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Diversity of *Anaplasma* species and importance of mixed infections in roe deer from Spain

Susana Remesar | Alberto Prieto | David García-Dios | Gonzalo López-Lorenzo | Néstor Martínez-Calabuig | José Manuel Díaz-Cao | Rosario Panadero | Ceferino Manuel López | Gonzalo Fernández | Pablo Díez-Baños | Patrocinio Morrondo | Pablo Díaz

Investigación en Sanidad Animal: Galicia (Grupo INVESAGA), Facultade de Veterinaria, Universidade de Santiago de Compostela, Lugo, Spain

Correspondence
Alberto Prieto, Facultad de Veterinaria, Pabellón I, Campus Universitario s/n. 27002, Lugo, Spain.
Email: alberto.prieto@usc.es

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Abstract

Although wildlife can act as reservoirs of some *Anaplasma* species, studies on the presence and distribution of *Anaplasma* spp. in wild cervids are mainly limited and focused on zoonotic species. In order to identify the *Anaplasma* species in roe deer from Spain and to detect co-infections, 224 spleen samples were tested for *Anaplasma* spp. using a commercial qPCR; positive samples were further characterized using generic 16S rRNA primers and species-specific primers targeting the *msp2* and *groEL* genes. *Anaplasma* DNA was detected in the 50.9% of samples, and four *Anaplasma* species were identified. *Anaplasma phagocytophilum* (43.8%) was predominant, followed by *Anaplasma bovis* (13.8%), *Anaplasma capra* (5.8%) and *Anaplasma ovis* (2.2%). In addition, strains similar to *Anaplasma platys* were found in nine animals. Most positive roe deer (71.9%) were infected with a single *Anaplasma* species, whereas co-infections with two (19.3%) or three (8.8%) *Anaplasma* species were also found. This study confirms the widespread occurrence of *Anaplasma* spp. in roe deer from Spain, being the first report of *A. platys*-like strains and *A. capra* in this cervid; it is also the first report of *A. capra* in Spain. The detection of *Anaplasma* species pathogenic for humans and/or domestic animals in roe deer suggests that this cervid may play a role in the sylvatic cycle of these bacteria contributing to the appearance of clinical anaplasmosis cases. In addition, co-infections are common in roe deer revealing that *Anaplasma* species specific PCR assays are essential for a reliable identification as well as for determining their real prevalence.

KEYWORDS
Anaplasma bovis, *Anaplasma capra*, *Anaplasma ovis*, *Anaplasma phagocytophilum*, *Anaplasma platys*, roe deer

1 | INTRODUCTION

Obligate intracellular bacteria of the family *Anaplasmataceae* have been reported worldwide in wildlife (García-Pérez et al., 2016). Among them, several species belonging to the genus *Anaplasma* are considered aetiological agents of a number of tick-borne diseases in mammalian hosts (Dumler et al., 2001) including bovine (*Anaplasma marginale*, *Anaplasma bovis* and *Anaplasma centrale*) and ovine anaplasmosis (*Anaplasma ovis*), human granulocytic anaplasmosis and tick-borne fever of ruminants (*Anaplasma phagocytophilum*) and canine anaplasmosis (*Anaplasma platys*) (Battilani et al., 2017; de la Fuente et al., 2005). Up to now, only *A. phagocytophilum*, *A. capra*, *A. ovis* and *A. platys* are considered zoonotic (Arraga-Alvarado et al., 2014; Breitschwerdt et al., 2014; H. Li, Zheng, et al., 2015).

The distribution of each *Anaplasma* species depends on several factors, mainly the presence of proper tick vector species as well as suitable hosts and competent reservoirs (Estrada-Peña & de la Fuente, 2014). In this regard, it has been reported that wildlife, especially cervids, can act as reservoirs or asymptomatic carriers of some *Anaplasma* species (Atif, 2016; Ben Said et al., 2015; de la Fuente et al., 2008; Overzier et al., 2013; Renneker et al., 2013). In Spain, *Ixodes ricinus*, *Rhipicephalus sanguineus* s.l., *Rhipicephalus bursa*, *Haemaphysalis punctata* and *Dermacentor reticulatus* are considered the main vectors of *Anaplasma* spp. (Dantas-Torres, 2010; Koenen et al. 2013; Portillo et al., 2011; Palomar et al., 2015). However, no transovarial transmission of the pathogen has been demonstrated in their vectors (Rikihisa, 2011), and thus wild animals may play an important role in maintaining the ecological cycle of these bacteria in nature (Di Domenico et al., 2016; Woldehiwet, 2010). In this regard, wild cervids such as roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus*), together with other domestic ruminants, are considered the main reservoirs of *A. ovis*, showing prevalences usually higher than 50% using molecular methods (de la Fuente et al., 2008; García-Pérez et al., 2016; Y. Q. Li, Yang, et al., 2015; Renneker et al., 2013). Similarly, *A. phagocytophilum* has been molecularly identified (6%–98%) in red deer, roe deer and fallow deer (*Dama dama*) (Hulínská et al., 2004; Hapunik et al., 2011; Overzier et al., 2013; Scharf et al., 2011; Teodorowski et al., 2020; Zeman & Pecha, 2008). *A. bovis* is a species less frequently found (4%–15%) in roe deer, white-tailed deer (*Odocoileus virginianus*), red deer or sika deer (*Cervus nippon*) (García-Pérez et al., 2016; Jilintai et al., 2009; Kawahara et al., 2006; Y. Q. Li, Yang, et al., 2015). In addition, it has been reported that deer can develop a persistent infection with *A. marginale* or *A. centrale*; the high seroprevalences detected reveal that these wild ungulates are frequently in contact with these pathogens (Atif, 2016). In recent years, new *Anaplasma* species have been proposed; *A. capra*, first reported in goats from China (H. Li, Zheng, et al., 2015), has also been detected in red deer from France (Jouglin et al., 2019). Finally, a molecular investigation performed in China also identified organisms similar to *A. platys* in red deer (9%) and sika deer (15%) (Y. Q. Li, Yang, et al., 2015). Unravelling the role of wild ruminants as reservoirs of different *Anaplasma* species is of major importance for a proper understanding of the epidemiology of these bacteria. Nevertheless, most *Anaplasma* investigations in wildlife are focused on few *Anaplasma* species, mainly *A. phagocytophilum*.

Molecular studies on the distribution of *Anaplasma* spp. in wild cervids from Spain are scarce and restricted to some areas (de la Fuente et al., 2008; García-Pérez et al., 2016; Portillo et al., 2011). Four *Anaplasma* species were previously detected in roe deer from Spain. In northern areas, *A. phagocytophilum* was the most prevalent (61%), followed by *A. ovis* (53%), *A. bovis* (3.81%) and *A. centrale* (0.95%) (García-Pérez et al., 2016). However, in southern areas, only *A. ovis* (53%) and *A. phagocytophilum* (18%) were detected (de la Fuente et al., 2008).

Therefore, the objective of the present study was to determine the prevalence of *Anaplasma* spp. in roe deer hunted in four ecological areas covering the geographical distribution of this wild cervid in Spain. In addition, the molecular identification of these species, their distribution throughout the country and the presence of co-infections were assessed. Finally, the possible influence of the age and sex of the animals on the prevalence of these pathogens was studied.

2 | MATERIAL AND METHODS

2.1 | Sample collection and preservation

Amongst the wild cervids in Spain, roe deer is the second most abundant and hunted species, with a population around 200,000 specimens (Escudero et al., 2020); available official sources showed that 66,737 roe deer were hunted in 2018 in the country (MAPA, 2021). Between 2013 and 2020, the whole spleen of 224 roe deer from all the distribution areas of this wild cervid in Spain (Figure 1a) was collected during field evisceration by the hunters of the Spanish Roe Deer Association (Asociación del Corzo Español). Most of these samples (n= 212) were included in a previous study on the molecular characterization of *A. phagocytophilum* in roe deer (Remesar et al., 2020). All samples were collected, classified and preserved as previously reported (Remesar et al., 2020). The location, age and sex of each deer were recorded. The age was estimated on the basis of teeth analysis (Høye, 2006). Regarding the location, four ecological areas (continental, Mediterranean, mountainous and oceanic) were established as previously described (Morrondo et al., 2017).

The approval of the Ethics Committee/Welfare Authority was not required since all samples were collected post-mortem.

2.2 | DNA extraction, detection and identification of *Anaplasma* species

DNA extraction from splenic tissue was performed as previously described (Remesar et al., 2020). Detection of *Anaplasma* spp. DNA was performed in those samples that tested negative for *A. phagocytophilum* (124/212) in the previous study (Remesar et al., 2020) as well as in 12 new samples using a commercial qPCR targeting the *msp4* gene (EXOone *Anaplasma* spp., Exopol, Zaragoza, Spain). qPCR was

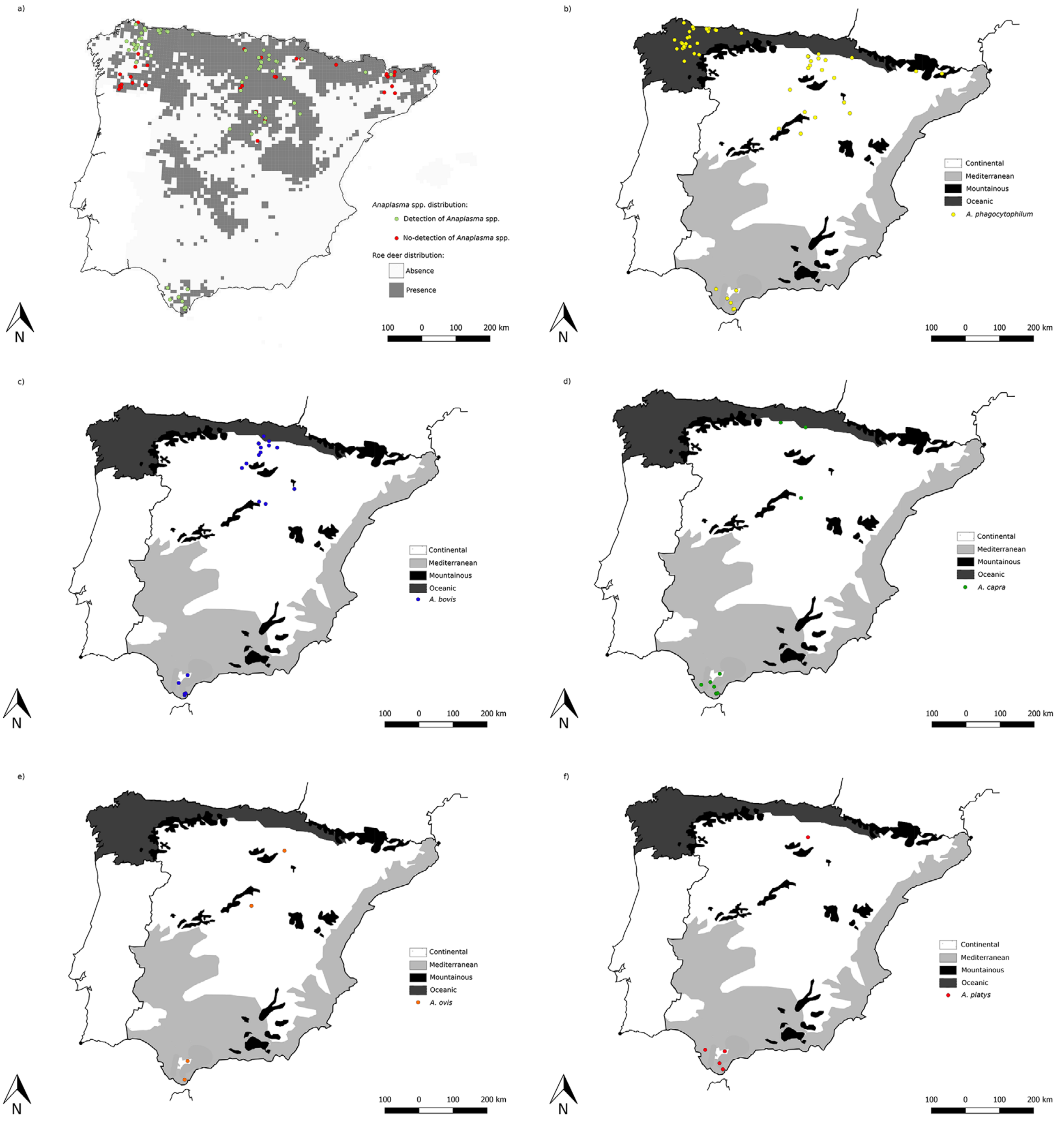

FIGURE 1 Maps showing roe deer distribution in Spain (a) and the four ecological areas (b-f). Dots represent the presence of *Anaplasma* spp. (a), *Anaplasma phagocytophilum* (b), *Anaplasma bovis* (c), *Anaplasma capra* (d), *Anaplasma ovis* (e) and *Anaplasma platys*-like (f)

performed in an Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Massachusetts, USA) following the manufacturer's instructions. All *Anaplasma* spp. qPCR positive samples, and those previously reported as positive to *A. phagocytophilum* (Remesar et al., 2020), were further tested using a PCR targeting the 16S rRNA gene of *Anaplasma* spp. (Table 1). A previously sequenced *A. phagocytophilum* sample and distilled water were included in each amplification reaction as positive and negative controls, respectively. PCR products were separated by electrophoresis on 1.5% agarose gels stained with RedSafe (iNtRON Biotechnology, South Korea) and then visualized using a Fluor-S MultiImager (Bio-Rad Laboratories, California, USA). The obtained 16S rRNA products were purified and sequenced in both senses on an ABI 3730xl (Applied Biosystems, Foster City, California, USA) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) at the Sequencing and Fragment Analysis Unit of the Santiago de Compostela University (Spain). Sequences were aligned and edited using ChromasPro (Technelysium, Brisbane, Australia) and consensus sequences were compared with sequences available from

TABLE 1 Primers and protocols used for detection and identification of *Anaplasma* spp

Gene target	Primer name	Primer sequence 5′-3′	Fragment size	Reference
16sRNA of *Anaplasma* spp.	AnaplsppF	AGA AGA AGT CCC GGC AAA CT	518 bp	(Zobba et al., 2014)
	AnaplR3	GAG ACG ACT TTT ACG GAT TAG CTC		
msp2 of *Anaplasma phagocytophilum*	msp2-3F	CCA GCG TTT AGC AAG ATA AGA G	334 bp	(Zeidner *et al*., 2000)
	msp2-3R	GCC CAG TAA CAA CAT CAT AAG C		
groEL of *Anaplasma bovis*	Ab groELF1	GTTCGCAGTATTTTGCCAGT	≈500 pb	(Guo et al., 2019)
	Ab groELR	CTGCRTTCAGAGTCATAAATAC		
	Ab groELF2	ATCTGGAAGRCCACTATTGAT		
	Ab groELR	CTGCRTTCAGAGTCATAAATAC		
groEL of *Anaplasma ovis*	Ao groELF	AGCAAAATAGCGCAATGCGTC	722 bp	(Belkahia et al., 2019)
	Ao groELR	TCAACTCTATCCTTAAGCTC		
groEL of *Anaplasma capra*	Ac groELF1	GCGAGGCGTTAGACAAGTCCATT	1264/1087 bp	(Jouglin et al., 2019)
	Ac groELR3	TCCAGAGATGCGAGCGTGTATAG		
	Ac groELF2	TGCACTGCTGGTCCAAAGGGGCT		
	Ac groELR2	CAACTTCGCTAGAGCCGCCAACC		
groEL of *Anaplasma platys*	Ap groELF	ATGGTATGCAGTTTGATCGC	624/515 bp	(Belkahia et al., 2019)
	Ap groELR1	TCTACTCTGTCTTTGCGTTC		
	Ap groELF	ATGGTATGCAGTTTGATCGC		
	Ap groELR2	CATAGTCTGAAGTGGAGGAC		

the GenBank database using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Five different species-specific PCR tests targeting the *msp2* gene of *A. phagocytophilum* and the *groEL* gene of *A. bovis*, *A. capra*, *A. platys* and *A. ovis* were also performed in all qPCR positive samples, including those previously positive to *A. phagocytophilum*, in order to determine the presence of co-infections. All PCR protocols were performed using previously reported protocols (Table 1), including positive and negative controls in each run assay.

Unique partial sequences identified in this study were deposited in GenBank under accession numbers MW759445-MW759459.

2.3 | Statistical analysis

The possible influence of the ecological area as well as both the age and sex of roe deer on the prevalence of *A. phagocytophilum* and *A. bovis* was analyzed using a logistic regression; risk analysis could not be performed on other *Anaplasma* species due to their low prevalence. The number of samples of each category is summarized in Table 2; information from six roe deer was incomplete, so they were not age-classified and one animal could not be sexed. Factors were eliminated from the initial model using a backward and forward conditional method based on Akaike information criterion (AIC) value until the best model was built. All pairwise interactions were evaluated. Odds ratio (OR) were computed by raising 'e' to the power of the logistic coefficient over the first category of each factor (reference category). The logistic analyses and the AIC selection were performed with glm() and step() functions in the R software (R Core Team, 2020). Level of significance was set at *p*-values < .05.

Phylogenetic analyses were 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 Hasegawa-Kishino-Yano (HKY+G) and a General Time Reversible substitution model (GTR + G), both with gamma-distributed rate variation across sites were used for the analysis of *Anaplasma* 16s rRNA and *A. bovis groEL* sequences, respectively. Both models were selected based on AIC value using the free software jModelTest v.2.1.10 (Darriba et al., 2012; Guindon & Gascuel, 2003). Trees were visualized and edited in FigTree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

3 | RESULTS

Most of the 224 analyzed roe deer were males (n = 157) and adults (n = 167). Regarding ecological areas, the highest number of samples originated from the oceanic area (n = 77), followed by Mediterranean (n = 55) and both mountainous and continental areas (n = 46 from each area) (Table 2).

First, qPCR results showed that 114 out of 224 (50.9%) spleen samples were positive to *Anaplasma* spp. Subsequently, amplification at the 16S rRNA gene was detected in 103 out of 114 *Anaplasma* spp. positive samples (90.4%). Sequence analysis at this gene allowed the

TABLE 2 Prevalence of *Anaplasma* species in roe deer from Spain when considering the hunting location, the age and sex of the roe deer

	Hunting location				Sex			Age		
	Oceanic (*n* = 77) (95% CI)	Mountain (*n* = 46) (95% CI)	Continental (*n* = 46) (95% CI)	Mediterranean (*n* = 55) (95% CI)	Female (*n* = 66) (95% CI)	Male (*n* = 157) (95% CI)	Unknown (*n* = 1) (95% CI)	Young (*n* = 51) (95% CI)	Adult (*n* = 167) (95% CI)	Unknown (*n* = 6) (95% CI)
Prevalence of each *Anaplasma* species detected on the total number of animals										
A. phagocytophilum	61.04% (49.22–71.74)	17.39% (8.32–31.95)	28.26% (16.45–43.68)	54.55% (40.66–67.80)	37.88% (26.47–50.70)	46.50% (38.56–54.60)	0.00% (0.00–94.53)	37.88% (34.95–63.23)	46.50% (36.13–51.59)	0.00% (0.00–48.32)
A. bovis	10.39% (4.91–19.97)	8.70% (2.82–21.69)	10.87% (4.07–24.36)	25.46% (15.09–39.27)	10.61% (4.73–21.23)	15.29% (10.23–22.09)	0.00% (0.00–94.53)	10.61% (3.67–22.19)	15.29% (10.10–21.50)	16.67% (0.88–63.62)
A. ovis	0.00% (0.00–5.92)	0.00% (0.00–21.69)	6.52% (17.00–48.93)	3.64% (0.63–13.60)	4.55% (1.18–13.56)	1.27% (0.22–5.00)	0.00% (0.00–94.53)	4.55% (1.53–17.23)	1.27% (00.21–4.71)	0.00% (0.00–48.32)
A. capra	1.30% (0.00–8.01)	0.00% (0.00–21.69)	6.52% (17.00–48.93)	16.36% (8.20–29.30)	0.00% (0.00–6.85)	8.28% (4.66–14.03)	0.00% (0.00–94.53)	0.00% (0.10–11.79)	8.28% (3.39–12.50)	0.00% (0.00–48.32)
A. platys-like	0.00% (0.00–5.92)	0.00% (0.00–21.69)	2.17% (0.11–12.97)	14.55% (6.93–27.22)	0.00% (0.00–6.85)	5.73% (2.82–10.93)	0.00% (0.00–94.53)	0.00% (0.10–11.79)	5.73% (2.24–9.55)	0.00% (0.00–48.32)
Prevalence of *Anaplasma* single species and coinfections detected on the total number of animals										
A. phagocytophilum	54.55% (42.84–65.79)	13.04% (5.42–26.95)	15.22% (6.84–29.48)	21.82% (12.25–35.36)	30.30% (19.91–43.00)	29.94% (23.03–37.84)	0.00% (0.00–94.54)	35.29% (22.80–50.00)	29.34% (22.69–36.96)	0.00% (0.00–48.32)
A. bovis	3.90% (1.01–11.73)	4.35% (0.76–16.04)	6.52% (1.70–18.93)	1.82% (0.00–10.99)	7.58% (2.82–17.50)	2.55% (0.82–6.81)	0.00% (0.00–94.54)	3.92% (0.68–14.59)	3.59% (1.47–8.01)	16.67% (0.88–63.52)
A. capra	0.00% (0.00–5.92)	0.00% (0.00–9.60)	6.52% (1.70–18.93)	5.45% (1.12–16.07)	0.00% (0.00–6.69)	3.82% (1.56–8.50)	0.00% (0.00–94.54)	1.96% (0.10–11.79)	2.99% (1.11–7.22)	0.00% (0.00–48.32)
A. phagocytophilum + *A. bovis*	5.19% (1.68–13.47)	4.35% (0.76–16.04)	4.35% (0.76–16.04)	7.27% (2.36–18.43)	3.03% (0.53–11.48)	6.37% (3.27–11.72)	0.00% (0.00–94.54)	5.88% (1.53–17.23)	5.39% (2.66–10.30)	0.00% (0.00–48.32)
A. phagocytophilum + *A. ovis*	0.00% (0.00–5.92)	0.00% (0.00–9.60)	6.52% (1.70–18.93)	0.00% (0.00–8.13)	4.55% (1.18–13.56)	0.00% (0.00–2.98)	0.00% (0.00–94.54)	5.88% (1.53–17.23)	0.00% (0.00–2.80)	0.00% (0.00–48.32)
A. phagocytophilum + *A. platys*-like	0.00% (0.00–5.92)	0.00% (0.00–9.60)	2.17% (0.11–12.97)	9.09% (3.40–20.71)	0.00% (0.00–6.69)	3.82% (1.56–8.50)	0.00% (0.00–94.54)	1.96% (0.10–11.79)	2.99% (1.11–7.22)	0.00% (0.00–48.32)
A. bovis + *A. capra*	0.00% (0.00–5.92)	0.00% (0.00–9.60)	0.00% (0.00–9.60)	1.82% (0.09–10.99)	0.00% (0.00–6.69)	0.64% (0.03–4.03)	0.00% (0.00–94.54)	0.00% (0.00–8.73)	0.60% (0.03–3.80)	0.00% (0.00–48.32)
A. phagocytophilum + *A. bovis* + *A. capra*	1.30% (0.07–8.01)	0.00% (0.00–9.60)	0.00% (0.00–9.60)	7.27% (2.36–18.43)	0.00% (0.00–6.69)	3.18% (1.18–7.66)	0.00% (0.00–94.54)	0.00% (0.00–8.73)	2.99% (1.11–7.22)	0.00% (0.00–48.32)
A. phagocytophilum + *A. bovis* + *A. platys*-like	0.00% (0.00–5.92)	0.00% (0.00–9.60)	0.00% (0.00–9.60)	5.45% (1.12–16.07)	0.00% (0.00–6.69)	1.91% (0.49–5.92)	0.00% (0.00–94.54)	0.00% (0.00–8.73)	1.80% (0.47–5.58)	0.00% (0.00–48.32)
A. phagocytophilum + *A. ovis* + *A. capra*	0.00% (0.00–5.92)	0.00% (0.00–9.60)	0.00% (0.00–9.60)	1.82% (0.09–10.99)	0.00% (0.00–6.69)	0.64% (0.03–4.03)	0.00% (0.00–94.54)	0.00% (0.00–8.73)	0.60% (0.03–3.80)	0.00% (0.00–48.32)
A. phagocytophilum + *A. bovis* + *A. ovis*	0.00% (0.00–5.92)	0.00% (0.00–9.60)	0.00% (0.00–9.60)	1.82% (0.09–10.99)	0.00% (0.00–6.69)	0.64% (0.03–4.03)	0.00% (0.00–94.54)	0.00% (0.00–8.73)	0.60% (0.03–3.80)	0.00% (0.00–48.32)
Total	64.94% (53.14–75.23)	21.74% (11.45–36.76)	41.30% (27.34–56.71)	63.64% (49.51–75.86)	45.45% (33.32–58.11)	53.50% (45.40–61.44)	0.00% (0.00–94.54)	54.90% (40.45–68.61)	50.90% (43.09–58.67)	16.67% (0.88–63.52)

Abbreviation: CI, confidence interval.

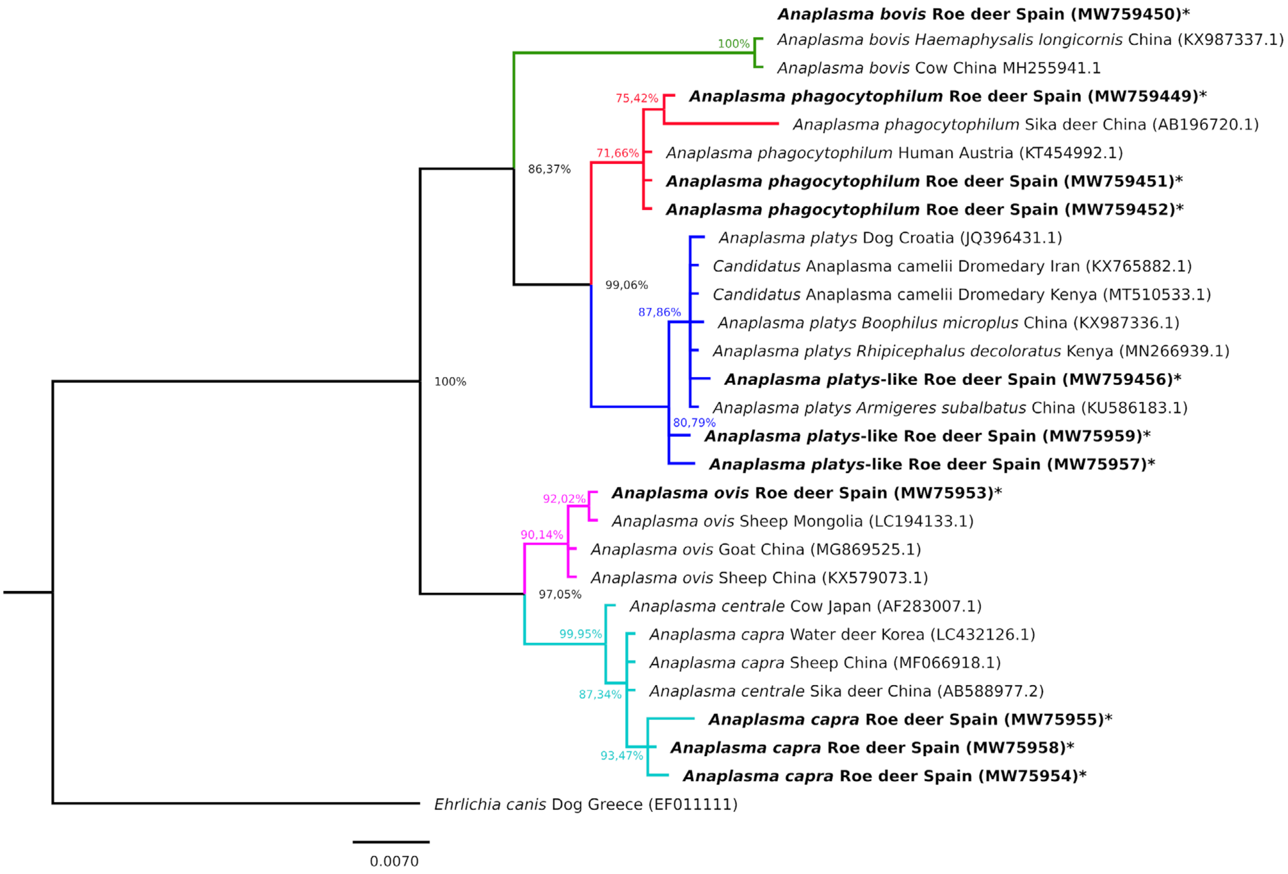

FIGURE 2 Phylogenetic tree clustering of the partial 16S rRNA gene of *Anaplasma* spp. The tree was obtained using a Hasegawa-Kishino-Yano with gamma-distributed rate variation across sites method (HKY+G) with the software MrBayes 3.2.7 (Ronquist et al., 2012) by Bayesian approach with Markov Chain Monte Carlo sampling (10,000,000 generations sampling every 1000 steps). The nucleotide sequence of *Ehrlichia canis* was used as an outgroup. Isolates identified in this study (*)

identification of five *Anaplasma* species: *A. phagocytophilum* (68/103), *A. bovis* (11/103), *A. capra* (11/103), *A. ovis* (4/103) and *A. platys*-like (9/103). Finally, and after performing the species-specific PCRs, 98 samples tested positive to the *msp2* gene of *A. phagocytophilum* (98/114) and 31 amplified through the study of the partial *groEL* gene of *A. bovis* (31/114). A low number of samples were positive to *A. capra* (13/114) and *A. ovis* (5/114) *groEL* gene specific PCRs. All *A. platys*-like samples were negative to the PCR assay targeting the specific *groEL* gene of *A. platys*. Sequence analysis of the species-specific PCRs at the *msp2* and *groEL* genes confirmed species identification in all 16S rRNA-positive samples except those identified as *A. platys*-like.

All *A. phagocytophilum*, *A. bovis*, *A. capra* and *A. ovis* 16S rRNA sequences showed a homology higher than 99.5% when compared to reference sequences (Supporting Information Material 1) and were clearly separated in four clades in the phylogenetic analysis of this gene (Figure 2). In addition, *A. platys*-like sequences were grouped in a clade including *A. platys* and *Candidatus* Anaplasma camelii reference sequences (Figure 2). Most of them (5/9) presented a homology higher than 99.5% with the *A. platys* sequence KX987336 obtained from ticks in China (Lu et al., 2017). The other four *A. platys*-like samples showed a homology higher than 99.4% when compared to sequences identified as *Candidatus* Anaplasma camelii (MT510533) and *A. platys* (MN266939) obtained from camels and cattle in Kenya, respectively (Kidambasi et al., 2020; Sang et al., 2006).

A. phagocytophilum sequences obtained through the study of *msp2* gene were identical to those deposited in GenBank (Supporting Information Material 1). However, *A. bovis groEL* obtained sequences exhibited a 93%–97% homology when compared to the deposited *A. bovis* sequences MH255909, MK340768, MK340781 MK340800 and MK340803 detected in goats and ticks from China (Guo et al., 2018). Finally, all *A. capra* (5.8%; 13/224) and *A. ovis* (2.2%; 5/224) *groEL* sequences showed a similarity higher than 99.3% to *A. capra* (MH084718) and *A. ovis* (MG869402) sequences obtained from a red deer in France (Jouglin et al., 2019) and from a goat in China (Guo et al., 2018), respectively.

When considering the overall prevalences for each *Anaplasma* species, the most frequent species was *A. phagocytophilum* (43.8%; 98/224), followed by *A. bovis* (13.8%; 31/224), *A. capra* (5.8%; 13/224) and *A. ovis* (2.2%; 5/224)*; Anaplasma platys*-like microorganisms were identified in 4% of samples (9/224). Infections with a single *Anaplasma* species were predominant, with *A. phagocytophilum* infections the most frequent, followed by *A. bovis* and *A. capra* infections (Table 2). The remaining *Anaplasma*-positive roe deer showed co-infections with two or three different *Anaplasma* species. The combination of

TABLE 3 Logistic regression model for the prevalence of *Anaplasma* spp.; *A. phagocytophilum; A. bovis* and *Anaplasma* spp. co-infections. Factors were removed following the Akaike information criterion value until the best model was built

	Estimate	*z*-Value	*p*-Value	OR	CI 95%
Anaplasma spp.					
(Intercept)	0.89475	2.220	.0265	2.4467287	1.12977872–5.5386893
Oceanic area	-	-	-	-	-
Mountainous area	−1.86743	−4.269	1.96e-05	0.1545197	0.06289031–0.3532744
Continental area	−0.88340	−2.231	.0257	0.4133771	0.18787941–0.8924070
Mediterranean area	-	-	-	-	-
Mountain area	−1.90887	−3.884	.000103	0.1482478	0.05439368–0.3771746
Continental area	−0.92483	−2.105	.035292	0.3965983	0.16509069–0.9298878
Anaplasma phagocytophilum					
(Intercept)	0.7141	0.4013	.07519	2.0422583	0.94101393–4.5823094
Oceanic area	-	-	-	-	-
Mountainous area	−1.9753	−4.289	1.79e-05	0.1387200	0.05310513–0.3283898
Continental area	−1.2296	−2.996	.00274	0.2923980	0.12759746–0.6427744
Mediterranean area	-	-	-	-	-
Mountain area	−1.7996	−3.523	.000426	0.1653665	0.05773717–0.4337461
Continental area	−1.7996	−2.334	.019583	0.3485644	0.14046084–0.8312083
Anaplasma bovis					
(Intercept)	−2.30777	−3.657	.000255	0.09948325	0.02514075–0.3075346
Oceanic area	-	-	-	-	-
Mediterranean area	1.04792	2.007	.044790	2.85170011	1.05189758–8.3249287
Anaplasma spp. coinfections					
(Intercept)	−0.23295	−0.310	.756305	0.7921971	0.17178867–3.3844292
Mediterranean area	-	-	-	-	-
Oceanic area	−2.13901	−3.617	.000298	0.1177710	0.03319924–0.3488534
Mountain area	−2.51042	−3.017	.002554	0.0812342	0.01145065–0.3436545
Continental area	−1.20123	−2.090	.036607	0.3008250	0.08979144–0.8790248

Abbreviations: CI, confidence interval; OR, odds ratio.

A. phagocytophilum/*A. bovis* and *A. phagocytophilum*/*A. platys*-like was the most prevalent dual co-infections. Finally, the most common triple co-infection was the association of *A. phagocytophilum*/*A. bovis*/*A. capra* (Table 2).

Using logistic regression, significant differences in the prevalence of *A. phagocytophilum* and *A. bovis* were only found when considering the ecological area (Table 3). Thus, roe deer from oceanic areas showed a lower probability of being positive to *A. phagocytophilum* than those from continental (OR = 0.29) and mountainous (OR = 0.14) areas; the risk of being positive was also lower in animals from Mediterranean areas than in those from continental (OR = 0.35) and mountainous areas (OR = 0.17) (Table 3). In addition, logistic regression showed that roe deer from Mediterranean areas presented a probability to be positive to *A. bovis* 2.9-fold higher than those from oceanic areas (Table 3). Due to the low number of *A. capra*, *A. ovis* and *A. platys*-like positive animals, risk analysis was not performed for these species.

4 | DISCUSSION

It has been demonstrated that wild ungulates can play an important role in the epidemiology of some tick-borne pathogens, mostly acting as carriers (Atif, 2016). In the past years, changes in land use and urbanization have led to an increased interaction between wildlife and humans and domestic animals, increasing the risk of transmission of these pathogens (Mackenstedt et al., 2015). Detection and identification of *Anaplasma* species present in wild ungulates is a major goal for assessing the role of these animals on their epidemiology.

Our data revealed that *Anaplasma* infections are very prevalent in roe deer from Spain, agreeing with previous molecular investigations performed in roe deer (65.7%–70.6%), red deer (50%) and fallow deer (50%) from northern areas of the country (de la Fuente et al., 2008; García-Pérez et al., 2016). Noticeable prevalence values have also been reported in other wild cervid species from other countries such as sika deer from Japan (39.7%; Kawahara et al., 2006) and China (50%; Y. Q.

Li, Yang, et al., 2015). All these data demonstrate that *Anaplasma* infections are very frequent in cervids, suggesting that these wild animals may act as reservoirs of this pathogen.

The results of this study show that wild cervids can be infected by a wide diversity of *Anaplasma* species. In this regard, three of the four *Anaplasma* species detected (*A. phagocytophilum*, *A. bovis* and *A. ovis*) have been previously reported in roe deer from Spain (de la Fuente et al., 2008; García-Pérez et al., 2016). In addition, this is the first report of *A. capra* and *A. platys*-like strains in European roe deer.

A. phagocytophilum has been reported in a wide range of animal species as well as in humans (Atif, 2016; Stuen et al., 2013); nevertheless, not all *A. phagocytophilum* strains are zoonotic since different variants adapted to particular geographical areas and hosts have been identified (Jahfari et al., 2014). Our results are consistent with previous investigations demonstrating that *A. phagocytophilum* is very prevalent in roe deer from different European countries (Atif, 2016; Teodorowski et al., 2020). Previous data from roe deer in Spain showed a high prevalence and variability of *A. phagocytophilum* strains, demonstrating that this wild ungulate is a reservoir for their own strains as well as some pathogenic *A. phagocytophilum* variants for humans and domestic animals (Remesar et al., 2020).

It is worth noting that most molecular investigations on the presence of *Anaplasma* spp. in wild ungulates were only focused on *A. phagocytophilum* because of its zoonotic potential, so data on the prevalence of other *Anaplasma* species is still limited. Our results revealed that *A. bovis* was the second most prevalent *Anaplasma* species in the sampled animals. This species is common in several domestic animals, being more prevalent in sheep and goats (16%–43%) than in cattle (≈4%) (Ben Said et al., 2015; Belkahia et al., 2015; Ceci et al., 2014; Liu et al., 2012; Nair et al., 2013; Ooshiro et al., 2008; Yang et al., 2015). It was also identified in a low-to-moderate percentage (4%–15%) of wild ruminants such as roe deer, white-tailed deer, red deer and sika deer (García-Pérez et al., 2016; Kawahara et al., 2006; Y. Q. Li, Yang, et al., 2015), agreeing with our results. Previous studies analyzing the *groEL* gene of *A. bovis* demonstrated a high genetic intraspecies diversity suggesting the existence of different lineages (Guo et al., 2018), as observed in a phylogenetic tree (Supporting Information Material 2); this fact may explain the percentages of identity between our sequences and other deposited *A. bovis* sequences (Supporting Information Material 1).

A. capra and *A. ovis* were detected in a low percentage of roe deer from Spain. *A. capra* was firstly reported in goats from China (H. Li, Zheng, et al., 2015) and since then it has been largely detected in Asian countries; thus, it has been found in sheep, cattle, Siberian roe deer, dogs, ticks and even humans with prevalences ranging from 6% to 12% (Peng et al., 2018; Shi et al., 2019; Seo et al., 2020; Yang et al., 2016). Nevertheless, a recent investigation also identified this species in some deer species such as red deer (3.4%) and swamp deer (*Rucervus duvaucelii*) (14.3%) from France, being the first report of *A. capra* outside Asia (Jouglin et al., 2019); these prevalence values agree with that detected in the present study. Thus, this is the first report of *A. capra* in Spain and the second report in Europe. It is worth noting the similarity of *A. capra* and *A. centrale* sequences at the 16S rRNA gene, as can be observed in Figure 2, that may lead to their misidentification. In fact, *A. centrale* isolates detected in deer from Japan by Kawahara et al. (2006) showed 16S rRNA sequences identical to the *A. capra* sequence MH762077 (Guo et al., 2018), suggesting that molecular analysis at more than one gene is required in order to achieve a reliable identification of both species. In addition, *A. ovis* was identified in a limited number of animals; this species has been detected in sheep and goats from Southern Europe (Italy and Portugal), Asia (Turkey, Iran, Iraq, Pakistan and China) and Africa (Kenya) with prevalence values ranging from 37% to 87% (Ahmadi-hamedani et al., 2012; Khan et al., 2015; Renneker et al., 2013; Torina et al., 2010; Yang et al., 2015). In contrast, data on the prevalence of *A. ovis* in deer is limited and restricted to a report in sika deer (20%) and red deer (32%) from China (Y. Q. Li, Yang, et al., 2015). It has been also found in a high percentage (53%) of roe deer from Spain (de la Fuente et al., 2008); the noticeable differences observed when compared to our data may be related to a decline in the number of sheep extensive farms in Spain in the last decade (Atif, 2016). In fact, a 22.4% reduction in the Spanish sheep population was reported in the last 10 years (Escudero et al, 2020).

In the past years, strains closely related to *A. platys* have been identified in several domestic animals such as cattle, goats, sheep, cats and camels (Ait Lbacha et al., 2017; Belkahia et al., 2015; Dahmani et al., 2015; H. Li, Zheng, et al., 2015; Y. Li, Chen, et al., 2015; Selmi et al., 2019; Wei et al., 2020; Zobba et al., 2014) as well as red deer and sika deer from China (Y. Q. Li, Yang, et al., 2015). Molecular data revealed percentages of identity ranging from 92% to 99% when compared to canine *A. platys* sequences at 16S rRNA and *groEL* genes (Belkahia et al., 2015). In this study, *A. platys*-like strains were detected in nine animals, most of them from the Mediterranean area (Figure 1f), being its first report in roe deer. It is also worth noting that all positive *A. platys*-like roe deer showed co-infections with *A. phagocytophilum*. It must be considered that only the 16S rRNA gene was studied since the results of all samples were negative when analyzed using an *A. platys* specific PCR targeting the *groEL* gene. These results might indicate that these strains may be more related to the strains previously detected in camels than to *A. platys* reported in dogs (Belkahia et al., 2015).

Risk analysis showed that the prevalence of some *Anaplasma* species was only influenced by the ecological area (Figure 1). *A. phagocytophilum* and *A. bovis* showed a wide geographical distribution since they were the only species detected in all the studied areas (Figure 1b,c), although it could be the result of their high prevalences. The significant differences observed in the prevalences of these species are probably related to the distribution of their major vectors (Remesar et al., 2020). The prevalence of *A. phagocytophilum* in roe deer (Figure 1b) was significantly highest in areas with oceanic climate located in the north of the country, where its main vector, *Ixodes ricinus*, is more abundant since it needs narrow diurnal temperature variations and high humidity (Estrada-Peña, 2017; Pérez-Latorre et al., 1999); in fact, *I. ricinus* is the most frequently reported tick species parasitizing deer from northern oceanic areas (Vázquez et al., 2011). In contrast, ticks of the genus *Rhipicephalus* and *Haemaphysalis*, which are considered the main vectors of *A. bovis* (Atif, 2016), are ecologically very adaptable and tolerate different climatic environments (Estrada-Peña et al., 2017).

In addition, previous studies showed that ticks from the genus *Rhipicephalus* are very common in ungulates from Mediterranean areas of Spain (Contreras et al., 2020; Ruíz-Fons et al., 2006). In the same way, *A. capra*, *A. ovis* and *A. platys*-like have also been reported in some species of the genus *Haemaphysalis* and *Rhipicephalus* (de la Fuente et al., 2007; Guo et al., 2018; Yang et al., 2016), which may explain their highest prevalences in continental and Mediterranean areas.

Information about *Anaplasma* co-infections in deer is currently limited. In this regard, it has been suggested that infections with a particular *Anaplasma* species may exclude infections with other *Anaplasma* spp. (de la Fuente et al., 2002; Stuen et al., 2005). Nevertheless, the use of species-specific primers in the present study allowed determining that *Anaplasma* co-infections were very frequent in roe deer since about 30% of animals were infected with two or three *Anaplasma* species. These results are consistent with the fact that deer is usually highly infested with ticks, showing high prevalences of tick-borne pathogens (Portillo et al., 2011; Remesar et al., 2020; Vázquez et al., 2011). The little information available on *Anaplasma* co-infections may be related to the frequent use of generic PCR protocols in *Anaplasma* molecular investigations, allowing the selective amplification of the dominant species (García-Pérez et al., 2016; Yu et al., 2020) and thus masking co-infections. Our results reveal that performing species-specific PCRs is strongly needed for detecting co-infections as well as for achieving a reliable identification of *Anaplasma* species, especially those less prevalent, and estimating their real prevalence.

5 | CONCLUSIONS

This study confirms that *Anaplasma* spp. is very prevalent in roe deer from Spain, being widespread in the distribution area of this cervid. In addition, our data support that roe deer can host a wide diversity of *Anaplasma* species. This study also represents the first report of *A. capra* and *A. platys*-like strains in Spain. Since some *Anaplasma* species detected are considered pathogenic for domestic animals (*A. phagocytophilum*, *A. bovis*, *A. ovis* and *A. capra*) or even zoonotic (*A. capra* and *A. phagocytophilum*), our results suggest that roe deer may play an important role in the sylvatic cycle of these pathogens contributing to the appearance of clinical anaplasmosis cases in both domestic animals and humans. The major factor influencing the presence of *A. phagocytophilum* and *A. bovis* was the geographical location, which could be related to the presence of their main vectors.

Finally, co-infections with two or three different *Anaplasma* species are frequent in roe deer and more common than expected; therefore, using *Anaplasma* species specific PCR protocols is essential for a reliable identification as well as for determining their real prevalence.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

No animals were culled for the purpose of this study. All samples were obtained post-mortem and kindly provided by the Spanish Roe Deer Association (ACE). Animals were hunted in accordance with the Spanish Hunter Code: (https://www.boe.es/biblioteca_juridica/codigos/abrir_pdf.php?fich=095_Codigo_de_Caza.pdf).

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

ORCID

Alberto Prieto https://orcid.org/0000-0002-3211-3494
José Manuel Díaz-Cao https://orcid.org/0000-0002-8119-7057
Pablo Díez-Baños https://orcid.org/0000-0003-2445-1095

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