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Molecular detection of *Rickettsia* spp. in wild ungulates and their ticks in Mediterranean areas of southwestern Spain

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Abstract

Wildlife is an important reservoir of zoonotic pathogens. The objective of the present study was to assess the importance of wild ungulates in the epidemiology of *Rickettsia* spp. Ticks and spleen samples were collected from 262 red deer (*Cervus elaphus*) and 83 wild boar (*Sus scrofa*) hunted in southwestern Spain over a 5-year period. DNA was extracted from tick pools ($n = 191$) and spleens ($n = 345$), and two nested PCR assays targeting the *rOmpA* and *rOmpB* genes were used to detect *Rickettsia* DNA. Five tick species were identified (*Hyalomma lusitanicum*, *Dermacentor marginatus*, *Ixodes ricinus*, *Rhipicephalus bursa* and *Haemaphysalis sulcata*). *Rickettsia* DNA was detected in 31 (16.2%) tick pools and two red deer spleen samples (0.8%). Four validated *Rickettsia* species (*R. slovaca*, *R. monacensis*, *R. helvetica* and *R. raoultii*), one uncultivated species (*Candidatus* R. rioja) and two uncharacterized *Rickettsia* spp. were detected in ticks. *R. helvetica* and *R. slovaca* were also detected in spleen samples from red deer. The overall prevalence in ungulate spleen samples was lower than in tick pools suggesting that these ungulates do not play a major role in the transmission of *Rickettsia* spp. However, their importance as spreaders of positive ticks cannot be ruled out. The results present a challenge for the veterinary and public health communities since most of the *Rickettsia* spp. detected are pathogenic. Furthermore, the new *Rickettsia* species could be potential pathogens. For these reasons, identifying *Rickettsia* species present in ticks and wildlife is of particular interest to clarify their sylvatic cycle and establish appropriate control measures.

KEYWORDS
red deer, *Rickettsia*, tick-borne diseases, wild boar, zoonoses

1 | INTRODUCTION

Wildlife is considered an important reservoir of zoonotic pathogens that represent a public health problem (Gonzalez-Barrio, 2022). An increasing number of studies have recently been conducted in wildlife to determine their role in the maintenance and transmission of tick-borne pathogens since these agents can have a notable impact on both human and animal health, as well as on livestock production (García-Pérez et al., 2016).

It has been suggested that some wild ungulates such as red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*) may play an important role in the ecology of tick-borne pathogens, acting as maintenance hosts for tick populations and, in some cases, as natural reservoirs of some tick-borne pathogens (Fabri et al., 2021). These species may also favour the geographical spread of tick populations (Fabri et al., 2021). The increased population densities of both red deer and wild boar observed in recent decades, as well as their distribution range expansion (Apollonio et al., 2010) have contributed to an increase in tick densities and, secondarily, to the increased prevalence of some tick-borne pathogens (Jaenson et al., 2012). The role of these wild species in the epidemiology of tick-borne pathogens depends on the selected pathogen. Ungulates are considered competent reservoirs for *Anaplasma phagocytophilum* (Díaz-Cao et al., 2022; Stuen et al., 2013) and some piroplasm species (Remesar et al., 2019; Zanet et al., 2014), but are unable to transmit *Borrelia burgdorferi* sensu lato (s.l.) to ticks (Hofmeester et al., 2016; Pacilly et al., 2014). On the other hand, the data on the role played by wild ungulates in the eco-epidemiology of many other pathogens, such as *Rickettsia* spp., is still very scarce.

Rickettsia species are obligate intracellular bacteria with complex lifecycles (Eremeeva & Dasch, 2015). They have been classified into several groups based on phenotype and phylogeny. The most important are the spotted fever group (SFG) and the typhus group (TG), which include most of the known zoonotic disease-causing species (Diop et al., 2019). The most prevalent rickettsioses in humans in Europe are Mediterranean spotted fever (MSF) and scalp eschar and neck lymphadenopathy after tick bite (SENLAT). MSF is caused by *Rickettsia conorii* subsp. *conorii* and mainly transmitted by *Rhipicephalus* ticks. SENLAT, also known as *Dermacentor*-borne necrosis erythema lymphadenopathy (DEBONEL), is caused by *Rickettsia slovaca*, *Rickettsia raoultii* and the uncultivated *Candidatus* Rickettsia rioja, with *Dermacentor* spp. being their main vectors (Oteo & Portillo, 2012; Parola et al., 2013). *Rickettsia aeschlimannii*, *Rickettsia sibirica* subsp. *mongolitimonae*, *Rickettsia monacensis*, *Rickettsia helvetica* and *Rickettsia massiliae* are other zoonotic species found in Europe, mainly transmitted by *Hyalomma* spp., *Ixodes* spp. and *Rhipicephalus* spp. (Oteo & Portillo, 2012; Parola et al., 2013).

Impacts

- *Rickettsia* spp. prevalence in ungulate spleen samples was lower than in tick pools.
- Most of the *Rickettsia* spp. detected are pathogenic.
- Two new *Rickettsia* spp. with unknown pathogenicity were detected.

In Spain, the presence of tick species positive to zoonotic *Rickettsia* spp. has been previously reported in ticks collected feeding on red deer and wild boar from northern and central areas, showing a percentage of positivity ranging from 10% to 68% depending on tick species and host (Castillo-Contreras et al., 2021; Márquez, 2009; Ortuño et al., 2006). These wild ungulate populations have expanded in this country in the past few years, moving closer to human populations and increasing the risk of transmission of zoonotic diseases (González-Crespo et al., 2018). Against this background, the aims of the present study were to identify tick species feeding on red deer and wild boar in Mediterranean areas of southwestern Spain, and to determine the presence of *Rickettsia* spp. in both of these wild ungulate species as well as in ticks feeding on them.

2 | MATERIALS AND METHODS

2.1 | Study area and sampling

The study was carried out in southwestern areas of Spain (39°N, 6°W) during the period 2016–2021. A total of 262 red deer and 83 wild boar, legally hunted on 31 hunting estates, were included in the study. Animals were classified by age (young: <1 year old, subadult: between 1 and 3 years old, and adult: >3 years old) and sex (male or female). The age of the animals was estimated on the basis of teeth replacement and wear (Sáenz de Buruaga et al., 2001). Each animal was individually examined in the field for the presence of ticks, which were then collected and bagged. In addition, a spleen sample was aseptically collected from each animal during on-field evisceration and placed in a plastic tube. Spleen samples and ticks were kept frozen at −20°C until further analysis.

2.2 | Tick identification

Ticks were identified using morphological keys (Pérez-Eid, 2007) and a subset of each species was selected for molecular confirmation of

identification. DNA was extracted from the ticks using a commercial kit (High Pure PCR Template Preparation Kit; Roche Diagnostics GmbH®), following the manufacturer's instructions. Before DNA extraction, the ticks were disrupted with a MagNa Lyser Instrument (Roche Diagnostic) at 6000 rpm for 60 s. Finally, a 460 bp fragment of the 16S rRNA gene of ticks was amplified, as previously described (Norris et al., 1996; Simon et al., 1994).

The PCR products were separated by electrophoresis on 1% agarose gel stained with RedSafe (iNtRON Biotechnology®) and visualized using the GelDoc Go Imaging System (Bio-Rad Laboratories®). Selected fragments were purified and sequenced on an ABI 3730xl sequencer (Applied Biosystems) at the Sequencing and Fragment Analysis Unit, University of Santiago de Compostela. The sequences were aligned and edited using ChromasPro (Technelysium) and consensus sequences were then scanned against the GenBank database using BLAST.

2.3 | Molecular detection of *Rickettsia* spp.

After identification, ticks from the same hunting estate were pooled according to ungulate species, tick species, and developmental stage (Table 1). DNA was extracted from tick pools and 200 mg of spleen tissue ($n = 345$), as above. The presence of *Rickettsia* DNA in tick samples was detected using two nested PCR assays targeting genes encoding two of the major outer membrane proteins of *Rickettsia* spp. (*rOmpA* and *rOmpB*), using previously reported primers and protocols (Choi et al., 2005; Regnery et al., 1991; Roux et al., 1996). Samples positive for *rOmpA* and/or *rOmpB* were further analysed by a third PCR assay targeting the *Rickettsia* spp. *gltA* gene (Labruna et al., 2004). In each assay, DNA from *Rickettsia amblyommatis*, a *Rickettsia* species not found in Europe, and nuclease free water were included as positive and negative controls, respectively. Detection of DNA amplification, sequencing and species identification were performed as above.

Phylogenetic analysis was carried out with MRBAYES 3.2.7 software (Ronquist et al., 2012), using a Bayesian technique with Markov chain Monte Carlo sampling (10,000,000 generations, sampling every 1000th generation). For the analysis of tick sequences in the *rOmpA* and *rOmpB* genes, a General Time Reversible substitution model with gamma-distributed rate variation across sites (GTR + G) was used. Model selection was based on the AIC (Akaike Information Criterion), using free JMODELTEST software, v.2.1.10 (Darriba et al., 2012). The tree was visualized and edited in FIGTREE software 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

2.4 | Statistical analysis

The prevalence of *Rickettsia* spp. in pooled ticks was estimated by maximum likelihood estimation (MLE), as previously described (Williams & Moffitt, 2005). This estimation takes into account the number of processed pools and the number of ticks processed in each pool. Due to the low prevalence of *Rickettsia* spp. found in wild ungulates, the associated risk factors could not be analysed for these species. The presence of significant differences in total tick numbers when considering tick species and developmental stage (nymph and adult) was assessed using an ANOVA test. The possible influence of certain variables (tick species and development stage, ungulate species, region, and hunting area) on the incidence of *Rickettsia* spp.

TABLE 1 Number of each tick species collected from red deer and wild boar in southwestern Spain and composition of pools analysed.

	Nymph		Adults		Total		
	No. ticks	No. pools	No. ticks	No. pools	No. ticks	%[a]	No. pools
Red deer							
Dermacentor marginatus	0	–	8	7	8	1.0	7
Hyalomma lusitanicum	4	4	733	106	737	93.8	110
Ixodes ricinus	0	–	19	8	19	2.4	8
Rhipicephalus bursa	21	6	1	1	22	2.8	7
Total	25	10	761	122	786	–	132
Wild boar							
D. marginatus	0	–	21	10	21	9.3	10
Haemaphysalis sulcata[b]	0	–	1	1	1	0.4	1
H. lusitanicum	1	1	196	40	197	87.2	41
I. ricinus	0	–	6	6	6	2.7	6
R. bursa	1	1	0	–	1	0.4	1
Total	2	2	224	57	226	–	59
Total	27	12	985	179	1012	–	191

[a]Percentage of collected ticks.

[b]This specimen was not included in the multivariate analysis.

in tick pools was analysed by multivariate analysis, using a multiple logistic regression model (Hosmer et al., 1989). This analysis was performed with the brglm2 R package (Kosmidis & Firth, 2021), which allows for estimation and inference from generalized linear models using implicit and explicit bias reduction methods (Kosmidis & Firth, 2021). Finally, factors were removed from the initial model using an AIC value-based method until the best model was built. All pairwise interactions were evaluated. Odds ratios were computed by raising e to the power of the logistic coefficient over the first category of each factor (reference category). All statistical analyses were performed with R statistical software (R Core Team, 2020). The significance level was set at p-values <0.05.

3 | RESULTS

3.1 | Tick species parasitising wild ungulates

A total of 1012 ticks were collected from red deer ($n=786$) and wild boar ($n=226$) (Table 1). In terms of developmental stage, adults predominated ($F=6.141$; $p=0.014$), and only 25 and two nymphs were collected, from red deer (3.2%) and wild boar (0.9%), respectively. No larvae were detected. Five tick species were morphologically identified (Table 1): *Hyalomma lusitanicum* was by far the most frequent (92.3%) in both ungulate species, followed by *Dermacentor marginatus* (2.9%), *Ixodes ricinus* (2.5%) and *Rhipicephalus bursa* (2.3%), and finally a single specimen of *Haemaphysalis sulcata* (0.1%). Sequence analysis confirmed the morphological identification of all tick species. *H. lusitanicum* sequences were identical to the *H. lusitanicum* sequence (MZ420716) obtained from ticks on wild rabbits in the same area (Remesar et al., 2022). The *D. marginatus* sequences were identical to sequence KX555656, obtained from *D. marginatus* ticks collected from sheep in China (Wang et al., 2015). Similarly, the sequences of *R. bursa*, *I. ricinus* and *H. sulcata* were identical to those (KR870983, MK620872 and MT799946) obtained from ticks in Turkey, France and Pakistan (Ghafar et al., 2020; Orkun & Cakmak, 2019).

All tick species were detected in both wild ungulates, except for *H. sulcata*, which was only detected in wild boar (Table 1). In both mammal species, the numbers of *H. lusitanicum* collected were significantly higher than those of *D. marginatus* ($F=6.185$; $p=0.014$ and $F=4.652$; $p=0.034$ for red deer and wild boar, respectively). The numbers of *H. lusitanicum* were also significantly higher than those of *I. ricinus* ($F=4.654$; $p=0.034$) and *R. bursa* ($F=9.223$; $p=0.003$) in red deer and wild boar, respectively. Co-infestations with different tick species were infrequently detected in the two ungulate species. Four co-infestations were detected in red deer: *H. lusitanicum*/*I. ricinus* (2.3%; 6/262), *H. lusitanicum*/*R. bursa* (1.9%; 5/262), *H. lusitanicum*/*D. marginatus* (1.5%; 4/262) and *H. lusitanicum*/*R. bursa* (0.4%; 1/262). In wild boar, the most common co-infestation detected was *H. lusitanicum*/*D. marginatus* (4.8%; 4/83), followed by *D. marginatus*/*I. ricinus* (3.6%; 3/83), *H. lusitanicum*/*I. ricinus* (1.2%; 1/83) and *H. lusitanicum*/*H. sulcata* (1.2%; 1/83). *H. lusitanicum* ticks at different developmental stages, including nymphs and adults, were found in four red deer (1.5%; 4/262). No co-infestations with the same tick species at different developmental stages were detected in wild boar.

3.2 | *Rickettsia* species in ticks and wild ungulates

Rickettsia DNA was detected in 31 of 191 (16.2%) tick pools and in two of 345 spleen samples (0.6%; 95% confidence intervals (95% CI): 0.00–1.38). *Rickettsia*-positive pools were found on 18 (58.1%) of 31 hunting estates. Positive samples were detected in ticks feeding on red deer from 9 out of the 24 (37.5%) and in ticks feeding on wild boar from 13 out of the 27 (48.1%) hunting estates sampled (Figure 1). The percentage of positive tick pools (Table 2) was higher in wild boar (27.1%; 16/59) than in red deer (11.4%; 15/132), although the differences were not statistically significant ($p>0.05$).

All *D. marginatus* pools were positive for *Rickettsia* spp. (100%; 17/17), and the percentage of *Rickettsia*-positive *I. ricinus* pools was also high (71.4%; 10/14). By contrast, *Rickettsia* DNA was detected in only 2.6% (4/151) of the *H. lusitanicum* pools, and none of the *R. bursa* (0%; 0/8) or *Haemaphysalis punctata* (0%; 0/1) pools (Table 2). Logistic regression showed that the percentages of *I. ricinus* and *D. marginatus* pools classed as positive were significantly higher than those for *H. lusitanicum* and *R. bursa* pools (Table 3). No significant differences were detected among the other tick species.

Rickettsia DNA was found in the spleens of two of 262 (0.8%; 95% CI: 0.00–1.82) red deer (US002 and US208; Figure 1). The two pools of *D. marginatus* and *I. ricinus* ticks collected from these two positive animals, respectively, also tested positive for *Rickettsia* infection. By contrast, no *Rickettsia*-positive spleens (0%; 0/83; 95% CI: 0.00–4.40) were detected in wild boar.

3.3 | *Rickettsia* species identified

Four validated *Rickettsia* species and one uncultivated species were molecularly identified in the tick pools (Table 2, Figures 2 and 3). In addition, two uncharacterized *Rickettsia* species were detected (Figure 2). The most common *Rickettsia* species detected in ticks was *Candidatus* Rickettsia rioja (5.2%; 10/191), followed by *R. slovaca* (4.2%; 8/191) and *R. monacensis* (3.1%; 6/191). Two uncharacterized *Rickettsia* species were detected in one pool each (1.0%; 2/191). *R. helvetica* and *R. raoultii* were both detected in a single pool (0.5%; 1/191). Finally, co-infection with *R. monacensis*/*R. helvetica* was detected in three pools (1.6%; 3/191) (Figure 1).

Candidatus R. rioja, *R. slovaca*, *R. monacensis* and *R. helvetica* were detected in tick pools from both ungulate species, as well as in the *R. monacensis*/*R. helvetica* co-infection. By contrast, *R. raoultii* and the two uncharacterized *Rickettsia* species were detected only in tick pools from wild boar and red deer, respectively (Table 2). The most common *Rickettsia* species in ticks from red deer was *R. slovaca* (3.0%; 4/132), followed by *R. monacensis* (2.3%; 3/132) and Ca. R.

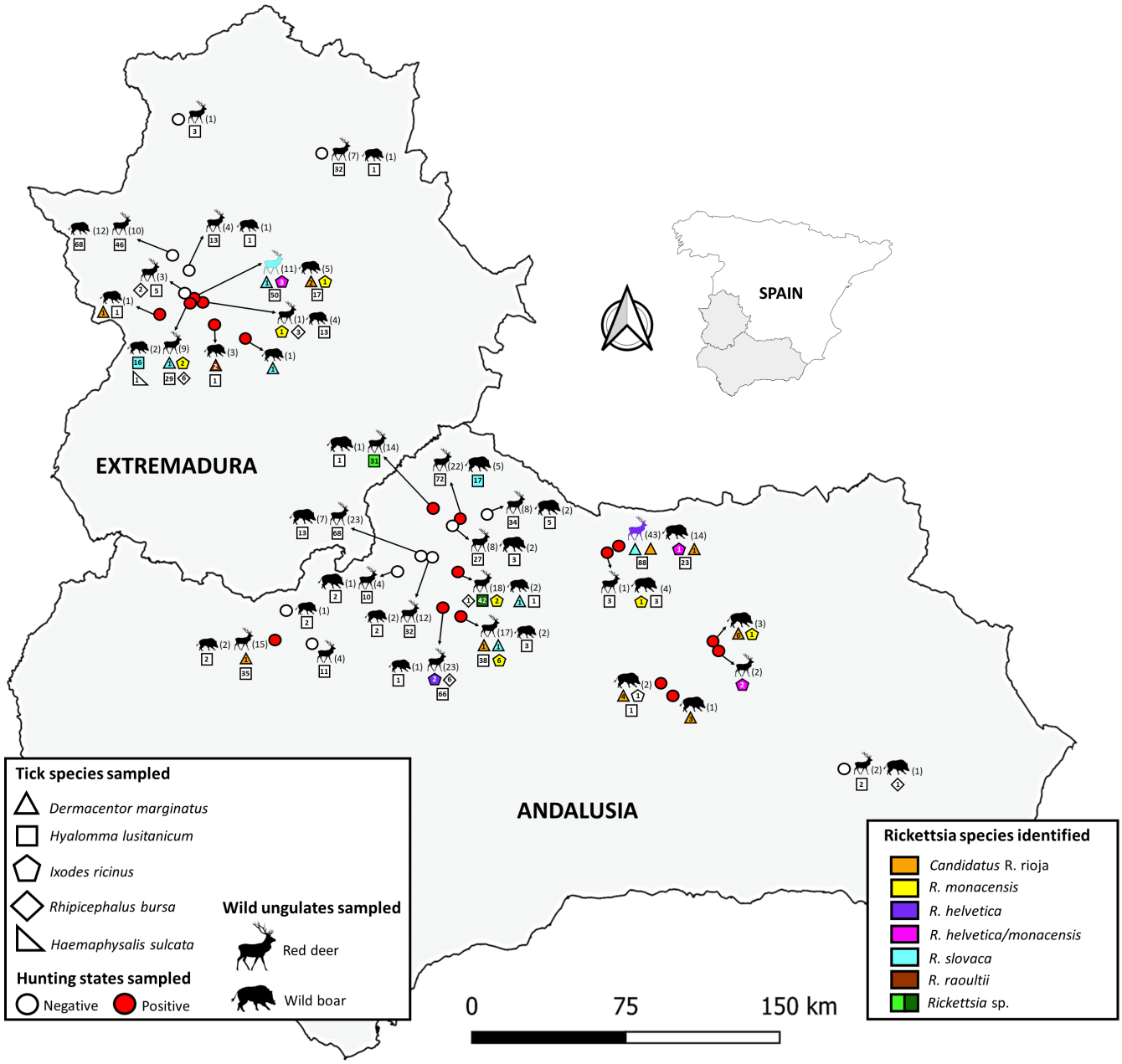

FIGURE 1 Map of southwestern Spain (Extremadura and Andalusia regions). Dots indicate the 31 hunting estates where red deer and wild boar were sampled. The presence of tick and spleen samples positive for *Rickettsia* spp. is indicated.

rioja (2.3%; 3/132) (Table 2). *R. monacensis*/*R. helvetica* co-infection was detected in two pools (1.5%; 2/132), and *R. helvetica* and each of the new *Rickettsia* species were detected in one tick pool (0.8%; 1/132) from this wild ruminant species (Table 2). In wild boar, the most abundant species was *Ca.* R. rioja (11.9%; 7/59), followed by *R. slovaca* (6.8%; 4/59) and *R. monacensis* (5.1%; 3/59). *R. raoultii* and *R. monacensis*/*R. helvetica* co-infection were detected once (1.7%; 1/59) (Figure 1).

Most of the *Rickettsia* species identified were found in a single tick species. Accordingly, *Ca.* R. rioja and *R. raoultii* were detected only in *D. marginatus*; *R. monacensis* and *R. helvetica* were detected only in *I. ricinus*, and the two uncharacterized *Rickettsia* species were detected only in *H. lusitanicum*. Only *R. slovaca* was detected in two different tick species, namely, *D. marginatus* and *H. lusitanicum* (Table 2).

The species identified in the two red deer spleen samples were *R. helvetica* (sample US002) and *R. slovaca* (sample US208), respectively. However, the *Rickettsia* species identified in the tick pools collected from each individual were *R. slovaca* (US002) and *R. helvetica* (US208), respectively.

Sequence data of *Rickettsia* spp. isolates in the *rOmpB*, *rOmpA* and *gltA* genes are shown in Table S1. The unique partial sequences identified were deposited under accession numbers OP729868–OP729890.

TABLE 2 Percentage of pools positive to *Rickettsia* species detected and maximum likelihood estimation (MLE) from red deer and wild boar.

	C. R. rioja		*Rickettsia slovaca*		*Rickettsia monacensis*		*Rickettsia helvetica*		*Rickettsia raoulti*		*Rickettsia monacensis/helvetica*		*Rickettsia* sp.		Total	
	% (pos/total)	MLE (95% CI)	% (pos/total)	MLE (95% CI)	% (pos/total)	MLE (95% CI)	% (pos/total)	MLE (95% CI)	% (pos/total)	MLE (95% CI)	% (pos/total)	MLE (95% CI)	% (pos/total)	MLE (95% CI)	% (pos/total)	MLE (95% CI)
Red deer																
Dermacentor marginatus	42.3 (3/7)	40.7 (12.0–75.4)	57.1 (4/7)	50.0 (19.1–80.9)	0 (0/7)	–	0 (0/7)	–	0 (0/7)	–	0 (0/7)	–	0 (0/7)	–	100 (7/7)	100 (73.5–100)
Hyalomma lusitanicum	0 (0/110)	–	0 (0/110)		0 (0/110)	–	0 (0/110)	–	0 (0/110)	–	0 (0/110)	–	1.8 (2/110)	0.7 (0.0–0.8)	1.8 (2/110)	0.7 (0.0–0.8)
Ixodes ricinus	0 (0/8)	–	0 (0/8)	–	37.5 (3/8)	18.6 (4.9–42.0)	12.5 (1/8)	5.3 (0.3–21.2)	0 (0/8)	–	25 (2/8)	12.3 (2.1–33.9)	0 (0/8)	–	75 (6/8)	10.8 (1.9–29.9)
Rhipicephalus bursa	0 (0/7)	–	0 (0/7)	–	0 (0/7)	–	0 (0/7)	–	0 (0/7)	–	0 (0/7)	–	0 (0/7)	–	0 (0/7)	–
Total	2.3 (3/132)	0.4 (0.1–1.0)	3 (4/132)	0.5 (0.2–1.2)	2.3 (3/132)	0.4 (0.1–1.0)	0.8 (1/132)	0.1 (0.0–0.6)	0 (0/132)	–	1.5 (2/132)	0.3 (0.0–0.8)	1.5 (2/132)	0.3 (0.0–0.9)	11.4 (15/132)	1.9 (1.1–3.1)
Wild boar																
D.marginatus	70 (7/10)	52.5 (25.3–80.7)	20 (2/10)	9.5 (1.7–26.6)	0 (0/10)	–	0 (0/10)	–	10 (1/10)	4.9 (0.3–19.8)	0 (0/10)		0 (0/10)	–	100 (10/10)	100 (71–100)
Haemaphysalis sulcata	0 (0/1)	–	0 (0/1)	–	0 (0/1)	–	0 (0/1)	–	0 (0/1)	–	0 (0/1)	–	0 (0/1)	–	0 (0/1)	
H. lusitanicum	0 (0/41)	–	4.9 (2/41)	1.1 (0.2–3.3)	0 (0/41)		0 (0/41)	–	0 (0/41)	–	0 (0/41)	–	0 (0/41)		4.9 (2/41)	1.1 (0.2–3.3)
I. ricinus	0 (0/6)	–	0 (0/6)	–	50 (3/6)	50 (15.6–84.4)	0 (0/6)	–	0 (0/6)	–	16.7 (1/6)	16.7 (1.0–55.4)	0 (0/6)	–	66.7 (4/6)	66.7 (28.1–93.5)
R.bursa	0 (0/1)	–	0 (0/1)	–	0 (0/1)	–	0 (0/1)	–	0 (0/1)	–	0 (0/1)	–	0 (0/1)	–	0 (0/1)	
Total	11.9 (7/59)	3.2 (1.4–6.0)	6.8 (4/59)	1.8 (0.6–4.2)	5.1 (3/59)	1.3 (0.3–3.4)	0 (0/59)	–	1.7 (1/59)	0.1 (0.0–1.9)	1.7 (1/59)	0.4 (0.0–1.9)	0 (0/59)		27.1 (16/59)	7.6 (4.5–11.8)

TABLE 3 Logistic regression model for the prevalence of *Rickettsia* spp. in ticks collected from red deer and wild boars in southwestern Spain.

	Estimate	*Z*-value	*p*-value	OR	95% CI
(Intercept)	−3.490	−7.268	<0.001		
Tick species[a]					
Hyalomma lusitanicum					
Ixodes ricinus	4.337	5.741	<0.001	76.481	17.400–336.177
Dermacentor marginatus	7.045	4.539	<0.001	1147.222	54.776–24,027.190
Rhipicephalus bursa	0.657	0.406	0.685	1.928	0.08–45.819
R. bursa					
I. ricinus	3.681	2.231	0.026	39.667	1.563–1006.712
D. marginatus	6.389	2.992	0.003	595.000	9.052–39,109.886
D. marginatus					
I. ricinus	−2.708	−1.706	0.088	0.067	0.003–1.495

[a]The only specimen of *Haemaphysalis punctata* was not included in the analysis.

4 | DISCUSSION

4.1 | Tick species parasitizing wild ungulates

All five tick species identified in this study (*H. lusitanicum*, *D. marginatus*, *I. ricinus*, *R. bursa* and *H. sulcata*) are commonly found in wild ungulates, although *R. bursa* tends to prefer domestic ungulates such as sheep and goats (Estrada-Peña et al., 2017). While all these species have previously been detected feeding on red deer and wild boar in Spain, their presence and/or predominance is dependent on the host and ecological preferences of each tick species. In general, *I. ricinus* and *Dermacentor reticulatus* are more abundant in areas of northern Spain, where the climate is mild and humid, since neither of them tolerate excessive heat or desiccation (Bowman & Nuttall, 2008; Remesar et al., 2019). This could explain their predominance in red deer and wild boar in northern Spain (Castillo-Contreras et al., 2021; Ortuño et al., 2006; Ruíz-Fons et al., 2006) and northern European countries (Kazimirova et al., 2018; Skotarczak et al., 2008). *H. lusitanicum*, *H. marginatus* and *D. marginatum* on the other hand are less sensitive to heat and all can be found in southern and western areas of Europe with a Mediterranean climate (Díaz-Cao et al., 2022; Estrada-Peña et al., 2017; Grech-Angelini et al., 2016; Márquez, 2009; Sgroi et al., 2021; Valcarcel et al., 2016).

Although several tick species belonging to genus *Rhipicephalus* (*Rhipicephalus annulatus*, *Rhipicephalus pusillus* and *R. bursa*) and *Haemaphysalis* (*H. punctata*, *H. sulcata* and *Haemaphysalis concinna*) have been reported in wild ungulates in Spanish Mediterranean ecosystems, they are less common and their distribution is not as restricted as the species cited above (Castillo-Contreras et al., 2021; Márquez, 2009; Ortuño et al., 2006; Ruíz-Fons et al., 2006; Valcarcel et al., 2016).

Regarding to the stage of development, adult ticks were the most abundant, which is consistent with previous studies carried out in wild ungulates (de la Fuente et al., 2004; Márquez, 2009; Ortuño et al., 2006; Ruíz-Fons et al., 2006). In this regard, it has been reported that the hosts of these tick species at more immature stages of development are most often small mammals and birds (Estrada-Peña et al., 2017). In most studies carried out in wild ungulates however, including our own, ticks were collected after direct examination in the field, so that the numbers at more immature stages may well be underestimated.

4.2 | *Rickettsia* species in wild ungulates and their feeding ticks

There was a lower percentage of *Rickettsia*-positive spleen samples than of *Rickettsia*-positive tick pools, even when a maximum likelihood estimation was carried out to approximate the true prevalence of the studied pathogens in tick population (MLE results indicated in Table 2). Our results are consistent with those obtained in other studies of ticks feeding on red deer and wild boar in Spain, which have reported higher prevalence values for *D. marginatus* (20%–68%) (Castillo-Contreras et al., 2021; Márquez, 2009; Ortuño et al., 2006) and *I. ricinus* (54.5%–60%) (Márquez, 2009) than for *H. lusitanicum* (3.2%) (Castillo-Contreras et al., 2021). Some specimens of *H. punctata*, *Rhipicephalus turanicus*, *R. bursa* and *R. annulatus* were also tested, although none were found to be *Rickettsia*-positive (Castillo-Contreras et al., 2021). Information on the prevalence of *Rickettsia* in ticks collected from red deer and wild boar in other European countries is limited, and most studies have been conducted in Italy, where the reported prevalences of *D. marginatus* and *I. ricinus* feeding on wild boar were 9.7%–35.7% and 0.6%, respectively (Chisu et al., 2017; García-Vozmediano et al., 2020; Selmi et al., 2017; Sgroi et al., 2021). In a study carried out in Germany, *Rickettsia* DNA was detected in 6.3% of the *I. ricinus* collected from wild boar (Silaghi et al., 2014). By contrast, no *Hyalomma excavatum* or *R. turanicus* ticks collected from red deer in Turkey were positive for this pathogen (Orkun & Emir, 2020).

In the present study, a wide variety of *Rickettsia* spp. were detected in ticks obtained from ungulates (Figures 2 and 3), including one uncultivated species and two uncharacterized *Rickettsia* species. In line with our results, *Rickettsia* spp. causing

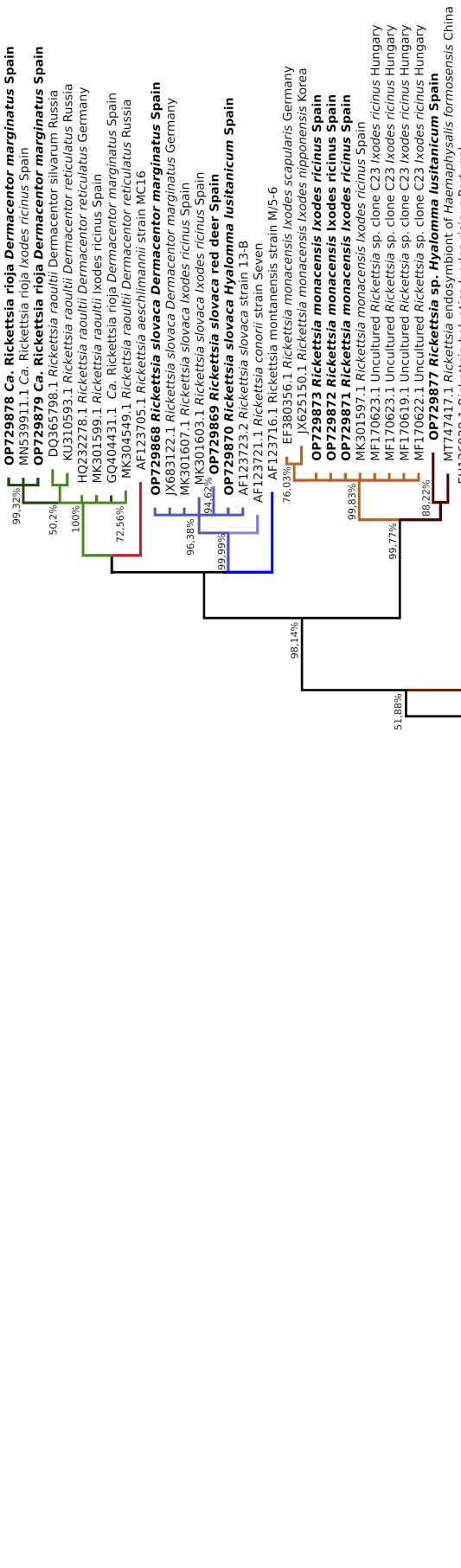

FIGURE 2 Phylogenetic tree clustering of the partial *rOmpB* of *Rickettsia* spp. The tree was obtained using a General Time Reversible substitution model with gamma-distributed rate variation across sites (GTR + G) with MRBAYES software 3.2.7 (Ronquist et al., 2012), using Bayesian inference with Markov Chain Monte Carlo sampling (10,000,000 generations, sampling every 1000th generation). This analysis involved 44 nucleotide sequences. The nucleotide sequence of *Rickettsia typhi* was used as an outgroup. Isolates in bold were identified, or identical to those identified in this study.

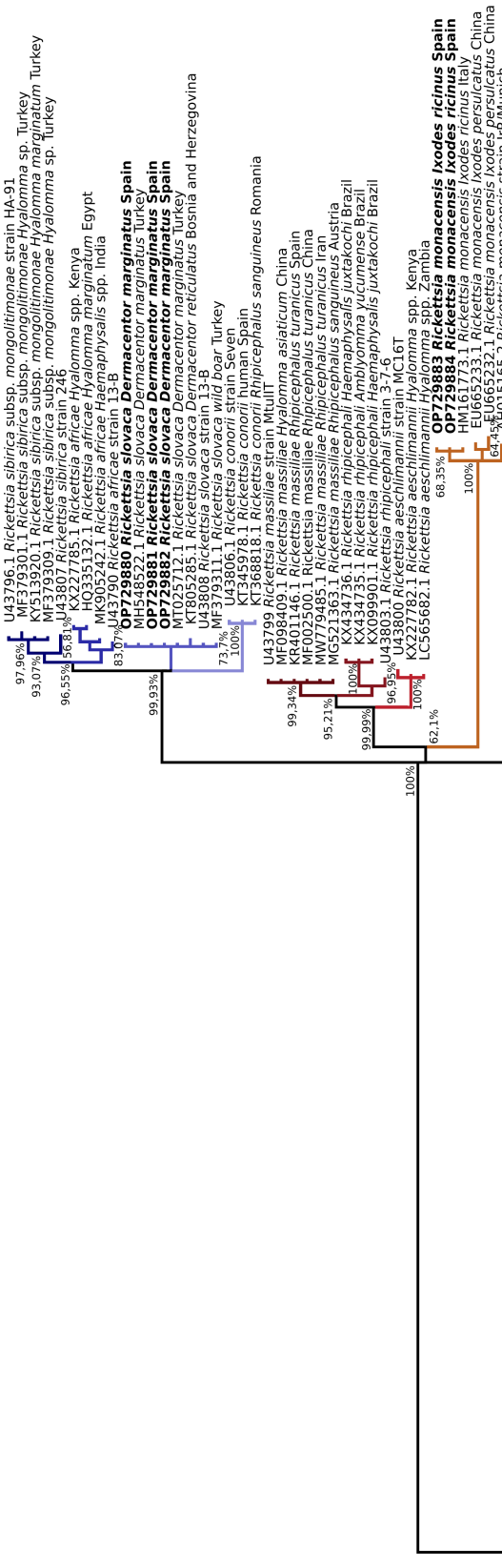

FIGURE 3 Phylogenetic tree clustering of the partial *rOmpA* of *Rickettsia* spp. The tree was obtained using a General Time Reversible substitution model with gamma-distributed rate variation across sites (GTR+G), with MRBAYES 3.2.7 software (Ronquist et al., 2012), using a Bayesian technique with Markov chain Monte Carlo sampling (10,000,000 generations, sampling every 1000th generation). This analysis involved 56 nucleotide sequences. The nucleotide sequence of *Rickettsia felis* was used as an outgroup. Isolates in bold were identified, or identical to those identified in this study.

DEBONEL were the most common species in previous studies on ticks captured on wild ungulates in south and northwestern Spain (Castillo-Contreras et al., 2021; Márquez, 2009; Ortuño et al., 2006). Castillo-Contreras et al. (2021) also detected an unidentified *Rickettsia* species in *D. marginatus* collected from wild boar. Meanwhile, the species identified in *I. ricinus* ticks collected from red deer and wild boar in southern Spain were *R. monacensis* and *R. helvetica* (Márquez, 2009).

It should be noted that *Ca*. R. rioja is an uncultivated *Rickettsia* species close to *R. raoultii*. Since the two can only be differentiated by sequence analysis of the *rOmpA* gene, most authors include it under *R. raoultii*. In our study, almost all tick pools positive for *Ca*. R. rioja, *R. slovaca* or *R. raoultii* were of *D. marginatus* (89.5%) and only two pools of *H. lusitanicum* were positive for *R. slovaca*. These *Rickettsia* species are of public health concern since they are the aetiological agents responsible for the human disease SENLAT (also known as DEBONEL), which is the most prevalent tick-borne rickettsiosis in Europe (Oteo & Portillo, 2012). These pathogens are usually associated with *Dermacentor* spp. ticks (Portillo et al., 2015). On the other hand, these *Rickettsia* species have also been reported in other tick species, such as questing *I. ricinus* (Chmielewski et al., 2009; Remesar et al., 2019; Reye et al., 2013; Stanczak et al., 2018) or, in agreement with our results, in *H. lusitanicum* (Castillo-Contreras et al., 2021) and *I. ricinus* (Sgroi et al., 2021) collected from wild boar. Nevertheless, the role of these tick species and/or ungulates in the epidemiology of these pathogens needs further investigation.

Rickettsia monacensis and *R. helvetica* were only detected in *I. ricinus* pools; notably, both species were detected in the same *I. ricinus* pool, which may be related to their high prevalence in these tick species. Both of these pathogens are zoonotic and have previously been detected in *I. ricinus* in more than half the countries of Europe (Parola et al., 2005). Although *I. ricinus* is considered to be the main vector and reservoir, recent research has detected *R. helvetica* in lizards (De Sousa et al., 2012), bats (Matei et al., 2021) and dogs (Banovic et al., 2021), suggesting that these vertebrate species could act as potential or transitory reservoirs for this *Rickettsia* species; however, this claim needs to be further evaluated. In fact, xenodiagnostic studies using rodents confirmed that these micromammals were unable to transmit *Rickettsia* species to ticks and concluded that ticks were probably the main reservoir of these pathogens (Burri et al., 2014; Sprong et al., 2009).

Two *H. lusitanicum* pools collected from red deer were found to be positive for two uncharacterized *Rickettsia* species by *rOmpB* gene sequence analysis. Phylogenetic analysis of *rOmpB* indicated that the sequence of one of these uncharacterized *Rickettsia* species (TPU029) fell outside the cluster including the main Spotted Fever Group *Rickettsia* (Figure 2). Nevertheless, it was clustered with other *Rickettsia* species, identified as endosymbionts of questing *H. punctata* and *H. sulcata* from northern Spain, which also failed to amplify in the *rOmpA* gene (Portillo et al., 2008). The other uncharacterized *Rickettsia* species (TPU050) was also clustered with another *Rickettsia* sequence identified as an endosymbiont of *Haemaphysalis formosensis*, detected in China (Zhu et al., 2020). Further molecular and culture studies of the two uncharacterized *Rickettsia* spp. should be performed to complete their characterization and determine their pathogenicity.

In the present study, *R. helvetica* and *R. slovaca* DNA was detected in the spleens of two red deer, whereas no wild boar spleen samples were positive for *Rickettsia* spp. Ticks have been considered the main reservoir of *Rickettsia* spp. as they can be transmitted transovarially (Socolovschi et al., 2009) and their prevalence in a number of wild and domestic animal species is generally low, except in certain rodents identified as reservoirs of *R. sibirica*, *R. helvetica* or *Rickettsia typhi* (Parola et al., 2013; Sprong et al., 2009). It has also been reported that dogs could transmit *R. conorii* and *Rickettsia rickettsii* to ticks (Levin et al., 2012; Piranda et al., 2011). Some studies have suggested that domestic ruminants, wild boar and Sika deer (*Cervus nippon*) could transmit *Rickettsia* species such as *Rickettsia africae*, *R. slovaca* and *R. helvetica* to ticks (Inokuma et al., 2008; Ortuño et al., 2007, 2012; Reye et al., 2012), although their role as reservoirs has not yet been demonstrated. The lower prevalence of *Rickettsia* spp. found in red deer and wild boar than in the ticks feeding on them, together with the detection of different *Rickettsia* species in ticks and spleen tissue from the same animal, suggests that these wild ungulates probably do not play an important role in the transmission of these pathogens. Nevertheless, it should be considered that these mammals may be important for maintaining and spreading positive tick populations. Indeed, these wild ungulates are often infested by ticks, and may have active *Rickettsia* spp. infections, which would explain previous reports of *Rickettsia* DNA in their tissue and blood (Inokuma et al., 2008; Ortuño et al., 2007, 2012; Reye et al., 2012). It should be noted that wild boar are abundant in urbanized areas, which could lead to increased risk of human exposure to ticks and hence to *Rickettsia* spp. (Castillo-Contreras et al., 2021).

5 | CONCLUSION

The present study found a remarkable prevalence and wide range of *Rickettsia* spp. in feeding ticks collected from wild boar and red deer in southwestern Spain. The detection of two uncharacterized *Rickettsia* spp. in *H. lusitanicum* feeding on red deer could represent a challenge for the medical, veterinary, and public health communities, since these bacteria may be potential pathogens and their epidemiology is not well understood. While the prevalence of *Rickettsia* in spleen samples taken from these wild ungulates suggests that these mammals do not play a major role in the transmission of this pathogen to ticks, their role in the maintenance and spread of *Rickettsia*-positive ticks cannot be ruled out. The populations of wild ungulates have shown an increasing trend in recent years and their presence in urbanized areas is becoming more common. For these reasons, identifying the *Rickettsia*

species present in ticks and wildlife is of particular interest for clarifying their sylvatic cycle and establishing appropriate control and prevention measures.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required since no animals were killed specifically for this study. Samples analysed in this study were collected from animals legally hunted in complete agreement with Andalusian and Spanish regulations. No ethical approval by an Institutional Animal Care and Use Committee was deemed necessary.

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