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Threat of cercarial dermatitis in Hungary: A first report of *Trichobilharzia franki* from the mallard (*Anas platyrhynchos*) and European ear snail (*Radix auricularia*) using molecular methods

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ABSTRACT

Cercarial dermatitis in humans is a re-emerging zoonotic disease caused by infectious larvae of avian blood flukes within the Schistosomatidae family. Upon water contact, these avian schistosome larvae directly penetrate human skin and cause irritation. Between September 2018 and September 2020, carcasses of 94 mallards (*Anas platyrhynchos*), two green-winged teals (*Anas crecca*) along with one ferruginous duck (*Aythya nyroca*) were examined. Birds were collected within 12 regions of Hungary, representative of 9 different counties. Inspecting both morphological characters and molecular data, the cytochrome oxidase subunit I (COI) and large subunit ribosomal DNA (LSU rDNA) sequences, *Bilharziella polonica* and *Dendritobilharzia pulverulenta* were each identified. Most importantly, *Trichobilharzia franki* was found for the first time in Hungary and in 5 dispersed counties in conjunction with of 3 counties where other avian schistosomes were found. Of note, these avian blood flukes were predominately encountered in wild mallards (~50%) and not in birds reared for hunting (~1%). In total, 245 European ear snails (*Radix auricularia*), a known intermediate host of *Trichobilharzia* spp., were collected from an urban pond in Eger, Hungary. Five snails (~2%) consistently shed numerous furcocercariae of *T. franki* that were confirmed by molecular methods. Our findings help to pinpoint a contemporary life cycle of this avian schistosome within an urban environment, a location regularly visited by various wild waterfowl and the possible species of avian schistosomes responsible for cercarial dermatitis in Hungary. Taken as a whole, we demonstrate the actual and potential risk zone for cercarial dermatitis, particularly with reference to *R. auricularia* distributions, within Hungary and across Central Europe.

1. Introduction

Avian schistosomes (Digenea: Schistosomatidae) are parasitic flatworms of medical relevance present worldwide (Chamot et al., 1998; Kourilova et al., 2004). They have a complex life cycle including freshwater snails of the Lymnaeidae family as intermediate hosts and various bird species as definitive or final hosts (Hertel et al., 2006; Kalbe et al., 2000). The free-living infectious stage (i.e. cercariae) may use humans as accidental hosts by penetrating the skin. In non-definitive hosts the larvae of the parasite usually die within tens of minutes within the dermis. The process of active penetration initiates a localised or topical inflammation of the skin (Żbikowska, 2003). Repeated invasion of cercariae cause overt vesiculo-maculo-papular skin eruptions giving rise to cercarial dermatitis (swimmer's itch), often accompanied by pathognomic intensive itching (Kolářová et al., 1997; Horák et al., 2002).

Experimental studies on immunocompetent mammalian models show that the majority of the cercariae die in the skin, although some schistosomula can migrate to the nervous system or lungs (Kolářová, 2007, Horák and Kolářová 2011). Consequently, monitoring of cercarial dermatitis outbreaks in Europe is now the subjects of many local and international research projects (Pilz et al., 1995; De Gentile et al., 1996; Kolářová et al., 1999; Thors and Linder 2001; Dubois et al., 2001; Canestri-Trotti et al., 2001; Zbikowska et al., 2001; Fraser et al., 2009; Soldánová et al., 2013; Marszewska et al., 2016; Al-Jubury et al., 2020). It is mostly species belonging to the genus of *Trichobilharzia* that have

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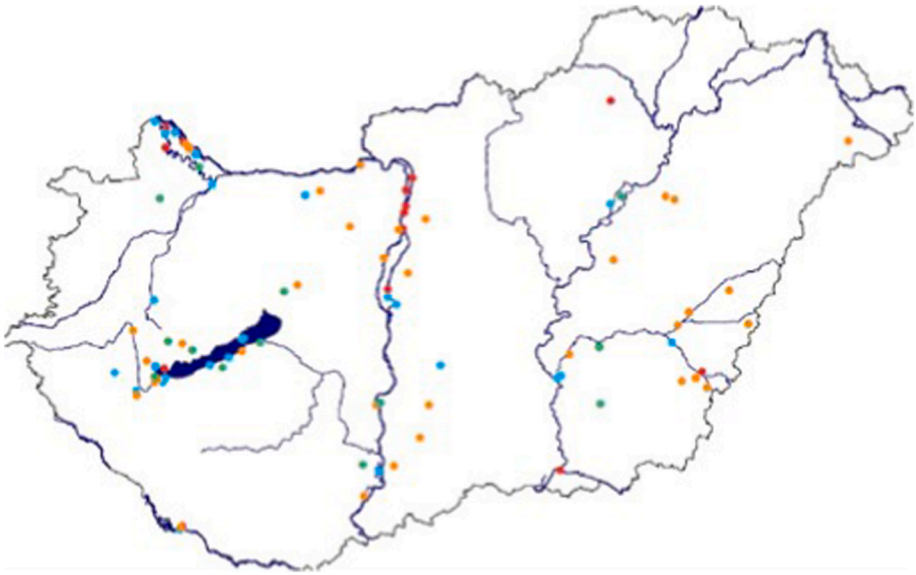

Fig. 1. Proved occurrence of *R. auricularia* snails in Hungarian habitats based on museum collections and own investigations. The species was detected in artificial ponds or canals (yellow dots: living specimens; green dots: shells) and also in natural habitats (red dots: living specimens; blue dots: shells). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

been identified as causative agents of outbreaks in Europe. This genus includes approximately 40 species worldwide (Brant and Loker, 2013). Currently six species are known in Europe, namely *Trichobilharzia szidati* Neuhaus (1952); *Trichobilharzia regenti* Horák et al. (1998); *Trichobilharzia franki* Müller and Kimmig (1994); *Trichobilharzia salmanticensis* Simon-Martin and Simon-Vicente, 1999; *Trichobilharzia anseri* Jouet et al., 2010; and *Trichobilharzia mergi* Kolářová et al. (2013) (Neuhaus, 1952; Simon-Martin and Simon-Vicente, 1999; Jouet et al., 2010; Kolářová et al., 2013; Horák et al., 1998, 2015; Christiansen et al., 2016). According to previous investigations *T. regenti* develops in *Radix balthica* snails as intermediate hosts, *T. szidati* and *T. franki* develop in *Lymnaea stagnalis, Radix auricularia* and *Radix peregra*, whereas *T. salamanticensis*, *T. anseri* and *T. mergi* can develop both in *Radix auricularia* and *R. balthica* snails (Horák et al., 2002; Jouet et al., 2010).

In temperate regions of Europe, three species were described in anseriform birds as zoonotic agents: *T. regenti, T. szidati* and *T. franki.* In Hungary, a limited number of scientific papers have been published on this subject despite the sporadic clinical occurrence of cercarial dermatitis across the country. In most of these cases, the origin of the larvae remains uncharacterised and the source of the infection is enigmatic, largely neglected by the medical or veterinarian practitioners such that the diversity and range of such schistosome species present in the country remains unknown.

The first Hungarian literature on cercarial dermatitis is associated with Kotlán and Kobulej who wrote briefly about this disease in the fourth edition of their veterinary handbook “Parasitology” (1972), mentioning a peculiar name of a unique disease as “water mange” of fishermen in Gemenc forest, a floodplain area of the Danube River. The authors assumed that this disease is also caused by various schistosomatid cercariae whereas Molnár et al. published the first scientific reports of cercarial dermatitis in Lake Balaton (Molnár et al., 2003). The first confirmed records of schistosomatid cercariae causing cercarial dermatitis came from Gemenc in South Hungary, that were shed from *R. auricularia* (Linnaeus 1758) and were assigned to *Schistosoma turkestanicum* (Skrjabin, 1913) which lives in red deer (*Cervus elaphus*) in that area. [*R. auricularia* is native to Europe and probably it occurs in all water sources in Hungary because former research proved its general presence in the country (Juhász et al., 2016; Juhász, 2018) (Fig. 1).] This assignment was based on serology and detailed morphological analyses of cercariae. These cercariae were proved to cause dermatitis in humans experimentally infected and indirectly upon contemporary reports of dermatitis witnessed in fishermen at the site of occurrence of infected snails.

In this paper, we characterise the likely species of avian schistosomes responsible for cercarial dermatitis in Hungary. To this end, we take advantage of molecular methods to provide representative DNA sequences to help with further studies in Hungary and Central Europe.

2. Materials and methods

2.1. Examination of carcasses, detection and preservation of adult flukes and eggs

From opportunist sampling, birds were investigated by necropsy in order to find blood-dwelling trematodes formerly detected in waterfowl. The birds were apparently healthy, 94 mallards shot during regular hunting and submitted for the survey by hunters. The two Eurasian green-winged teals (*Anas crecca*) along with one ferruginous duck (*Aythya nyroca*) were collected as road-kill or from wildlife rescue stations where animals died from complications after injury. The nasal cavity and the viscera of these animals were investigated at the Department of Parasitology and Zoology, at University of Veterinary Medicine. Mallards were investigated from 12 regions of 9 different counties of Hungary between September 2018 and September 2020 (Table 1). All birds were collected during legal hunts or from veterinary hospitals therefore no ethical approval was requested.

We received 79 frozen and 18 freshly killed carcasses of waterfowls. To identify trematode infection, the nasal area was cut open and the epithelial tissue and the underlying vascular tissue removed as Horák et al., 2002 suggested. The liver was cut into small pieces and lacerated apart to smaller pieces, the intestine was cut up and sliced. The removed tissues were soaked in a bucket of tap water separately by organs and individuals, gently squeezed and eventually macerated, while the remaining liquid was decanted and rinsed several times. The sediment was fixed in 70% ethanol and examined under a stereo microscope and light microscope. Recovered trematodes were counted, sorted and stored in 70% ethanol for subsequent DNA analysis. Adult flukes or their pieces were freshly mounted on slides for measurements and morphological observation according to the morphological features described by Mcdonald, 1981). Remaining small segments of liver washed with tap

Table 1
Occurrence of trematodes in the investigated birds.

Investigated species of birds, their origin and number	Areas in Hungary from where the samples were collected and the date of shooting	Trematode species in infected organs (number of infected birds)
Anas platyrhynchos (wild, 8)	Csenderes,January 14, 2019.	Heart: *Bilharziella polonica* eggs Liver: *Bilharziella polonica* adults and eggs (1)
Anas platyrhynchos (wild, 1)	Ercsi,February 05, 2019.	Nasal sinus: *Trichobilharzia* sp. eggs (1)
Anas platyrhynchos (reared, 13)	Noszlop,August 06, 2019.	–
Anas platyrhynchos (reared, 10)	Alap,September 10, 2019.	–
Anas crecca (wild, 2)	Mekszikópuszta, 09.2019.	Liver: *Bilharziella polonica* adults and eggs (1)
Anas platyrhynchos (reared, 5)	Hajdúnánás, 10.10.2019.	–
Anas platyrhynchos (reared, 12)	Hajdúnánás, 12.12.2019.	–
Anas platyrhynchos (wild, 5)	Ercsi,January 09, 2020.	Liver: *Bilharziella polonica* adults and eggs, *Trichobilharzia* sp. adult (1)
Anas platyrhynchos (reared, 18)	Szentes,January 10, 2020.	Liver: *Bilharziella polonica* adult (1)
Anas platyrhynchos (wild, 5)	Ádánd,February 13, 2020.	Liver: *Trichobilharzia* sp. adults and eggs
Anas platyrhynchos (wild, 4)	Kápolna,February 14, 2020.	Liver: *Trichobilharzia* sp. adults, *Bilharziella polonica* adults (1)
Anas platyrhynchos (wild, 1)	Szabadszállás,February 14, 2020.	Liver: *Trichobilharzia* sp. adults (1)
Aythya nyroca (wild, 1)	Budapest, 28.02.2020.	Liver: *Bilharziella polonica* adults, eggs (1)
Anas platyrhynchos (wild, 9)	Hortobágy,February 28, 2020.	Nasal sinus: *Trichobilharzia* sp (2) Lung: *Bilharziella polonica* adults Liver: *Trichobilharzia* sp. adults and eggs (2), *Bilharziella polonica* adult (2), *Dendritobilharzia pulverulenta* adults (1) Body cavity: *Bilharziella polonica* adults (2)
Anas platyrhynchos (wild, 1)	Budapest,September 09, 2020.	–
Anas platyrhynchos (wild, 2)	GyulajSeptember 15, 2020.	Liver: *Trichobilharzia* sp. adults (2)

water were placed in a household blender of 2-litre capacity and made into a suspension with tap water. Depending on the amount of suspension, 200–300 mL of 80 g/L sodium hydroxide (NaOH) was added to the suspension to achieve alkali concentration of approximately 1–2% in the mixture. The tissue suspension was then incubated at room temperature for 24 h. The solubilised tissue was filtered through a mesh screen with a mesh of 63 μm aperture in order to filter out the tissue residues larger than the size of fluke eggs. The filtered mixture was divided into conical glass and was allowed to settle. The sediment was inspected by a binocular compound microscope for schistosome eggs.

2.2. Collection of intermediate hosts

European ear snails, *Radix auricularia*, were collected by hand from an urban pond around which mallards had been frequently observed in summer and early autumn. This ornamental pool was fed by warm water of an artificial well all the year and a stable stock of *R. auricularia* snails inhabits the half-acre lake densely overgrown with plants. Living snails were kept cool and moist until returned to the laboratory. Adult snails

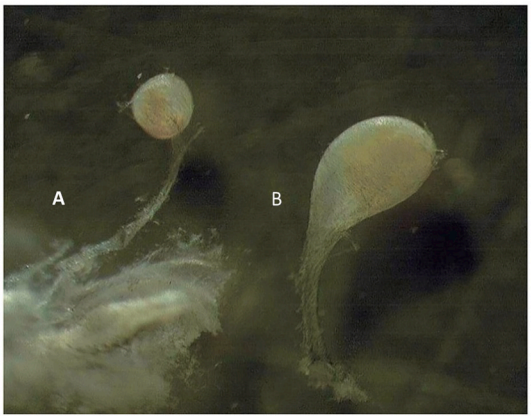

Fig. 2. The shape of bursa copulatrix of the adult *R. auricularia* is spherical and the stalk is long (A), while the bursa copulatrix of *Radix balthica* is oval and stalk is short (B). This anatomical structure seems the most reliable morphological difference to distinguish of the two most common *Radix* species in Hungary, but can only be studied on sexually mature and non-trematode infected specimens (Juhász, 2018) The length of the dissected organs is about 1 cm.

were identified by the anatomy of their genital organs (Fig. 2) and shell morphology with the aid of comparative specimens from the collections of the senior author (G.M.) and the Malacological Department of Natural History Museum of Hungary.

2.3. Detection and identification of cercariae

R. auricularia (245 collected specimens) snails were separated individually into a 6-well tissue culture plate in distilled water, at room temperature and exposed to natural light. Five *R. auricularia* regularly shed ocellate furcocercariae during every morning at least 5 days. Live cercariae shed from snails spontaneously can be collected and investigated under a light microscope in a wet smear. The snails were monitored and studied for the emergence of cercariae for 1 week. After this period all snails were dissected in order to detect trematode sporocysts in their organs with microscopical examination. The fork-tailed cercariae leaving the naturally infected snails were identified according to the description of Müller and Kimmig (1994); Podhorský et al. (2009), Jouet et al., (2010). Recovered cercariae were saved in 70% ethanol for further analyses. Approximately 50 individual cercariae from an infected snail, were pooled and centrifuged in a 1.5-ml microfuge tube for 1 min at 13,000 rpm.

2.4. Molecular methods

For DNA extraction, samples preserved in 70% ethanol were centrifuged at 8000g for 5 min, Excess ethanol was pipetted from the worms and pelleted cercariae and the tubes were left open at room temperature for 10 min to aid evaporation of any further ethanol from the samples. The DNA was extracted using a Genomic DNA Mini Kit (Geneaid) and eluted in 100 μL AE buffer. The COI and LSU rDNA genes were amplified via PCR. The primers Cox1_SchistF (5′ TCTTTRGATCATAAGCG3′) and Cox1_SchistoR (5′ TAATGCATMGGAAAAAAACA3′) (Lockyer et al., 2003) were used for amplifying the COI in a 25-μL reaction mixture comprised of 2 μL of extracted genomic DNA, 5 μL of 1 mM dNTPs (MBI Fermentas, Burlington, Canada), 0.25 μL of each primer, 2,5 μL of 10 × Taq buffer (MBI Fermentas), 0.1 μL of DreamTaq polymerase (0.5 U) (MBI Fermentas) and 14,9 μL of water. The PCR profile consisted of an initial denaturation step of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 44 °C for 30 s and 72 °C for 2 min, and was finished with a terminal extension at 72 °C for 7 min, then stored at 4 °C. The primers U178 (5′

Table 2
List of the sequenced cercariae and adult samples of *Trichobilharzia franki* and *Bilharziella polonica*.

Sample	Morphological identification	Host	Developmental stage	Date of collection	Locality of collection	LSU rDNA sequence	COI sequence
6	*Trichobilharzia franki*	*Radix auricularia*	cercaria	September 06, 2019.	Eger	MZ560932	–
8	*Trichobilharzia franki*	*Radix auricularia*	cercaria	September 06, 2019.	Eger	MZ560933	–
9	*Trichobilharzia franki*	*Radix auricularia*	cercaria	September 06, 2019.	Eger	MZ560934	–
10	*Trichobilharzia franki*	*Radix auricularia*	cercaria	September 06, 2019.	Eger	MZ560935	–
12	*Bilharziella polonica*	*Anas platyrhynchos*	adult	February 28, 2020.	Hortobágy	MZ560936	MZ562959
13	*Bilharziella polonica*	*Anas platyrhynchos*	adult	February 28, 2020.	Hortobágy	MZ560937	MZ562960
14	*Trichobilharzia franki*	*Anas platyrhynchos*	juvenile	February 28, 2020.	Hortobágy	–	MZ562961
15	*Trichobilharzia franki*	*Anas platyrhynchos*	adult	February 28, 2020.	Hortobágy	MZ560938	MZ562962
16	*Trichobilharzia franki*	*Radix auricularia*	cercaria	September 17, 2020.	Eger	–	MZ562963
17	*Bilharziella polonica*	*Anas platyrhynchos*	adult	September 15, 2020.	Gyulaj	MZ560939	MZ562964
18	*Trichobilharzia franki*	*Anas platyrhynchos*	adult	September 15, 2020	Gyulaj	MZ560940	MZ562965
19	*Trichobilharzia franki*	*Anas platyrhynchos*	adult	September 15, 2020.	Gyulaj	MZ560941	MZ562966

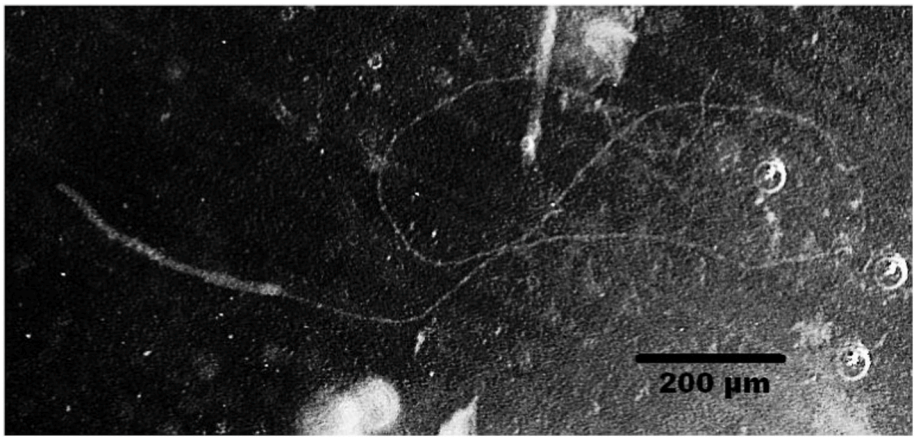

Fig. 3. A complete specimen of native *Trichobilharzia* male in cell suspension from the liver of a mallard.

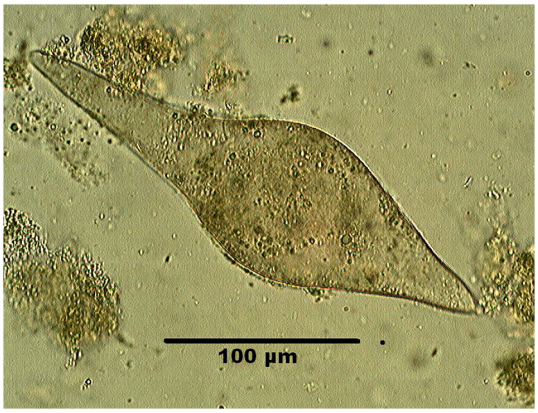

Fig. 4. Egg of *Trichobilharzia* in cell suspension of liver of a mallard.

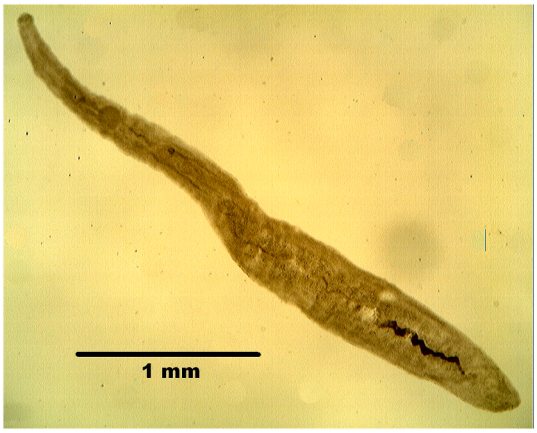

Fig. 5. Male *Bilharziella polonica* from the liver of a mallard.

GCACCCGCTGAAYTTAAG3′) and L1642 (5′ CCAGCGCCATCCATTTTCA 3′) (Lockyer et al., 2003) were used for amplifying the LSU rDNA in 50 µL of reaction mixture comprised of 4 µL extracted genomic DNA, 10 µL of 1 mM dNTPs (MBI Fermentas), 0.5 µL of each primer, 5 µL of 10 × Taq buffer (MBI Fermentas), 0.5 µL of DreamTaq polymerase (1 U) (MBI Fermentas) and 28,5 µL of water. The PCR consisted of an initial denaturation step of 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 2 min and a final extension step at 72 °C for 7 min, then stored at 4 °C.

PCR products were electrophoresed in 1.0% agarose gels in Tris-Acetate-EDTA (TAE) buffer gel, 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 COI and LSU rDNA were sequenced with the PCR primers and with six additional inner primers 300F (5′ CAAGTACCGTGAGGGAAAGTTG3′), 300R (5′ CAACTTTCCCTCACGGTACTTG3′), EDC2 (5′CCTTGGTCCGTGTTTCAA GACGGG3′), 900F (5′ CCGTCTTGAAACACGGACCAAG3′), 1200F (5′ CCCGAAAGATGGTGAACTATGC3′), 1200R (5′ GCATAGTTCACCAT CTTTCGG3′) (Lockyer et al., 2003) in the case of LSU rDNA. ABI BigDye Terminator v3.1 Cycle Sequencing Kit was used for sequencing, and the sequences read using an ABI 3100 Genetic Analyser.

2.5. Phylogenetic analysis

The sequenced fragments were assembled using MEGA X (Kumar

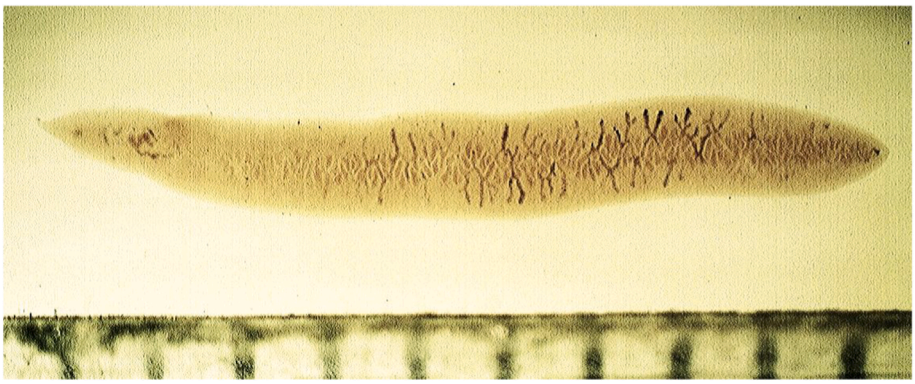

Fig. 6. A hemalaun-stained *Dendritobilharzia* male from the liver of a mallard. Every scale under the specimen is equal to a millimetre.

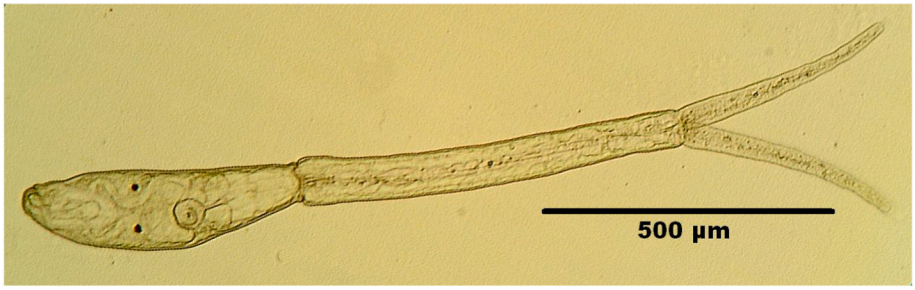

Fig. 7. Ocellata-type of furcocercaria emerged from *Radix auricularia*.

et al., 2018). Nucleotide sequence fragments were assembled with the software Bioedit (Hall, 1999) and corrected manually. The two alignments (LSU rDNA and COI) were generated in MEGA X by Clustal W algorithm (Thompson et al., 1994). Alignments were also corrected with GBlocks (Castresana, 2020) to eliminate poorly aligned positions and divergent regions. Sequences were deposited in the GenBank under the accession numbers (MZ560932-MZ560941, MZ562959-MZ562966). DNA pairwise distances were calculated with the MEGA software using the Tamura-Nei substitution model. Maximum likelihood (ML) and Bayesian inference (BI) analyses were performed for both alignments. The analysed samples are listed in Table 2. The dataset was tested using MEGA X for the nucleotide substitution model of best fit, and the model, shown by the Akaike Information Criterion (AIC) as the best-fitting one, was chosen for each partition. ML analyses were performed in MEGA X under the GTR + G model for the LSU rDNA region and for the COI. Bootstrap values based on 1000 re-sampled datasets were generated. BI was computed using Topali 2.5 (Milne et al., 2004). Posterior probabilities (PP) were estimated over 1,000,000 generations via two independent runs of 4 simultaneous MCMCMC chains, with every 100th tree saved. The first 25% of the sampled trees were discarded as 'burn in'. The phylogenetic trees were visualised using the tree explorer of MEGA X.

3. Results

From the 97 waterfowls examined between September of 2018 and September of 2020, 7 mallards were found infected at least with single male or female specimens of *Trichobilharzia* sp., due to extreme fragility of the worms after the destroying effect of freezing only one fully intact specimen was collected from an unfrozen carcass of mallard (Fig. 3) and recognizable fragments of other 14 specimens of *Trichobilharzia* sp. were found in the organs of hosts. The majority of these parasites resembled adults and mostly males by their shape and because they had no egg in the body. Unfortunately, in consequence of severe destruction of tissues of hosts we could not observe precisely the inner structure of worms. Therefore, these worms were identified to generic level only by microscope according to description of Mcdonald, 1981). Probably, the number of *Trichobilharzia* worms in the infected livers ranged between 1 and 10 but supposedly we were not able to find all individuals during necropsy.

Trichobilharzia worms were usually found in the lacerated livers and sometimes in the abdominal cavity. As the organs of shot birds were damaged by bullets and blood released in the cavities the original dwelling place of blood parasites in the host was not be ascertained in all cases. More or less intact specimen thread like trematodes were accepted as *Trichobilharzia* sp. and used for molecular testing. In the nasal sinuses *Trichobilharzia* sp. eggs were identified (Fig. 4), in two cases. Ten mallards, 2 Eurasian green-winged teals and 1 ferruginous duck were found infected with *Bilharziella polonica* (Fig. 5) and an adult *Dendritobilharzia pulverulenta* (Fig. 6), was identified in the liver of one mallard (Table 1). As these worms are much bigger than the *Trichobilharzia,* their identification at species level was possible by their morphology also by according to description of Mcdonald (1981). They occurred sometimes together with the *Trichobilharzia* thereby proving that a bird can carry mixed infection of blood fluke species. All of these parasites were found only in birds had lived free in the wild, but no such worms were found in artificially reared mallards, which were released promptly before hunt.

Radix auricularia (Linnaeus, 1758) [syn: *Lymnaea auricularia*] snails living in a small ornamental pond in an urban park were recognized to release ocellated form of furcocercariae. Snails were identified and separated from other Hungarian lymnaeid snails according Glöer (2019), by their characteristic ear-like shells, colourless circular spots on the dark pigmented mantle and the long stalked, completely spherical bursa copulatrix on the genital organ (Fig. 2.). However, the conchological similarity of Radix representatives makes it best to carry out molecular diagnostics of bird schistosome snail hosts. *R. auricularia* is the only snail species which lives at this permanent habitat. Five individuals of *R. auricularia* (out of 245) released spontaneously schistosomatid cercariae (Table 1). Live furcocercariae were morphologically identified to genus level using light microscopy. The morphology of

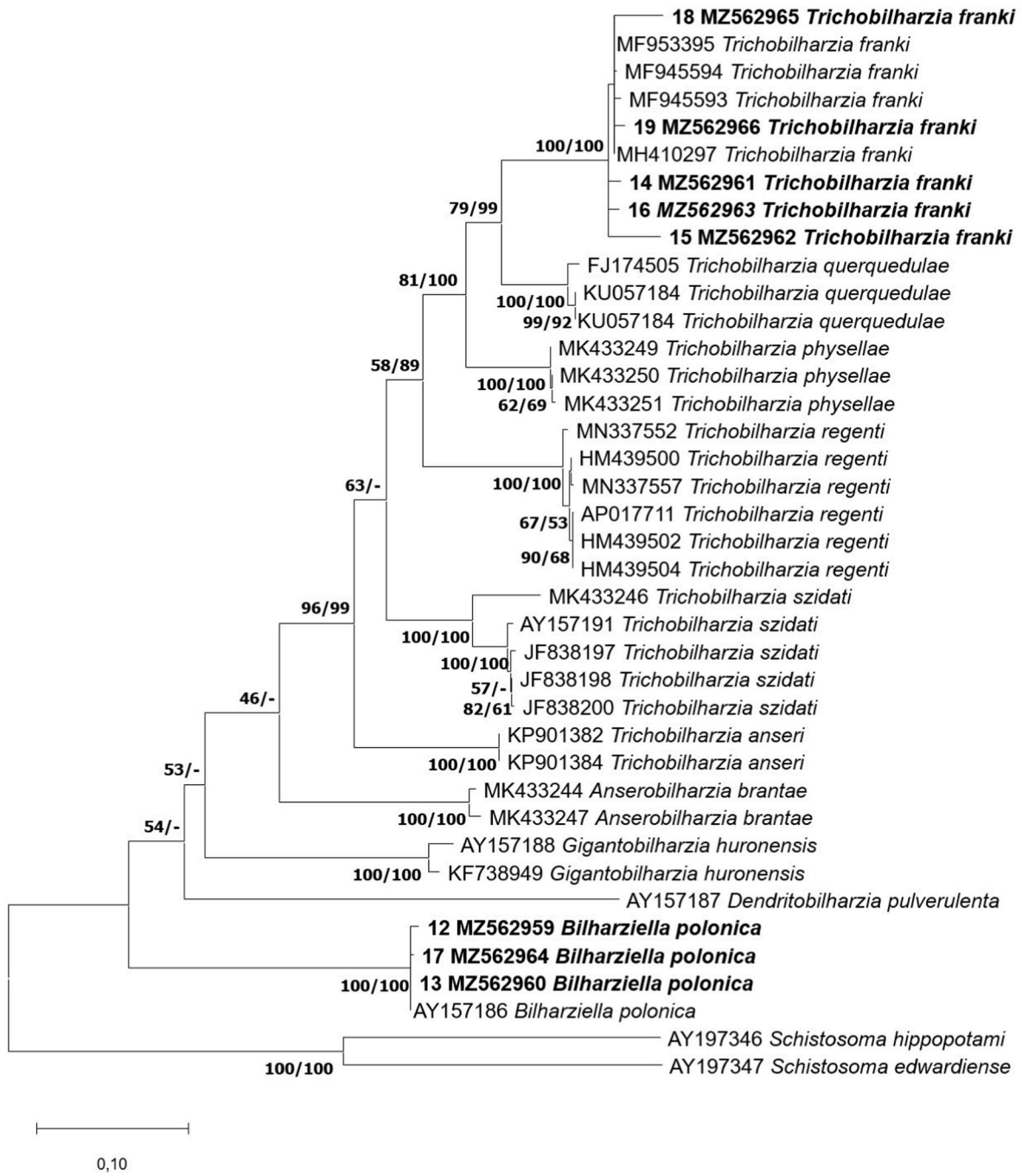

Fig. 8. a, b. Maximum likelihood tree of the samples of *Trichobilharzia franki* and *Bilharziella polonica* from the present study (a COI, b 28S) in relation to other schistostomatid sequences deposited in GenBank. Bootstrap values are given at the nodes; posterior probabilities for Bayesian inference are shown behind the bootstrap values. Unsupported nodes by BI are marked with a hyphen. Samples from the present study are in bold. The scale bar indicates the expected number of substitutions per sit.

isolated cercariae (Fig. 7) mostly matched the description of *Trichobilharzia* larvae found in the literature (Müller and Kimmig 1994; Podhorský et al., https://doi.org/10.2478/s11686-009-0011-9 ">2009; Jouet et al., 2010).

Trichobilharzia worms from 5 mallards, *Bilharziella* adults from 3 mallards and pooled cercariae from 5 snails, yielded enough DNA for molecular testing (Table 2). Sequencing of the amplified PCR fragments yielded approximately for 1100 and 1600 bp long nucleotide sequences for COI and LSU rDNA, respectively. After removing poorly aligned positions and divergent regions, the alignment of COI sequences LSU rDNA was consisted of 1164 bps with 544 conservative and 598 variable (429 of them parsimony-informative) sites. The alignment of LSU rDNA was consisted of 1277 bps, including 801 conservative and 476 variable (286 of them parsimony-informative) sites. ML analysis of COI resulted in a robust phylogenetic tree with maximum bootstrap support at the terminal ends (Fig. 8a). *Trichobilharzia* and other investigated species formed distinct clades. Sequences of samples 14, 15, 16, 18 and 19 were placed into the monophyletic clade of *T. franki* samples, while samples 12, 13, 17 were put into the clade of *B. polonica*. Pairwise distances showed an average 96,7% identity of samples 14, 15, 16, 18 and 19 with

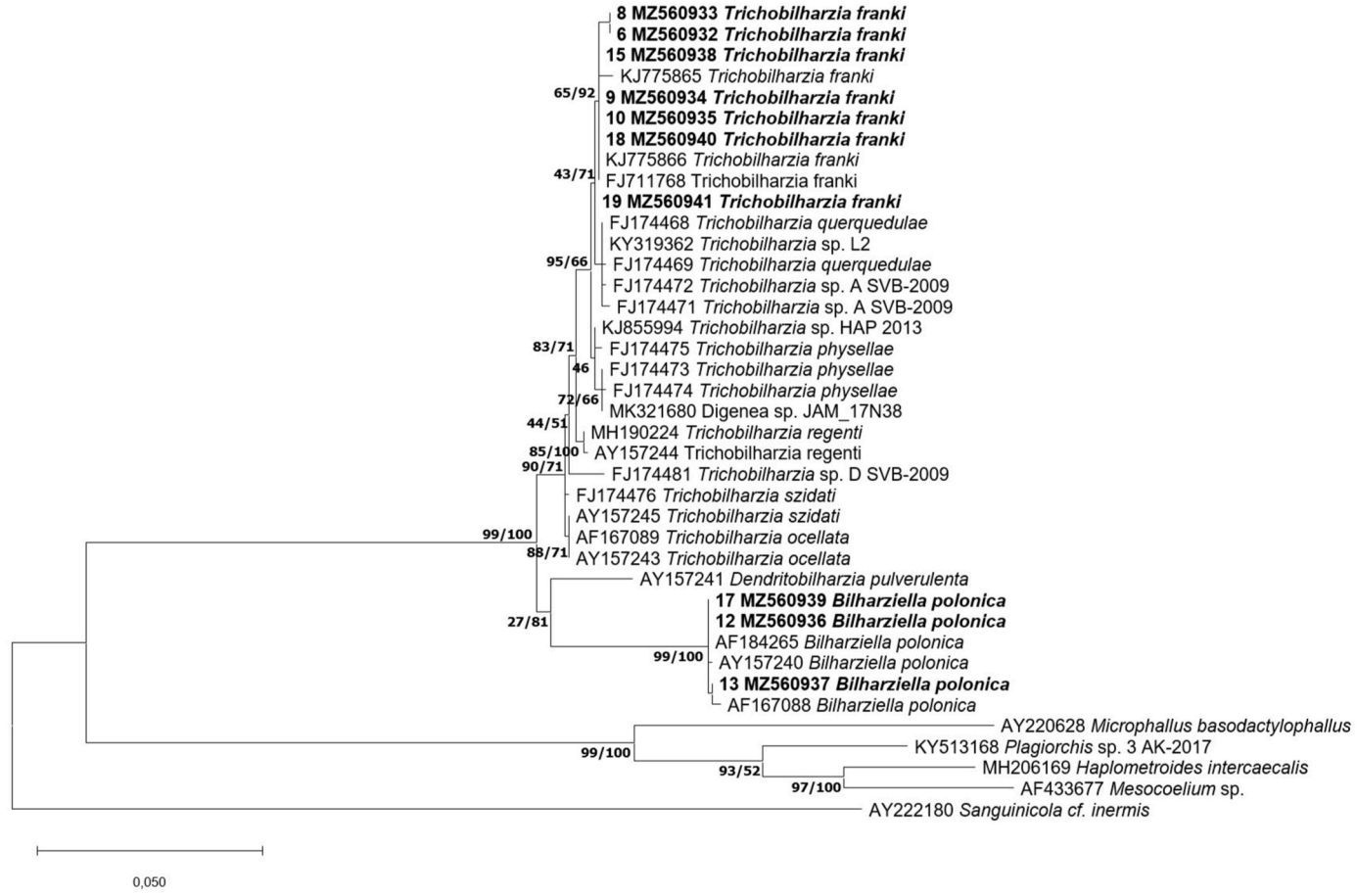

Fig. 8. (*continued*).

the *T. franki* (MF945593-95, MH410297). There were no remarkable differences between the different developmental stages of the collected samples, the within mean group distance was 4.0%. The sister taxon of the *T. franki* clade was the species *Trichobilharzia querquedulae* with an average 9.1% difference. *B. polonica* samples showed only a 0.9% difference to the *B. polonica* sequence (AY157186) from the Genbank. The closest relative was *D. pulverulenta* (AY157187) but with only a 79.4% similarity. BI analysis resulted in a highly similar topology to the ML analysis, only a few basal nodes with low support differed from ML analysis, therefore BI tree is not presented here, only posterior probabilities are indicated at the nodes. ML analysis of LSU sequences resulted in similar topology to the COI sequences, however bootstrap values were significantly lower at the terminal nodes. Samples 1, 6, 8, 9, 10, 15 and 18 formed a clade with the published *T. franki* sequences (FJ71176, KJ775865-66) while 12, 13 and 17 were put with the *B. polonica* samples together as it was presented in the case of COI sequences (Fig. 8b). The within mean group distance was 0.1–0.2% in the case of the above-mentioned groups. Clade of *T. franki* showed a 0.4% distance from *T. querquedulae,* while *B. polonica* was 5.1% far from *D. pulverulenta.* BI analysis of LSU also resulted in a highly similar topology to the ML analysis, therefore only ML phylogenetic tree presented here with posterior probabilities of BI indicated at the nodes. COI and LSU rDNA sequences of the Hungarian isolates were submitted to GenBank under the accession numbers MZ560932-MZ560941, MZ562959-MZ562966.

4. Discussion and conclusions

Our study first describes the specific and detailed record of *T. franki* in Hungary. Based on the obtained results, it can be stated that at least three species of avian blood-flukes of anseriform birds likely cause human cercarial dermatitis in Hungary. While detection of *B. polonica* and *D. pulverulenta* can be achieved by morphological analysis, the precise identification of *T. franki* required recourse to molecular methods.

There is a difficulty in finding the blood flukes at their adequate predilection site in shot birds, parasites sometimes are swept from the damaged organs by the blood or translocated across organs due to gunshot trauma. Trematode eggs which are originally embedded in those organs where the worms live, release and drifted away to other places by bloodstream in consequence of tissue laceration. Because birds have a very fast heartbeat and blood flow, this process may takes seconds. Moreover, the occurrence of worms in not preferred organs may also be due to the migration of the flukes to unusual places. In any case, the ectopic occurrence of flukes suggests that when examining shot birds, it is worth examining all the viscera if we want to find blood-flukes in them. It is also advisable to inspect the presence of eggs in tissues because we found such host that did not have any worms but only eggs in tissue of the head (Fig. 4).

Adapting molecular techniques described by Lockyer et al., (2003), there was possible to confirm the presence *T. franki* in Hungary. The morphological description of *T. franki*, shows that the adults and cercariae have considerable similarity to other *Trichobilharzia* species. A molecular approach was therefore considered essential for identification. Analysing two genes, a mitochondrial (COI) and a nuclear (LSU rDNA) resulted in confirming our samples as *T. franki* and *B. polonica*. LSU rDNA sequences showed less divergence between the species than COI which is a consequence of the less variable feature of LSU rDNA compared to the COI. This is also the reason why bootstrap values and posterior probabilities are lower in the case of LSU rDNA. The pairwise distances of COI between the samples collected in Hungary and the

T. franki samples available in the Genbank showed less than 5% difference which is considered by Ashrafi et al. (2018) as a reasonable cut-off for separating schistosome species.

The difference between the infestation of the reared and wild mallards can be explained by the young (~half-year-old) age and stationary lifestyle of those birds which are kept on shore of fishpond till hunting. Therefore, it seems that those birds can get the blood-fluke infection more easily which live free and can visit several lakes and rivers during their lifespan. Furcocercariae drawn from infected intermediate hosts collected from an urban pond prove that potential sites of infection are not limited to natural waters but should also be calculated even in urban environment. Cercarial dermatitis can develop either through contact with the water of artificial lakes, such as boating, or even during hand or foot cooling, if certain conditions (presence of intermediate hosts, regular visits by definitive hosts) exist (Appleton et Lethbridge, 1979; Ley Vesque et al., 2002; Valdovinos and Balboa, 2008; Cipriani et al., 2011).

Due to the heterogeneity of the samples in space and time, we could not investigate the real prevalence of trematodes, but to try to find as many avian blood-fluke species as we can. The study of wild birds and snails living in natural habitats involves many technical difficulties yet we were able to find *T. franki* for the first time in Hungary and we have detected *B. polonica* and *D. pulverulenta* in Hungary again since first report on these trematodes in the last century (Edelényi, 1974). Neither of these avian blood-flukes or cercariae have been collected in the country since then and this is the first time that molecular techniques have been used to study them in Hungary.

Although the disease of cercaria dermatitis is specifically a human disease, it is also addressed in the Hungarian veterinary literature as a zoonosis (Kassai, 2001). Our work is an initial step in recognizing diseases caused by avian blood-flukes in Hungary and more widely across Central Europe. The search for cercarial dermatitis causing blood-flukes would be more effective if it not depended on the hunting of game alone. Supposedly, parasitological monitoring of *R. auricularia* snail can reveal more potential sites of infection than the random inspection of migratory birds and identify transmission points of putative risk. Snail populations persist more or less constantly on their locality while migratory birds spend less time on these habitats. This is the reason why the distribution of *R. auricularia* snails in Hungary was mapped formerly. The data of this map come mainly from museum collections and serve as a plan for sites of further research (Juhász, 2018).

Ethical approval

No birds were sacrificed for this study. The mallard (*Anas platyrhynchos*) used for molecular analysis here was submitted for diagnostic purposes to the Department of Parasitology and Zoology, University of Veterinary Medicine.

Declaration of competing interest

All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.

This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.

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