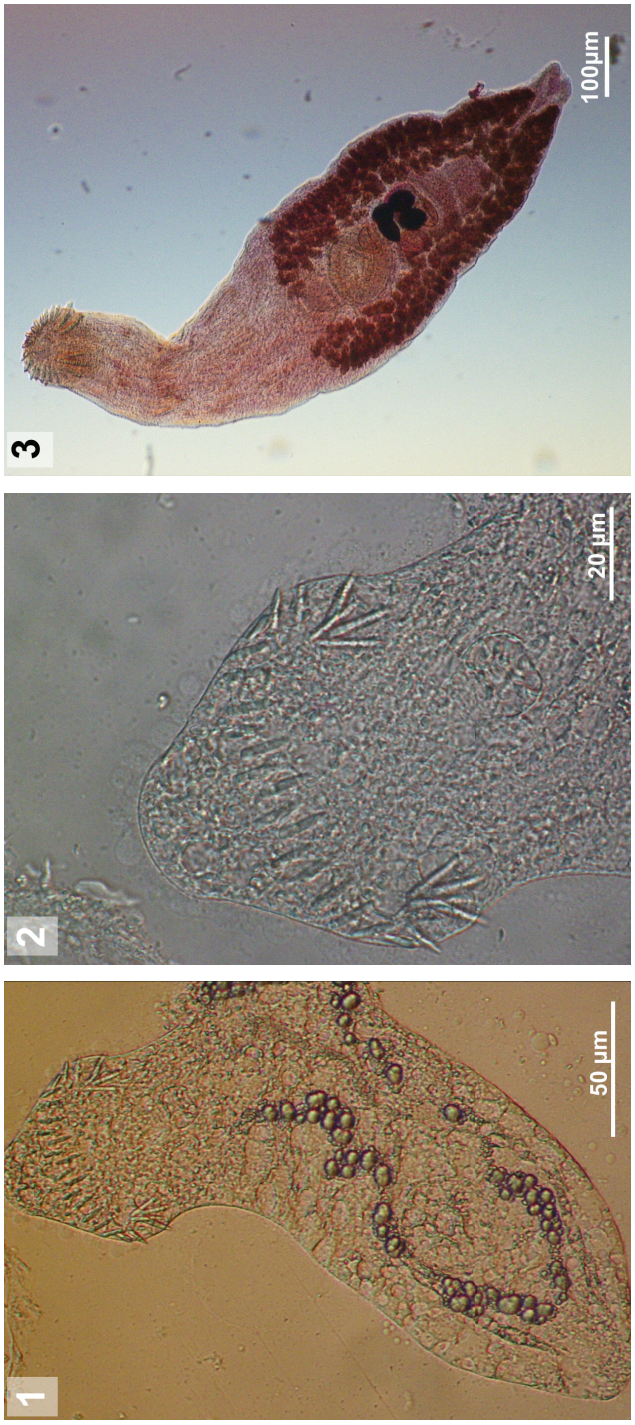


Fig. 1. Excysted specimen of a Type 2 echinostomatid metacercaria
Fig. 2. Collar spines of a Type 2 echinostomatid metacercaria, showing all of the dorsal spines of similar size
Fig. 3. Adult specimen of *Petasiger exaeretus* collected from the gut of a cormorant *(Phalacrocorax carbo)*

Table 1

List of the sequenced metacercariae and adult samples

Sample	Species	Host	Developmental stage	Collection date	Collection site	ITS	28S rDNA
PA3	***Pet. exaeretus***	**roach**	**metacercaria (Type 2)**	28 April 2014	**Keszthely, Lake Balaton, Hungary**	**KY283996**	**KY284001**
KM2	***Pet. exaeretus***	**cormorant**	**adult**	22 April 2014	**Hortobágy, Hungary**	**KY283997**	**KY284007**
KM4	***Pet. exaeretus***	**cormorant**	**adult**	22 April 2014	**Hortobágy, Hungary**	**KY283998**	**KY284009**
CK1	*Pet. phalacrocoracis*	roach	metacercaria (Type 2)	10 August 2012	Keszthely, Lake Balaton, Hungary	KJ720683	**KY284004**
CK2	*Pet. phalacrocoracis*	rudd	metacercaria (Type 2)	31 May 2012	Keszthely, Lake Balaton, Hungary	KJ720684	**KY284005**
PA1	*Pet. phalacrocoracis*	roach	metacercaria (Type 2)	06 May 2014	Keszthely, Lake Balaton, Hungary	KM972991	**KY283999**
PA2	*Pet. phalacrocoracis*	roach	metacercaria (Type 2)	28 April 2014	Keszthely, Lake Balaton, Hungary	KM972992	**KY284000**
PA4	*Pet. phalacrocoracis*	white bream	metacercaria (Type 2)	05 May 2014	Keszthely, Lake Balaton, Hungary	KM972993	no data
PH1	*Pet.* sp.	roach	metacercaria (Type 1)	28 April 2014	Keszthely, Lake Balaton, Hungary	KM972994	**KY284002**
PH2	*Pet.* sp.	roach	metacercaria (Type 1)	06 May 2014	Keszthely, Lake Balaton, Hungary	KM972995	**KY284003**
KM1	*Pet. phalacrocoracis*	cormorant	adult	22 April 2014	Hortobágy, Hungary	KM972996	**KY284006**
KM3	*Pet. phalacrocoracis*	cormorant	adult	22 April 2014	Hortobágy, Hungary	KM972997	**KY284008**
KM5	*Pa. radiatum*	cormorant	adult	22 April 2014	Hortobágy, Hungary	KM972998	**KY284010**
KM6	*Pa. radiatum*	cormorant	adult	23 April 2014	Lake Balaton, Hungary	KM972999	no data
KM7	*Pa. radiatum*	cormorant	adult	24 April 2014	Lake Balaton, Hungary	KM973000	no data

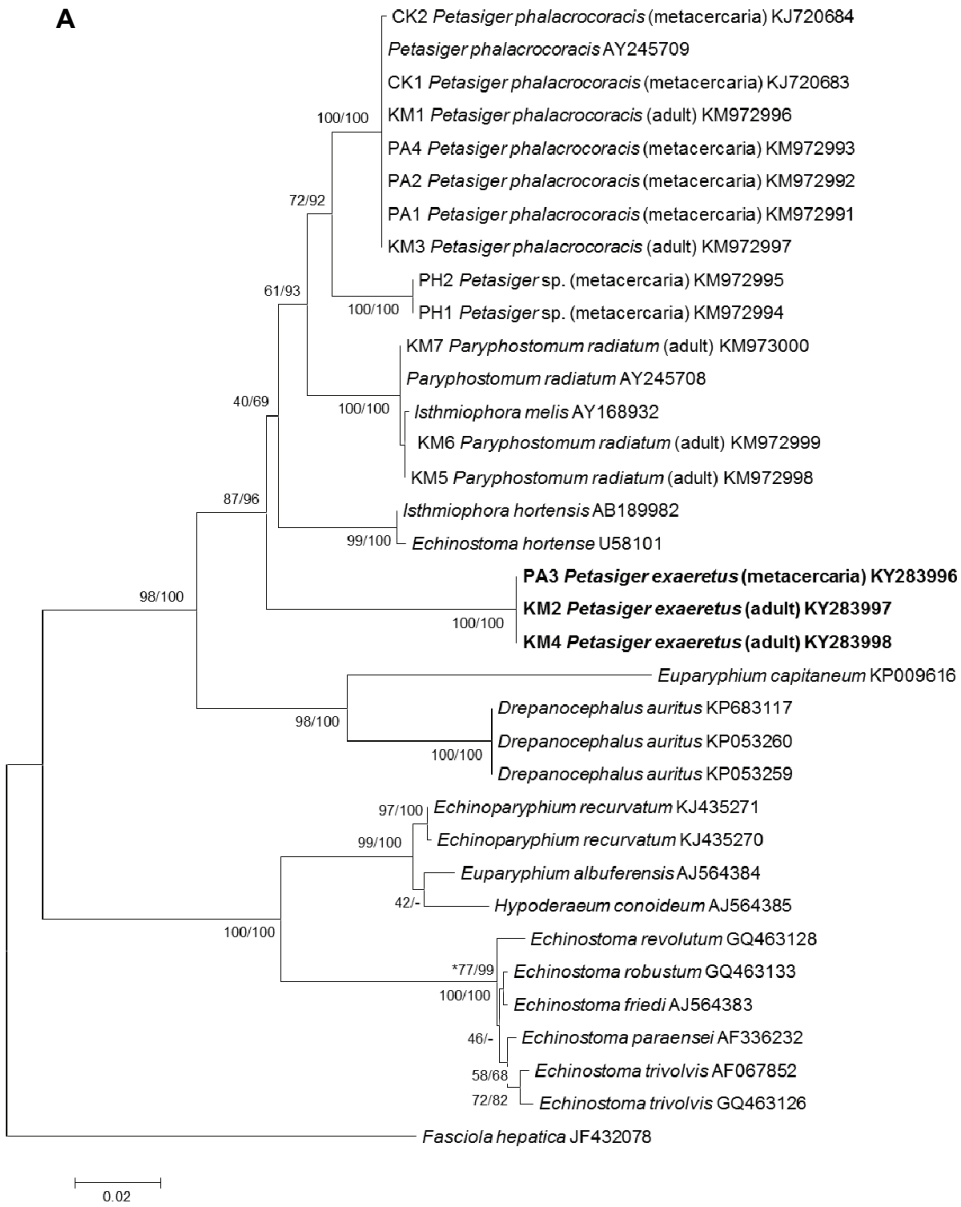
The new samples and sequences acquired by this study are written in bold, while the rest were published by Molnár et al. (2015) (*Pet.: Petasiger, Pa.: Paryphostomum*)

During an additional study of 12 *Phalacrocorax carbo* specimens caught during 2016, further echinostomatid trematodes were collected from the gut (Fig. 3). At this time, in addition to the previously reported species *Paryphostomum radiatum* and *Pet. phalacrocoracis*, the presence of a third species, *Pet. exaeretus,* was confirmed in these cormorants.

The ITS region and the 28S rDNA were sequenced for samples PA3, KM2 and KM4, in addition to the 28S rDNA for the samples previously reported by Molnár et al. (2015) (Table 1). The sequences of the ITS region were more than 1,300 bps long and the final alignment, containing the examined sequences and those downloaded from GenBank, was 1,088 bps long. Partial sequences of the 28S rDNA were about 1,230 bps long, with the corresponding alignment being 1,231 bps long. Phylogenetic analysis showed that the ITS region and the partial 28S rDNA region of metacercariae and adult stages of *Petasiger* and *Paryphostomum* spp. corresponded (Figs 4A and 4B) and proved that the sequences of one of the Type 2 metacercariae (PA3) were virtually identical with those of adult specimens of *Petasiger exaeretus* (KM2, KM4) isolated from the gut of cormorants. The samples were topologically separated from other *Petasiger* or *Paryphostomum* (*Pa.*) species with high bootstrap. The ITS sequences of the three *Pet. exaeretus* sequences showed remarkable differences from the species *Pet. phalacrocoracis* (7.8–7.9%), *Pa. radiatum* (7.4–7.5%) and the as yet unidentified Type 1 metacercariae (8.2–8.3%). The partial 28S rDNA sequences gave similar results, i.e. the *Pet. exaeretus* samples collected by us were identical with the specimen deposited in GenBank (KT956923) and differed from *Pet. phalacrocoracis* by 1.6–1.7%, from *Pa. radiatum* by 1.6% and from the Type 1 metacercaria by 1.6%. *Pet. phalacrocoracis* and *Pa. radiatum* samples were identical or nearly identical (0.0%–0.3% differences) with the deposited sequences (JQ425593, KT956926, KTKT956927). The sequences of the Type 1 metacercariae did not match those of any other genetically characterised species. The partial 28S rDNA sequences of all samples studied by us exhibited a relatively great difference from the sequences of *Pet. islandicus* Kostadinova & Skírnisson, 2007 (3.1%) and the cercariae of *Petasiger* spp. (3–4%) described by Selbach et al. (2014).

Discussion

The ITS and 28S rDNA sequences of adult specimens of *Petasiger exaeretus* and a single metacercaria (PA3) yielded an unexpected result. Previously, we had supposed that the sequences of *Pet. exaeretus* might correspond to those of the Type 1 metacercariae with uneven dorsal spines. Instead of this, a metacercaria (PA3) which corresponded morphologically to the metacercariae of *Pet. phalacrocoracis* proved to be identical with the adult specimens of *P. exaeretus*, whereas the Type 1 metacercaria (PH1, PH2) remained unidentified. There are other


A
CK2 Petasiger phalacrocoracis (metacercaria) KJ720684
Petasiger phalacrocoracis AY245709
CK1 Petasiger phalacrocoracis (metacercaria) KJ720683
KM1 Petasiger phalacrocoracis (adult) KM972996
PA4 Petasiger phalacrocoracis (metacercaria) KM972993
PA2 Petasiger phalacrocoracis (metacercaria) KM972992
PA1 Petasiger phalacrocoracis (metacercaria) KM972991
KM3 Petasiger phalacrocoracis (adult) KM972997
PH2 Petasiger sp. (metacercaria) KM972995
PH1 Petasiger sp. (metacercaria) KM972994
KM7 Paryphostomum radiatum (adult) KM973000
Paryphostomum radiatum AY245708
Isthmiophora melis AY168932
KM6 Paryphostomum radiatum (adult) KM972999
KM5 Paryphostomum radiatum (adult) KM972998
Isthmiophora hortensis AB189982
Echinostoma hortense U58101
PA3 Petasiger exaeretus (metacercaria) KY283996
KM2 Petasiger exaeretus (adult) KY283997
KM4 Petasiger exaeretus (adult) KY283998
Euparyphium capitaneum KP009616
Drepanocephalus auritus KP683117
Drepanocephalus auritus KP053260
Drepanocephalus auritus KP053259
Echinoparyphium recurvatum KJ435271
Echinoparyphium recurvatum KJ435270
Euparyphium albuferensis AJ564384
Hypoderaeum conoideum AJ564385
Echinostoma revolutum GQ463128
Echinostoma robustum GQ463133
Echinostoma friedi AJ564383
Echinostoma paraensei AF336232
Echinostoma trivolvis AF067852
Echinostoma trivolvis GQ463126
Fasciola hepatica JF432078
100/100
72/92
61/93
100/100
40/69
100/100
87/96
99/100
98/100
100/100
98/100
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97/100
99/100
42/-
100/100
*77/99
100/100
46/-
58/68
72/82
0.02

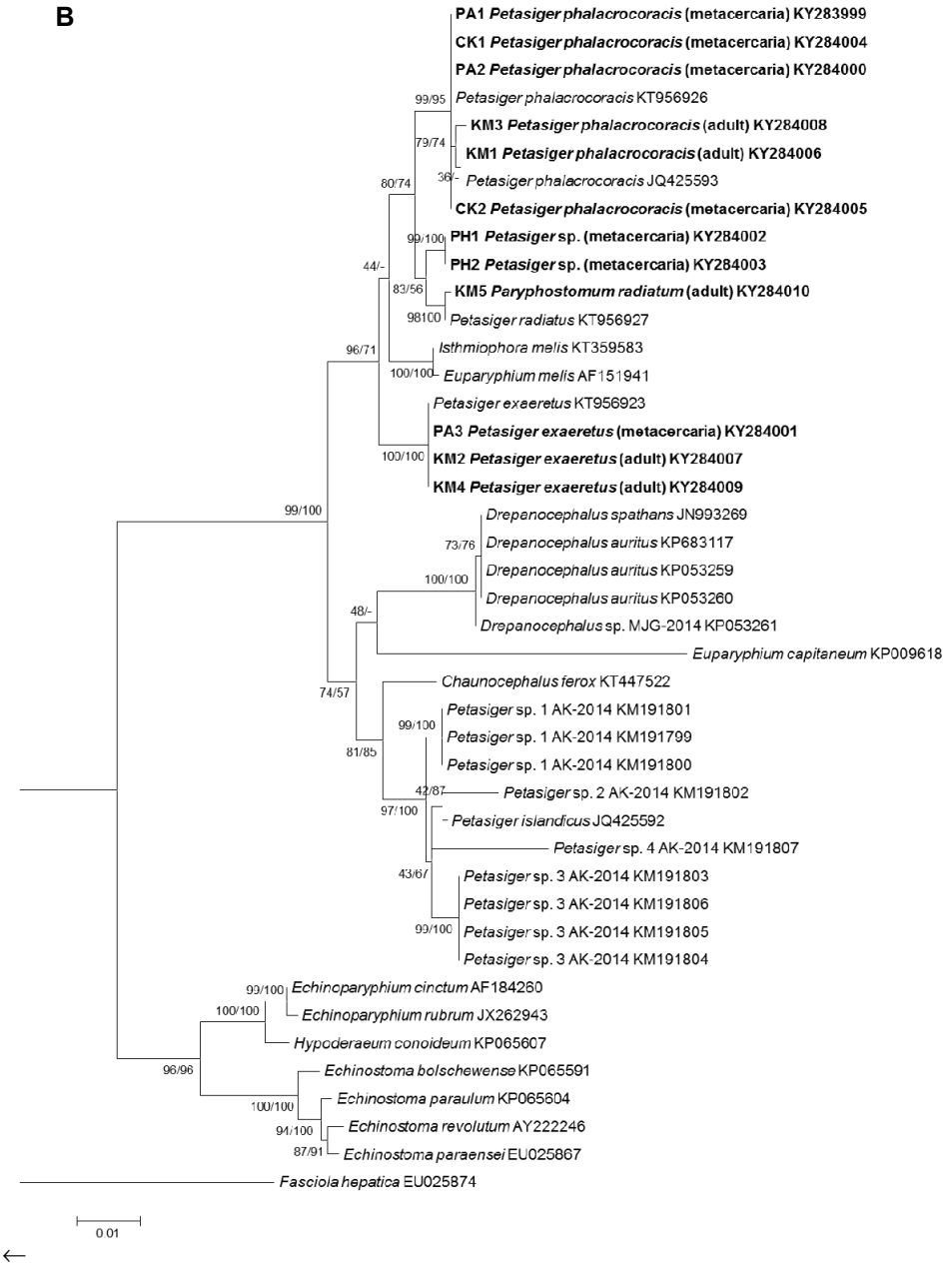

Fig. 4. Maximum likelihood tree (A: ITS; B: 28S rDNA) of the commonly found echinostomatid metacercariae from the lateral line scales of roach and adult worms from cormorants in relation to echinostomatid material deposited in GenBank. Posterior probabilities for Bayesian inference (BI) are given behind the bootstrap values for ML (hyphen means unsupported by BI). New samples (PA3, KM2, KM4) and newly acquired 28S rDNA sequences of the previously published samples (Molnár et al., 2015) are in bold

Petasiger species described from Europe (Kostadinova, 2005) for which sequence data are not yet available; these include *Pet. grandivesicularis* Ishii, 1935, *Pet. megacanthus* (Kotlán, 1922) and *Pet. pungens* (Linstow, 1893). A fifth species, *Pet. neocomense* Fuhrmann, 1927, has only nadI sequences in GenBank and, consequently, could not be compared with our samples. The above-mentioned five species have 19 collar spines rather than 27, and it is unlikely, therefore, that the Type 1 metacercaria belongs to any of these. Those species with 19 collar spines were elevated to full generic rank, as *Neopetasiger* Baschkirova, 1941, by Tkach et al. (2016), which would represent the morphological and molecular separateness of these species. The cercaria samples isolated by Selbach et al. (2014) also had 19 collar spines, so it is expected that the 28S rDNA sequences of *Pet. exaeretus, Pet. phalacrocoracis, Pa. radiatum* and the Type 1 metacercariae will not match any of them. Tkach et al. (2016) also proposed that *Pa. radiatum* should be transferred from the genus *Paryphostomum* to *Petasiger*. We agree with the opinion of Selbach et al. (2014) that there could well be a much higher diversity of *Petasiger* species based on the number of described cercariae as compared with adult forms (nine *vs.* five). Selbach et al. (2014) only discussed species with 19 collar spines, but the same diversity can be assumed for species having 27 collar spines. The Type 1 metacercaria cannot yet be regarded as a developmental stage of any of the genetically characterised species, as the nucleotide sequences of two genes contradict this; therefore, it likely indicates the presence of an as yet unknown species of *Petasiger*. Further studies on the *Petasiger* spp. of birds other than cormorants might help explain this result.

Acknowledgements

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