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Research paper

Diversity of *Babesia* spp. in cervid ungulates based on the 18S rDNA and cytochrome *c* oxidase subunit I phylogenies

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ABSTRACT

Free ranging ungulates, represented in Europe mostly by several deer species, are important hosts for ticks and reservoirs of tick-borne infections. A number of studies have focused on the prevalence of tick borne pathogens in deer chiefly with the aim to determine their potential role as reservoir hosts for important human and livestock pathogens. However, genetic similarity of *Babesia* spp. forming a group commonly termed as a clade VI that accommodates the deer piroplasms, complicates this task and has led to the description of a bewildering array of poorly characterised strains. This study aims to resolve this issue by using two independent genetic loci, nuclear 18S rRNA and mitochondrial cytochrome *c* oxidase subunit I genes, used in parallel to identify *Babesia* isolates in free-ranging red, sika, and roe deer in two areas of their co-occurrence in the Czech Republic. The COX1 loci, in contrast to 18S rRNA gene, shows a clear difference between interspecific and intraspecific variation at the nucleotide level. The findings confirm *B. divergens*, *Babesia* sp. EU1 and *B. capreoli* in studied deer species as well as common presence of another unnamed species that matches a taxon previously referred to as *Babesia* sp. or *Babesia* cf. *odocoilei* or *Babesia* CH1 group in several other sites throughout Europe. The invasive sika deers enter the life cycle of at least three piroplasmid species detected in native deer fauna. The presence of *B. divergens* in both sika and red deer in an area where bovine babesiosis is apparently absent raises important questions regarding the epidemiology, host specificity and taxonomic status of the parasite.

1. Introduction

Blood parasitic protists of the genus *Babesia* are among the frequently studied tick-borne pathogens, because of their broad distribution, remarkable importance for human and animal health and only partially described diversity. Since description of the first species at the end of 19th century, methodological concepts and resulting knowledge moved from a single host/single pathogen approach through single host/single pathogen/single vector to current research, aiming at understanding of the *Babesia* diversity and epidemiology of babesiosis at a multi-host/multi-pathogen research platform. Several species of the genus *Babesia* were found zoonotic, however, with remarkable differences in epidemiology between geographic regions (Gray et al., 2010; Yabsley and Shock, 2013; Zintl et al., 2003). At the same time, a plethora of molecular studies revealed high diversity of piroplasms and resulting problems in their classification both at genus and species levels. The 18S rRNA gene (referred further as 18S) is a most commonly used marker in diagnostic assays as well as in phylogenetic and taxonomic studies and enables delineation of the deeper as well as of more recent phylogenetic relationships of piroplasmids. Molecular studies

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Table 1
Detailed characterizations of examined animals. For each category, number of positive animals (based on COX1 sequencing results) and total number of animals are stated. M – male, F – female, N/A – data not available

		Gender			Age		
	Locality	M	F	N/A	≤1 year	> 1 year	N/A
Cervus elaphus (n = 143)	Hradiště	19/38	18/32	1/1	15/24	18/39	5/8
	Libavá	16/26	30/43	3/3	15/20	31/48	3/4
Capreolus capreolus (n = 80)	Hradiště	12/24	0/4		2/7	10/19	0/2
	Libavá	12/42	1/7	1/3	8/23	4/26	2/3
Cervus nippon (n = 32)	Hradiště	1/10	6/22		2/8	5/23	0/1
Bos taurus (n = 100)	Hradiště		0/100			0/100	

based on 18S revealed a high diversity and allowed to classify this group previously into at least six and recently into ten different lineages (Jalovecka et al., 2019; Lack et al., 2012; Schnittger et al., 2012). However, the 18S persists as a dominant marker also in studies addressing the circulation of piroplasmids and the epidemiology of piroplasmoses, regardless of its obvious limits to distinguish between closely related taxa. As a result, species are continuously being described/renamed and discussed without a clear species concept, life cycle knowledge, and strict adherence to taxonomic rules (see critical comments in Uilenberg et al., 2018) and recent studies are more and more blurred by a number of doubtful taxa, usually bearing the popular suffix "-like".

The castor bean tick, *Ixodes ricinus*, represents a predominant tick species in most of the ecosystems of Central Europe. As such, this tick species is involved as dominant vector in a transmission of surprisingly diverse spectrum of *Babesia* species (Castro et al., 2015; Dumitrache et al., 2012; Lempereur et al., 2012), including species of apparent importance for cattle (*B. divergens*) as well as for humans, inflicting severe disease in immunocompromised human patients (*B. divergens, Babesia* sp. EU1[1]). The uniformity of 18S obscures clear delimitation of individual species, impeding their accurate diagnostic and a better understanding of the circulation of deer *Babesia* spp. in sylvatic life cycles, including description of reservoir hosts and tick vectors.

In this study, we analyzed the species diversity of the *Babesia* 'clade VI' sensu Schnittger et al. (2012) in Central European deer using the cytochrome *c* oxidase subunit I (COX1) mitochondrial gene (Gou et al., 2012; Hrazdilová et al., 2019) as a target for species identification. In order to relate our results to the large body of work that has previously been done in this field (Lempereur et al., 2012; Razanske et al., 2019; Silaghi et al., 2011), the 18S gene was analyzed in parallel. The host specificity of identified *Babesia* spp. in system that involves three syntopic deer species (accompanied by cattle) and the reservoir role of studied hosts for zoonotic *Babesia* species were evaluated.

2. Material and methods

2.1. Source of deer and cattle samples and reference isolates

Blood samples were collected from 255 free ranging deer including 143 red (*Cervus elaphus*), 32 sika (*Cervus nippon*), and 80 roe deer (*Capreolus capreolus*) from two localities, Libavá and Hradiště, between August 2014 and November 2015 (Table 1, Fig. 1). Both study sites are extensive natural forested areas used for military training and commercial hunting. At Hradiště, a population of introduced *C. nippon* (since 1960s) occurs spatially mixed with *C. elaphus* (Anděra, 2019; Borkovcová et al., 2009; Dvořák and Čermák, 2008). Blood samples were collected either directly in the field by individual hunters or during meat inspection. Blood (or blood coagulum) was collected from the heart, thoracic or abdominal cavity into sterile plastic tubes, cooled immediately on ice and frozen within few hours after collection. In addition, coccygeal venous blood was collected from 100 adult beef cattle (Simmental and Hereford breeds) at Hradiště. All samples were stored at −20 °C until DNA isolation. *B. odocoilei* DNA isolated from a Saskatchewan elk (Pattullo et al., 2013) and *B. divergens* DNA isolated from Irish cattle and a human case (O'Connell et al., 2017) were used as reference isolates.

2.2. DNA isolation and PCR protocols

DNA was isolated from 200 µl of whole blood using the Genomic DNA Mini Kit (Geneaid Biotech, Taiwan) following the manufacturers' instructions and stored in −20 °C. All samples were screened using nested PCR assays targeting the COX1 gene (Table 2). Subsequently, selected COX1-positive samples were analyzed using two nested PCR assays aimed at the 18S gene. All PCR amplifications were performed in 25 µl reaction volume, consisting of 1 µl template DNA or 1 µl of PCR product from the first run, 10 pmol of each primer, 12.5 µl of 2× PCRBIO Taq Mix Red (PCR Biosystems, UK) and 9.5 µl of PCR H_2O (Top-Bio, Czech Republic). DNA isolated from a horse naturally infected with *Theileria equi* was used as positive control. PCR products were visualized on 1.5% agarose gel stained with GoldView (Beijing SBS Genetech, China).

2.3. Sequencing and sequence analysis

All PCR products of expected size were excised from the agarose gels, purified using the Gel/PCR DNA Fragment Extraction Kit (Geneaid Biotech, Taiwan) and sequenced by Macrogen capillary sequencing services (Macrogen Europe, the Netherlands) using the amplification primers. For large PCR amplicons (~1700 bp) additional internal sequencing primers, 600F and 1200F, were used (Bartley et al., 2016).

Samples that yielded low-quality sequencing results or clearly mixed chromatogram signals, were cloned using the TOPO-TA Cloning Kit (Thermo Fisher Scientific, USA). Cloned plasmid DNA was - purified from bacterial culture using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, USA) and sequenced using universal T7/SP6 primers. DNA sequences were edited using Geneious 9.1.2 (Kearse et al., 2012) and compared with sequences available in the GenBank database by BLASTn analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For the COX1 gene, where a single sample yielded multiple conspecific clones (labeled as "_clA, B, …"), a consensus sequence was generated (labeled as "_1, 2"). Alignments of non-coding (18S) sequences were generated using the ClustalW algorithm. For coding COX1 sequences, the nucleotide alignment was guided by amino acid translation (TransAlign, Geneious 9.1.2). Phylogenetic trees were inferred by the maximum likelihood method using IQ-TREE v. 1.6.beta4 (Nguyen et al., 2015). For each dataset, the best-fit evolution model 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 et al.,

[1] Commonly referred to as *Babesia venatorum*; this taxon was never formally described according to nomenclatural rules; adhering to International Code of Zoological Nomenclature (ICZN, 1999), we do not consider it a valid name.

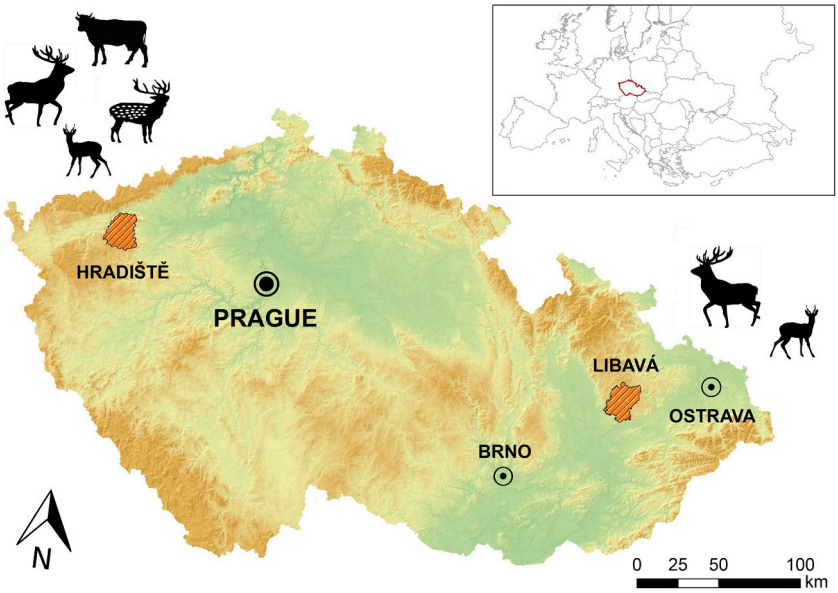

Fig. 1. Map of model localities and the distribution of studied species.

Table 2
Nucleotide sequences of PCR primers used for amplification and sequencing, length of products and annealing temperatures used

Target gene	Primer name	Sequence (5′-3′)	Length of product (bp)	Annealing (°C)	Reference
COX1	BaFor1	ATWGGATTYTATATGAGTAT	~1250	45	Modified, Gou et al., 2012
	BaRev1	TCTCTWCATGGWTTAATTATGATAT			
	BaFor2	ATAATCWGGWATYCTCCTTGG	~975	49	
	BaRev2	TAGCTCCAATTGAHARWACAAAGTG			
18S rDNA	BT1 F	GGTTGATCCTGCCAGTAGT	~1730	55	Criado-Fornelio et al., 2003
	BT outer R	GGAAACCTTGTTACGACTTCTC			Bartley et al., 2016
	PIRO OF-2	GCCAGTAGTCATATGCTTGTCTTA	~1670	49	Tuvshintulga B et al., 2016
	BT inner R	TTC TCC TTC CTT TAA GTG ATA AG			Bartley et al., 2016
	BTH 1F	CCTGAGAAACGGCTACCA CATCT	~685	60	Criado-Fornelio et al., 2003
	BTH 1R	TTGCGACCATACTCCCCCCA			
	GF2	GTCTTGTAATTGGAATGATGG	~560	62	Zintl et al., 2011
	GR2	CCAAAGACTTTGATTTCTCTC			Bonnet et al., 2007
	600F	AGTTAAGAAGCTCGTAGTTG	Sequencing		Bartley et al., 2016
	1200F	AGGATTGACAGATTGATAGC	Sequencing		Bartley et al., 2016

2010). Trees were visualized and edited using FigTree v1.4.1 and Inkscape 0.91. A map of localities was constructed in ArcGIS 10.3 (ESRI 2011. ArcGIS Desktop: Release 10. Redlands, CA: Environmental Systems Research Institute).

3. Results

3.1. PCR amplification and sequencing

Based on the nested PCR assay targeted at a ~975 bp fragment of the COX1 region, 99 red deer (out of 143), 51 roe deer (out of 80), and 15 sika deer (out of 32), but none of 100 cattle showed a band of expected size. Using combined direct sequencing and cloning approaches, a total of 213 COX1 sequences were obtained from 121 individual animals (61.5% red deer, 32.5% roe deer and 21.9% sika deer were confirmed positive by sequencing, Table 3).

Analysis using protocols aimed at a large fragment of the 18S gene, yielded products of expected size in 103 red deer (70.2%, out of 143), 37 roe deer (46.3%, out of 80) and 7 sika deer (21.9%, out of 32) samples. Direct sequencing resulted exclusively in low quality, mixed signal. Sequencing of 172 clones from 45 individual deer yielded 16 long, high quality *Babesia* spp. sequences (Table 3), the remaining

Table 3
Comparison of effectivity of direct sequencing and cloning of COX1 and 18S PCR products. “On gel positive” samples displayed band of expected size on 1.5 % agarose gel after PCR; „direct seq“ samples provided high quality sequence of purified PCR product by direct sequencing using amplification primers and subsequent assembly; „clones/samples“ displays number of sequenced individual clones from number of samples; „final seq/samples“ represents final number of acquired *Babesia* spp. sequences (direct or cloned) from number of samples.

		COX1				18S rDNA (1700 bp fragment)			
	n	On gel positive	Direct seq	Clones/samples	Final seq/samples	On gel positive	Direct seq	Clones/samples	Final seq/samples
Cervus elaphus	143	99	57	114/30	163/87	103	0	147/40	9/8
Capreolus capreolus	80	51	21	18/5	40/26	37	0	20/4	2/4
Cervus nippon	32	15	5	8/3	10/7	7	0	5/1	2/1
Total	255	165	83	140/40	213/121	147	0	172/45	16/15

clones were identified as unspecific PCR products (forest plants, rumen microflora).

Amplification of the short (~560 bp) 18S fragment performed on a subset of 52 samples representing all host species and all COX1-defined *Babesia* clades as well as 5 COX1 negative samples, yielded 34 *Babesia* sequences (65.4%). Six 300 bp fragments (11.5%) matched with rumen microflora while 12 samples of approx. 600 bp fragment size were identified as *Sarcocystis* spp. (23.1%). Of the 5 COX1-negative samples, two yielded bands of the expected size, which were identified as *B. capreoli* and *Sarcocystis* sp. None of the 100 cattle samples were positive using any of the PCR assays.

3.2. Phylogeny and taxonomy

All analyzed forty five 18S sequences of either ~1700 bp or ~560 bp (Supplementary Table S1) together with representative sequences of species of *Babesia* spp. 'clade VI' sensu Schnittger et al. (2012) retrieved from GenBank (Supplementary Table S3) were included in the phylogenetic analysis (Supplementary Fig. S1). The dataset comprised of 123 GenBank sequences (including two *Theileria parva* sequences used as an outgroup), and 45 sequences from deer *Babesia* spp. obtained in our study. All of the deer *Babesia* isolates amplified in this study integrated into a monophyletic clade formed by *B. divergens*, *B. capreoli*, *B. odocoilei*, *B.* cf. *odocoilei* and *Babesia* sp. EU1, in a sister position to canine *Babesia* species. Together with annotated sequences from the GenBank database (Supplementary Table S3) and sequences from reference samples of *B. divergens* and *B. odocoilei*, five separate *Babesia* clades were distinguished of which four could be assigned to recognized species (Supplementary Fig. S1).

The phylogenetic analysis of the COX1 locus was based on 245 *Babesia* nucleotide sequences belonging to clade VI (sensu Schnittger et al., 2012), 27 of which were retrieved from GenBank and 218 sequenced in this study (Supplementary Table S2). Three *Theileria* species sequences were included as an outgroup (Supplementary Figs. S2 and S3). The overall topology of the resulting phylogenetic tree corresponds well to trees constructed based on the 18S gene (Corduneanu et al., 2017; Lack et al., 2012; Schnittger et al., 2012), but provides a better resolution of the taxa. All *Babesia* sequences amplified from deer and cattle form a separate and well-supported clade (100%/100%) indicating that they are a monophyletic group, in a sister position to canine *Babesia* species with 95.6%/99% support values. The group is further divided into four subclades *B. divergens*, *B. capreoli*, 'deer clade', *B.* sp. EU1 and a single *B. odocoilei* reference sequence (from *Cervus elaphus canadensis*, Canada) (Fig. 2, in red). The single available GenBank sequence of COX1 from *Babesia* species in this group belonging to *B. divergens* strain:Bd Rouen 1987 (accession no. LK935355) acquired by shot gun sequencing (Cuesta et al., 2014), confirms the identity of one of the four COX1 clades. This *B. divergens* clade includes 6 clones from 3 reference samples (A1, A5, A7 isolated from a human and two cattle babesiosis cases respectively, Supplementary Table S2), as well as 70 cloned sequences originating from 37 red deer samples and 4 sequences from sika deer.

Previous affiliation of our sample sequences in 18S analysis to respective *Babesia* species enabled us to assign two (*B. capreoli*, *Babesia* sp. EU1) out of four COX1 cervid clades. *Babesia* sp. EU1 clade was the least numerous, consisting of eight clones from four samples of roe deer with very low branch support (30%/73%). The greatest host species diversity was observed in the *B. capreoli* clade, which included isolates from all three deer species although most of them (32 clones/23 samples) originated from roe deer. The remaining sequences, almost all of which originated from red deer, formed the 4th clade labeled 'deer clade' (87 clones/60 samples from red deer and a single sequence from sika deer).

Analysis of COX1 sequences offers a clear difference between interspecific and intraspecific variation on the nucleotide level, where the intraspecific variation ranges from zero to 6.7% while the interspecific

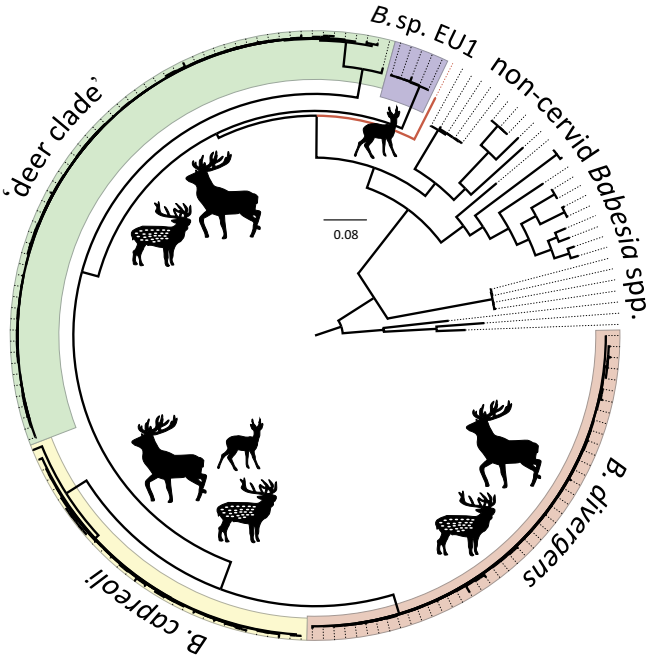

Fig. 2. Simplified visualization of phylogenetic tree based on consensus COX1 sequence dataset inferred using Maximum Likelihood analysis. Clade names assigning follows the 18S rDNA phylogenetic analysis of representative samples from each COX1 clade with GenBank retrieved sequences (Supplementary Table S3, Supplementary Figs. S2, S3). Sequence from reference sample of *B. odocoilei* is highlighted in red. For detailed view of the tree see Supplementary Fig. S2, S3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

one from 7.1 to 16.1% (Table 4) across the analyzed 900 nt long fragment. The herein presented variability of COX1 sequence can slightly reduce in future with growing number of obtained sequences. Our approach included the cloning step to solve the mixed PCR products from individuals co-infected by two different *Babesia* spp.; this can contribute to higher diversity of obtained sequences caused by the sequencing of single molecules containing random polymerase errors, rather than representing the major PCR product. On the contrary, in case of approx. 1800 nt of the 18S rRNA gene, the intervals for intraspecific (0–1.44%) and interspecific (0.81–4.65%) variability overlap (GenBank retrieved sequences analyzed, data not shown). Both 18S and COX1-based phylogenetic trees show a close relationship between *B. divergens* and *B. capreoli*. Although more 18S sequences were available for each clade, lower branch support values were observed in the 18S derived tree compared to the one based on COX1.

3.3. Host-specificity and coinfections

All four putative species identified in this study were present in both localities (Supplementary Fig. S4). *Babesia* sp. EU1 was identified exclusively in roe deer samples, *B. divergens* and isolates belonging to the 'deer clade' only in red and sika deer (Fig. 3, Supplementary Fig. S5). *B. capreoli* was isolated from all 3 deer species, albeit at a much lower prevalence in red deer than in the other two species. Fifteen COX1-positive individuals (eleven red, two roe, two sika deer respectively) were co-infected with two *Babesia* species. In red deer mixed infections of *B. divergens*/'deer clade' and *B. capreoli*/'deer clade' were detected; whereas mixed infections of *B. capreoli*/*B. divergens* and *B. capreoli*/*Babesia* sp. EU1 detected in sika deer and roe deer, respectively.

4. Discussion

Ungulates of the family Cervidae in the Palaearctic realm are

Table 4
Pairwise sequence identities (%) of nucleotide and amino acid (bold) sequences based on 927 nt long COX1 alignment used for inferring phylogeny displayed on Supplementary Fig. S2

	B. divergens	*B. capreoli*	*B. odocoilei*	*Babesia* sp. EU1	'deer clade'
B. divergens	96.4–100.0/**96.4–100.0**	**95.2–99.0**	**91.6–93.5**	**92.7–95.9**	**91.3–97.1**
B. capreoli	90.1–92.9	96.2–100.0/**96.4–100.0**	**91.9–93.8**	**92.7–95.2**	**90.9—96.8**
B. odocoilei	85.7–87.1/	86.3–87.6	N/A	**94.8–95.6**	**90.9–93.5**
Babesia sp. EU1	84.8–86.4/	84.6–87.3	87.8–88.3	98.8–99.9/**98.4–100.0**	**92.4–95.9**
'deer clade'	83.9–91.2	83.5–88.7	85.9–87.3	85.7–87.9	93.3–100.0/**96.1-100.0**

important hosts for range of tick species, which contributes to the diversity of detected piroplamids, including taxa of zoonotic importance. In contrast to canine *Babesia* spp., where closely related species of large *Babesia* probably evolved through processes of allopatric speciation (as they differ in geographic range and in their vectors), related *Babesia* spp. in various deer species frequently co-occur in mixed infections (Hoby et al., 2009; Michel et al., 2014; Razanske et al., 2019; Silaghi et al., 2011; Zanet et al., 2014). Moreover, they are all transmitted by the same tick vectors, chiefly *I. ricinus* - a ubiquitous tick with an extremely broad host specificity (Blaschitz et al., 2008; Øines et al., 2012; Overzier et al., 2013b; Venclikova et al., 2015).

In deer, *Babesia* species are usually reported at high prevalence rates, typically as asymptomatic infections detected by molecular diagnostics, although there are some notable exceptions of fatal cases reported in wild roe deer (reviewed by Malandrin et al., 2010), captive/semicaptive reindeer (Holman et al., 1994; Langton et al., 2003; Petrini et al., 1995; Silaghi et al., 2011) and chamois (Hilpertshauser et al., 2006; Hoby et al., 2009; Michel et al., 2014; Schmid et al., 2008; Zanet et al., 2014).

Our results, confirming the high prevalence of *Babesia* spp. and mixed infections in red, roe and sika deer, are broadly in line with previous studies; the three *Babesia* species identified in our study, *B. capreoli*, *Babesia* sp. EU1 and *B. divergens*, have frequently been reported throughout Europe (Andersson et al., 2016; Bonnet et al., 2007; Kauffmann et al., 2017; Malandrin et al., 2010; Michel et al., 2014; Overzier et al., 2013a; Razanske et al., 2019; Silaghi et al., 2011; Tampieri et al., 2008; Zintl et al., 2011). A fourth species, referred here as 'deer clade', matched sequences previously referred to as *Babesia* sp. or *Babesia* cf. *odocoilei* or *Babesia* CH1. This species, appearing as a sister taxon to North American *B. odocoilei*, seems to be common throughout European populations of red deer (Hilpertshauser et al., 2006; Kauffmann et al., 2017; Razanske et al., 2019; Silaghi et al., 2012; Zintl et al., 2011), sika deer (this study), moose (Puraite et al., 2016) and *I. ricinus* (Hilpertshauser et al., 2006; Øines et al., 2012).

In our material, there was no evidence of other piroplasmids occasionally reported in other studies, such as *Theileria capreoli* (Hornok et al., 2017; Sawczuk et al., 2008), *B. ovis*, *B. bigemina* or *B. microti*-like species (as reviewed by Silaghi et al., 2011). *Babesia pecorum* was never reported in Central Europe and is probably transmitted by ticks with more Mediterranean affinities (Hornok et al., 2017; Jouglin et al., 2014). The apparent absence of *Theileria* is noteworthy but not surprising. This species was suggested to be transmitted by *Haemaphysalis* ticks (Hornok et al., 2017), which have a strong association with lowland ecosystems in Central Europe (Nosek, 1971). Both study sites were located at medium to high altitude, precluding the presence of *Haemaphysalis concinna*, the major *Haemaphysalis* species occurring in the Czech Republic. Future investigation of piroplasmids in deer in lowland localities of the Czech Republic is warranted.

Our goal was to address the host specificity of *Babesia* of deer in localities of tight spatial coexistence of more host species, anticipating a homogenous infections pressure by the ubiquitous vector *Ixodes ricinus*. In roe deer, *B. capreoli* was the dominant *Babesia* species. The only other species detected in this host was *Babesia* sp. EU1, which was present in a relatively small proportion, though in both sampling sites. Interestingly, a search of the GenBank database reveals that the majority of sequences identified as EU1 (or as its synonym *B. venatorum*) were isolated from *I. ricinus* (or other ticks), not from mammalian hosts, suggesting that this zoonotic species may have other, as yet unknown reservoir hosts in Europe. Alternatively, its presence in deer may be overshadowed by co-occurring species making its detection less likely. As described elsewhere, also in our study the *Babesia* sp. EU1 was detected in roe deer exclusively.

Sika deer, native to East Asia, were introduced into many parts of Europe during the last century and one of our model sites was chosen for reason of its common presence (Borkovcová et al., 2009; Dvořák and Čermák, 2008). While sika deer in Europe are well established as competent hosts for *B. capreoli* (Gray et al., 1991; Gray et al., 1990), this is the first record of *B. divergens* in this deer species. Genetically similar (in the 18S and b-tubulin loci), *Babesia* '*divergens*-like' parasites have been reported in Japan (Zamoto-Niikura et al., 2014), however, these records are well outside the recognized geographical range of *B. divergens*, considered a European species (Zintl et al., 2003). Apparently,

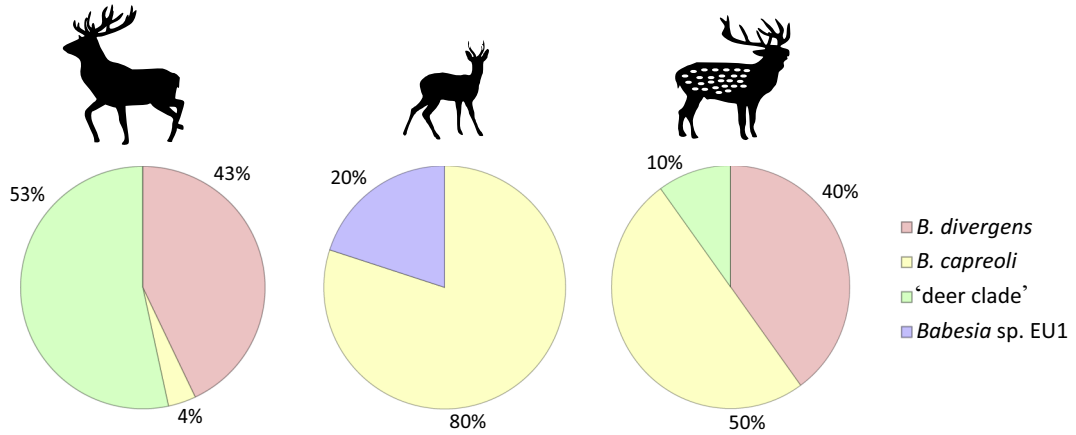

Fig. 3. *Babesia* spp. distribution in individual host species based on clones sequenced and used for inferring COX1 phylogeny.

invasive sika deers at our study locality enter the life cycle of at least three piroplasmid species detected in native deer fauna.

While the number of published molecular records of *B. divergens* in red deer is steadily growing, to our knowledge the parasite has never been successfully isolated and cultured from this host. Comprehensive biological and molecular characterization of the *B. divergens* isolates investigating the intraspecific variability will be necessary to unequivocally confirm their identity and epidemiology in various ecosystems. The present study, comparing the nuclear 18S rRNA and the mitochondrial COX1 genetic loci, provide strong evidence that *Babesia* isolates from deer and cattle are in fact both pertaining to *B. divergens*. However, the apparent widespread presence of *B. divergens* in red deer and, to a lesser extent in sika deer, in the Czech Republic is puzzling, considering that the parasite is apparently absent from cattle. No bovine babesiosis case has been reported in this country for many decades. Moreover, all 100 cattle samples investigated during this study were PCR-negative. It is questionable whether electric fencing separating pastures from deer-inhabited areas together with regular ivermectin treatment against ticks represent an effective barrier against transmission of *B. divergens*. On the other hand, the decline of bovine babesiosis has apparently occurred in some countries in spite of persistent infections in deer and ticks (Egyed et al., 2012; Zintl et al., 2011). It remains to be seen whether extensification of cattle farming favored under environmental policy will lead to the (re)emergence of the disease in cattle.

5. Conclusion

Similar to studies on other Apicomplexa, the majority of studies focused on the phylogeny and molecular epidemiology of *Babesia* spp. are based on partial 18S sequences. In contrast to the 18S, the COX1 gene possesses considerable interspecific sequence diversity and much reduced intraspecific sequence variation rendering it a better target for the differentiation of closely related species. Our results strongly support the usefulness of the COX1 locus for determining the phylogenetic relationships and clear discrimination of *Babesia* species. However, this study also raises important questions concerning the ubiquitous presence of *B. divergens* in red and sika deer in spite of its absence in the local cattle populations. The identity of a new *Babesia* sp. circulating in deer across Europe previously labeled *Babesia* sp. or *B.* cf. *odocoilei* or *Babesia* CH1 group and designated in this study as 'deer clade' is also a matter requiring further research. Application of advanced genomic approaches is seriously complicated by ubiquitous mix-infections and very low parasitaemia in naturally infected deer. Future *in vitro* and *in vivo* studies on deer isolates of *Babesia* spp. are warranted to answer persisting questions.

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Declarations of Competing Interest

None.

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