

Comparison of mitochondrial *cox1* gene in *Fascioloides magna* from different host species

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ABSTRACT

Large American liver fluke (*Fascioloides magna*) was introduced to Europe at least twice, via invaded North American deer species. These imports resulted in formation of three permanent foci of fascioloidosis - Italian, Czech and Danube floodplain forests. Molecular analysis revealed that flukes in Italian foci represent separate genetic group, and that flukes from Czech and Danube foci are genetically identical. Since previous studies of genetic structure of *F. magna* from Croatia were based on samples collected from red deer in one limited area (Baranja), the aim of this study was to analyse genetic diversity of 111 fluke samples collected from various hosts (red, roe and fallow deer, wild boar) and geographic locations in Croatia. On 355 base pair long cytochrome oxidase subunit 1 sequence, three haplotypes were identified - previously published CO1-Ha3 and CO1-Ha4, and one new haplotype named CO1-Ha36. Results obtained in our study confirmed as expected, that *F. magna* in Croatia has a Danube origin and that haplotypes are shared among different final host species.

Key words: *Fascioloides magna*; *cox-1* gene; genetic diversity; red deer; roe deer; wild boar

Introduction

Following a necropsy of a wapiti deer (*Cervus elaphus canadensis*) from Royal Park La Mandria near Turin in Italy, BASSI (1875) described a new trematode species. He named it *Distomum magnum*. Some forty years later the parasite will be given its current name - *Fascioloides magna* (WARD, 1917). Originally, a parasite of the North American deer species, this fluke was introduced to Europe in the 19th century mainly by wapiti and white-tailed deer

(*Odocoileus virginianus*). The import occurred at least twice, resulting in establishment of the three permanent foci of invasion - 1) north - western Italy, 2) middle Europe (Czech Republic, Poland and Germany), and 3) Danube floodplain forests (KRÁLOVÁ-HROMADOVÁ et al., 2011, 2015; HUSCH et al., 2017). Favourable environmental conditions and a variety of available intermediate and final hosts have resulted in relatively rapid

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spread of *F. magna* to new geographic areas in Europe. Therefore, invasion of wild and domestic ruminants was so far confirmed in Italy, Czech Republic, Slovakia, Hungary, Germany, Poland, Austria, Croatia and Serbia (BAZSALOVICSOVÁ et al., 2015; HUSCH et al., 2017). Currently, final wild hosts for *F. magna* in Europe are defined as definitive (red, *Cervus elaphus* and fallow deer, *Dama dama*), aberrant (roe deer, *Capreolus capreolus* and mouflon, *Ovis musimon*) and dead-end (wild boar, *Sus scrofa*).

In Croatia, the parasite was detected for the first time in a red deer that was sanitary culled due to the poor physical appearance in Šprešhat region in Baranja, east Croatia (MARINCULIĆ et al., 2002). The fluke probably entered Croatia some time before by natural migration of cervids from neighbouring Hungary (MARINCULIĆ et al., 2002) and by intermediate hosts flooded by the Danube river (SLAVICA et al., 2006). Such spread was confirmed genetically, showing Danube origin of the parasite in eastern Croatia (KRÁLOVÁ-HROMADOVÁ et al., 2011; BAZSALOVICSOVÁ et al., 2013). Despite treatment programme with triclabendazole and occasionally albendazole, the invasion continued to spread and today the parasite is distributed from Baranja to Zagrebačka County, representing a serious problem for game management. A high rate of red deer invasion was confirmed by several authors (SLAVICA et al., 2006; RAJKOVIĆ-JANJE et al., 2008) and detrimental effects on roe deer population were described by KONJEVIĆ et al. (2021). Also, invasions in wild boars were studied in Croatia to investigate potential role of wild boar in the maintenance of fascioloidosis (KONJEVIĆ et al. 2017, 2019).

The origin of European *F. magna* populations was studied using two mitochondrial markers - cytochrome c oxidase subunit I (*cox1*) and nicotinamid dehydrogenase subunit I (*nad1*) (KRÁLOVÁ-HROMADOVÁ et al., 2011). Two independent phylogenetic lineages were found clearly indicating that *F. magna* was introduced to Europe at least twice; first time to Italy and then to second European focus — Czech Republic. Spreading from Italy to other European countries

was excluded and research of the microsatellite loci showed that *F. magna* spread from Czech Republic to Slovakia, followed by Hungary and later from Hungary to Croatia (JUHÁSOVA et al., 2016).

In this paper we have analysed *cox-I* gene of flukes originating from different types of hosts and from different regions of Croatia to gain deeper insight into genetic structure of this important alien parasite.

Material and methods

A total of 111 *Fascioloides magna* samples originating from red deer (58 flukes collected from 22 individuals), roe deer (40/6), fallow deer (12/1) and wild boar (1/1) were collected in Croatia from animals hunted during the regular game management activities. Samples originated from different continental regions - Vukovarsko – srijemska (22 samples), Požeško – slavonska (26), Virovitičko – podravska (3), Bjelovarsko – bilogorska (36), Sisačko – moslavačka (21), and Zagrebačka county (3), covering the current area of *F. magna* distribution in Croatia (Fig. 1). Livers were extracted and transported to the Faculty of Veterinary Medicine University of Zagreb. Intact flukes were extracted from livers and frozen till further analysis.



Fig. 1. Map of Croatia with locations of *F. magna* samples included in the study

DNA was extracted from individual parasites using a Wizard Genomic DNA Purification Kit (Promega, USA), following the manufacturer's protocol. The *cox1* gene was amplified using primers FM-*cox1*-F (5' GGTCATGGGGTTATAATGA 3') and FM-*cox1*-R (5' ACAGCATAGTAATAGCCGC 3') (KRÁLOVÁ-HROMADOVÁ et al., 2008). PCR was carried out using a total volume of 20 µl Platinum® PCR SuperMix (Invitrogen) (consisting of *Taq* DNA polymerase with Platinum® *Taq* antibody, 22 mM Tris-HCL (pH 8.4), 55 mM KCl, 1.65 mM MgCl₂, 220 µM dNTP mix), 5 µl of extracted parasite DNA and 0.2 mM of each primer. PCR conditions were 2 min at 95°C; 40 cycles at 94°C for 30 s, 30 s at 45°C, 72°C for 30 s and a final elongation stage at 72°C for 7 min. The amplicons were sequenced by the commercial company MacroGen Europe B.V. Sequences were analysed using software CLC Genomics Workbench (Qiagen), while DNASP (ROZAS et al., 2017) was used to calculate haplotype frequencies.

Results

We have successfully amplified 111 *Fascioloides magna* *cox-1* sequences corresponding to positions

7 086 – 7 440 of the *F. magna* complete mitochondrial genome (GenBank accession number KU060148). Among 355 base pair (bp) long sequences seven polymorphic sites were identified yielding three haplotypes. The most frequent haplotype was found in 105 individual parasites and was shared among all the host species (Table 1). The frequency of two other haplotypes was significantly lower as they were found in four and two samples. When compared with 46 *F. magna* sequences available in the Genbank in the length of 335 bp we found that our most frequent haplotype, found in all sampled species, corresponds to the reference haplotype CO1-Ha3 (also reported by KRÁLOVÁ-HROMADOVÁ et al., 2008; RADVANSKY et al., 2011; KRÁLOVÁ-HROMADOVÁ et al., 2015; JUHASOVÁ et al., 2016; BAZSALOVICSOVÁ et al., 2017), while haplotype found in two red deer samples corresponds to the reference haplotype CO1-Ha4 (RADVANSKY et al., 2011). The third haplotype identified in this study was not previously reported and was named CO1-Ha36, following the numbering strategy of KRÁLOVÁ-HROMADOVÁ et al. (2011) and BAZSALOVICSOVÁ et al. (2015).

Table 1. Haplotypes identified among individual *F. magna* parasites extracted from the red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*) and wild boar (*Sus scrofa*), haplotype frequencies and Genbank accession numbers of matching haplotypes

Haplotype	No of individual parasites (host species)	Haplotype frequency	Matching haplotypes
CO1-Ha3	105 (53 <i>Cervus elaphus</i> , 39 <i>Capreolus capreolus</i> , 12 <i>Dama dama</i> , 1 <i>Sus scrofa</i>)	0.94	EF534996 KU060148 GU599864 KY793001 KX279954 KP635008
CO1 – Ha36	4 (1 <i>Capreolus capreolus</i> , 3 <i>Cervus elaphus</i>)	0.04	
CO1 – Ha4	2 (<i>Cervus elaphus</i>)	0.02	GU599868

Discussion

Danube floodplain forests have been recognized as dynamically expanding European focus of fascioloidosis (KRÁLOVÁ-HROMADOVÁ et al. 2016). Giant liver flukes originating from the Danube foci form an independent phylogenetic lineage (KRÁLOVÁ-HROMADOVÁ et al., 2011), and so far molecular studies were published for parasites originating from Poland (KRÁLOVÁ-

HROMADOVÁ et al. 2015, JUHASOVA et al 2016), Germany (BAZSALOVICSOVÁ et al. 2017), Austria (HUSCH et al. 2017) and Croatia (BAZSALOVICSOVÁ et al. 2013).

Previous research of Croatian *F. magna* genetic structure revealed presence of CO1-Ha3 and CO1-Ha4 reference *cox1* haplotypes, which were also identified in flukes from the

Czech Republic and Danube floodplain foci (KRÁLOVÁ-HROMADOVÁ et al., 2011; BAZSALOVICSOVÁ et al., 2013). Both studies were based on flukes extracted from red deer in eastern Croatia - Baranja region. Actually, samples used in KRÁLOVÁ-HROMADOVÁ et al. (2011) and BAZSALOVICSOVÁ et al. (2013) studies originated from the same hunting ground (Podunavlje – Podravlje, including the area around village Tikveš) bordering the Danube river. Differently, our research included samples of significantly larger geographic and host origin. Results obtained in our study confirmed as expected, that *F. magna* in Croatia has a Danube origin but we also found one previously unidentified haplotype. This is probably the result of analysing a larger sample set covering the entire current *F. magna* distribution area in Croatia and all wildlife host species. Until now, in Europe only HUSCH et al. (2017) and BAZSALOVICSOVÁ et al. (2017) analysed flukes originating from other final host species besides the red deer. Same as our study, they found that *F. magna* haplotypes are shared amongst different European final host species. This corresponds to the findings of BAZSALOVICSOVÁ et al. (2015) that in North America genetic status of *F. magna* is not related to the cervid hosts species.

Considering that the large American liver fluke is allochthonous parasite with large potential for spreading to new areas and causing significant damages in wildlife management, research of all aspects of its biology and pathology are the foundation for its prevention and eradication. Our study presents novel data about genetic structure of this invasive parasite. However, in order to gain better insight, it is necessary to expand the analysis to another genetic marker – mitochondrial *nad1*, and preferably, the microsatellite markers.

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SAŽETAK

Veliki američki metilj (*Fascioloides magna*) unesen je u Europu najmanje dva puta, uvozom sjevernoameričkih vrsta jelena. Ovi su unosi rezultirali stvaranjem triju trajnih žarišta fascioloidoze, u Italiji, Češkoj i u dolini rijeke Dunav. Molekularne analize potvrdile su da metilji iz Italije čine zasebnu skupinu, dok su oni s područja ostalih dvaju žarišta genetski identični. Kako su prijašnja istraživanja genske strukture metilja *F. magna* u Hrvatskoj temeljena na uzorcima prikupljenima od jelena običnih isključivo s područja Baranje, cilj je ovog istraživanja bio istražiti gensku raznolikost 111 uzoraka metilja prikupljenih iz različitih nositelja (jelen obični, srna, jelen lopatar i divlja svinja) i s različitih lokacija. Na slijedu citokrom-oksidge podjedinice 1 dugoj 355 parova baza utvrđena su tri haplotipa, od čega su dva poznata – CO1-Ha3 i CO1-Ha3, te jedan novi haplotip koji do sada nije opisan u literaturi. Kao što je i očekivano, naše je istraživanje potvrdilo da metilji u Hrvatskoj potječu iz dunavskog žarišta, a identifikacija novog haplotipa ističe važnost daljnjih istraživanja.

Ključne riječi: *Fascioloides magna*; gen *cox-1*; genska raznolikost; različiti nositelji
