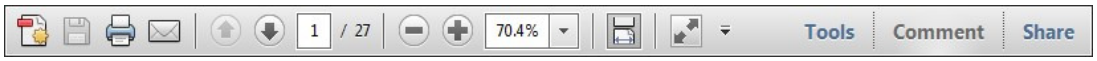
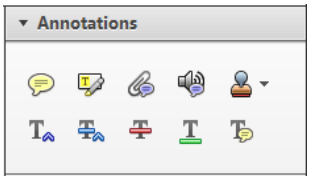
Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

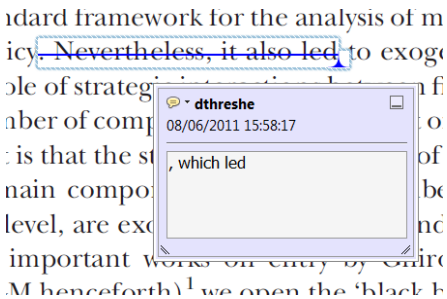


1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

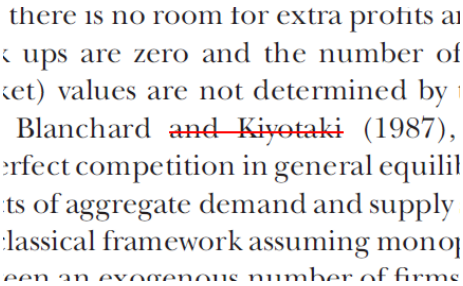


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

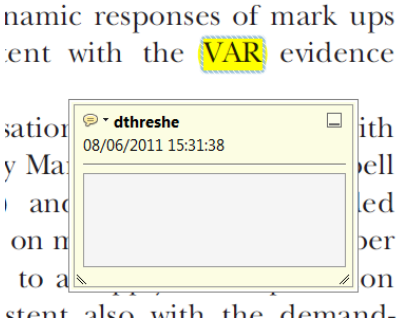


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

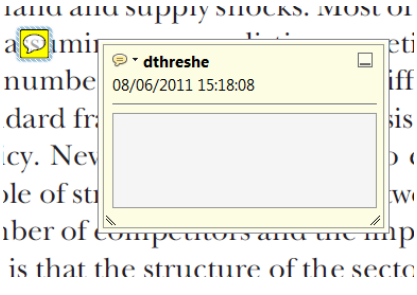


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.

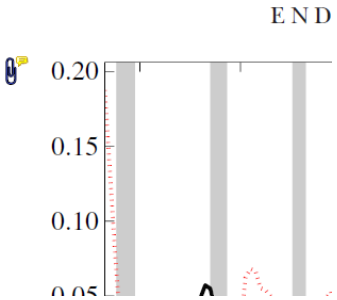


5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate place in the text.

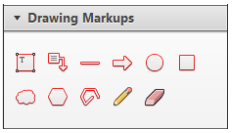
How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.



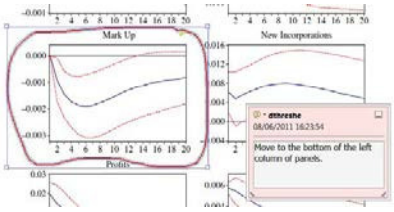
6. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks.



How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.



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Malformations of the gill filaments of the ruffe *Gymnocephalus cernuus* (L.) (Pisces) caused by echinostomatid metacercariae

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Abstract

In parasite surveys of fishes from Lake Balaton and its tributaries in Hungary, infections with metacercariae of a species of the digenean genus *Echinochasmus* (Trematoda: Echinostomatidae) were found in seven species of fish. In ruffe, *Gymnocephalus cernuus,* malformations of the gill filaments apparently caused by these infections were observed. These malformations were in the form of bifurcations of the filaments at about their mid-length. At the point where the filaments bifurcate, an *Echinochasmus* metacercaria was always embedded in the cartilaginous ray of the gill filament. All specimens of the ruffe were found to be infected by these metacercariae, and each ruffe specimen was infected by 30–300 metacercariae. Such a bifurcation was found in all of the ruffe specimens, but, apart from these gill malformations, the metacercariae produced only local changes in the cartilage. In the other six infected fish species, only local signs were observed in the cartilage. Experimental infections of chicks with metacercariae resulted in the finding of the sexual adult (marita) of an unidentified species of *Echinochasmus*. ITS sequences of the adult and metacercaria corresponded with each other, and also with a cercaria isolated from a gravel snail (*Lithoglyphus naticoides*), with a 99.5–100% similarity.

Keywords: Digenea, gill filaments, *Gymnocephalus cernuus*, ITS region, malformation.

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Introduction

During regular veterinary surveys of fishes in Lake Balaton (46°50′N; 17°44′E) and its tributaries in Hungary, our research group has been collecting data on their parasitic fauna for many years. During these surveys, several hundreds of Lake Balaton fish were caught as part of a general parasitological study (Molnár & Székely 1995, 1998, 1998; Molnár & Székely 2003; Molnár *et al.* 2001, 2002) and several records of the occurrence of *Echinochasmus* metacercariae in the gills of some of these fish were recorded. After finding malformations in a ruffe, *Gymnocephalus cernuus* (L.), which appeared to have been caused by an *Echinochasmus* infection, during the spring of 2014, a special research project was initiated.

Metacercariae of species of the digenean genus *Echinochasmus* Dietz, 1909 (Echinostomatidae) are common parasites of the gills of fishes (Skrjabin & Bashkirova 1956; Hoffman 1999; Kostadinova 2005). They frequently infect a series of freshwater fishes. Most papers written on *Echinochasmus* spp. infections deal with their occurrence (e.g. Violante-Gonzalez, Aguirre-Macedo & Vidal-Martinez 2008; Brock & Font 2009; Mierzejewska

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7 *et al.* 2012), with their life cycle (e.g. Besprozvannykh 1991; Scholz *et al.* 1994, 1995; Ditrich, Scholz & Vargas-Vazquez 1996; Scholz, Ditrich & Vargas-Vazquez 1996; Choi *et al.* 2006) and with their possible zoonotic role (e.g. Chai & Lee 2002; Chai *et al.* 2009; Sayasone *et al.* 2009; Sohn *et al.* 2009).

Little is known of the pathological effects of *Echinochasmus* spp. on the gills, although Bass & Weis (2009) reported that a heavy infection with *Ascocotyle* and *Echinochasmus* metacercariae caused a conspicuous change in the behaviour of *Fundulus heteroclitus* (L.). The pathogenic effect of other fish parasites on their host is, however, well documented, and cases are reported of parasitic infections causing malformations of different organs. The best known example is whirling disease in trout caused by the myxosporean *Myxobolus cerebralis* (Hofer, 1903), which commonly causes a distortion of the vertebral column (Hedrick *et al.* 1998). Vertebral deformities are also reported as being caused by metacercariae of *Apophallus* sp. in northern pikeminnows, *Ptychocheilus oregonensis* (Richardson), and chiselmouths, *Acrocheilus alutaceus* Agassiz & Pickering (Kent *et al.* 2004). It is also known that *Diplostomum* metacercariae can cause a herniation of the lens in catfishes (Larson 1965). A distinctive malformation has been described for a metacercarial infection of amphibians. Metacercariae of *Ribeiroia ondatrae* (Price, 1931) cause malformations in the limbs of leopard frogs. These are manifest, among other signs, in the form of polymely (duplication of limbs) or polydactyly (duplication of digits) in the Pacific chorus frog, *Pseudacris regilla* (Baird & Girard), and northern leopard frog, *Rana pipiens* Schreber (e.g. Johnson *et al.* 2002; Schotthoefer *et al.* 2003; Goodman & Johnson 2011). The pathogenic effect of *R. ondatrae* has also been documented in long-toed salamanders, *Ambystoma macrodactylum* Baird, by Johnson *et al.* (2006). Similar deformities have been produced experimentally in tadpoles of the common hourglass tree frog, *Polypedates cruciger* Blyth, by Rajakaruna *et al.* (2008) and Jayawardena *et al.* (2010). Kelly *et al.* (2010) also reported spinal malformations in a New Zealand fish, *Galaxias anomalus* Stokell, after infecting it with cercariae of the trematode *Telogaster opisthorchis* MacFarlane, 1945.

In this paper, our aims are to report malformations in the gills of ruffe, *Gymnocephalus cernuus* (L.), where the gill filaments have been caused to bifurcate at the encystment site of a digenean metacercaria, and to attempt an identification of this pathogen. Experimental infections in chicks are used to obtain adult specimens of the parasite, and the sequences of the ITS region are employed to link life-history stages and help confirm the identification of the metacercaria at the generic level.

Materials and methods

The survey took place between 15 April 2014 and 15 August 2015 in the south-western part of Lake Balton, during which only the gills of the examined fish were checked for metacercarial infection. In addition to 47 ruffe, 10 specimens each of roach, *Rutilus rutilus* (L.), white bream, *Abramis bjoerkna* (L.), common bream, *Abramis brama* (L.), bleak, *Alburnus alburnus* (L.), stone moroko, *Pseudorasbora parva* (Temminck et Schlegel) (all Cyprinidae), river goby, *Neogobius fluviatilis* (Pallas) (Gobiidae), and perch, *Perca fluviatilis* (L.) (Percidae), were examined. Of the less common fishes from the lake, the gills of seven pumpkin seeds, *Lepomis gibbosus* (L.) (Centrarchidae), and two tench, *Tinca tinca* (L.) (Cyprinidae), were also checked for metacercarial infection. In addition to fishes from Lake Balaton, five Chinese sleepers, *Percottus glehnii* Dybowski (Odontobutidae), and two mudfishes, *Umbra krameri* Walbaum (Umbridae), were studied for metacercarial infections from a tributary of the River Zala close to its entry into the lake.

Various sized fishes of these species were caught using a small seine net. They were carried to the laboratory alive in oxygenated plastic bags and held in aerated aquaria for several days. The fish were then sedated by adding a few drops of clove oil to their water (this dose represents an effective, practically non-toxic, empirically tested drug). The fish were killed with a cervical cut and subjected to a complete parasitological examination. Samples from different organs were examined under a dissecting microscope and the results recorded. In cases where a rare or unidentified parasite species was found, a more detailed examination under a compound microscope was undertaken. After making a complete parasitological investigation of the first five ruffe specimens, examinations were restricted to the gills. Pieces of gill filaments found to be infected with metacercariae were placed on a slide using a glass pipette, covered with a coverslip

and slightly compressed. They were then studied under a Zeiss compound microscope. Metacercariae were photographed with an Olympus DP10 digital camera, and measurements were taken from digitized images using IMAGO® software. As we were unable to excyst the metacercariae from their cartilaginous capsules, an experimental infection was undertaken in which 10 one-day-old chicks were force-fed with gill tissues containing about 100 metacercariae per chick. These chicks had been purchased from a commercial supplier (Hegyhát BR Kft.) and fed on a non-medicated chick starter diet. Formal ethical approval was given by the Pest Megyei Kormányhivatal (permit PEI/001/1004-4/2015). Five chicks served as a control. After infection, on each consecutive day, a chick was killed by neck dislocation and its intestine studied for trematode infections. For the identification of the parasite species, the keys given by Skrjabin & Bashkirova (1956) and Faltynková, Gibson & Kostadinova (2008) were used. For molecular studies, pieces of the gills of four ruffes and a stone moroko infected with at least 20 metacercariae were collected in Eppendorf tubes containing 70% alcohol (Table 1). In addition, four specimens of adult *Echinochasmus* specimens from the experimental chicks were studied. Molecular studies were extended to include a sample of cercariae collected from the snail *Lithoglyphus naticoides* (Pfeiffer) and identified tentatively as *Echinochasmus* sp. Tissue samples from the infected gill hemibranchs exhibiting the unusual bifurcations of the filaments were fixed in Bouin's solution, embedded in paraffin wax, sectioned at 4–5 μm and stained with haematoxylin and eosin. Ten ruffe specimens were selected for estimating the rate of bifurcation in the gill filaments and the number of encysted metacercariae and malformations counted.

For DNA extraction, samples preserved in 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) 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′-CAACTTTCCCTCACGGTAC TTG-3′) (Jousson, Bartoli & Pawlowski 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), 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 and then stored at 4 °C. The primers D1 (5′-AGG AATTCCTGGTAAG-TGCAA-3′) and D2 (5′-CGT TAC TGA GGG AAT CCT GGT-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 and 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.). Purified PCR products were sequenced with the PCR primers D1 and D2 and with two additional inner primers 5.8Sr (5′-TGTCGATGAAGAGCGCAGC-3′) and 5.8S2 (5′-TAAGCCGACCCTCGGA-CAGG-3′) (Tkach *et al.* 2000) using an ABI BigDye Terminator v3.1 Cycle Sequencing Kit with an ABI 3100 Genetic Analyser.

The sequence 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, Higgins & Gibson 1994). The alignment was corrected manually using the alignment editor of the software MEGA 6.06. Sequences were deposited in the GenBank under the accession numbers KT989660-KT989667. 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. The samples examined are listed in Table 1. *Diplostomum spathaceum* (Rudolphi, 1819) was chosen as the outgroup. The data set was tested using MEGA 6.06 for the

Table 1 Xxxxx

Sample ID	Developmental stage	Host	Collection site	Collection date	GenBank accession number
SD44	Cercaria	Gravel snail (*Lithoglyphus naticoides*)	Keszthely	2014.viii.06	KT989660
MK5	Metacercaria	Ruffe (*Gymnocephalus cernuus*)	Keszthely	2014.xii.01	KT989661
MK7	Metacercaria	Ruffe	Keszthely	2014.viii.07	KT989662
MK16	Metacercaria	Stone moroko (*Pseudorasbora parva*)	Zala tributary	2013.vii.29	KT989663
AE1	Adult	Ruffe (host of metacercaria); chicks (host of adult)	Keszthely	2015.vi.30 (metacercariae)	KT989664
AE2	Adult			2015.vii.06 (infection)	KT989665
AE3	Adult			2015.vii.16 (adults)	KT989666
AE4	Adult				KT989667

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. Bootstrap values based on 1000 resampled data sets were generated. BI was computed by Topali 2.5 (Milne *et al.* 2004). The likelihood parameters for BI were based on the GTR + G model. Posterior probabilities (PP) were estimated over 1 000 000 generations via two independent runs of four simultaneous MCMCMC chains, with every 100th tree saved. The first 25% of the sampled trees were discarded as 'burn-in'. The ML tree was visualized using the tree explorer of MEGA 6.06.

Results

During the course of general surveys carried out between 1995 and 2014, metacercariae of a species of *Echinochasmus* were occasionally found in fishes from Lake Balaton (our unpublished data). In 2014–2015, when a special survey of fish gills for *Echinochasmus* infection was undertaken in the south-western part of Lake Balaton and in the lower reaches of the Zala River close to where it enters the lake, seven fish species belonging to five families, namely *Pseudorasbora parva* and *Tinca tinca* (Cyprinidae), *Perca fluviatilis* and *Gymnocephalus cernuus* (Percidae), *Percottus glehnii* (Odontobutidae), *Umbra krameri* (Umbridae) and *Neogobius fluviatilis* (Gobiidae), proved to be infected with this parasite. Of the cyprinids, only *Ps. parva* (7 specimens) and *T. tinca* (2 specimens) were infected and the other species examined exhibited no signs of infection. Of the non-cyprinid fishes, the gills of all of the *G. cernuus, Pe. fluviatilis* and *N. fluviatilis* exhibited relatively high levels of infection with *Echinochasmus* metacercariae. In these fishes, small, ellipsoidal metacercariae were encysted in the gill filaments closely associated with the cartilaginous part of the filament (referred to here as the ray). These metacercariae measured 0.79–0.90 (0.83) mm in length and 0.32–0.37 (0.35) mm in width. We were unable to see collar spines at the anterior end of these larvae (Fig. 1b). The intensity of the infections ranged from a single to several hundred metacercariae. In ruffe, *G. cernuus* between 40 and 400 specimens were usually found. These worms appeared to infect the gills randomly, occurring both close to the tip and to the base of the filaments. In most fishes, the cysts are attached to the cartilage of the filaments on one side only (Fig. 2). Consequently, these ellipsoidal cysts become aligned perpendicularly in relation to the gill filaments and were surrounded by a thin layer of cartilaginous cells and a layer of collagenous connective tissue (Fig. 3). Less frequently, they were incorporated into the cartilaginous tissue of the filament rays (Fig. 4). In the majority of cases, only local changes were seen at the attachment sites; these involved a proliferation of the cartilaginous and connective tissues. In ruffe, however, major distortions of the gill filaments were also observed. In this fish, a bifurcation of some of the gill filaments was recorded. These filaments, at about their half length, branched, causing a complete duplication of the distal part of the filament (Fig. 5). This bifurcation started at the point where an *Echinochasmus* metacercaria was located inside the cartilage of the filament (Fig. 6). The invasion of the gill with metacercariae and the number of bifurcations did not correlate. In a

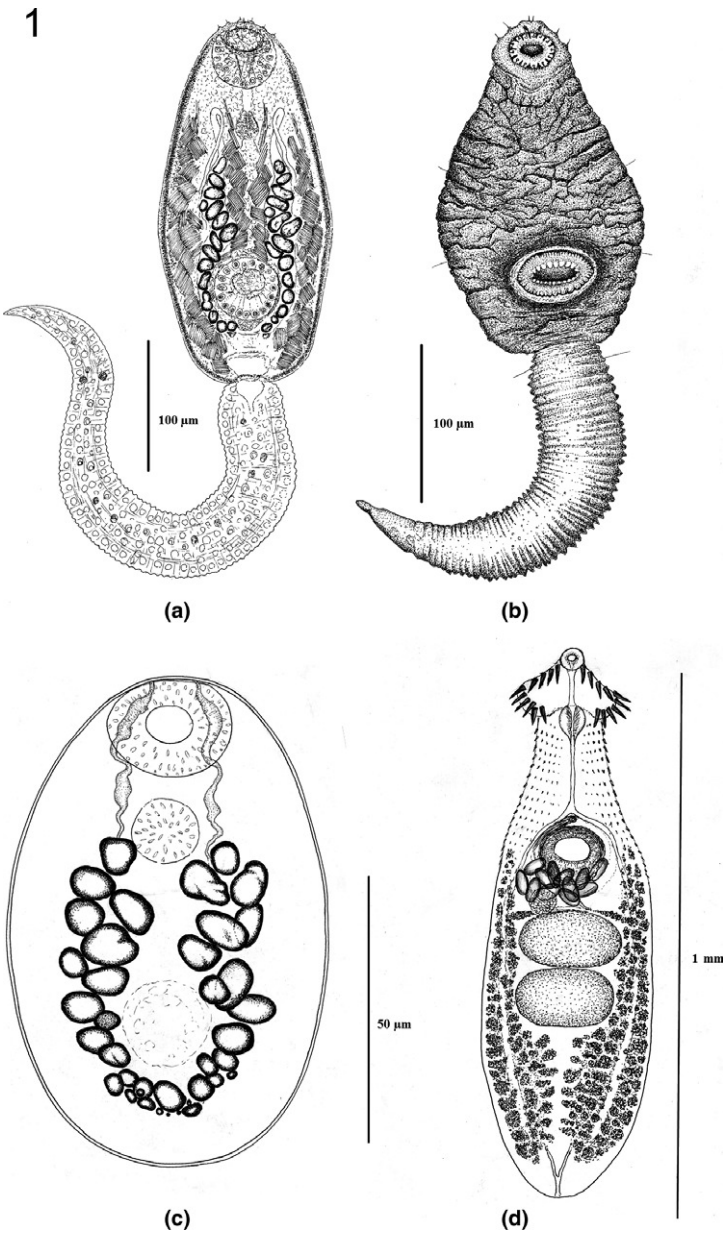

Figure 1 Diagrammatic illustrations of some of the developmental stages of *Echinochasmus* sp.: (a) cercaria, internal features; (b) cercariae, external features; (c) metacercaria; (d) adult (marita).

specimen where about 17–22 bifurcated filaments were found, a great number of metacercariae were recorded which did not cause this malformation (Figs 5 and 6). In a survey estimating the rate of malformation, in 10 ruffe specimens 740 metacercariae were counted and of these 135 (19.2%) caused a bifurcation of the filament. The duplicated parts of the damaged filaments were similar in length and morphology and exhibited a similar structure to uninfected filaments.

Histological sections of ruffe gills supported observations based on the examination of fresh gills. Most metacercariae were attached on one side to the cartilaginous part of the gill filaments and became surrounded by young cartilaginous cells, but otherwise caused no major alteration of the filaments (Fig. 7). Some metacercariae, however, do deform the cartilaginous centre of the gill filaments more seriously, affecting their linear course. In addition to cartilaginous cells, these cysts were also surrounded by a thick layer of collagenous connective tissue (Fig. 8). Some of these metacercariae can be seen associated with the bifurcation of the gill filaments in ruffe (Fig. 9).

Trematodes were found in the first part of the gut in each of the 10 experimentally infected chicks. The number of trematodes recovered varied from 5 to 27 specimens. Fully developed sexual adults (maritae), containing eggs, appeared during the seventh day post-infection (Figs 1d and 10). The trematodes collected had 20 collar spines, eight of which were located orally and 12 laterally; 18 of these spines were similar in size and shape, measuring 45–52 μm in length, but the last pair of lateral spines were somewhat smaller. The adult specimens exhibited typical characters of a species of *Echinochasmus,* and most closely resembled *E. dietzevi* Issaitschikov, 1927. (The full identification of the species, its detailed description and experimental data will be the topic of another paper). None of the control chicks exhibited any infection with trematodes.

A sample of cercariae was isolated from the gravel snail *Lithoglyphus naticoides* (Pfeiffer) at one of the collection sites where the metacercariae were found in fish. They exhibited a characteristic gymnocephalus form (Figs 1a,b and 11) and were thought to be possibly conspecific with the *Echinochasmus* metacercariae from fishes. The body of this cercaria was oval and 160 by 90 μm in size. At 140 by 45 μm in size, the tail was almost as long as the body. Oral and ventral suckers were observed on the ventral surface and granular cells were apparent around the ventral sucker, but the crown of collar spines was not recognizable.

Eight samples of the present material, tentatively considered to represent a species of *Echinochasmus*, were studied by molecular methods (Table 1). The amplified ITS region (with additional parts of the 18S rDNA and 28S rDNA) of the *Echinochasmus* samples was more than 1300 bps. ITS sequences of two metacercarial samples from ruffe (KT989661, KT989662) proved to be identical but showed 0.3% differences to metacercariae from the stone moroko (KT989663). There were only small differences (0.0–0.5%) between the metacercariae and the adult (marita) developed experimentally in chicks (KT989664-KT989667). The single cercarial sample (KT989660) exhibited a 0.0–0.2% difference from the metacercariae and 0.0–0.5% from the adult specimens. In general, the samples exhibited a great similarity with each other, but differed significantly (6.7–7.0%) from sequences of *Echinochasmus* sp. (FJ756940) from Lithuania deposited in the GenBank, which was the closest match. The *Echinochasmus* sp. (FJ756940) sample was only 659 bp long (containing only the 5.8S rDNA, ITS2 and partial 28S rDNA), and consequently, the alignment used for the phylogenetic analyses (Fig. 12) was only 691 bp long. All of the samples from the present study (*Echinochasmus* sp. cercaria, metacercaria and adult) formed a distinct clade with a high bootstrap value, whereas *Echinochasmus* sp. (FJ756940) and *Stephanoprora uruguayense* (KJ957828) and *S. pseudoechinata* (KJ542638) represented a sister group. Other echinostomatid and fasciolid species showed only a distant connection, occurring on the second main clade of the phylogenetic trees (Fig. 12).

Discussion

In the broadest sense, most parasitic infections cause malformations of some kind or other in the organs or tissues of the host's body. *Thelohanellus nikolskii* Achmerov, 1955, a myxosporean parasite of the common carp, *Cyprinus carpio* L., for example, forms large nodules in the fins of the carp fingerlings surrounded by a thick, cartilaginous capsule and connective tissue; such cysts can deform the fin rays or result in a breaking of the fins (Molnár 1972). Infections with metacercariae 11
of *Apophallus* spp. are also so known to cause cartilaginous distortions mainly in the fin rays, but Kent *et al.* (2004) observed severe deformations of the vertebrae in infected fishes. Although prolifera- 12
tion in the form of nodules, pigmentation, etc., around metacercarial cysts is often readily visible (e.g. in the case of *Cryptocotyle* spp. in fish skin and fins), the functionality of these organs is not usually affected, as the changes are due to the host's defence mechanism and often result in the death encapsulation of the parasite, such that the organ regains its original shape and function. In the case of the present *Echinochasmus* metacercarial infection in ruffe, however, the bifurcation of the gill filament results in a permanent change to the gill structure. This bifurcation process corresponds, to some extent, with those malformations of the hindlimbs of amphibians caused by a *Ribeiroia ondatrae* metacercarial infection (e.g. Goodman & Johnson 2011). Johnson *et al.* (2002) remarked that different amphibian species were differently affected by *R. ondatrae* infections and some species exhibited high frequencies of abnormalities. In the present case, of

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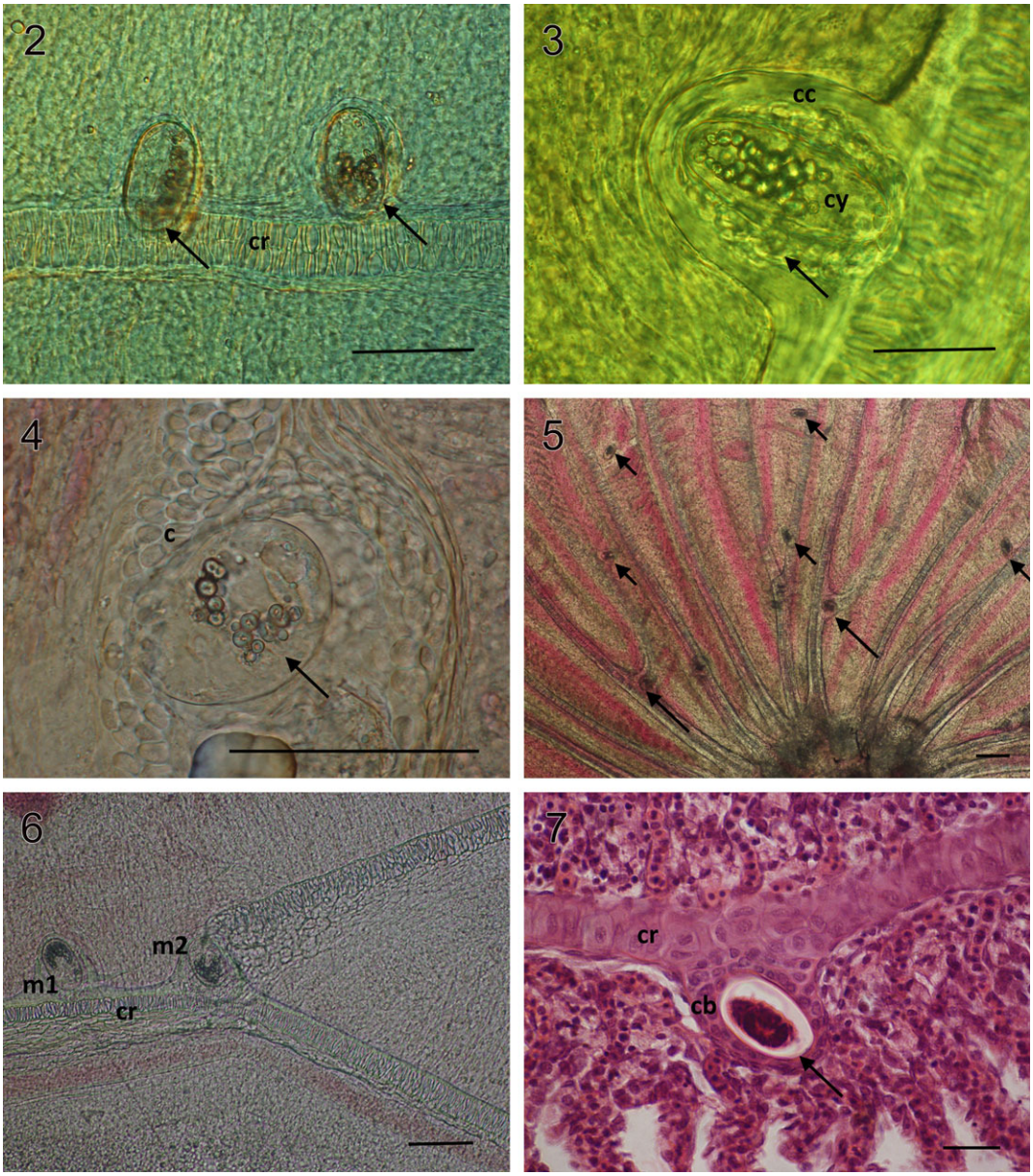
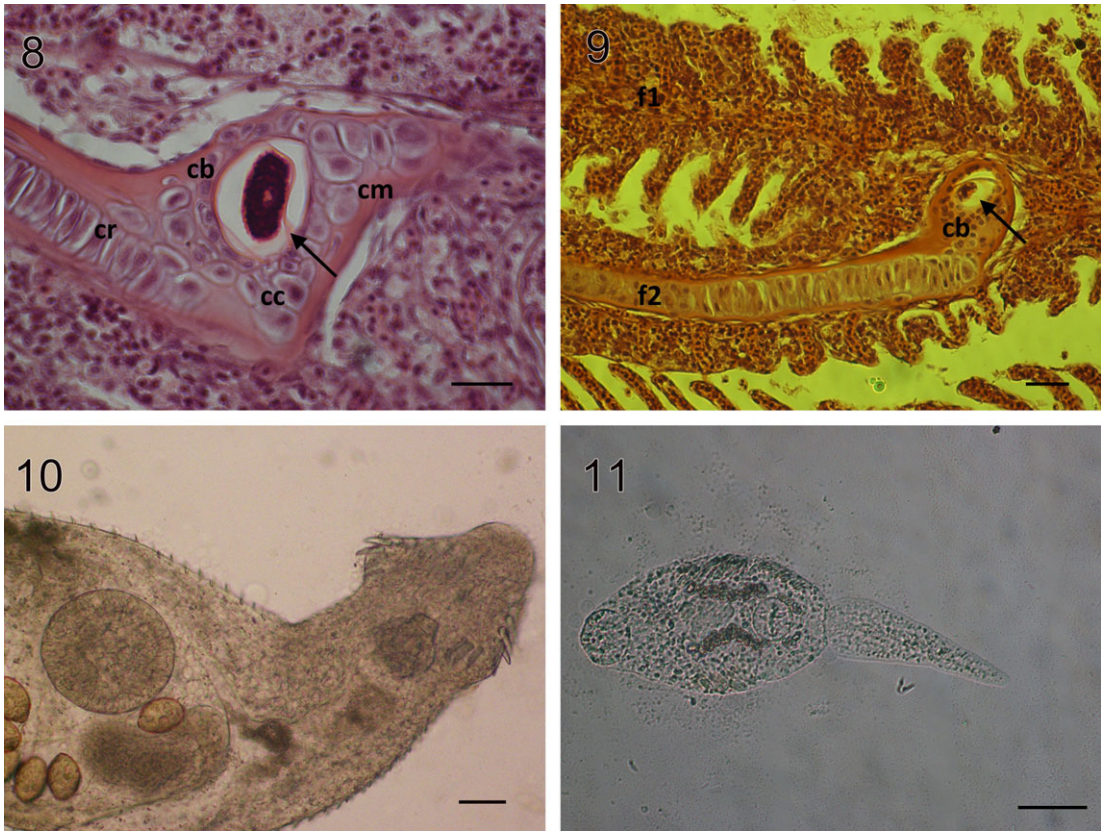


Figures 2–7 Micrographs of *Echinochasmus* sp. metacercariae in ruffe. **2.** Metacercariae (arrows) attached on one side to the cartilaginous ray (cr) of a gill filament. Scale bar = 500 μm. **3.** Micrographs of *Echinochasmus* sp. metacercariae in ruffe. Metacercaria located perpendicularly to the cartilaginous ray of the gill filament. The cyst (cy), which cause only local changes in the filament ray, is surrounded by young chondrocytes (arrow) and a thick layer of collagenous connective tissue (cc). Scale bar = 500 μm. **4.** Micrographs of *Echinochasmus* sp. metacercariae in ruffe. Metacercaria (arrow) located in the cartilaginous filament ray and surrounded by cartilaginous cells (c). Scale bar = 1mm. **5.** Micrographs of *Echinochasmus* sp. metacercariae in ruffe. Hemibranch of a ruffe with bifurcate gill filaments. At the point of bifurcation, there is always a metacercaria (long arrows) associated with the cartilaginous gill ray. Other metacercariae (short arrows) evoke no duplication. Scale bar = 2 mm. **6.** Micrographs of *Echinochasmus* sp. metacercariae in ruffe. Two metacercariae associated with the cartilaginous gill ray (cr). One metacercaria (m1) appears to be causing only local changes in the ray to which it is attached by one end. The second metacercaria (m2) is located inside the cartilage and appears to have caused the bifurcation of the filament. Scale bar = 1 mm. **7**. Micrographs of *Echinochasmus* sp. metacercariae in ruffe. Histological section showing a local deformation of the cartilaginous filament ray (cr) caused by the presence of a metacercaria (arrowed). The metacercaria is surrounded by young chondroblasts (cb) but does not greatly alter the linear orientation of the ray. Scale bar = 500 μm.

the seven infected fish species, bifurcation of the gill filaments was observed only in ruffe. In this host, however, all 47 specimens examined during 2014–2015 from the Lake Balaton exhibited this malformation. The cause of the bifurcation of the gill filaments caused by the *Echinochasmus* infection in ruffe is not known, but it may be a similar mechanism to that of *R. ondatrae* infection in amphibians, where Szuroczki *et al.* (2012) have suggested that an increased level of retinoic acid might result in the malformations.

The fact that the two branches of the bifurcated gill filament of infected ruffes are similar in length suggests that the cercariae initially attach to the filament terminally, or almost so, and this would certainly be the most exposed part of the gill available to an invading larva. Whether or not the bifurcation of the gill filaments affects the host is problematical; one could argue that the duplication of the filament increases the area available for gas exchange, but on the other hand more tightly packed filaments may be less efficient in this respect. However, it seems reasonable to suppose that the concentration of gill filaments has evolved to represent the optimum condition and that any change would represent a suboptimal situation, which would make respiration less efficient to the disadvantage of the host and the advantage of the parasite in terms of transmission.

The generic and species identification of the metacercarial stage could not be determined for certain, because the characteristic collar spines were not observed either in cercariae or in the metacercariae. However, typical *Echinochasmus* adults (maritae) developed in experimental infections of chicks, which proved that the



Figures 8–11 Micrographs of *Echinochasmus* sp. **8.** Histological section of a more severe case of metacercarial infection. Here, a metacercaria (arrow) has caused a distinct break in the linear orientation of the cartilaginous ray (cr). The metacercaria has been surrounded by young chondroblasts (cb), fragments of cartilage cells (cc) from the ray and thick collagenous material (cm). Scale bar = 500 μm. **9.** Micrographs of *Echinochasmus* sp. Histological section of metacercaria (arrow) surrounded by young chondroblast cells (cb) at the point where the gill filament divides into two (f1 and f2). Scale bar = 1 mm. **10**. Micrographs of *Echinochasmus* sp. Adult (marita) collected from the gut of an experimentally infected chick at 9 days post-infection. Scale bar = 100 μm. **11.** Micrographs of *Echinochasmus* sp. Cercaria collected from a gravel snail (Lithoglyphus naticoides). Scale bar = 50 μm.

12

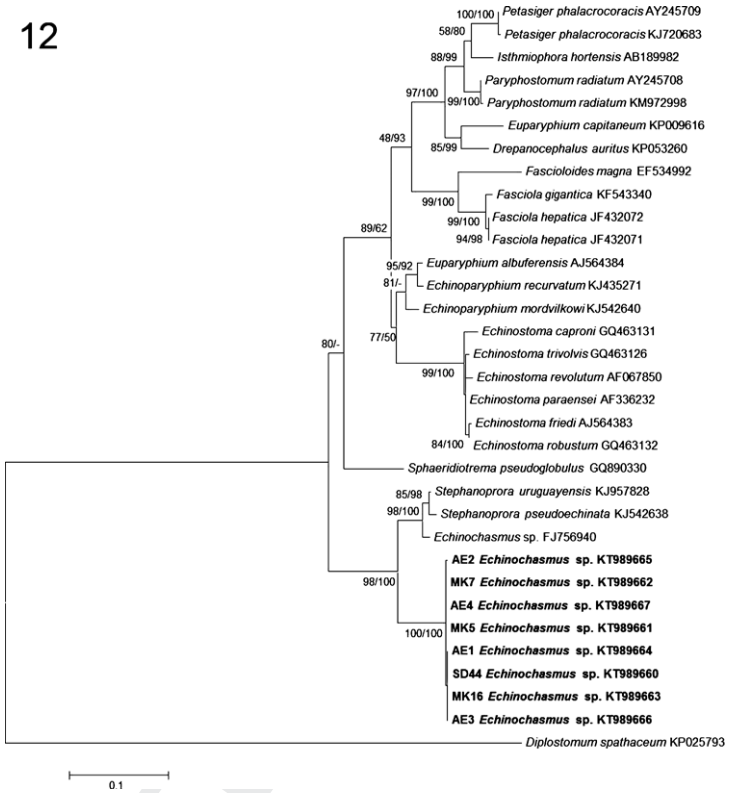


Figure 12 Maximum likelihood tree of the samples of *Echinochasmus* sp. from the present study (SD44: cercaria; MK5, MK7, MK16: metacercaria; AE1-AE4: adult) in relation to echinostomatid and fasciolid sequences deposited in GenBank. Bootstrap values are given at the nodes; posterior probabilities for Bayesian inference are shown behind the bootstrap values. Samples from this study are in bold. The scale-bar indicates the expected number of substitutions per site.

malformations in ruffe were caused by metacercariae of a species of *Echinochasmus*. These adult worms resembled to *E. dietzevi,* but a definitive identification of the species requires further studies (these will be reported in a later publication). Sequences of *Echinochasmus* in the GenBank are available only for *E. coaxatus* Dietz, 1909, *E. japonicus* Tanabe, 1926 and an *Echinochasmus* sp. from *Lithoglyphus naticoides,* but ITS sequences are available only for the latter. Sequences of our specimens showed, however, distinct differences from sequences of these *Echinochasmus* spp., but clearly proved that the adult samples from the experimental final host, the metacercaria from the two fish hosts and the cercaria from the snail *Lithoglyphus naticoides* all have matching ITS sequences, demonstrating that they represent one and the same species of *Echinochasmus*.

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