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ORIGINAL PAPER

Molecular detection and phylogenetic analysis of *Hepatozoon* spp. in questing *Ixodes ricinus* ticks and rodents from Slovakia and Czech Republic

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Abstract By amplification and sequencing of 18S rRNA gene fragments, *Hepatozoon* spp. DNA was detected in 0.08 % (4/5057) and 0.04 % (1/2473) of questing *Ixodes ricinus* ticks from Slovakia and Czech Republic, respectively. *Hepatozoon* spp. DNA was also detected in spleen and/or lungs of 4.45 % (27/606) of rodents from Slovakia. Prevalence of infection was significantly higher in *Myodes glareolus* (11.45 %) than in *Apodemus* spp. (0.28 %) ($P < 0.001$). Sequencing of 18S rRNA *Hepatozoon* spp. gene amplicons from *I. ricinus* showed 100 % identity with *Hepatozoon canis* isolates from red foxes or dogs in Europe. Phylogenetic analysis showed that at least two *H. canis* 18S rRNA genotypes exist in Slovakia of which one was identified also in the Czech Republic. The finding of *H. canis* in questing *I. ricinus* suggests the geographical spread of the parasite and a potential role of other ticks as its vectors in areas where *Rhipicephalus sanguineus* is not endemic. Sequencing of 18S rRNA gene amplicons from *M. glareolus* revealed the presence of two closely related genetic variants, *Hepatozoon* sp. SK1 and *Hepatozoon* sp. SK2, showing 99–100 % identity with isolates from *M. glareolus* from other European countries. Phylogenetic analysis demonstrates that 18S rRNA variants SK1 and SK2 correspond to previously described genotypes UR1 and UR2 of *H. erhardovae*, respectively. The isolate from *Apodemus flavicollis* (*Hepatozoon* sp. SK3b) was 99 % identical with isolates from reptiles in Africa and Asia. Further studies are necessary to identify the taxonomic status of *Hepatozoon* spp. parasitizing rodents in Europe and the host-parasite interactions in natural foci.

Keywords Apicomplexa · *Hepatozoon canis* · *Myodes glareolus* · *Apodemus* spp. · Ticks · Central Europe

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Introduction

The genus *Hepatozoon* Miller, 1908 (Adeleorina: Hepatozoidae) comprises apicomplexan parasites infecting a wide range of vertebrate species (Smith 1996). The life cycle of *Hepatozoon* spp. involves sexual reproduction and sporogony in arthropods that serve as vectors and definitive hosts and merogony followed by gamogony in vertebrate intermediate hosts. Arthropods acquire the infection by feeding on vertebrate blood-containing blood cells infected with parasite gametocytes. Vertebrates do not become infected through tick or insect bites, but after the ingestion of infected arthropods (Smith 1996).

In Europe, canine hepatozoonosis is caused by *Hepatozoon canis* (James, 1905) Wenyon, 1926 (Baneth et al. 2003). The occurrence of *H. canis* has been restricted to southern parts of the continent and linked to the distribution area of its primary vector, the *Rhipicephalus sanguineus* (Latreille, 1806) tick. Dogs usually become infected by ingestion of ticks containing oocysts of the parasite, or of prey infested with parasitized ticks (Baneth et al. 2003). In addition, vertical transmission of the parasite has been confirmed in dogs (Murata et al. 1993). Wild-living carnivores (e.g., foxes, golden jackals) may also be involved in the epidemiology of the disease (e.g., Criado-Fornelio et al. 2003a; Gabrielli et al. 2010; Duscher et al. 2013; Imre et al. 2015). Recently, *H. canis* DNA detections in dogs and wild-living carnivores have been reported from temperate and northern latitudes of Europe, i.e., from areas outside the distribution range of the vector tick (Majláthová et al. 2007; Karbowiak et al. 2010; Hornok et al. 2013; Farkas et al. 2014; Najm et al. 2014). These findings suggest a potential role of other tick species as definitive hosts for *H. canis* in areas where no permanent populations of *R. sanguineus* are established (Otranto et al. 2015). *Hepatozoon canis* DNA has been detected in engorged *Ixodes ricinus* (L., 1758), *Dermacentor* spp., or *Haemaphysalis* spp., removed from dogs or red foxes in Hungary and Germany (Hornok et al. 2013; Najm et al. 2014). Molecular detections of *H. canis* in questing *I. ricinus* have also been reported, e.g., in Luxembourg (Reye et al. 2010) and Italy (Gabrielli et al. 2010). However, *I. ricinus* is currently not considered to be a vector of *H. canis* as so far laboratory experiments have failed to confirm sporogony of the parasite in this tick species (Giannelli et al. 2013a, b).

Rodents in Europe have been found to be infected with *Hepatozoon* spp., with over 80 % prevalence of infection in some bank vole, *Myodes glareolus* (Schreber, 1780), populations (Frank 1977; Skofitsch 1980; Laakonen et al. 2001; Bajer et al. 2014; Rigó et al. 2016). Based on morphology, *Hepatozoon erhardovae* Krampitz, 1964 was described from the bank vole (Krampitz 1964), whereas different species of *Hepatozoon*, but with lower prevalence, were found to infect other small mammals (Šebek 1975, 1978; Laakonen et al. 2001; Pawelczyk et al. 2004). Recently, the presence of closely related genetic variants of *Hepatozoon* spp. were revealed in bank voles from Spain, Poland, Germany, and Hungary by employing molecular methods (Criado-Fornelio et al. 2006; Bajer et al. 2014; Rigó et al. 2016). Although parasite gametocytes were observed in larvae of *I. ricinus* feeding on infected rodents (Frank 1977), fleas, mites, or lice are considered as primary vectors of the rodent-associated *Hepatozoon* spp. (Göbel and Krampitz 1982; Smith 1996; Rigó et al. 2016).

By molecular screening of ticks and rodents from southwestern Slovakia for apicomplexan parasites (Hamšíková et al. 2016), *Hepatozoon* spp. DNA was identified in questing *I. ricinus* and rodents. Moreover, a *Hepatozoon*-infected questing *I. ricinus* was found in the Czech Republic. The aim of the present study was the molecular characterisation of the *Hepatozoon* spp. isolates from ticks and rodents from the two Central European countries.

Materials and methods

Rodents and ticks

Rodents and questing ticks were collected from southwestern Slovakia during a previous study carried out in 2011–2014 (for details see Svitálková et al. 2015). The questing tick samples from the Czech Republic were obtained during 2011–2014 (Venclíková et al. 2016). Both studies were conducted within the framework of FP7 EDENext project (No. 261504).

DNA was extracted from 606 rodent spleen samples and selected samples, from other organs (blood, lungs, skin) and 5057 (3158 nymphs and 1899 adults) questing *I. ricinus*, from Slovakia, and from 2473 questing ticks from the Czech Republic by commercial isolation kits as described in Svitálková et al. (2015) and Venclíková et al. (2016), respectively.

Polymerase chain reaction (PCR) and sequence analysis

Rodent and tick samples were screened for *Babesia* spp. DNA by PCR targeting a 450-bp fragment of the 18S rRNA gene (Casati et al. 2006), followed by sequencing (Hamšíková et al. 2016; Venclíková et al. 2016). After BLAST analysis (www.ncbi.nlm.nih.gov/BLAST/), some sequences showed 99–100 % identity with *Hepatozoon* spp. sequences deposited in the GenBank database. A part of these samples was further analysed by PCR using a set of *Hepatozoon*-specific primers HepF_for (5′-ATA CAT GAG CAA AAT CTC AAC-3′) and HepR_rev (5′-CTT ATT ATT CCA TGC TGC AG-3′) to amplify a 660-bp fragment of the 18S rRNA gene (Inokuma et al. 2002). PCR reactions were performed in a volume of 50 μl (including 5 μl of DNA

template) by using a HotMaster Taq DNA Polymerase kit (5 Prime, Hamburg, Germany) according to manufacturer's instructions. The reaction was set at the following parameters: initial denaturation step of 94 °C for 2 min, 33 cycles of a denaturation period at 95 °C for 30 s, a 30 s annealing period at 57 °C, and an extension period of 90 s at 72 °C. Amplification was completed by a final extension step of 5 min at 72 °C. PCR products were electrophoresed in 2 % agarose gel stained with GelRed™ (Biotium, Hayward, USA). PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced by a commercial company (Eurofins Operon, Ebersberg, Germany) (for details see Najm et al. 2014). Analysed sequences were submitted to the GenBank (Accession numbers: KU597235–KU597254). The list of sequences with GenBank accession numbers, their source (host, location), and numbers of analysed sequences are given in Tables 1 and 2.

Phylogenetic analysis

The 18S rRNA gene sequences analysed in the present study were used as query in a BLASTn search to identify and download closely related well-defined *Hepatozoon* spp. 18S rRNA gene sequences deposited in NCBI GenBank database http://www.ncbi.nlm.nih.gov/. A multiple alignment of the hypervariable region of 95 18S rRNA gene sequences comprising selected and analysed sequences including the 18S rRNA gene of *Adelina bambarooniae*, used as outgroup in the subsequent tree building, was done using MUSCLE (Whipp et al. 2012; Edgar 2004). Positions containing gaps and missing data were eliminated from the 584-bp nucleotide-alignment to result in 542 positions in the final dataset. After estimation of the shape parameter (rate difference of 5 categories, $G = 0.29$), the T92+G parameter model was applied to generate a maximum likelihood tree (Tamura 1992). Bootstrap values were estimated based on 1000 replicates. Phylogenetic analysis was carried out using the MEGA6 software (Tamura et al. 2013).

Statistical analysis

Chi-square test was used to analyse the association of *Hepatozoon* spp. with certain rodent species. $P < 0.05$ was considered as significant. Rodents positive in spleen and/or lungs were included in the analyses. Species represented by single specimens were excluded.

Results

Hepatozoon spp. in ticks

Hepatozoon spp. DNA was detected in four questing adult *I. ricinus* (2 females, 2 males; total prevalence 0.08 %) out of the 5057 tested ticks from Slovakia and in one adult *I. ricinus* male (total prevalence 0.04 %) out of the 2473 screened ticks from the Czech Republic. The *Hepatozoon*-positive ticks originated from a sylvatic habitat (2 females, 1 male; site Fúgelka) and Bratislava forest park (1 male), both located in the Small Carpathian Mountains (southwestern Slovakia), and from pasture land (1 male; site Suchov: 48°53′ N, 17°34′E) in the Czech Republic.

Table 1 Accession numbers (GenBank database) of *Hepatozoon* spp. 18S rRNA gene sequences from questing *Ixodes ricinus* and rodents from Slovakia, amplified by using *Babesia*-specific primers (Casati et al. 2006)

Source	Species, variant	Accession no.	Name of isolate	Bp	Number of analysed samples with identical sequences
Ixodes ricinus, questing female from Bratislava	*Hepatozoon canis*	KU597235	F2B_Bab	499	1
Ixodes ricinus, questing female from Fúgelka	*Hepatozoon canis*	KU597236	F2F_Bab	499	1
Ixodes ricinus, questing male from Fúgelka	*Hepatozoon canis*	KU597237	M16F_Bab	499	1
Ixodes ricinus, questing male from Fúgelka	*Hepatozoon canis*	KU597238	M17F_BabV2	499	1
Myodes glareolus from Bratislava	*Hepatozoon* sp. SK1b	KU597243	SHB172_Bab	502	7 (spleen)
Myodes glareolus from Bratislava	*Hepatozoon* sp. SK2b	KU597244	SHB174_Bab	502	3 (spleen)
Myodes glareolus from Fúgelka	*Hepatozoon* sp. SK1b	KU597245	SHF266_Bab	502	3 (spleen)
Myodes glareolus from Fúgelka	*Hepatozoon* sp. SK2b	KU597246	SHF24_Bab	502	8 (spleen)
Myodes glareolus from Fúgelka	*Hepatozoon* sp. SK1b	KU597247	PHF135_Bab	502	2 (lungs)
Myodes glareolus from Fúgelka	*Hepatozoon* sp. SK2b	KU597248	PHF172_Bab	502	2 (lungs)
Myodes glareolus from Fúgelka	*Hepatozoon* sp. SK2b	KU597249	UHF266_Bab	502	1 (skin)
Apodemus flavicollis from Fúgelka	*Hepatozoon* sp. SK3b	KU597250	SHF302_Bab	502	1 (spleen)
Apodemus flavicollis from Fúgelka	*Hepatozoon* sp. SK3b	KU597251	PHF302_Bab	502	1 (lungs)

Table 2 Accession numbers (GenBank database) of *Hepatozoon* spp. 18S rRNA gene sequences from questing *Ixodes ricinus* and rodents from Slovakia and the Czech Republic (*), amplified by using *Hepatozoon*-specific primers (Inokuma et al. 2002)

Source	Species, variant	Accession no.	Name of isolate	Bp	Number of analysed samples with identical sequences
Ixodes ricinus, questing female from Fúgelka	*Hepatozoon canis*	KU597239	F2F_Hep	625	1
Ixodes ricinus, questing male from Fúgelka	*Hepatozoon canis*	KU597240	M16F_Hep	625	1
Ixodes ricinus, questing male from Fúgelka	*Hepatozoon canis*	KU597241	M17F_HepV2	625	1
Ixodes ricinus, questing male*	*Hepatozoon canis*	KU597242	Czech_Hep	666	1
Myodes glareolus from Bratislava	*Hepatozoon* sp. SK1	KU597252	SHB173_Hep	575	1 (spleen)
Myodes glareolus from Fúgelka	*Hepatozoon* sp. SK2	KU597253	SHF70_Hep	625	4 (spleen)
Myodes glareolus from Fúgelka	*Hepatozoon* sp. SK1	KU597254	SHF216_Hep	625	2 (spleen)

Two genetic variants of *Hepatozoon canis* in Slovakian *I. ricinus*, differing in nucleotides at four positions, were revealed by analysing sequences of a 450-bp 18S rRNA gene fragment amplified by using *Babesia*-specific primers (Table 1). Three isolates were identical (KU597235–KU597237) and showed 100 % identity with *H. canis* isolates from foxes in Bosnia and Hercegovina (KP216454) and Austria (KM115991) and 99 % identity with an isolate from questing *I. ricinus* in Luxemburg (GU827130.1). One isolate (KU597238) showed 100 % identity with *H. canis* isolates from foxes in Bosnia and Herzegovina (KP216462), Austria (KM116003), and Spain (AY150067).

Analysis of sequences of a 660-bp fragment of the 18S rRNA gene amplified by using *Hepatozoon*-specific primers confirmed the presence of *H. canis* in the studied isolates from *I. ricinus*. Two genetic variants of *H. canis* could be distinguished in the Slovakian ticks (Table 2). The first variant was identified in two isolates (KU597239, KU597240) and showed 100 % identity with *H. canis* from foxes in Hungary (KJ572978) and Croatia (HM212625), and from a dog in Croatia (FJ497009). It was also identified in an *I. ricinus* male (KU597242) captured in the Czech Republic. The second variant which differed in four nucleotide positions was identified in a Slovakian isolate (KU597241) and was found to be 100 % identical with *H. canis* isolates from dogs in Malaysia (KT267962), Japan (AF418558), and Brazil (FJ743476) and from foxes in Spain (AY150067) and Brazil (AY461375).

The phylogenetic analysis distinguishes the existence of at least three strongly supported clades comprising evolutionary lineages of *H. canis* 18S rRNA gene sequences that have been designated groups 1, 2, and 3 (Fig. 1). The two *Hepatozoon* variants KU597239/KU597240 vs. the variant KU597242 place in groups 2 and 3, respectively. Whilst the *H. canis* variants comprising group 2 have been found to be endemic in dogs and foxes in Central European countries like Slovakia, Czech Republic, Croatia, and Hungary, corresponding variants representing group 3 have been identified in dogs and foxes in widely distant geographic regions like Brazil, Slovakia, Spain, Sudan, and Malaysia.

Hepatozoon spp. in rodents

Altogether 606 rodents of six species, 356 *Apodemus flavicollis* (Melchior, 1834), two *Apodemus sylvaticus* (Linnaeus, 1758), one *Micromys minutus* (Pallas, 1771), 227 *Myodes glareolus*, 19 *Microtus arvalis* (Pallas, 1778), and one *Microtus subterraneus* (de Selys-Longchamps, 1836) were screened for the presence of *Babesia* spp. *Hepatozoon* spp. DNA was detected in 26 *M. glareolus* and in one *A. flavicollis* (total prevalence 4.45 %). Species-specific differences in prevalence were observed ($\chi^2 = 41.538$; $P < 0.001$), with significantly higher prevalence in *M. glareolus* (11.45 %) than in *Apodemus* spp. (0.28 %). No *Hepatozoon* spp. DNA was detected in *M. arvalis*. Out of the 27 *Hepatozoon*-positive rodents, 22 (81.48 %) were positive in spleen and lungs, four (14.81 %) were positive in lungs only, and one specimen (3.70 %) was positive in spleen, lungs, and skin from ear biopsy (ESM 1: Online Resource 1). Rodent blood was negative for *Hepatozoon* spp. DNA. The majority (84.62 %) of *Hepatozoon*-positive bank voles was trapped during September–October; 53.85 % were adult females, and 46.15 % were adult males.

Sequences of a 450-bp 18S rRNA gene fragment amplified by using *Babesia*-specific primers were analysed in 22, four and one isolate from spleen, lungs, and skin of *M. glareolus*, respectively, and in isolates from spleen and lungs of one *A. flavicollis* specimen. In *M. glareolus*, two genetic variants could be distinguished (Table 1, ESM 1: Online Resource 1). Sequences of variant *Hepatozoon* sp. SK1b (KU597243, KU597245, KU597247) from ten isolates from spleen and two isolates from lungs (Table 1) were identical to each other and showed 100 % identity with a sequence of *Hepatozoon* sp. from blood of *M. glareolus* in Spain (*Hepatozoon* sp. BV1: AY600626) and 99 % identity with an isolate from spleen of

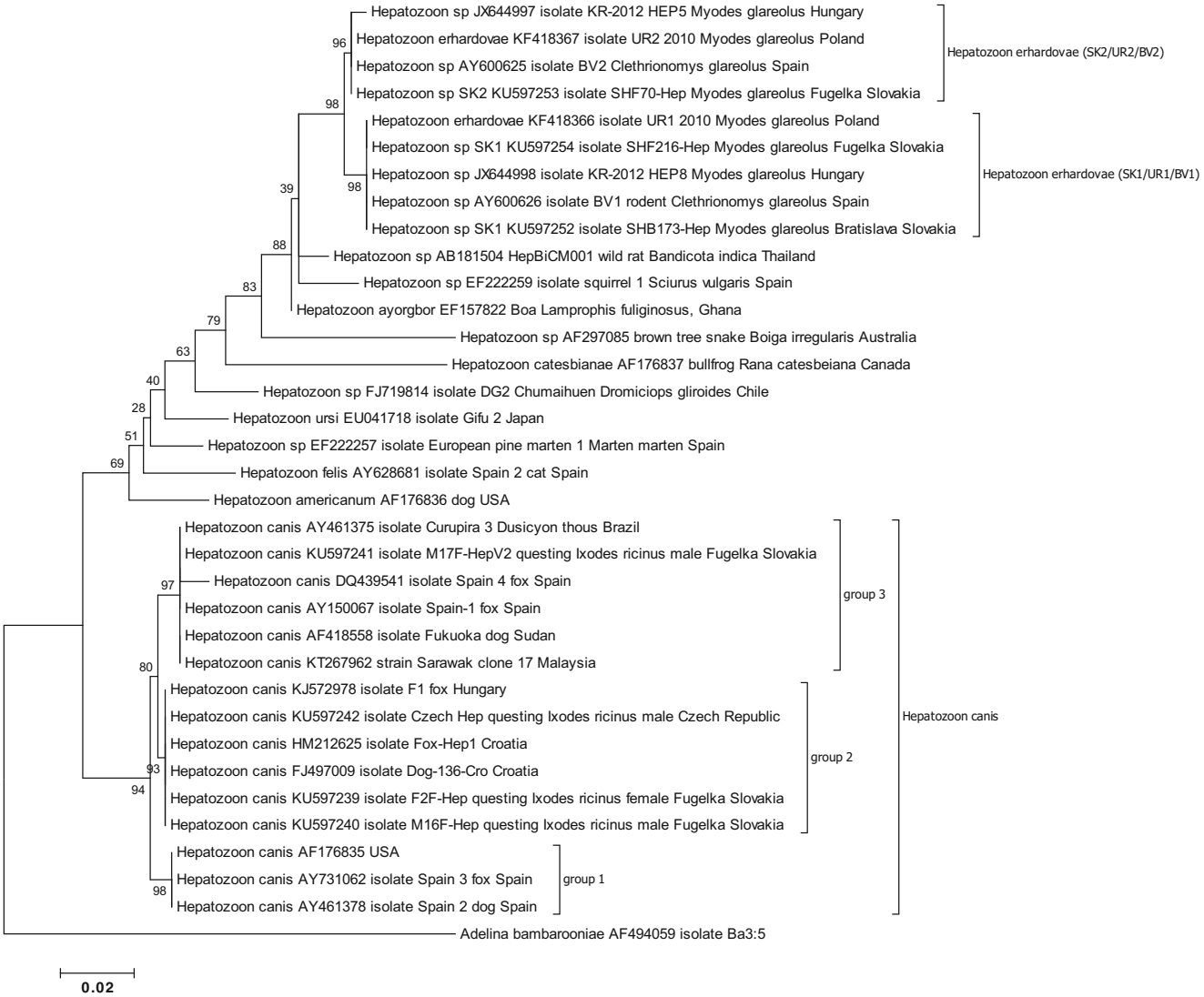

Fig. 1 Phylogenetic tree of hypervariable 18S rRNA gene sequences of *Hepatozoon* parasites using maximum likelihood. The sequence of each isolate is labelled with its GenBank accession number, isolate designation, host, and geographic origin. The bootstrap values based on 1000 replicates are displayed next to the branches. The tree is rooted using *Adelina bambarooniae* as outgroup (Whipps et al. 2012). Clades marked by *brackets* display a highly significant bootstrap value (≥85). The evolutionary distance is shown in the units of the number of base substitutions per site

M. glareolus in Hungary (*Hepatozoon* sp. KR-2012: JX644997) and an isolate from *M. glareolus* ear biopsy in eastern Slovakia (*Hepatozoon* sp. 22118: KJ649313). The other variant, *Hepatozoon* sp. SK2b (KU597244, KU597246, KU597248, KU597249), was identified in 11 isolates from spleen, two isolates from lungs, and one isolate from skin (Table 1). It showed 100 % identity with sequences of *Hepatozoon* sp. from blood of *M. glareolus* in Spain (*Hepatozoon* sp. BV2: AY600625) and from spleen of *M. glareolus* in Hungary (*Hepatozoon* sp. KR-2012: JX644997) and with an isolate from *M. glareolus* ear biopsy in eastern Slovakia (*Hepatozoon* sp. 22118: KJ649313). In one *M. glareolus* specimen, variant *Hepatozoon* sp. SK1b was identified in spleen and variant *Hepatozoon* sp. SK2b in skin (ESM 1: Online Resource 1). A distinct variant, *Hepatozoon* sp. SK3b (KU597250, KU597251), was identified in spleen and lungs of *A. flavicollis* (Table 1, ESM 1: Online Resource 1). Comparison with sequences in GenBank revealed 99 % identity with sequences of *Hepatozoon* sp. YLW-2014 (KF939627) from snakes in China, *Hepatozoon* sp. DB2231 (KC696569) from snakes in North Africa, or *Hepatozoon* sp. pty01po (HQ734790) from lizards in Algeria.

Seven *Hepatozoon* spp. isolates from *M. glareolus* spleen were analysed after amplification of a 660-bp fragment of the 18S rRNA gene by using *Hepatozoon*-specific primers (Table 2, ESM 1: Online Resource 1). Two genetic variants were revealed by comparison with sequences deposited in the

GenBank database. Variant *Hepatozoon* sp. SK1 (KU597252, KU597254) was detected in three isolates (Table 2). The sequences were identical to each other and showed 100 % identity with sequences of *Hepatozoon* sp. from spleen of *M. glareolus* in Hungary (*Hepatozoon* sp. KR-2012: JX644998) and from blood of *M. glareolus* in Spain (*Hepatozoon* sp. BV1: AY600626) and 99 % identity with *Hepatozoon erhardovae* (KF418366) from *M. glareolus* in Poland. Variant *Hepatozoon* sp. SK2 (KU597253) was found in four isolates (Table 2). The sequences were identical to each other and showed 100 and 99 % identity to *Hepatozoon* sp. BV2 (AY600625) and *Hepatozoon* sp. KR-2012 (JX644997) from blood and spleen of *M. glareolus* in Spain and Hungary, respectively.

The inferred tree (Fig. 1) places 18S rRNA gene sequence variants *Hepatozoon* sp. SK1 and SK2 each in a strongly supported clade with corresponding sequences of *Hepatozoon erhardovae* isolate UR1 and UR2 originating from Poland, *Hepatozoon* sp. isolate BV1 and BV2 from Spain, and *Hepatozoon* sp. isolate KR-2012 HEP8 and KR-2012 HEP5 from Hungary.

Discussion

Hepatozoon canis has been detected in engorged *Ixodes ricinus* and other hard tick species indigenous to Central Europe (e.g., *Haemaphysalis* spp., *Dermacentor* spp.) that were removed from infected dogs or foxes (Hornok et al. 2013; Najm et al. 2014). For example, over 20 % of *I. ricinus* nymphs feeding on infected red foxes in Germany were found to contain *H. canis* DNA (Najm et al. 2014). In addition, a few detections of *H. canis* in questing *I. ricinus* have been reported (e.g., Reye et al. 2010; Gabrielli et al. 2010). However, the presence of *H. canis* DNA in a tick does not necessarily mean that the tick is a competent vector for the parasite. Conventional PCR does not reveal if reproduction and trans-stadial transmission of the parasite take place in the tick, or only remnants of the parasite DNA from a previous blood-meal are detected. Nevertheless, the findings of *H. canis* in questing *I. ricinus* adults, including males, may indicate a potential role of other tick species as vectors of the parasite in areas where *R. sanguineus* is not endemic. Although experimental studies failed to demonstrate sporogony of *H. canis* in *I. ricinus* (Giannelli et al. 2013), more extensive studies are necessary to confirm or disprove the vector/reservoir status of *I. ricinus*. The presence of *H. canis* has been reported from red foxes in eastern Slovakia (Majláthová et al. 2007) and in Poland (Karbowiak et al. 2010). However, there are no reports on the occurrence of *H. canis* in wild-living carnivores from other parts of Slovakia or Czech Republic. Thus, the findings of *H. canis* in new areas of Europe may suggest a geographical spread of the parasite, probably by wild-living carnivores serving as feeding hosts for *I. ricinus*. The assumptions are supported by analyses of *H. canis* DNA sequences from our study, showing high degree of identity with isolates from red foxes in Hungary (Farkas et al. 2014), Austria (Duscher et al. 2014), Bosnia, and Hercegovina (Hodžić et al. 2015) and from red foxes and dogs in Croatia (Vojta et al. 2009; Dezdek et al. 2010) or Spain (Criado-Fornelio et al. 2003a, b). The strong support between clades in the phylogenetic tree supports the assumption that *H. canis* does not represent a single species but a species complex and that the two *Hepatozoon* variants found in Slovakia constitute two different co-existing species which both infect dogs and foxes.

Infection of rodents with *Hepatozoon* parasites has frequently been reported from Europe (e.g., Krampitz 1964; Frank 1977; Sebek 1978; Skofitsch 1980; Healing 1981; Laakkonen et al. 2001; Pawelczyk et al. 2004; Karbowiak et al. 2005; Criado-Fornelio et al. 2006; Bajer et al. 2001, 2014; Rigó et al. 2016). Ticks are not considered as definitive hosts of rodent-associated *Hepatozoon* species (Krampitz 1964, 1981; Frank 1977; Smith 1996), but gametocytes of the parasites were detected in ticks feeding on the blood of parasitemic rodents (e.g., Frank 1977). We failed to detect the presence of DNA of the parasites in the blood of rodent specimens positive in spleen and/or lungs. We did not find *Hepatozoon* spp. in rodent-attached ticks (data not shown), and our results agree with recent findings from Hungary (Rigó et al. 2016). Rodents from our study were found to be infested with different groups of ectoparasitic arthropods (Mojšová et al. 2013). Similarly, as in a recent study from Hungary (Rigó et al. 2016), we assume that fleas may serve as definitive hosts for *Hepatozoon* spp. parasitizing rodents also in our study area, although they have not been investigated for the presence of the parasites.

Generally, the estimated prevalence of infection of rodents with *Hepatozoon* spp. was found to depend on the detection method used and on the examined organ. Morphological observations of *Hepatozoon* spp. in organs derived from European rodents have been common, but molecular studies have been scarce (e.g., Criado-Fornelio et al. 2006; Bajer et al. 2014; Rigó et al. 2016). Application of molecular tools may reveal infection in a higher number of specimens than microscopic examinations (Criado-Fornelio et al. 2003a, 2006), although the same prevalence was found in spleen of bank voles from Hungary that were examined microscopically and by PCR (Rigó et al. 2016). Furthermore, inner organs of rodents, especially lungs, were found to be infected by *H. erhardovae* at higher rates than blood (Krampitz 1964; Frank 1977; Šebek 1978; Šebek et al. 1980; Skofitsch 1980; Laakkonen et al. 2001; Bajer et al. 2001). Thus, the real prevalence of infection

in rodent populations may be higher than that estimated based on examination of blood samples. Nevertheless, the prevalence of infection with *Hepatozoon* spp. in rodents from our study was lower than the prevalence previously reported from the majority of sites in Europe (Krampitz 1964; Šebek 1975; Frank 1977; Skofitsch 1980; Laakkonen et al. 2001; Karbowiak et al. 2005; Bajer et al. 2001, 2014; Rigó et al. 2016). Furthermore, *Hepatozoon* infections were found to be more prevalent in voles than in mice and amongst voles, and infection rates were higher in *M. glareolus* than in *Microtus* spp. (Šebek 1975; Laakkonen et al. 2001; Karbowiak et al. 2005). These findings are corroborated by our results.

To the best of our knowledge, this is the first report on molecular detections of *Hepatozoon* spp. in rodents from southwestern Slovakia. Analysis of the DNA sequences derived from bank voles and their comparison with the GenBank database revealed the presence of two different *Hepatozoon* genetic variants, each of which showed a high degree of identity to *Hepatozoon* spp. isolates obtained from bank voles in Spain (Criado-Fornelio et al. 2006), Poland and Germany (Bajer et al. 2014), and Hungary (Rigó et al. 2016), respectively. Interestingly, so far in all studied regions, both genetic variants seem to co-exist as both genotypes have been identified in Poland, Germany, Slovakia, Hungary, and Spain. In our phylogenetic analysis, both these genotypes are placed in two different strongly supported clades each including a genotype variant of *H. erhardovae*. Thus, *Hepatozoon* sp. SK1 and SK2 seem to correspond to genotypes UR1 and UR2 of the species *H. erhardovae*, respectively. This observation raises the question whether two orthologous 18S rRNA gene variants correspond to different *H. erhardovae* subspecies or whether two paralogous 18S rRNA genes are encoded in the *H. erhardovae* genome. Additional detailed studies are necessary to show whether *H. erhardovae* represents a single or two different species or subspecies. Molecular studies supported by morphological examinations will also be necessary to reveal the taxonomic status of the distinct *Hepatozoon* sp. in *A. flavicollis*.

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Compliance with ethical standards The experiments presented in this paper comply with current laws of the Slovak Republic and the Czech Republic. Rodent trapping and handling were approved by the Regional Environmental Office in Bratislava (licence ZPO-594/2012-SAB).

Conflict of interest The authors declare that they have no conflicts of interest.

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