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Identification of *Hepatozoon erhardovae* Krampitz, 1964 from bank voles (*Myodes glareolus*) and fleas in Southern Hungary

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Abstract In order to investigate the prevalence and life cycle of apicomplexan parasites, small mammals were live-trapped with modified Sherman traps in Southern Hungary between 2010 and 2012. Altogether, 528 rodents (*Apodemus flavicollis* Melchior, 1834, *Apodemus agrarius* Pallas, 1771, *Myodes glareolus* Schreber, 1780, *Microtus agrestis* Linnaeus, 1761, *Mus musculus* Linnaeus, 1758 and *Micromys minutus* Pallas, 1771) were collected and four shrews (*Sorex* spp.) were by-catched. Captured animals belonging to non-protected species were euthanized, and spleen samples were preserved for histological and molecular analyses. During the examination of spleen smears, *Hepatozoon* parasites were observed in eight out of 48 bank voles (*M. glareolus*). DNA was isolated from altogether 221 spleen samples, and 18S rDNA was amplified using two different PCR protocols. The eight bank vole samples were positive with PCR, but none of the other *M. glareolus* spleen samples or any of the tissue samples from

other species were found to be infected. Sequenced amplicons were very similar to *Hepatozoon* spp. detected in *M. glareolus* in Spain and Poland. Ectoparasites were collected from the small mammal carcasses and from the vegetation. *Hepatozoon* DNA was not found in the 181 ticks removed from the small mammals or in the 162 ticks collected with flagging, but was detected in all three flea species (4/43 *Megabothris turbidus* Rothschild, 1909, 3/10 *Ctenophthalmus assimilis* Taschenberg, 1880 and 7/78 *Ctenophthalmus agyrtes* Heller, 1896). Based on gamont morphology, vertebrate and arthropod host species and DNA sequences, the parasites in our study can be identified as *Hepatozoon erhardovae*.

Keywords *Hepatozoon erhardovae* · *Myodes glareolus* · Bank vole · Rodent · Flea · Hungary

Krisztina Rigó and Gábor Majoros contributed equally to this work.

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Introduction

The genus *Hepatozoon* (Hepatozoidae) consists of haemoparasites that have been described from a wide range of animals. The heteroxenous life cycle of these intracellular blood parasites takes place in a vertebrate intermediate host and a haematophagous invertebrate definitive host, which also serves as a vector. Asexual reproduction (schizogony) can occur in different organs of mammalian hosts and gamonts are found in blood cells. Sexual reproduction (sporogony) takes place in the hemocoel of the invertebrate definitive host. As there are no observed occurrences of the migration of *Hepatozoon* sporozoites to the salivary gland of the arthropod host, it is assumed that the ingestion of the definitive host containing the sporulated oocysts is required for transmission (Smith 1996; Craig 2001; Laakkonen et al. 2001).

In the last 50 years, *Hepatozoon* infection of small mammals was found in several studies, in different parts of Europe. The differentiation of these species—when it was even attempted—was based on the vertebrate host, the geographical region where the samples were collected and the morphology of the bloodstream developmental forms (Laakkonen et al. 2001; Criado-Fornelio et al. 2003; Karbowiak et al. 2005). The life cycle and host range of most of these species are still unknown. Our objective was to collect data on the species composition and prevalence of different arthropod-borne apicomplexans from multiple small mammal species and to gather information about some of the arthropod vectors that play a part in the life cycles of these parasites, using a combination of histological and molecular methods.

Materials and methods

Between 2010 and 2012, small mammals were live-trapped with 100 modified Sherman traps at three different locations within the Gemenc area, which is a forest-covered floodplain near the river Danube, in Southern Hungary (46° 07' N, 18° 46' E). The total number of trap nights (the sum of the total number of nights each trap was used) was 2200. The species and sex of trapped rodents were identified (Aulagnier et al. 2009), and animals belonging to protected species were then released. All non-protected rodents were euthanized. The carcasses were checked for ticks and fleas, and spleen samples were collected. In addition to tissue sample collection, impression smears were prepared by tapping the freshly cut surface of the spleen repeatedly to a microscope slide. These smears were then air-dried at room temperature, fixed with 70 % ethanol, stained with Giemsa and examined with a light microscope for the presence of blood parasites.

In May 2012, ticks were collected with flagging from the vegetation in several different locations within the Gemenc area. Ticks and fleas found on the trapped animals and ticks collected from the vegetation were stored in 70 % ethanol, and were later identified using standard identification keys (Rosický 1957; Nosek and Sixl 1972; Szabó 1975; Hillyard 1996). After identifying the species and developmental stage of ticks and fleas, DNA was extracted from the ectoparasite samples by alkaline hydrolysis (Guy and Stanek 1991), the extracted DNA samples were examined for the presence of apicomplexan parasites with polymerase chain reaction either individually (fleas) or using pools (1–10 tick samples/pool).

Based on the preliminary results provided by the thorough examination of the spleen smears, altogether 200 samples were selected for examination by molecular methods. All bank vole (*Myodes glareolus*) spleen samples and all samples from less common species were examined. In the case of more common species (i.e. *Apodemus flavicollis* and *Apodemus*

agrarius), since they were negative by microscopy, samples were selected randomly for the molecular biological analyses.

DNA was isolated from the selected tissue samples with a modified Miniprep Express Matrix protocol (MP Biomedical, Santa Ana, USA). Samples not originating from bank voles were examined in pools containing DNA extracted from one to five animals. All bank vole samples were analysed individually. To determine whether the tissue or arthropod samples contained any kind of apicomplexan parasite, first, primers RLB-F (5'-GAGGTAGTGACAAGAAATAACAATA-3') and RLB-R (5'-TCTTCGATCCCCTAACTTTC-3') were used to detect an approximately 500-bps length fragment of the V4 region of the 18S rRNA gene (Gubbels et al. 1999). From the positive samples, the complete 18S rRNA gene was amplified with a second pair of primers (CRYPTO F [5'-AACCTGGTTGATCCTGCCAGT-3'] and CRYPTO R [5'-GCTTGATCCTTCTGCAGGTTACCTAC-3']) (Herwaldt et al. 2003). The first PCR reaction was used because of its higher specificity; based on our previous experiences, in some cases, especially when a tissue type containing a high amount of DNA is used (e.g. spleen), the CRYPTO F–CRYPTO R primer pair can amplify mammal (or even tick) DNA. All PCR-positive samples were sequenced with capillary sequencing. Amplicons of the whole 18S rDNA reaction were sequenced from both directions, and where it was possible, sequences from the two DNA strands were assembled. Forward, reverse and assembled sequences were all blasted against the NCBI Nucleotide Database.

Confidence intervals for the prevalence of *Hepatozoon* infection were calculated using the R statistical software (The R Development Core Team 2010).

The phylogenetic tree was created using selected complete (and near complete) 18S rDNA *Hepatozoon* sequences originating from different mammal species. The multiple sequence alignment was generated using MUSCLE (Edgar 2004). Conserved blocks from the alignment were selected with Gblocks (Castresana 2000). The phylogenetic tree was created using a maximum likelihood approach with PhyML (Guindon et al. 2010). The Hasegawa-Kishino-Yano 85 (HKY85) nucleotide substitution model was selected for the analysis. Branch support was calculated by running 500 non-parametric bootstrap steps.

Results

Altogether, 528 small mammals (Table 1) were trapped throughout the duration of our study. During the examination of spleen smears, ellipsoidal-shaped intra- and extraerythrocytic stages (gamonts) of *Hepatozoon* parasites were observed (G. M.) from eight of the 36 trapped bank voles (*Myodes glareolus*) (Fig. 1). These were also found positive with apicomplexan-specific primers. All

Table 1 Number of captured and tested small mammals during our study at Gemenc area, Hungary

Species	2010 Captured/tested	2011	2012	Total
<i>Apodemus flavicollis</i>	91/22	69/20	24/2	184/44
<i>Apodemus agrarius</i>	73/29	181/72	14/0	268/101
<i>Myodes glareolus</i>	37/37	3/3	7/7	47/47
<i>Microtus arvalis</i>	12/12	4/4	0/0	16/16
<i>Micromys minutus</i>	2/2	2/2	0/0	4/4
<i>Mus musculus</i>	5/5	0/0	0/0	5/5
<i>Sorex</i> spp.	0/0	4/4	0/0	4/4
Total	220/96	263/99	45/5	528/221

spleen samples from other small mammal species were found negative with both methods.

Out of all the captured animals, only 40 were infested with ticks. Ticks were not found on any of the collected *Micromys minutus*, *Mus musculus* or *Sorex* spp. Three different tick species (*Haemaphysalis concinna*, *Ixodes ricinus* and *Dermacentor marginatus*) were collected from both small mammals, and the vegetation. *Ixodes acuminatus* was only found on trapped animals and *Dermacentor reticulatus* was only collected from the vegetation. Apicomplexan parasites were not found in the 181 ticks removed from the small mammals or in the 162 ticks collected with flagging. Details about tick species and tick-borne pathogens of the same study have recently been published (Szekeres et al. 2015a, 2015b). Altogether, 131 fleas belonging to three different species (*Ctenophthalmus agyrtes*, *Ctenophthalmus assimilis* and *Megabothris turbidus*) were collected from 81 small mammals. Thirteen fleas (including all three species) were found to be infected with *Hepatozoon* spp (Table 2) but none of the tick samples (data not shown). Prevalence was as follows: *C. agyrtes*, 8.97 % [95 % CI 4.41–17.38 %], *C. assimilis*, 30 % [95 % CI (confidence interval) 10.78–60.32 %] and *M. turbidus*: 9.3 % [95 % CI 3.68–21.6 %].

The most similar sequences in the NCBI GenBank only showed 95–96 % similarity to our sequenced amplicons

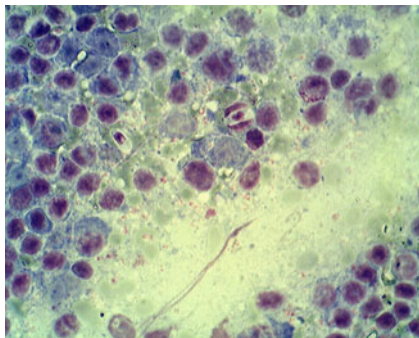


Fig. 1 Ellipsoidal-shaped intra- and extraerythrocytic stages (gamonts) in a Giemsa-stained spleen impression of a bank vole

created with primers RLB-F and RLB-R. Amplicons of the whole 18S rDNA reaction (accession numbers: JX644996, JX644997, JX644998) proved to be very similar to *Hepatozoon* sp. detected in *Myodes glareolus* in Spain (accession numbers: AY600625.1, AY600626.1) (Criado-Fornelio et al. 2006) and Poland (accession numbers: KF418366 and KF418367) (Bajer et al. 2014) and also to the sequence of a *Hepatozoon ayorgbor* sample collected from *Python regius* snakes imported from Ghana (EF157822.1) (Sloboda et al. 2007). Unfortunately, 18S rDNA sequencing was not successful for any of the PCR-positive flea samples. Therefore, in this case, partial 18S sequences sequenced using primers RLB-F and RLB-R have been submitted to the NCBI Genbank (accession numbers: KJ634066 and KJ608372). These partial sequences were almost identical with the corresponding regions of the whole 18S sequences from tissue samples. Based on gamont morphology and 18S rDNA sequences (Fig. 2), the bank vole as the exclusive host and fleas (and not ticks) as probable vectors, we identified the parasite as *Hepatozoon erhardovae*.

Discussion

During the present survey on wild living small mammals, only tissue samples from *M. glareolus* were found to be infected with *Hepatozoon erhardovae*. The prevalence of the infection was relatively high (17.02 % [95 % CI 8.89–30.14 %]) (especially compared to the latest report by Šebek (Sebek 1978) of *Hepatozoon* from *M. glareolus* in Hungary, which was ~7 %), but even higher values have been reported previously: 18–57 % in Northern Europe (Laakkonen et al. 2001) and 18.9–64.2 % in Poland (Karbowski et al. 2005; Bajer et al. 2014). Bank voles seem to have a unique role in maintaining *Hepatozoon* species in Europe. Not only they had been found to be infected with these parasites in several different geographical locations and often at a high prevalence, but unlike most other examined mammal species, not only one, but multiple *Hepatozoon* species have been reported from this host (e.g. *Hepatozoon griseisciuri*, *Hepatozoon sylvaticus* and *H. erhardovae*) (Krampitz and Wongchari 1980; Krampitz 1981; Craig 2001). Unfortunately, there are currently no available DNA sequences from these species in the NCBI GenBank.

The similarity of 18S genes of *Hepatozoon* spp. detected in *M. glareolus* in Europe and in a *Python regius* originating from Ghana has already been pointed out by Sloboda et al. (Sloboda et al. 2007). The occurrence of these apicomplexans, which seem to be genetically very similar, but found in taxonomically and geographically very diverse hosts, raises a question about the host and vector specificity of these parasites. The published data about this issue are somewhat controversial. Apparently, in some cases, genetically very similar parasites can infect a range of different hosts and vectors

Table 2 Number of collected and tested fleas with apicomplexan PCR from small mammals at Gemenc area, Hungary (2010–2013)

Species	Flea-infected individuals	<i>Ctenophthalmus agyrtes</i> Positive/tested (prevalence)	<i>Ctenophthalmus assimilis</i>	<i>Megabothris turbidus</i>	Total per host species
<i>Apodemus flavicollis</i>	30	3/24 (13 %)	1/4 (25 %)	1/19 (5 %)	5/47 (11 %)
<i>Apodemus agrarius</i>	41	3/46 (7 %)	1/2 (50 %)	2/14 (14 %)	6/62 (10 %)
<i>Myodes glareolus</i>	8	0/5	0/0	1/10 (10 %)	1/15 (7 %)
<i>Microtus arvalis</i>	2	1/3 (33 %)	1/4 (25 %)	0/0	2/7 (29 %)
Total	81	7/78 (9 %)	3/10 (30 %)	4/43 (9 %)	13/131 (10 %)

(Criado-Fornelio et al. 2006; Sloboda et al. 2007). On the other hand, infection experiments using closely related host or vector species often fail for some *Hepatozoon* parasites but not for others (Harkness et al. 2010). Some genetically diverse *Hepatozoon* species infecting the same host species has also been reported (Harris et al. 2011). A recent study involving snakes from various continents found neither geographical pattern nor congruency in the host association of the studied *Hepatozoon* sp. (Haklová et al. 2014). Although information deriving from single gene analyses has to be treated with caution, the results of our phylogenetic analysis also support the theory that genetically very similar *Hepatozoon* spp. can occur in geographically diverse hosts (see Fig. 2). To clarify the phylogenetic relationship between these parasites detected in different hosts at different locations, a multi-gene, multi-species phylogenetic study is needed.

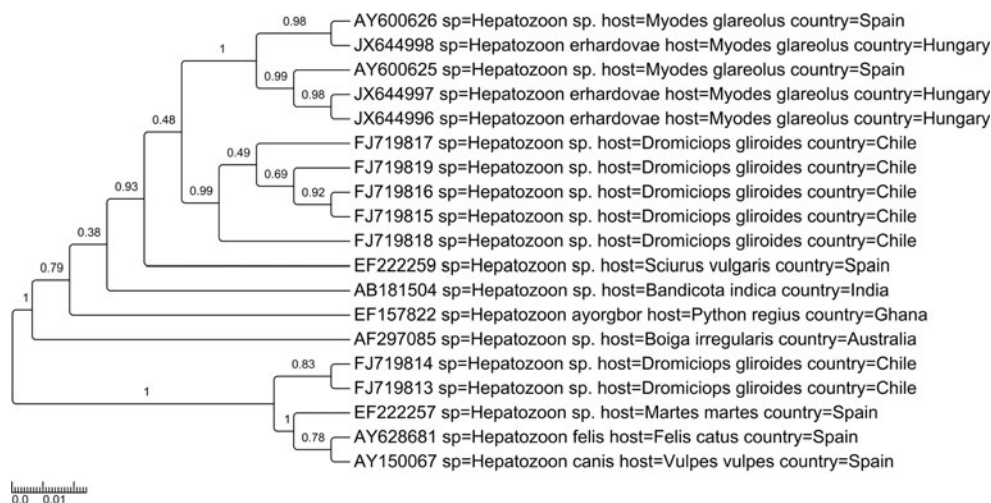
Hepatozoon species were found in all three collected flea species. These have been reported previously as vectors of *H. erhardovae* (Krampitz and Wongchari 1980) and likely play an important part in the maintenance of this apicomplexan parasite. As most of the members of this parasitic insect order, these three species have a wide host range among rodents and even insectivores and have been reported in Hungary before (Szabó 1975; Rigó et al. 2011). The lack of *H. erhardovae* infection in the large number of other rodent

species analysed and the presence of the parasite DNA in all three flea species removed from these supports the known host specificity of this blood parasite.

Little is known about the pathogenicity of *H. erhardovae*. Although it is considered as an apathogenic parasite for voles, a recent study observed the parasites in the majority of *Bordatella bronchiseptica* cases in association with active inflammatory processes (Forbes et al. 2015).

Identification of *Hepatozoon* species of mammals proves to be a challenge, as most of the species have been described in the 1920s to 1980s, and the description was based solely on morphological features of a single developmental stage (Smith 1996; Craig 2001). Although, in the last few years, a number of studies were published, that used DNA based methods to characterize and compare *Hepatozoon* samples collected from different intermediate and definitive hosts, in most cases, the species remain unidentified and unnamed (Criado-Fornelio et al. 2006; Sloboda et al. 2007). Recent studies highlighted that phylogenetic position of closely related genera like *Karyolysus* and *Hemolivia* towards *Hepatozoon* needs also further clarification (Haklová-Kočíková et al. 2014; Kvičarová et al. 2014). A combination of morphological examination of multiple developmental forms of each species and the creation of credible, species-specific

Fig. 2 Phylogenetic tree of selected (near) complete 18S rDNA sequences. Note the similarity between samples originating from geographically and/or taxonomically very distant hosts



reference sequence collection would be highly beneficial for further studies, as it would provide a useful tool for screening and identification of *Hepatozoon* species in a wide range of vertebrates and arthropods.

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Compliance with ethical standards

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

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