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Molecular identification of zoonotic *Rickettsia* species in Ixodidae parasitizing wild lagomorphs from Mediterranean ecosystems

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Abstract

A survey study was carried out to identify tick species parasitizing wild lagomorphs in Mediterranean ecosystems in southern Spain and to determine the occurrence of *Rickettsia* species present in these ticks in this region. A total of 1304 European wild rabbits (*Oryctolagus cuniculus*) and 58 Iberian hares (*Lepus granatensis*) were individually examined for the presence of ticks. Ticks were found in 42.9% and 50% of the wild rabbits and hares sampled, respectively. A total of 1122 ticks were collected and five species, including *Rhipicephalus pusillus*, *Hyalomma lusitanicum*, *Haemaphysalis hispanica*, *Ixodes ventalloi* and *Rhipicephalus sanguineus* sensu lato (s.l.), were microscopically and molecularly identified at the 16S rRNA gene. This is the first study on Ixodidae parasitizing Iberian hares. The presence of *Rickettsia* DNA was assessed in 254 tick pools (according to hunting states, lagomorph species, tick species and tick development stage) using PCR assays targeting the *rOmpA*, *rOmpB* and *gltA*. Twenty-seven pools (10.6%) were positive to *Rickettsia* DNA. Five zoonotic *Rickettsia* species were identified, being *Rickettsia massiliae* the most frequent (4.7%), followed by *Rickettsia sibirica* subsp. *mongolitimonae* (2.8%), *Rickettsia slovaca* (2.0%), *Rickettsia aeschlimannii* (0.8%) and *Rickettsia africae* (0.4%). The results suggest that wild rabbits and Iberian hares are parasitized by a wide range of tick species and that these lagomorphs may play an important role in the sylvatic cycle of some zoonotic *Rickettsia* species in Mediterranean ecosystems. Our data represent the first report of *R. massiliae*, *R. aeschlimannii*, *R. slovaca* and *R africae* in ticks collected in wild lagomorphs in Europe, and the first report of not imported *R. africae* in this continent. Since *R. slovaca* and *R. africae* DNA was detected in tick species different to their main vectors, further studies are warranted to unravel the role of wild lagomorphs in the epidemiology of these vector-borne pathogens.

KEYWORDS
Iberian hare, Rickettsia africae, Rickettsia slovaca, Spain, wild rabbit, zoonotic

1 | INTRODUCTION

The European wild rabbit (*Oryctolagus cuniculus)* and the Iberian hare *(Lepus granatensis)* are native mammals of the Iberian Peninsula (Amori et al., 2008). They play an important role in the ecology and diversity of the Spanish Mediterranean ecosystems since they are staple preys for more than 40 predators including the threatened Spanish imperial eagle (*Aquila adalberti*) and the Iberian lynx (*Lynx pardinus*) (Delibes-Mateos et al., 2008). In addition, these lagomorphs may also represent significant animal and public health concerns for several reasons. First, both species show a wide distribution in Spain since they are very adaptable to different ecological conditions, sharing habitat and resources with other sympatric domestic and wild species, as well as humans. Second, the wild rabbit and the Iberian hare are among the main small game species in Spain, being generally consumed without sanitary inspection (Gortázar et al., 2015). Third, wild lagomorphs have shown to be natural carriers or reservoirs of different zoonotic pathogens, including vector-borne pathogens (González-Barrio et al., 2015; Jiménez et al., 2014).

Previous investigations reported that *Rhipicephalus pusillus*, *Hyalomma lusitanicum*, *Haemaphysalis hispanica* and *Ixodes ventalloi* are the major tick species detected in wild rabbits from Spain (Ciceroni et al., 1988; Gilot & Aubert, 1985; Gonźalez et al., 2016; Lópes de Carbalho et al., 2016; Pereira et al., 2018; Varcárcel et al., 2015). Up to now, available information on the presence of ectoparasites in Iberian hares is lacking. Two previous studies carried out on European hares (*Lepus europaeus*) from northern Spain revealed that *Ixodes ricinus* is the most common tick species detected in this hare species, followed by *Rhipicephalus* spp. and *Haemaphysalis* spp. (Alzaga et al., 2009; Astobiza et al., 2011). All these results have an important epidemiological significance since some of these ticks are considered competent vectors of zoonotic pathogens such as *Anaplasma phagocytophilum*, Crimean–Congo haemorrhagic fever virus and some *Rickettsia* species (Estrada-Peña et al., 2012; Márquez, 2008).

Concerning the presence of *Rickettsia* infections in humans in Europe, Mediterranean spotted fever (MSF), which is endemic in the Mediterranean basin, is the most frequent rickettsiosis (Parola et al., 2005). Although *Rickettsia conorii* subspecies are the main etiological agents of MSF, other *Rickettsia* species such as *Rickettsia monacensis*, *Rickettsia massiliae* and *Rickettsia aeschlimannii* have also been reported in Spanish patients with similar clinical manifestations (Oteo & Portillo, 2012). In addition, other zoonotic *Rickettsia* species such as *Rickettsia slovaca* and *Rickettsia sibirica* subsp. *mongolitimonae* have been detected in humans in Spain (Aguirrebengoa et al., 2008; Oteo & Ibarra, 2002). However, data on the presence and identification of *Rickettsia* species in ticks from wild lagomorphs in Europe are very limited, and the role of these mammals on the sylvatic epidemiological cycles of these tick-borne pathogens is unknown. In this respect, previous studies carried out in America suggested that wild lagomorphs may play an important role in the maintenance of some *Rickettsia* species due to their long-lasting asymptomatic rickettsiaemia (Rovery et al., 2008). Even though in this continent some *Rickettsia* strains detected in ticks collected from rabbits were identified as avirulent (Eremeeva et al., 2018; Roth et al., 2017), *Rickettsia rickettsii* strains identical to those previously detected in human clinical samples were also identified in *Haemaphysalis leporispalustris* collected from these lagomorphs in Costa Rica (Hun et al., 2008). The aims of the present study were (a) to identify tick species parasitizing wild rabbits and Iberian hares in Mediterranean ecosystems in southern Spain and (b) to detect and identify the *Rickettsia* species present in ticks parasitizing these wild lagomorph species.

2 | MATERIALS AND METHODS

2.1 | Study area

The present study was carried out in Andalusia (southern Spain) (36 °N-38° 60′ N, 1° 75′ W-7° 25′ W) between 2018 and 2020. This region has a Mediterranean climate characterized by dry summers and mild, humid winters; the average annual temperature is 16°C and the average annual precipitation is 590 mm (CMAOT, 2009). However, the presence of noticeable climatic differences in this region allowed establishing six bioclimatic regions: Mediterranean oceanic (MedOc), sub-continental Mediterranean with warm winters (SubMedWa), sub-continental Mediterranean with cold winters (SubMedCo), Mediterranean sub-desert (MedDes), Mountainous (Mount) and Mediterranean sub-tropical (MedSubTro) (CMAOT, 2009).

2.2 | Wild lagomorphs sampling

A total of 1304 wild rabbits and 58 Iberian hares were individually examined in the field for the presence of ticks. Rabbits and hares were legally hunted in 60 and 29 hunting states (limited geographical areas where recreational hunting activities are allowed under the government legislation), respectively, located in four of the six bioclimatic regions: MedOc (seven and five hunting states for rabbits and hares, respectively), SubMedWa (10 and 10 hunting states), SubMedCo (34 and 13 hunting states) and MedDes (nine and one hunting states) (Figure 1a,d).

2.3 | Tick collection and identification

A total of 1122 ticks were collected using tweezers, individually bagged up and labelled. Samples were kept frozen at −20°C until examination. Tick specimens were identified to species level using morphological keys (Pérez-Eid, 2007). In order to confirm the microscopic identifications, a subset of each tick species was further molecularly analyzed (Table 1). First, ticks were disrupted using a MagNaLyser Instrument (Roche Diagnostic, Manheim, Germany) at 6000 rpm for 60 s. DNA was then extracted using a commercial kit (High Pure PCR Template Preparation Kit, Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions. A 460 bp fragment of the 16S rRNA gene of ticks was amplified using previously reported PCR protocols

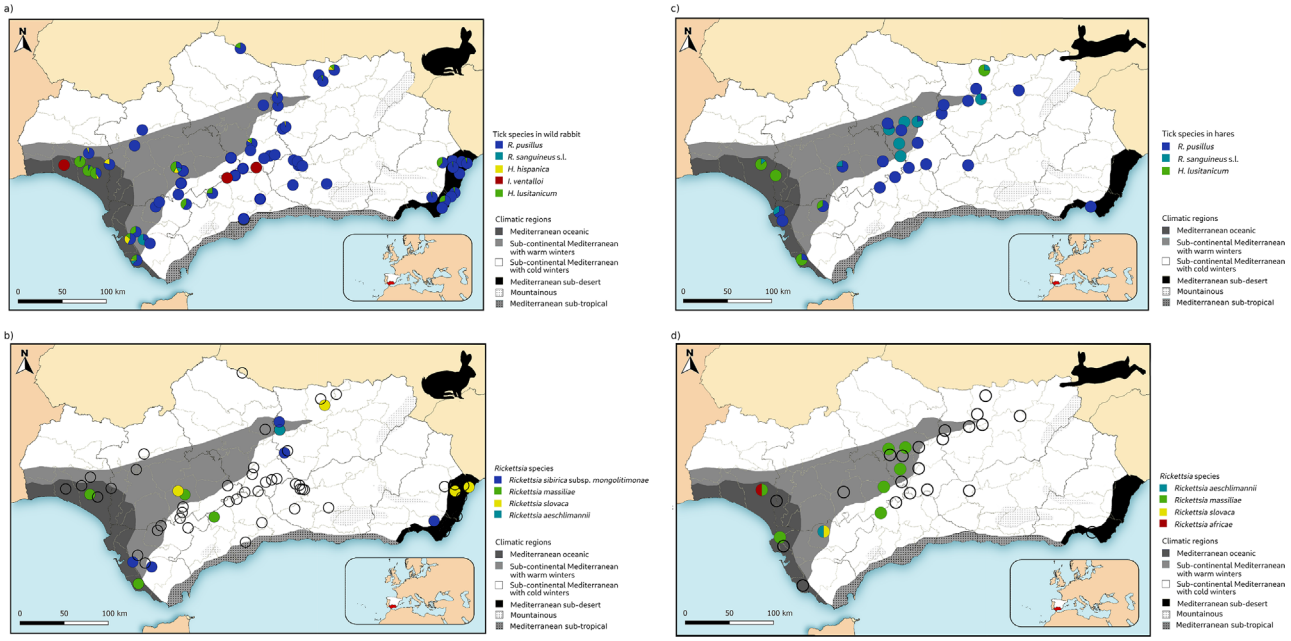

FIGURE 1 Distribution of tick (a and c) and *Rickettsia* (b and d) species in wild rabbits (*Oryctolagus cuniculus*) (a and b) and Iberian hares (*Lepus granatensis*) (c and d) from southern Spain

TABLE 1 Total number of ticks captured, and pools processed from wild rabbits (*Oryctolagus cuniculus*) and Iberian hares (*Lepus granatensis*) from southern Spain

	Larvae		Nymph		Female		Male		Total	
Host species	Number of ticks	Number of pools	Number of ticks	Number of pools	Number of ticks	Number of pools	Number of ticks	Number of pools	Number of ticks	Number of pools
Wild rabbit										
Rhipicephalus pusillus	18	–	407	65	251	54	221	50	897	169
Rhipicephalus sanguineus s.l.	0	–	0	–	0	–	3	2	3	2
Haemaphysalis hispanica	0	–	26	13	0	–	0	–	26	13
Hyalomma lusitanicum	0	–	93	23	0	–	0	–	93	23
Ixodes ventalloi	0	–	0	–	7	4	3	2	10	6
Total	18	–	526	101	258	58	227	54	1029	213
Iberian hare										
Rhipicephalus pusillus	1	–	5	3	20	11	22	10	48	24
Rhipicephalus sanguineus s.l.	0	–	0	–	0	–	25	11	25	11
Haemaphysalis hispanica	0	–	0	–	0	–	0	–	0	0
Hyalomma lusitanicum	0	–	16	4	4	2	0	–	20	6
Ixodes ventalloi	0	–	0	–	0	–	0	–	0	0
Total	1	–	21	7	24	13	47	21	93	41
Total	19	–	547	108	282	71	274	75	1122	254

(Norris et al., 1996; Simon et al., 1994). PCR products were separated by electrophoresis on 1.5% agarose gels stained with RedSafe (iNtRON Biotechnology, South Korea) and visualized using a Fluor-S MultiImager (Bio-Rad Laboratories, California, USA). Selected fragments were purified and sequenced on an ABI 3730xl sequencer (Applied Biosystems, Foster City, CA, USA) at the Sequencing and Fragment Analysis Unit of the Santiago de Compostela University. Sequences were aligned and edited using ChromasPro (Technelysium, Brisbane, Australia), and consensus sequences were then scanned against the GenBank database using BLAST. Unique 16S rRNA sequences identified in this study were deposited in GenBank under accession numbers MZ420711–MZ420717.

A phylogenetic analysis was carried out using MrBayes 3.2.7 software (Ronquist et al., 2012) by Bayesian approach with Markov Chain Monte Carlo sampling (10,000,000 generations sampling every 1000 steps). A general time reversible substitution model with gamma-distributed rate variation across sites (GTR + G) was used for the analysis of tick sequences at the 16s rRNA gene. The model was selected based on Akaike information criterion (AIC) value using the free software jModelTest v.2.1.10 (Darriba et al., 2012). The tree was visualized and edited using FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

2.4 | *Rickettsia* detection in ticks

For *Rickettsia* spp. detection, ticks from the same hunting state were pooled including those specimens of the same species and development stage. Ticks from rabbits and hares were independently pooled. In addition, if the number of ticks of the same hunting state, species and development stage was higher than 10, more than one pool was prepared. The number of ticks included in each pool is summarized in Table 1.

DNA was extracted using the same commercial kit previously indicated (High Pure PCR Template Preparation Kit; Roche Diagnostics GmbH, Mannheim, Germany). The presence of *Rickettsia* DNA in tick samples was detected by using two nested PCR assays targeting the genes encoding for two 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). All samples were analyzed in parallel using both PCR protocols. In addition, in order to increase the diagnostic sensibility, positive samples to *rOmpA* and/or *rOmpB* were further analyzed using a third PCR assay targeting the *gltA* of *Rickettsia* spp. (Labruna et al., 2004). In each assay, DNA of *Rickettsia amblyommatis*, a species absent in Europe, and nuclease free water were included as positive and negative controls, respectively. PCR products were processed and sequenced as previously indicated. Partial *Rickettsia* spp. sequences identified in this study were deposited under accession numbers MZ420707–MZ420710 and OK205202–OK205222.

Finally, the obtained sequences were scanned against the GenBank database. A phylogenetic analysis of the partial *rOmpA* was carried out as previously indicated. A Hasegawa–Kishino–Yano substitution model with gamma-distributed rate variation across sites (HKY+G) was used for the analysis of *Rickettsia* spp. sequences at the *rOmpA*. The model was selected based on AIC value using the software jModelTest v.2.1.10 (Darriba et al., 2012).

2.5 | Statistical analysis

The association between the total number of tick specimens when considering the development stage (larvae, nymph and adult) was assessed using an analysis of variance (ANOVA) test.

The maximum likelihood estimation (MLE) was used to estimate the prevalence of *Rickettsia* spp. in pooled ticks (Williams & Moffitt, 2005). The possible influence of tick species and development stage, lagomorph species and bioclimatic area on the *Rickettsia* spp. infection was analyzed by multivariate analysis using a multiple logistic regression model as described by Hosmer and Lemeshow (1989). Factors were eliminated from the initial model using a method based in AIC value until the best model was built. All pairwise interactions were evaluated. Odds ratio 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 using the statistical software R (R Core Team, 2020), and the level of significance was set at p-values $< .05$.

A quantum geographic information system software was used to depict spatial distribution of ticks and *Rickettsia* species in the different hunting states and bioclimatic regions (QGIS.org, 2021).

3 | RESULTS

3.1 | Tick species parasitizing wild lagomorphs

Ticks were found in 560 of the 1304 (42.9%) and 29 of the 58 (50.0%) wild rabbits and Iberian hares examined, respectively. A total of 1029 tick specimens were collected in wild rabbits; nymphs and adults were predominant, whereas larvae were only occasionally found (Table 1). Significant differences were only detected between the number of nymphs and larvae ($F = 8.225$; $p = .004$). Ninety-three Ixodidae were recovered from hares, being the number of adults higher than that of nymphs and larvae (Table 1), but these differences were not statistically significant ($p > .05$).

Five different tick species were identified in the lagomorphs examined; all of them were found in rabbits and three in hares (Table 1, Figure 1a,c). The number of specimens collected according to the tick species and the development stage is summarized in Table 1. *R. pusillus* was the most abundant tick species in both wild rabbits and Iberian hares. Co-infestations with two types of tick species were detected in both lagomorph species. In rabbits, two co-infestations were detected *R. pusillus/H. lusitanicum* (6.6%; 86/1304) and *R. pusillus/H. hispanica* (2.0%; 26/1304). In hares, the most commonly co-infestation detected was *R. pusillus/Rhipicephalus sanguineus* s.l. (8.6%; 5/58) followed by the associations *H. lusitanicum/R. pusillus* (3.4%; 2/58) and *H. lusitanicum* s.l./*R. sanguineus* s.l. (3.4%; 2/58). Sequence analysis confirmed the morphological identification of all tick species except for *H. hispanica* because no sequences of this species are currently deposited in the GenBank database. The sequence obtained in the present study showed a homology of 91.8% and 91.3% with those of *Haemaphysalis sulcata* (MT799946.1) and *Haemaphysalis longicornis* (MT555306.1), respectively. In addition, the phylogenetic analysis showed that our sequence formed a clade separated from other *Haemaphysalis* species deposited in GenBank (Figure 2).

R. pusillus was the only species found in different development stages in the same rabbit being the co-infestation nymph/adult (19.5%; 254/1304) the most commonly detected, followed by larvae/nymph (3.5%; 45/1304) and larvae/adult (3.5%; 45/1304). The

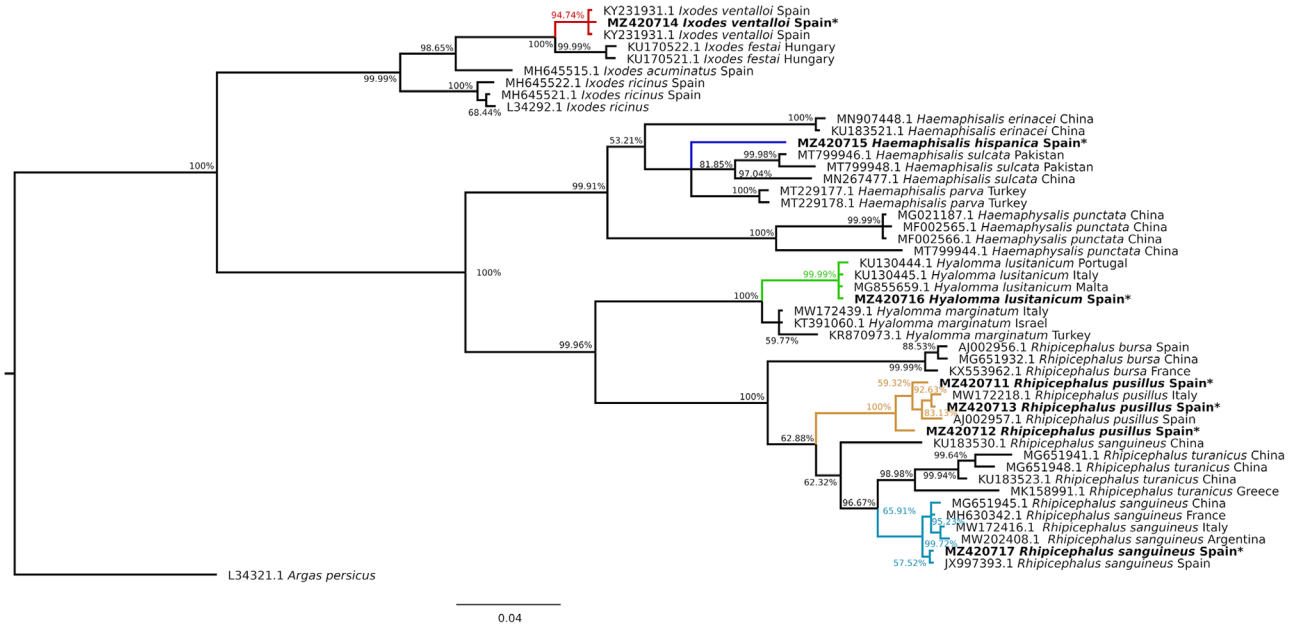

FIGURE 2 Phylogenetic tree clustering of the partial 16S rRNA gene of the ticks. The tree was obtained using a general time reversible substitution model with gamma-distributed rate variation across sites (GTR + G) with the software MrBayes 3.2.7 (Ronquist et al., 2012) by Bayesian approach with Markov Chain Monte Carlo sampling (10,000,000 generations sampling every 1000 steps). This analysis involved 48 nucleotide sequences. The nucleotide sequence of *Argas persicus* was used as an outgroup. Isolates identified in this study (*)

percentage of rabbits parasitized by all the development stages of this species was low (1.2%; 15/1304). In hares, no co-infestation with different development stages of the same tick species were observed.

Ticks were collected from wild rabbits and hares from all sampled hunting states, but differences in the geographical distribution of tick species were found (Figure 1a,c). Thus, *I. ventalloi* was the less spread tick since it was only detected in hunting states with MedOc and SubMedCo climate (Table 2). The remaining tick species were found in MedOc, SubMedCo and SubMedWa areas, but only *R. pusillus* and *H. lusitanicum* were collected in areas with MedDes climate (Figure 1a,c;). *R. pusillus* shown the widest distribution on wild lagomorphs from southern Spain, since it was found in the 95% and 80% of the hunting states where rabbits and hares were sampled, respectively (Figure 1a,c).

3.2 | Rickettsia species in ticks from wild lagomorphs

3.2.1 | Percentage of pools positive to *Rickettsia* spp

Rickettsia DNA was detected in 27 out of 254 (10.6%) tick pools. *Rickettsia*-positive pools were found in 22 out of 79 (27.9%) hunting states (Figure 1b,d). The percentage of positive pools was higher in Iberian hares (11/41; 26.8%) than in wild rabbits (16/213; 7.5%), and the logistic regression results (Table 3) showed that these differences were statistically significant [OR = 5.5; 95% confidence interval (CI): 1.5–22.1; $p = .012$].

Only four (14.8%) of the 27 *Rickettsia*-positive pools were positive to all the three studied genes. Most positive pools (92.6%; 25/27) were detected using the PCR protocol targeting the *rOmpA* gene, whereas amplification was only observed in 8 (29.6%) and 14 (51.9%) pools when analyzed at the *rOmpB* and *gltA* genes, respectively. The highest percentage of positivity was detected in pools composed of *R. sanguineus* s.l. (38.5%; 5/13) and *H. lusitanicum* (37.9%; 11/29), followed by *H. hispanica* (7.7%; 1/13) and *R. pusillus* (5.2%; 10/193). No *I. ventalloi* pools were positive to *Rickettsia* spp. (Table 2). The logistic regression showed that the number of *H. hispanica* positive pools was significantly lower than *H. lusitanicum* positive pools (OR = 0.1; 95%CI: 0.03–0.4; $p < .001$). Similarly, a significantly lower percentage of *R. pusillus*-positive pools was detected when compared to *H. lusitanicum*-positive pools (OR = 0.09; 95%CI: 0.01–0.6; $p = .040$). The multivariate analysis (Table 3) also identified a significantly higher number of *Rickettsia*-positive pools in SubMedWa area than in SubMedCo (OR = 5.4; 95%CI: 1.7–20.2; $p = .007$).

3.2.2 | *Rickettsia* species identified

Five different *Rickettsia* species were molecularly identified in the tick pools (Table 4, Figure 1b,d). The most commonly detected *Rickettsia* species was *R. massiliae* (4.7%; 12/254), followed by *R. sibirica* subsp. *mongolitimonae* (2.8%; 7/254), *R. slovaca* (2.0%; 5/254), *R. aeschlimannii* (0.8%; 2/254) and *Rickettsia africae* (0.4%; 1/254). No coinfections were found in the tick pools analyzed.

All the *Rickettsia* species identified were found in ticks collected from wild rabbits except *R. africae*, being *R. sibirica* subsp.

TABLE 2 Percentage of pools positive to *Rickettsia* spp. and maximum likelihood estimation (MLE) from wild rabbits (*Oryctolagus cuniculus*) and Iberian hares (*Lepus granatensis*) when considering the tick development stage and the climatic region

	Stage of development						Sampling area								Total
	Nymphs		Female		Male		MedOc		SubMedWa		SubMedCo		MedDes		
	%	MLE (95%CI)	%	MLE (95%CI)	%	MLE (95%CI)	%	MLE (95%CI)	%	MLE (95%CI)	%	MLE (95%CI)	%	MLE (95%CI)	%
Wild rabbit															
Rhipicephalus pusillus	5/65 (7.7%)	1.3 (0.5–2.8)	1/54 (1.9%)	0.40 (0.0–1.8)	1/50 (2%)	0.5 (0.0–2.0)	0/13 (0%)	–	5/32 (15.6%)	2.53 (0.9–5.4)	2/87 (2.3 %)	0.52 (0.1–1.6)	0/37 (0%)	–	7/169 (4.1%)
Rhipicephalus sanguineus s.l.	–	–	–	–	0/2 (0%)	–	–	–	0/2 (0%)	–	–	–	–	–	0/2 (0%)
Haemaphysalis hispanica	1/13 (7.7%)	3.9 (0.2–16.2)	–	–	–	–	0/5 (0%)	–	1/5 (20%)	7.42 (0.4–28.9)	0/3 (0%)	–	–	–	1/13 (7.7%)
Hyalomma lusitanicum	8/23 (34.8%)	9.2 (4.3–16.5)	–	–	–	–	2/8 (25%)	4.37 (0.7–12.9)	2/2 (100%)	100 (25.2–100)	2/9 (22.2%)	6.90 (1.2–19.8)	2/4 (50%)	21.18 (3.9–52.5)	8/23 (34.8%)
Ixodes ventalloi	–	–	0/4 (0%)		0/2 (0%)		0/4 (0%)		–	–	0/2 (0%)		–	–	0/6 (0%)
Iberian hares															
Rhipicephalus pusillus	1/3 (33.3%)	26.3 (1.7–78.0)	2/11 (18.2%)	10.90 (1.9–30.2)	0/10 (0%)	–	1/4 (25%)	14.50 (0.9–50.9)	2/6 (33.3%)	29.5 (5.5–68.0)	0/11 (0%)	–	0/3 (0%)	–	3/24 (12.5%)
Rhipicephalus sanguineus s.l.	–	–	–	–	5/11 (45.5%)	20.5 (7.9–39.0)	2/2 (100%)	100 (38.3–100)	2/6 (33.3%)	11.5 (2.0–31.4)	1/3 (33.3%)	20.00 (1.3–62.8)	–	–	5/11 (45.5%)
Hyalomma lusitanicum	3/4 (75%)	52.4 (12.0–96.3)	0/2 (0%)	–	–	–	1/3 (33.3%)	20.63 (1.2–73.7)	–	–	2/3 (66.7%)	29.00 (4.9–73.1)	–	–	3/6 (50%)

Abbreviations: CI, confidence interval; MedDes, Mediterranean sub-desert; MedOc, Mediterranean oceanic; SubMedCo, sub-continental Mediterranean with cold winters; SubMedWa, sub-continental Mediterranean with warm winters.

TABLE 3 Logistic regression model for the prevalence of *Rickettsia* spp. in ticks collected in both wild rabbits (*Oryctolagus cuniculus*) and Iberian hares (*Lepus granatensis*) from southern Spain

	Estimate	*Z*-value	*p*-Value	OR	CI 95%
(Intercept)	1.881	1.968	.050	–	–
Lagomorph species					
Wild rabbit	–	–	–	–	–
Iberian hare	1.698	2.512	.012	5.46	1.49–22.11
Tick species					
H. lusitanicum	–	–	–	–	–
R. pusillus	−2.449	−2.055	.040	0.09	0.01–0.64
H. hispanica	−2.161	−3.340	<.001	0.12	0.03–0.40
Bioclimatic area					
SubMedCo	–	–	–	–	–
SubMedWa	1.693	2.703	.007	5.44	1.67–20.15

Abbreviations: CI, confidence level; OR, odds ratio; SubMedCo, sub-continental Mediterranean with cold winters; SubMedWa, sub-continental Mediterranean with warm winters.

mongolitimonae the most frequently detected in this lagomorph species (Table 4). In contrast, *R. massiliae* was the major *Rickettsia* species detected in ticks collected from Iberian hares; it is worth noting that *R. sibirica* subsp. *mongolitimonae* was not detected in this lagomorph (Table 4). All the identified *Rickettsia* species were found in *H. lusitanicum* and all but *R. africae* were detected in *R. pusillus*. In addition, only a single *Rickettsia* species was identified in *R. sanguineus* s.l. *(R. massiliae)* and *H. hispanica (R. sibirica* subsp. *mongolitimonae)*. *Rickettsia*-DNA was not detected in *I. ventalloi* (Table 4).

Through the study of *rOmpA* (Figure 3), *R. massiliae* and *R. aeschlimannii* sequences showed a percentage of identity higher than 99.6% with the sequences obtained in Greece (MG521363), China (MF098409, KU365969 and MF098409) and Kenya (KX227782) and a percentage of identity higher than 99.4% with the reference sequences U43799 and U43800 of *R. massiliae* and *R. aeschlimannii*, respectively (Fournier et al., 2003). The obtained sequence of *R. africae* was identical to that obtained in Kenya (KX227785) and showed a percentage of identity of 99.0% with the reference sequence U43790 (Fournier et al., 2003). The sequence of *R. sibirica* subsp. *mongolitimonae* was identical to the obtained in Turkey (MF379309) and to the reference sequence U43796 (Fournier et al., 2006). Finally, the sequences of *R. slovaca* were identical to those obtained in Turkey (MH548522), Italy (HM161798) and China (MG598413) and showed a percentage of identity higher than 99.6% with the reference sequence U43808 (Fournier et al., 2003). Sequence analysis at the *rOmpB* and *gltA* showed that all sequences were identical to those obtained in questing *R. turanicus* (KY233281, KY233273 and MF002497) and *Hyalomma* spp. (KX227786, HM050273, KY233273 and KU961540) from Lebanon, Senegal, Kenya and China; in *I. ricinus* (MK301603 and MK301607) from Spain and in *Dermacentor nuttalli* (MF002541) from China. In addition, *rOmpB* sequences showed a percentage of identity higher than 99.4% with the reference sequences AF123714, AF123705, AF123706, DQ097083 and AF123723 of *R. massiliae, R. aeschlimannii, R. africae, R. sibirica subsp. mongolitimonae* and *R. slovaca* (Fournier et al., 2003).

4 | DISCUSSION

4.1 | Ixodidae fauna of wild lagomorphs

The results obtained in the present study reveal that tick infestation is very common in the wild rabbit and Iberian hare populations from southern Spain since more than 42% of the examined animals were parasitized, and tick-positive lagomorphs were found in all sampled hunting states. Previous investigations carried out in northern Spain reported lower prevalences than those observed in our study since ticks were only recovered from the 17% of wild rabbits and the 14–31% of European hares (Alzaga et al., 2009; Astobiza et al., 2011). In contrast, a higher prevalence has been reported in Sicily (southern Italy), where ticks were found on the 75.2 % of the sampled wild rabbits (Napoli et al., 2021).

It is worth noting that no significant differences were observed between the number of adults and immature ticks found in both wild lagomorph species, whereas most investigations performed in other European countries reported a predominance of tick immature stages (Dantas-Torres et al., 2011; González et al., 2016; Pereira et al., 2018; Talleklint & Jaenson, 1997). Nevertheless, it is important to point out that the tick species detected, their ecological preferences and behaviour, the time of the year when the animals were sampled (Napoli et al., 2021; Sobrino et al., 2012) as well as the difficulties in capturing tick immature stages due to its size in field conditions, are possible factors that may explain the discrepancies observed among studies.

Five different tick species were found in wild rabbits and Iberian hares in the present study. Our results are consistent with the data

TABLE 4 Percentage of pools positive to *Rickettsia* species detected and maximum likelihood estimation (MLE) from wild rabbits (*Oryctolagus cuniculus*) and Iberian hares (*Lepus granatensis*) from southern Spain

	Rickettsia sibirica subsp. *mongolitimonae*		*Rickettsia massiliae*		*Rickettsia slovaca*		*Rickettsia africae*		*Rickettsia aeschlimannii*		Total	
	%	MLE (95%CI)	%	MLE (95%CI)	%	MLE (95%CI)	%	MLE (95%CI)	%	MLE (95%CI)	%	MLE (95%CI)
Wild rabbit												
Rhipicephalus pusillus	5/169 (3.0%)	0.6 (0.2–1.2)	1/169 (0.6%)	0.1 (0.0–0.5)	0/169 (0%)	–	0/169 (0%)	–	1/169 (0.6%)	0.1 (0.0–0.5)	7/169 (4.1%)	0.8 (0.3–1.5)
Rhipicephalus sanguineus s.l.	0/2 (0%)	–	0/2 (0%)	–	0/2 (0%)	–	0/2 (0%)	–	0/2 (0%)	–	0/2 (0%)	–
Haemaphysalis hispanica	1/13 (7.7%)	3.9 (0.2–16.2)	0/13 (0%)	–	0/13 (0%)	–	0/13 (0%)	–	0/13 (0%)	–	1/13 (7.7%)	3.9 (0.2–16.2)
Hyalomma lusitanicum	1/23 (4.4%)	1.1 (0.1–4.7)	3/23 (13.0%)	3.4 (0.9–8.5)	4/23 (17.4%)	4.4 (1.4–9.4)	0/23 (0%)	–	0/23 (0%)	–	8/23 (34.78%)	9.20 (4.3–16.5)
Ixodes ventalloi	0/6 (0%)	–	0/6 (0%)	–	0/6 (0%)	–	0/6 (0%)	–	0/6 (0%)	–	0/6 (0%)	–
Iberian hares												
Rhipicephalus pusillus	0/24 (0%)	–	2/24 (8.3%)	4.4 (0.7–12.8)	1/24 (4.2%)	2.2 (0.1–9.4)	0/24 (0%)	–	0/24 (0%)	–	3/24 (12.5%)	6.68 (1.7–16.4)
Rhipicephalus sanguineus s.l.	0/11 (0%)	–	5/11 (45.5%)	20.5 (7.9–39.0)	0/11 (0%)	–	0/11 (0%)	–	0/11 (0%)	–	5/11 (45.5%)	20.45 (7.9–39.0)
Hyalomma lusitanicum	0/6 (0%)	–	1/6 (16.7%)	5.0 (0.3–20.2)	0/6 (0%)	–	1/6 (16.7%)	5.8 (0.3–23.4)	1/6 (16.7%)	6.19 (0.4–25.3)	3/6 (50%)	25.5 (6.5–59.5)

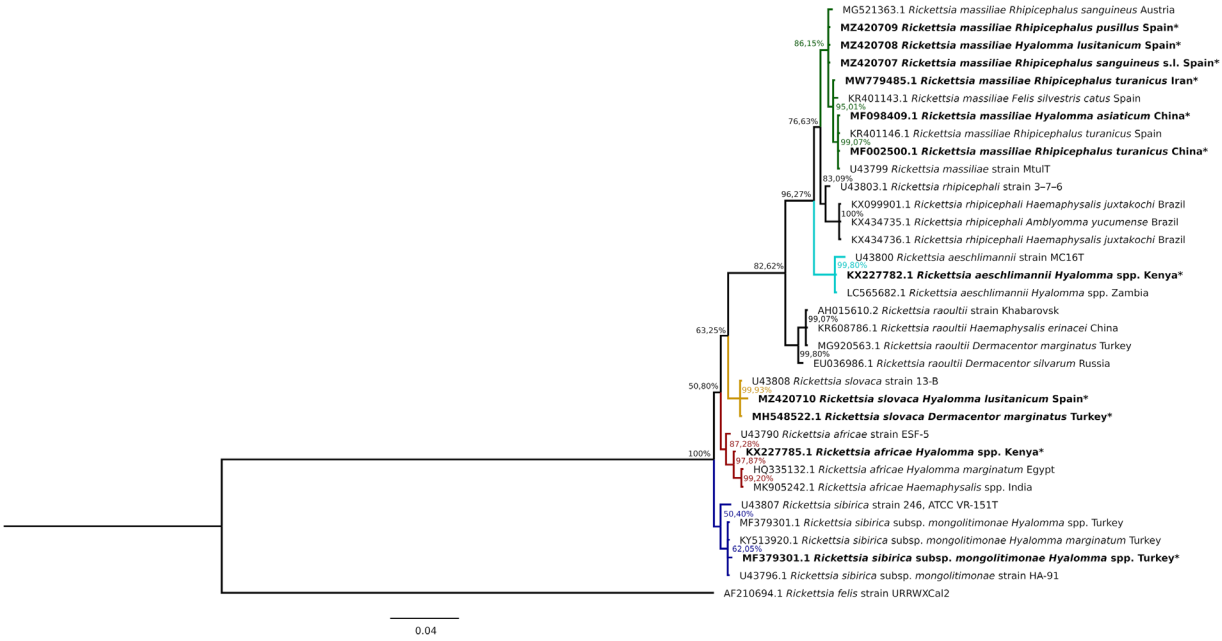

FIGURE 3 Phylogenetic tree clustering of the partial *rOmpA* of *Rickettsia* spp. The tree was obtained using a Hasegawa–Kishino–Yano substitution model with gamma-distributed rate variation across sites (HKY + G) with the software MrBayes 3.2.7 (Ronquist et al., 2012) by Bayesian approach with Markov Chain Monte Carlo sampling (10,000,000 generations sampling every 1000 steps). This analysis involved 34 nucleotide sequences. The nucleotide sequence of *Rickettsia felis* was used as an outgroup. Isolates identified or identical to those identified in this study (*)

available for wild rabbits and European hares from Europe (Ciceroni et al., 1988; Gilot & Aubert, 1985; Gonźalez et al., 2016; Lópes de Carbalho et al., 2016; Pereira et al., 2018; Varcárcel et al., 2015). Nevertheless, noticeable differences on the presence and abundance of tick species in wild lagomorphs have been reported in Europe, probably related to their environmental preferences. Thus, *I. ricinus* is the most frequent Ixodidae in wild lagomorphs from northern European countries (Buttler et al., 1994; Gilot & Aubert, 1985; Talleklint & Jaenson, 1997) since it is sensitive to desiccation and extreme heat and needs a moist microclimate to live (A. R. Walker et al., 2009). In contrast and agreeing with our findings, the most abundant tick species in wild lagomorphs from the Mediterranean basin were *H. lusitanicum* and *R. pusillus* (Astoriza et al., 2011; Ciceroni et al., 1988; Dantas-Torres et al., 2011; González et al., 2016; Lopes de Carballo et al., 2016; Orkun & Karaer, 2017; Pereira et al., 2018; Psaroulaki et al., 2014; Varcarcel et al., 2015), which are adapted to dry Mediterranean ecosystems (J. B. Walker et al., 2000).

The most abundant and distributed tick species in wild rabbits and Iberian hares from the study area, *R. pusillus* and *H. lusitanicum*, feed mainly on lagomorphs at least in one stage of development (Estrada-Peña et al., 2017). However, while all development stages of *R. pusillus* primary feed on lagomorphs (Guglielmone & Nava, 2014), only immature stages of *H. lusitanicum* feed on these species (Varcárcel et al., 2020). This could explain the large number of nymphs of the latter species found in the present study. Although *H. hispanica* and *I. ventalloi* are also endophilic species, which mainly parasite lagomorphs, they were not found in MedDes regions since they are adapted to Iberian Mediterranean sclerophyllous and mixed ecoregion (Estrada-Peña et al., 2017), which are not present in MedDes bioclimatic regions of the study area. *R. sanguineus* s.l. is a nidicolous tick well adapted to a wide range of climatic conditions which feed mainly in dogs; however, it can also be found in other hosts (Dantas-Torres, 2010).

The diversity of Ixodidae found in Iberian hares was lower than in wild rabbits since *I. ventalloi* and *H. hispanica* were not detected in this hare species. *I. ventalloi* has been previously found in European hares from Cyprus (Psaroulaki et al., 2014). Although this tick species is mainly a parasite of wild rabbits (Estrada-Peña et al., 2017), in the present study, a low number of specimens were found in this lagomorph. Therefore, its absence in Iberian hares may be related to the low number of examined individuals. It should be noted that the number of hares hunted in the studied area is 10-fold lower than the number of rabbits (CAPDS, 2019). To the best of our knowledge, *H. hispanica* has not been previously detected in hares. This tick species has an endophilic and monotropic behaviour and all stages feed predominantly on rabbits (Estrada-Peña et al., 2017).

The identification of some of these tick species may be difficult, especially for those belonging to the genus *Rhipicephalus* spp., and misidentifications may exist (Estrada-Peña et al., 2017). In addition, no sequences of *H. hispanica* are currently deposited in GenBank. For these reasons, the identification of all tick species detected was confirmed through the study of the 16S rRNA gene and a phylogenetic analysis was performed. In the obtained phylogenetic tree (Figure 2), *R. sanguineus* s.l., *R. pusillus* and *H. hispanica* sequences were included in different and independent clades close to other *Rhipicephalus* spp. and *Haemaphysalis* spp. deposited sequences confirming the morphological identifications.

4.2 | *Rickettsia* spp. in ticks feeding on wild lagomorphs

Our data represent the first report of *R. massiliae*, *R. aeschlimannii*, *R. slovaca* and *R. africae* in ticks collected in wild lagomorphs in Europe. The results indicate that *Rickettsia* spp. is a common pathogen in tick species parasitizing wild lagomorphs in Mediterranean ecosystems in southern Spain, since 7.5%–26.8% of tick pools from rabbits and hares, respectively, were positive, and a MLE up to 25% was detected for some tick species. This finding is in accordance with those previously obtained in Turkey, where *Rickettsia* DNA was found in the 23.1% of the pooled ticks collected from European hares (Orkun & Çakmak, 2019), although the tick species found in this study (*H. marginatum*, *Hyalomma aegyptium*, *R. turanicus* and *Haemaphysalis parva*) were different to those reported in the present study.

In the present study, the identification of all *Rickettsia* species was confirmed by at least two genes, and all positive samples were identical or almost identical to previously deposited sequences of *Rickettsia* spp. obtained in questing ticks from Europe, Africa and Asia and to their reference sequences (Figure 3). These results are of public health concern since all the *Rickettsia* species identified are zoonotic pathogens. In this respect, *R. massiliae* and *R. aeschlimannii* cause clinical signs in humans similar to the MSF-like, and *R. sibirica* subsp. *mongolitimonae* and *R. slovaca* are related to the lymphangitis-associated rickettsiosis and the *Dermacentor*-borne necrosis erythema lymphadenopathy, respectively. In addition, *R. africae* is the etiological agent of the African tick-bite fever.

R. slovaca is the etiological agent of the second most prevalent rickettsiosis in Europe being only surpassed by the MSF caused in this area by *R. conorii* subsp. *conorii* (Mediannikov et al., 2008). In addition, human rickettsiosis caused by *R. massiliae* and *R. sibirica* subsp. *mongolitimonae* are sporadically reported in the European Mediterranean basin (Aguirrebengoa et al., 2008; Caron et al., 2008; de Sousa et al., 2008; García-García et al., 2010; Vitale et al., 2006). However, no autochthonous clinical cases caused by *R. aeschlimannii* or *R. africae* have been reported in this continent (Oteo & Portillo, 2012). In fact, our study represents the first report of *R. africae* in ticks from Europe.

The *Rickettsia* species were found in four of the five tick species collected from wild rabbits and Iberian hares. It is worth noting that DNA of these *Rickettsia* species was only previously identified in a number of tick species in Europe (Chisu et al., 2018; de Sousa et al., 2006; Fernández de Mera et al., 2009; Fernández-Soto et al., 2006; Márquez, 2008; Orkun & Çakmak, 2019; Ortuño et al., 2018), including some considered as their main vectors such as *R. pusillus* and both *R. sibirica* subsp. *mongolitimonae* and *R. massiliae* or *Hyalomma* spp. and *R. aeschlimannii* (Chisu et al., 2018; de Sousa et al., 2006; Hendershot & Sexton, 2009; Mediannikov et al., 2008; Palomar et al., 2016). Nevertheless, our results have important epidemiological significance since, to the best of the authors' knowledge, they represent the first report of *R. massiliae* in *H. lusitanicum; R. aeschlimannii* in *R. pusillus; R. sibirica* subsp. *mongolitimonae* in both *H. hispanica* and *H. lusitanicum; R. slovaca* in *H. lusitanicum* and *R. pusillus* and *R. africae* in *H. lusitanicum*. It is important to point out that this study was carried out in ticks removed from animals; therefore, it is not surprising to detect some *Rickettsia* species in ticks not considered their vectors. Wild lagomorphs can be parasitized by a wide variety of Ixodidae and the joint presence of the main vectors of some *Rickettsia* species and other tick species feeding on the same host has been detected. For these reasons, the infection of the host or even the transmission through co-feeding from *Rickettsia* spp. positive ticks to those which are simultaneously feeding on the same host in the absence of systemic infection (Zemtsova et al., 2010) may be the cause of the detection of *R. aeschlimannii*, *R. massiliae* and *R. sibirica* subsp. *mongolitimonae* in tick species not considered their main vectors. Nevertheless, this hypothesis is not valid for *R. slovaca* and *R. africae*. In fact, *Dermacentor* spp., considered the major vector of *R. slovaca* (Mediannikov et al., 2008), was not detected in the examined lagomorph populations. In the same way, *Amblyomma* species, which are absent in Europe, are the main vectors and reservoirs of *R. africae* (Hendershot & Sexton, 2009; Jensenius et al., 2003). Some investigations have reported this *Rickettsia* species in imported *H. aegyptium* and *Amblyomma variegatum* ticks in Turkey, France and Italy (Cicculli et al., 2019; Gargili et al., 2012; Pintore et al., 2021). However, these authors hypothesized that the infected Ixodidae were introduced by migratory birds from Sub-Saharan Africa, as has been previously suggested for *R. africae* (Pintore et al., 2021; Wallménius et al., 2014) and for other *Rickettsia* species such as *R. aeschlimannii* (Wallménius et al., 2014). Our results are consistent with this hypothesis since the ticks included in the *R. africae*-positive pool were from lagomorphs sampled in Doñana National Park, which is the largest natural reserve in Europe and the transit point for thousands of migratory birds. Considering that *H. lusitanicum* immature stages usually parasite lagomorphs and are not commonly detected in birds (Estrada-Peña et al., 2017), the detection of *R. africae* in *H. lusitanicum* nymphs feeding on hares suggests that this *Rickettsia* species might have found a competent reservoir in Spain. Further studies are warranted to assess the role of wild lagomorphs in the epidemiology of *R. africae*.

The multiple logistic regression analysis identified, in addition to lagomorph species, two other potential risk factors associated with *Rickettsia* spp. infection in ticks from wild lagomorphs in the study area. Thus, the probability of being infected with *Rickettsia* spp. was significantly higher in *H. lusitanicum* than in *R. pusillus* and *H. hispanica*. Since *Rickettsia*-positive *H. lusitanicum* was detected in all studied climatic regions, this tick species may be involved in the sylvatic cycle of these zoonotic pathogens. In this regard, *Hyalomma* species have been previously identified as natural vectors of *Rickettsia* species (Estrada-Peña et al., 2017) such as *R. aeschlimannii* and *R. sibirica* subsp. *mongolitimonae* (Oteo & Portillo, 2012). In addition, Orkun and Çakmak (2019) detected *Rickettsia* spp. in *Hyalomma* spp. collected from European hares in Turkey. The multivariate analysis also showed that *Rickettsia* spp. was significantly more frequent in SubMedWa than in SubMedCo areas. These differences may be mainly attributed to the climatic conditions of each area which could affect the presence and abundance of suitable tick-vectors and competent reservoirs.

Several limitations should be noted in this study. The present investigation is a cross-sectional study since animals were captured

during the hunting seasons (August–December), and ticks were collected under field conditions. In this way, the number of some tick species or development stages could be underestimated, particularly for immature stages which are smaller and more difficult to detect than adults. Finally, for logistic and economic reasons, ticks removed from wild lagomorphs were pooled, so this investigation cannot be understood as an individual prevalence study, and the role of both wild lagomorph and tick species in the epidemiology of the selected pathogens should be further assessed.

In summary, our results indicated that wild lagomorphs from southern Spain are parasitized by up to five different tick species. This is the first study on Ixodidae parasitizing Iberian hares. The results obtained indicate that *Rickettsia* species are widespread distributed in wild lagomorph populations in in the study region. To the best of the authors' knowledge, this is also the first report of *R. massiliae* in *H. lusitanicum; R. aeschlimannii* in *R. pusillus; R. sibirica* subsp. *mongolitimonae* in both *H. hispanica* and *H. lusitanicum; R. slovaca* in *H. lusitanicum* and *R. pusillus* and *R. africae* in *H. lusitanicum*. We also report for first time the presence of *R. africae* in ticks from Europe.

Even though the presence of *Rickettsia* spp. in tick species different to their main vectors may be the consequence of the infection of the host, the role of these tick species and their hosts in the sylvatic cycle of these pathogens cannot be ruled out. Our results suggest that both, tick and lagomorph species, may play an important role in the sylvatic cycle of several zoonotic *Rickettsia* species, which is of public health concern. Further studies are needed to unravel epidemiological role of wild lagomorphs and their ticks in the maintenance and transmission of *Rickettsia* spp. in Mediterranean ecosystems in southern Spain.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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. Ticks analyzed in this study were collected from wild rabbits legally hunted in complete agreement with Andalusian and Spanish regulations. No ethical approval by an Institutional Animal Care and Use Committee was deemed necessary.

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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