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Research Article

Ophiotaenia europaea Odening, 1963 (Cestoda: Onchoproteocephalidea) adopts a North American brown bullhead catfish *Ameiurus nebulosus* Lesueur, 1819 as intermediate/paratenic host in Europe

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Abstract

Natural infection of non-native brown bullhead catfish *Ameiurus nebulosus* Lesueur, 1819 (Siluriformes: Ictaluridae) with larvae of the cestode parasite *Ophiotaenia europaea* Odening, 1963 (Onchoproteocephalidea) was confirmed at several localities in the Czech Republic, this representing the first record of *O. europaea* larvae in the country. Adult cestodes infect the intestinal tract of colubrid snakes [predominantly grass snakes *Natrix natrix* (Linnaeus, 1758) and dice snakes *Natrix tessellata* (Laurenti, 1768)], with cyclopoid copepods and fish and/or frogs as the first and second intermediate hosts, respectively. Brown bullhead was introduced to Europe from North America during the last century and has since become widely distributed in many European countries. Larval cestodes were found encysted in the brown bullhead mesentery and on the surface of the intestine and kidney; all parasites were alive. Species identification was confirmed using sequencing of partial 18S and 28S rDNA and COX1 mtDNA. Brown bullhead are only the second natural intermediate/paratenic host of *O. europaea* reported, and the first confirmed by molecular genetics. Its wide distribution within different populations of *A. nebulosus* indicates that this non-native fish species has been successfully included into the life cycle of *O. europaea*. Dice snakes, one of the two natural definitive hosts of *O. europaea*, are rare in the Czech Republic and classified as an endangered species; and larvae of *O. europaea* were predominantly found in localities close to the distribution area of this snake species. Possible impacts of bullhead catfish introduction into regions with dice snakes are discussed further.

Key words: Ophiotaeniidae, Ictaluridae, non-native species, natricine snakes, larval cestode

Introduction

The introduction of non-native species has the potential to disturb natural host-parasite associations in the recipient area, particularly in cases where the species is included into the life cycle of the parasite. For parasites with complex life cycles, the new alternative host may serve as a "sink", leading to a reduction in infection levels, particularly in cases where the parasite cannot develop in the host after having infected it (Keesing et al. 2006). Alternatively, parasite occurrence may be reduced when the new host, though competent for the parasite, is not consumed by the parasite's definitive hosts (Gendron and Marcogliese 2017). On the other hand, if the parasite successfully develops and its new host is consumed by the parasite's definitive host, the total number of parasites to which native hosts are exposed may be amplified, potentially leading to an emerging disease in the affected environment (Hemmingsen et al. 2005; Poulin et al. 2011). In such cases, parasite amplification may be stronger in habitats with a high abundance of the new competent hosts (Ondračková et al. 2015). As a secondary effect, this could potentially lead to increased levels of infection in all hosts involved in the parasite's life cycle, an indirect effect of particularly importance when the host species is endangered.

The brown bullhead catfish *Ameirus nebulosus* Lesueur, 1819 (Ictaluridae), a native of Canada and the USA, was first introduced to Europe in the late 1880s and is now common in most European countries (Rutkayová et al. 2013). Occurrence of brown bullhead in the Czech Republic was first documented in 1890 (Hanel and Lusk 2005) and it reached maximum population density in the Elbe River basin during the mid-20th century. While the species' distribution remains rather patchy, it has increased its range in recent years, particularly in the basins of the rivers Vltava, Elbe and Morava. In Europe, parasites reported from non-native brown bullhead populations mostly include co-introduced monogenean species of the genera *Gyrodactylus* and *Ligictaluridus* (summarised in Ondračková et al. 2020). Reports on acquired parasites in the species' European range are few and far between (see Chechina et al. 1953 for an exception), though more data is available for the related black bullhead *Ameiurus melas* Rafinesque, 1820 (e.g. Scholz and Cappellaro 1993; Oros and Hanzelová 2009). In its native range, the brown bullhead is a common definitive host for a range of proteocephalid cestodes, especially species of the genera *Corallotaenia*, *Megathylacoides* and *Proteocephalus* (Hoffman 1999; de Chambrier et al. 2017).

Ophiotaenia europaea Odening, 1963 (Onchoproteocephalidea) is a widespread proteocephalid cestode infecting semi-aquatic snakes of the family Colubridae in Europe and western Asia (Sharpilo and Monchenko 1971), with grass snakes *Natrix natrix* (Linnaeus, 1758) and dice snakes *N. tessellata* (Laurenti, 1768) the main definitive hosts (Freze 1965; Sharpilo and Monchenko 1971). Both snake species occur naturally in the Czech Republic, with widely overlapping distributions. The grass snake is regularly distributed over the whole country while dice snake populations are more fragmented (Figure 1; Jeřábková et al. 2015). Overall, the dice snake is more common in southern parts of Europe, the northernmost, western and central European populations (Czech and German) being considered relicts of the postglacial warm period (Gruschwitz et al. 1999).

The first intermediate hosts for *O. europaea* are cyclopoid copepods, with both fish and amphibians serving as suitable second intermediate hosts (Biserkov and Genov 1988). According to Freze (1965), the Ophiotaeniid

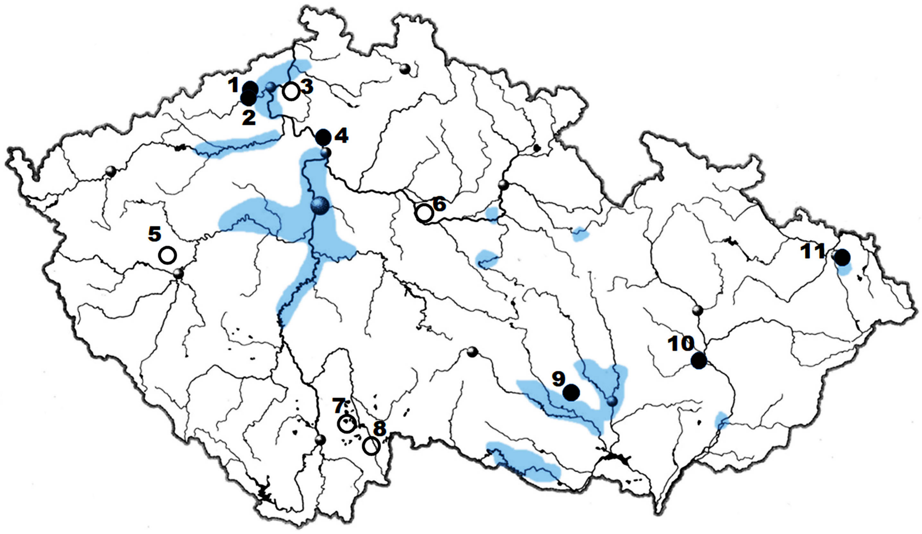

Figure 1. Map of the Czech Republic, showing the distribution area of dice snake *Natrix tessellata* (blue areas; according to Jeřábková et al. 2015) and brown bullhead catfish *Ameiurus nebulosus* sampling sites. As grass snake *Natrix natrix* is distributed more-or-less regularly throughout the country, it is not marked on the map. Circles representing brown bullhead sampling sites are marked as full (presence of *Ophiotaenia europaea*) or empty (absence of *O. europaea*). 1 = Barbora pond, 2 = Ruzovy pond, 3 = Svadov side arm, 4 = Libechov restoration pool, 5 = Hamr pond, 6 = Kolin pond, 7 = Stary pond, 8 = U Janu artificial pool, 9 = Skrinka pond, 10 = Medek pond, 11 = Skrecon pond.

life cycle reflects the evolution of this parasite group alongside their amphibian and reptile hosts, with the introduction of the second intermediate hosts evolving due to ecological features typical of tropical and subtropical regions, where the hosts are mainly distributed. Despite the cestode's wide distribution in Europe, and its presumed low host specificity for second intermediate hosts, natural records of *O. europaea* larvae in second intermediate host are rare, with just a few recent findings reported from the invasive Chinese sleeper *Perccottus glenii* Dybowski, 1877 (e.g. Sokolov et al. 2015; Kvach et al. 2017) and none from amphibians (summarised in Reshetnikov et al. 2013).

During a recent parasite survey of wild brown bullhead in the Czech Republic (Central Europe), we found larval *O. europaea* at several sampling sites. Therefore, we evaluate the distribution of this parasite in brown bullhead populations. As morphological description of proteocephalid larvae is insufficient for species determination, we then provide genetic identification using 18S, 28S rDNA and COX1 fragment of mitochondrial DNA. We also discuss potential consequences for the parasite's definitive hosts, natricine snakes.

Materials and methods

Brown bullhead were sampled at 11 water bodies in the Czech Republic (eight fish ponds, one artificial pool, one restoration pool and one river side arm) between June 2018 and October 2020 using electrofishing gear (Figure 1, Table 1). The fish were transported alive in aerated source water

Table 1. List of brown bullhead catfish *Ameiurus nebulosus* sampling sites in the Czech Republic (N. Sea = North Sea drainage, Bl. Sea = Black Sea drainage, Ba.Sea = Baltic Sea drainage), showing number of fish sampled (N), standard length (SL in mm; mean ± S.D.) and presence (+) or absence (–) of *Ophiotaenia europaea*.

Locality	Habitat	Coordinates	Basin	Date	N	Fish SL	*O. europaea*
Barbora	pond	50.604417; 13.753694	Bilina (N.Sea)	November 2019	20	146 ± 7	+
Ruzovy	pond	50.624889; 13.752833	Bilina (N.Sea)	September 2019	20	159 ± 4	+
Svadov	river side arm	50.666694; 14.107500	Elbe (N.Sea)	November 2018	20	129 ± 4	–
Libechov	restoration pool	50.433361; 14.467278	Elbe (N.Sea)	June 2018	21	143 ± 4	+
Hamr	pond	49.853083; 13.328639	Berounka (N.Sea)	September 2019	20	48 ± 4	–
Kolin	pond	50.093972; 15.154306	Elbe (N.Sea)	September 2018	20	155 ± 4	–
Stary	pond	49.009194; 14.731306	Luznice (N.Sea)	July 2019	22	95 ± 4	–
U Janu	artificial pool	48.980028; 14.861833	Luznice (N.Sea)	August 2018	20	115 ± 4	–
Skrinka	pond	49.305250; 16.211222	Svratka (Bl.Sea)	July 2019	20	129 ± 4	+
Medek	pond	49.305667; 17.412472	Morava (Bl.Sea)	October 2018	17	85 ± 4	+
Skrecon	pond	49.902889; 18.387639	Oder (Ba.Sea)	October 2020	20	158 ± 3	+

to the laboratory, where they were measured for standard length (SL; Table 1) and dissected within two days of capture (Kvach et al. 2016). Larval cestodes were extracted from the host tissue, photographed alive under a light microscope, then preserved in either 96% ethanol, for further molecular analysis, or hot 4% formaldehyde followed by staining with iron acetic carmine and mounted in Canada balsam. Parasites were identified and measured using an Olympus BX51 light microscope and QuickPhoto 3.2 image analysis (Olympus Optical Co., Japan). Prevalence, intensity of infection and mean abundance were calculated according to Bush et al. (1997).

Nine larval cestodes were used for genetic analysis (Table 1). In addition, adult *O. europaea* (collected by M. Jirků in 2003 from grass snake in Nová Osada, Komárno, Slovakia) was obtained from the helminthological collection at the Institute of Parasitology, Czech Academy of Sciences, České Budějovice. Total genomic DNA was extracted using the Invisorb® Spin Forensic Kit (STRATEC Molecular, Germany) following the standard protocol. Three fragments of nuclear ribosomal DNA and one fragment of mitochondrial DNA were used for molecular characterisation: (a) the partial 28S rDNA gene amplified using the primers LSU5 (TAGGTCGACCCGCT GAAYTTAAGCA) (Littlewood 1994) and ECD2 (CCTTGGTCCGTGTTT CAAGACGGG) (Littlewood et al. 1997); and two fragments of the partial 18S rDNA gene amplified using the primers (b) 81 (TTCACCTACGGAA ACCTTGTTACG) and 83 (GATACCGTCCTAGTTCTGACCA) and (c) 82 (CAGTAGTCATATGCTTGTCTCAG) and 84 (TCCTTTAAGTTTCA GCTTTGC), according to Mariaux (1998), and (c) the partial cytochrome *c* oxidase I (COX1) gene amplified using primers PBI-cox1F_PCR (CATTT TGCTGCCGGTCARCAYATGTTYTGRTTTTTTGG) and PBI-cox1R_PCR (CCTTTGTCGATACTGCCAAARTAATGCATDGGRAA) (Scholz et al. 2013). The reaction mix contained 4 µl of extracted DNA, 0.3 µl primer, 2 µl buffer A, 0.2 µl dNTPs, 0.2 µl $MgCl_2$, 0.1 µlTaq polymerase and 2.9 µL ddH2O. PCR was undertaken using the KAPA2G Robust HotStar PCR Kit (Kapabiosystems, USA). Amplification took place in a Mastercycler ep gradient S thermocycler (Eppendorf) with an annealing temperature of 58 °C

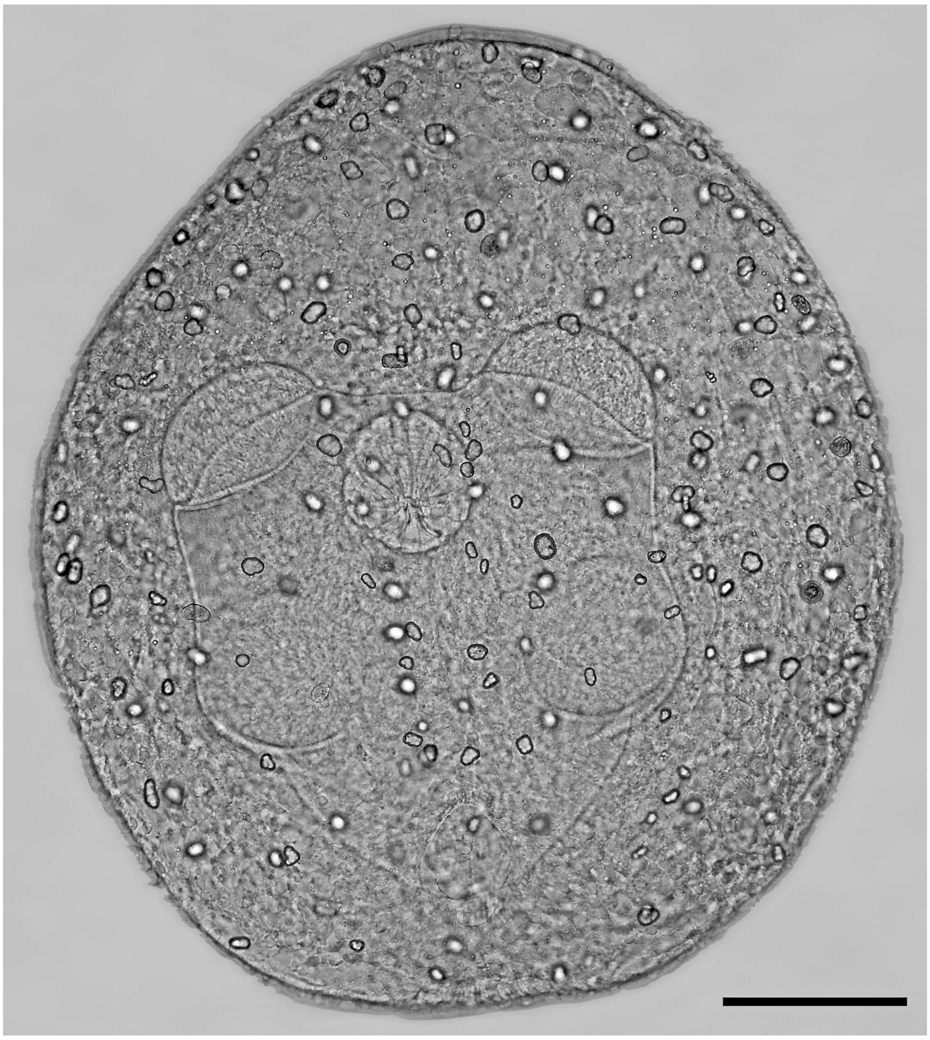

Figure 2. Microphotograph of larval *Ophiotaenia europaea* cestode from the mesentery of brown bullhead *A. nebulosus*, native slide. Microphotograph by M. Ondračková.

for the 28S rDNA gene, 51 °C for both fragments of the18S rDNA gene, and 61 °C for the COX1 gene. All PCR products were purified using ExoSAP-IT (Affymetrix Inc., Santa Clara, USA), according to the manufacturer's instructions. PCR products were sequenced commercially at Eurofins Genomics GmbH (Germany). Additionally, COX1 PCR products were sequenced using PBI-cox1F_seq (CATTTTGCTGCCGGTCA) and PBI-cox1R_seq (TAATGCATDGGRAAAAAAC) to yield clean sequence data as suggested by Scholz et al. (2013). The sequences were checked and aligned using Geneious v. 9.0.5. software (http://www.geneious.com). The newly generated sequences were then compared with the NCBI database using BLASTn to assess sequence similarity.

Results

Larvae of *Ophiotaenia europaea* were found in the brown bullhead mesentery, on the surface of kidney tissue and the intestine wall, with all parasites encysted and alive (Figure 2). The cestodes were observed at six of the 11 sampling localities, i.e. in the Ruzovy, Barbora, Skrinka, Medek and

Table 2. Infection parameters of *Ophiotaenia europaea* larvae in brown bullhead catfish *A. nebulosus.*

Locality	N	Prevalence %	Abundance	Intensity range	Number sequenced
Barbora	20	30%	0.80	1–5	1
Libechov	21	19%	0.57	1–9	–
Ruzovy	20	40%	0.75	1–5	2
Skrinka	20	35%	0.65	1–3	3
Skrecon	20	25%	0.55	1–4	3
Medek	17	6%	0.18	3	–

Skrecon ponds and in the Libechov restoration pool (Figure 1, Table 1), with all sites showing comparable infection parameters (Table 2). The majority of fish were infected with 1–3 parasites, while prevalence and abundance ranged from 6 to 40% and 0.2 to 0.8, respectively. A maximum intensity of infection of nine larvae per fish was observed at the Libechov restoration pool (Table 2). Larval cestodes were observed throughout the year, from June to November.

Cestode larvae morphology was typical, with the presence of a scolex with four suckers and an apical organ (Figure 2). Measurements (in μm) were as follows (N = 8): cyst oval with length and width: 358–573 × 256–458, suckers oval: 93–128 × 75–101, and apical organ: 57–64 × 64–77, width representing *c.* 60% of sucker width.

Sequences of partial 28S rDNA were obtained from larval (1008 bp) and adult (376 bp) cestodes. No intraspecific variance was observed between larvae collected from brown bullhead in the Danube, Elbe or Oder basins or in adult from Slovakian grass snake. The first fragment of 18S rDNA (covering the V5–V8 regions) was obtained from both larval (834 bp, consensual sequence) and adult (898 bp) cestodes. While there was no sequence variation between larvae from all three river basins, the sequence obtained from adult cestodes differed from that of the larvae by 0.25% (two substitutions). The second fragment of 18S rDNA (covering the V2–V4 regions), also obtained from larval (981 bp) and adult (938 bp) cestodes, showed no intraspecific variance. The partial sequence of COX1 mtDNA gene were also found for both, larval (553 bp) and adult (543 bp) cestodes. No intraspecific variance among sequences was detected. Because of lacking intraspecific variance, only unique sequences have been deposited in GenBank (accession numbers: 28S: MZ675524; 18S V5–V8: MZ675532 (adult), MZ675533 (larva); 18S V2–V4: MZ675534; COX1: MZ675527). The BLASTn search showed maximum (99.5%) similarity (28S rDNA; differing in five gaps) to *O. europaea* from natricine water snakes *Natrix maura* (Linnaeus, 1758) collected in France (AJ388598; Zehnder and Mariaux 1999).

Discussion

The present study provides evidence for the recent acquisition of a non-native intermediate/paratenic host by a proteocephalid cestode. The North-American brown bullhead catfish was found to be infected with larvae of a native European cestode, *O. europaea*, at multiple sites across the Czech Republic. These results are important for two different reasons, both of which may be interrelated. First is the addition of a non-native species as a competent host for a local parasite with a complex life cycle, potentially increasing the risk of trophically-mediated parasite "spillback" (see Kelly et al. 2009) with a potential of subsequent amplification of parasite numbers in the environment. This is of particular importance considering there have been no reports of naturally infected native intermediate or paratenic hosts to date. Secondly, this may have important consequences for the conservation status of the parasite's definitive reptile host in the study region (Jeřábková et al. 2017).

Our results showing regular infection of brown bullhead at distant localities throughout the year (from late spring to late autumn) indicate that this non-native fish species has become an appropriate second intermediate or paratenic host for *O. europaea*. The position of the second intermediate host requires consumption of the first intermediate hosts, i.e. copepods, while the paratenic host preys on the infected second intermediate host (in this case, fish or anuran). The brown bullhead feeds on both copepods (Collier et al. 2018; personal observation) and fish and anurans (Snow et al. 2017; El Balaa and Blouin-Demers 2013), suggesting its likely position as both the second intermediate and paratenic host in the cestode life cycle. Interestingly, in its native range, brown bullhead are known as intermediate or paratenic hosts for a range of endoparasitic species, excluding cestodes (Muzzall and Whelan 2011). As bullhead catfish are common prey of American reptiles, including snakes and turtles (Moldowan et al. 2015; Kalki et al. 2018), involvement of these fish in the life cycle of reptile parasites such as ophiotaeniid cestodes might be expected.

While *O. europaea* is a common parasite of natricine snakes in Europe (Sharpilo and Monchenko 1971; Reshetnikov et al. 2013), larval cestodes have never been reported in native second intermediate hosts, including the Czech Republic (Moravec 2001). To date, only non-native fish such as the Chinese sleeper (Kvach et al. 2013, 2017; Reshetnikov 2013; Sokolov et al. 2015) or brown bullhead (this study) have been found naturally infected with *Ophiotaenia* larvae. In this case, the non-native brown bullhead is only the second documented species serving as an intermediate/paratenic host for *O. europaea*. Why only non-native species have become intermediate/paratenic hosts for this cestode remains unknown.

As *O. europaea* includes second intermediate hosts (fish, amphibians) in its life cycle (Biserkov and Genov 1988), infection by this parasite will reflect the feeding behaviour of the snake species considered here, i.e. while the diet of dice snakes mainly comprises fish, the grass snake has a wider dietary spectrum, including both fish and anurans (Corti et al. 2010). In some studies (e.g. Filippi et al. 1996), grass snakes appear to show a preference for amphibians; however, it is more likely that their feeding reflects food availability in the environment, with the most common and easily accessible prey dominant in the diet (Stellati et al. 2019). As such, *Natrix* snakes would be expected to switch to feeding on non-native fish species in those habitats where they are found at high densities. Over two hundred years ago, Lindaker (1790) noted that "grass snakes especially like smooth animals", a description that fits the bullhead catfish perfectly, and, to a certain extent, the Chinese sleeper also. As suggested above, both these fish species form an important dietary element of *Natrix* snakes in those regions where these invasive species are common (Reshetnikov et al. 2013; Stellati et al. 2019). As these non-native species appear to be suitable intermediate hosts for the cestode, their predation increases the risk of snake infection, as found Reshetnikov et al. (2013), who recorded up to 100% prevalence in the Russian Federation. In comparison, a much lower prevalence of up to 50% has been recorded in other regions, such as the Czech Republic (Borkovcová and Kopřiva 2005) and Armenia (Sargsyan et al. 2014), where Chinese sleeper is absent. Moreover, natricine snakes may show a significant decrease in body condition following predation of fish with long and sharp fin rays, especially those with poison glands such the bullhead catfishes (Stellati et al. 2019). These authors found that almost all snakes captured were in very poor body condition, with some having been injured by fin spines or even having spines piercing their bodies. Though cestode infection is not expected to harm the snake host, even at higher intensities (T. Scholz *personal communication*), secondary effects of parasites on snakes in poor condition due to injury cannot be excluded, though this requires further investigation in those areas affected.

The data presented in this study indicate that occurrence of *O. europaea* in aquatic habitats may be linked to the occurrence of the dice snake, as most brown bullhead populations infected with the cestode were located in close proximity to the patchy distribution area of this snake species (see Figure 1). These isolated populations are typically found in the northern part of the country, e.g. in the lower River Ohre, with one population of unclear origin near the town of Havirov, approximately 12 km from the Skrecon pond. While the grass snake is the most common snake in the Czech Republic, representing an important part of the fauna in wetlands, pond systems and wet biotopes along most rivers (Berec et al. 2015), both snake species are protected under law (Jeřábková et al. 2017). In comparison, the dice snake is one of the rarest reptiles in the Czech Republic (Vlček et al. 2011) and is classified as an endangered species. The dice snake is also registered under the Endangered Category under IUCN Red List criteria, while the grass snake has recently been transferred to the 'near threatened' category due to its decreasing area of distribution (Jeřábková et al. 2017). As such, the inclusion of bullhead catfish species into the diet of these threatened *Natrix* snakes may represent an additional endangering factor, with a subsequent drop in body condition due to injury (Stellati et al. 2019) exacerbated by infection with the *O. europaea* cestode.

As species identification for most larval cestodes is very difficult using classical morphological methods, researchers have tended to use combined morphological and genetic methods for many parasite species in recent years (e.g. Tanaka et al. 2014). Basic morphometrical characters of *O. europaea* larvae observed in this study correspond to that presented by Biserkov and Genov (1988), but these traits are insufficient for species identification within the family. This study then represents the first genetically supported identification of *Ophiotaenia* larvae in Europe, all previous studies reporting *O. europaea* in Chinese sleeper (Kvach et al. 2013, 2017; Reshetnikov 2013; Sokolov et al. 2015) having used morphology only for species determination. Though *O. europaea* is the only cestode infecting European natricine snakes as their definitive host (as followed from Rego 1994), Freze and Sharpilo (1967) have described two other species of *Ophiotaenia* in Ukraine and Russia, *O. dubinini* Freze and Sharpilo, 1967 from the smooth snake *Coronella austriaca* Laurenti, 1768 (Colubrinae) and *O. spasskyi* Freze and Sharpilo, 1967 from the common European adder *Vipera berus* (Linnaeus, 1758) (Viperidae). Shortly after their description, these species were suggested as synonyms of *O. europaea* and both snake species denoted as unsuitable accidental hosts (Sharpilo 1976). According to de Chambrier et al. (2017), the number of undescribed species of *Ophiotaenia* remains high, particularly in the Neotropical region and possibly also in the Palaearctic. Moreover, based on the assumption of strict host specificity in this cestode genus (de Chambrier et al. 2021), it is possible that the two *Ophiotaenia* species mentioned above remain valid species with larvae hardly distinguishable without the help of molecular genetics. In our study, species determination was supported by analysis of the 18S, 28S rDNA and COX1 mtDNA genes, using our own comparative material of adult cestodes extracted from naturally infected grass snakes and data deposited in the NCBI database. All larvae originating from the three different river basins covered in this study were identical, though there was a slight variance (0.25%, two substitutions) at the V5–V8 region of 18S rDNA between larvae and an adult cestode (no variance found at V2–V4 18S, 28S rDNA and COX1 mtDNA). Unfortunately, we only had one adult cestode available for genetic analysis, preventing any serious explanation for this difference. Further research, including a comparison of sequences from adult cestodes obtained from the typical host species (i.e. grass and dice snakes, and possibly viperine water snakes) could help elucidate this intraspecific genetic variance.

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Authors' contribution

The study was conceived by M.O. Material was collected and processed by M.O. and Y.K. Molecular analyses were carried by V.B. The manuscript was written by M.O., with comments from all authors.

Ethics and permits

This research was undertaken in line with the ethical requirements of the Czech Republic and has been approved by the appropriate ethics committee. The maintenance and care of fish prior dissection, as well as the method of fish killing, complied with the legal requirements of the Czech Republic (§ 7 law No. 114/1992 on the Protection of Nature and Landscape and § 6, 7, 9 and 10 regulation No. 419/2012 on the Care, Breeding and Use of Experimental Animals). MO is certified according to Czech legal requirements (§15, Law No. 246/1992 on Animal Welfare) to work with experimental animals.

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