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Molecular characterization of European *Pygorchis* Looss, 1899

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Short Communication

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Abstract

Adult trematodes of the genus *Pygorchis* Looss, 1899 (Trematoda: Philophthalmidae) parasitize the cloaca of birds. The genus contains three species, all of which are rarely reported and molecular phylogenetics of which have not been applied. The absence of reference DNA sequences limit studies of their indistinct larval forms. Based on the materials that were obtained from birds of the Czech origin, we performed a molecular characterization of both currently known *Pygorchis* spp., which are known from the Palearctic, the type species *Pygorchis affixus* Looss, 1899 and *Pygorchis alakolensis* Zhatkanbaeva, 1967, and provided morphological description of the examined *P. alakolensis* specimen. We found that the two species were of similar dimensions; the only difference was in the position of testes and in the extent of vitelline follicles. However, the position of testes in *P. affixus* was variable, and approximately 10% of examined *P. affixus* individuals had testes positioned obliquely. The second feature that allows differential diagnostic, the extent of vitelline follicles, was more reproducible as the vitelline follicles of *P. affixus* did not extend beyond the intestinal caeca, or, in exceptional cases, they extended them at only one side. In the examined *P. alakolensis* individual, the testes were positioned obliquely, and the vitelline follicles extended beyond the intestinal caeca. We reported *P. alakolensis* for the first time from Europe; previously, it was known only from Central Asian lakes and rivers. We confirmed the classification of *Pygorchis* into Philophthalmidae.

Introduction

Species of the genus *Pygorchis* Looss, 1899 are rare and little understood trematodes that occur in the cloaca of birds. Based on their morphological characters, the representatives of the genus *Pygorchis* and morphologically similar *Cloacitrema* Yamaguti, 1935, *Oswaldotrema* Muniz-Pereira & Pinto, 2000 and *Pittacium* Szidat, 1939 form the subfamily Cloacitrematinae Yamaguti, 1958 within the Philophthalmidae Looss, 1899. The genus *Pygorchis* consists of three species, the type species *Pygorchis affixus* Looss, 1899 and *Pygorchis alakolensis* Zhatkanbaeva, 1967, which are known from the Palearctic, and the Nearctic species *Pygorchis americanus* Dronen, 1985. Concerning host specificity, *P. affixus* is known in hooded crows *Corvus cornix* Linnaeus, 1758, common kestrels *Falco tinnunculus* Linnaeus, 1758, Eurasian marsh harriers *Circus aeruginosus* (Linnaeus, 1758) and pied avocets *Recurvirostra avosetta* Linnaeus, 1758 from Egypt (Looss 1899), in grey herons *Ardea cinerea* Linnaeus, 1758 from the Czech Republic (eight records; Sitko, 1999, 2012; Sitko *et al.*, 2006; Sitko & Heneberg, 2015), in black-winged stilts *Himanthopus himanthopus* from Ukraine (Iskova *et al.*, 1995) and in white wagtails *Motacilla alba* Linnaeus, 1758 from Russia (Bykhovskaya-Pavlovskaya, 1974). Concerning the other two less frequently reported species, *P. alakolensis* was found in representatives of the Laridae Rafinesque, 1815 – namely, in black-headed gulls *Chroicocephalus ridibundus* (Linnaeus, 1758), Caspian terns *Hydroprogne caspia* Kaup, 1829, common terns *Sterna hirundo* Linnaeus, 1758 in Kazakhstan (Zhatkanbaeva, 1967) and in European herring gulls *Larus argentatus* Pontoppidan, 1763 in Russia (Semenova, 1983; Semenova & Ivanov, 1985; Nekrasov *et al.*, 1999); and *P. americanus* was described in roseate spoonbills *Platalea ajaja* Linnaeus, 1758 in Texas (Dronen, 1985). There are no molecular data available for the *Pygorchis*.

In the present study, we performed a molecular characterization of two of the three currently known *Pygorchis* spp., report the extension of the distribution area of *P. alakolensis* (previously known only from Central Asian lakes and rivers) and used molecular phylogenetics to confirm of the classification of *Pygorchis* into Philophthalmidae.

Material and methods

Sampling

For the molecular analyses, we examined the *Pygorchis* spp. that were obtained from the adult male of *S. hirundo* collected on July 20, 2014 and from the adult female of *A. cinerea* collected

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Table 1. Prevalence (*n*), relative prevalence (%) and intensity (I) of infections by *Pygorchis* spp. in analysed birds of Czech origin.

Host species	Helminth species	Number of host individuals examined	*N*	%	I
Sterna hirundo	*Pygorchis alakolensis*	125 adults examined in March through May	1	0.8	2
Ardea cinerea	*Pygorchis affixus*	142 males examined in February through May	3	2.1	1, 1, 2
Ardea cinerea	*Pygorchis affixus*	88 females examined in February through May	5	5.7	1, 2, 4, 5, 6

The data are based only on individuals, which were obtained until May of the respective year, because it is likely that the *Pygorchis* spp. lifecycles include intermediate hosts that are absent in the Czech Republic. We applied this limitation due to the extrapolation of our data that we obtained on a local strictly migratory *C. ridibundus* population (J. Sitko, pers. obs.). In *C. ridibundus*, the trematodes of the gastrointestinal tract, which have their intermediate hosts at *C. ridibundus* wintering sites only, usually did not survive beyond May, whereas such trematodes of the liver and kidneys usually survived until the autumn migration.

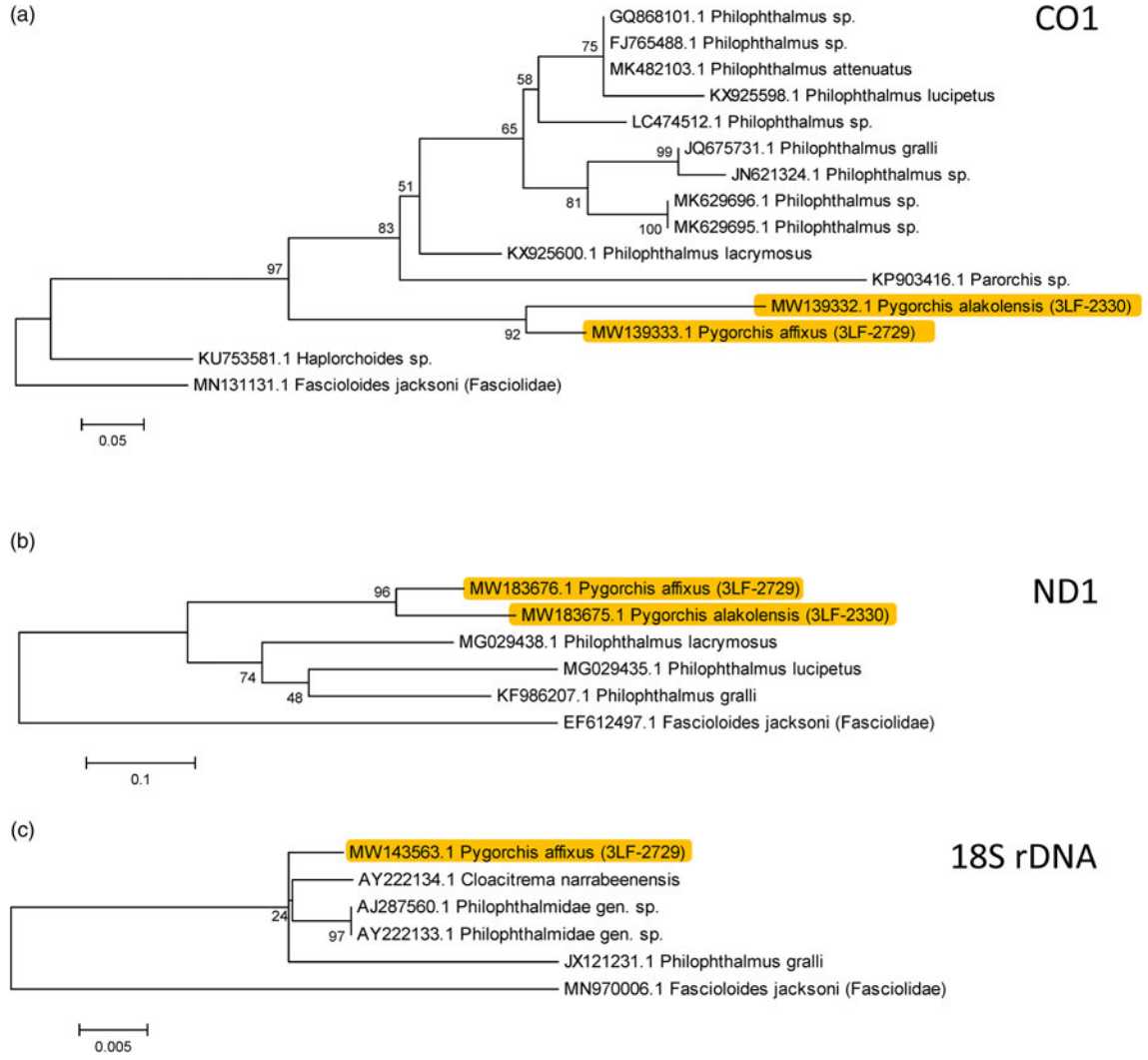


Fig. 1. Maximum likelihood analyses of sequences of mitochondrial and nuclear DNA loci of Philophthalmidae. (a) CO1; (b) ND1; (c) 18S rDNA. The bars indicate the number of substitutions per nucleotide. Sequences of *Pygorchis* spp. are highlighted.

on May 10, 2015; both were found in the vicinity of Záhlinice (district Kroměříž, Czech Republic; 49°16′N, 17°28′E). The molecular analyses involved one specimen of each examined species. For the morphological analyses, the examined birds originated from various locations throughout the Czech Republic. The morphological analyses included one specimen from *S. hirundo* and 16 specimens from *A. cinerea*. We obtained all specimens of *Pygorchis* from birds provided as dead from various causes for deposition in the Comenius Museum in Přerov, Czech Republic. We immediately examined all the birds upon receipt, or we froze the specimens and subsequently examined within the next two months. For the molecular analyses, we fixed representative individuals of *Pygorchis* in 96% ethanol. For the comparative morphological analyses, we stained the freshly obtained *Pygorchis* in Semichon's carmine, followed by dehydration through an alcohol series, clearing with xylene-alcohol and pure xylene, and we then mounted them in Canada balsam. For the analyses of egg length, we measured the longest egg present within each examined adult individual. The dimensions are shown as a range (mean ± standard deviation), indicated in μm.

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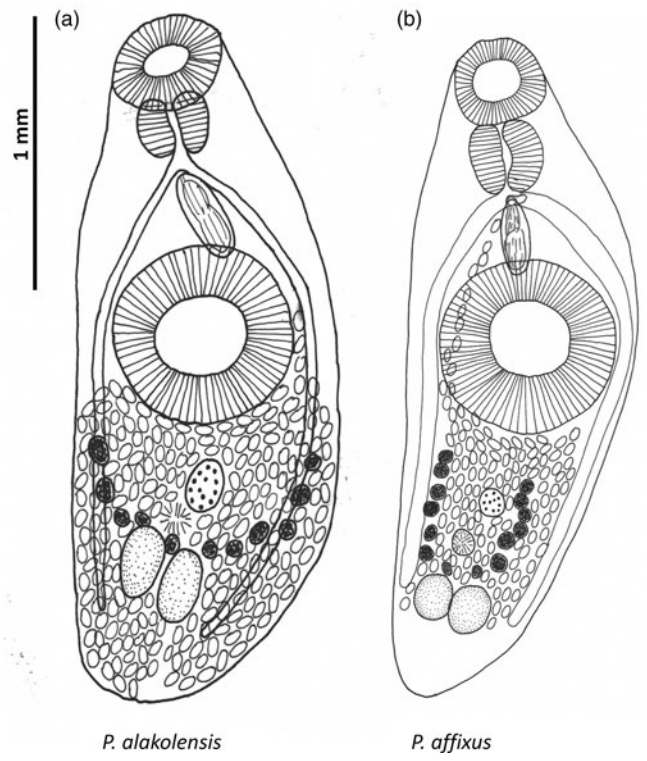


Fig. 2. Representative drawings of the *Pygorchis alakolensis* (a) and *Pygorchis affixus* (b) analysed in this study.

DNA extraction, amplification and sequencing

We extracted, amplified and sequenced the DNA using the primers that targeted two mitochondrial loci (cytochrome *c* oxidase I (CO1) and NADH dehydrogenase subunit 1 (ND1)) and one nuclear ribosomal DNA locus (18S rDNA) using the reaction conditions as described (Sitko *et al.*, 2017; Heneberg *et al.*, 2018). We used the primers JB3 and JB4.5 to amplify the CO1 locus (Bowles *et al.*, 1992), ND1J and ND1J2A to amplify the ND1 locus (Morgan & Blair, 1998; Bray *et al.*, 1999) and C for and A rev to amplify the 18S rDNA locus (Routtu *et al.*, 2014). We submitted the consensus sequences to the National Center for Biotechnology Information (NCBI) GenBank database under the accession numbers MW139332–MW139333 (CO1), MW183675–MW183676 (ND1) and MW143563 (18S rDNA).

Phylogenetic analyses

We aligned the newly generated sequences with those of Philophthalmidae obtained from NCBI GenBank as of October 31, 2020, and sequences of the corresponding outgroups by using MUSCLE. We corrected the alignments for any inconsistencies, trimmed the sequences to cover the same extent of the analysed loci and removed short-length sequences from the alignments. The trimmed CO1 locus (partial CO1 coding sequence) corresponded to nt 107–351 (245 bp) of *Philophthalmus gralli* JQ675731. The trimmed ND1 locus (partial ND1 coding sequence) corresponded to nt 1–378 (378 bp) of *P. gralli* KF986207. The trimmed 18S rDNA locus (partial small-subunit ribosomal ribonucleic acid coding sequence) corresponded to nt 50–763 (714 bp) of *P. gralli* JX121231. For each locus, we calculated the maximum likelihood fits of 24 nucleotide substitution models. To determine the tree inference and to obtain tree nodal support values, we used a bootstrap procedure at 1000 replicates and the nearest-neighbour-interchange as the maximum likelihood heuristic method, when the initial tree was formed using a neighbour-joining algorithm. We used best-fit models for the follow-up maximum likelihood phylogenetic analyses. We also estimated the pairwise distances expressed as the number of base differences per site obtained by averaging over all sequence pairs between groups in order to analyse the evolutionary divergence between the *Pygorchis* spp. using the bootstrap procedure at 1000 replicates.

Results

Central European Pygorchis

During the examination of Czech birds, we identified two species of the genus *Pygorchis*. These species were represented by *P. affixus*, which was an infrequent parasite of adult *A. cinerea*, and by *P. alakolensis*, which we report for the first time from Europe, and which parasitized *S. hirundo*. The two species were of similar dimensions; the only difference was in the position of testes and in the extent of vitelline follicles. In the majority of *P. affixus* individuals, the testes were in parallel position; however, approximately 10% of examined individuals had testes positioned obliquely. The vitelline follicles did not extend beyond the intestinal caeca, or, in exceptional cases, they extended them at only one side. In the examined *P. alakolensis* individual, the testes were positioned obliquely, and the vitelline follicles extended beyond the intestinal caeca, which resembled the situation that is characteristic for *P. americanus*. We provide the prevalence and intensities of infection by the *Pygorchis* spp. in table 1.

Because the morphological support for the validity of *P. alakolensis* description was weak, we sequenced the DNA of one of the two individuals of *P. alakolensis* found, and sequenced a representative *P. affixus* individual. The phylogenetic analysis of CO1 and ND1 (fig. 1) suggested that the two sequenced specimens represented two independent species that jointly formed a *Pygorchis* clade within Philophthalmidae. The phylogenetic analysis, therefore, revealed *P. alakolensis* as a valid species, which is properly classified into the genus *Pygorchis*.

Description of the analysed specimen of *P. alakolensis*:

Pygorchis alakolensis Zhatkanbaeva, 1967.
Synonym. *Pygorchis alacolensis* Zhatkanbaeva, 1967 (Semenova & Ivanov, 1985; Ivanov *et al.*, 2003; Schuster, 2013).
Collection Comenius Museum Přerov (J. Sitko), one specimen, number P-P-1870/6 (fig. 2a).
Host. Charadriiformes – *Sterna hirundo*.
Locality. Czech Republic: Záhlinice (49.29°N, 17.48°E).

Description

Based on one specimen from *S. hirundo*. Egg-shaped body tapering anteriorly, broader in rear part. Body 2543 × 857, body length/width ratio 1:2.97. Suckers distinct. Oral sucker subterminal, broadly oval 290 × 365. Pharynx 290 × 244, large, muscular. Intestinal bifurcation just posterior to pharynx, caeca extend slant to lateral edge of ventral sucker, pass along ventral sucker edge and extend slightly up to level of ovary, form loop around testes and end blind posterior to testes at about half of testes width. Ventral sucker oval 598 × 561, in the middle of the body. Suckers' length ratio 1:2.06, width ratio 1:1.54. Oral sucker/pharynx

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Table 2. Measurements of *Pygorchis* spp. based on the adult individuals collected in Czechia (*P. affixus*, *P. alakolensis*; this study and Sitko, 2012) and in the USA (*P. americanus*; Dronen, 1985).

Measure	Species		
	Pygorchis alakolensis ex *Sterna hirundo*	*Pygorchis affixus* ex *Ardea cinerea*	*Pygorchis americanus* ex *Platalea ajaja*
Body length	2543	1637–2714 (2102)	2084–3060 (2580)
Body width	857	629–1000 (855)	979–1195 (1128)
Body length/width ratio	2.97		
Oral sucker length	290	241–387 (317)	315–360 (339)
Oral sucker width	365	253–462 (372)	358–393 (378)
Pharynx length	290	204–323 (274)	285–315 (301)
Pharynx width	244	216–323 (264)	233–311 (287)
Ventral sucker length	598	482–839 (658)	638–728 (690)
Ventral sucker width	561	482–860 (691)	698–831 (761)
Oral/ventral suckers length ratio	2.06	1.82–2.66 (2.08)	2.0
Oral/ventral suckers width ratio	1.54	1.68–2.79 (1.86)	2.0
Oral sucker/pharynx length ratio	2.04		
Oral sucker/pharynx length ratio	1.50		
Ventral sucker/pharynx length ratio	2.04		
Ventral sucker/pharynx length ratio	2.30		
Cirrus sac length	368	90–430 (249)	
Cirrus sac width	129	72–151 (113)	
Ovary length	162	90–116 (104)	114–143 (127)
Ovary width	128	90–116 (101)	102–169 (135)
Testes length	232–249	90–210 (144)	115–221 (174)
Testes width	174–180	72–168 (127)	106–180 (142)
Mehlis' gland length	116	81–116 (90)	
Mehlis' gland width	139	81–116 (93)	
Number of vitelline field follicles	7 (left), 8 (right)	7–8 per side	4–6 per side
Egg length	67	59–71 (68)	72–90 (83)
Egg width	38	35–37 (37)	36–45 (41)

The host species of the individuals described are: *P. alakolensis* – one adult individual from *S. hirundo*; *P. affixus* – 16 adult individuals from *A. cinerea*; *P. americanus* – eight adult individuals from *Platalea ajaja*. The data are shown as a range (mean).

length ratio 1:2.04, width ratio 1:1.5. Ventral sucker/pharynx length ratio 1:2.06, width ratio 1:2.3. Genital pore medial, posterior to pharynx. Cirrus sac oval, 368 × 129, between ventral sucker and pharynx. Testes oblong, between intestinal caeca, in oblique position at posterior body end; right testis 232 × 180, left testis 249 × 174. Ovary 162 × 128, oval or globular. Mehlis' gland 116 × 139, oval, medial, between ovary and testes. Vitelline fields consist of seven (left side) or eight (right side) large follicles cutting through intestinal branches at level of ovary in mediolateral direction. Uterus fills whole body posterior to ventral sucker and terminates onto surface of body in zone of intestinal bifurcation. Eggs 67 × 38, contain miracidium with pigment stain.

Molecular features

CO1 (MW139332) and ND1 (MW183675) sequences. The lowest interspecific evolutionary divergence (in base differences per nucleotide): CO1: 0.140 ± 0.030 compared to *P. affixus* (0.322 ± 0.049 and 0.297 ± 0.049 compared to *Philophthalmus lucipetus* and *Philophthalmus lacrymosus*, respectively). ND1: 0.128 ± 0.026 compared to *P. affixus* (0.259 ± 0.054 and 0.287 ± 0.059 compared to *P. lucipetus* and *P. lacrymosus*, respectively).

Discussion

The morphology of *P. affixus* is highly variable, and previous descriptions do not allow distinguishing *P. affixus* unequivocally from *P. alakolensis* based on morphological features (see table 2 for comparative measurements of the three *Pygorchis* spp.). Schuster (2013) summarized the state-of-the-art identification features of the *Pygorchis* and *Cloacitrema* spp. as follows: '[t]he position of testes to each other even within one species may vary from parallel to slightly oblique … for *P. alakolensis* and *P. americanus* while in *P. affixus* the testes are in a parallel

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position' and '[v]itelline fields extending extracaecally can be seen in *P. alakolensis* and *P. americanus* but not in the type species, *P. affixus*.' However, as an example of the variability found, Bykhovskaya-Pavlovskaya (1974) reported three adult *P. affixus* in *M. alba* that were examined at the Curonian spit. The mentioned publication did not contain any description; however, it contained a drawing where *P. affixus* has testes in serial order and the left field of vitelline follicles extends towards the body edge – that is, beyond the caeca. Therefore, both key morphological identification features of *P. affixus* cannot be applied to all individuals of this species. Based on our own examinations and based on published data, we estimate that only approximately 80% of *P. affixus* fit the characteristic description of this species (fig. 2b). Therefore, the validity of *P. alakolensis* was questionable until the molecular data provided a strong independent support of the existence of both *P. affixus* and *P. alakolensis* as two independent species.

The descriptions of *P. alakolensis* are also variable and all (including the one that is provided in the present study) are based on a small number of examined specimens. Semenova & Ivanov (1985) examined one specimen of *P. alakolensis* from a cloaca of *L. argentatus*. It was of similar dimensions as the presently examined specimen, oral sucker was subterminal, ovary was slightly larger than testes, vitelline fields consisted of six fields at each side, reaching diagonally the body edges, and eggs were thinner, 60–67 × 45–52. The size of the ovary was twice larger, and the testes twice smaller compared to the original description by Zhatkanbaeva (1967), but matched the dimensions of the specimen examined in the present study. The cirrus sac (190 × 90) was three-times smaller compared to Zhatkanbaeva (1967) and twice shorter compared to the present study. The oral sucker (400 × 360) was larger compared to Zhatkanbaeva (1967) and somewhat larger compared to the presently examined specimen. The number of vitelline fields reported by Semenova & Ivanov (1985) was lower (six) compared to Zhatkanbaeva (1967) and compared to the present specimen (seven to eight). The type specimen that was described by Zhatkanbaeva (1967) was slightly larger than the one examined in the present study, with correspondingly larger oral sucker; the eggs were of the similar size.

Sitko & Heneberg (2015) noticed that they were able to retrieve *P. affixus* only from adult herons in their study area in Central Europe; moreover, seven of the eight records they report originated from April or May. Therefore, they suggested that this species must be introduced by migrating herons from Africa. The distribution of intermediate hosts of *P. alakolensis* is unclear; the Central European populations of their host, *S. hirundo*, migrate to Mosambique and South Africa (Kralj *et al.*, 2020); it also cannot be excluded that some *S. hirundo* individuals migrate to the Caspian Sea, where the previously known area of distribution of *P. alakolensis* is located (Semenova & Ivanov, 1985).

In conclusion, we provided the first conclusive molecular evidence on the validity of *P. alakolensis*, which we report for the first time from Europe. To complement previously published data, we provide the description of a third specimen of this species. We confirmed the classification of *Pygorchis* into Philophtalmidae.

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of animals.

Author contributions. PH and JS conceived the study, JS examined the host birds and performed the morphological analyses, PH performed the molecular and phylogenetic analyses and wrote the manuscript. Both authors revised the manuscript and agreed on its final version.

Data and materials availability. Representative specimens of the helminths analysed in this study are available in the collections of the Comenius Museum in Přerov. All data are available in the main text.

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