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Digenean trematodes (Trematoda: Digenea) parasitizing the digestive system of the great cormorant (*Phalacrocorax carbo*) in Hungary

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Summary

Great cormorants (*Phalacrocorax carbo*) are widespread piscivorous birds, which cause huge economic losses in the fish fauna worldwide. As a consequence of their predatory behaviour, they serve as definitive hosts for many digenean trematodes, and their digestive systems usually contain several parasite species.

Between 2019 and 2022, 131 bird carcasses were collected from Biharugra (Hungary) as culling on the cormorant population. Their digestive systems were subjected to parasitological examination. The studied organs (131 intestines, 44 stomachs and 21 pharynxes) were opened, and their contents were settled in water, filtered, and sorted under microscope. For species identification, sequence analysis of the ITS region was performed. Of the 131 birds, 118 were infected by tapeworms or nematodes, 105 with trematodes, and 10 were parasite-free.

The vast majority of obtained sequences (57 of the 105 trematodes) belonged to the genus *Petasiger* (43/57) and *Hysteromorpha triloba* (13/57). A single specimen of *Metorchis* sp. (1/57) as a zoonotic trematode was recorded during our survey. The results confirmed the high trematodes prevalence of the examined cormorants. However, most of them are not considered as human pathogens.
Keywords: great cormorant; *Phalacrocorax carbo*; Digenea; trematodes; ITS region; Hungary

Introduction

Great cormorants (*Phalacrocorax carbo*) (Pelecaniformes: Phalacrocoracidae) are resident piscivorous birds of several countries on different continents (except for South America and Antarctica), and are predators of the local fish fauna (Moravec & Scholz, 2016). The presence of the great cormorant in high number in the Czechia has a very negative impact for fisheries, mostly in South Bohemia and South Moravia (Moravec & Scholz, 2016). They are excellent definitive hosts of digenean trematodes, to which the primary intermediate hosts are fish. Thus the digestive system of these birds is infected generally with a variety of parasitic worms (flatworms, tapeworms, and flukes).

The digenetic flukes (Trematoda: Digenea) belong to the phylum Platyhelminthes and are considered to be one of the most significant animal and zoonotic human pathogens. Among many other piscivorous birds, (great) cormorants are definitive hosts for different digenean trematodes, so they easily transfer these parasites between different water bodies. Edelényi (1972) had already described several species of flukes colonizing cormorants at Hortobágy (Hungary), such as *Hysteromorpha* Lutz, 1931 and *Petasiger* Dietz, 1909. The parasitological results of Moravec and Scholz (2016) in Czechia reported the high prevalence of *Petasiger*, and the occasional occurrence of *Hysteromorpha* and *Metorchis* Looss, 1899 species in the digestive system. A parasitological survey on a large number of great cormorants in Poland, resulted

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in the identification of 9 digenean trematode species, belonging to the genera *Petasiger*, *Hysteromorpha* and *Metorchis* flukes (Kanarek *et al.*, 2003).

The main aim of this study was to identify the trematode fauna of great cormorants in a region of Eastern Hungary, where pond cultures and native freshwater habitats are situated together, and to assess the possible role of cormorants in spreading of the trematodes.

Materials and Methods

Sampling

Between October 2019 and April 2020, 131 carcasses of great cormorant (*Phalacrocorax carbo*) were collected from Biharugra (46°58'N, 21°34'E) during a population reduction via hunting (conducted by local fishermen) and processed under laboratory conditions. The stomachs were opened up, and the fish inside were identified in 83 cases. The digestive systems of the birds, including 131 intestines, 44 stomachs and 21 pharynxes were obtained from frozen cadavers.

After thawing, the organs were cut longitudinally with scissors and immersed in beakers filled with tap water. Removing the soaked organs, the residual solution with parasites was filtered through a hand sieve (with mesh size 200 µm), and the sediment with intestinal content was rinsed back into Petri dishes. The adult trematodes were separated based on size and morphology under dissecting (Olympus SZX16) and light microscopes (Olympus BX53), and photographed using DP74 digital camera (4×, 10× and 20× magnifications). The collected samples were preserved in Eppendorf tubes filled with 70 % ethanol for molecular investigations.

Molecular methods

The DNA was extracted using a Geneaid DNA Mini Kit (Geneaid, Taipei City, Taiwan) and eluted in 100 µL AE buffer according to the manufacturers' recommendations. The ITS region (part of 18S rDNA, ITS1, 5.8S rDNA, ITS2, and part of 28S rDNA) was amplified via a nested PCR. The primers S18 (5'-TAACAGGTCT-GTGATGCC-3') and L3T (5'-CAACTTTCCCTCACGGTACTTG-3') (Jousson *et al*., 1999) were used in the first run, and the primers D1 (5'-AGGAA-TTCCTGGTAAGTGCAA-3') and D2 (5'-CGT-TACTGAGGGAATCCTGGT-3') (Galazzo *et al*., 2002) - in the second run. The concentrations and conditions in the PCR reaction mainly followed the protocols by Sándor *et al.* (2017), and in some cases based on the methods by by Ai *et al.* (2010) with BD1 (Bowles & McManus, 1993) and BD2 (Morgan & Blair, 1995) primers.

PCR products were electrophoresed in 1.0 % agarose gels in Tris-Acetate-EDTA (TAE) buffer, stained with 1 % ethidium bromide, and then purified with an EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc., Markham, Canada). Purified PCR products of the ITS region were sequenced bidirectionally with primers used in the preceding PCR using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit. The sequences were read at the Delta Bio 2000 Kft in Szeged, Hungary, using an ABI Prism 3100 Genetic Analyser (Thermo Fisher Scientific, Waltham, USA).

Table1. The list of fish species from the stomachs of great cormorants.

Fish species	Fish family	Number of individuals	The number of cormorants in which the fish species was found
Prussian carp (*Carassius gibelio*)	Cyprinidae	57	35
Common bream (*Abramis brama*)		11	9
White bream (*Blicca bjoerkna*)		7	2
Bleak (*Alburnus alburnus*)		15	3
Barbel (*Barbus barbus*)		1	1
Asp (*Leuciscus aspius*)		1	1
Carp (*Cyprinus carpio*)		9	7
Grass carp (*Ctenopharyngodon idella*)		3	3
*Hypophthalmichthy*s sp.		2	2
Pikeperch (*Sander lucioperca*)	Percidae	34	13
Perch (*Perca fluviatilis*)		4	3
Ruffe (*Gymnocephalus cernua*)		1	1
Schraetzer (*Gymnocephalus schraetser*)		1	1
Wels catfish (*Silurus glanis*)	Siluridae	1	1
Black bullhead catfish (*Ameiurus melas*)	Ictaluridae	21	6
Northern pike (*Esox lucius*)	Esocidae	2	2

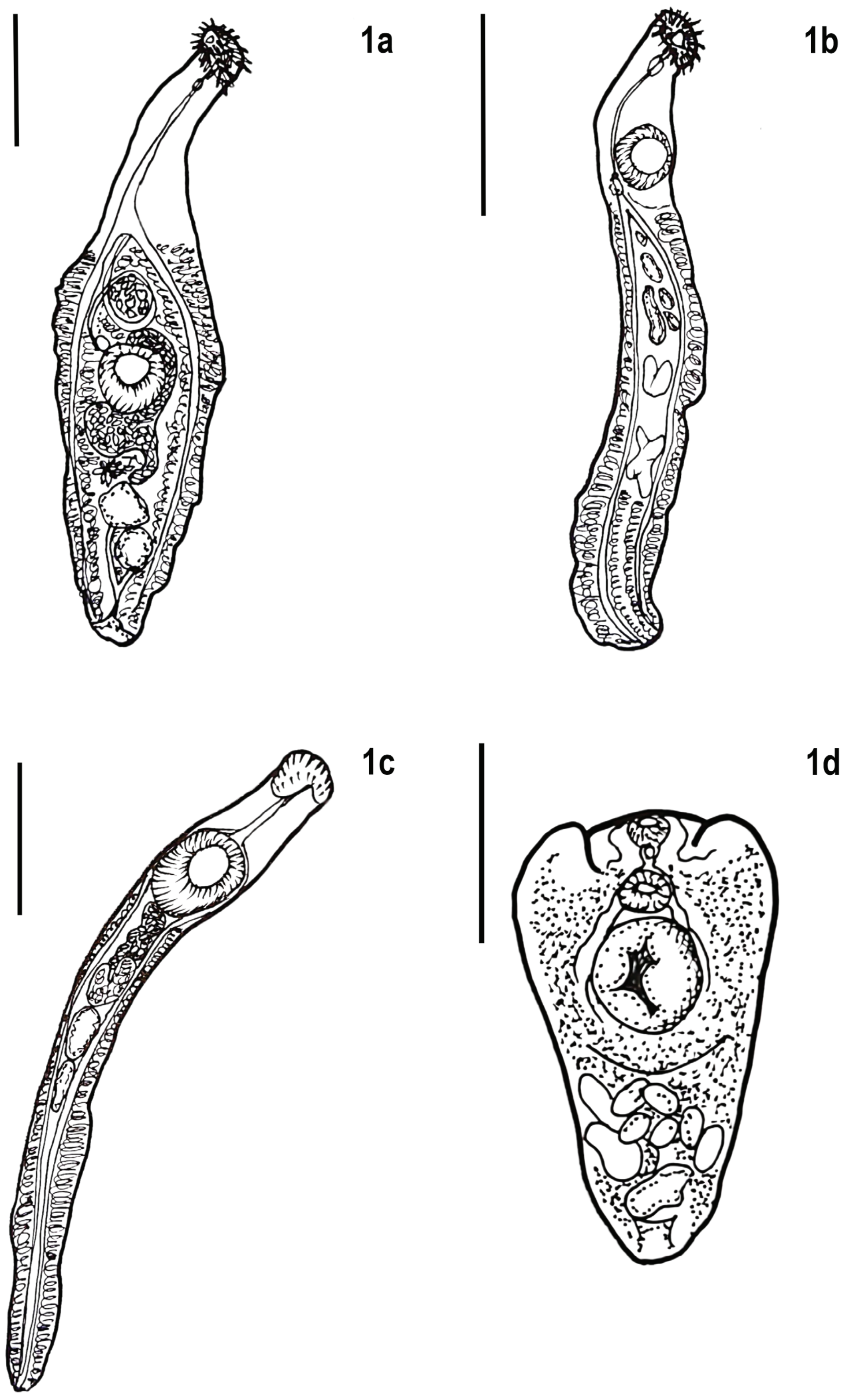

Fig. 1. Line drawing of the trematode species commonly found in the intestines of cormorants: a – *Petaseiger exaeretus*, b – *Petasiger radiatus*, c – *Petasiger phalacrocoracis*, d – *Hysteromorpha triloba*. Scale bars: a, d – 200 μm; b, c – 500 μm.

The sequenced fragments of the ITS region manually edited and assembled using Geneious Prime v.11.1 (Kearse *et al.*, 2012) and ambiguous bases were clarified using overlapping parts of the ABI chromatograms. Assembled sequences were identified by nucleotide BLAST search in the NCBI GenBank database. Pairwise distances between our samples and reference sequences from GenBank were calculated with the MEGA X (Tamura *et al.*, 2021) software using the p-distance model. Representative sequences (12) of the identified species were deposited in GenBank under the accession numbers (PP188695-PP188706). Maximum likelihood (ML) analyses were performed in MEGA X for the three alignments (*Petasiger*, *Hysteromorpha* and *Metorchis* species and their close relatives). The datasets were tested using 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. ML analyses were performed under the GTR + I for the datasets of *Petasiger* species and TN93 G + I for the other two alignments. Bootstrap values based on 1 000 resampled datasets were generated. The ML trees were visualised using the tree explorer of MEGA X. *Fasciola hepatica* (JF432078), *Cotylurus marcogliesei* (MH521248) and *Apophallus muehlingi* (MF438052) were chosen as outgroups for the three analyses, respectively.

Ethical Approval and/or Informed Consent

The article does not contain any studies involving animals in experiments performed by any of the authors.

Results

Gross and Microscopic observations

In this study, the digestive systems of 131 frozen great cormorants were processed and subjected to parasitological investigation. Different parts of gastrointestinal tract (pharynx, stomach, intestine) were studied. Parasitic worms were detected only in the intestine. The stomachs of the cormorants were mostly (83 individuals) filled with remnants of fish, 48 of 131 were found empty. In the content of stomachs, overall 170 fish from 16 species were identified, their number varied from 0 up to 11 per cormorant. The biggest was a 36.1 cm long grass carp (*Ctenopharyngodon idella*). The ingested fish were mostly members of the family of Cyprinidae, Prussian carp (*Carassius gibelio*) was the most common species (Table 1)

Table 2. Prevalence of trematodes isolated and identified by molecular analysis from the digestive system of great cormorants from Biharugra

Trematoda species	Prevalence
Petasiger exaeretus	17/57 (29.8%)
Petasiger radiatus	23/57 (40.4%)
Petasiger phalacrocoracis	3/57 (5.2%)
Hysteromorpha triloba	13/57 (22.8%)
Metorchis orientalis	1/57 (1.7%)

Of the 131 birds, 118 were infected by tapeworms or nematodes, 105 with trematodes, and 10 were parasite-free. Although, both tapeworms and nematodes were abundant in the digestive tract of cormorants, only few samples were preserved, but no morphological and molecular analyses were performed on them.

The presence of various fluke species in one specimen was uncommon, but in some cases, the co-existence of three different *Petasiger* species could also be detected.

Pharynxes were also examined for the presence of certain parasites such as the zoonotic *Clinostomum complanatum* (Rudolphi, 1814) but all samples were negative.

Molecular analysis

Molecular identification was performed by sequence analysis of the 800-1200 bp long ITS region of digenetic trematodes. There were 57 samples where required region sequences were successfully obtained. According to the results (Table 2), the vast majority of collected flukes belonged to the genus Petasiger (43/57), including *Petasiger phalacrocoracis* (Yamaguti, 1939) (3/57), *Petasiger radiatus* (Dujardin, 1845) (23/57) and *Petasiger exaeretus* Dietz, 1909 (17/57). About a quarter of the specimens were *Hysteromorpha triloba* (Rudolphi, 1819) (13/57) which is also a highly common parasite of the intestinal tract of cormorants (Fig. 1). In addition, one of the samples showed the closest similarity to *Metorchis orientalis* Tanabe, 1920 (1/57) a proven zoonotic species with cyprinids as the primary intermediate hosts, could be detected in one great cormorant.

The nucleotide sequences of *P. palacrocoracis* showed an 99.4 % similarity overall to previously submitted ones into GenBank. The overall sequence similarities for *P. exaeretus* and *P. radiatus* to the sequences recorded in GenBank were 99.8 % and 98.7 %, respectively. The obtained sequences from *H. triloba* showed an overall 99.4 % similarity. The single *Metorchis* sample showed 99.5 % identity to the *Metorchis orientalis* sequences (MW001041, MW001043-45), 99.6 % to *Metorchis xanthosomus* (JQ716400) and 99.7 % to *Metorchis ussuriensis* (KP222468). On the other hand, additional annotated *Metorchis orientalis* sequences showed remarkably lower similarities (ranging from 96.9 to 97.9 %).

Maximum likelihood analyses (Fig. 2) confirm species identity in the case of the *Petasiger* and *Hysteromorpha* samples, where the samples were clustered into monophyletic clades with maximum bootstrap values. In the case of the *Metorchis* sample, however, the situation is more complex, as the corresponding clade includes isolates of *M. orientalis*, *M. ussuriensis,* and *M. xanthosomus*. On the other hand, the samples of *M. orientalis* also group with other *Metorchis* species in sister clades, which makes identification to species level questionable. Therefore, we restrict the identification to the genus level and use the name *Metorchis* sp.

Discussion

There are more than 40 digenean species have been reported from cormorants in Europe (Table 3) with the most data originating from Czechia. The parasitic fauna of the great cormorants found in this study agreed with the majority of previous publications that *Petasiger* and *Hysteromorpha* species are dominant in the intestines (Vojtěchovská-Mayerová, 1952; Ryšavý, 1958; Moravec *et al*., 1988; Kanarek & Zaleśny, 2014; Moravec & Scholz, 2016). The first records of trematodes in these birds were reported by Vojtěchovská-Mayerová (1952) and Ryšavý (1958), from the former Czechoslovakia, River Danube from a colony population so-called '*Cormorant Island*'. In their research, they reported two species of trematodes, *Petasiger radiatus* and *Hysteromorpha triloba*, beside a cestode, *Paradilepis scolecina* (Rudolphi, 1819) and a

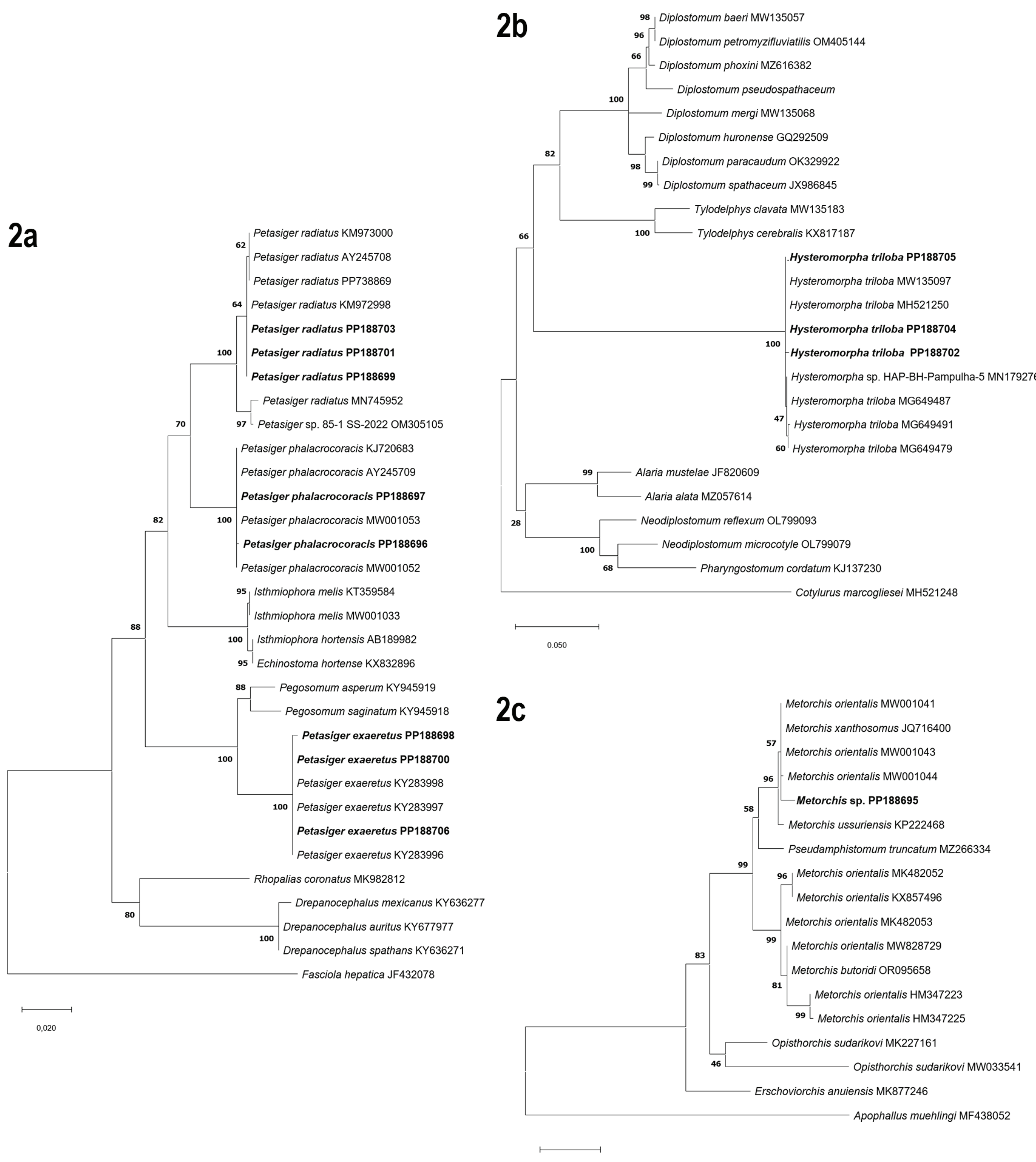

Fig. 2. Phylogenetic analysis by maximum likelihood estimation of *Petasiger* (a) *Hysteromorpha* (b) and *Metorchis* (c) samples with related sequences deposited inGenBank. Samples from the present study are in bold. The scale bar indicates the expected number of substitutions per site.

Table 3. The list of digenean trematode species documented from great cormorants (*Phalacrocorax carbo*) in Europe. Some of the references use former species names, therefore the listed names here are not necessarily identical with names in the original references. References: [1]Biedunkiewicz *et al*. (2012), [2]Cech *et al* (2017), [3]Kanarek *et al*. (2003), [4]Kanerek and Rokicki (2005), [5]Kanarek and Zaleśny (2014), [6]Kornyushin (2008), [7]Molnár *et al*. (2015) [8]Moravec and Scholz (2016), [9]Našincová *et al*. (1993), [10]Ossmann (2008), [11]Sitko *et al*. (2006), [12]Sonin (1985, 1986), [13]Švažas *et al*. (2012), [14]Yakovleva *et al*. (2020), [15]this study

Species	**References**
Apatemon gracilis (Rudolphi, 1819)	5
Apophallus mühlingi (Jägerskjöld, 1899)	6, 9, 11
Aporchis croaticus (Stossich, 1889)	12
Ascocotyle longa Ransom, 1920	8, 9, 11
Cercarioides aharonii Witenberg, 1929	5, 8, 9, 11
Ciureana cryptocotyloides Issaitschikov, 1923	6
Clinostomum complanatum (Rudolphi, 1814)	10, 12
Cryptocotyle concava (Creplin, 1825)	3, 4, 5, 6
Cryptocotyle lingua (Creplin, 1825)	6
Echinochasmus amphibolus Kotlán, 1922	12
Echinochasmus coaxatus Dietz, 1909	4, 5, 13
Echinochasmus mergi (Cannon, 1939)	12
Echinochasmus mordax (Looss, 1899)	6
*Echinochasmus muraschkinzev*i Baschkirova, 1941	12
Echinochasmus spinulosus (Rudolphi, 1809)	4, 5
Echinoparyphium recurvatum (von Linstow, 1873)	12
Echinostoma revolutum (Fröhlich, 1802)	12
Galactosomum cochleariforme (Rudolphi, 1819)	6
Galactosomum lacteum (Jägerskiöld, 1896)	6, 8, 9, 11
Galactosomum phalacrocoracis Yamaguti, 1939	6
Galactosomum puffini Yamaguti, 1941	6
Heterophyes aequalis Looss, 1902	8, 9, 11
Holostephanus dubinini Vojtek & Vojtkova, 1968	3, 4, 5, 8, 9, 10, 13
Hysteromorpha triloba (Rudolphi, 1819)	3, 4, 5, 6, 8, 9, 10, 13, 15
Maritrema linguilla Jägerskiöld, 1908	12
Maritrema subdolum Jägerskiöld, 1909	12
Metagonimus yokogawai (Katsurada, 19129	5, 6
Metorchis xanthosomus (Creplin, 1846)	3, 4, 5, 6, 8, 9, 10, 11, 13, 15
Neoharvardia shigini Iskova, Stenko & Sudarikov, 1992	6
Patagifer parvispinosus Yamaguti, 1933	12
Petasiger exaeretus Dietz, 1909	2, 3, 4, 5, 6, 7, 8, 9, 11, 13, 14, 15
Petasiger phalacrocoracis (Yamaguti, 1939)	2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15
Petasiger radiatus (Dujardin, 1845)	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15
Petasiger testitrifolius (Gogate, 1934)	12
Posthodiplostomum cuticola (Nordmann, 1832)	5
Prosthogonimus cuneatus (Rudolphi, 1809)	12
Pygidiopsis genata Looss, 1907	6
Renicola secundus Skrjabin, 1924	11
Stephanoprora polycestus (Dietz, 1909)	6
Stephanoprora pseudoechinata (Olsson, 1876)	4, 5, 12
Tylodelphys clavata (von Nordmann, 1832)	3, 4, 11, 13,
Uroproctepisthmium bursicola (Creplin, 1937)	12

nematode (*Contracaecum rudoplhi*). Moravec *et al.* (1988) also reported trematodes from great cormorants. At that study covering South Bohemia, they isolated 5 species of trematodes, including *Desmidocercella incognita* Solonitsin, 1932, *H. triloba, Paradilepis scolecina, Petasiger exaeretus* and *Petasiger radiatus*. Subsequently, Našincová *et al*. (1993) reported 11 species: *P. exaeretus, P. phalacrocoracis, P. radiatus, Metorchis xanthosomus, Heterophyes aequalis, Apophallus muehlingi Galactosomum lacteum Cercarioides aharonii Ascocotyle longa, Holostephanus dubinini* and *Hysteromorha triloba.* The Checklist of Trematodes (Digenea) of birds (Sitko *et al*. 2006) shows greater diversity of species and lists 13 species from cormorants in Czechia and Slovakia, beside the formerly mentioned 11 species. Even *Renicola secundus* and *Tylodelphys clavata* were also recorded. Later also from South Bohemia, Moravec and Scholz (2016) investigated 46 freshly shot great cormorants, in which *H. triloba* (4), *M. xanthosomus* (Creplin, 1846), *P. radiatus*, *P.r exaeretus* and *P. phalacrocoracis* were found. The 56 great cormorant samples from South Moravia were more diverse regarding to trematodes: beside the above mentioned species, the authors found adult species of *A. muehlingi* (Jägerskiöld, 1899), *A. longa*, *C. aharonii*, *G. lacteum, H. aequalis* and *H. dubinini*. Our result compared to the data by Moravec and Scholz (2016) from the Czechia, showed a high similarity in the diversity and abundance of taxa.

In Poland, Kanarek *et al.* (2003) reported the abundancy of *Petasiger* flukes and 8 species in total: *P. radiatus, P. exaeretus, P. phalacrocoracis, M. xanthosomus, Cryptocotyle concava* (Creplin, 1825), *H. triloba, H. dubinini* and *T. clavata* (von Nordmann, 1832). The latter species was the first record from cormorants. Kanarek and Rokicki (2005) extended the list with *Echinochasmus coaxatus* and *Monilifer spinulatus*. Biendunkiewicz *et al.* (2012) did not report a diverse digenean fauna, however demonstrated a high prevalence of *P. radiatus*. Data reported by Kanarek *et al.* (2014) demonstrated the presence of many more trematode species during a complete helminthological examination of 90 cormorant specimens from the brackish waters of Poland between 2000 and 2001. They detected 9 digenetic trematoda species from 83 birds: *P. radiatus*, *P. exaeretus*, *P. phalacrocoracis*, *Mesorchis pseudoechinatus*, *M. xanthosomus*, *C. concava*, *H. triloba*, *T. clavata* and *H. dubinini*. Then, Kanarek and Zaleśny (2014) documented the occurrence of "so-called" cormorant specific digenetic trematodes (helminth species that reach their maturity in members of the family Phalacrocoracidae) such as *H. triloba*, *H. dubinini*, *P. radiatus*, *P. exaeretus*, *P phalacrocoracis* and the generalist species (*Stephanoprora pseudoechinata*, *Cercarioides aharonii*, *C. concava*, *Metagonimus yokogawai, M. xanthosomus*) from north-eastern Poland.

There are some additional data form the rest of Europe, Sonin (1985, 1986) listed several species from cormorant in the territory of the former Soviet Union (Table 3). In Germany, Oßmann (2008) also found the dominance of *Petasiger* species in cormorants beside a considerable amount of *H. triloba* and *M. xanthosomus*, as well as few members of *H. dubinini* and one specimen of *Clinosotomum complanatum*. Švažas *et al.* (2012) reported 7 species from Lithuania, *Petasiger* sp. individuals were also found in abundance similarly to the above mentioned resources.

In Hungary, there are only scarce data about the trematodes of cormorants. Previous results come from the Hortobágy (approximately 80 km far from our sampling area), where Edelényi (1972) performed an autopsy on wild water birds; however, apparently, no cormorants were dissected as they were much less abundant at that time. He reported the presence of several typical trematodes of cormorants in other birds, like *Metorchis intermedius* in the gall bladder of a coot (*Fulica atra*) from Hortobágy, and also *H. triloba* (42) in the intestinal tract of a spoonbill (*Platalea leucorodia*). However, *P. radiatus, P. exaeretus* or *P. phalacrocoracis* were not recovered during his survey.

Molnár *et al.* (2015) observed *P. radiatus*, *P. phalacrocoracis* and a third, unidentified *Petasiger* sp. metacercariae in the lateral line scales of several cyprinids, some percid and centrarchid species in the Kis-Balaton area (located 340 km west from Biharugra) of Hungary. Parallel, the digestive tracts of 12 great cormorants originating from the Hortobágy and Lake Balaton were examined for echinostomatid trematodes, which confirmed that the intestinal tracts of the birds are commonly infected with *Petasiger* species. Subsequent molecular studies based on the ITS region (18S rDNA, ITS1, 5.8S rDNA, ITS2) and partial 28S rDNA sequences determined the third, previously unidentified *Petasiger* species as *P. exaeretus* (Cech *et al.*, 2017). These earlier data are consistent with our recent results, the genus *Petasiger* is present commonly in Hungarian pond aquacultures and natural freshwaters: metacercariae in the lateral line of fishes and adults in the intestinal tract of great cormorants.

Hysteromorpha is also a typical species of cormorant intestinal tract, whose unique trilobulated forebody is easily recognised under the microscope. Adults of all these trematodes are commonly found in the small intestine of great cormorants. Metacercariae of *Petasiger* are embedded in the lateral line scales of cyprinid fish, and metacercariae of *Hysteromorpha* are found in the muscle tissue of same host species. While members of the genus *Metorchis* parasitize the gall-bladder of birds explaining why only one *Metorchis* specimen have been found in the intestinal tract besides many *Petasiger* flukes.

In the examined great cormorants, only a single zoonotic sample, *Metorchis* sp*.* was found. Although species level identity could not be determined unambiguously, however based on the sequence data it seems proven that our sample belonged to the genus *Metorchis*. These trematodes develop into metacercariae by embedding in the muscle tissues of the secondary intermediate host (fishes) but the adult flukes live mainly in the bile ducts and gall bladder of the definitive hosts (piscivorous mammals and birds), where they can cause chronic inflammation, gastritis and carcinomas (Sohn, 2009; Sitko *et al.*, 2016; Gao *et al.*, 2021).

Conflict of Interest

The authors declare that they have no conflicts of interest.

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