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PROTOZOOLOGY - SHORT COMMUNICATION

Hepatozoon in Eurasian red squirrels *Sciurus vulgaris,* its taxonomic identity, and phylogenetic placement

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Abstract

Adeleorid apicomplexan parasites of the genus *Hepatozoon* Miller, 1908 are broadly distributed among the rodents. Broader molecular data on *Hepatozoon* from Palaearctic squirrels are necessary for evaluation of diversity and origin of *Hepatozoon* in Eurasian red squirrel *Sciurus vulgaris* populations, considering ongoing invasion by Gray squirrel *S. carolinensis*. Our report brings a set of molecular data from a population of *S. vulgaris* in the Czech Republic, non-invaded by any invasive squirrel species. Cadavers of 41 Eurasian red squirrels were examined using nested PCR targeting 18S rRNA gene; 30 animals tested positive for the presence of *Hepatozoon* spp. DNA in at least one tissue. Phylogenetic analysis of obtained sequence types revealed relatedness to sequences of *Hepatozoon* sp. from *S. vulgaris* from Spain and the Netherlands, forming a sister clade to *Hepatozoon* isolates from other European rodents. The fact that all available 18S rRNA gene sequences form a monophyletic clade is interpreted as a presence of a single *Hepatozoon* species in *S. vulgaris* in continental Europe, most probably *Hepatozoon sciuri*. The presented molecular data on the *Hepatozoon* from European squirrels provides a basis for future studies on possible exchange of *Hepatozoon* species between Eurasian red and gray squirrels.

Keywords *Hepatozoon* · Squirrel · *Sciurus* · 18S rRNA gene · Europe · Hemoparasites

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Introduction

Adeleorid apicomplexan parasites of the genus *Hepatozoon* Miller, 1908 are broadly distributed among the terrestrial vertebrates, representing the most diversified and complex assemblage of haemogregarines (Smith 1996). Ubiquitous presence and unique appearance of gametocytes within white blood cells of mammalian hosts make *Hepatozoon* commonly reported and discussed vector-borne pathogen since the beginning of twentieth century. European squirrel *Hepatozoon* was originally described as *Haemogregarina sciuri* Coles, 1914 in mononuclear leucocytes of captive Eurasian red squirrels *Sciurus vulgaris* Linnaeus, 1758 in England (Coles 1914); later, the same name was used for a haemogregarine reported from Italian *S. vulgaris* (Franchini 1932). At the North American continent, *Hepatozoon griseisciuri* Clark, 1958 was described, including its life cycle, from gray squirrels *S. carolinensis* Gmelin, 1788 from Maryland (Clark 1958). The fact that the original description by Coles (1914) from England mentioned that the examined squirrels were caged with captive gray squirrels *S. carolinensis* opens a question of possible introduction

of squirrel *Hepatozoon* to British Isles by invasive *S. carolinensis*. Under such scenario, the pathogenicity of *Hepatozoon* observed in the original study as well as in recent accounts from British Isles (Simpson et al. 2006, 2013) can relate to its origin from other host species, similar to fatal infections in European squirrels by squirrel pox virus spread by invasive *S. carolinensis* (Sainsbury et al. 2000). Broader molecular data on *Hepatozoon* from Palaearctic squirrels (both from areas invaded and not invaded by gray squirrels) are necessary for evaluation of diversity and origin of *Hepatozoon* in Eurasian red squirrel populations. Our report brings a set of molecular data from a population of *S. vulgaris* in the Czech Republic, non-invaded by any invasive squirrel species.

Material and methods

Cadavers of 41 Eurasian red squirrels *Sciurus vulgaris*, which were accidentally killed (mostly road kills) or had died in rescue centers, were examined, originating from more than 30 localities throughout the Czech Republic (Table S1, Supplementary material). At necropsy, tissues were removed under sterile conditions, and a piece (5 × 5 mm) of each tissue was placed into a 2-ml Eppendorf tube, where it was cut into 1-mm pieces; the liver, spleen, and blood were the only tissues used in this study. Blood was obtained from heart of the cadaver with sterile Pasteur pipette, and 50–100 µl of the blood was placed into a 2-ml Eppendorf tube. Blood and organ samples were stored at − 20 °C until further analyses. The blood samples did not require any mechanical processing; however, liver and spleen samples were homogenized in RLT buffer (Qiagen) with addition of beta-mercaptoethanol using sterile stainless steel beads (Qiagen) and Tissue Lyzer II as 30% (w/v) suspensions. After addition of 20 µl of proteinase K, the samples were incubated 30 min at 57 °C. The lysate was cleared by centrifugation, and the supernatant collected to a clean sterile microtube. The blood samples were only treated with 20 µl of proteinase K for 30 min at 57 °C. DNA was isolated from 180 µl of the lysate using DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions. The elution volume was 200 µl. All samples were screened using sensitive nested PCR assay targeting nuclear 18S rRNA gene. For the first round of PCR amplifying ~ 1800 bp, reactions were performed in 15 µl reaction volume, consisting of 1 µl template DNA, 400 nM of each primer (HAM 1F: 5´-GCCAGT AGTCATATGCTTGTC-3´, HPF 2R: 5´- GACTTCTCCTTC GTCTAAG-3´ (Criado et al. 2006)), and 7.5 µl of 2 × PCRBIO Taq Mix Red (PCR Biosystems, UK). The second round of nested PCR was done in 25 µl using 1 µl of PCR product from the first round as template, 400 nM of each primer (EF-M: 5´-AAAACTGCAAATGGCTCATT-3´, ER-M: 5´-CTYGCG CTTACTAGGCATTC-3´, modified for this study (Kvičerová et al. 2008), and 12.5 µl of 2 × PCRBIO Taq Mix Red (PCR Biosystems, UK). Cycling conditions were identical for both reactions following manufacturer´s recommendations with annealing temperature 52 °C and elongation time of 45 s. PCR products were visualized on 1% agarose gel stained by MIDORIGreen Advance (Nippon Genetics Europe, Germany).

All PCR products of expected size were purified from the gel using the Gel/PCR DNA Fragment Extraction Kit (Geneaid Biotech, Taiwan) and directly sequenced by Macrogen capillary sequencing services (Macrogen Europe, the Netherlands) using the amplification primers in both directions. All obtained sequences were carefully edited using Geneious 11.0.2 (Kearse et al. 2012) and compared with those available in GenBank by the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Unique acquired sequences were deposited in GenBank (Acc. No. MN104636–40). Phylogenetic analysis was conducted on dataset of all 18S rRNA gene sequences available in public databases (available by the end of 2018) for the proposed *Bartazoon* clade (Karadjian et al. 2015; Maia et al. 2016). In order to reduce the dataset, only sequences longer than 1000 nt and differing > 5 bp from each other were included in the final analyses. All (three) available sequences from *Sciurus* spp. host were included in analyses. In total, 69 sequences were aligned using MAFFT v7 with the Q-INS-I iterative method applying parameters by default (Katoh et al. 2019); poorly aligned regions were eliminated using the least stringent Gblocks options (Talavera and Castresana 2007) resulting in an alignment of 1570 nt in length. Phylogenetic trees were inferred by maximum likelihood method using IQ-TREE v. 1.6.beta4 (Nguyen et al. 2015). A best-fit evolution model (TVM + F + R3, transversion model AG = CT, using empirical base frequencies and relaxing the assumption of Gamma-distributed rates using 3 categories) was chosen based on the Bayesian information criterion (BIC) computed by ModelFinder (Kalyaanamoorthy et al. 2017). Branch supports were assessed by the ultrafast bootstrap (UFBoot) approximation (Minh et al. 2013) and by the SH-like approximate likelihood ratio test (SH-aLRT) (Guindon and Gascuel 2003). Trees were visualized and edited using FigTree v1.4.1 (http://tree.bio.ed.ac.uk/software/figtree/); final graphic outputs were processed by InkScape v.092.1 (http://www.inkscape.org/). Differences in presence of *Hepatozoon* spp. DNA in examined tissues were computed using Fisher Exact test in the GraphPad Prism 8 software (GraphPad Software, La Jolla California, USA).

Results and discussion

Of the 30 individuals positive for the presence of *Hepatozoon* spp. DNA, 56 high-quality sequences of 18S rRNA gene were acquired from different tissues (Table S1), making

the infection prevalence 73%; the prevalence was higher in males (80%) than in females (66%). To reveal the most suitable tissue for *Hepatozoon* spp. detection, only animals with all three tissues available ($n = 34$) were considered for statistical analysis. The prevalence (at least one tissue positive) in this subset was 79.4% (27/34). Blood and spleen tissues (67.6 and 64.7%, respectively) were significantly more positive ($p = 0.015$ and $p = 0.028$, respectively) than liver (35.3%, 12/34).

High-quality sequence of 18S rRNA gene was acquired for 56 samples (Supplementary Table 1); no double peaks suggesting the mix infections were observed in chromatograms. Comparing results of sequencing with GenBank database confirmed 98.59–98.86% (17–20 nucleotide differences) identity with *Hepatozoon* sp. from a squirrel from Spain (Acc. No. EF222259). Five sequence types (with 99.71–99.93% identity) were represented in our dataset. In majority of samples, two dominant sequence types were identified: SV12-7 in 45 samples and SV146-6 in 8 samples differing in one nucleotide. Additionally, three other sequence types (SV52-4, SV174-7, SV194-7) were represented by a single sequence only. These sporadic sequences differed by a single nucleotide from the two main sequence types as well as from sequences acquired from other tissue of the same individual and were not included in phylogenetic analyses. The biological relevance of this variability needs to be confirmed. Phylogenetic analysis of the two major sequence types from our study confirmed their relatedness to already published sequences of *Hepatozoon* sp. from *Sciurus vulgaris* from Spain and the Netherlands, forming a sister clade to *Hepatozoon* isolates from other European rodents (Fig. 1). A single available sequence from *Sciurus carolinensis* from the USA (Acc. No. JF491240) remained well-separated from all *S. vulgaris* sequences.

PCR detection followed by Sanger sequencing revealed a common presence of the *Hepatozoon* throughout the population of Eurasian red squirrels in the Czech Republic. Similarly, using histopathological examination and detection in blood smears, Simpson et al. (2006) confirmed *H. sciuri* in 37% of the examined red squirrels in the UK, reporting a single partial 18S rRNA gene sequence obtained from a squirrel from Isle of Wight, with 97.9% identity to *H. erhardovae*. Sequence identity within the squirrel and rodent subclades ranged between 98.1 and 99.9%, while between these

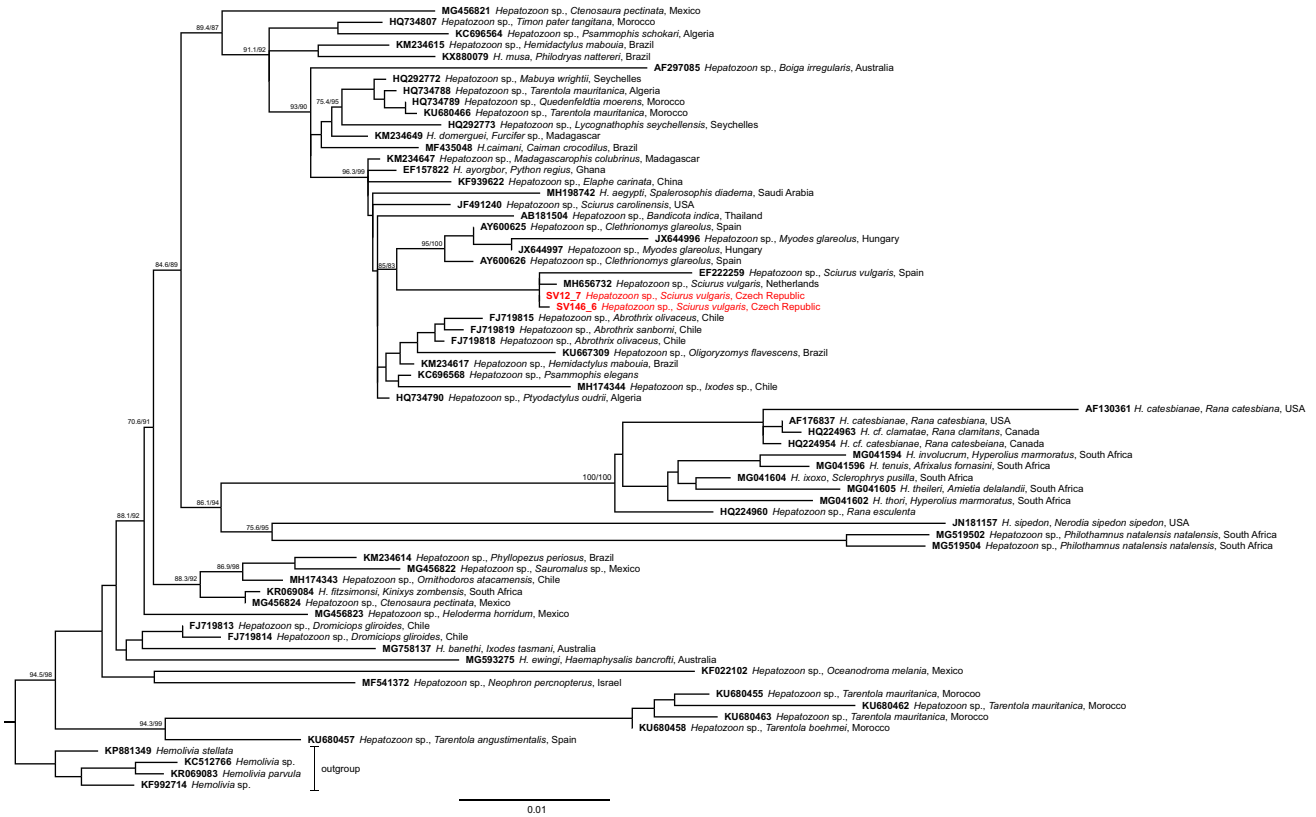

Fig. 1 Phylogenetic analysis assessed by maximum likelihood method based on > 1,000 nt long and differing > 5 bp from each 18S rRNA gene sequences representing genus *Bartazoon* as proposed by Karadjian et al. (2015). Node support values (SH-aLRT/UFB) above the threshold 80%/95% are displayed. Sequences are marked by their accession numbers, species, host, and country of origin as retrieved from GenBank; newly described sequences from this study are highlighted in red

two clades reached 95.1–98.1%. In our analyses, obtained sequences clustered with other sequences from red squirrel *Hepatozoon*, forming a clearly monophyletic group within the subclade of European rodent *Hepatozoon* spp. Interpretation of observed variability is complicated by virtual absence of a species concept in haemogregarine taxonomy. Observed sequence variability fails within variability range of 18S rRNA gene sequences of so far accepted taxa as closely related *Hepatozoon erhardovae* or more distant *Hepatozoon canis*. Considering these facts, we believe that observed monophyletic clade of squirrel *Hepatozoon* can be tentatively interpreted as a presence of a single but variable *Hepatozoon* species in *S. vulgaris* in continental Europe. Sequencing of more molecular markers including mitochondrial genes as a cytochrome b (Léveillé et al. 2020), morphological data and knowledge of vectors involved as definitive hosts in the life cycle are the only ultimate approach to solve the taxonomy of Eurasian red squirrel *Hepatozoon* genetic lineages.

At least in two European regions, populations of native Eurasian red squirrels *S. vulgaris* are threatened by invasive Gray squirrel *S. carolinensis*. Latter species is replacing the native squirrel in large part of British Isles from the end of nineteenth century (Lawton et al. 2010) and, more recently, also in northern Italy (Bertolino et al. 2008). In past decades, competition between native and invasive squirrels in the UK involved a squirrel poxvirus, creating a situation classified as disease-mediated invasion (Strauss et al. 2012). The presented molecular data on *Hepatozoon* spp. from European squirrels provides a basis for future studies on possible exchange of *Hepatozoon* species between Eurasian red and gray squirrel in areas of their recent co-occurrence and for understanding of possible impact on native red squirrel populations.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00436-021-07229-1.

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Declarations

Conflict of interest The authors declare no competing interests.

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