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56 Abstract

Metacercariae of species of the genus *Apophallus* Lühe, 1909, infecting the fins and skin of freshwater fishes, frequently cause black spot disease. Two species, *Apophallus muehlingi* (Jägerskiöld, 1899) and *A. donicus* (Skrjabin & Lindtrop, 1919), are known to occur in Hungarian fishes. It has generally been thought that metacercariae of *A. muehlingi* infect cyprinid fishes, whereas those of *A. donicus* develop in percids. As part of a morphological, experimental and molecular study, metacercariae were collected from 99 infected specimens of five cyprinid hosts (*Abramis brama*, *Blicca bjoerkna*, *Chondrostoma nasus*, *Squalius cephalus*, *Scardinius erythrophthalmus*) and 18 infected specimens of two

		percid hosts (*Gymnocephalus cernua*, *Perca fluviatilis*) in Hungarian natural waters (Lake Balaton, River Danube). Moreover, 1024 common carp (*Cyprinus carpio*) specimens collected from Hungarian fish ponds were investigated for *Apophallus* infection, but without positive results. For reliable species identification, experimental infections of chicks were carried in order to produce adult specimens from metacercariae collected from the fins and skin of the cyprinid and percid hosts. Within 8 days, adult specimens of both *A. muehlingi* and *A. donicus* developed in chicks infected with metacercariae from the cyprinid common bream (*Abramis brama*) and the white bream (*Blicca bjoerkna*) and the ruffe (*Gymnocephalus cernua*), a percid, respectively. The morphology of the collected metacercariae and adult individuals developed in the feeding experiments was characterised. A molecular analysis was extended to cercarial samples from the snail *Lithoglyphus naticoides* and to a single adult specimen of *Apophallus* from a fox. Sequences of 28 specimens were analysed using molecular methods (sequencing the internal transcribed spacer region and the cytochrome oxidase I subunit). Phylogenetic analysis was executed, and the *Apophallus* samples clustered into three distinct branches using both genes, *A. muehlingi* from cyprinids, *A. donicus* from percids and, a third, previously unknown, *Apophallus* clade, also from cyprinids.
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ORIGINAL PAPER

An investigation of the host-specificity of metacercariae of species of *Apophallus* (Digenea: Heterophyidae) in freshwater fishes using morphological, experimental and molecular methods

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Abstract Metacercariae of species of the genus *Apophallus* Lühe, 1909, infecting the fins and skin of freshwater fishes, frequently cause black spot disease. Two species, *Apophallus muehlingi* (Jägerskiöld, 1899) and *A. donicus* (Skrjabin & Lindtrop, 1919), are known to occur in Hungarian fishes. It has generally been thought that metacercariae of *A. muehlingi* infect cyprinid fishes, whereas those of *A. donicus* develop in percids. As part of a morphological, experimental and molecular study, metacercariae were collected from 99 infected specimens of five cyprinid hosts (*Abramis brama*, *Blicca bjoerkna*, *Chondrostoma nasus*, *Squalius cephalus*, *Scardinius erythrophthalmus*) and 18 infected specimens of two percid hosts (*Gymnocephalus cernua*, *Perca fluviatilis*) in Hungarian natural waters (Lake Balaton, River Danube). Moreover, 1024 common carp (*Cyprinus carpio*) specimens collected from Hungarian fish ponds were investigated for *Apophallus* infection, but without positive results. For reliable species identification, experimental infections of chicks were carried in order to produce adult specimens from metacercariae collected from the fins and skin of the cyprinid and percid hosts. Within 8 days, adult specimens of both *A. muehlingi* and *A. donicus* developed in chicks infected with metacercariae from the cyprinid common bream (*Abramis brama*) and the white bream (*Blicca bjoerkna*) and the ruffe (*Gymnocephalus cernua*), a percid, respectively. The morphology of the collected metacercariae and adult individuals developed in the feeding experiments was characterised. A molecular analysis was extended to cercarial samples from the snail *Lithoglyphus naticoides* and to a single adult specimen of *Apophallus* from a fox. Sequences of 28 specimens were analysed using molecular methods (sequencing the internal transcribed spacer region and the cytochrome oxidase I subunit). Phylogenetic analysis was executed, and the *Apophallus* samples clustered into three distinct branches using both genes, *A. muehlingi* from cyprinids, *A. donicus* from percids and, a third, previously unknown, *Apophallus* clade, also from cyprinids.

Keywords Metacercariae · Black spot disease · *Apophallus* infections

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Introduction

Metacercariae of species of the genus *Apophallus* Lühe, 1909 (Digenea: Heterophyidae) are known to cause heavy infections in cyprinid, percid and salmonid fishes, which include deformities of the vertebral column (Kent et al. 2004), ectopic bone formation (Pike and Burt 1983; Taylor et al. 1993, 1994) and infections of the skeletal muscles (Cameron 1937, 1945; Rodnick et al. 2008; Ferguson et al. 2010, 2012). Its most common manifestation is black spot disease. The formation of black pigments around metacercariae and signs of black spot disease are known for several species of *Apophallus*, *Posthodiplostomum* and *Uvulifer* (Dönges 1964; Odening 1970, 1973; Quist et al. 2007; Tobler and Schlupp 2008). In the case of *Apophallus*, several species are known to cause such discolouration of tissues on the course of infections in fish. Of these, three species, *A. muehlingi* (Jägerskiöld, 1899)

in cyprinids (Odening 1970; Wierzbicka and Wierzbicki 1973), *A. donicus* (Skrjabin & Lindtrop, 1919) in percids (Odening 1973; Ivanov and Semenova 2004) and *A. brevis* (Ransom, 1920) in salmonids (Lyster 1940; Miller 1941, 1942), are the most often studied. Metacercariae of *Apophallus* spp. can develop anywhere in the fish body, but they most commonly infect the cartilaginous fin rays. At an early stage of infection, metacercariae are found as unpigmented cysts, after which melano-macrophage cells appear around the cyst wall, causing black spots on the fish body (Dönges 1964, 1967), which in most cases result in black spot disease. This disease was first described from Hungary by Molnár (1963), and its effects on Lake Balaton fishes were discussed by Molnár et al. (2001).

The host-specificity of the metacercariae has been a matter of debate. Generally, metacercariae of digeneans are considered to have a low host-specificity (Paperna 1995), but experimental (Hoffman 1958) and molecular (Locke et al. 2010) studies have shown that species of *Posthodiplostomum* and *Diplostomum* do exhibit some specificity, infecting only certain fish families or species. In the case of *Apophallus* spp., Odening (1973) suggested that they are host-specific to fishes at the family level, namely *A. muehlingi* infects cyprinids, whereas *A. donicus* occurs in percids. Several authors have shared this view (Bykhovskaya-Pavlovskaya 1962; Wierzbicka and Wierzbicki 1973). However, other authors have reported that *A. donicus* also infects cyprinid species (Yamaguti 1971; Bykhovskaya-Pavlovskaya and Kulakovskaya 1987; Vojtek 1989; Moravec 2001). It is generally accepted (Paperna 1995) that the host-specificity of the redial and cercarial developmental stages in molluscs is much more strict than that of metacercariae; however, heterophyids are reported to be more plastic in their affinity towards their snail intermediate hosts. For example, Niemi and Macy (1974) and Villeneuve et al. (2005) indicated that a species of *Fluminicola* (Lithoglyphidae) act as the first intermediate hosts for a species of *Apophallus*, and other *Apophallus* spp. have been shown by Malek (1980) to use species of *Juga* (Semisulcospiridae). These molluscan genera represent taxonomically very distinct clades of freshwater snails.

Odening (1970, 1973) first described the life cycle of both *A. muehlingi* and *A. donicus* in Germany. The most specific morphological characteristics of the cercariae of these species are the two rectangular black eyespots in the cephalic region and dorsoventral finfolds which run the length of the tail (Fig. 1). In both species, Odening found the rediae and cercariae in the gravel snail *Lithoglyphus naticoides* (Pfeiffer, 1828). Similarly, according to the checklist of Cichy et al. (2011), based on the findings of Chernogorenko (1977) and Mastitsky (2007), both species have been found in other parts of Europe (Ukraine and Belarus) in this same species of gravel snail. Furthermore, Izvekova and Tyutin (2011) and Tyutin and Izvekova (2013) have also indicated that *L. naticoides* is the first intermediate host of *A. muehlingi* in Russia, as has Vojtek (1989) in the former Czechoslovakia.

There is only one previous article, focusing on *Apophallus* spp., which involves experimental and molecular methods; while describing *Apophallus* cf. *microsoma* from North America and studying its development, Ferguson et al. (2012) carried out experimental infections of chicks with metacercariae and analysed the COI (cytochrome oxidase I subunit) sequences. Moreover, these metacercarial COI sequences were added to a phylogenetic analysis and were identified as *A. donicus.* Their material was collected from the cyprinids *Alburnoides bipunctatus* (Bloch, 1782) and *Rutilus rutilus* (L., 1758) caught in the Bega River, Romania.

The aim of our study was to use morphological, experimental and molecular methods to determine for certain whether *Apophallus* infections from cyprinid and percid fishes were caused by two distinct species, namely *A. muehlingi* and *A. donicus*. The collected metacercarial and cercarial samples were analysed based on sequences of the internal transcribed spacer (ITS) region and COI. Due to the difficulties in identifying metacercariae morphologically, experimental infections in chicks were used to obtain adult specimens for study. These, in association with the sequences of the ITS region and COI, enabled the linking of the different life history stages and facilitated confirmation of the identification of the metacercariae at the species level.

Materials and methods

Sample collection

During the present work, 99 black-spotted specimens out of 150 individuals of three cyprinid species, common bream *Abramis brama* (L., 1758), white bream *Blicca bjoerkna* (L., 1758) and rudd *Scardinius erythrophthalmus* (L., 1758), and 18 specimens out of 52 individuals of two percid species, ruffe *Gymnocephalus cernua* (L., 1758) and perch *Perca fluviatilis* (L., 1758), were collected from three regions of Lake Balaton in Hungary, namely Keszthely, Siófok and Tihany (Table 1). In addition, 23 nase *Chondrostoma nasus* (L., 1758) and 5 chub *Squalius cephalus* (L., 1758) were collected from the River Danube close to the city of Szentendre. Moreover, 1024 common carp (*Cyprinus carpio*) specimens collected from four geographically distinct Hungarian fish ponds (258 specimens by farm) were investigated for *Apophallus* infection. Fishes of various sizes were caught with a small, 15-m long, seine net and taken live to the laboratory in oxygenated plastic bags. The infected fishes were sedated by adding a few drops of clove oil to their water and were killed within a few days by a cervical cut. Metacercariae were collected either manually from the skin and fins or by using a digestive pepsin solution that contained 1 L of tap water, 1:10,000 NF pepsin

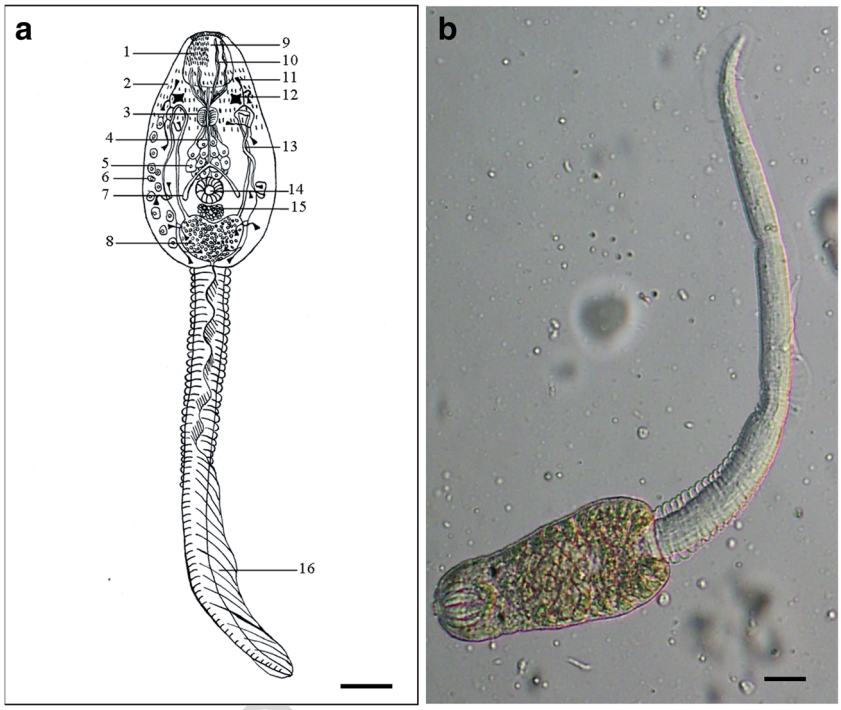

Fig. 1 **a** Diagrammatic illustration of an *Apophallus* cercaria based on the illustrations of Odening (1970) 1: oral sucker with spines, 2: body-spines, 3: pharynx, 4: oesophagus, 5: penetration gland-cells, 6: cystogenous glands, 7: caecum, 8: excretory bladder, 9: oral sucker, 10: canals of gland-cells, 11: flame-cells, 12: rectangular eye-spots, 13: protonephridial excretory system, 14: ventral sucker, 15: genital primordium, 16: tail with undulating fin-fold. Scale bar = 50 μm. **b** Micrograph of the pleurolophocercous cercaria of *Apophallus* sp. collected from a gravel snail (*Lithoglyphus naticoides*). Scale bar = 30 μm

powder (Molar Chemicals, Halásztelek, Hungary) and 8 mL 25% HCl. These ingredients were mixed in a 1 L beaker, after which the solution was heated on a magnetic stirrer to 37 °C. Excised black-spotted fins (Fig. 2) were immersed in the solution; after 20 min, the fins had dissolved and intact metacercariae were collected following filtration. They were examined under a dissecting microscope, studied morphologically and preserved for both molecular investigations in 70% ethanol and experimental infections in physiological saline solution. After the pepsin digestion, several *Apophallus* metacercariae were excysted from their capsules using a solution containing 50 mL distilled water, 2.5 g pancreatin and 0.25 g $NaHCO_3$ (Fried 1994). The excystment was carried out at 27 °C for 5–10 min, after which they were placed in physiological saline solution to avoid over-digestion.

Cercariae were collected from about 50 gravel snails *Lithoglyphus naticoides* from Lake Balaton at the city of Keszthely. The snails were placed in separate dishes for some days and, following water filtration or crushing the shell, released cercariae were examined under a microscope and preserved for molecular studies in 70% ethanol.

Due to the lack of a suitable final host, experiment infections of chicks were used to obtain the adult stages of *Apophallus muehlingi* and *A. donicus*. In addition, an adult *Apophallus* specimen was also examined which was derived from the small intestine of a fox that had been shot on the

Table 1 The average size of the examined fishes and the frequency of metacercaria infection

Species of fishes		Total number (*N*)	Average size of fishes in cm (min-max)	Infected	Non-infected	Average number of metacercariae (min-max)
Cyprinids	Common bream	42	14.8 (5.5–24)	23	19	38.2 (0–400)
	White bream	59	6.8 (4.5–16.5)	45	14	10.3 (0–200)
	Common nase	38	9.6 (7.5–11.5)	23	15	15 (0–95)
	Chub	7	10.3 (7–14)	5	2	13.9 (0–40)
	Rudd	5	7.5 (7–8)	3	1	1.5 (0–3)
	∑	150	–	99	51	–
Percids	Perch	14	5.3 (4–6.5)	6	8	4.4 (0–18)
	Ruffe	38	6.1 (4.8–7.5)	12	26	1.7 (0–18)
	∑	52	–	18	34	–

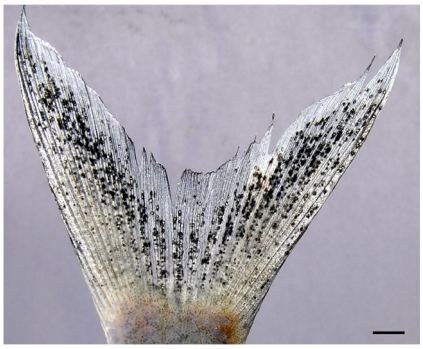

Fig. 2 Caudal fin of a common bream (*Abramis brama*) infected with *Apophallus* metacercariae, showing the typical appearance of black spot disease. Scale bar = 1 cm

course of obligatory culling of fox population by professional hunters in the Bakony Mountains in the Pannonia region of western Hungary (North of Balaton Lake).

Experimental infection

Two experimental infections of chicks were performed during 06–15 July 2015 and in 09–22 February 2016 (Table 2, 3). In the first case, seven chicks were fed with fins of common bream containing about 50 metacercariae, while three other chicks were infected with 50 metacercariae from a ruffe. The chicks had been purchased from a commercial supplier (Hegyhát BR Kft., Szentgotthárd-Rábafüzes, Hungary) and kept on a non-medicated chick starter diet. Formal ethical approval was given by the Government Office of Pest County (permit PEI/001/1004-4/2015). Chicks were killed with a cervical cut and their intestines studied under a Zeiss stereo microscope for trematode infections. In the second experiment, two chicks were each fed one by one with about 50 metacercariae from white bream, another chick was given metacercariae from common bream and three other chicks were each infected one by one with about 30 metacercariae collected from ruffe. In both experiments, five uninfected chicks served as controls.

In the first experiment, chicks infected with metacercariae from the common bream were sacrificed on days 2 (n = 1), 3 (n = 1), 4 (n = 1), 7 (n = 1), 8 (n = 1) and 9 (n = 2), whereas chicks infected with metacercariae from the ruffe were necropsied on days 3 (n = 1), 4 (n = 1) and 7 (n = 1) post-infection. In the second experiment, chicks infected with metacercariae from one common bream were sacrificed on day 8 (n = 1), whereas that fed with metacercariae from two white bream were necropsied on days 10 (n = 1) and 13 (n = 1) post-infection. Chicks infected with metacercariae from ruffe were necropsied on days 8 (n = 1) and 10 (n = 1) and on day 13 (n = 1). Specimens of *Apophallus* were collected from the duodenum of the chicks and were regarded as adults when they were ovigerous.

For the identification of the parasite species, the keys given by Morozov (1952), Odening (1970, 1973) and Niemi and Macy (1974) were used.

All of the collected developmental stages were investigated using a dissecting microscope and a Zeiss compound microscope. Fresh samples were photographed using an Olympus DP20 digital camera, and measurements (in micrometres) were taken from digitized images using IMAGO® software.

Molecular methods

For DNA extraction, samples preserved in 80% ethanol were centrifuged at 8000*g* for 5 min, after which the ethanol was removed. The DNA was extracted using a QIAGEN DNeasyTM 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 amplified via a nested PCR. The primers S18 (5′-TAACAGGTCTGTGATGCC-3′) and L3T (5′-CAAC TTTCCCTCACGGTACTTG-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), 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 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 was finished with a terminal extension at 72 °C for 5 min, then stored at 4 °C. The primers D1 (5′-AGGAA-TTCCTGGTAAGTGCAA-3′) and D2 (5′-CGTTACTGAGGGAATCCTGGT-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), 0.5 μL of each primer, 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. The COI was amplified via a semi-nested PCR using the primers by Van Steenkiste et al. (2015), Dice1F and Dice14R in the first round and Dice1F and Dice11R in the second round. The reaction condition and thermal profile were set as recommended by van Steenkiste et al. (2015). In the case of some samples, the PCR reaction did not yield sufficient PCR products; therefore, selective COI primers were designed for *Apophallus* samples using Primer3Plus (Untergasser et al. 2007): Apom1f (5′-GATGATTTATATGGTTTTAGGTTTGTG-3′) and Apom1r (5′-CGATCAAAAAGCAA-CATAGTAATCC-3′). The reaction mixture was the same as applied for the ITS PCR and the thermal conditions were as follows: initial denaturation step of 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 49 °C

Q4 **Table 2** The results of the first chick infections (06 July 2015–15 July 2015)

Intermediate host of metacercariae	Beginning of the infection	Date of chick dissection	Number of elapsed days after infection	Number of sacrificed chicks	Number of adult *Apophallus* specimens recovered
1. Bream	06 July 2015	08 July 2015	2	1	0
2. Bream	06 July 2015	09 July 2015	3	1	1
3. Bream	06 July 2015	10 July 2015	4	1	1
4. Bream	06 July 2015	13 July 2015	7	1	10
5. Bream	06 July 2015	14 July 2015	8	1	2
6. Bream	06 July 2015	15 July 2015	9	2	1
7. Bream	06 July 2015	15 July 2015	9		0
Total *Apophallus muehlingi* specimens from chick infections 15					
1. Ruffe	06 July 2015	09 July 2015	3	1	1
2. Ruffe	06 July 2015	10 July 2015	4	1	2
3. Ruffe	06 July 2015	13 July 2015	7	1	2
Total *Apophallus donicus* specimens from chick infections 5					

for 30 s and 72 °C for 45 s, and finished with a terminal extension at 72 °C for 7 min.

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 ITS region and COI were sequenced with the PCR primers and with two additional inner primers 5.8Sr (5′-TGTCGATGAAGAGCGCAGC-3′) and 5.8S2 (5′-TAAGCCGACCCTCGGACAGG-3′) (Tkach et al. 2000) in the case of ITS region. ABI BigDye Terminator v3.1 Cycle Sequencing Kit was used for sequencing, and the sequences read using an ABI 3100 Genetic Analyser.

Phylogenetic analysis

The sequenced fragments were assembled using MEGA 6.06 (Tamura et al. 2013) and ambiguous bases clarified using corresponding ABI chromatograms. Nucleotide sequences were aligned with the software CLUSTAL W (Thompson et al. 1994). The two alignments (ITS region and COI) were corrected manually using the alignment editor of the software MEGA 6.06. Alignments were also corrected with GBlocks (Castresana 2000) to eliminate poorly aligned positions and divergent regions. Sequences were deposited in the GenBank under the accession numbers (MF438049-101 and MF447672). DNA pairwise distances were calculated with the MEGA 6.06 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 4. *Fasciola gigantica* (KX198618 and GQ398050) was chosen as the outgroup for both genes. The dataset was tested using MEGA 6.06 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 6.06 under the GTR + G + I model

Table 3 The result of the second chick infections (09 February 2016–17 February 2016)

Intermediate host of metacercariae	Beginning of the infection	Date of chick dissection	Numbers of elapsed days after infection	Numbers of sacrificed chicks	Number of adult *Apophallus* specimens recovered
1. Bream	09 February 2016	17 February 2016	8	1	0
2. White bream	09 February 2016	19 February 2016	10	1	1
3. White bream	09 February 2016	22 February 2016	13	1	0
Total *Apophallus muehlingi* specimens from chick infections 1					
1. Ruffe	09 February 2016	17 February 2016	8	1	25
2. Ruffe	09 February 2016	19 February 2016	10	1	10
3. Ruffe	09 February 2016	22 February 2016	13	1	2
Total *Apophallus donicus* specimens from chick infections 37					

Table 4 List of the sequenced cercarial, metacercarial and adult samples of *Apophallus* spp.

	Sample	Morphological identification	Host	Developmental stage	Date of collection	Site of collection	ITS sequence	COI sequence
1.	CC	*Apophallus* sp.	Gravel snail (*Lithoglyphus naticoides*)	Cercaria	21 May 2009	Lake Balaton (Keszthely)	MF438062	MF438088
2.	C45	*Apophallus* sp.	Gravel snail (*Lithoglyphus naticoides*)		13 November 2013	Lake Balaton (Tihany)	MF438049	No data
3.	AD3	*Apophallus* sp.	Chub (*Squalius cephalus*)	Metacercaria	13 August 2015	River Danube (Szentendre)	MF438050	MF438076
4.	AD4	*Apophallus* sp.	Chub (*Squalius cephalus*)		13 August 2015	River Danube (Szentendre)	MF438051	MF438077
5.	AP2	*Apophallus* sp.	Ruffe (*Gymnocephalus cernua*)		24 March 2015	Lake Balaton (Siófok)	MF438055	MF438081
6.	AP3	*Apophallus* sp.	Perch (*Perca fluviatilis*)		24 March 2015	Lake Balaton (Siófok)	MF438056	MF438082
7.	AP4	*Apophallus* sp.	Perch (*Perca fluviatilis*)		24 March 2015	Lake Balaton (Siófok)	MF438057	MF438083
8.	AP5	*Apophallus* sp.	Perch (*Perca fluviatilis*)		24 March 2015	Lake Balaton (Siófok)	MF438058	MF438084
9.	DK1	*Apophallus* sp.	Common bream (*Abramis brama*)		30 June 2015	Lake Balaton (Keszthely)	MF438064	MF438090
10.	DK3	*Apophallus* sp.	Common bream (*Abramis brama*)		30 June 2015	Lake Balaton (Keszthely)	MF438065	MF438091
11.	KK5	*Apophallus* sp.	White bream (*Blicca bjoerkna*)		30 June 2015	River Danube (Keszthely)	MF438066	MF438092
12.	MK8	*Apophallus* sp.	Perch (*Perca fluviatilis*)		18 June 2014	River Danube (Szentendre)	MF438067	MF438093
13.	MK9	*Apophallus* sp.	Common bream (*Abramis brama*)		01 December 2014	Lake Balaton (Siófok)	MF438068	MF438094
14.	MK11	*Apophallus* sp.	Common bream (*Abramis brama*)		01 December 2014	Lake Balaton (Siófok)	MF438069	MF438095
15.	MK15	*Apophallus* sp.	Perch (*Perca fluviatilis*)		19 March 2014	Lake Balaton (Siófok)	MF447672	MF438096
16.	MK21	*Apophallus* sp.	Ruffe (*Gymnocephalus cernua*)		12 March 2015	Lake Balaton (Siófok)	MF438070	No data
17.	MK23	*Apophallus* sp.	Perch (*Perca fluviatilis*)		12 March 2015	Lake Balaton (Siófok)	MF438071	MF438097
18.	PU2	*Apophallus* sp.	Nase (*Chondrostoma nasus*)		12 May 201	River Danube (Szentendre)	MF438072	MF438098
19.	PU3	*Apophallus* sp.	Nase (*Chondrostoma nasus*)		12 May 2016	River Danube (Szentendre)	MF438073	MF438099
20.	VK1	*Apophallus* sp.	Rudd (*Scardinius erythrophthalmus*)		17 February 2016	Lake Balaton (Siófok)	MF438075	MF438101
21.	AM1	*Apophallus muehlingi*	Chick (1. infection)	Adult	16 July 2015	Infection (06 July 2015)	MF438052	MF438078
22.	AM2	*Apophallus muehlingi*	Chick (1. infection)		16 July 2015	Infection (06 July 2015)	MF438053	MF438079
23.	AM3	*Apophallus muehlingi*	Chick (1. infection)		16 July 2015	Infection (06 July 2015)	MF438054	MF438080
24.	AV1	*Apophallus donicus*	Chick (2. infection)		17 February 2016	Infection (09 February 2016)	MF438059	MF438085
25.	AV2	*Apophallus donicus*	Chick (2. infection)		17 February 2016	Infection (09 February 2016)	MF438060	MF438086
26.	AV3	*Apophallus donicus*	Chick (2. infection)		17 February 2016	Infection (09 February 2016)	MF438061	MF438087
27.	DA1	*Apophallus donicus*	Chick (1. infection)		16 July 2015	Infection (06 July 2015)	MF438063	MF438089
28.	RK2	*Apophallus* sp.	Red fox (*Vulpes vulpes*)		April 2015	Tata	MF438074	MF438100

for the ITS region and GTR + G 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 6.06.

Results

Heterophyid larval stages (cercaria, metacercaria) collected from snails and fish could reasonably be identified to the generic level as *Apophallus*, but the two species *A. muehlingi* and *A. donicus* could not be differentiated based solely on morphology. In other words, pleurolophocercous cercariae (sensu Seymour Sewell 1922) found in the gravel snail *Lithoglyphus naticoides* were identified as heterophyid-like based on their two rectangular black eyespots in the cephalic region and the dorsoventral finfolds which extend along the tail (Fig. 1), as described by Odening (1970) for *A. muehlingi* and by Odening (1973) and Niemi and Macy 1974for *A. donicus.* In the case of the metacercariae, encystment in pancreatin solution resulted access to free specimens whose anatomy could be studied in physiological saline solution. Their morphological characteristics and the size of their organs (Fig. 3) corresponded with those of *Apophallus* metacercariae described by Odening (1970, 1973) and Mödlinger (1934).

Metacercarie were found in higher proportion of the investigated cyprinid (99/150) and percid fish species (18/34) in the natural fresh waters in great number (Table 1); however, all the analysed carp specimens were uninfected by *Apophallus* metacercariae.

In the first infection experiment of seven chicks, 15 adult worms (Fig. 4) and developing individuals of *A. muehlingi* were found in five of the chicks (Table 2), whereas in the second experiment (Table 3), only a single adult *A. muehlingi* was collected from the three infected chicks. In the case of those chicks in the first experiment which were infected with metacercariae from ruffe, five adult *A. donicus* (Fig. 4) were recovered from three chicks, whereas in the second experiment, 37 adult individual of *A. donicus* were found in three chicks. Specimens found in chicks 8 days after infections were regarded mature when they were ovigerous. The morphology and measurements of mature specimens of the two *Apophallus* species corresponded with the morphological data for these species presented by Morozov (1952) and Odening (1970, 1973).

Twenty-eight *Apophallus* samples were analysed for the ITS region and COI genes, including cercarial, metacercarial and adult developmental stages (Table 3). The amplified ITS region (with additional parts of the 18S rDNA and 28S rDNA) of the samples was more than 1500 bps, with the alignment being 1493 bps long, after removing poorly aligned positions and divergent regions, and containing 954 conservative and 539 variable (336 of them parsimony-informative) sites. The COI fragments exceeded 500 bps, and the alignment consisted of 528 bps, including 257 conservative and 271 variable (213 of them parsimony-informative) sites. The sequences of the ITS region and COI genes of this material corresponded with the results from the morphological and experimental studies. In the case of both genes, the various developmental stages of *Apophallus* (cercaria, metacercaria, adult) were located in a monophyletic branch, with strong bootstrap support (Fig. 5), and were subdivided into three groups. Sequences of metacercariae collected from the bream (Cyprinidae), cercariae collected from gravel snails in Lake Balaton and the adult specimen from a fox resulted an identical pattern with adult *A. muehlingi* samples developed in the gut of experimental chicks (Fig. 5, Table 4). Therefore, those metacercariae from cyprinid fishes and cercariae form *Litoglyphus naticoides* could be unambiguously identified as *A. muehlingi*. On the other hand, sequences of metacercariae collected from perch and ruffe (Percidae) were identical with the adult stages of *A. donicus* which developed in chicks from metacercariae taken from ruffe (Fig. 5, Table 4). Surprisingly, a third branch of *Apophallus* sequences was also present; these came from metacercariae taken from chub and nase in the River Danube and from a rudd in Lake Balaton (all Cyprinidae).

The pairwise distances of the ITS region indicated clear differences between the three groups; the overall mean distance between *A. muehlingi* and *A. donicus* was 2.0%, whereas the third unknown group exhibited 2.8 and 3.1% distances from *A. muehlingi* and *A. donicus*, respectively. The mean distances within groups had a much lower value, i.e. 0.5% in *A. muehlingi* and 0.1% in both *A. donicus* and *Apophallus* sp.

The pairwise distances resulted in higher values in the case of the COI due to its greater variability. The mean distance between *A. muehlingi* and *A. donicus* was 20.6%. The third *Apophallus* group included the five metacercarial samples collected by ourselves and *A. donicus* samples (JQ241154-58) from Romania deposited in GenBank by Ferguson et al. (2012) (Fig. 5). Its distance from *A. muehlingi* was 18.2% and from *A. doncius* 13.5%. The distances within the groups were very low, being 0.4% for *A. muehlingi*, 0.2% for *A. donicus* and 0.2% for *Apophallus* sp., which indicates the homogeneity of the three species.

North American *Apophallus* spp. in GenBank also showed a clear divergence from our analysed samples (Fig. 5). *Apophallus brevis* Ransom, 1920 (JQ241151-53) was distinguishable from *A. muehlingi* by 21.7%, from *A. donicus* by 12.8% and from *Apophallus* sp. by 16.9%. The sole sequence of *A. microsoma* Ferguson et al. 2012 (JQ241159) differed by 18.8, 11.8 and 14.2%, and an unidentified *Apophallus* sp. (KM538077) by 16.0, 11.9 and 16.3%, respectively.

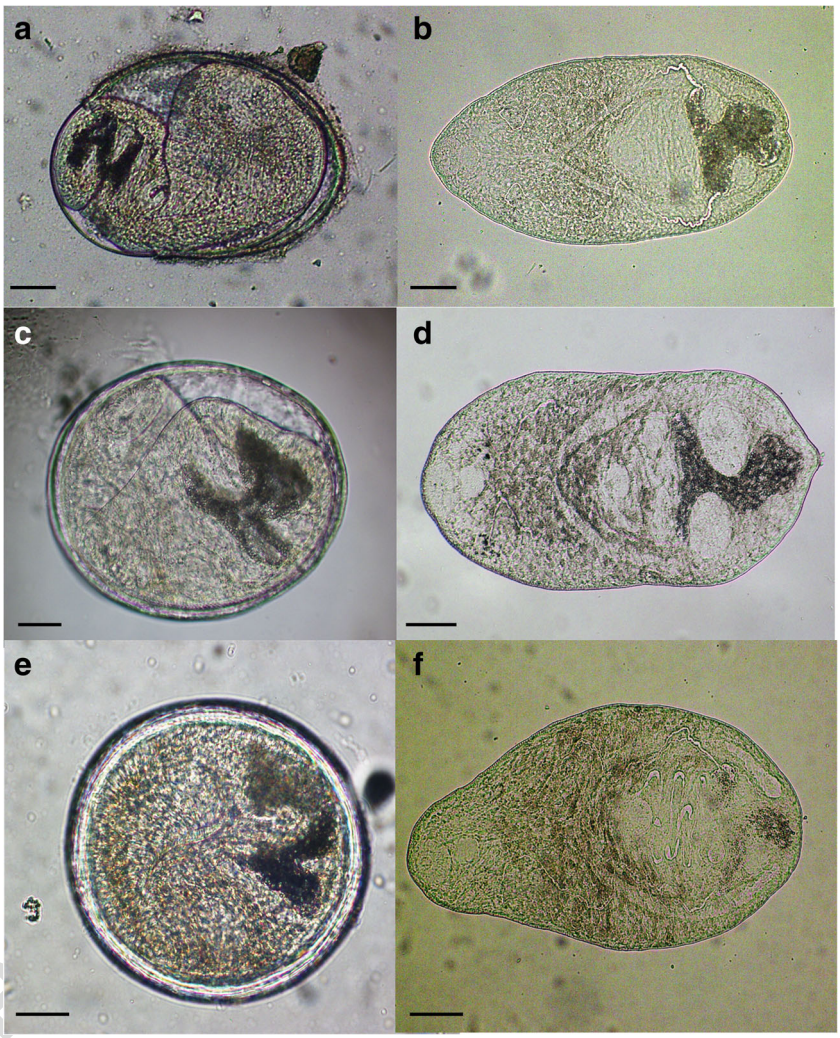

Fig. 3 Encysted and excysted metacercariae of *Apophallus muehlingi* (**a**, **b**), *Apophallus donicus* (**c**, **d**) and *Apophallus* sp. (**e**, **f**) showing dark excretory, y-shaped, vesicle, isolated from the skin of common bream (*Abramis brama*), perch (*Perca fluviatilis*) and rudd (*Scardinius erythrophthalmus*). Scale bar = 40 μm

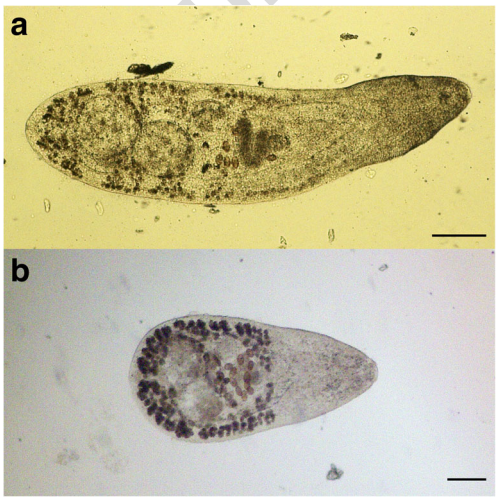

Fig. 4 Micrographs of adults of *Apophallus muehlingi* (**a**) and *A. donicus* (**b**) collected from the guts of experimentally infected chicks at post-mortem. Scale bar = 100 μm

Discussion

Pleurolophocercous cercariae found in the gravel snail *Lithoglyphus naticoides* agreed with the morphological characteristics of heterophyid cercariae described by Seymour Sewell (1922) and were identified as *A. muehlingi* by the sequences of their ITS region and COI. These results are in agreement with the conclusions of previous studies (Odening 1970, 1973; Chernogorenko 1977; Izvekova and Tyutin 2011; Tyutin and Izvekova 2013) and conclusively confirm *Lithoglyphus naticoides* as the first intermediate host of this species. Unfortunately, cercariae of *A. donicus* were not found during our investigation, with the result that further sampling and sequencing is necessary to support the results of Odening (1973) and Mastitsky (2007).

It can be observed that *Apophallus* metacercariae were found in abundance in the natural fresh waters both in cyprinid and percid fishes; however, the investigated Hungarian carp farms showed no infection at all. It can be assumed that the significant infection in the natural waters are caused by the

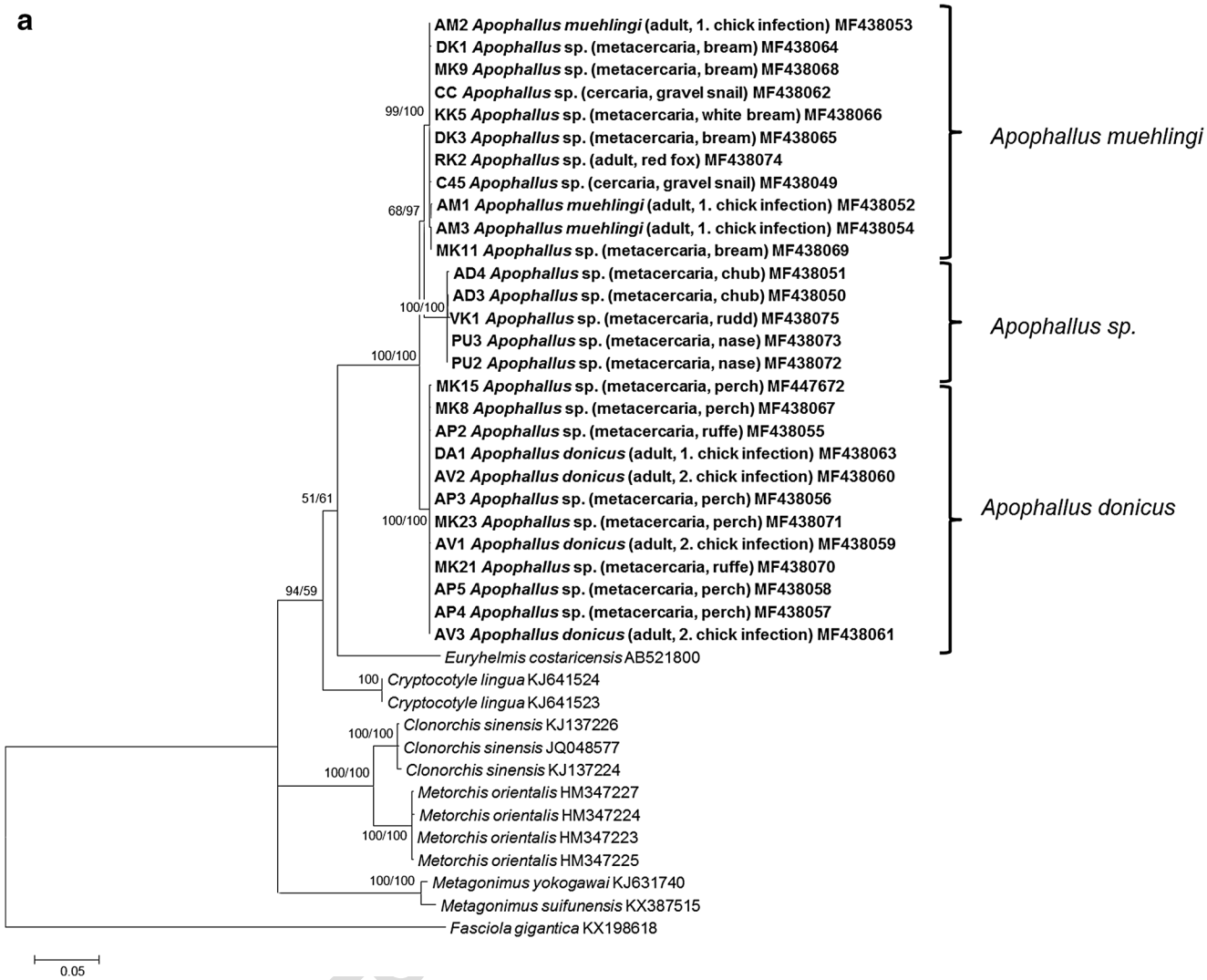

Fig. 5 Maximum likelihood tree of the samples of *Apophallus* spp. from the present study (**a** ITS region, **b** COI) in relation to other heterophyid and opistorchiid sequences deposited in GenBank. Bootstrap values are given at the nodes; posterior probabilities for Bayesian inference are shown behind the bootstrap values. Samples from the present study are in bold. The scale bar indicates the expected number of substitutions per site

presence of *Lithoglyphus naticoides* as well as wild living water birds also inhabit these areas; therefore, every circumstance is available for the trematodes to finish their life cycles. However, the applied farming methods might influence the snail fauna in the fish ponds causing the lack of the first intermediate host and the prevention of spreading the infection.

Generally, our studies on *Apophallus* metacercariae in Hungarian fishes support Odening's (1973) hypothesis that metacercariae of *A. muehlingi* infect cyprinid fishes, whereas those of *A. donicus* infect percid fishes. Experimental infections of day-old chicks resulted in the development of the adult stage of *A. muehlingi* from metacercariae taken from bream and white bream (Cyprinidae), whereas typical adults of *A. donicus* developed from metacercariae taken from ruffe (Percidae). Sequence data of adult, metacercarial and cercarial life history stages of *Apophallus* specimens corresponded with the morphological and experimental findings. These data, supported by both genes, clearly showed that sequences of metacercariae developing in the two bream species were identical with the sequences of adult specimens corresponding morphologically with *A. muehlingi*, as characterised by Odening (1970, 1973), whereas sequences of metacercariae from the percids ruffe and perch corresponded with adults of *A. donicus,* as described by Morozov (1952) and Odening (1973).

Unexpectedly, our molecular studies also revealed a third species of *Apophallus*. Samples of metacercariae from chub, nase and rudd resulted in a well-defined phylogenetic clade; this was indicated by both genes as being distinct from both *A. muehlingi* and *A. donicus*, with bootstrap values and pairwise distances strongly supporting its phylogenetic position. Since all of the three groups were placed in a monophyletic clade, it can be assumed that a third *Apophallus* taxon exists in central

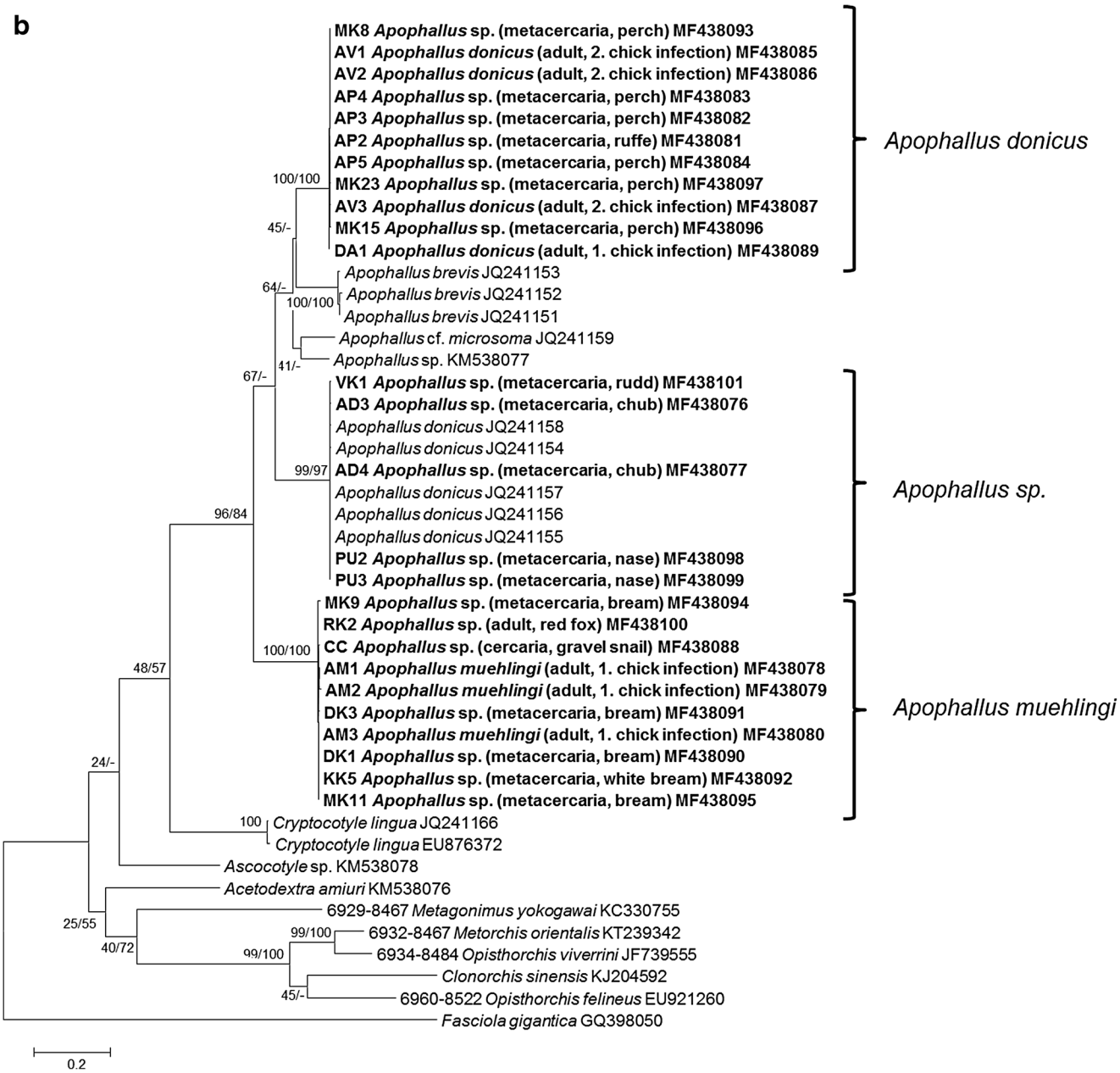

Fig. 5 continued.

Europe and likely represents a previously unknown and undescribed species. However, only metacercariae of this species have been found, and these lack the morphological characteristics required for the erection of new species. It is hoped that adult worms can be cultured by experimental infection and fully described in a near future.

Interestingly, the metacercarial samples of the third species were grouped together with the *A. donicus* sequences of Ferguson et al. (2012), whereas our *A. donicus* samples were positioned on a different branch. This incongruity can be resolved by accepting that the '*A. donicus*' metacercariae from Romania acquired by Ferguson et al. (2012) represent specimens of the previously unknown species of *Apophallus*. This notion is supported by the fact that these samples would have lacked unambiguous morphological characteristics which would have aided identification and that their host species were cyprinids, roach *Rutilus rutilus* and schneider *Alburnoides bipunctatus*, rather than generally accepted percids. On the other hand, *A. donicus* metacercariae in the present study originated from the percids perch *Perca fluviatilis* and ruffe *Gymnocephalus cernua*, and adult specimens from chick infections also exhibited characteristics of *A. donicus*, as indicated in the keys by Morozov (1952), Odening (1973) and Niemi and Macy (1974). There is, therefore, clear evidence that morphologically indistinguishable, or almost indistinguishable, metacercariae can belong to different

species (Galazzo et al. 2002; Locke et al. 2010; Cech et al. 2017). Consequently, the identification of metacercariae based solely on morphological grounds should be treated with caution, and it is advisable to have either supporting sequence data or morphological characteristics from adult individuals directly linked to the metacercariae under investigation.

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AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES.

Q1. The abbreviation "ITS" was expanded to "internal transcribed spacer". Please check if the provided expansion is correct.
Q2. Please check if the provided keywords are correct; otherwise, please amend.
Q3. The citation “Bykhovskaya-Pavlovskaya et al. 1962” has been changed to “Bykhovskaya-Pavlovskaya, 1962” to match the author name/date in the reference list. Please check if the change is fine in this occurrence and modify the subsequent occurrences, if necessary.
Q4. Please check if the presentation of dates is correct.
Q5. The sentence "It can be assumed, that the significant infection..." was modified for clarity. Please check if the intended meaning is retained.
Q6. Please provide complete bibliographic details of this reference.