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Molecular identification of tick-borne pathogens (*Rickettsia* spp., *Anaplasma phagocytophilum, Borrelia burgdorferi* sensu lato, *Coxiella burnetii* and piroplasms) in questing and feeding hard ticks from North-Western Spain

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ABSTRACT

The occurrence of tick-borne pathogens (TBPs) of human and veterinary interest was studied in questing and feeding ticks collected from wild animals in a region in North-Western Spain. A total of 529 ticks (489 questing, 40 feeding) of seven different species (386 *Ixodes ricinus*, 53 *Haemaphysalis concinna*, 27 *Haemaphysalis punctata*, 25 *Dermacentor marginatus*, 21 *Haemaphysalis inermis*, 15 *Dermacentor reticulatus*, and two *Rhipicephalus bursa*) were analyzed. Molecular analysis of the 16S rRNA gene in *I. ricinus* ticks, revealed the presence of two phylogenetic groups in the region. Most of the sequenced ticks (96%) were assigned to *I. ricinus* haplogroup and 4% of the ticks were phylogenetically related to *I. inopinatus* haplogroup. Feeding ticks were removed from 17 animals from seven wild species (seven roe deer -*Capreolus capreolus*-, three wolves -*Canis lupus*-, two Iberian red deer -*Cervus elaphus hispanicus*-, two European wild boar -*Sus scrofa*-, one Cantabrian brown bear -*Ursus arctos*-, one Eurasian badger -*Meles meles*-, and one red fox -*Vulpes vulpes*-). Presence of *Rickettsia* spp., *Anaplasma phagocytophilum*, piroplasms, *Borrelia burgdorferi* sensu lato (s.l.) and *Coxiella burnetii* were tested in ticks by specific PCR. A total of 92 (17.4%) of the 529 ticks analyzed were positive for at least one of the TBPs tested. Sequencing revealed the presence of the genospecies "*Candidatus* Rickettsia rioja", *Rickettsia raoultii*, and *Anaplasma phagocytophilum* in both questing and feeding ticks. *Rickettsia slovaca, Borrelia lusitaniae, Borrelia afzelii, Borrelia garinii, Borrelia burgdorferi* sensu stricto and *Babesia bigemina* were only detected in questing ticks, while *Babesia* sp. badger type A, *Theileria* OT3 and *Hepatozoon canis* occurred only in engorged ticks. None of the ticks were positive for *C. burnetii*. The analysis of the 16S rRNA gene sequences of *A. phagocytophilum* revealed the presence of three variants (I, X and W) circulating in the region. New host-tick-pathogen interactions have been revealed, finding for the first time the human pathogen *R. raoultii* in *D. reticulatus* removed from a Cantabrian brown bear. Co-occurrence between different TBPs were detected in 4.3% of the ticks. The association *B. burgdorferi* s.l./*Rickettsia* spp. was detected in questing ticks; and *Rickettsia* spp./piroplasms and *A. phagocytophilum/Theileria* OT3 in feeding ticks. The presence of pathogenic agents constitutes a threat to human and animal health, and should be considered in the diagnosis and treatment after a tick bite. This study increases the knowledge on TBPs diversity of medical and veterinary interest circulating between ticks and their hosts in North-Western Spain.

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1. Introduction

Ticks are important vectors of human and animal pathogens worldwide. Among these, *Rickettsia* spp., *Anaplasma phagocytophilum, Borrelia burgdorferi* sensu lato (s.l.), *Coxiella burnetii* and piroplasms are tick-borne pathogens (TBPs) of veterinary and public health interest that can infect a wide range of hosts, including humans and wild and domestic animals.

Human rickettsioses are considered an emergent global threat. In Europe, most rickettsial infections are caused by tick-borne rickettsiae of the spotted fever group (SFG). This group includes *Rickettsia conorii* subsp. *conorii*, the causative agent of Mediterranean spotted fever (MSF), and other *Rickettsia* spp. that cause MSF-like illness such as *Rickettsia helvetica, Rickettsia monacensis, Rickettsia massiliae* or *Rickettsia aeschlimannii* (Tomassone et al., 2018b). DEBONEL/TIBOLA (*Dermacentor* spp.-borne necrosis-erythema-lymphadenopathy/tick-borne lymphadenopathy) or SENLAT (scalp eschar and neck lymphadenopathy after a tick bite) are recently described syndromes of SFG, caused by *Rickettsia slovaca, Rickettsia raoultii* and "*Candidatus* Rickettsia rioja", which can be transmitted by *Dermacentor* ticks (Portillo et al., 2015).

Anaplasma phagocytophilum is a zoonotic bacterium widely distributed in the Northern Hemisphere. In Europe, it is mainly transmitted by the bite of *Ixodes ricinus* ticks, and is the cause of human granulocytic anaplasmosis (HGA), tick-borne fever of ruminants and equine and canine granulocytic anaplasmosis (de la Fuente et al., 2008).

The genus *Borrelia* contains several genospecies that affect humans and animals, and some species cause Lyme borreliosis. In Europe, at least five species of *B. burgdorferi* s. l. can cause this disease (*Borrelia afzelii, Borrelia garinii, Borrelia burgdorferi* sensu stricto (s. s.), *Borrelia spielmanii*, and *Borrelia bavariensis*) and three species (*Borrelia bissettiae, Borrelia lusitaniae*, and *Borrelia valaisiana*) have occasionally been detected in patients, nonetheless they are not recognized as important pathogens (Stanek et al., 2012). *Borrelia garinii* is the genospecies mostly involved in human clinical cases in Spain (Portillo et al., 2014).

Coxiella burnetii is the causative agent of Q fever in humans, but it also affects domestic and wild ruminants (Eldin et al., 2017). Despite the epidemiological wild cycle of Q fever is not fully known, ticks have been suggested to act as a reservoir of *C. burnetii* and to play a role in the transmission to vertebrate hosts in natural environments, mainly through tick feces (Duron et al., 2015).

Piroplasmids of the genera *Babesia* and *Theileria* are almost worldwide distributed tick-borne protozoan that cause babesiosis or theileriosis respectively (Gray et al., 2010). *Babesia microti* and *Babesia divergens* are known to cause human babesiosis (Antunes et al., 2017), while *Theileria* spp. have an impact on animal health (Tirosh-Levy et al., 2020). The diversity of piroplasms present in Spain has been widely reported in horses (Nagore et al., 2004), domestic ruminants (García-San Martín et al., 2006; Ros-García et al., 2013), wildlife (Barandika et al., 2016; Millán et al., 2016) and ticks (García-Sanmartín et al., 2008; Remesar et al., 2021).

The tick *I. ricinus* is the most widely distributed and abundant tick species in Atlantic Iberian Peninsula (Barandika et al., 2011) and is considered the main vector of *B. burgdorferi* s.l. and *A. phagocytophilum* in Europe (de la Fuente et al., 2008). *Dermacentor reticulatus* and *Dermacentor marginatus* are proven or potential vectors of *Babesia canis, Babesia caballi, R. raoultii* and *R. slovaca* (Földvári et al., 2016; Samoylenko et al., 2009; Socolovschi et al., 2009), and both tick species are present in Spain (Barandika et al., 2011).

Wild animals are recognized reservoirs of zoonotic pathogens and they can serve as amplifying hosts for both pathogens and vectors, and can act as dispersers of ticks over long distances (Tomassone et al., 2018a). Several epidemiological surveys have investigated the presence of TBPs in wildlife in Northern Spain (García-Sanmartín et al., 2007; Ortuño et al., 2007; Gimenez et al., 2009; Remesar et al., 2020), but only a few are available for the region of study (Barandika et al., 2016; García-Pérez et al., 2016; Espí et al., 2021).

Thus, in order to better understand the eco-epidemiology of TBPs circulating in North-Western Spain, the prevalence of five taxonomic groups of TBPs of medical and veterinary importance (*Rickettsia* spp., *A. phagocytophilum, B. burgdorferi* s.l., *C. burnetii* and piroplasms) in questing and feeding ticks collected from vegetation and wild animals respectively was investigated.

2. Materials and methods

2.1. Study area

Asturias is an autonomous community of 10,604 km^2 located in North-Western coastal Spain, bordered by the Bay of Biscay to the north and the Cantabrian Mountain range to the south. The region can be divided into three different geographical areas: western, central and eastern Asturias, which are separated by large north-to-south oriented valleys running through the Cantabrian mountain range. The predominant climate is temperate oceanic with abundant rainfall throughout the year and mild temperatures in winter (Peel et al., 2007), which favors the development of deciduous and mixed forests interspersed with open pastures and meadows shared by livestock and wildlife. This diverse habitat is suitable for a wide range of mammalian species, including the endangered Cantabrian brown bear (*Ursus arctos*), other carnivore species such as the wolf (*Canis lupus*), the red fox (*Vulpes vulpes*) and the Eurasian badger (*Meles meles*), and several wild ungulates (Iberian red deer -*Cervus elaphus hispanicus*-, fallow deer -*Dama dama*-, roe deer -*Capreolus capreolus*-, Cantabrian chamois -*Rupicapra pyrenaica parva*- and European wild boar -*Sus scrofa*-).

2.2. Tick collection and identification

Questing ticks were collected from January 2012 to March 2019 at 33 sampling points that represented most of the diverse geographic and ecological areas in Asturias (Fig. 1). Questing ticks were collected by dragging a blanket (1 × 1 m) over the vegetation, which was inspected every 10 m, and the gathered specimens were then collected in plastic tubes for morphological identification. Feeding ticks from wildlife were collected from January 2015 to October 2018, except for hunting species, which were collected throughout the year during hunting periods (Fig. 1). Seventeen animals were examined after being hunter-harvested, and the ticks which were present sent to the Regional Agrifood Research and Development Service (SERIDA) for species identification and molecular analyses. Ticks were identified using taxonomic keys (Gil Collado et al., 1979; Manilla, 1998) and stored at −20 °C until being tested. In order to investigate the occurrence of the recently described *Ixodes inopinatus* species (Estrada-Peña et al., 2014), ticks morphologically identified as *I. ricinus* were subjected to molecular identification using primers targeting the mitochondrial 16S rRNA gene as previously described (Black and Piesman, 1994).

2.3. DNA extraction, PCR amplification and sequencing analysis

Total DNA was extracted from ticks using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Adult ticks were analyzed individually; questing nymphs were pooled by species, collection date and sampling site into groups of five individuals. In each DNA extraction round, a negative control sample free of any template was included to rule out contamination in the extraction process. Five µl of tick DNA were used in all PCR reactions. Samples were screened for the presence of *Rickettsia* spp, *Anaplasma* spp. (*A. phagocytophilum* and *A.marginale/A. ovis*), piroplasms (*Babesia* spp./*Theileria* spp.), *C. burnetii* and *B. burgdorferi* s.l. by previously reported PCR assays (Table 1). All PCR primers and probes used in the study and their respective references are provided in Table 1. Real-time PCR amplifications were performed in a StepOne Plus™ system (Applied Biosystems, ThermoFisher Scientific, USA) with TaqMan Universal PCR Master Mix (Applied Biosystems,

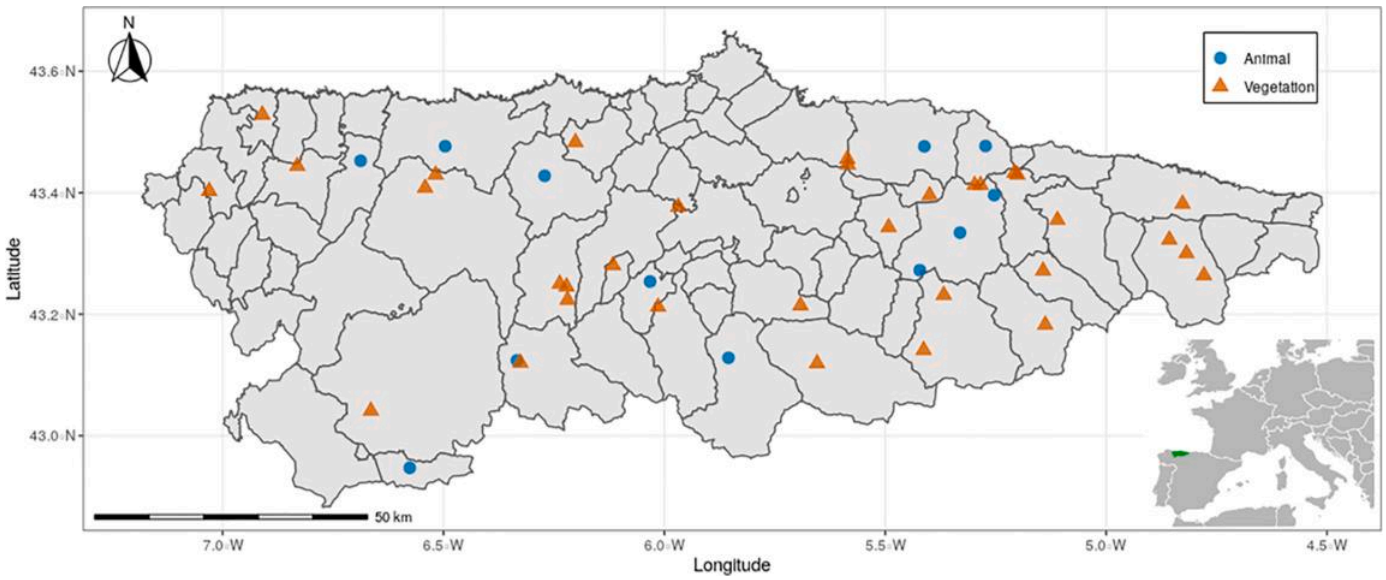

Fig. 1. Location map of the study area in Spain (green) and sampling sites of the ticks analyzed in the present study. Locations for ticks collected from animals are marked with blue dots (n = 12) and locations for ticks from vegetation with orange triangles (n = 33).

Table 1
PCR primers and probes used for amplification and sequencing of tick-borne pathogens in ticks.

Target	Target gene	Oligo name	Sequence (5′–3′)	Refs.
Ixodidae	16S rRNA	16S+3	ATACTCTAGGGATAACAGCGT	Black and Piesman (1994)
		16S-3	AAATTCATAGGGTCTTCTTGTC	
Anaplasma spp.	16S rRNA	Aspp16S-F	GCTATGCCGCGTGAGTGAG	Hurtado et al. (2015)
		Aspp16S-R	AGTTTGCCGGGACTTCTTCTG	Hurtado et al. (2015)
		Aspp16S-Pr	FAM-CTTAGGGTTGTAAAACTC[a]	Hurtado et al. (2015)
Anaplasma phagocytophilum	16S rRNA	ge3a	CACATGCAAGTCGAACGGATTATTC	Massung et al. (1998)
		ge10r	TTCCGTTAAGAAGGATCTAATCTCC	Massung et al. (1998)
		ge9f	AACGGATTATTCTTTATAGCTTGCT[b]	Massung et al. (1998)
		ge2	GGCAGTATTAAAAGCAGCTCCAGG[b]	Massung et al. (1998)
	msp2	ApMSP2-FN1	AAGGCAGTGTTGGKTAYGGTATT	Hurtado et al. (2015)
		ApMSP2-R	TTGGTCTTGAAGCGCTCGTA	Courtney et al. (2004)
		ApMSP2-Pr	Cy5-TGGTGCCAGGGTTGAGCTTGAGATTG-BHQ3	Courtney et al. (2004)
Anaplasma marginale/ Anaplasma ovis	msp4	AomMSP4-F	ACGGGCAGTACGCRAAAAG	Hurtado et al. (2015)
		AomMSP4-R	CATGACTGARGTGTTTGTGATTTCA	Hurtado et al. (2015)
		AmarMSP4-Pr	HEX-CCCGCGACGCTAACATTACTGAGACC-BHQ1	Hurtado et al. (2015)
		AovMSP4-Pr	FAM-CCACAAAGTAATTGTTCTC[a]	Hurtado et al. (2015)
Coxiella burnetii	IS1111	sIS1pri_f	CGGGTTAAGCGTGCTCAGTAT	Schets et al. (2013)
		sIS1pri_r	TCCACACGCTTCCATCACCAC	Schets et al. (2013)
		Tqpro sIS1	FAM-AGC CCA CCT TAA GAC TGG CTA CGG TGG AT-BHQ1	Schets et al. (2013)
Babesia spp./*Theileria* spp.	18S rRNA	TB-F3	TTACTTW[+*G*]AG[+*A*][+*A*]AAYTAGAGTG[c]	Hurtado et al. (2015)
		TB-R3	CTAAGAATTTCA[+C]CTCTGACA[c]	Hurtado et al. (2015)
		TB-Pr3	TAMRA-CCAA[+C]Y[+*G*]TT[+C][+C]TATTAA[+C][+C]ATTA-BHQ2[c]	Hurtado et al. (2015)
		BJ1	GTCTTGTAATTGGAATGATGG[b]	Casati et al. (2006)
		BN2	TAGTTTATGGTTAGGACTACG[b]	Casati et al. (2006)
Borrelia burgdorferi s.l	flaB	Outer 1	AARGAATTGGCAGTTCAATC	Clark et al. (2005)
		Outer 2	GCATTTTCWATTTTAGCAAGTGATG	Clark et al. (2005)
		Inner 1	ACATATTCAGAGCAGACAGAGGTTCTA[b]	Clark et al. (2005)
		Inner 2	GAAGGTGCTGTAGCAGGTGCTGGCTGT[b]	Johnson et al. (1992)
Rickettsia spp.	gltA[d]			Genesig, Primerdesign Ltd, UK
	gltA	RpCs.877p	GGGGGCCTGCTCACGGCGG[b]	Regnery et al. (1991)
	ompA	RpCs.1258n	ATTGCAAAAAGTACAGTGAACA[b]	Regnery et al. (1991)
		Rp190.70p	ATGGCGAATATTTCTCCAAAA[b]	Regnery et al. (1991)
		Rp190.701n	GTTCCGTTAATGGCAGCATCT	Roux et al. (1996)
		Rp190.602n	AGTGCAGCATTCGCTCCCCCT[b]	Regnery et al. (1991)
IAC[e]	plasmid	IACyers-F1	GGAGGAAGGGTTAAGTGTTA	Lund et al. (2004)
		IACyers-R1	GAGTTAGCCGGTGCTTCTT	Lund et al. (2004)
		IACyers-Pr3	Cy5-TGCGAGTAACGTCAATGTTCAGTGC-BHQ3	Lund et al. (2004)

[a] MGB probe, Applied Biosystems.
[b] The primer was used for PCR and/or the sequencing reaction.
[c] LNA™ modified oligos, Locked nucleic acids are in brackets with a plus sign.
[d] No information on the primer sequence available.
[e] IAC: Internal Amplification Control.

ThermoFisher Scientific, USA) and end-point PCR assays were carried out in a 2720 Thermal cycler (Applied Biosystems, ThermoFisher Scientific, USA) with Amplitools Master Mix (Biotools, Madrid, Spain). Positive (DNA from the corresponding pathogen tested) and negative controls were included in each assay run. All the PCR products of the expected size were purified using NZYGelpure (NZYTech Lda, Lisboa, Portugal) and sent to Eurofins Genomics (Köln, Germany) for Sanger sequencing with the corresponding forward and reverse PCR primers (Table 1). Nucleotide sequences were aligned with Clustal W (Thompson et al., 1994) algorithm using MEGA X package (Kumar et al., 2018) and the obtained sequences were compared with GenBank® database by using the Basic Local Alignment Search Tool (BLASTN) at the National Center for Biotechnology Information (NCBI). Representative nucleotide sequences obtained in this study were submitted to GenBank® under accession numbers from MW800885 to MW800890 [16S rRNA *A. phagocytophilum*], from MW817092 to MW817147 [*fla*B *B. burgdorferi* s.l. and *omp*A *Rickettsia* spp.], from MW829613 to MW829622 [18S rRNA piroplasmids], from ON116366 to ON116376 [16S rRNA Ixodidae] and from ON149673 to ON149676 [16S rRNA Ixodidae].

For phylogenetic analyses, obtained sequences were aligned with related sequences available in GenBank® using Muscle algorithm (Edgar, 2004), and the phylogenetic tree was constructed using the Maximum-Likelihood method based on the Kimura 2-parameter distance method (Tamura et al., 2013), with bootstrap analysis of 1000 replicates (Brown, 1994). The phylogenetic tree obtained was edited using a suite of R packages, *tidytree, treeio* and *ggtree*.

2.5. Statistical analysis

A 2 × 2 Chi square test or Fisher's exact test were performed using R (http://www.r-project.org/) to compare prevalence for a given pathogen regarding tick species and origin (vegetation or wild animal). The differences were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. Tick samples

A total of 529 ticks (489 collected from the vegetation and 40 from the animals) were analyzed for TBP detection. Spatial distribution of the sampled ticks is shown in Fig. 2. Ticks collected from the vegetation included 119 adults and 370 nymphs belonging to seven species: *I. ricinus* (n = 356), *Haemaphysalis concinna* (n = 53), *Haemaphysalis punctata* (n = 27), *D. marginatus* (n = 24), *Haemaphysalis inermis* (n = 20), *D. reticulatus* (n = 7) and *Rhipicephalus bursa* (n = 2). Most of the ticks were collected during spring (32.9%), followed by winter (27.4%), autumn (22.5%) and summer (17.2%). The seasonal variation of tick species analyzed in the study is shown in Fig. 3.

A total of 40 feeding ticks (39 adults and 1 nymph) were collected from 17 individuals of seven wild animal species: seven roe deer (n = 14), three wolves (n = 10), two red deer (n = 5), two wild boar (n = 4), one brown bear (n = 3), one Eurasian badger (n = 2), and one red fox (n = 2). *Ixodes ricinus* represented 75% (30/40) of the ticks removed from animals, *Dermacentor* spp. represented 22% (9/40) of the ticks and were only found attached to wild boar, brown bear or wolf; only one *H. inermis* (2%, 1/40) was found on a red deer. Co-feeding of *I. ricinus*/*H. inermis* and *I. ricinus*/*D. reticulatus* was observed on one red deer and one wolf respectively.

Sequencing of a fragment of the 16S rRNA gene (460 pb) was successfully achieved in 76 *I. ricinus* ticks (75 adults and one feeding nymph collected from a red deer). According to the molecular analyses of the sequences 15 unique haplotypes were identified. The most represented haplotypes were H2 (14 ticks), H7 (12 ticks), H8 (7 ticks) and H13 (27 ticks), followed by haplotypes H3 (4ticks), H11 (2 ticks) and H15 (2 ticks). The remaining eight haplotypes include a single specimen each. Based on the nucleotides at position 184/185 of the sequences, two haplotypes (comprising 2 adults and 1 nymph) were assigned to a group phylogenetically close to *I. inopinatus* (AG bases at position184/185, KM211789 reference sequence). The remaining 13 haplotypes (comprising 73 ticks) with CT bases at position 184/185 (KM211785 reference sequence) were assigned to *I. ricinus* group (Fig. 4).

3.2. Pathogen detection

Questing ticks

Overall, 7.8% (38/489, CI95%: 5.6–10.6) of the questing adult ticks and nymphs analyzed were positive for *Rickettsia* spp., 3.7% (18/489; CI95%: 2.3–5.9) for *B. burgdorferi* s.l., 3.5% (17/489; CI95%: 2.1–5.6) for *Anaplasma* spp., and 0.2% (1/489; CI95%: 0.01–1.3) for piroplasms (Table 2). None of the samples tested positive for *C. burnetii*. These prevalences were significantly higher (p<0.05) in *Dermacentor* spp. (29/31, 93.5%; CI95%: 77.2–98.9) than in the other taxa (9.8% in *Ixodes* and

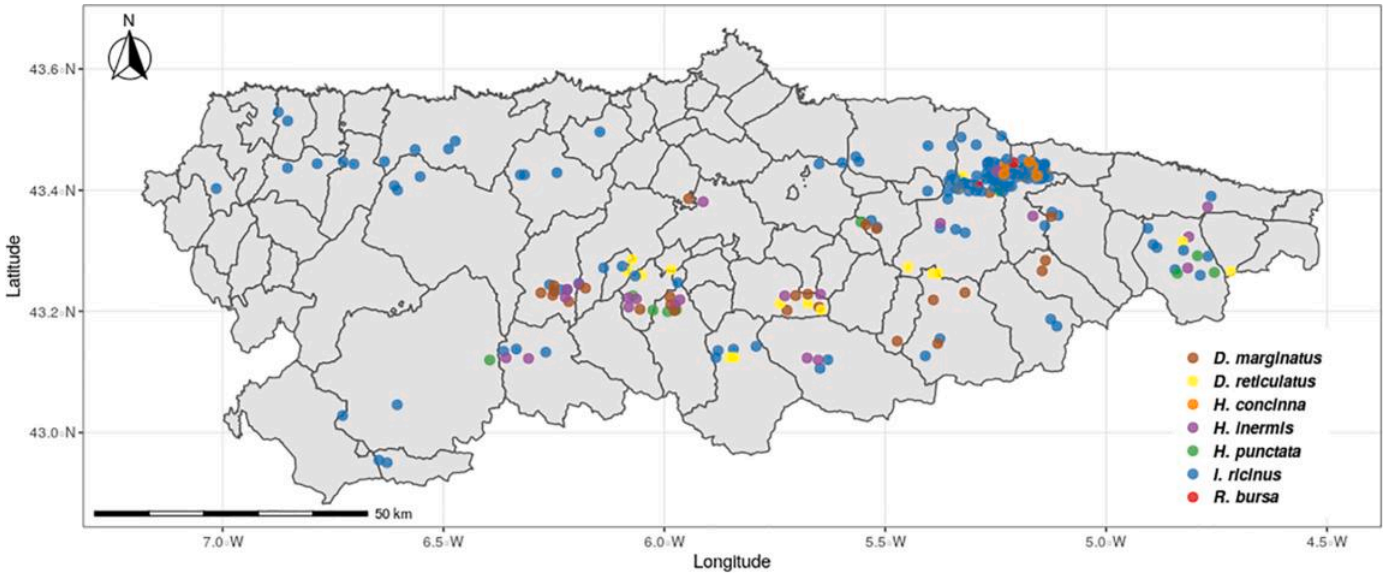

Fig. 2. Geographic distribution of tick samples collected in the area of study. Tick species are represented by different colors (brown = *D. marginatus,* yellow= *D. reticulatus*, orange= *H. concinna*, purple= *H. inermis*, green= *H. punctata*, blue= *I. ricinus*, red= *R. bursa*).

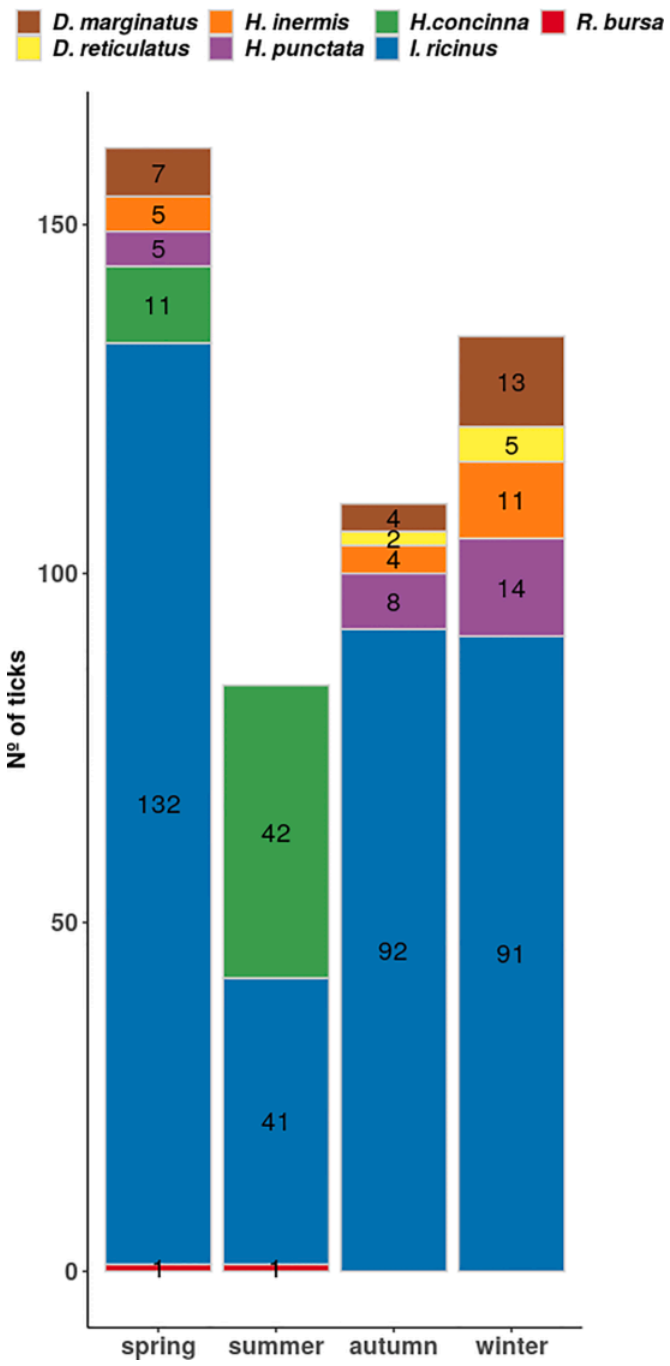

Fig. 3. Stacked bar-plot showing seasonal variation (diversity and abundance) of questing ticks analyzed in the present study. The number in each bar represent the number of ticks for that species.

8.5% in *Haemaphysalis*). Except in *R. bursa,* both *Rickettsia* spp. and *Borrelia* spp. were detected in all tick species. The presence of these pathogens was significantly higher ($p < 0.05$) in adults than in nymphs. *Anaplasma phagocytophilum* was more frequently found in nymphs than in adult stages of *I. ricinus* ($p = 0.48$). Piroplasms were only detected in a single *R. bursa* and no TBPs were detected in *H. concinna*. (Table 2). Co-infections of *Rickettsia* spp. and *Borrelia* spp. were detected in *Dermacentor* spp. [9.7% (3/31; CI95%: 2.5–26.9)] and *I. ricinus* [0.6% (2/356; CI95%: 0.1–2.2)] ($p < 0.05$) (Table 3).

Feeding ticks

In feeding ticks, 32.5% were positive for piroplasms (13/40; CI95%: 19.1–49.2), 20.0% (8/40; CI95%: 9.6–36.1) for *Rickettsia* spp., and 12.5% (5/40; CI95%: 4.7–27.6) for *Anaplasma* spp. None of the feeding ticks were positive for *C. burnetii* or *Borrelia* spp. (Table 4). Co-occurrence of two different species of bacteria was also detected in 12.5% of the ticks (5/40; CI95%: 4.7–27.6) (Table 3).

3.3. Sequencing and genotypes

Questing ticks

Sequencing analysis of a fragment of the *omp*A gene (532 bp) obtained in 37 *Rickettsia* spp. positive samples (37/38), revealed the presence of the species "*Candidatus* Rickettsia rioja" [55.3% (CI95%: 38.5–71.0; 21/38)], *R. raoultii* [36.8% (CI95%: 22.3–54.0; 14/38)] and *R. slovaca* [5.3% (CI95%: 0.9–19.0; 2/38)]. All "*Candidatus* Rickettsia rioja" sequences from positive samples were identical to each other, showing a very high identity (>99%) with sequences associated with human cases (EF0282011, GQ404429) and questing ticks (MK301595) previously reported in Spain. *Rickettsia raoultii* sequences were nearly identical (99–100%) to sequences described in *D. marginatus* from Turkey (MH548521) and Italy (HM161794). The only *R. slovaca* detected was 100% identical to a sequence reported in questing ticks from Turkey (MK922650). The three *Rickettsia* species were present in *D. marginatus* ticks, while *D. reticulatus* only harboured *R. raoultii.* Both "*Candidatus* Rickettsia rioja" and *R. raoultii* were also found in *I. ricinus*.

In 17 out of 18 *B burgdorferi* s.l. positive questing ticks, genospecies were identified based on the amplification and sequencing of 338 bp of the *fla*B gene (Table 2). The predominant species was *B. lusitaniae* [58.8% (CI95%: 32.9–81.5; 10/17)] and sequences shared >99–100% identity to *B. lusitaniae* from *I. ricinus* parasitizing lizards in Slovakia (DQ788618). Three of the *Borrelia* positive samples [17.6% (CI95%: 3.7–43.4; 3/17)] showed >98–100% identity to *B. afzelii* (MH102390, EU220780) and other two [11.7% (CI95%:1.4–36.4; 2/17)] were similar (>99% identity) to *B. garinii* (MK604262, MF150067). Other genospecies detected in single samples were *B. valaisiana*, with a sequence identical to one described in ticks collected in the Chernobyl Exclusion zone (MK790205), and *B. burgdorferi* s.s., showing >99% identity with sequences of this species reported in *Ixodes* spp. from Poland (MK604273, MH807139 and MH807138) (Table 2). All *Borrelia* genospecies identified were detected in *I. ricinus* ticks, whereas in adult *D. reticulatus* ticks only *B. lusitaniae* and *B. afzelii* were detected. *Borrelia lusitaniae* was the single species present in *D. marginatus, H. punctata* and *H. inermis* ticks.

Based on the 16S rRNA gene sequencing (546 bp), three different variants, i.e., "I", "X" and "W" were identified in 15 out of 16 *A. phagocytophilum* positive samples (Table 5). Variant "W" was the most frequent (11/16) followed by variant "X" (3/16), while variant "I" was only identified in a single questing *I. ricinus* adult tick. Identification of the variant was not possible in one sample due to the poor quality of the sequencing result.

The piroplasm detected in an adult *R. bursa* tick was identified as *B. bigemina* (Table 2), showing high identity (>99%) with a sequence described in engorged *R. bursa* from France (MK732475).

Feeding ticks

The most prevalent *Rickettsia* species found in feeding ticks was "*Candidatus* Rickettsia rioja" [50% (CI95%: 21.5–78.4; 4/8)], followed by *R. raoultii* [25% (CI95%:3.1–65.0; 2/8)]. "*Candidatus* Rickettsia rioja" was detected in *I. ricinus* and *D. marginatus* adult ticks collected from ungulates (red deer, roe deer and wild boar), while *R. raoultii* was found in adults of *I. ricinus* and *D. reticulatus* ticks collected from carnivores (wolf and brown bear respectively) (Table 4).

Two *A. phagocytophilum* 16S rRNA variants were identified in four ticks collected from cervids. Regarding host isolation source, variant "W" was found in ticks from two red deer (two adults of *I. ricinus* and *H. inermis* species, and one nymph phylogenetically close to the *I. inopinatus* group), while variant "X" was identified in one *I. ricinus* parasitizing a roe deer.

Identification of *Babesia/Theileria* by sequencing a fragment

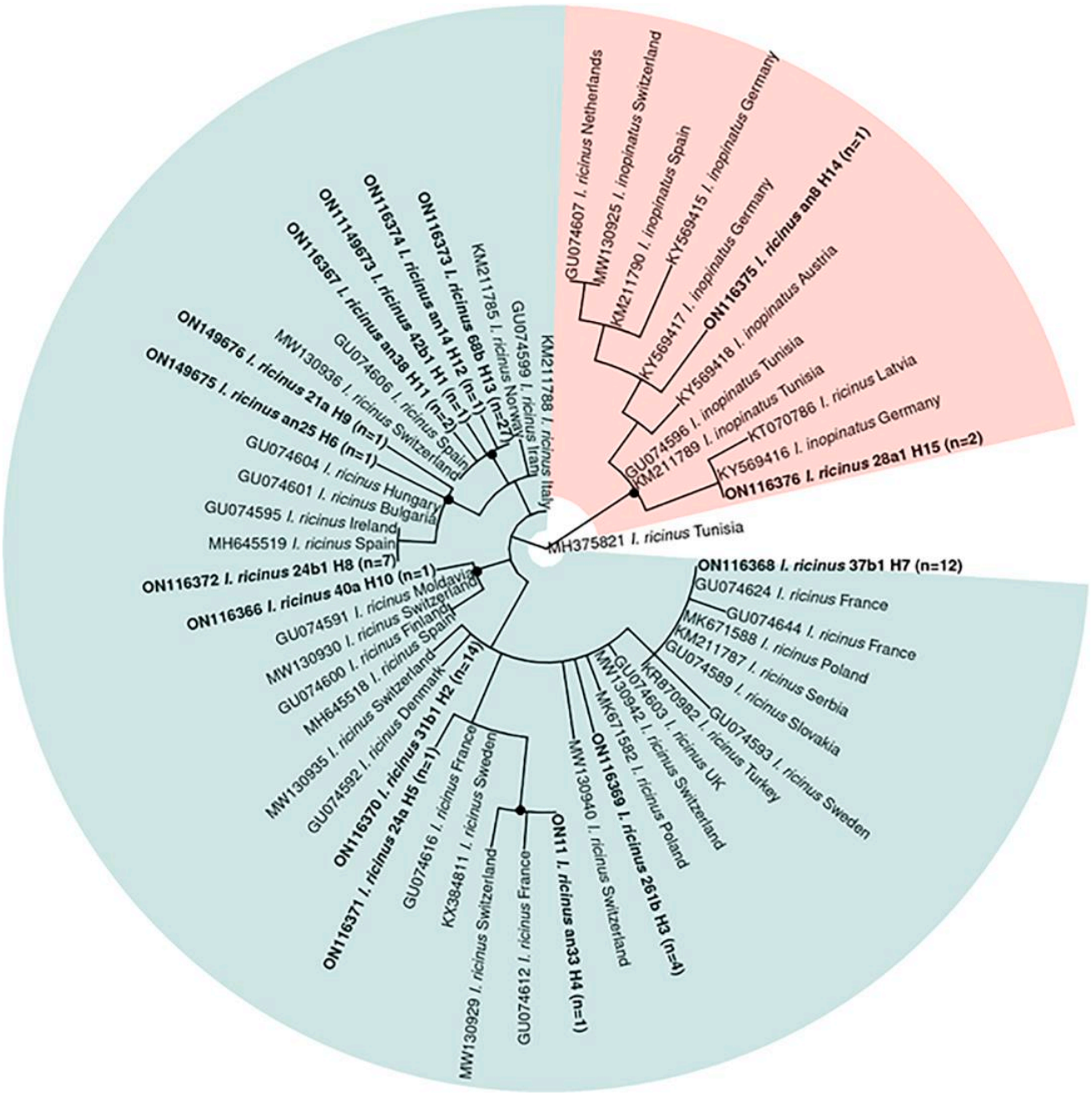

Fig. 4. Maximum likelihood (ML) phylogenetic tree inferred from the partial sequence of 16S rRNA gene (395 bp) with MEGA X software. Sequences from *I. ricinus* and *I. inopinatus* species retrieved from the GenBank database were included. The evolutionary model was GTR+ G + I and numbers at nodes correspond to bootstrap (1000 replicates), values ≥40% are shown. *I. ricinus* and *I. inopinatus* sequences obtained in the present study are highlighted in bold and the number of analyzed ticks included in each haplotype is shown. The two haplogroups are color-coded: pink for *I. inopinatus* haplogroup and green for *I. ricinus* haplogroup.

(411–452 bp) of the V4 region of the 18S rRNA gene, was successfully achieved in 10 piroplasmid PCR-positive samples, including a nymph belonging to the *I. inopinatus* group. Most sequences were identified as *Theileria* OT3 [46.15% (CI95%:19.2–74.8; 6/13]. Ticks harbouring this pathogen (three *I. ricinus,* one *H. inermis* and two *D. reticulatus*) were feeding on four different ungulates and one brown bear (Table 4). These sequences were identical among themselves and to other *Theileria* OT3 from sheep (AY533145) and cervids from Northern Spain (DQ866840 and MH522433) and Portugal (LC131068). *Babesia* sp. badger type A was identified in two *I. ricinus* collected from one red deer and one roe deer, showing 100% identity with a sequence described in European badgers from Spain (KT223484). The tick-borne protozoan *Hepatozoon canis* was identified in two *I. ricinus* collected from two wolves. Both sequences were identical (100% identity), and showed high identity (>99%) with other sequences from dogs and wild canids from Europe (KU893127 and MN791089) (Table 4).

4. Discussion

As could be expected from previous studies in the region (Espí et al., 2016), *I. ricinus* was the predominant species in both questing (72.8%) and feeding (75.0%) ticks. Molecular-phylogenetic analysis of the 16S rRNA sequences revealed the genetic diversity of *I. ricinus* in the region and the occurrence of haplotypes phylogenetically close to the recently described tick species *I. inopinatus*, although in lower abundance compared to *I. ricinus.* This tick species, originally described in the

Table 2
Tick-borne pathogens (TBPs) detected in questing ticks.

Identified TBPs	N° of positive ticks (prevalence%)									
	Total[a]		***Ixodes ricinus***		***Dermacentor marginatus***	***Dermacentorreticulatus***	***Haemaphysalis punctata***		***Haemaphysalisinermis***	***Rhipicephalusbursa***
	Adults(n = 119)	Nymphs(n = 370)	Adults (n = 56)	Nymphs(n = 300)	Adults(n = 24)	Adults(n = 7)	Adults (n = 7)	Nymphs(n = 20)	Adults(n = 20)	Adults(n = 2)
***Rickettsia* spp.**	**34 (28.6)**	**4 (1.1–5.5)** [b]	**5 (9.0)**	**3 (1.0–15.0)**	**24 (100.0)**	**4 (57.1)**	**0**	**1 (5.0–25.0)**	**1 (5.0)**	**0**
"Candidatus Rickettsia rioja"	20 (17.0)	1 (0.3–1.5)	5 (9.0)	1 (0.3–1.5)	14 (58.3)	4 (57.1)			1 (5.0)	
Rickettsia raoultii	12 (10.1)	2 (0.5–2.5)		2 (0.7–3.5)	8 (33.3)					
Rickettsia slovaca	2 (1.7)				2 (8.3)					
Anaplasma phagocytophilum	**4 (3.4)**	**12 (4–20)**	**4 (7.1)**	**12 (4–20)**	**0**	**0**	**0**	**0**	**0**	**0**
Piroplasms	**1 (0.8)**		**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1 (50.0)**
Babesia bigemina	1 (0.8)									1 (50.0)
***Borrelia burgdorferi* s.l.**	**16 (13.4)**	**2 (0.5–2.5)**	**10 (17.9)**	**2 (0.6–3)**	**1 (4.2)**	**3 (42.9)**	**1 (14.3)**	**0**	**1 (5.0)**	**0**
Borrelia lusitaniae	10 (8.4)	1 (0.3–1.5)	6 (10.7)	1 (0.3–1.5)	1 (4.2)	1 (14.3)	1 (14.3)		1 (5.0)	
Borrelia garinii	1 (0.8)	1 (0.3–1.5)	1 (1.8)			1 (14.3)				
Borrelia afzelii	2 (1.7)		1 (1.8)							
Borrelia burgdorferi s.s.	1 (0.8)		1 (1.8)							
Borrelia valaisiana	1 (0.8)		1 (1.8)							

[a] All *Haemaphysalis concinna* ticks analyzed (3 adults and 50 nymphs) were negative for all the pathogens tested therefore the results are not shown, but they were included in the calculation of prevalence data.
[b] "Minimum expected prevalence": percentage of positives is calculated assuming that any pool would contain one infected tick.

Table 3
Co-occurrence of tick-borne pathogens by tick species and origin.

Pathogen associations	N° of positve ticks (prevalence%)							
	Total[a]		*Ixodes ricinus*		*Dermacentor reticulatus*		*Dermacentor marginatus*	*Haemaphysalis inermis*
	V(n = 489)	A(n = 40)	V(n = 356)	A(n = 30)	V(n = 7)	A(n = 8)	V(n = 24)	A(n = 1)
"*Candidatus* Rickettsia rioja"/*Borrelia lusitaniae*	1 (0.2)		1 (0.28)					
" *Candidatus* Rickettsia rioja "/*Borrelia garinii*	1 (0.2)	1 (2.5)	1 (0.28)	1 (3.3)	1 (14.3)	1 (12.5)	1 (4.2)	1 (100.0)
" *Candidatus* Rickettsia rioja " / *Babesia badger* type A	1 (0.2)	1 (2.5)		1 (3.3)	1 (14.3)			
" *Candidatus* Rickettsia rioja " / *Theileria* OT3	1 (0.2)	2 (5.0)						
Rickettsia raoultii/Borrelia lusitaniae								
Rickettsia raoultii/Borrelia afzelii								
Rickettsia slovaca / Borrelia lusitaniae								
Rickettsia sp. / *Theileria* OT3								
Anaplasma phagocytophilum / *Theileria* OT3								
Total	**5 (1.0)**	**5 (12.5)**	**2(0.6)**	**3 (10.0)**	**2 (29.0)**	**1 (12.5)**	**1 (4.2)**	**1 (100.0)**

V: questing ticks collected from the vegetation; A: feeding-ticks collected from animals.
[a] Questing and feeding ticks negative for co-infections are not shown, but they were included in the calculation of prevalence data.

Mediterranean area (Estrada-Peña et al., 2014), has already been detected in other parts of Europe (Chitimia-Dobler et al., 2018; Hornok et al., 2022). In Spain, the distribution of *I. inopinatus* was described as allopatric with *I. ricinus* (Estrada-Peña et al., 2014), but the findings of the present study support the idea that the two species may share a common distribution area in northern regions (Chitimia-Dobler et al., 2018; Hauck et al., 2019). However, in this work, the identification of *I. inopinatus* species was only based on a single mitochondrial gene marker, which does not allow the detection of hybrid specimens. Thus, for a more in-depth study of the genetic relationship between *I. ricinus* and *I. inopinatus* in the region of study, a correct morphological identification of the specimens and complementary molecular markers (Kovalev et al., 2016) are needed.

Ixodes ricinus were found feeding on ungulates and carnivores, whereas *Dermacentor* spp. were only found on wild boar, wolf and brown bear at much lower intensity. The wild boar has been described as one of the main hosts in the wild cycle of *D. marginatus* in North-Eastern Spain, where this species is overabundant and is considered an important hunting target (Ortuño et al., 2007). The information about ticks parasitizing brown bear is very scarce. The presence of four different tick species (*Rhipicephalus turanicus, Hyalomma marginatum, I. ricinus* and *Haemaphysalis parva*) has been described on brown bears from Turkey (Orkun et al., 2020), but there is no prior evidence of *D. reticulatus* feeding on this host.

In this study, the overall occurrence of *Rickettsia* spp. in questing ticks was significantly higher in the genus *Dermacentor* than in the other tick species. The results obtained for *D. marginatus* and *D. reticulatus*, are in accordance with previous studies carried out in questing ticks in other Northern Spanish regions, where prevalences of 100% for *D. marginatus* (Remesar et al., 2019a) and 49.5% for *D. reticulatus* (Barandika et al., 2008) have been reported. In contrast, results for *I. ricinus* are far from the high prevalences (20.7%) reported in a nearby region (Remesar et al., 2019a). Although only few engorged ticks were analyzed, our results are consistent with other studies reporting the presence of *Rickettsia* spp. in *Dermacentor* spp. and *I. ricinus* collected from wild animals in Spain (Millán et al., 2016; Ortuño et al., 2007).

Sequence analyses revealed that "*Candidatus* Rickettsia rioja" and *R. raoultii* were the most abundant *Rickettsia* species in our study area, as has been previously reported in Northern Spain by Remesar et al. (2019a). *Dermacentor marginatus* was described as the main vector of these species (Portillo et al., 2015). However, these results highlighted that other tick species like *I. ricinus* and *D. reticulatus* could act as vectors of "*Candidatus* Rickettsia rioja" and *R. raoultii* in Northern Spain (Remesar et al., 2019a). Both species have been previously described in questing and feeding *Dermacentor* spp. ticks attached to different hosts (Spitalská et al., 2012; Reye et al., 2013; García-Vozmediano et al., 2020), which is in agreement with our results. In addition, "*Candidatus* Rickettsia rioja" was detected for the first time in adult *I. ricinus* ticks collected from cervids, and *R. raoultii* in adult *D. reticulatus* and *I. ricinus* feeding on a brown bear and a wolf respectively. In Asturias, both hosts share not only the habitat, but also certain food sources, the top position of the food chain and pathogens (Oleaga et al., 2021). Indeed, they share the habitat with a remarkable density of both people and dogs (Millán et al., 2016) which makes the study of many pathogens affecting these two wild predators very interesting from an epidemiological point of view. *Rickettsia slovaca* was identified at a lower rate in questing adult *D. marginatus* ticks, below the 10.6% and 11.1% reported in Central and Northern Spain, respectively (Toledo et al., 2009; Remesar et al., 2019a). All *Rickettsia* spp. identified in this work are considered the main agents of the emerging tick-borne rickettsiosis DEBONEL/TIBOLA/SENLAT (Portillo et al., 2015).

In this study piroplasms were associated to ticks collected from wildlife, while the presence of this pathogen in questing ticks is reduced to the detection of *B. bigemina* in a single *R. bursa. Theileria* OT3 and *B*abesia sp. badger type A were identified in feeding ticks collected from ungulates and carnivores. The genotype *Theileria* OT3 is mainly associated with small ruminants (Nagore et al., 2004; Giangaspero et al., 2015) and other ungulates (García-Sanmartín et al., 2007; Remesar et al., 2019b; Díaz-Cao et al., 2021), and there is evidence of its presence in *I. ricinus* (Remesar et al., 2021). As far as we know, our results revealed the presence of *Theileria* OT3 in adult *D. reticulatus* and *H. inermis* ticks feeding on wild ungulates and a brown bear for the first time. Since the presence of DNA in a tick does not prove competence as a vector, further investigations are needed to unravel the role of these and other tick species in the transmission of *Theileria* OT3. The presence of *Babesia* sp. badger type A was described for the first time in European badgers from Northern Spain (Barandika et al., 2016). Here, the association between DNA of this *Babesia* and *I. ricinus* from cervids is reported for the first time. The hemoprotozoan *H. canis* was identified in two of the adult ticks collected from three wolves. The presence of this

Table 4
Tick-borne pathogens (TBPs) detected in ticks collected from wildlife.

Identified TBPs	N° of positive ticks (prevalence%)									
		Red deer (n = 2)		Roe deer (n = 7)	Wild boar (n = 2)		Brown bear (n = 1)	Wolf (n = 3)	
	Total[a] (n = 40)	*Ixodes ricinus*[b] (n = 4)	*Haemaphysalis inermis*(n = 1)	*Ixodes ricinus*(n = 14)	*Dermacentor reticulatus*(n = 3)	*Dermacentor marginatus*(n = 1)	*Dermacentor reticulatus*(n = 3)	*Ixodes ricinus*(n = 8)	*Dermacentor reticulatus*(n = 2)
***Rickettsia* spp.**	**8 (20.0)**	**1 (25.0)**	**0**	**2 (14.3)**	**2 (66.7)**	**1 (100.0)**	**1 (33.3)**	**1 (12.5)**	**0**
"Candidatus Rickettsia rioja"	4 (10.0)	1 (25.0)		2 (14.3)		1 (100.0)	1 (33.3)	1 (12.5)	
Rickettsia raoultii	2 (5.0)								
Anaplasma phagocytophilum	**4 (10.0)**	**2 (50.0)**	**1(100.0)**	**1 (7.14)**	**0**	**0**	**0**	**0**	**0**
Piroplasms	**13 (32.5)**	**2 (50.0)**	**1 (100.0)**	**3 (21.4)**	**1 (33.3)**	**0**	**2 (66.7)**	**3(37.5)**	**1 (50.0)**
Babesia badger type A	2 (5.0)	1 (25.0)	1 (100.0)	1 (7.1)	1 (33.3)		1 (33.3)	2 (25.0)	
	6 (15.0)	1 (25.0)		2 (14.3)					
Theileria OT3	2 (5.0)								
Hepatozoon canis									

[a] Ticks collected from Eurasian badger (2 *I. ricinus*)) and red fox (2 *I. ricinus*) were negative for all the pathogens tested therefore the results are not shown, but they were included in the calculation of prevalence data.
[b] All feeding ticks were adults, except one *I. ricinus* nymph collected from one red deer.

parasite is common in foxes and beech martens –*Martes foina*- (Gimenez et al., 2009; Ortuño et al., 2021), and it has been recently detected in wolves from Italy (Battisti et al., 2020). Although animal samples were not included in the study, the finding of TBPs that have been reported in wildlife (Díaz-Cao et al., 2021; Ortuño et al., 2021) in engorged ticks but not in questing ticks, indicates the possible role of these animals in the epidemiology of the TBPs in Northern Spain.

The overall presence of *Borrelia* infecting questing *I. ricinus* is higher than previously reported for adults in the same region but lower for nymphs (Espí et al., 2016). These different results between studies could be explained by the different sampling areas for tick collection. In this case, *B. lusitaniae* was the most common genospecies detected, and to a lesser extent *B. afzelii, B. garinii, B. burgdorferi* s.s. and *B. valaisiana*. The Lyme borreliosis agents *B. afzelii* and *B. garinii* have been previously identified in the studied region (Espí et al., 2016), however this is the first report on pathogenic species *B. burgdorferi* s.s and *B. lusitaniae* prevalence in ticks from Asturias.

Ixodes ricinus is the main vector of *A. phagocytophilum*, which causes human and animal granulocytic anaplasmosis, and the reservoir role of wild ruminants for this pathogen have been widely investigated in Europe (Adamska, 2020; de la Fuente et al., 2008; Di Domenico et al., 2016; Kazimirová et al., 2018; Overzier et al., 2013a; Pereira et al., 2016; Remesar et al., 2020). In the current study, the presence of this pathogen was higher in feeding ticks collected from cervids (10.0% for adults and one nymph) than in questing ticks (7.1% for adults and 4.0% for nymphs), as was also observed in Germany (Overzier et al., 2013a). The finding of DNA of *A. phagocytophilum* in deer was previously reported in Northern Spain (Portillo et al., 2011; Remesar et al., 2020). Indeed, the detection of this pathogen in one *I. ricinus* nymph and one *H. inermis* adult cofeeding on the same red deer, suggest the contribution of the deer population to the maintenance of *A. phagocytophilum* in this geographic area. In the case of the prevalences reported for questing ticks, the current results are in line with those reported for adults *I. ricinus* from vegetation in Northern Spain (5.6%) (Barandika et al., 2008) but contrast with the rate (0.7%) reported in questing ticks from a neighboring region (Remesar et al., 2021). Genetic variants of *A. phagocytophilum* are associated to different epidemiological cycles (Jahfari et al., 2014). Our results revealed that the "W" variant, associated to livestock (Scharf et al., 2011), cervids (Remesar et al., 2020) and ticks (Overzier et al., 2013b), is circulating in most of the *A. phagocytophilum*-positive tick samples (73.7%), including questing and feeding adult ticks and nymphs. This variant is described as causing tick-borne fever in domestic ruminants (Nieder et al., 2012). Albeit at lower rates, variants "X" and "I" were detected in the region associated to adult *I. ricinus* collected from roe deer and questing adults and nymphs. These non-pathogenic variants were also identified on *I. ricinus* and roe deer in previous studies (Overzier et al., 2013a; Remesar et al., 2020).

Concerning the negative results of *C. burnetii* in analyzed samples, they are in accordance with the low prevalences (0.1%) reported in Northern Spain (Barandika et al., 2008). However, due to the widespread presence of this pathogen in humans and animals in Asturias (Rodríguez-Alonso et al., 2020; Espí et al., 2021), future studies of the sylvatic cycle of *C. burnetii* in the region should be addressed.

In line with other studies, we found the coexistence of two pathogens in both *I. ricinus* and *Dermacentor* spp. ticks collected from vegetation and animal hosts (Kazimirová et al., 2018). In this study, the combination *Rickettsia* spp./*Borrelia* spp. was found in questing ticks, while *A. phagocytophilum*/*Theileria* OT3 and *Rickettsia* spp./piroplasmids combinations were observed in feeding ticks. This could be due to co-transmission of the pathogens during blood feeding on the host (Randolph et al., 1996) or as result of infection during different feedings. Coexistence of several pathogens indicate a potential risk for humans and animals to be infected by multiple pathogens, and should be considered in the diagnosis and treatment of tick-borne diseases. The circulation of ticks harbouring the variant "W" of *A. phagocytophilum* and

Table 5
16S rRNA gene variants of *Anaplasma phagocytophilum* amplified from ticks in this study compared with human granulocytic anaplasmosis agent GenBank accession number U02521.

Sequence variant[a]	GenBankaccesion n°	Tick origin(n° of samples)	Nucleotide at indicated position[b]								
			74	**76**	**78**	**80**	**84**	**170**	**175**	**376**	**405**
B	U02521	human	A	A	A	A	G	C	C	G	A
I	MW800890[c]	vegetation (1)	A	**G**	A	A	**A**	C	**A**	G	A
W	MW800885[c]	red deer (3)	A	A	A	A	**A**	C	C	G	A
	MW800889[c]	vegetation (11)									
X	MW800887[c]	roe deer (1)	A	**G**	A	A	**A**	C	C	G	A
	MW800888[c]	vegetation (3)									

[a] Nomenclature used in previous studies (Silaghi et al., 2011; Overzier et al., 2013a).
[b] Single-nucleotide position in relation to the 497-pb partial 16S rRNA gene of the HGA agent (GenBank U02521). Differences to the reference genome are indicated in bold.
[c] Representative sequences of each variant for questing and feeding ticks obtained in this study.

Theileria OT3, could be relevant for animal health. It is worth noting, that this pattern of co-occurrence was detected in a nymph associated with the *I. inopinatus* haplogroup. Despite the fact that DNA of different TBPs have been detected in this new tick species (Hauck et al., 2019), there are still few and limited studies to know if the genetic differences between these two species of the genus *Ixodes* imply epidemiological differences in the transmission cycles of TBPs. Overall, the pathogen diversity was higher in *I. ricinus* than in the other tick species analyzed, with 11 pathogens identified. All of them, except piroplasmids and *H. canis,* are pathogenic to humans. Fewer species were detected in the genus *Dermacentor*, but it is worth noting the strong association found between *Dermacentor* spp. and SFG rickettsiae which would require further investigations to identify other possible natural reservoirs of this pathogen. Most prevalent tick species in the area are those with the highest burden of zoonotic TBPs, which would entail a high health risk for humans. Indeed, the present study demonstrates the occurrence of *I. ricinus* haplotypes phylogenetically related to *I. inopinatus* in Northern Spain. The detection of pathogen DNA in one of these samples indicates that the role of this species in the transmission of TBDs should be investigated, which underlines the importance of a correct identification of the different haplogroups of *I. ricinus*. Therefore, further studies whould be advisable to assess the natural reservoirs of these zoonotic agents.

5. Conclusions

In light of the results obtained, the range of tick-borne pathogens circulating in this region is relevant from a human and animal health point of view and they must be taken into account in the differential diagnosis and treatment of clinical cases. This study also revealed some novel tick-host and tick-borne pathogens associations. Finally, wildlife could have an important role not only as TBPs reservoirs but also in maintaining and spreading tick populations and tick-borne pathogens.

Declaration of Competing Interest

The authors declare no conflicts of interest. The funders had no role in the study design, data collection and interpretation, or the decision to submit the work for publication.

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