

An epidemiological study in wild carnivores from Spanish Mediterranean ecosystems reveals association between *Leishmania infantum*, *Babesia* spp. and *Hepatozoon* spp. infection and new hosts for *Hepatozoon martis*, *Hepatozoon canis* and *Sarcocystis* spp.

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Abstract

The aetiology and epidemiology of vector borne apicomplexan *Babesia* and *Hepatozoon* and kinetoplastid *Leishmania infantum* infections in wildlife have not been explored in wide areas of southern Spain. We investigated these infections in 151 wild carnivores, including foxes, badgers, beech martens, hedgehogs, wild cats, Egyptian mongooses, otters, genet and racoons. Overall, *Hepatozoon*, *Babesia* and *L. infantum* infections were detected in 68%, 48% and 23% of the wild animals surveyed, respectively. *L. infantum*-infected wildlife were more likely to be also infected with the apicomplexan *Hepatozoon* and *Babesia* spp. compared to the non-infected counterparts ($p < .05$). We report for the first time *Hepatozoon martis* in badgers and wild cats and *H. canis* in beech martens, and a *Babesia* sp. in wild cats from Spain. Our results also indicate the widespread distribution of *H. canis* in foxes (91%) and beech martens (13%), *H. martis* in beech martens (81%), wild cats (20%) and badgers (13%), *H. felis* in wild cats (60%), *B. vulpes* in foxes (64%), *Babesia* sp. type A closely related to *B. vulpes*, in badgers (58%) and *Babesia* sp. in wild cats (20%). Moreover, *L. infantum* infection was found in foxes (29%), beech martens (13%), badgers (8%) and 1/3 Egyptian mongooses. We also detected *Cytauxzoon* sp. in a wild cat and the first *Sarcocystis* sp. in a genet. This study provided evidence of increased risk of *L. infantum* infection in wildlife animals co-infected with *Babesia* spp. or *Hepatozoon* spp. and indicated that these infections are widespread in wild carnivores from Spanish Mediterranean ecosystems.

KEYWORDS

Babesia, *Cytauxzoon*, *Hepatozoon*, *Leishmania*, *Sarcocystis*, Spain, wildlife

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1 | INTRODUCTION

Vector-borne infections (VBIs) are an emerging problem worldwide, associated with factors influencing vector distribution, such as climate warming, and increased host exposure to vectors associated with modern lifestyle. Most VBIs are transmitted by ticks and insects, and affect humans and animals in interconnected domestic and sylvatic transmission cycles (Ortuño et al., 2019; Tomassone et al., 2018). *Babesia* and *Hepatozoon* spp. are widespread apicomplexan protozoans transmitted by ticks during their feeding process in the case of *Babesia* spp. and by ingestion of infected arthropod vectors in the case of *Hepatozoon* spp. Parasites of these two genera show a variable degree of host specificity. *Babesia* and *Hepatozoon* species infecting carnivores are characterized by molecular methods and display morphological differences in some cases. *Babesia* spp. infection may cause severe haemolytic syndromes but latent infections are also possible, and their virulence varies between species and the susceptibility of their different hosts (Irwin, 2010; Solano-Gallego & Baneth, 2011). Infection with *Hepatozoon* spp. is sometimes mild or asymptomatic, but severe illness has been reported (Baneth, 2011; Baneth et al., 2007). On the other hand, *Leishmania* is a kinetoplastid protozoan parasite infecting macrophages and monocytes, transmitted by sand flies whose vector transmission is restricted to the warm latitudes of the planet, including Spain, where *Leishmania infantum* is the only endemic *Leishmania* species. The dog is the primary domestic reservoir of this parasite, but infection has been described in a wide range of host species, including humans. While most *Leishmania*-infected animals remain asymptomatic, infection may be life threatening particularly to dogs and people (Solano-Gallego & Baneth, 2008).

Babesia, *Hepatozoon* and *Leishmania* coinfections have been reported and are to be expected where ticks, sand fly vectors and their hosts share a habitat (Medkour, et al., 2020; Medkour, et al., 2020; Yisaschar-Mekuzas et al., 2013). Yet, few studies have investigated the clinical and immunopathological mechanisms of coinfections with these parasites (De Tommasi et al., 2014), and the risk of coinfections has rarely been studied to evaluate the epidemiological relationships between them (Mekuzas et al., 2009; Toepp et al., 2019). Th1 cell-mediated immune responses are considered the most effective line of defence against intracellular parasites (Day, 2011). Indeed, animals with a limited capacity to elicit a cellular response are more susceptible to develop clinical leishmaniosis, and it is influenced by inheritable traits (Iborra et al., 2007; Solano-Gallego et al., 2000). Coinfections may also be facilitated by immunosuppression resulting from these parasites targeting immune response mechanisms and modifying them.

The present study investigated *Babesia*, *Hepatozoon* and *Leishmania* infections in wild carnivores from southern Spain. This is a typical *L. infantum* endemic area and infection with this parasite has been reported in humans, dogs and wildlife (Chitimia et al., 2011; Ortuño et al., 2017; Risueño et al., 2018; Sobrino et al., 2008), but information on infection with haemoprotozoa apicomplexan parasites in wildlife from this area is scarce. Therefore, the objectives of this study were to estimate the spatial and temporal distribution of infection with these parasites, characterize the piroplasmid and *Hepatozoon* spp. found by

DNA sequencing and investigate the risk of coinfection between these and *L. infantum*.

2 | MATERIALS AND METHODS

2.1 | Study population and experimental design

The study included samples from 151 wild animals from the autonomous communities of Murcia and Valencia in southeast Spain, Andalusia in the south and Extremadura in the southwest (Figure 1), collected between 2011 and 2018. It included 89 red foxes (*Vulpes vulpes*), 24 badgers (*Meles meles*), 16 beech martens (*Martes foina*), 6 hedgehogs (*Erinaceus europaeus*), 5 wild cats (*Felis silvestris*), 3 Egyptian mongooses (*Herpestes ichneumon*), 3 genets (*Genetta genetta*), 3 otters (*Lutra lutra*) and 2 raccoons (*Procyon lotor*). Except for raccoons which are introduced and are an invasive species, the rest of the species are native to Spain, including genets and Egyptian mongooses, which were introduced centuries ago and considered naturalized. Animals were either found dead by local authorities, legally hunted (foxes) or had died while being cared for at wildlife rescue centres following injury.

All 151 animals were tested for *Babesia* spp. and *Hepatozoon* spp. using DNA extracted from spleen samples, which provides a high diagnostic sensitivity to detect infection by these parasites (Cardoso et al., 2014). In contrast, *L. infantum* diagnosis in this study was performed for 75 animals from south and southwest Spain (31 foxes, 20 badgers, 7 beech martens, 2 wild cats, 6 hedgehogs, 3 Egyptian mongooses, 3 genets and 3 otters), and the remaining 76 animals included had already been analysed for *Leishmania* infection (Risueño et al., 2018). *L. infantum* tests results from all 151 animals were used in the present study to evaluate risk factors and coinfection with other parasites. *L. infantum* diagnoses were performed using DNA from spleen samples, all of which provide a high diagnostic sensitivity for detecting *L. infantum* infection (Chitimia et al., 2011; de Andrade et al., 2006). Data from animals included the following demographic variables: sex, age, date and place of capture.

2.2 | DNA extraction

DNA was extracted using an automatized system (Maxwell® Promega, Madison, WI, USA) or a commercial kit ('HigherPurity™ Tissue DNA Purification Kit', Canvax®, Spain). DNA concentration and purity were analysed using the NanoDrop 2000® spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA).

2.3 | Screening for *Babesia* spp. and *Hepatozoon* spp. by generic PCR amplification

Conventional PCR was performed to amplify an approximately 360 base pair (bp) of the common *Babesia* spp. and *Hepatozoon* spp. 18S ribosomal RNA sequence (rRNA) using primers Piroplasmid F (5'-CCA



Figure 1 Areas in southern Spain where the study was performed

GCA GCC GCG GTA ATT C-3') and Piroplasmid R (5'-CTT TCG CAG TAG TTY GTC TTT AAC AAA TCT-3') (Tabar, 2008). PCR was performed using the Syntezza PCR-Ready High Specificity kit (Syntezza Bioscience, Israel), and 1 µl of each primer (10 µM), 19 µl Ultra-Pure Water (UPW) and 4 µl DNA. The following conditions were used for amplification: 95°C for 5 min; 35 cycles of 95°C for 30 s, 64°C for 45 s and 72°C for 30 s; and 72°C for 5 min (Margalit Levi et al., 2018).

PCR assays included negative and positive DNA controls from a laboratory-bred pathogen-free dog and from dogs naturally infected with *Babesia vogeli* or *Hepatozoon canis*, and non-template DNA controls (NTC). PCR products were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide and evaluated under UV light for the size of amplified fragments by comparison to a 100 bp DNA molecular weight marker.

2.4 | Specific *Babesia* spp. and *Hepatozoon* spp. PCR amplifications

An additional PCR using specific primers for *Babesia* spp. or *Hepatozoon* spp. 18S ribosomal RNA gene was performed in samples testing positive to the Piroplasmid PCR. The *Babesia* species-specific PCR amplified a 408-bp 18S RNA sequence with PIRO-A (5'-AAT ACC CAA TCC TGA CAC AGG G-3') and PIRO-B (5'-TTA AAT ACG AAT GCC CCC AAC -3')

primers (Olmeda et al., 1997). The PCR was run with the same water, DNA and primer concentrations as described above, and the conditions used for amplification were: 95°C for 5 min; 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s; and 72°C for 5 min (Barbosa et al., 2020).

The *Hepatozoon* species-specific PCR amplified a 574-bp 18S RNA sequence with the *Hepatozoon* 18S forward (5'-GGT AAT TCT AGA GCT AAT ACA TGA GC -3') and reverse (5'-ACA ATA AAG TAA AAA ACA YTT CAA AG -3') primers (Almeida et al., 2012). The PCR was run with the same water, DNA and primer concentrations as described above, and the conditions used for amplification were: 95°C for 5 min; 35 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 30 s; and 72°C for 5 min (Sarma et al., 2019).

2.5 | DNA sequencing

All positive PCR products were sequenced at the Center for Genomic Technologies, Hebrew University of Jerusalem with both forward and reverse primers.

The sequences were aligned and analysed using the ClustalW alignment program (Larkin et al., 2007) and the MEGA X software (Kumar et al., 2018), respectively, and compared to sequences deposited in GenBank, using the Basic Local Alignment Search Tool (BLAST,

<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). A result was considered positive for a certain pathogen if it was the first match on BLAST and had at least a 97% identity with a known GenBank accession.

2.6 | *Leishmania infantum* detection

Samples from animals analysed by Risueño et al. (2018) had been previously tested using the TaqMan probe real-time PCR (rt-PCR) assay for kinetoplast DNA (kDNA) described by Mary et al. (2004). Samples analysed for the present study were tested using a *Leishmania*-FAST15 TaqMan probe rt-PCR assay similarly targeting kDNA sequences, with primers LEISH-1 (5'-AACTTTTCTGGTCTCCGGGTAG-3') and LEISH-2 (5'-ACCCCCAGTTTCCCGCC-3'), and TaqMan-MGB probe (FAM-5'-AAAAATGGGTGCAGAAAT-3'-non-fluorescent quencher-MGB) (Dantas-Torres et al., 2017; Francino et al., 2006). The *L. infantum* parasite load was estimated by considering the rt-PCR amplification threshold cycle (CT) at which a near logarithmic PCR product generation is detected (Gomes et al., 2008). Samples with a Ct > 38 were considered negative.

2.7 | Phylogenetic and genetic distance analyses

Phylogenetic and genetic distance analyses were performed using the Molecular Evolutionary Genetics Analysis software MEGAX (Kumar et al., 2018). The maximum likelihood method was used to infer tree topology. Confidence values for individual branches of the resulting trees were determined by bootstrapping analysis considering a 70% or higher value significant. Homologous sequence of *Plasmodium falciparum* (accession number MF155937) was used as outgroup sequences for *Babesia*, *Cytauxzoon* and *Sarcocystis* spp. phylograms, while *Sarcocystis cymruensis* (MG564723) was used for the *Hepatozoon* spp. trees.

Genetic distance (expressed in %) among a selection of representative sequences was calculated using the Kimura 2-parameter substitution model with gamma distributed (G) rates and pairwise deletion to treat gaps and/or missing data (Kimura, 1980), implemented in the MEGA X software.

2.8 | Statistical analysis

The proportion of infected foxes and badgers (the most abundant species tested) and median *L. infantum* PCR CTs among positive animals were compared according to levels of categorized explanatory variables using Fisher's exact test and the non-parametric Kruskal–Wallis test, respectively. Explanatory variables were age (young or adult), collection years (two equal size categories: 2013–15 and 2016–18 for foxes, and 2011–14 and 2015–17 for badgers), season and geographical origin of the animals (southeast and south-southwest). Statistical significance was taken for $p < .05$ for a two-tailed test. All analyses were performed with R software (<http://cran.r-project.org/>).

3 | RESULTS

3.1 | Frequency of *Babesia* spp., *Hepatozoon* spp. and *L. infantum* infection

Among the 151 animals analysed, 123 (81%) were positive to at least one pathogen. The overall infection prevalence (range in positive host species) was 48% (20–64%) for *Babesia* spp., 68% (13–94%) for *Hepatozoon* spp. and 21% (8–33%) for *L. infantum* (Table 1). *Leishmania infantum* prevalence in samples from the south and southwest of Spain tested here for the first time was 5% (3–33%). Median (range) PCR CTs among *L. infantum* positive animals were 34 (10–38) for all host species, and 34 (10–38), 37 (37–38), 38 (37–38) and 38 (38–38) for foxes, badgers, beech martens and the Egyptian mongoose, respectively ($p < .05$).

In foxes, the prevalence of *B. vulpes*, *H. canis* and *L. infantum* was higher in the southeast compared to south and southwest of Spain (78% vs. 39%, 100% vs. 74% and 46% vs. 3%, respectively) ($p < .05$) and in 2013–2015 compared to 2016–2018 (80% vs. 42%, 98% vs. 79% and 52% vs. 12%, respectively) ($p < .05$). Similarly, in badgers, *H. martis* prevalence was higher in the southeast compared to the south and southwest (75% vs. 0%) ($p < .05$). None of the other associations between infection and explanatory variables were significant ($p > .05$).

In addition, PCR amplification with the piroplasmid primers allowed identifying *Sarcocystis* spp. infection in one of three genets and *Cytauxzoon* sp. in one of five wild cats.

3.2 | DNA sequence characterization of *Babesia* spp., *Hepatozoon* spp., *Cytauxzoon* spp. and *Sarcocystis* spp., and phylogenetic and genetic distance analyses

The 57 fox *Babesia* spp. sequences (isolates BV1–3) corresponded to *B. vulpes*, with a 99.7–100% identity with *B. vulpes* from a red fox from Iraq (MK957183) and *B. vulpes* from a dog from Galicia in NW Spain (MK585200) (Table 2). The fox *B. vulpes* BV1–3 sequences from this study clustered together in a phylogenetic tree with homologous GenBank *B. vulpes* sequences from dogs and foxes and were separated from sequences of other close *Babesia* spp. with a bootstrap value of 93% (Figure 2). *Babesia* spp. sequences from 14 badgers (BSP1 and BSP3) were 100% similar to *Babesia* sp. badger type A from badgers in China (MG799845) and the United Kingdom (MT077002). In the phylogenetic analysis, *Babesia* sp. types A and B from badgers clustered separately (98% and 99% bootstrap values, Figure 2) and were close to *B. vulpes* sequences (0.9–5.5% nucleotide divergence, Table 1S). The wild cat *Babesia* sequence (BSP2) had a 98.7% similarity to *Babesia* sp. from foxes in Israel and golden jackals from Iraq (KJ956782 and MK957184), clustering together (91% bootstrap value), and separated to *Babesia* sp. in wild carnivores from Africa but closely related to them (4.1–5.3% nucleotide divergence; Figure 2, Table 1S).

Hepatozoon spp. from all foxes (isolates HC1–2, HC4–13 and HC15) and two beech martens (HC3 and HC14) were 99.1–100% identical to *H. canis* (MN628317, MN628318, MK645967, MN791089,

TABLE 1 Number (%) of *Babesia* spp., *Hepatozoon* spp. and *Leishmania infantum* PCR-positive wildlife animal species detected from spleen samples

	N	<i>Babesia</i>			<i>Hepatozoon</i>				<i>Leishmania infantum</i> ^a	Animals infected by at least one pathogen
		<i>vulpes</i>	other <i>Babesia</i> spp.	all	<i>canis</i>	<i>felis</i>	<i>martis</i>	all		
Foxes	89	57 (64)	0 (0)	57 (64)	81 (91)	0 (0)	0 (0)	81 (91)	26 (29)	87 (98)
Badgers	24	0 (0)	14 (58)	14 (58)	0 (0)	0 (0)	3 (13)	3 (13)	2 (8)	15 (63)
Beech martens	16	0 (0)	0 (0)	0 (0)	2 (13)	0 (0)	13 (81)	15 (94)	2 (13)	15 (94)
Wild cats	5	0 (0)	1 (20)	1 (20)	0 (0)	3 (60)	1 (20)	4 (80)	0 (0)	5 (100)
Hedgehogs	6	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Egyptian mongooses	3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (33)	1 (33)
Otters	3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Genets	3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Raccoons	2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
All	151	57 (38)	15 (10)	72 (48*)	83 (55)	3 (2)	17 (11)	103 (68*)	31 (21)	123 (81)

*Significant differences between species ($p < .001$).

^aSeventy-six of these animals were previously analysed for *L. infantum* in the study of Risueño et al. (2018).

MN628329, KP216425, MK957188 and MK757802) (Table 2). The *Hepatozoon* spp. samples from the remaining 13 beech martens (HM2-3 and HM-6) and from 3 badgers (HM4 and HM5) and 1 wild cat (HM1) were 99.4–100% identical to *H. martis* (MG136688 and MH656728). The *Hepatozoon* sp. from the three other wild cats (HF1-3) had a 99.1–100% identity with *H. felis* (MG386483, MG386484 and AY628681) (Table 2). In the phylogenetic trees, *H. canis* sequences from foxes and beech martens clustered together with homologous sequences found in canids, presenting less than 3% of nucleotide divergence (Table 2S), and were found to segregate with a 81–96% bootstrap into a different clade from other *Hepatozoon* spp. (Figures 3 and 4). *H. felis* sequences from wild cats grouped together with homologous sequences from domestic and wild felids (bootstrap value 85–99%; Figures 3 and 4) and presented less than 1% nucleotide divergence (Table 3S). The *H. martis* sequences retrieved from badgers, wild cats and beech martens presented less than 2% nucleotide divergence (Table 3S), and clustered together in a separate clade from *H. felis* sequences with a bootstrap value of 76–84% (Figures 3 and 4).

The *Cytauxzoon* sp. sequence found in a wild cat was 100% identical to a common fragment in three new *Cytauxzoon* species (*C. europaeus*, *C. otrantorum* and *C. banethi*) described in wild felids from European countries, named by Panait et al. (2021) (MT904044, MT904033 and MT904029) (Table 2). They all clustered together (99% bootstrap value; Figure 5) and were different from *C. felis* (3.1–8.2% of nucleotide divergence; Table 4S).

A *Sarcocystis* sp. sequence found in a genet was 100% identical to *S. cymruensis* from brown rats from China and Grenada (West Indies) and to *S. muris* from a cat from Egypt, and 99.1% identical to *S. muris* from mice (MT372786, MG564723, KC877996 and M64244). Consequently, it clustered together with *Sarcocystis* spp. sequences from these rodents (100% bootstrap value), and separately to those retrieved from wild carnivores (Figure 6). The estimated evolutionary

divergence for the *Sarcocystis* sp. sequence from the genet was 0% when compared to *S. cymruensis* from brown rats, *S. muris* from a cat and *S. rodentifelis* from a vole, and 0.6% compared to *S. muris* (M64244) from a mouse (Table 5S).

3.3 | Coinfections

The prevalence of *Babesia* spp. or *Hepatozoon* spp. was significantly higher in *L. infantum*-positive wild animals compared to *L. infantum*-negative individuals ($p < .05$, Table 3). When considering individual host species, this relationship was marginally significant only in foxes between *L. infantum* and *B. vulpes* or *H. canis* ($p < .10$) (Table 6S).

Thirty foxes were coinfecting with *H. canis* and *B. vulpes*, 5 foxes with *H. canis* and *L. infantum* and 21 foxes were coinfecting with all 3 three pathogens. Two badgers had *Babesia* sp. type A and *H. martis*, and another two had *Babesia* sp. type A and *L. infantum*. One beech marten was infected with *H. canis* and *L. infantum* and another one had *H. martis* and *L. infantum* infections. Finally, one wild cat was coinfecting with *H. felis* and *Cytauxzoon* sp. (Table 4).

4 | DISCUSSION

This study describes for the first time the prevalence and characterization of *Babesia* spp., *Hepatozoon* spp. and *L. infantum* in wildlife in previously uncharted areas of southern Spain and provides an account of the risk of coinfections between these parasites. *Hepatozoon* spp. and piroplasmid spp. infections were widespread among wildlife. *L. infantum* prevalence was comparatively lower, and those animals infected were also more likely to have *Babesia* spp. and *Hepatozoon* spp. compared to those not infected with the apicomplexans. Furthermore, it

TABLE 2 Results of the BLAST analysis for the *Babesia* spp. and *Hepatozoon* spp. positive samples

New GenBank accession No.	Isolate	Parasite species	Wildlife mammalian host (N)	Primers	Sequence length (bp)	Closest GenBank accession No.	Identity (%)	Query cover (%)
MW578971	BSP1	<i>Babesia</i> sp. badger type A	Badger (8)	Piropl-F/R	311	MT077002	100	100
MW578972	BSP2	<i>Babesia</i> sp.	Wild cat (1)	Piropl-F/R	312	MK957184	98.7	100
MW578973	BSP3	<i>Babesia</i> sp. badger type A	Badger (6)	Piro-A/B	370	MG799845	100	100
MW578974	BV1	<i>Babesia vulpes</i>	Fox (4)	Piropl-F/R	316	MK957183	100	100
MW578975	BV2	<i>Babesia vulpes</i>	Fox (51)	Piro-A/B	375	MK585200	100	100
MW578976	BV3	<i>Babesia vulpes</i>	Fox (2)	Piro-A/B	375	MK585200	99.7	100
MW578979	HC1	<i>Hepatozoon canis</i>	Fox (18)	Piropl-F/R	337	MN628317	100	100
MW578980	HC2	<i>Hepatozoon canis</i>	Fox (2)	Piropl-F/R	337	MN628318	100	100
MW578981	HC3	<i>Hepatozoon canis</i>	Beech marten (1)	Piropl-F/R	337	MN628318	100	100
MW578982	HC4	<i>Hepatozoon canis</i>	Fox (1)	Piropl-F/R	337	MN791089	99.7	100
MW578983	HC5	<i>Hepatozoon canis</i>	Fox (44)	Piropl-F/R	337	MN791089	100	100
MW578984	HC6	<i>Hepatozoon canis</i>	Fox (1)	Piropl-F/R	337	MN628317	99.1	100
MW578985	HC7	<i>Hepatozoon canis</i>	Fox (1)	Piropl-F/R	337	MK645967	99.1	100
MW578986	HC8	<i>Hepatozoon canis</i>	Fox (1)	Piropl-F/R	337	MN628317	99.4	100
MW578987	HC9	<i>Hepatozoon canis</i>	Fox (1)	Piropl-F/R	337	MN628317	99.7	100
MW578988	HC10	<i>Hepatozoon canis</i>	Fox (4)	Piropl-F/R	337	MN628329	99.7	100
MW578989	HC11	<i>Hepatozoon canis</i>	Fox (1)	Piropl-F/R	337	MK645967	100	100
MW578990	HC12	<i>Hepatozoon canis</i>	Fox (1)	Piropl-F/R	337	MK645967	99.4	100
MW578991	HC13	<i>Hepatozoon canis</i>	Fox (3)	Piropl-F/R	337	KP216425	100	100
MW578992	HC14	<i>Hepatozoon canis</i>	Beech marten (1)	Hep18S-F/R	515	MK957188	99.6	100
MW578993	HC15	<i>Hepatozoon canis</i>	Fox (3)	Hep18S-F/R	515	MK757802	99.8	100
MW578994	HF1	<i>Hepatozoon felis</i>	Wild cat (1)	Piropl-F/R	337	MG386483	100	100
MW578995	HF2	<i>Hepatozoon felis</i>	Wild cat (1)	Piropl-F/R	337	MG386484	99.1	100
MW578996	HF3	<i>Hepatozoon felis</i>	Wild cat (1)	Hep18S-F/R	515	AY628681	100	100
MW578997	HM1	<i>Hepatozoon martis</i>	Wild cat (1)	Piropl-F/R	337	MG136688	99.4	100
MW578998	HM2	<i>Hepatozoon martis</i>	Beech marten (2)	Piropl-F/R	337	MG136688	99.4	100
MW578999	HM3	<i>Hepatozoon martis</i>	Beech marten (10)	Piropl-F/R	337	MG136688	100	100
MW579000	HM4	<i>Hepatozoon martis</i>	Badger (1)	Piropl-F/R	337	MG136688	100	100
MW579001	HM5	<i>Hepatozoon martis</i>	Badger (2)	Hep18S-F/R	515	MH656728	100	100
MW579002	HM6	<i>Hepatozoon martis</i>	Beech marten (1)	Hep18S-F/R	515	MH656728	100	100
MW578977	CYT1	<i>Cytauxzoon</i> sp.	Wild cat (1)	Piropl-F/R	307	MT904044	100	100
MW578978	SC1	<i>Sarcocystis</i> sp.	Genet (1)	Piropl-F/R	325	MG564723	100	100

is the first report of *H. canis* infection in a beech marten and of *H. martis* in wild cats. We also detected *H. martis* in three badgers, which agrees with the finding of Barandika et al. (2016), who reported infection with a *Hepatozoon* sp. in a badger from northern Spain which, due to the sequence identity, was probably the species described later as *H. martis* in martens by Hodžić et al. (2018). *Hepatozoon* spp. infection has been scarcely and only locally investigated in wildlife in Spain. The overall 68% prevalence of infection found in the present study suggests that these parasites are very common and efficiently transmitted in sylvatic environments. Moreover, based on the present study and other reports, *Hepatozoon* spp. seem to have low host specificity

(Criado-Fornelio et al., 2006, 2007; Smith, 1996). However, there were significant differences in the prevalence of *Hepatozoon* spp. infection between host species, ranging from 80% to 91% in foxes, wild cats and beech martens, 13% in badgers and 0% in all other species. Similarly, high prevalences were detected in foxes in the Iberian Peninsula, including 76% in Portugal and 90% in Guadalajara, central Spain (Cardoso et al., 2014; Criado-Fornelio et al., 2006). Likewise, *Hepatozoon* spp. infection in badgers was less common in previous studies, and estimated prevalence values were 1% in northern Spain (Barandika et al., 2016) and 0% in central Spain and northern Italy (Battisti et al., 2020; Gimenez et al., 2009). The reasons for such diverging differences

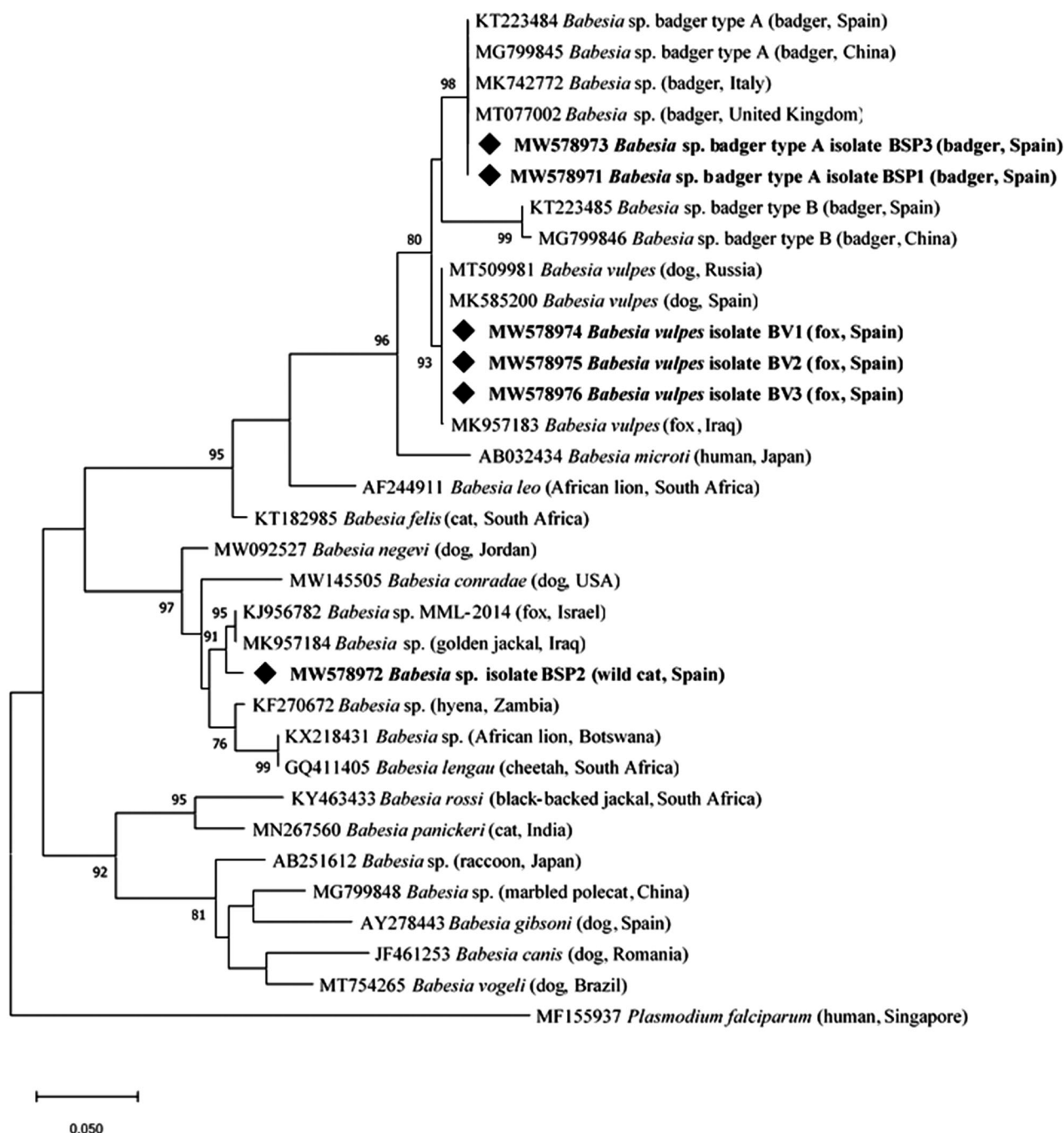


Figure 2 Phylogenetic tree of *Babesia* spp. sequences from wild carnivores in this study (diamond-shaped) together with homologous sequences from GenBank. The evolutionary history was inferred by using the maximum likelihood method and Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap values below 70% are not shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated, that is, fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position (partial deletion option). There were a total of 236 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

between host species could be related to the epidemiology of each *Hepatozoon* sp., with relation to the abundance of its competent vectors in different areas, other possible modes of transmission, such as transmission by carnivorous or transplacentally, and the abundance of other sympatric species infected with the same species in the study region. In addition to ticks, certain *Hepatozoon* spp. are transmitted

by other arthropods (Smith, 1996) as well as transplacentally (Murata et al., 1993) and by carnivorous (Johnson et al., 2009; Smith, 1996). However, the relative importance of these modes of transmission is unknown. Moreover, no transmission route has yet been described for *H. felis* and *H. martis* (Baneth et al., 2013; Hodžić et al., 2018). *H. canis* is efficiently transmitted by *Rhipicephalus sanguineus*, which is the most

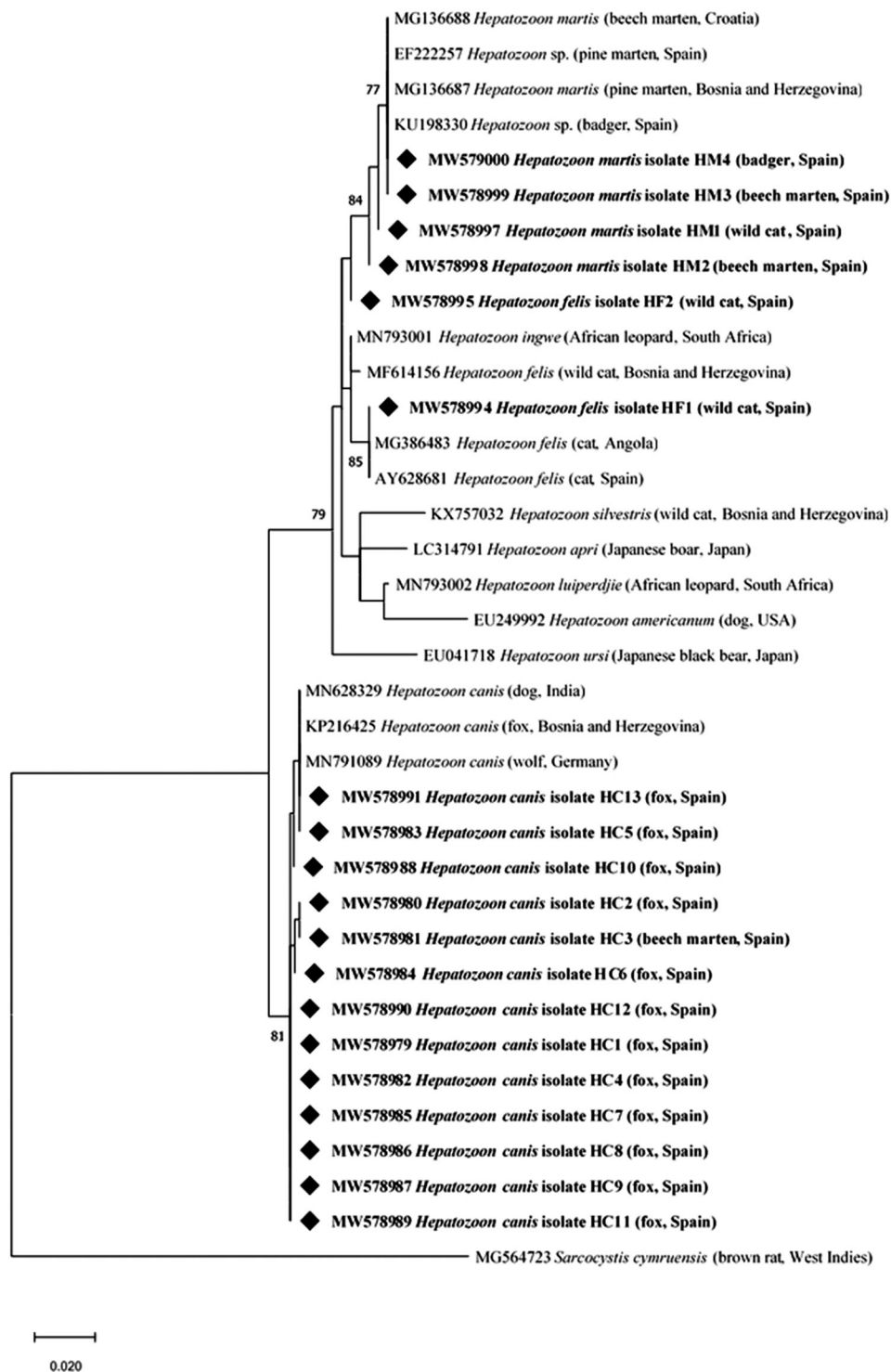


Figure 3 Phylogenetic tree of *Hepatozoon* spp. sequences with Piroplasmid F/R primers from wild carnivores in this study (diamond-shaped) together with homologous sequences from GenBank. The evolutionary history was inferred by using the maximum likelihood method and Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap values below 70% are not shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated, that is, fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position (partial deletion option). There were a total of 332 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

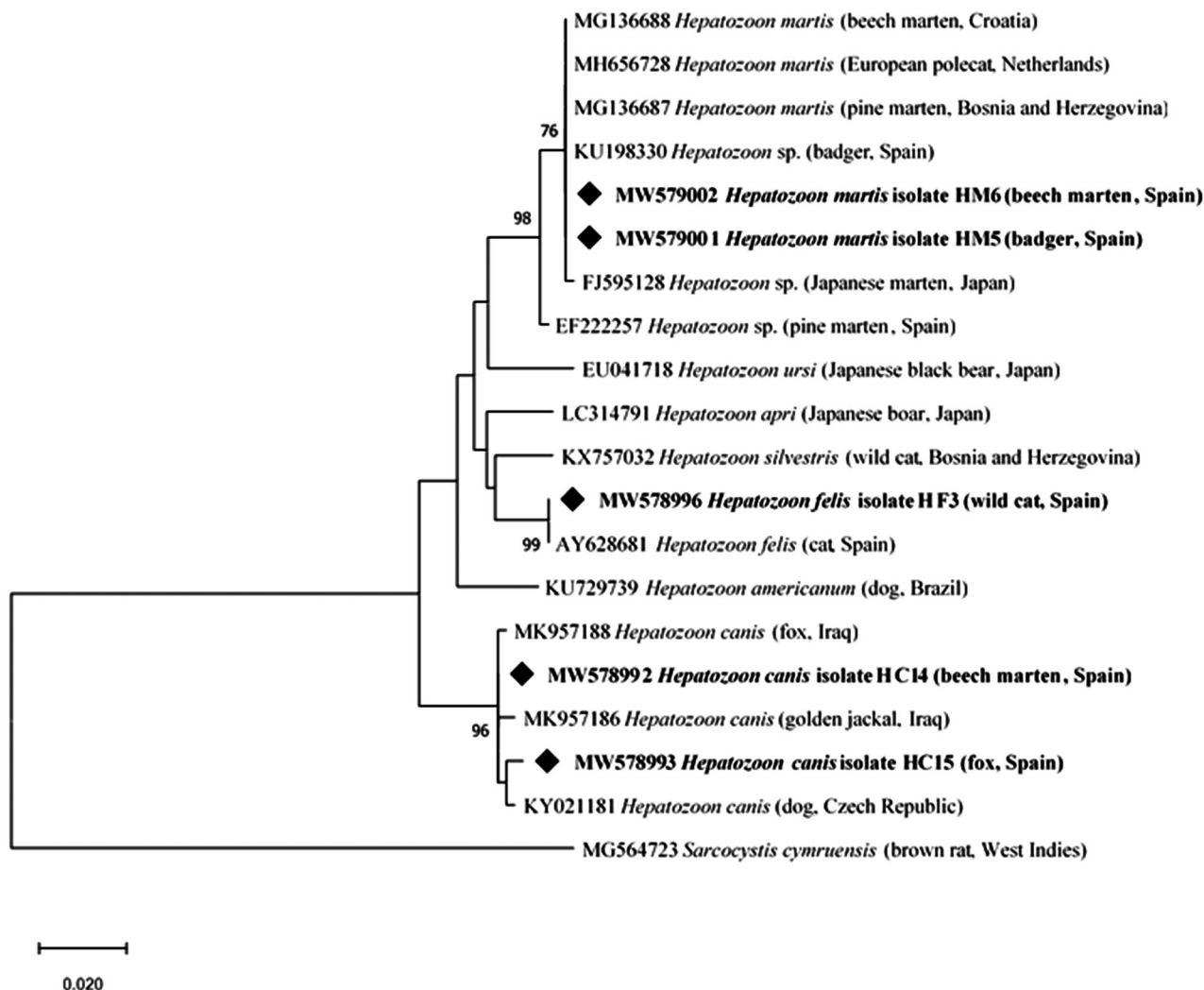


Figure 4 Phylogenetic tree of *Hepatozoon* spp. sequences with Hep18S F/R primers from wild carnivores in this study (diamond-shaped) together with homologous sequences from GenBank. The evolutionary history was inferred by using the maximum likelihood method and Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap values below 70% are not shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated, that is, fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position (partial deletion option). There were a total of 515 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

prevalent and abundant tick species in dogs in urban and rural areas in Spain (Checa et al., 2019; Estrada-Peña et al., 2017), but much less so in foxes in Spain (< 4% prevalence and < 1% abundance) (Checa et al., 2018; Sanchis Monsonís, 2016; Serrano Barrón, 2004), except in Murcia, where 55% of foxes were shown to be infested with *R. sanguineus* (Martínez-Carrasco et al., 2007).

The high prevalence (64%) of *B. vulpes* in foxes is in agreement with other studies from the Atlantic northwest of the Iberian Peninsula (63–100%) and central Spain (50%) (Cardoso et al., 2013; Criado-Fornelio et al., 2007; García, 2006; Miró et al., 2015). Our results indicate that this parasite is also efficiently transmitted in southern Spain. Animals may become infected with *Babesia* spp. by tick vectors, congenitally and possibly by blood exchange through dog fighting (Yeagley et al., 2009). *Ixodes hexagonus* was considered a potential vector of *B. vulpes* in northwest Spain based on its abundance and the detection of DNA

of the parasite in this tick species (Camacho et al., 2003; Checa et al., 2018). However, *I. hexagonus* was not detected in a previous study in Murcia (Martínez-Carrasco et al., 2007) or in other Southern and Mediterranean regions in Spain (Estrada-Peña et al., 2017), except in the Autonomous Community of Valencia where it was found in 20% of 272 foxes analysed (Sanchis, 2016). *B. vulpes* is also prevalent in areas where *I. hexagonus* is not a common tick, including the United States (Birkenheuer et al., 2010). Clearly, other transmission modes exist for *B. vulpes* that need to be investigated (Barash et al., 2019; Simões et al., 2011).

Babesia spp. infection was also widespread in badgers and it was detected in one wild cat for the first time in Spain. Prevalence in badgers (58%) was higher than in a previous study in northern Spain, where 35% (43/122) of badgers were *Babesia* sp. positive (Barandika et al., 2016). Barandika et al. (2016) described two variants of

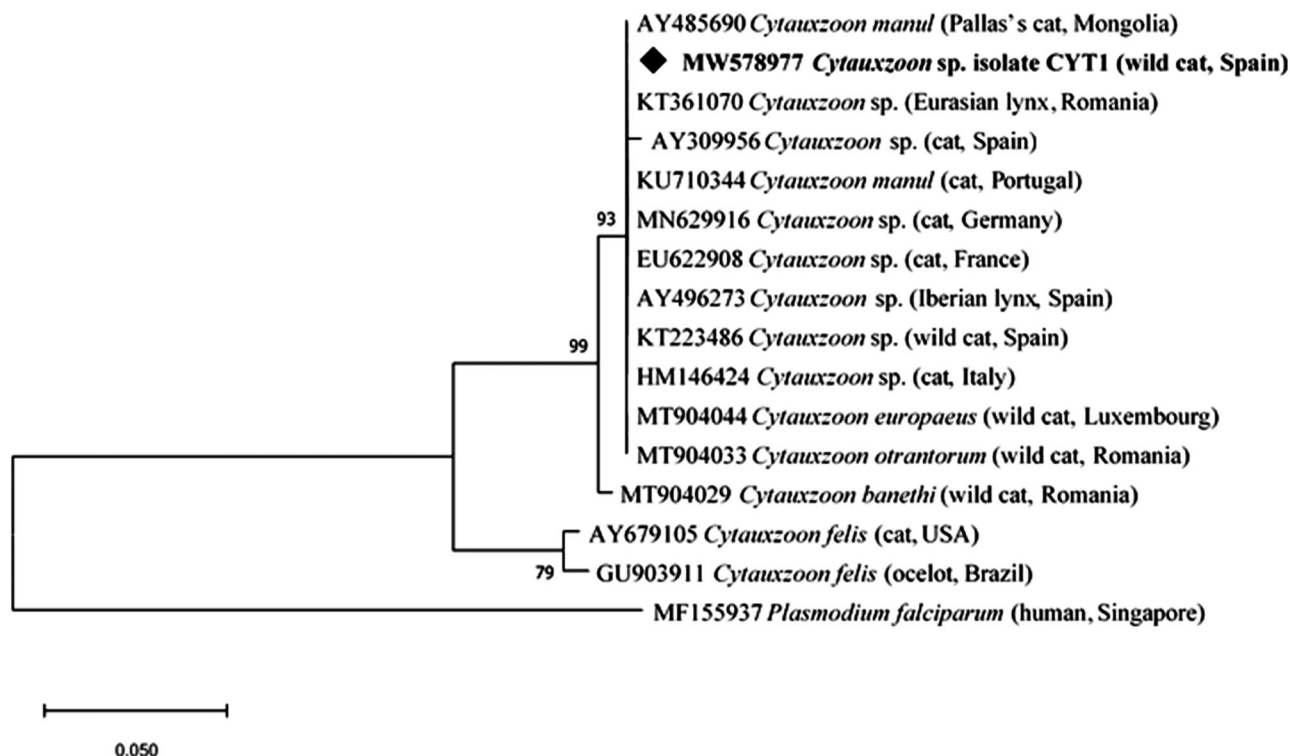


Figure 5 Phylogenetic tree of *Cytauxzoon* spp. sequence from a wild cat in this study (diamond-shaped) together with homologous sequences from GenBank. The evolutionary history was inferred by using the maximum likelihood method and Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap values below 70% are not shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated, that is, fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position (partial deletion option). There were a total of 260 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

TABLE 3 Relationship between *Babesia*, *Hepatozoon* and *L. infantum* infections

Parasite	Infected	No.	Babesia spp.		Hepatozoon spp.		L. infantum ^a	
			% positive	p-value	% positive	p-value	% positive	p-value
Babesia spp.								
	Yes	72	–	–	74	.2209	32*	.0011
	No	79	–		63		10	
Hepatozoon spp.								
	Yes	103	51	.2209	–	–	27*	.0024
	No	48	40		–		6	
L. infantum ^a								
	Yes	31	74 [*]	.0011	90 [*]	.0024	–	–
	No	120	41		63		–	

^aData of *L. infantum* infection in wild carnivores from southwest (N = 75) and southeast (N = 76) Spain. The *L. infantum* results from southeast Spain were part of a previous study (Risueño et al., 2018) and included here because they were analysed for additional pathogens in the present study.

*Significant differences (p < .01).

badger-associated *Babesia* sp., named 'type A' and 'type B', which have been reported elsewhere in Europe and China (Barandika et al., 2016; Bartley et al., 2017; Guardone et al., 2020; Hornok et al., 2018; Santoro et al., 2019). *Babesia* sp. type A found here in badgers is closely

related to *B. vulpes*. In contrast, the sequence from the wild cat is close to *Babesia* sp. from golden jackals from Iraq and red foxes from Israel, and more distantly related to *B. lengau* from cheetahs and domestic cats in South Africa (Bosman et al., 2010; Margalit Levi et al., 2018;

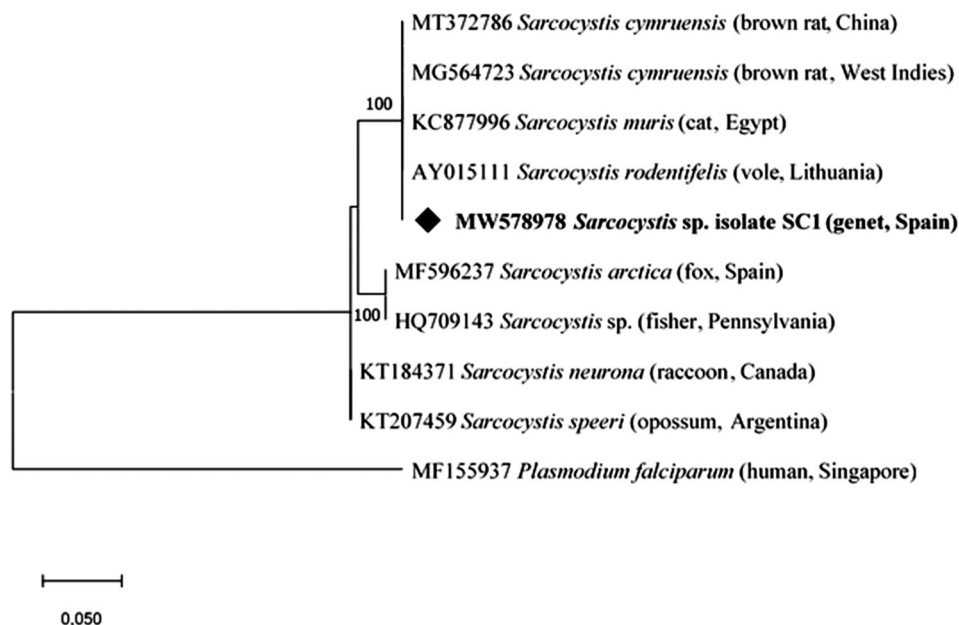


Figure 6 Phylogenetic tree of *Sarcocystis* spp. sequence from a genet in this study (diamond-shaped) together with homologous sequences from GenBank. The evolutionary history was inferred by using the maximum likelihood method and Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bootstrap values below 70% are not shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions with less than 95% site coverage were eliminated, that is, fewer than 5% alignment gaps, missing data and ambiguous bases were allowed at any position (partial deletion option). There were a total of 323 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

TABLE 4 Relationship between *Babesia*, *Hepatozoon* and *Leishmania* spp. infections in different wildlife hosts

	Fox	Badger	Beech marten	Wild cat	Genet	Egyptian mongoose	Hedgehog	Otter	Raccoon	All
Parasites	N = 89	N = 24	N = 16	N = 5	N = 3	N = 3	N = 6	N = 3	N = 2	N = 151
Double infections										
<i>B. vulpes</i> + <i>H. canis</i>	30 (34)	0	0	0	0	0	0	0	0	30 (20)
<i>Babesia</i> sp. type A + <i>H. martis</i>	0	2 (8)	0	0	0	0	0	0	0	2 (1)
<i>Babesia</i> sp. type A + <i>L. infantum</i>	0	2 (8)	0	0	0	0	0	0	0	2 (1)
<i>H. canis</i> + <i>L. infantum</i>	5 (6)	0	1 (6)	0	0	0	0	0	0	6 (4)
<i>H. martis</i> + <i>L. infantum</i>	0	0	1 (6)	0	0	0	0	0	0	1 (1)
<i>H. felis</i> + <i>Cytauxzoon</i> sp.	0	0	0	1 (20)	0	0	0	0	0	1 (1)
Triple infections										
<i>B. vulpes</i> + <i>H. canis</i> + <i>L. infantum</i>	21 (24)	0	0	0	0	0	0	0	0	21 (14)

Otranto et al., 2019). The low prevalence of *Babesia* spp. in wild cats and its absence in stone martens, genets, otters, hedgehogs, Egyptian mongooses and raccoons in the present study is in agreement with other surveys in some of these wildlife species in Europe (Millán et al., 2016; Santoro et al., 2019). However, the number of animals belonging to some of these species that we tested was small.

The high apicomplexan infection prevalence in wildlife described here, along with common contact between wildlife and domestic animals in periurban environment (Mackenstedt et al., 2015), highlights

the potential risk of infection spill-over from sylvatic to domestic environments, which may have major health consequences. Examples of this include *B. vulpes* causing severe infection in dogs in Portugal and Spain (Camacho et al., 2004), *H. americanum* in coyotes severely affecting dogs in the United States (Panciera & Ewing, 2003) and *C. felis* from bobcats causing serious illness in domestic cats (Birkenheuer et al., 2006).

We also report the detection of *Cytauxzoon* sp. in a wild cat and *Sarcocystis* spp. in a genet. The wild cat came from Jaén province

(Andalusia), where Iberian lynxes are also infected with *Cytauxzoon* spp. (García-Bocanegra et al., 2010; Meli et al., 2009; Millán et al., 2007), and this parasite is considered a potential threat to this endangered feline species (Millán et al., 2009). *Cytauxzoon* spp. has also been reported in domestic (0.8–23%) and wild cats (67–79%) in Spain and other European countries (Barandika et al., 2016; Carli et al., 2012; Criado-Fornelio et al., 2009; Díaz-Regañón et al., 2017; León et al., 2017). However, *Cytauxzoon* sp. infection in Europe is less pathogenic than *C. felis* in the United States, transmitted by *Amblyomma americanum*, a tick species from North America not present in Europe (Birkenheuer et al., 2006; Reichard et al., 2010). It was recently reported that there are at least three different species of *Cytauxzoon*, which infect wild felids in Europe and are genetically distinct (Panait et al., 2021). *Sarcocystis* spp. have not been previously reported in genets and the sequence found in this study was identical to *S. cymruensis* from brown rats from China and the West Indies, and to *S. muris* from a cat from Egypt (Al-Kappany et al., 2013; Murata et al., 2018). *Sarcocystis* spp. have an indirect host-prey life cycle. The definite host of *S. cymruensis* has not been identified yet and could be genets, as they feed on rodents (Amroun et al., 2014; Sánchez et al., 2008). This hypothesis would need to be confirmed by the detection of intestinal sexual stages and the presence of oocysts or sporocysts in the genet's faeces. *S. cymruensis* in this study was found in a spleen sample, which is a possible location for extra-intestinal *Sarcocystis* spp. stages, which are increasingly reported in muscle tissue from carnivore definitive hosts (Máca, 2018; Prakas et al., 2019).

L. infantum infection prevalence in southern and southwestern Spain (5%) was lower than in other PCR surveys in wild carnivores in this part of Spain, specifically in Huelva (15%) (Sobrino et al., 2008) and Extremadura (75%) (Muñoz-Madrid et al., 2013). Differences in *L. infantum* prevalence would be associated with variable sandfly infection rates and distribution, which is related to macro- and micro-environmental factors, and the presence of other susceptible hosts (Muñoz et al., 2017; Muñoz et al., 2019; Risueño et al., 2017). Canine leishmaniosis is widespread in southern Spain but seroprevalence is similarly variable depending on the area and population examined (Gálvez et al., 2020).

The high prevalence of *L. infantum*, *Babesia* spp. and *Hepatozoon* spp. in the southeast of Spain compared to other regions in this study indicates a greater infection pressure in this region to the three parasites and possibly, synergism between them. Coinfections with these parasites have been described before (Miró et al., 2015; Tabar, 2009), but the immunopathological mechanisms facilitating *Leishmania*, *Babesia* and *Hepatozoon* spp. coinfection remain unknown. Previous studies suggested synergism between *L. infantum* and *Ehrlichia canis*, due to common target cells and overlapping immunopathologic pathways, and *Ehrlichia* spp., *Anaplasma* spp. and other VBI increased the risk of *Leishmania* spp. seropositivity and clinical disease in dogs from the United States and Brazil (Attipa et al., 2018; Toepf et al., 2019). Therefore, wildlife mammals infected with *L. infantum* in southern Spain are likely to be infected also with other VBIs, such as *Babesia* and *Hepatozoon* spp., and should be tested and potentially treated for co-infection.

5 | CONCLUSIONS

This study indicated that *Hepatozoon* spp., *Babesia* spp. and *L. infantum* infections are widespread in wild carnivores from Spanish Mediterranean ecosystems, and provided strong evidence that there is an increased risk of *L. infantum* infection in wildlife infected with *Babesia* spp. and *Hepatozoon* spp. *H. canis* is highly prevalent in foxes and so is *H. martis* in beech martens. Furthermore, it was shown for the first time that *H. canis* infects beech martens, *H. martis* infects wild cats and genets are a target species for *Sarcocystis* spp. and may act as a definite host of *S. cymruensis*. *B. vulpes* is highly prevalent in foxes and so is *Babesia* sp. badger type A in badgers. Moreover, *Babesia* sp. also infects wild cats in Spain. Therefore, wildlife mammals may be an important source of *Babesia* spp. and *Hepatozoon* spp. infections for domestic animals.

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CONFLICTS OF INTEREST

The authors declare that they do not have conflicts over data-ownership or of interest on the information here presented, and all contributions have been appropriately attributed.

ETHICAL APPROVAL

The study was approved by the University of Murcia Animal Ethics Committee (<http://www.um.es/web/vic-investigacion/contenido/vicerrectorado/estructura/comisiones/etica-investigacion>) and Murcia's Regional Government.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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