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Integrative taxonomy of European parasitic flatworms of the genus *Metorchis* Looss, 1899 (Trematoda: Opisthorchiidae)

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ABSTRACT

Metorchis spp. are flukes (Platyhelminthes: Digenea) that infect vertebrates, including humans, dogs, cats, poultry and wild game, with cyprinid freshwater fish serving as typical second intermediate hosts. In their definitive hosts, the *Metorchis* spp. are difficult to identify to species. We provide and analyze sequences of two nuclear (18S rDNA and ITS2) and two mitochondrial (CO1 and ND1) DNA loci of four morphologically identified European species of the *Metorchis*, namely *Metorchis albidus*, *Metorchis bilis*, *Metorchis crassiusculus* and *Metorchis xanthosomus*, and of another opisthorchiid, *Euamphimerus pancreaticus*. DNA analysis suggests that the *Metorchis* specimens identified morphologically as *M. albidus* (from *Lutra lutra*), *M. bilis* (from *Phalacrocorax carbo*) and *M. crassiusculus* (from *Aquila heliaca* and *Buteo rufinus*) represent a single species. Thus, *M. albidus* (Braun, 1893) Loos, 1899 and *M. crassiusculus* (Rudolphi, 1809) Looss, 1899 are recognized as junior subjective synonyms of *M. bilis* (Braun, 1790) Odening, 1962. We also provide comparative measurements of the Central European *Metorchis* spp., and address their tissue specificity and prevalence based on the examination of extensive bird cohort from 1962 to 2015. *M. bilis* and *M. xanthosomus* can be morphologically diagnosed by measuring the extent of genitalia relative to body length and by the size ratio of their suckers. They also differ in their core definitive hosts, with ducks (*Anas*, *Aythya*) and coots (*Fulica*) hosting *M. xanthosomus*, and cormorants (*Phalacrocorax*), the birds of prey (*Buteo*, *Aquila*, etc.), piscivorous mammals (*Lutra*, *Vulpes*, *Ursus*, etc.) and humans hosting *M. bilis*. Previous reports on the *Metorchis* spp. contain numerous suspected misidentifications.

1. Introduction

Liver flukes of the family Opishorchiidae Looss, 1899 are considered causative agents of serious disease worldwide, with the clinical pathology associated principally with chronic infection. They cause choledocholithiasis, ascending cholangitis, pancreatitis and cholangiocarcinoma of the liver due to duct irritation and subsequent host immune reaction [1]. Compared to *Fasciola* spp., opisthorchiids cause only limited host morbidity due to liver infections, because they enter the bile ducts via the ductus choledochus instead of causing mechanical damage, and the observed local necrotic foci are considered a result of the formation of periductular inflammative infiltrates of monocytes and lymphocytes [2–4]. Routine medical and parasitological examination of affected patients usually does not allow distinguishing between opisthorchiasis and metorchiasis, and the usual diagnosis "opisthorchiasis" is thus thought to include the pathologies caused by infection by any of the Opisthorchiidae species, particularly in Russia [5–8]. Whereas the infections caused by *Clonorchis* and *Opisthorchis* spp. were extensively studied, there is an incomplete understanding of the epidemiology, taxonomy and pathology of infections caused by *Metorchis* Looss, 1899.

The *Metorchis* spp. are parasites of carnivorous vertebrates. Of particular importance is the North American *Metorchis conjunctus*, the zoonotic parasite of carnivorous mammals, causing infections particularly in aboriginal populations in Canada and the Eastern coast of Greenland [1, 9–10]. The focal prevalence in humans consuming raw freshwater fish can be quite high. The native Canadians examined in Fort Hope, NW Ontario, displayed 20% prevalence of acute *Metorchis* infections [11], but later the prevalence in the same community decreased to just 3% in humans (but remained at 73% in their sledge dogs and 100% in bears hunted within the same area). This was probably due to the change in food supply to the community, which was associated with a rapid decrease in consumption of smoked suckers *Catosomus commersoni* [10]. The infection is usually asymptomatic, but may lead to acute febrile illness with epigastric pain and eosinophilia [1]. The disease is transmitted by the consumption of raw freshwater fish, including *Catostomus* spp., *Salvelinus fontinalis* and *Perca flavescens*. In certain areas the fish may be

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heavily infested. For example, in Canadian Lac Edouard, the prevalence of infection was reported to reach up to 100% in *Catosomus commersoni*, and only slightly less in other fish species [12]. The infections may be fatal in dogs due to liver damage associated with the infection [13–14]. Another distinct *Metorchis* species is represented by *Metorchis orientalis*, the East Asian zoonotic parasite of piscivorous birds and mammals. The first infection of humans by *M. orientalis* was only reported as late as 2001 in four Chinese residents [15]. The disease is transmitted by the consumption of raw cyprinid freshwater fish, including *Cyprinus carpio*, *Carassius carassius*, *Pseudogobio esocinus* and *Pseudorasbora parva* [16]. In Europe, several *Metorchis* spp. have been described, some of which are rejected in this study. Of particular importance is *Metorchis bilis*, which is a zoonotic parasite of piscivorous birds and mammals. Mordvinov et al. suggested previously in this journal that *M. bilis* (together with *Opisthorchis felineus*) is the main agent of liver fluke infection in Russian residents [8]. The infections of both humans and piscivorous vertebrates by *M. bilis* are probably under-diagnosed. In Russia, large-scale serological examination revealed that 50% of clinically diagnosed "opisthorchiases" were in fact metorchiases caused by *M. bilis*. In another 38% of cases, the patients were positive for both, *O. felineus* and *M. bilis*, suggesting frequent combined infection [17]. Another study, also from Russia, confirmed that metorchiasis is neglected and confused with opisthorchiasis, reporting 7% of clinically diagnosed "opisthorchiases" being caused by *M. bilis*, and another 63% of them caused by the combined infections by *O. felineus* and *M. bilis* [18]. Similarly, in Husky dogs, Schuster et al. [2] found 93% of those examined in Germany to be positive for antibodies against *M. bilis*. They found the opisthorchiid eggs being shed by only a minor fraction of the examined dogs. Despite this, the treatment with 20–50 mg of praziquantel led to the disappearance of the anti-*Metorchis* antibodies in most of the dogs [2], suggesting that their high titers were not a result of the infection in the past or some non-specific products. *M. bilis* is considered one of the dominant helminths of otters *Lutra lutra*, in which the infection rates may reach up to 31% as recorded in Denmark [19]. Clinicopathological data obtained for the otters found that all the otter gall bladders in poor condition characterized by extremely fibrous and thickened tissue were infected by *M. bilis*, whereas less than 10% of healthy gall bladders were infected [20]. This association of otter gall bladder damage with the infection by *M. bilis* was later confirmed by the same research group [21]. In white-tailed sea eagles *Haliaeetus albicilla*, *M. bilis* is considered a dominant helminth, with the frequency of infection reaching 40% in Finland and 51% in Germany, and is considered a causative agent of up to 11% of reported deaths [22–24]. However, *M. bilis* was reported absent in *H. albicilla* from Greenland, where the birds were probably feeding on marine fish [25]. In cormorants, another core host of *M. bilis*, the frequency of infection oscillates around 40%, a finding reported by multiple authors from the German state of Saxony (40% [26], and 9.1% and 46.3% in two subsequent years [27]), Czech and Slovak Republics (33% [28]) and Poland (43% [29]). The typical life cycle of *M. bilis* includes *Bithynia tentaculata* (or other *Bithynia* spp.) as the first intermediate hosts and a broad range of freshwater cyprinid fish as the second intermediate hosts [8].

Molecular data supporting the taxonomy and identification of *Metorchis* spp. are limited. Kang et al. sequenced ITS1 of a single specimen of *Metorchis* (termed *bilis*) from an undisclosed host species from Spain [30]. Ai et al. sequenced the ITS locus, and the mitochondrial CO1 and ND1 loci from *M. orientalis* metacercaria isolates from China [31]. Shekhovtsov et al. provided partial sequences of the paramyosin gene of *Metorchis xanthosomus* and two additional *Metorchis* specimens unidentified to species [32]. Several other unpublished DNA sequences are available in the NCBI GenBank database, including the recently released full mitochondrial genome sequence of *M. orientalis* (NCBI GenBank acc. No. KT239342). Collectively, the hitherto available DNA sequences of *Metorchis* spp. did not allow us to draw any definitive conclusions on the status of the previously proposed European species of this genus analyzed in this study, i.e., *Metorchis albidus*, *M. bilis*, *M. crassiusculus* and *M. xanthosomus*.

Here we use integrative taxonomy to analyze European *Metorchis* spp. parasitizing piscivorous birds and mammals. We focus on the prevalence of *Metorchis* spp. across a broad range of their definitive bird hosts. We perform the first conclusive phylogenetic analysis of the taxonomic position of European *Metorchis* spp. based on four independent nuclear and mitochondrial DNA loci. The phylogenetic analysis also includes all the hitherto sequenced *Metorchis* spp. We then provide comparative measurements of the *Metorchis* spp. found.

2. Material and methods

2.1. Sampling

To analyze the prevalence, we examined over 17,000 individuals of 240 bird species for the presence of *Metorchis* spp. between years 1962 and 2015. All birds examined originated from the Czech Republic (48°39'N–50°59'N, 12°19'E–18°29'E), primarily from eastern parts of the country.

For the purpose of phylogenetic analyses, we examined representative specimens of the *Metorchis* spp. collected in the Czech Republic (Strachotín 48.91°N, 16.65°E, Záhlinice 49.17°N, 17.28°E), Slovakia (Pieniny 49.39°N, 20.39°E) and Poland (Zalew Wislany 54.28°N, 19.26°E) on May-2013–Apr-2015. All the helminths originated from birds supplied dead for preparation in the Comenius Museum in Přerov, Czech Republic (Czech specimens), Institute of Parasitology, Slovak Academy of Sciences (Slovak specimens) or in the Museum and Institute of Zoology, Polish Academy of Sciences (Polish specimens), and consisted of the individuals found dead or hunted. For the phylogenetic analyses, we examined the *Metorchis* spp. from *Anas platyrhynchos* (2 specimens), *Aquila heliaca* (1 specimen), *Aythya fuligula* (1 specimen), *Buteo rufinus* (3 specimens) and *Phalacrocorax carbo* (2 specimens). We also examined a single specimen of *Euamphimerus pancreaticus*, another opisthorchiid with yet unknown DNA sequence, obtained from *Turdus philomelos*.

For the morphological analyses, we stained representative specimens in Semichon's carmine, dehydrated them by alcohol series, and mounted each specimen in Canada balsam. For the phylogenetic analyses, we fixed and stored the specimens in 96% ethanol. Dimensions are shown in μm as a range (mean ± SD). All other data are shown as mean ± SD unless otherwise stated. The significance of morphological differences between *M. bilis* and *M. xanthosomus* was examined using *t*-tests with Bonferroni correction at $n = 33$. Individual variability in the key identification signs is shown by a dot plot.

2.2. DNA extraction, amplification and sequencing

We extracted and amplified the DNA as described [33], using the primers targeting nuclear 18S rDNA and ITS2 loci, and mitochondrial CO1 and ND1 loci. The primers used were identical with those used by Heneberg et al. [34]. The generated DNA fragments were purified using USB Exo-SAP-IT (Affymetrix, Santa Clara, CA) and sequenced bidirectionally using an ABI 3130 DNA Analyzer (Applied Biosystems, Foster City, CA). The resulting consensus DNA sequences were submitted to the GenBank database under the accession numbers KT740961–KT740992 (Table 1).

2.3. Alignments and phylogenetic analyses

We imported the DNA sequences obtained in the course of this study into the program MEGA5, along with comparable sequences of *Metorchis* spp. and corresponding outgroups available in the NCBI GenBank database as of 11-Sep-2015, and aligned sequences by ClustalW (gap opening penalty 7, gap extension penalty 2 for both pairwise and multiple alignments, DNA weight matrix IUB, transition weight 0.1). We also included sequences identified during the course of the Cardiff University's "Otter Project", the results of which were analyzed in detail in a paper published

Table 1
Newly generated sequences during the course of this study from Opisthorchiidae collected in the Czech Republic, Slovakia and Poland. NCBI GenBank accession numbers (KT740961–KT740992) are indicated.

Specimen	Species, host, sampling site	Locus			
		18S rDNA	ITS2	CO1	ND1
3LF-1832	*Metorchis bilis*, host *Aquila heliaca*, Strachotín, Czech Rep.	KT740961	KT740976	KT740966	KT740985
3LF-1868	*Metorchis xanthosomus*, host *Anas platyrhynchos*, Zalev Wislany, Poland	KT740962	KT740977	KT740968	
3LF-1869	*Metorchis bilis*, host *Phalacrocorax carbo*, Záhlinice, Czech Rep.		KT740978	KT740969	KT740986
3LF-1870	*Metorchis bilis*, host *Phalacrocorax carbo*, Záhlinice, Czech Rep.	KT740963	KT740979	KT740970	KT740987
3LF-1912	*Metorchis bilis*, host *Buteo rufinus*, Pieniny, Slovakia		KT740980	KT740971	KT740988
3LF-1913	*Metorchis bilis*, host *Buteo rufinus*, Pieniny, Slovakia		KT740981	KT740972	KT740989
3LF-1914	*Metorchis bilis*, host *Buteo rufinus*, Pieniny, Slovakia	KT740964	KT740982	KT740973	KT740990
3LF-2430	*Metorchis xanthosomus*, host *Aythya fuligula*, Záhlinice, Czech Rep.	KT740965	KT740983	KT740974	KT740991
3LF-2432	*Euamphimerus pancreaticus*, host *Turdus philomelos*, Záhlinice, Czech Rep.		KT740984	KT740975	KT740992

in parallel in this journal [19]. We trimmed the aligned sequences, and removed short-length sequences to create the alignment that was used for all subsequent analyses. We sequenced slightly different parts of the DNA loci than those that were used in the otter project [19], thus we performed the comparison of isolates from the birds to those from the mammals separately. The trimmed 18S rDNA locus corresponded to nt. 286–747 (462 bp) of JF314771 of *Metorchis orientalis*, coding for the partial SSU rRNA (Table S1). The trimmed ITS2 locus corresponded to nt. 2798–3445 (648 bp) of AB521800 of *Euryhelmis costaricensis*, which consisted of partial ITS2 sequence and 28S rDNA (Table S2). The trimmed CO1 locus corresponded to nt. 202–518 (317 bp) of HM347234 of *M. orientalis*; the full extent of this locus consisted of partial CO1 coding sequence (Table S3). The trimmed ND1 locus corresponded to nt. 18–412 (395 bp) of HQ659766 of *M. orientalis*; the full extent of this locus consisted of partial ND1 coding sequence (Table S4). For the comparison of mammalian and bird isolates, the trimmed ITS2 locus corresponded to nt. 2853–3186 (334 bp) of AB521800 of *E. costaricensis*, which consisted of a partial ITS2 sequence and 28S rDNA (Table S5), and the trimmed CO1 locus corresponded to nt. 141–301 (161 bp) of HM347234 of *M. orientalis*; the full extent of this locus consisted of partial CO1 coding sequence (Table S6).

Maximum likelihood fits of 24 nucleotide substitution models were performed as described [35], with all sites used for the analyses, including the gaps. For each model, we calculated the Bayesian information criterion, Akaike information criterion (corrected) and maximum likelihood values. For the 18S rDNA locus, we analyzed 10 sequences with a total of 462 positions in the final dataset (Table S7). For the ITS2 locus, we analyzed 10 sequences with a total of 649 positions in the final dataset (Table S8). For the CO1 locus, we analyzed 11 sequences with a total of 317 positions in the final dataset (Table S9). For the ND1 locus, we analyzed 12 sequences with a total of 418 positions in the final dataset (Table S10). For the ITS2 locus of mammalian and bird isolates, we analyzed 21 sequences with a total of 335 positions in the final dataset (Table S11). For the CO1 locus of mammalian and bird isolates, we analyzed 30 sequences with a total of 100 positions in the final dataset (Table S12).

Next we used the best fit model to construct a tree. For the 18S rDNA data, for the ITS2 data and for the comparison of mammalian and bird ITS2, we used the Kimura 2-parameter model [36]. For the comparison of mammalian and bird CO1, we also used the Kimura 2-parameter model, but with the non-uniformity of evolutionary rates among sites modeled using a discrete Gamma distribution (+G) with 5 rate categories. For the CO1 and ND1 data, we used the Hasegawa–Kishino–Yano model [37], with the non-uniformity of evolutionary rates among sites modeled by using a discrete Gamma distribution (+G) with 5 rate categories. We employed the bootstrap procedure at 1000 replicates. For the tree inference, we used nearest-neighbor-interchange as the maximum likelihood heuristic method of choice, with the initial tree formed by the neighbor joining algorithm.

We next used the maximum likelihood method to estimate inter- and intrasite evolutionary divergence in European *Metorchis* spp. We calculated the number of base differences per site by averaging over all sequence pairs between groups (distance) ± SE, and employed the bootstrap procedure at 1000 replicates. The models used to estimate inter- and intrasite evolutionary divergence based on the 18S rDNA, mammalian and bird ITS2, and mammalian and bird CO1 loci, were identical with those used to construct the respective trees. To analyze the ND1 locus, we employed the Tamura–Nei model [38] with the non-uniformity of evolutionary rates among sites modeled by using a discrete Gamma distribution (+G) with 5 rate categories. The Tamura–Nei model was selected because the model used for the construction of the ND1 tree (Hasegawa–Kishino–Yano) does not allow the calculation of inter- and intrasite evolutionary divergence to be made when using MEGA5 [39].

3. Results

3.1. Central European *Metorchis* spp.

During our extensive long-term examination of Czech birds, we confirmed the presence of two *Metorchis* spp. (*M. bilis* and *M. xanthosomus*). The initial species identification based on the morphological examination and host association suggested that the specimens found represent *M. bilis*, *M. xanthosomus*, and also previously recognized *M. crassiusculus* (found typically in the birds of prey; synonymized with *M. bilis* in this study). Moreover, *M. bilis* re-defined by this study also includes the specimens recognized previously as *M. albidus* (found typically in the mammals).

The prevalence of *Metorchis* spp. in Czech birds is shown in Table 2. The differences in prevalence suggest strict separation of ecological niches for the two species. The more abundant species, *M. bilis*, was highly prevalent in great cormorants (*Phalacrocorax carbo*), in fish-eating birds of prey (*Aquila*, *Buteo*, *Haliaeetus*), and it was also identical

Table 2
Prevalence of the *Metorchis* spp. in the Czech Republic in years 1962–2015.

Helminth species: host species (n examined)	*Metorchis bilis*	*Metorchis xanthosomus*
	Number/relative share [%] of positive individuals	
Anas platyrhynchos (534)		8/1.5
Anas crecca (72)		1/1.4
Netta rufina (14)		1/7.1
Aythya ferina (157)		2/1.3
Aythya fuligula (232)		3/1.3
Gavia arctica (10)		1/10
Phalacrocorax carbo (148)	30/20	
Platalea leucorodia (1)	1/100	
Podiceps cristatus (534)		2/0.4
Haliaeetus albicilla (5)	1/20	
Aquila heliaca (2)	1/50	
Buteo buteo (453)	1/0.2	
Buteo rufinus (3)	1/33	
Fulica atra (495)		11/2.2

with *Metorchis* specimens found in mammals such as otters across multiple European countries as revealed by the comparison of its ITS2 and CO1 loci (see below). Contrary to that, *M. xanthosomus* was limited to ducks (Anatidae) and Eurasian coots (*Fulica atra*), with rare findings also in grebes (Podicipediformes) and loons (Gaviiformes). The prevalence of *M. xanthosomus* in the Czech Republic was low in contrast to *M. bilis* in its core host species, reaching only 1.3–1.5% in ducks and 2.2% in coots, which is probably caused by its presence only in birds migrating from abroad since the 1980s. The newly suggested spectrum of *Metorchis* spp. host–parasite interactions was corroborated by DNA sequencing as described below.

3.2. Phylogenetic analyses of specimens isolated from birds

Maximum likelihood analysis of both nuclear (18S rDNA and ITS2) and mitochondrial (CO1 and ND1) DNA loci revealed the existence of only two distinct, well-defined species among the *Metorchis* specimens isolated from various bird hosts, including those identified initially as *M. crassiuscullus* (Fig. 1). Three of the DNA loci tested (ITS2, CO1 and ND1) differentiated between the specimens from ducks (*Anas*, *Aythya*) and from other bird hosts (*Phalacrocorax*, *Buteo*, *Aquila*); the fourth DNA locus tested (18S rDNA) was identical for all opisthorchiids tested, including those with 18S rDNA sequences publicly available in the NCBI GenBank database. The ITS2, CO1 and ND1 loci also displayed intraspecific variability, but there were no host-specific haplotypes. Instead, the particular haplotypes were shared, e.g., by *M. bilis* isolates from *Phalacrocorax carbo* and *Buteo rufinus* (Fig. 1).

The sequences of DNA from another opisthorchiid species, *Euamphimerus pancreaticus*, were very distant from other opisthorchiids, including all the hitherto sequenced *Metorchis*, *Opisthorchis* and *Clonorchis* spp. (Fig. 1). The classification of the other opisthorchiids (*M. orientalis*, *O. felineus*, *O. viverrini* and *Clonorchis sinensis*) was not supported based on their publicly available DNA sequences (Fig. 1). The sequences of *M. orientalis* were very distinct from *M. bilis* and *M. xanthosomus* and due to the long branches associated with their mitochondrial sequences, the ND1 and CO1 genes cannot be used to classify the *M. orientalis* within Opisthorchiidae. Analysis of more DNA loci of these species is needed to provide any conclusive reclassification of the Opisthorchiidae.

3.3. Synonymization of *M. bilis* with *M. albidus* from mammals and *M. crassiusculus* from birds

Maximum likelihood analysis of both nuclear (ITS2) and mitochondrial (CO1, ND1) DNA loci revealed that the *Metorchis* specimens identified as *M. albidus*, *M. bilis* and *M. crassiusculus* represent a single species. Based on the priority of publication, *Metorchis albidus* (Braun,

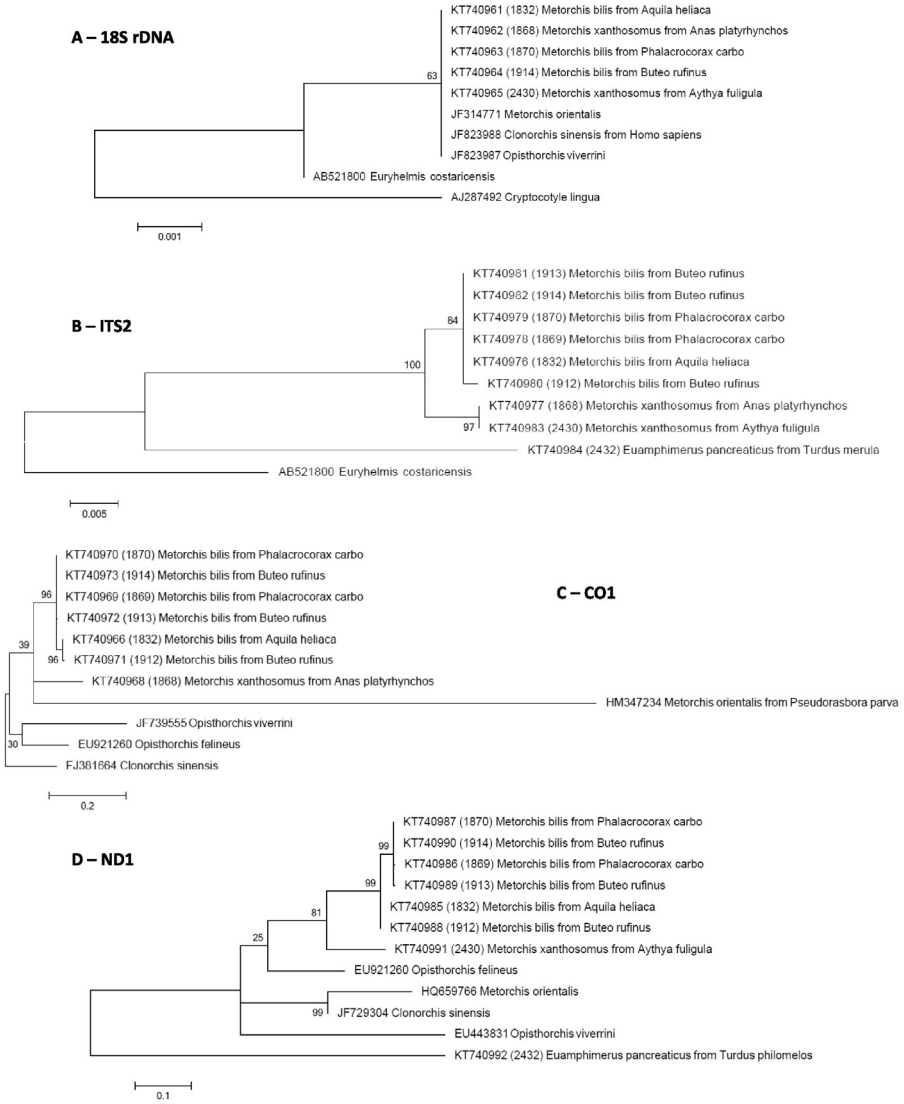

Fig. 1. Maximum likelihood analysis of sequences of nuclear (18S rDNA (A) and ITS2 (B)) and mitochondrial DNA loci (CO1 (C) and ND1 (D)) of *Metorchis* spp. isolated from birds.

1893) Loos, 1899 and *Metorchis crassiusculus* (Rudolphi, 1809) Looss, 1899 are recognized as junior subjective synonyms of *Metorchis bilis* (Braun, 1790) Odening, 1962. Besides the well-known morphologic similarities, the following supporting DNA-based evidence was used to propose this synonymization:

1) All the nine haplotypes of CO1 of *M. albidus* identified in *Lutra lutra* from eight European countries (Czech Republic, Denmark, France, Germany, Norway, Poland, Sweden and United Kingdom, analyzed in detail in the study published in parallel in this journal [19]) were identical or clustered with the *M. bilis* specimens identified in birds in the Czech Republic (Fig. 2). The haplotypes were not species- or region-specific.
2) All the four haplotypes of CO1 of *M. crassiusculus* identified in *Buteo rufinus* and *Aquila heliaca* from the Czech Republic and Slovakia were identical or clustered with the *M. bilis* specimens identified in

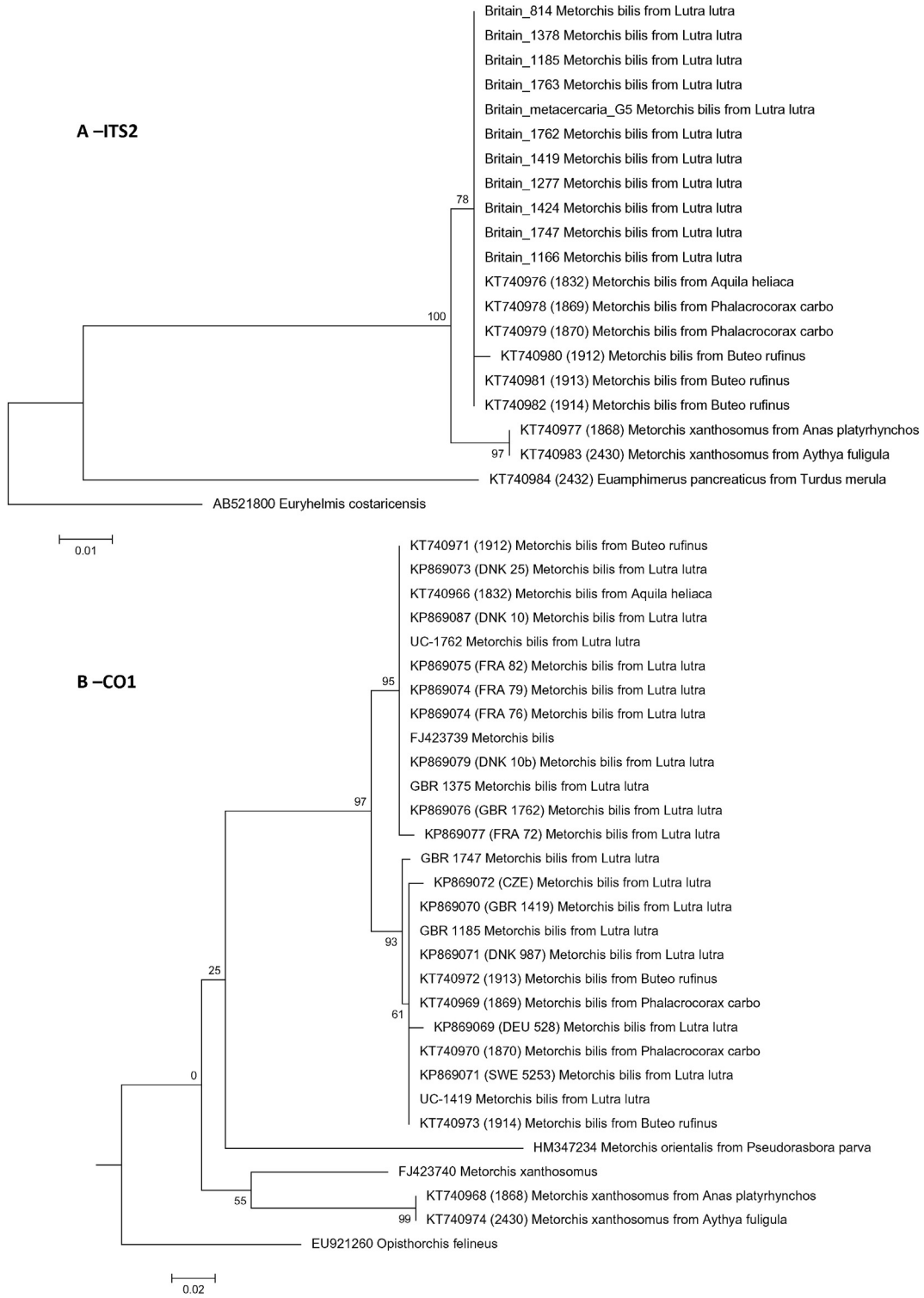

Fig. 2. Maximum likelihood analysis of sequences of nuclear (ITS2 (A)) and mitochondrial DNA loci (CO1 (B)) of *Metorchis bilis* isolated from bird and mammalian hosts reveals the overlap of haplotypes obtained from specimens classified previously as *M. albidus* (mammalian hosts), *M. bilis* and *M. crassiusculus* (bird hosts).

Table 3
Estimates of the intra- and inter-specific evolutionary divergence of the *Metorchis* spp. from various bird and mammalian hosts collected across Europe as analyzed in Figs. 1A, D and 2A, B. The estimates are based on sequences of the 18S rDNA, ITS2, CO1 and ND1 DNA loci. Distance: the number of base differences per site generated by averaging over all sequence pairs between groups.

Species 1	Species 2	Distance ± SE			
	Locus	18S rDNA	ITS2	CO1	ND1
M. bilis (former *albidus*)	*M. bilis* (former *bilis*)	N/D	0.000 ± 0.000	0.016 ± 0.007	N/D
M. bilis (former *albidus*)	*M. bilis* (former *crassiuscullus*)	N/D	0.001 ± 0.001	0.014 ± 0.007	N/D
M. bilis (former *albidus*)	*M. xanthosomus*	N/D	0.015 ± 0.007	0.144 ± 0.033	N/D
M. bilis (former *albidus*)	*M. orientalis*	N/D	N/D	0.175 ± 0.041	N/D
M. bilis (former *albidus*)	Intra-specific divergence	N/D	0.000 ± 0.000	0.016 ± 0.007	N/D
M. bilis (former *bilis*)	*M. bilis* (former *crassiuscullus*)	0.000 ± 0.000	0.001 ± 0.007	0.013 ± 0.007	0.013 ± 0.005
M. bilis (former *bilis*)	*M. xanthosomus*	0.000 ± 0.000	0.015 ± 0.007	0.150 ± 0.034	0.189 ± 0.036
M. bilis (former *bilis*)	*M. orientalis*	0.000 ± 0.000	N/D	0.179 ± 0.043	0.518 ± 0.096
M. bilis (former *bilis*)	Intra-specific divergence	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
M. bilis (former *crassiuscullus*)	*M. xanthosomus*	0.000 ± 0.000	0.016 ± 0.007	0.144 ± 0.033	0.186 ± 0.034
M. bilis (former *crassiuscullus*)	*M. orientalis*	0.000 ± 0.000	N/D	0.174 ± 0.041	0.517 ± 0.091
M. bilis (former *crassiuscullus*)	Intra-specific divergence	N/D	0.002 ± 0.002	0.017 ± 0.009	0.018 ± 0.006
M. xanthosomus	*M. orientalis*	0.000 ± 0.000	N/D	0.197 ± 0.042	0.416 ± 0.005
M. xanthosomus	Intra-specific divergence	0.000 ± 0.000	0.000 ± 0.000	0.084 ± 0.023	N/D
M. orientalis	Intra-specific divergence	N/D	N/D	N/D	N/D

cormorants in the Czech Republic (Fig. 2B). The haplotypes were not species- or region-specific.

3) All the DNA sequences of ITS2 of *M. albidus* identified in *Lutra lutra* from eight European countries (Czech Republic, Denmark, France, Germany, Norway, Poland, Sweden and United Kingdom, analyzed in detail in the study published in parallel in this journal issue [19]) represent the single haplotype, which is identical with the major haplotype identified in the *M. bilis* specimens from the Czech and Slovak birds (Fig. 2A).
4) Both haplotypes of ITS2 of *M. crassiusculus* identified in *Buteo rufinus* and *Aquila heliaca* from the Czech Republic and Slovakia were identical with or highly similar to *the M. bilis* specimens identified in cormorants in the Czech Republic (Fig. 2B).
5) All the three haplotypes of ND1 of *M. crassiusculus* identified in *Buteo rufinus* and *Aquila heliaca* from the Czech Republic and Slovakia were identical or clustered with the two haplotypes of *M. bilis* specimens identified in cormorants in the Czech Republic (Fig. 1D).
6) The only additional species of *Metorchis* known to occur in Europe (*M. xanthosomus*) can be identified by the use of any of the three above DNA loci (ITS2, CO1 and ND1); its haplotypes do not overlap with those of *M. bilis* or the synonymized species.

Collectively, the combined morphologic and genetical analysis revealed the existence of only two distinct, well-defined species among the *Metorchis* specimens isolated from various bird and mammalian hosts collected across Europe. There is a considerable genetic variability among both *M. bilis* and *M. albidus* (Table 3), but we did not find any host species- or region-specific patterns of the observed intraspecific variability.

3.4. Species description of Metorchis spp. based on the material collected in birds in Central Europe

3.4.1. Family Opisthorchiidae Looss, 1899

3.4.1.1. Metorchis bilis (Braun, 1790) Odening, 1962. Hosts: Suliformes: *Phalacrocorax carbo* (intensity of infection 4.0; range 1–19); Pelecaniformes: *Platalea leucorodia* (4; 4); Accipitriformes: *Aquila heliaca* (68; 68), *Buteo buteo* (21; 21), *Buteo rufinus* (5; 5), *Haliaeetus albicilla* (3; 3) (Table 2).

Site: gall bladder.

Localities: Brno (49.23°N, 16.51°E, 6765), Hodonín (48.86°N, 17.07°E, 7169), Strachotín (48.90°N, 16.65°E, 7065).

Life cycle (based on previously published data [40–45]): 1 — Gastropoda: *Bithynia inflata*, *Bithynia tentaculata*; 2 — Osteichthyes: *Alburnus alburnus*, *Blicca bjoerkna*, *Carassius carassius*, *Gasterosteus aculeatus*, *Gobio gobio*, *Leuciscus idus*, *Leuciscus leuciscus*, *Pelecus cultratus*, *Pungitius pungitius*, *Rutilus rutilus*; 3 — Aves (birds of prey and cormorants) and Mammalia (broad spectrum of species, which regularly or occasionally feed on fish; incidental infections of humans were reported as well). Note that previous definitive host records in anseriform, podicipediform and gruiform hosts need further verification due to the likely misidentification with *M. xanthosomus*.

Specimens deposited: P-P-1860/4 (Comenius Museum, Přerov, Czech Republic) (Fig. 3A).

Description: (*Buteo buteo* 25 specimens, *Aquila heliaca* 5 specimens; measurements provided in Table 4). Body pear-shaped, gradually broadening towards posterior end, with maximum width anteriorly from rear testes. Tegument spined posteriorly up to anterior edge of anterior testes. Oral sucker terminal, broadly oval, larger than ventral sucker. Ventral sucker in forebody, at end of anterior third of body, ventral:oral sucker width ratio 1:1.00-1:1.40 (1.18 ± 0.11) (Fig. 4). Pharynx round or oval, near posterior edge of oral sucker or slightly posteriorly. Esophagus very short or absent. Caeca extend posteriorly alongside lateral body margins, terminate posterior to posterior testes.

Genitalia fill up 36–46% (41 ± 3) of body length (Fig. 4). Testes two, oval, slightly or deeply lobate, diagonally behind one another at posterior body end, anterior testis always smaller than posterior one. Mehlis gland at level of anterior testis, forming broadly opened letter U, exceeding size of ovary. Ovary oval, anterior to testes. Vitellaria very fine, divided into individual follicles, running along lateral body margins, anteriorly to pharynx, posteriorly anterior to testes. Uterus loops filled with eggs occupy whole space from reproductive organs to pharynx and frequently cover ventral sucker.

3.4.1.2. Metorchis xanthosomus (Creplin, 1846). Hosts: Anseriformes: *Anas crecca* (intensity of infection 5; range 5), *Anas platyrhynchos* (6.1; 1–30),[1] *Aythya ferina* (1.5; 1–2), *Aythya fuligula* (2.3; 2–3), *Netta rufina* (3; 3); Gaviiformes: *Gavia arctica* (1; 1); Podicipediformes: *Podiceps cristatus* (1.5; 1–2); Gruiformes: *Fulica atra* (8.6; 1–65)[1] (Table 2).

Site: gall bladder.

Localities: Bartošovice (49.67°N, 18.05°E, 6374), Dolní Věstonice (48.89°N, 16.64°E, 7165), Strachotín (48.90°N, 16.65°E, 7065); Tovačov (49.41°N, 17.30°E, 6569); Záhlinice (49.29°N, 17.48°E, 6770).

[1] The infections in *A. platyrhynchos* and *F. atra* displayed site-specific intensity, with the highest intensities experienced at the largest Central European periodical wetland, which was termed Pansee and was located between Strachotín and Dolní Věstonice. The wetland Pansee was the only site where the *M. xanthosomus* completed its life cycle in the Czech Republic; it no longer exists due to the dam construction. All other infection events were recorded in the periods of bird migration.

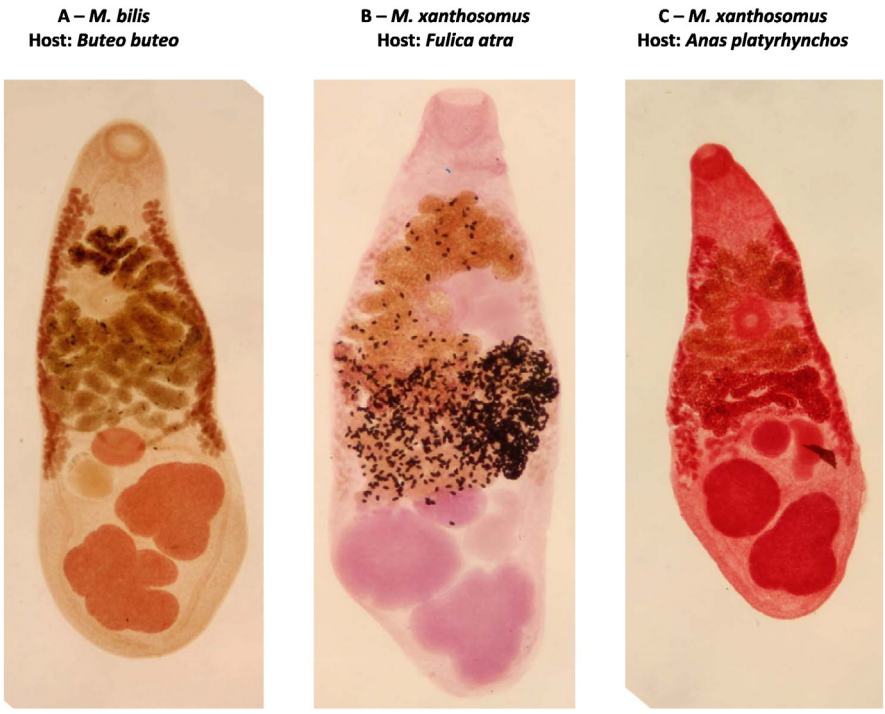

Fig. 3. Representative photographs of *Metorchis bilis* (A) and *M. xanthosomus* (B–C) stained in Semichon's carmine. (A) *M. bilis*, host: *Buteo buteo*, adult male, sampling site and date: Hodonín, Czech Republic, 15-Jan-1976. (B) *M. xanthosomus*, host: *Fulica atra*, juvenile, sampling site and date: Strachotín, Czech Republic, 10-Aug-1962. (C) *M. xanthosomus*, host: *Anas platyrhynchos*, juvenile, sampling site and date: Strachotín, Czech Republic, 28-Jun-1967.

Table 4
Body measures (in μm) of the bird *Metorchis* spp. based on the adult individuals collected in the Czech Republic in years 1962–2015. The significance of differences between the measures of the two species was examined using *t*-tests with Bonferroni correction at n = 33. Data are shown as range (mean ± SD). The identification features, the combination of which is considered appropriate for differential diagnosis of *M. bilis* and *M. xanthosomus*, are shown in bold.

Measure	Species		P (*t*-test; Bonferroni correction: $p < 0.05$ equals to $p < 1.52E-03$ at n = 33	Significance of the differences observed (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, n.s. = not significant)
	Metorchis bilis (Braun, 1790) Odening, 1962	*Metorchis xanthosomus* (Creplin, 1846)		
Body length	1800–3140 (2408 ± 320)	2057–3850 (2760 ± 564)	0.9E−04	*
Body width	686–1000 (808 ± 84)	571–1429 (916 ± 176)	4.3E−04	*
Length:width ratio	2.1–4.4 (3.0 ± 0.5)	2.2–4.4 (3.1 ± 0.5)	0.44	n.s.
Forebody length	514–943 (726 ± 111)	685–1429 (934 ± 181)	4.6E−07	***
Hindbody length	1086–1771 (1414 ± 175)	1057–2229 (1640 ± 353)	1.5E−02	n.s.
Forebody:hindbody length ratio	1.5–3.0 (2.0 ± 0.4)	1.2–2.2 (1.8 ± 0.2)	7.3E−03	n.s.
Spines length	17–19 (19 ± 1)	17–19 (19 ± 0.4)	0.32	n.s.
Spines width	1–2 (2 ± 0)	1–2 (2 ± 0)	0.50	n.s.
Extent of genitalia relative to the body length [%]	**36–46 (41 ± 3)**	**20–38 (27 ± 4)**	**3.9E−24**	***
Oral sucker length	162–249 (206 ± 21)	174–365 (239 ± 36)	3.7E−05	**
Oral sucker width	197–249 (228 ± 15)	203–365 (255 ± 39)	3.8E−04	*
Ventral sucker length	145–215 (186 ± 17)	203–319 (249 ± 33)	2.9E−13	***
Ventral sucker width	145–232 (193 ± 16)	203–360 (258 ± 39)	6.3E−12	***
Ventral: oral sucker length ratio	**1.00–1.38 (1.11 ± 0.10)**	**0.79–1.16 (0.96 ± 0.09)**	**1.3E−07**	***
Ventral: oral sucker width ratio	**1.00–1.40 (1.18 ± 0.11)**	**0.93–1.16 (0.99 ± 0.05)**	**1.4E−13**	***
Esophagus	0–22 (1 ± 4)	27–74 (58 ± 12)	2.7E−33	***
Pharynx length	58–96 (68 ± 9)	58–129 (79 ± 21)	1.1E−02	n.s.
Pharynx width	48–87 (70 ± 8)	58–151 (74 ± 15)	2.7E−02	n.s.
Anterior testes length	222–510 (342 ± 62)	244–736 (447 ± 119)	4.1E−05	**
Anterior testes width	270–566 (383 ± 72)	302–828 (500 ± 134)	5.6E−05	**
Posterior testes length	232–453 (322 ± 62)	232–782 (440 ± 126)	1.6E−05	***
Posterior testes width	348–626 (503 ± 68)	261–1222 (550 ± 179)	9.8E−02	n.s.
Mehlis gland length	232–540 (379 ± 85)	232–493 (321 ± 82)	5.4E−03	n.s.
Mehlis gland width	145–456 (275 ± 59)	110–598 (251 ± 113)	0.16	n.s.
Ovary length	90–203 (136 ± 30)	87–302 (187 ± 41)	7.4E−07	***
Ovary width	90–215 (159 ± 30)	168–319 (247 ± 43)	7.4E−13	***
Vitelline reservoir length	116–122 (119 ± 3)	87–203 (131 ± 41)	1.3E−03	*
Vitelline reservoir width	29–41 (35 ± 3)	29–35 (30 ± 2)	0.20	n.s.
Left vitellarium length	1000–1714 (1369 ± 205)	914–2286 (1564 ± 403)	1.2E−02	n.s.
Right vitellarium length	1057–1857 (1394 ± 202)	924–2486 (1551 ± 394)	3.1E−02	n.s.
Extent of vitellaria relative to the body length [%]	46.0–71.3 (59.5 ± 6.4)	40.0–68.5 (57.3 ± 5.9)	8.2E−02	n.s.
Egg length	27–31 (29 ± 1)	27–34 (29 ± 1)	2.8E−02	n.s.
Egg width	16–19 (18 ± 1)	17–22 (21 ± 2)	4.5E−06	***

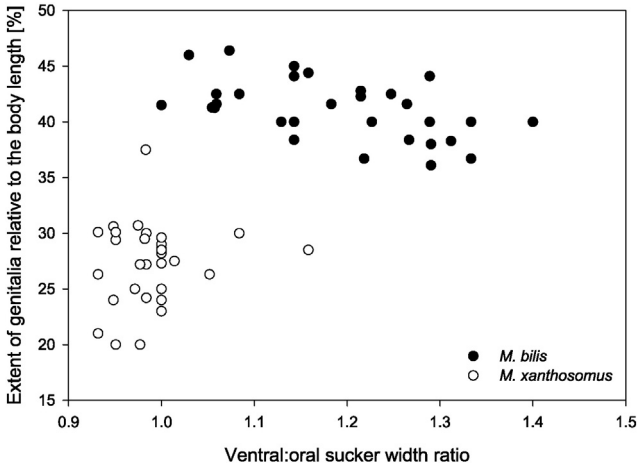

Fig. 4. Individual variability in key identification measures of *M. bilis* and *M. xanthosomus*. The populations of adult *M. bilis* and *M. xanthosomus* differ in their ventral:oral sucker width ratio and extent of genitalia relative to the body length [%], as revealed based on the examination of adult individuals collected in the Czech Republic in years 1962–2015, n = 30 each.

Life cycle (based on previously published data [46–49]): 1 — Gastropoda: probably *Bithynia tentaculata*; 2 — Osteichthyes: *Cobitis taenia*, *Coregonus lavaretus*, *Gymnocephalus cernua*, *Leucaspius delineatus*, *Leuciscus idus*, *Phoxinus phoxinus*, *Sander lucioperca*; 3 — Aves: various Anseriformes, Podicipediformes and Gruiformes species, particularly *Anas* spp., *Aythya* spp. and *Fulica atra*. Note that previous definitive host records in other than anseriform, podicipediform and ralliform hosts need further verification due to the likely misidentification with other *Metorchis* spp.

Specimens deposited: P-P-1860/5 (Comenius Museum, Přerov, Czech Republic) (Fig. 3B–C).

Description: (*Anas platyrhynchos* 15 specimens, *Fulica atra* 15 specimens; measurements provided in Table 4). Body elongate, with maximum width at level of ovarium or anterior testes. Tegument spined. Oral sucker terminal, smaller or equally sized as ventral sucker. Ventral sucker in forebody, at end of anterior third or fourth of body, ventral:oral sucker width ratio 0.93–-1.16 (0.99 ± 0.05) (Fig. 4). Pharynx long oval, in region of oral sucker or slightly posterior to it. Esophagus short. Caecal bifurcation just posterior to pharynx, caeca extend posteriorly alongside lateral body margins, terminate posterior to posterior testes.

Genitalia fill up 20–38% (27 ± 4) of body length (Fig. 4). Testes two, oval, smooth or slightly lobed, in tandem at posterior body end. Vitellaria in small follicles running along lateral body margins, anteriorly posterior to caecal bifurcation, posteriorly anterior to testes. Ovary widely oval, anterior to testes. Laurer's canal developed. Uterus loops occupy whole space at level of vitellaria and frequently cover ventral sucker.

4. Discussion

Three of the four species of *Metorchis* analyzed in this study, *M. albidus*, *M. bilis* and *M. xanthosomus*, were originally described in bird hosts. The earliest described species, *M. bilis* (originally *Planaria bilis* Braun, 1790), infected the gall bladder of *Falco melanferus* [50]. In 1809, Rudolphi described *M. crassiusculus* (as *Distomum crassiusculum* Rudolphi, 1809) based on specimens collected from gall bladders of birds of prey [51]. In 1902, Braun described *M. xanthosomus* based on the specimen named as "*Distomum xanthosomum*" (nomen nudum) by Creplin (1846), who obtained it from *Gavia stellata*. Braun also observed that the opisthorchiids of waterfowl were typically *M. xanthosomus*, whereas birds of prey typically harbored *M. crassiusculus* [52]. Only the last of the four species analyzed in this study, *M. albidus*, was described in a mammal, with Braun discovering it as *Distomum albidum* in a cat liver [53]. This formed the first record of *Metorchis* spp. in mammals. Later, Odening in his above-mentioned study [54] suggested that all the *Metorchis* spp. actually belong to a single species based on the highly similar morphology of adult stages. However, the metacercariae of *M. xanthosomus* are spheroid and have a thick hyaline cyst wall, thus differing morphologically from the remaining three above-named species [55]. During the 20th century, several dozens of *Metorchis* spp. were proposed, but in this study, we dealt only with the four above-mentioned taxa because of the availability of the geographically and host-matched specimens.

Besides our data presented in this study, numerous independent pieces of evidence support the synonymy of *M. crassiusculus* and *M. albidus* with *M. bilis*. First, Razmashkin [55] found that the experimental host spectrum of *M. bilis* includes both birds (terns, gulls) and mammals (cats, golden hamsters and mice), whereas the larvae of *M. xanthosomus* developed in ducks only during his experiments, and failed to do so in mice, golden hamsters or black terns. Of note is that there was a methodical problem with his pioneering experiments, because diet was not controlled in the experimental animals used. Thus, the animals used might have acquired infections from the ingested food, and thus while the negative results can be trusted, the positive results should be taken with caution. This likely explains why Razmashkin found *Metorchis* infection also in ducks fed by *M. bilis* larvae. Based on the body measurements provided in this paper (Table 4, Fig. 4) and supported by the analysis of DNA sequences, our records of *Metorchis* from over a thousand of examined ducks and coots included *M. xanthosomus* only (Table 2).

Second, Pauly et al. [56] noticed that their newly developed PCR test aimed to be used for the diagnosis of mammalian *M. bilis* specimens also amplified the control *Metorchis* specimen isolated from *Circus aeruginosus*, which was morphologically indistinguishable from the mammalian *Metorchis* specimens used to develop the test.

Third, Odening [54] already suggested half a century ago that the extent of morphologic similarities between adult forms of *M. bilis* and *M. albidus* calls for their treatment as a single species. However, this suggestion was not reflected by other authors (cf., e.g., [57]).

The two species analyzed in this study can be morphologically diagnosed by measuring the extent of genitalia relative to the body length and by the ratio of the size of their suckers (Table 4, Fig. 4). Previous reports on the *Metorchis* spp. contain numerous suspected misidentifications (cf., e.g. [28,58–59]) and/or the use of now synonymized species names. It seems that both the species discriminate well among their core hosts, with ducks (*Anas*, *Aythya*) and coots (*Fulica*) hosting *M. xanthosomus*, and cormorants (*Phalacrocorax*), the birds of prey (*Buteo*, *Aquila*), piscivorous mammals (*Lutra*, *Vulpes*, *Ursus*, etc.) and humans hosting *M. bilis*. *M. xanthosomus* seems to be limited in its host spectrum, whereas *M. bilis* is a generalist. Considering that *M. bilis* is a zoonotic species, broadening of the spectra of its zoonotic core hosts has direct implications for the knowledge of disease spread and disease reservoirs in areas, where humans are infected. The prevalence of *M. bilis* infection may increase with global warming, because its prevalence in the core hosts was previously shown to increase with higher temperatures, less rainfall and fewer days of ground frost [21]. Further research is needed to confirm the validity and classification of other *Metorchis* spp., which occur outside Europe.

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