Digenean trematodes in Hungarian freshwater aquacultures

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ABSTRACT

Occurrence of metacercariae of potentially zoonotic trematodes (Platyhelminthes: Digenea) in the musculature of common carp (*Cyprinus carpio* L. 1758) was monitored in four Hungarian aquacultures. Four geographically distinct fish farms (located in the Northwestern, Southwestern, Northeastern and Southeastern parts of Hungary) were selected for the investigation. From each farm, a total of 258 one-summer-old fingerlings were sampled and examined in the years 2016 and 2017. In addition, in 2017, we examined 60 market size specimens (30 two-summers and 30 three-summers) sampled from the most infected aquaculture in the Northeastern part of Hungary. The fish were euthanized and decapitated whereafter their musculature (fillets) was digested in a pepsin solution to isolate metacercariae from the tissue whereafter morphological and molecular analyses (PCR and sequencing of ITS region) were performed. Opisthorchiid metacercariae were not recovered but in one of the farms numerous metacercariae were detected in the musculature of carp. They were identified as cyathocotylid trematodes based on their morphological characteristics and by sequencing the ITS region. The infection levels proved to be remarkably different among the four fish farms. Carps from the Northeastern farm were infected by large numbers of cyathocotylid metacercariae, while 8 *Posthodiplostomum cuticola* metacercariae were detected in the Northwestern aquaculture. In the other two farms (Southwestern and Southeastern) no infection was recorded. The infected farm is located close to a protected natural wetland habitat populated by a rich fauna of aquatic birds (potential final hosts) and snails (first intermediate host) which may create a higher risk of infection in the neighbouring fish farms.

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1. Introduction

An extensive monitoring project (as part of the Horizon 2020 ParaFishControl project) involving marine and freshwater fish farms in Europe has been conducted aiming at assessing the occurrence of zoonotic flukes in the six most important farmed fish species in EU. Aquaculture enterprises included reared Atlantic salmon (*Salmo salar* L. 1758), European seabass (*Dicentrarchus labrax* (L. 1758), gilthead seabream (*Sparus aurata* L. 1758), turbot, (*Scophthalmus maximus* (L. 1758), rainbow trout (*Oncorhynchus mykiss* (Walbaum, 1792) and common carp (*Cyprinus carpio* L. 1758). Monitoring activity was conducted in several EU countries: Denmark, Greece, Italy, Norway, Spain and Hungary. The present study aimed at investigating common

carp farms in Hungary for occurrence of zoonotic flukes. In addition, sampling of freshwater snails (possible intermediate hosts) was also carried out to record the infection status and their release of infective cercariae.

Fish-borne zoonotic trematode (FZT) infections are known throughout the globe. In endemic regions, people acquire infections repeatedly due to regular consumption of raw, marinated, cold smoked or inadequately cooked fish products. It is estimated that up to 40 million people are infected with trematodes worldwide (WHO, 2011) and mainly opisthorchiid liver flukes, heterophyid or echinostomatid intestinal species are involved in these trematodiases (Pozio and Morales, 2014). Southeast and East Asia exhibit the highest prevalence of diseases caused by flukes including *Clonorchis sinensis* in East Asia (Chen et al., 1994; Hong, 2003; Rim, 1990; Sohn et al., 2011; Sohn et al., 2012; Yu et al., 2003), *Opisthorchis viverrini* and *Haplorchis taichui* from Thailand (Kaenjampa et al., 2017; Onsurathum et al., 2016), and *Haplorchis pumilio* from Vietnam (Tran et al., 2009). Infections reported outside Asia are caused by *Metorchis conjunctus* in Canada (Behr et al., 1998; MacLean et al., 1996; Yamaguti, 1958) and *Opisthorchis felineus* in Eastern Europe and Siberia (Erhardt et al., 1962; Mordvinov et al., 2012; Mordvinov et al., 2012) and in 13 countries within the European Union (Pozio et al., 2013). In most cases the fluke-infection causes mild symptoms (fever, abdominal pain, weakness) but generally no serious health issues (Armignacco et al., 2008). However, if the raw fish contains large number of metacercariae, heavy infections of the consumer may result. These cases may be associated with chronic hepatobiliary diseases including cholangitis, biliary calculi, hepatomegaly, liver cirrhosis, cholelithiasis, pancreatitis and cholangiocarcinoma (Sripa, 2003). Monitoring of fishborne zoonotic metacercariae in aquaculture products should therefore be conducted as a preventive health care measure. Common carp (*Cyprinus carpio* L. 1758) is the most important aquaculture species in Hungary (annual production 9–10,000 metric tonnes) and in the year 2015 freshwater fishes accounted for 20.4% (70,000 t) of the entire aquaculture production in the European Union. Although common carp is a major species in the European aquaculture, the largest producers are China and Indonesia with 72% and 10%, respectively (EUFOMA, 2016).

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Despite the focus on zoonoses it is noteworthy that also non-zoonotic metacercariae can occur in the musculature of freshwater fishes. The globally distributed digenean trematode family Cyathocotylidae Mühling, 1896, comprises species with the adult flukes often infecting the intestine of birds, and in more rare cases reptiles, mammals and fishes. The taxon is considered monophyletic representing an ancient digenean lineage (Achatz et al., 2019). The presence of cyathocotylid metacercarie in various freshwater fishes was documented in Czechia (Kvach et al., 2016), Finland (Näreaho et al., 2017), France (Gettová et al., 2016), Slovakia (Ondračková et al., 2009) and Russia (Kvach et al., 2015). Human infections have not been documented but mammals (dogs) have been suggested as potential hosts of adult cyathocotylids (Chandler, 1950; El-Assal et al., 1986).

Zoonotic helminths in wild fish populations in Europe have been documented in several countries (Armignacco et al., 2008; Borges et al., 2015; Keiser and Utzinger, 2005; Mordvinov et al., 2012; Murell and Pozio, 2017; Pozio et al., 2013; Skov et al., 2008). It is therefore relevant to conduct an extensive epidemiological survey of the freshwater aquacultures of the European Union. The present study contributes to our understanding of the occurrence of digenan metacercariae in European fish farms.

2. Materials and methods

2.1. Sample collection

Four fish farms in Hungary (located in the Northwestern, Southwestern, Northeastern and Southeastern parts of the country) were selected (Fig. 1). These traditionally constructed paddy pond fish farms apply the regular technology of carp polycultures in Central Europe as described by Woynarovich et al. (2010). Northeastern aquaculture is considerably larger than the other three aquacultures and it is located inside the territory of a national park. In the years 2016 and 2017, 258 one-year-old specimens of common carp were examined from each farm. In order to assure a high probability of detecting a parasite, even when rare, the sample size was estimated using the formula described by Daniel (1999): n = [Z^{2}P (1-P)]/d^2, where n = sample size, Z = Z statistic for a level of confidence, P = expected prevalence (in proportion of one), d = precision (in proportion of one) with a 99% level of confidence. A *P* value for the worst-case percentage (50%), and a precision of 5% was adopted as a good compromise. Moreover, during October 2017, 30 one-summer-old, 30 two-summers and 30 three-summers carp specimens and 25 Lister's river snails (*Viviparus contectus* Millet, 1813) were collected from the Northeastern farm, where fish sampled during the first year were positive for metacercariae in the fillets. The sampling was restricted to the pond which was positive during the previous year. Fish and snails were brought into the laboratory in oxygenated plastic bags and examined for parasites.

2.2. Artificial digestion

The fish were sedated by adding a few drops of clove oil into the water and euthanized by a cervical cut. After measuring body weight and standard length (from the tip of the lower jaw to the posterior end of the hypural bone) the body musculature was
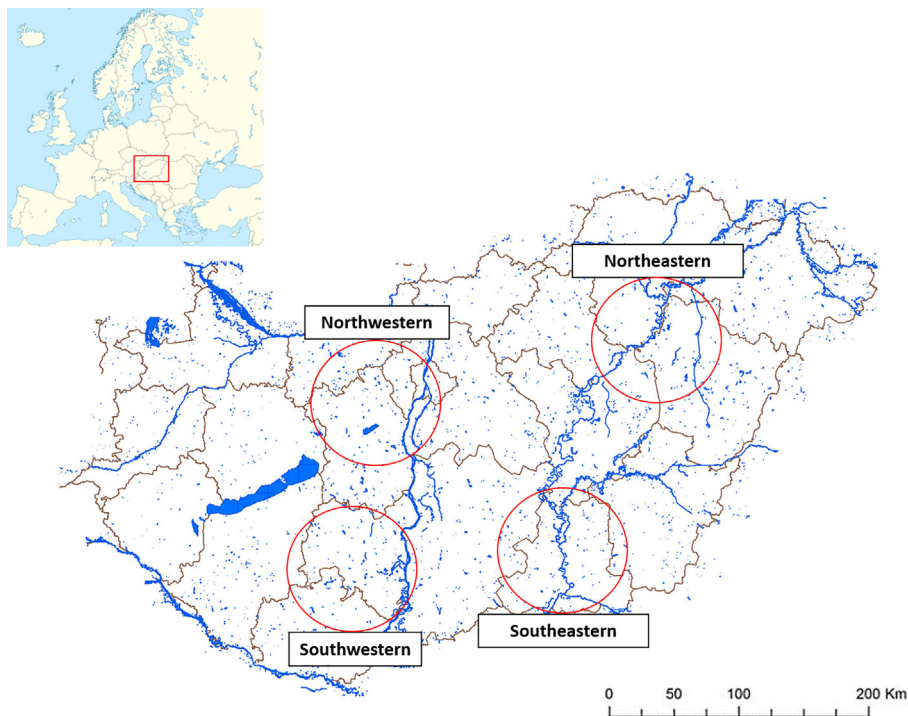
Fig. 1. Location of the four sampled farms in Hungary.

recovered (Table 1). During the artificial digestion, the whole fillets of each carp were immersed separately in glass beakers containing the digestive pepsin-HCl-solution (2 l of tap-water, 10 g 1:10000 NF pepsin powder - Molar Chemicals, Halásztelek, Hungary - and 16 ml 25% HCl) and incubated on a magnetic stirrer at 37 °C to simulate the physiological condition of the final host's crop. Following 20 min incubation the fillets were completely digested and the metacercariae were collected by filtration.

2.3. Light microscopic examination

The encysted metacercariae were collected with glass pipette and their morphology studied under dissecting and light microscopes. Morphological and morphometric characters (body length and width, size of pharynx, oral and ventral suckers, length of caecum) of 15 metacercariae (live condition) were measured and documented (Erasmus, 1962). The fresh samples were photographed using an Olympus BH2 equipped with DP20 digital camera (4×, 10× and 20× magnifications) and subsamples preserved in 70% ethanol for molecular investigations.

2.4. Molecular methods

In addition to the samples collected in Hungary, seven cyathocotylid metacercariae from rudd (*Scardinius erythrophthalmus* (L. 1758) and tench (*Tinca tinca* (L. 1758) specimens sampled in Italy were added to the molecular analysis for comparison (Table 2). The isolation, PCR and sequencing processes were conducted differently as described below.

2.4.1. Hungarian samples
A total of 20 single metacercariae preserved in 70% ethanol were centrifuged at 8,000*g* for 5 min, and the ethanol removed (vacuum centrifuge) whereafter DNA was extracted with the QIAGEN DNeasy™ tissue kit (animal tissue protocol; Qiagen, Hilden, Germany) and eluted in 100 μl AE buffer. The ITS region (part of 18S rDNA, ITS1, 5.8S rDNA, ITS2 and part of 28S rDNA) was

Table 1
The average body weight, standard length and musculature weight of the one-, two- and three-year-old carp specimens.

Measured parameters (average ± SD)			
One-summer carps (N = 1062)	Body weight (g)	Weight of examined fillets (g)	Standard length (cm)
	28.3 ± 8.7	19.3 ± 5.9	9.5 ± 1.2
Two-summers carps (N = 30)	464.3 ± 158.1	267.9 ± 90	22.7 ± 3.7
Three-summers carps (N = 30)	2154.3 ± 305.8	1680.4 ± 238.5	39.4 ± 2.2

Table 2
List of the sequenced metacercariae of *Holostephanus* sp.

	Sample	Morphological identification	Host	Developmental stage	Date of collection	Site of collection	Accession number
1.	HS1	Cyathocotylid metacercaria	common carp (*Cyprinus carpio*)	Metacercaria	29/08/2016	Northeastern part of Hungary	MT668947
2.	HS3	Cyathocotylid metacercaria	common carp (*Cyprinus carpio*)	Metacercaria	18/04/2016	Northeastern part of Hungary	MT668948
3.	HS5	Cyathocotylid metacercaria	common carp (*Cyprinus carpio*)	Metacercaria	26/09/2016	Northeastern part of Hungary	MT668949
4.	HS11	Cyathocotylid metacercaria	common carp (*Cyprinus carpio*)	Metacercaria	20/10/2016	Northeastern part of Hungary	MT668950
5.	HS17	Cyathocotylid metacercaria	common carp (*Cyprinus carpio*)	Metacercaria	22/09/2016	Northeastern part of Hungary	MT668951
6.	72/14 6	Cyathocotylid metacercaria	rudd (*Scardinius erythrophthalmus*)	Metacercaria	11/03/2014	Iseo Lake, Italy	MT668944
7.	71/14	Cyathocotylid metacercaria	Tench (*Tinca tinca*)	Metacercaria	11/03/2014	Iseo Lake, Italy	MT668945
8.	80/14 1	Cyathocotylid metacercaria	Tench (*Tinca tinca*)	Metacercaria	12/03/2014	Como Lake, Italy	MT668943
9.	199/11 2P	Cyathocotylid metacercaria	Tench (*Tinca tinca*)	Metacercaria	14/11/2011	Garda Lake, Italy	MT668942
10.	199/11 4G	Cyathocotylid metacercaria	Tench (*Tinca tinca*)	Metacercaria	14/11/2011	Garda Lake, Italy	MT668941
11.	8/14	Cyathocotylid metacercaria	Tench (*Tinca tinca*)	Metacercaria	07/01/2014	Iseo Lake, Italy	MT668946
12.	214/13	Cyathocotylid metacercaria	Rudd (*Scardinius erythrophthalmus*)	Metacercaria	18/07/2013	Maggiore Lake, Italy	MT668940

amplified through nested PCR. The primers S18 (5′-TAACAGGTCTGTGATGCC-3′) and L3T (5′-CAACTTTCCCTCACGGTACTTG-3′) (Jousson et al., 1999) were used in the first run in a 25 μl reaction mixture comprised of 2 μl of extracted genomic DNA, 5 μl of 1 mM dNTPs (MBI Fermentas, Burlington, Canada), 12.5 pmol of each primer (0.5 μM final concentration), 2.5 μl of 10× Taq buffer (MBI Fermentas), 0.1 μl of DreamTaq polymerase (0.5 U) (MBI Fermentas) and 15 μl of water. The PCR profile consisted of an initial denaturation step of 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 2 min, and finished with a terminal extension at 72 °C for 5 min, then stored at 4 °C. The primers D1 (5′-AGGAATTCCTGGTAAGTGCAA-3′) and D2 (5′-CGTTACTGAGGGGAATCCTGGT-3′) (Galazzo et al., 2002) were used in the second run in 50 μl of reaction mixture comprised of 1 μl PCR product from the first run, 10 μl of 1 mM dNTPs (MBI Fermentas), 25 pmol of each primer (0.5 μM final concentration), 5 μl of 10× Taq buffer (MBI Fermentas), 0.2 μl of DreamTaq polymerase (1 U) (MBI Fermentas) and 33 μl of water. The second PCR consisted of an initial denaturation step of 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 2 min and a final extension step at 72 °C for 5 min, then stored at 4 °C.

PCR products were electrophoresed in 1% 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 ITS region were sequenced with D1 and D2 primers and 5.8Sr (5′-TGTCGATGAAGAGCGCAGC-3′) and 5.8S2 (5′-TAAGCCGACCCTCGGACAGG-3′) internal primers (Tkach et al., 2000). ABI BigDye Terminator v3.1 Cycle Sequencing Kit was used for sequencing and the sequences read using an ABI 3100 Genetic Analyser (MTA SZBK Szekvenáló Platform, Szeged, Hungary).

2.4.2. Italian samples

The DNA was extracted using Chelex100 (Sigma-Aldrich, Saint Louis, MO, USA) at 5% concentration. Briefly, 300 μl of 5% Chelex100 were added to one metacercaria, incubated in heat block at 95 °C for 5 min and centrifuged at full speed for 5 min. The supernatant containing the DNA was transferred into a clean tube and diluted at least at 1:10 for downstream use. For the amplification of the ITS rDNA the protocols and primers of Gustinelli et al. (2010) were used. The products were resolved on a 1% agarose gel stained with SYBR Safe DNA Gel Stain in 0.5× TBE (Invitrogen – Thermo Fisher Scientific, Carlsbad, CA, USA). For sequencing, bands were excised and purified by NucleoSpin Gel and PCR Cleanup (Mackerey-Nagel, Düren, Germany) and sequenced in both direction with an ABI 3730 DNA analyser at StarSEQ GmbH (Mainz, Germany).

2.5. Phylogenetic analysis

In the case of Hungarian samples, the sequenced fragments of the ITS region were assembled by MEGA X (Kumar et al., 2018) and ambiguous bases clarified using corresponding ABI chromatograms, while the Italian samples were assembled with Vector NTI AdvanceTM 11 software (Thermo Fisher Scientific, Carlsbad, CA, USA). Nucleotide sequences of the ITS rDNA region were aligned with the software CLUSTAL W (Thompson et al., 1994). The alignments were corrected manually using the alignment editor included in MEGA X software. Pairwise distances were calculated with the MEGA X using the p-distance model. The dataset was tested using MEGA X for the best fitting nucleotide substitution model and the model predicted by the Akaike Information
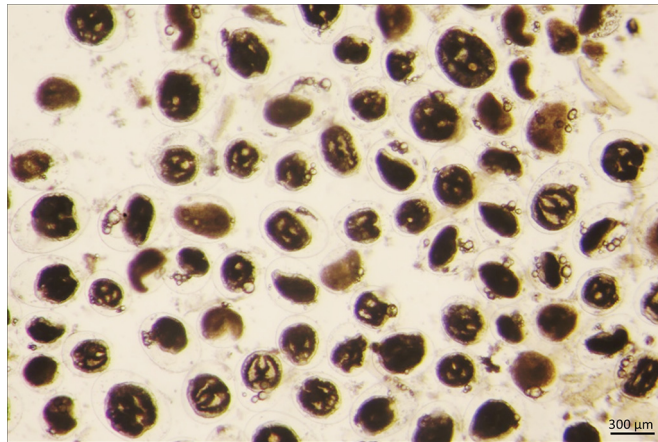
Fig. 2. Cyathocotylid metacercariae from common carp (*Cyprinus carpio*) of the Northeastern farm isolated following successful artificial digestion.

Criterion (AIC) was chosen. ML analyses of the ITS region and was performed under the GTR + G model. Bootstrap values based on 1000 resampled datasets were generated. The ML tree was visualised using the tree explorer of MEGA X. *Metagonimus yokogawai* (Katsurada, 1912) (sequence KJ631740) was used as outgroup.

## 3. Results

3.1. Monitoring

During the 2016–2017 monitoring activities, all the 1032 one-year-old carps were negative for zoonotic metacercariae in the fillets. However, non-zoonotic cyathocotylid metacercariae (Fig. 2.) were detected in the Northeastern farm, showing a prevalence of 13.9% and a mean intensity of infection of 12.8 ± 9.4 metacercariae per fish. Two carps from the Northwestern farm were infected by *Posthodiplostomum cuticola* (Fig. 3.) metacercariae (prevalence 0.77%, mean intensity: 4 ± 1.4) while all Southwestern and Southeastern carps were negative.

The fish examined in the second survey, carried a higher parasite load. Cyathocotylid metacercariae were found in all 30 one-summer-old carp (100%, mean intensity: 41.3 ± 33.21), in 24 of 30 two-summers (80%, mean intensity: 291.1 ± 226.34) and in 27 of 30 three-summers carps (90%, mean intensity: 130 ± 43).

Hundreds of cercariae were detected in the hepatopancreas and body cavities all of the 25 Lister's river snails (*Viviparus contectus*). The morphological features indicated a gymnocephalic cercarial type and not a furcocercarial type which is characteristic for cyathocotylid cercariae.

Fig. 3. Isolated *Posthodiplostomum cuticola* metacercaria from common carp (*Cyprinus carpio*) skin in the area of the Northwestern farm.
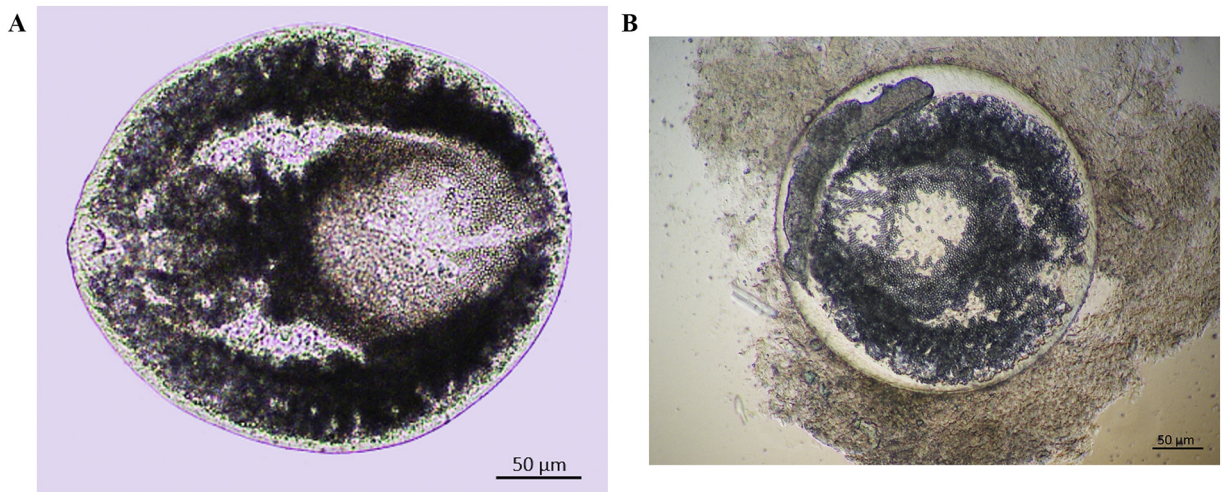
Fig. 4. A/B: Micrographs of encysted prohemistomulum metacercaria type (Cyathocotylidae) from the musculature of common carp (*Cyprinus carpio*) collected from Northeastern farm.

3.2. Identification

The metacercariae collected complied with the prohemistomulum type (Fig. 4 A/B) characteristic for the family Cyathocotylidae (Digenea), based on the following morphological features: round or oval body with a thick outer wall and a thin inner membrane; oral and ventral suckers were present whereas pseudosuckers were absent (Niewiadomska, 2002). The complete morphological description of the examined metacercariae was published by Sándor et al. (2020): The body surface of encysted metacercariae was smooth with no spines. Body length was 324.7 ($\pm$ 35.6) μm and body with 245.3 ($\pm$ 52.2) μm. The oral sucker was 32.3 ($\pm$ 7.3) μm long and 32.3 ($\pm$ 7.3) μm wide. After a short prepharynx, the oral sucker was closely followed by the small-sized pharynx, which was 39.3 ($\pm$ 4.6) μm long and 33.4 ($\pm$ 4.8) μm wide. The ventral sucker was 75 ($\pm$ 9.3) μm long and 75 ($\pm$ 9.3) μm wide. The length of the caecal branches was 274.7 ($\pm$ 33.1) μm. In order to identify the metacercariae at species level we performed additional molecular diagnostics.

3.3. Molecular examination

The ITS rDNA region of 5 samples (HS1, HS3, HS5, HS11, HS17) were successfully amplified and sequenced extending 1400 bp (Table 2, MT668947-51). Moreover, ITS sequences (72/14 6, 71/14, 80/14 1, 199/11 2P, 199/11 4G, 8/14, 214/13) of cyathocotylid metacercariae collected in Italy from different hosts (rudd and tench) were also included in the analysis (MT668940-46). The alignment of the sequences from collected samples and related sequences retrieved from GenBank was 1545 bps long and contained 828 conservative and 704 variable (596 of them parsimony-informative) sites. Based on the Maximum Likelihood phylogenetic analysis (Fig. 5) and the pairwise distance comparison of the samples and the related sequences available in GenBank, there were three different trematode species in the musculature of the carps, all of them belonging to a clade supported by 100% bootstrap, representing the trematode family Cyathocotylidae.

Three specimens (HS1, HS5 and HS17) were identical to each other, with a small difference (0–0.4%). The cyathocotylid samples collected in Italy (72/14 6, 71/14, 80/14 1, 199/11 2P, 199/11 4G, 8/14) were also identical showing only a minimal genetic difference (0.1–0.5%). The closest matches in the GenBank were two sequences, JF911799 and JF911800, identified as *Carassius auratus* with just a pairwise distance between 0.2 and 2.0% towards our samples and an another sequence of a silurid fish, *Ompok pabda* is also present among the close sequence matches with difference values above 10%. Due to the possible mistake in the annotation, these three samples were not included in the phylogenetic tree. The sample HS3 clustered with *Cyathocotyle prussica* (MH521249), *Cyathocotyle* sp. 1 (MN723852), *Cyathocotyle* sp. 2 (MN723853, MN723854) and *Holostephanus dubini* (AY245707) even with a marked sequence divergence (6.8% and 7.0%). The sample HS11 remained also unidentified being basal to the *Holostephanus*/cyathocotylid clade, showing genetic distance above 15%. Sample 214/13 grouped together and was identical to *Holostephanus dubini* (AY245707). Other cyathocotylid species were observed in the clade together with two *Mesostephanus* sp. samples (HM064922 and HM064924), but all of them showed a distinct separation from the metacercariae found in the examined carp individuals.

The data retrieved from the morphological and molecular (ITS) analyses of metacercariae did not allow a full genus identification of all specimens. Only one sample (214/13) was identical with *Holostephanus dubini* (AY245707), and in the present study we therefore refer to the parasites as cyathocotylid metacercariae.
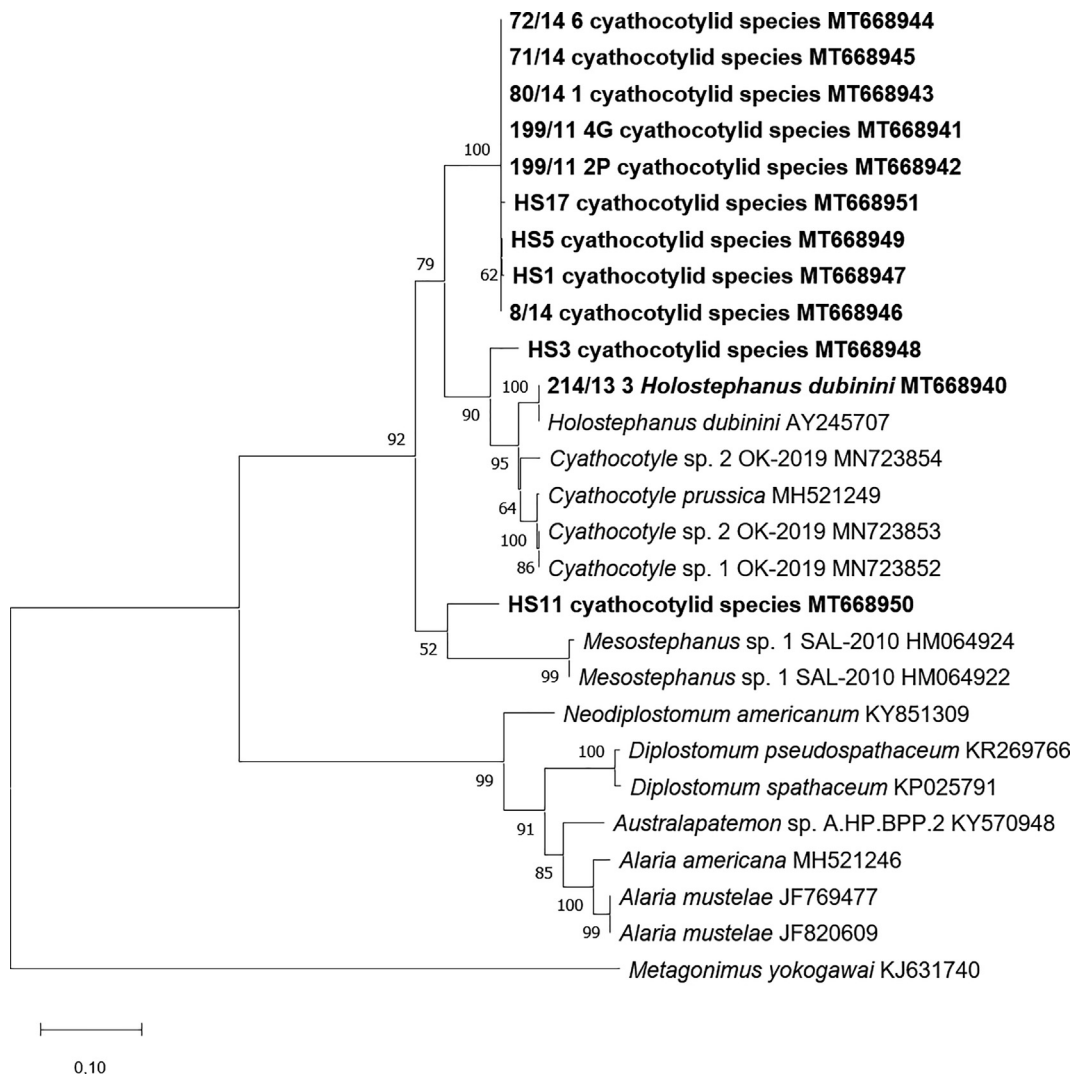
Fig. 5. Maximum likelihood tree of the ITS region sequences of the samples (HS1, HS3, HS5, HS11, HS17 + 72/14 6, 71/14, 80/14 1, 199/11 2P, 199/11 4G, 8/14) from the present study in relation to other cyathocotylid and related sequences deposited in GenBank. Bootstrap values are given at the nodes. Samples from the present study are in bold. The scale bar indicates the expected number of substitutions per site.

4. Discussion

Farmed fish in Europe have not previously been monitored for occurrence of zoonotic flukes until the examinations conducted during the EU supported Horizon 2020 project ParaFishControl. The present study on Hungarian farms is part of this program and demonstrated the absence of zoonotic parasites in the examined fish comprising far more than 1000 carp. This confirms the notion that European (including Hungarian) aquacultures are free from zoonotic trematodes. Two Hungarian farms (Southwestern and Southeastern) were free of any kind of metacercarial infection. Eight *Posthodiplostomum cuticola* metacercariae (non-zoonotic bird trematodes) (Nähreaho et al., 2017) were detected in the Northwestern farm. Infections may lead to deformations of fingerlings (Lucký, 1970; Ondračková et al., 2004a; Tobler and Schlupp, 2008; Zrnčić et al., 2009) and as the cysts may appear as black spots on the body surface, fins and the scales of older fish, their presence may affect the commercial value of the fish negatively. In the Northeastern aquaculture farm, cyathocotylid metacercariae were present in all age groups with a relatively high prevalence and intensity of infection. However, based on solely the morphology of the metacercariae it is impossible to decide if they belong to either of the genera *Holostephanus* or *Cyathocotyle*. In addition, basic molecular data are scarce, or not available, whereby also sequence data cannot solve the question at present. The maximum likelihood phylogeny based on the ITS sequences suggests that *Cyathocotyle* and *Holostephanus* genera do not form distinct monophyletic clades calling for a future phylogenetic revision. Achatz et al. (2019), based on nuclear 28S rDNA sequences, presented the molecular phylogeny of the family Cyathocotylidae but unfortunately only two *Cyathocotyle* and one *Holostephanus* species were present in the analysis, which is not sufficient for a precise description of the relationship between the two genera.

The difference between the prevalence recorded in the two sampling years (13.9% in 2016 vs. 100% in 2017) may be explained by the fact, that only one pond (known to be infected in 2016) was sampled in 2017. Thereby no confounding effect of sampling non-infected ponds occurred in 2017. At present no studies have documented a possible adverse effect of these infections on the fish and it is worthwhile to initiate studies on parasite induced effects on carp culture. The are no available prevalence data about cyathocotylid metacercariae in fish, only FZTs were monitered in the past. As they a lot of them is also muscle-infecting species (like *O. felineus*), it makes sense to compare against their prevalence. In Europe, the most important species of fish borne zoonotic trematodes in wild freshwater fish is *Opisthorchis felineus*. Several studies on wild fish in natural waters have documented the oc-currence of *O. felineus*. Thus, De Liberato et al. (2011) found an overall prevalence of 88.5% (116/131) in tench (*Tinca tinca* L. 1758) from two lakes in Central Italy whereas other fish species inhabiting the lakes were free of *O. felineus.* Hering-Hagenbeck and Schuster (1996) observed corresponding prevalences (76% and 74%, respectively) in common roach (*Rutilus rutilus* L. 1758) and common bleak (*Alburnus alburnus* L. 1758) in German waterbodies. Infection of humans by FZTs is more widespread in East and South-East Asia. Investigations of wild and farmed fish in this geographic region are therefore available. Phan et al. (2010) reported the prevalence of fishborne zoonotic trematodes in cyprinids (mostly *Haplorchis pumilio*, a species absent from Europe) as 64.3% in cultured fish and 68.9% in wild-caught fish. Skov et al. (2009) found also recorded high prevalences of zoo-notic metacercariae (*Haplorchis pumilio*, *Haplorchis taichui* and *Procervum* sp.) in two cyprinid species (*Hypophthalmichthys molitrix* and *Labeo rohita*) and in climbing perch (*Anabas testudineus*) collected from two Vietnamese fish ponds. The significant prevalence in farmed fish can be explained by the fact that there are many small family farms applying non-intensive breeding and rearing methods. Other studies reported lower prevalence, as Chi et al. (2008) observed 42.7% prevalence and Thien et al. (2007) ascertained only 10% prevalence in carp. Different prevalences might be due to seasonal variation (Madsen et al., 2015; Ondračková et al., 2004b) or by the distance of farms from wild waters (Phan et al., 2010).

The Northeastern farm is in close proximity to a protected natural conservation area therefore it is reasonable that metacercariae were found in large numbers. In this wetland potential intermediate and final hosts are present. Thus, freshwater snails (*Viviparus contectus*) and water birds (*Ardea alba*, *Phalacrocorax carbo*, *Ardea cinerea*) are abundant. Several studies have shown a positive correlation between the richness of host species and the diversity and abundance of parasites (Hechinger and Lafferty, 2005; Lebarbenchon et al., 2007). Our preliminary snail shedding study of *V. contectus* individuals from the monitored farms detected only an another cercarial type (gymnocephalous), which is the developmental stage of other trematodes. However, these molluscs may also serve as the first intermediate host of cyathocotylid trematodes (Niewiadomska, 2002). In addition, other studies reported *Bithynia* sp. (common in Hungarian freshwaters, but not present in fish ponds) as the first intermediate host of cyathocotylid trematodes in freshwaters (Besprozvannykh et al., 2013; Erasmus, 1962; Serbina, 2014). The source of the infection in the Hungarian cyprinid fish may be non-detected infected snails in ponds.

Metacercariae were identified based on morphological features after the artificial digestion and manual isolation of the cysts. Exact species identification was not possible due to the developmental status of the metacercaria (lack of reproductive organs). The results obtained suggest that the trematodes in the carp musculature belong to the family Cyathocotylidae, and their morpho-logical characteristics show a high similarity to *H. luehi* presented by Erasmus (1962). Based on the molecular results there are at least 3 species infecting aquacultured carp in the Northeastern farm. All of them are the members of the same clade that is mostly representing cyatocotylid trematodes. Comparison with cyathocotylid trematode sequences from Italy suggested identity for the samples HS1, HS5 and HS17 whereas samples HS3 and HS11 presumably are different species. The NCBI search gave an unex-pected result that the samples HS1, HS5, and HS17 a close match with some misidentified sequences, namely two sequences of *Carassius auratus* and of a silurid fish, the Asian *Ompok pabda.* The connecting publication to the "*Carassius auratus* sequences" does not discuss or even mention these sequences only AFLP analysis of *Carassius* hybrids is presented. The sequence of "*Ompok pabda*" has no publication information therefore it was not possible to obtain further information. As all of the other re-lated sequences belong to digenetic flukes, it makes highly possible that those three sequences were erroneously annotated as fish. The reason may be that the DNA extracted from fish tissues were heavily infected with cyathocotylid metacercarie and a sub-optimal PCR reaction amplified the parasite DNA. Other sequences placed in the same clade are all belonging to family Cyathocotylidae, comprising the genera *Cyathocotyle, Holostephanus* and *Mesostephanus.* Species level identification was only pos-sible in the case of *H. dubini* (sample 214/13 from Italy), however species level identity of the remaining samples remain unclear. There are several genera and species within the family Cyathocotylidae (*Holostephanus, Mesostephanus, Cyathocotyle*) but only few with associated with DNA sequence records. Future molecular studies should therefore elucidate this problem by including addi-tional sampling areas and life cycle studies. Although these samples are placed somewhat distant from the sequence of *Holostephanus dubini* while others (like *Mesostephanus*) are positioned closer, it does not contradict morphological observations. Forthcoming sequence data and analyses might rearrange the recent taxonomy of Cyathocotylidae which is only based on mor-phology at this point.

According to the results of this survey, the examined Hungarian freshwater fish products are free of zoonotic flukes; one carp farm was infected by trematodes but only with non-zoonotic cyathocotylid species. These parasites have been demonstrated un-able to infect mammalian model animals (mice and Syrian hamsters) reflecting their non-zoonotic nature (Sándor et al., 2020). Accordingly, there is no information in the scientific literature that cyathocotylid species can cause human infection (Niewiadomska, 2002) although a related species such as *Mesostephanus longisaccus* may infect dogs (Chandler, 1950). Moreover, according to El-Assal et al. (1986) cyathocotylid flukes may be isolated from dogs fed fish. Culinary practices and preservation methods commonly used in Hungary and in most European countries can prevent the survival and possible transfer of metacercariae present in fish fillets. Some traditional practices in Hungary, including smoked fish, were once common, but during the 20th century, its production and consumption has become sporadic. Other European countries may have fish dishes with a

potential risk, such as marinated tench in Italy, a known source of *O. felineus* (Armignacco et al., 2008; Pozio et al., 2013). Consumers in some areas in South-Asia may prefer raw or undercooked fish products. As culinary trends are known to migrate through countries, it seems important to assess the risk of zoonoses by monitoring the occurrence of FZTs in wild and farmed fish throughout Europe. According to Yossepowitch et al. (2004) *O. felineus* metacercariae may under certain conditions survive smoking, freezing and marinating. It is therefore noteworthy that Sándor et al. (2020) showed that metacercariae of the non-zoonotic cyathocotylid species were highly vulnerable to different physical and chemical conditions, which frames the safety of Hungarian carp products.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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