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Zoonotic *Enterocytozoon bieneusi* genotypes in free-ranging and farmed wild ungulates in Spain

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Abstract

Microsporidia comprises a diverse group of obligate, intracellular, and spore-forming parasites that infect a wide range of animals. Among them, *Enterocytozoon bieneusi* is the most frequently reported species in humans and other mammals and birds. Data on the epidemiology of *E. bieneusi* in wildlife are limited. Hence, *E. bieneusi* was investigated in eight wild ungulate species present in Spain (genera *Ammotragus*, *Capra*, *Capreolus*, *Cervus*, *Dama*, *Ovis*, *Rupicapra*, and *Sus*) by molecular methods. Faecal samples were collected from free-ranging ($n = 1058$) and farmed ($n = 324$) wild ungulates from five Spanish bioregions. The parasite was detected only in red deer (10.4%, 68/653) and wild boar (0.8%, 3/359). *Enterocytozoon bieneusi* infections were more common in farmed (19.4%, 63/324) than in wild (1.5%, 5/329) red deer. A total of 11 genotypes were identified in red deer, eight known (BEB6, BEB17, EbCar2, HLJD-V, MWC_d1, S5, Type IV, and Wildboar3) and three novel (DeerSpEb1, DeerSpEb2, and DeerSpEb3) genotypes. Mixed genotype infections were detected in 15.9% of farmed red deer. Two genotypes were identified in wild boar, a known (Wildboar3) and a novel (WildboarSpEb1) genotypes. All genotypes identified belonged to *E. bieneusi* zoonotic Groups 1 and 2. This study provides the most comprehensive epidemiological study of *E. bieneusi* in Spanish ungulates to date, representing the first evidence of the parasite in wild red deer populations worldwide. Spanish wild boars and red deer are reservoir of zoonotic genotypes of *E. bieneusi* and might play an underestimated role in the transmission of this microsporidian species to humans and other animals.

Lay Summary

The fungal-related intracellular parasite *Enterocytozoon bieneusi* is a worldwide public health and veterinary problem. Here we demonstrated that it was present in wild boar, and wild and farmed red deer in Spain, with genotypes potentially capable of infecting humans, posing a public health risk.

Keywords: *Enterocytozoon bieneusi*, wild ungulates, molecular diversity, Spain

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Introduction

Microsporidia is a diverse group of obligate, intracellular, and spore-forming parasites related to fungi that infect a wide range of vertebrate and invertebrate hosts.[1] At least 220 genera and 1700 species of Microsporidia have been described so far, of which 17 species are able to infect humans. Among them, *Enterocytozoon bieneusi* is regarded as the most frequent species causing human microsporidiosis.[2] *Enterocytozoon bieneusi* is primarily identified in immunocompromised (including HIV+) patients associated with chronic diarrhoea and, to a lesser extent, extra-intestinal clinical manifestations.[3,4] However, its presence has been increasingly reported in apparently healthy individuals in recent years.[5–8] The routes of transmission of this parasite have not been fully elucidated yet; it is likely that the major route is via faecal-oral transmission of spores through direct contact with infected animals (including humans), or by ingestion of contaminated food and water. Indeed, *E. bieneusi* has been identified as the causative agent of a foodborne outbreak of microsporidiosis in Denmark.[9]

To date, over 600 *E. bieneusi* genotypes have been identified based on the analysis of the ITS region of the parasite.[10] These genetic variants have been allocated into 11 phylogenetic major groups, which Group 1 and 2 containing most genotypes with zoonotic potential, whereas the remaining (Groups 3–11) include mostly host-adapted genotypes associated to specific animals.[11]

Along human history, wildlife has been an important source of infectious diseases for humans.[12] Currently, zoonotic pathogens with a wildlife reservoir constitute a major public health problem, affecting all continents. Several viruses, bacteria and parasites have been able to cross the host species (e.g., fox, red deer, wild boar) barrier to emerge as zoonoses.[13]

During the last few decades, wild ungulates species in Europe have spatially expanded due to different factors: the intensification of game management practices, human depopulation of rural areas, changes in land use, introduction of individuals outside their native range, or reintroductions of endangered species.[14–16] Data on the epidemiology of *E. bieneusi* in ungulates species are limited. Most of the studies conducted globally have focused on the presence of this microsporidia in farmed or captive ungulate species,[11] but occurrence and molecular data in wild ungulates remain largely unknown. *Enterocytozoon bieneusi* has been documented in wild ungulate species of the genera *Axis*, *Capreolus*, *Cervus*, *Dama*, *Hydropotes*, *Kobus*, *Muntiacus*, *Odocoileus*, *Rangifer*, *Rusa*, and *Sus* at prevalence rates ranging from 0–42% in farmed animals, and from 0–54% in free-living animals, with sporadic cases identified in captive animals at zoological institutions (Table 1).[17–38] Most of the *E. bieneusi* genotypes identified in those hosts belong to the zoonotic Groups 1 and 2, but others are included in host-adapted Group 3 and Group 8[11] (Table 1). Only three studies have reported *E. bieneusi* in free-living wild boar populations, with infection rates ranging from 2–14% (Table 1). All available data on this host come from studies conducted in European (Austria, Czech Republic, Poland, Slovakia, and Spain) and Asian (South Korea) countries. These studies revealed a limited *E. bieneusi* genetic diversity in wild boars, being EbpA and EbpC (Group 1) the most prevalent genotypes described in this host (Table 1). There are no reports of *E. bieneusi* in wild red deer; all studies in this host were conducted in farmed or captive animals in China (Table 1). Infection rates ranged from 8–38% with five *E. bieneusi* genotypes identified (BEB6, JLD-IV, JLD-XIII, HLJD-V, and HLJD-VI).

In Spain, wild ungulate species including wild boar and red deer are well-known suitable hosts of zoonotic infectious pathogens such as *Mycobacterium bovis*, hepatitis E virus, and *Coxiella burnetii*.[39–42] However, little information is currently available about the epidemiology of *E. bieneusi* in wild ungulates in the country. Just a single study has previously reported *E. bieneusi* in Spanish wild boars, but this survey was conducted only at regional scale.[38] Hence, this study was carried out to determine the prevalence, genetic diversity, and zoonotic potential of *E. bieneusi* in a large population of free-ranging and farmed wild ungulates from different Spanish regions to gain national representativeness.

Materials and methods

Study area and sampling strategy

Between 1999 and 2021, a retrospective nationwide survey was performed. Faecal samples from the eight wild ungulate species present in Spain: Barbary sheep (*Ammotragus lervia*), Iberian wild goat (*Capra pyrenaica*), roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), fallow deer (*Dama dama*), mouflon (*Ovis aries musimon*), Southern chamois (*Rupicapra pyrenaica*), and wild boar (*Sus scrofa*), were collected throughout the five bioregions (BRs, see below) of mainland Spain (Table 1, Fig. 1).

Based on landscape structure, major ecosystems, game management practices, and socio-political aspects, the Spanish Wildlife Disease Surveillance Scheme splits mainland Spain into five different BRs (Fig. 1) sharing similar epidemiological features.[43] BR1 comprises the Northern areas of temperate Atlantic climate with almost no game management; meanwhile, the remaining BRs present a Mediterranean climate with an increasing drought gradient from BR2 to BR54. In the Mediterranean BRs, game management is not the norm except for BR3 and the Southwest of BR5, where the highly productive savannah-like or oak forest landscapes are frequently profited for large game production. Mountain habitats are more dominant in BRs 1, 2, and 5, while cereal plains are predominant in BR4. This zoning has been previously exploited to facilitate disease surveillance efforts in wild ungulates in Spain.[39,44–47] From each sampling site, that is, hunting states or game reserves ($n = 65$; Fig. 1) selected by simple random sampling throughout the study area, the animals (15–20 whenever possible) were also randomly sampled.

All animals were legally harvested by hunters or culled as part of population control programmes on game reserves. Faecal samples were collected directly from the rectum of each animal during field necropsies after hunting using disposable gloves and placed in individual sterile tubes with records of the date, location, and host. Collected samples were transported in cooled boxes to each participating institution responsible for the sampling and stored at –20°C. Aliquots of these faecal samples were shipped to the Spanish National Centre for Microbiology, Majadahonda (Spain) for subsequent molecular analyses.

Aliquots of faecal samples from farmed red deer belonging to a semi-extensively bred red deer population located in

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Table 1. Infection rates and molecular diversity of *E. bieneusi* reported in wild, farmed, and captive ungulate (cervid and wild boar) species worldwide. Bolded genotypes belong to zoonotic Group 1 and Group 2.

Host common name	Host scientific name	Country	Population type	Occurrence rate (no. pos./total no.)	Genotype(s) (*n*)	Reference
Barking deer	*Muntiacus muntjak*	Bangladesh	Captive[a]	0 (0/6)	–	17
Chinese water deer	*Hydropotes inermis inermis*	China	Wild	7 (3/40)	HLJD-V (1), HND-I (1), BJCWD (1)	18
Fallow deer	*Dama dama*	China	Captive[a]	ND	HND-1 (1)	19
			Captive	27 (15/55)	HLJD-V (2), BEB6 (2), MWC_d1 (1), BJFD (10)	18
		Australia	Wild	0 (0/17)	–	20
Hog deer	*Axis porcinus*	China	Captive[a]	ND (3/ND)	BEB6 (2), CHS9 (1)	21
Korean water deer	*Hydropotes inermis argyropus*	Korea	Wild	54 (52/97)	D (29), WL1 (12), WL2 (5), WL4 (1), WL6 (1)	22
Musk deer	*Moschus berezovskii*	China	Farmed	17 (38/223)	EbpC (38)	23
Père David's deer	*Elaphurus davidianus*	China	Wild	35 (45/128)	HLJD-V (42), MWC_d1 (3)	24
			Farmed	34 (16/47)	Type IV (4), EbpC (4), EbpA (4), BEB6 (2), COS-I (1), COS-II (1)	25
			Wild	30 (24/80)	HLJD-V (12), MWC_d1 (4), BEB6 (1), BJED-I to BJED-V (7)	18
Red deer	*Cervus elaphus*	China	Farmed	38 (6/16)	BEB6 (2), JLD-IV (3), JLD-XIII (1)	26
			Farmed, captive	20 (1/5)	HLJD-V (1)	27
			Farmed	8 (8/104)	BEB6 (7), HLJD-VI (1)	28
			Captive[a]	ND (1/ND)	BEB6 (1)	21
		Australia	Wild	0 (0/77)	–	20
		Spain	Wild	2 (5/329)	EbCar2 (1), S5 (2), BEB17 (1), Type IV (1)	This study
		Spain	Farmed	19 (63/324)	HLJD-V (43), BEB6 (3), MWC_d1 (1), Wildboar3 (6), DeerSpEb1 (7), DeerSpEb2 (13), DeerSpEb3 (1)	This study
Reindeer	*Rangifer tarandus*	China	Farmed	17 (21/125)	CHN-RD1 (12), Peru6 (6), CHN-RD2 (1), CHN-RD3 (1), CHN-RD4 (1)	29
Roe deer	*Capreolus capreolus*	Korea	Wild	50 (1/2)	WL1 (1)	22
Sambar deer	*Rusa unicolor*	Australia	Wild	5 (25/516)	MWC_d1 (19), D (3), Type IV (1), J (1), MWC_d2 (1)	20
Siberian roe deer	*Capreolus pygargus*	China	Farmed	11 (2/18)	BEB6 (2)	28
Sika deer	*Cervus nippon*	China	Farmed	7 (23/326)	J (11), BEB6 (4), EbpC (1), CHN-DC1 (1), CAF-1 (1), JLD-1 (2), JLD-2 (2), JLD-3 (1)	30
			Farmed	14 (111/818)	BEB6 (84), EbpC (3), I (1), JLD-III (1), JLD-VIII (3), JLD-IX(1), JLD-XV (2), JLD-XVI (2), JLD-XVII (2), JLD-XVII (2), JLD-XIX (2), JLD-XX (2), JLD-XXI (2), JLD-XXII (1), JLD-XXIII (2), LND-I (1)	31

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Table 1. Continued

Host common name	Host scientific name	Country	Population type	Occurrence rate (no. pos./total no.)	Genotype(s) (*n*)	Reference
			Farmed	36 (215/599)	BEB6 (129), HLJDI (18), EbpC (3), HLJD-IV (2), COS-I (1), EbpA (1), D (1), JLD-I (7), JLD-II (5), HND-I (4), JLD-III (2), HND-II (1), JLD-IV (3), JLD-V (2), JLD-VI (5), HND-III (1), JLD-VII (1), JLD-VIII (16), JLD-IX (1), JLD-X (1), HND-IV (1), JLD-XI (2), JLD-XII (1), JLD-XIV (7)	26
			Farmed, captive	33 (28/86)	BEB6 (20), HLJD-V (4), HLJD-I (1),HLJD-II (1), HLJD-III (1), HLJD-IV (1)	27
			Captive[a]	ND (1/ND)	BEB6 (1)	21
			Captive[a]	ND	BEB6 (5), HND-1 (5), CD7 (2)	19
			Captive	12 (5/40)	CGC2 (3), JLD-XV (2)	18
Spotted deer	*Axis axis*	Bangladesh	Captive[a]	27 (8/30)	BAN5 (8)	17
Waterbuck	*Kobus ellipsiprymnus*	Bangladesh	Captive[a]	0 (0/7)	–	17
White-lipped deer	*Cervus albirostris*	China	Captive[a]	ND	CD7 (1), CHWD1 (1)	19
White-tailed deer	*Odocoileus virginianus*	USA	Wild	33 (26/80)	WL4 (11), I (7), LW1 (1), DeerEb1-DeerEb13 (1 each), J (1)	32
			Wild	12 (6/49)	WL18 (2), WL19 (2), WL4 (2)	33
Wild boar	*Sus scrofa*	Austria	Wild	14 (6/44)	D (1) EbpC (2), Henan-I (3)	34
		China	Captive[a]	42 (108/257)	CAM5 (5), CHS12 (9), CM8 (8), CTS3 (14), EbpC (35), Henan-IV (11), pigEBITS5 (18), Wildboar12 (5)	35
			Farmed	41 (147/357)	CHC5 (10), CHG19 (11), D (1), EbpA (22), EbpC (85), PigEBITS5 (1), RWSH4 (1) SC02 (1), Wildboar7 (1), Wildboar8 (5), Wildboar9 (2) Wildboar10 (6), Wildboar11 (1)	36
		Czech Republic	Wild	9 (20/231)	D (5), EbpA (10), EbpC (1), G (1), Wildboar3 (1), Wildboar4 (2)	34
		Poland	Wild	8 (10/129)	EbpA (2), EbpC (3), Wildboar2 (1), Wildboar3 (1), Wildboar5 (2), Wildboar6 (1)	34
		Slovakia	Wild	4 (2/56)	D (1), Wildboar1 (1)	34
		Korea	Wild	3 (13/502)	D (4), EbpC (3), KWB1 (3), KWB2 (1), KWB3 (1), KWB4 (1)	37
		Spain	Wild	2 (3/142)	EbpA (2), PigSpEb1(1)	38
		Spain	Wild	1 (3/359)	Wildboar3 (1), WildboarSpEb1 (2)	This study

[a]Animals kept in zoological institutions.
ND. Not determined.

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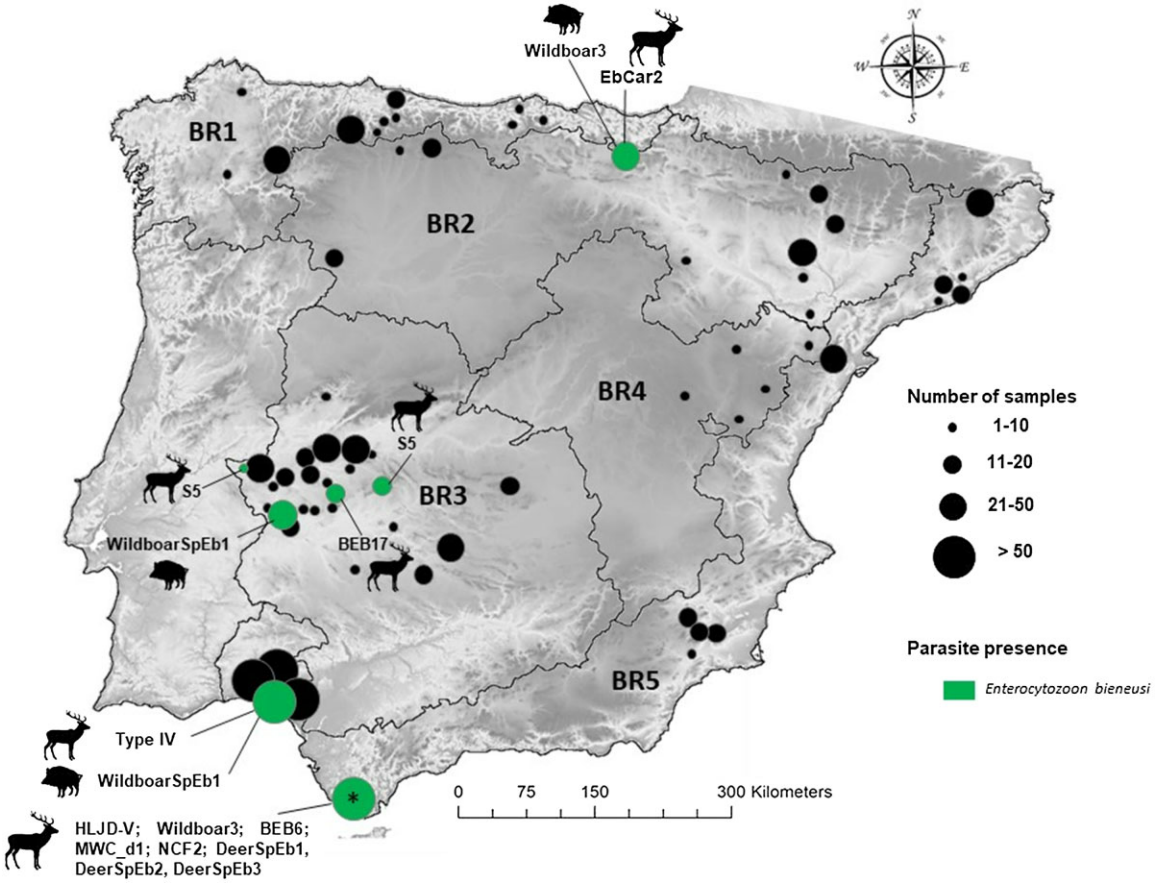

Figure 1. Map of Spain showing the sampling areas and the geographical distribution of *E. bieneusi* DNA detected in wild and farmed (*) ungulate species according to established bioregions (BR1-5) as described in reference 44.

southern Spain were obtained from a previous work.[48] The deer were semi-extensively bred in a forest-shrub prairie habitat divided into different plots by high-wire fencing. The animals were kept in separate batches according to their sex and productive status. They were kept within large fenced (6–8 ha) enclosures in batches of 60–80 reproductive females; the males were kept in separate enclosures. The animals were identified with individual ear tags. Faecal material was collected directly from the rectum using sterile disposable latex gloves during health veterinary inspections.

DNA extraction and purification

Genomic DNA was isolated from about 200 mg of each faecal specimen of wild ungulate origin by using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, except that samples mixed with InhibitEX buffer were incubated for 10 min at 95°C. Extracted and purified DNA samples were eluted in 200 μl of PCR-grade water and kept at 4°C until further molecular analysis.

PCR and sequence analysis

To identify *E. bieneusi*, a nested PCR protocol was used to amplify the ITS region as well as portions of the flanking large and small subunit of the ribosomal RNA gene as previously described.[49] The outer (EBITS3 and EBITS4) and inner (EBITS1 and EBITS2.4) primer sets were used to generate PCR products of 435 and 390 bp, respectively. Negative and positive controls were included in every PCR run. The amplicons of the second PCR were examined on 2% D5 agarose gels stained with Pronasafe (Conda, Madrid, Spain). All amplicons of the expected size were directly sequenced in both directions with the internal primer pair in 10 μl reactions using Big Dye^{TM} chemistries and an ABI 3730xl sequencer analyser (Applied Biosystems, Foster City, CA, USA). Raw sequences were examined with Chromas Lite version 2.1 software (http://chromaslite.software.informer.com/2.1) to generate consensus sequences. These sequences were compared with reference sequences deposited at the National Center for Biotechnology Information (NCBI) using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The established nomenclature system based in ITS nucleotide sequence was used to determine *E. bieneusi* genotypes.[50] Sequences generated in the present study were deposited in the GenBank public repository database under accession numbers ON819430–ON819442.

Cloning of *E. bieneusi* DNA

When *E. bieneusi* mixed genotype infection within a specimen was suspected from the chromatogram sequence traces, the secondary PCR products were cloned using the TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA, USA). Transformants (eight clones from each specimen) were selected, PCR-amplified, and sequenced in both directions using M13

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Table 2. Occurrence rates of *E. bieneusi* in wild free-ranging and farmed ungulates ($n = 1382$) according to host species and established bioregions (BR1-5) describes in reference 44. 95% Confidence Intervals (CI) are indicated.

Variable	Animals examined (*n*)	Positive samples (*n*)	Infection rate % (95% CI)
Host			
Barbary sheep	20	0	0.0 (0.00–16.1)
Fallow deer	96	0	0.0 (0.00–3.8)
Mouflon	10	0	0.0 (0.00–27.7)
Red deer	653	68	10.4 (8.3–12.9)
Roe deer	93	0	0.0 (0.00–3.9)
Iberian wild goat	89	0	0.0 (0.00–4.1)
Southern chamois	62	0	0.0 (0.00–5.8)
Wild boar	359	3	0.8 (0.3–2.4)
Bioregion			
1	103	0	0.0 (0.00–3.6)
2	164	2	1.2 (0.3–4.3)
3	335	4	1.2 (0.5–3.0)
4	32	0	0 (0.00–10.7)
5	748	65	8.7 (6.9–10.9)
Total	1382	71	5.1 (4.1–6.4)

forward and reverse primers. Briefly, amplicons were purified using Exonuclease I/Shrimp Alkaline Phosphatase (ExoSAP-IT Express, Affymetrix Inc., Santa Clara, CA, USA), and sequenced in both directions using primers utilized for PCR screening in 10 μl reactions, Big Dye^{TM} chemistries, and an ABI 3130 sequencer analyser (Applied Biosystems).

Sequence and phylogenetic analysis

Sequence chromatograms of each strand were aligned and examined with Lasergene software (DNASTAR, Inc., Madison, WI, USA). Sequences obtained in this study as well as *E. bieneusi* sequences previously identified in livestock, wildlife, and companion animals in Spain, and appropriate reference sequences retrieved from GenBank were aligned with the Clustal W algorithm. Phylogenetic analysis was performed using the Neighbour–Joining (NJ) method, and genetic distance was calculated with the Kimura parameter-2 model using MEGA X.[51,52]

Statistical analysis

The Pearson's χ^2 test was used to assess differences in *E. bieneusi* occurrence rates according to host species, population type (wild vs. farmed), and bioregion (BR1-5) of origin. Analyses were carried out using the R Statistical Package version 2.15.3.[53]

Results

Occurrence of *E. bieneusi*

A total of 1382 faecal samples were collected from wild ungulates (76.6%, 1058/1382) and farmed red deer (23.4%, 324/1382) from different Spanish regions during the period 1999–2021 (Supplementary Table S1). Overall, 5.1% (71/1382; 95% CI: 4.0–6.4) of the faecal samples from ungulates analysed were positive for *E. bieneusi* by PCR. Parasite DNA was detected in red deer (10.4%, 68/653; 95% CI: 8.3–12.9) and wild boars (0.8%, 3/359; 95% CI: 0.3–2.4), but not in fallow deer (0/96; 95% CI: 0.00–3.8), roe deer (0/93; 95% CI: 0.00–3.9), mouflons (0/10; 95% CI: 0.00–27.7), Iberian wild goat (0/89; 95% CI: 0.00–4.1), Barbary sheep (0/20; 95% CI: 0.00–16.1), or Southern chamois (0/62; 95% CI: 0.00–5.8) (Table 2, Fig. 1). Among the red deer populations, the occurrence of *E. bieneusi* was statistically higher in farmed red deer (19.4%, 63/324) than in wild red deer (1.5%, 5/329) [χ^2 (1, $n = 653$) = 56.2, $P < 0.001$].

Regarding spatial distribution by bioregion, *E. bieneusi* was detected in both red deer and wild boar in the three BR regions, BR5 (8.7%, 66/748), BR3 (1.2%, 4/335), and BR2 (1.2%, 2/164), without statistically significant differences (Table 2, Fig. 1).

The full dataset of this study showing sampling, epidemiological, diagnostic, and molecular data can be found in Supplementary Table 2.

Molecular characterization of *E. bieneusi*

In wild boars, sequence analysis of the ITS revealed the presence of two genotypes, a previously reported genotype in wild boar (Wildboar3) and a novel genotype (named WildboarSpEb1) (Table 3). WildboarSpEb1 differed by a single nucleotide polymorphism (SNP) at ITS region with genotype EbpA (AF076040) at nucleotide site 113 (C→T). Wildboar3 was detected in one animal and WildboarSpEb1 in two animals, one each in BR3 and BR5 (Supplementary Table 1).

In red deer, analysis of the nucleotide sequences at the ITS region revealed a high genetic diversity with 11 distinct *E. bieneusi* genotypes circulating alone or in combination. Out of the 11 genotypes, 8 were known genotypes (HLJD-V, BEB6, BEB17, MWC_d1, S5, EbCar2, Type IV, and Wildboar3) and 3 novel genotypes (named DeerSpEb1, DeerSpEb2, and DeerSpEb3) (Table 3). Mixed infections involving two or more genotypes were identified in 15.9% (10/63) of the farmed red deer samples analysed (Supplementary Table S1). DeerSpEb1 differed by a single SNP from genotype FJL (MK357781) at nucleotide site 78 (G→T); DeerSpEb2 differed by a SNP from genotype LND-I (MN056217) at nucleotide site 144 (A→G); and DeerSpEb3 differed by a SNP from nucleotide sequence with no genotype information isolated from a Père David's deer (MG703260) at nucleotide site 144 (A→G).

HLJD-V was the most prevalent genotype identified in red deer (52.9%, 36/68), followed by DeerSpEb2 (10.3%, 7/68), Wildboar3 (5.9%, 4/68), and DeerSpEb1 (5.9%, 4/68)

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Table 3. Frequency and molecular diversity of *E. bieneusi* genotypes identified in the wild and farmed ungulates investigated in the present study.

Host species	Genotype (ITS)	Isolates (*n*)	Frequency (%)	GenBank accession number
Wild boar	WildboarSpEb1	2	66.7	ON819430
	Wildboar3	1	33.3	ON819431
Red deer	HLJD-V	36	52.9	ON819432
	DeerSpEb2	7	10.3	ON819433
	Wildboar3	4	5.9	ON819434
	DeerSpEb1	4	5.9	ON819435
	HLJD-V+DeerSpEb2	3	4.4	–
	S5	2	2.9	ON819436
	HLJD-V+BEB6	2	2.9	–
	DeerSpEb1+DeerSpEb2	1	1.5	–
	DeerSpEb2+DeerSpEb3	1	1.5	ON819437[a]
	BEB6	1	1.5	ON819438
	BEB17	1	1.5	ON819439
	MWC_d1	1	1.5	ON819440
	EbCar2	1	1.5	ON819441
	Type IV	1	1.5	ON819442
	HLJD-V+DeerSpEb1	1	1.5	–
	Wildboar3+DeerSpEb1	1	1.5	–
	Wildboar3+HLJD-V+DeerSpEb2	1	1.5	–

[a]Sequence for genotype DeerSpEb3 only.

(Table 3). Genotypes EbCar2, BEB17, S5, and Type IV were only observed in wild red deer, whereas genotypes HLJD-V, BEB6, MWC_d1, Wildboar3, DeerSpEb1, DeerSpEb2, and DeerSpEb3 were found in farmed red deer only (Table 3). Regarding the bioregion of origin, EbCar2 was only found in wild red deer populations from BR2, BEB17 and S5 in BR33 and Type IV in BR5 (Supplementary Table 3).

Phylogenetic analysis

Phylogenetic analysis of ITS sequences using the NJ method demonstrated that novel genotypes belonged to groups of *E. bieneusi* that contain zoonotic genotypes. DeerSpEb1 and WildboarSpEB1 clustered within the Group 1, and DeerSpEb2 and DeerSpEb3 within the Group 2 (Fig. 2).

Discussion

In Spain, information on the occurrence and molecular diversity of *E. bieneusi* in wildlife is limited. Recent studies have identified this microsporidian species in wild boars and Iberian pigs sharing the same habitat,[38] wild and domestic carnivores,[54–56] lagomorphs,[57] urban pigeons,[58,59] and wild micromammals.[60] In our study, *E. bieneusi* was identified in 10.4 and 0.8% of the investigated red deer and wild boar populations, respectively. Of note, infection rates were significantly higher in farmed (19.4%) than in wild (1.5%) red deer. This large discrepancy can be explained by two reasons: (i) farmed animals confined in limited enclosures have higher group sizes, densities, and interaction rates (all favouring parasite transmission) than free-living animals, and (ii) the surveyed deer farm features a great faunal biodiversity and is located in the European-African migration route, factors that promote inter-species parasite transmission. Indeed, higher nematodal parasite burdens have been previously found in farmed deer raised at high densities than in wild deer populations in Argentina.[61] Similarly, a direct relationship between host density and parasite burdens has been demonstrated in white-tailed deer in the USA[62] and in wild cervids (*Cervus elaphus* and *Dama dama*) in Spain.[63]

To date, *E. bieneusi* has been reported in farmed and captive (zoo) red deer in China[26,27] (Table 1). Present survey reports for the first time the presence of *E. bieneusi* in wild red deer populations worldwide. The infection rate found in farmed red deer (19.4%) was similar to that reported in farmed red deer in China (20.0%),[27] but lower than that identified in farmed and zoo animals (37.5%) in the same country.[26] Although *E. bieneusi* has also been reported in wild roe deer in Korea,[22] we did not detect this parasite in roe deer in this study. This could be related to the relatively low number of roe deer samples collected in the study ($n = 93$) coupled with fact that a low infection rate was observed in wild red deer in this study (1.5%). This will also explain the negative results for presence of *E. bieneusi* for the other ungulates including in the study: fallow deer ($n = 96$), mouflons ($n = 10$), Iberian wild goat ($n = 90$), Barbary sheep ($n = 20$), and Southern chamois ($n = 62$). Clearly, more studies including higher number of samples appear to be required to investigate this parasite in wild populations due to the expected low prevalence.

Enterocytozoon bieneusi was detected at low infection rates (0.8%, 3/359) in the investigated wild boar population. This figure was slightly lower than that (2.1%) recently reported in wild boars in Southern Spain.[38] Comparatively higher occurrence rates (8–14%) have been documented in Central European countries including Austria, Czech Republic, Poland, Slovak Republic,[34] and in South Korea (3%).[37]

An interesting contribution of this study is the demonstration that red deer are suitable hosts for a very large diversity ($n = 11$) of *E. bieneusi* genotypes. Besides the eight already known genotypes (BEB6, BEB17, EbCar2, HLJD-V, MWC_d1, S5, and Wildboar3), three novel genotypes named DeerSpEb1, DeerSpEb2, and DeerSpEb3 were additionally described. In previous studies, only genotypes BEB6, HLJD-V, HLJD-VI, JLD-IV, and JLD-XIII were found circulating in red deer populations (see Table 1). Therefore, this study constitutes the first report of genotypes BEB17, EbCar2, S5, Wildboar3, DeerSpEb1, DeerSpEb2, and DeerSpEb3 in this host. Furthermore, we identified mixed infections in farmed red deer involving two or more genotypes in a single faecal sample, suggesting that these infections were common in

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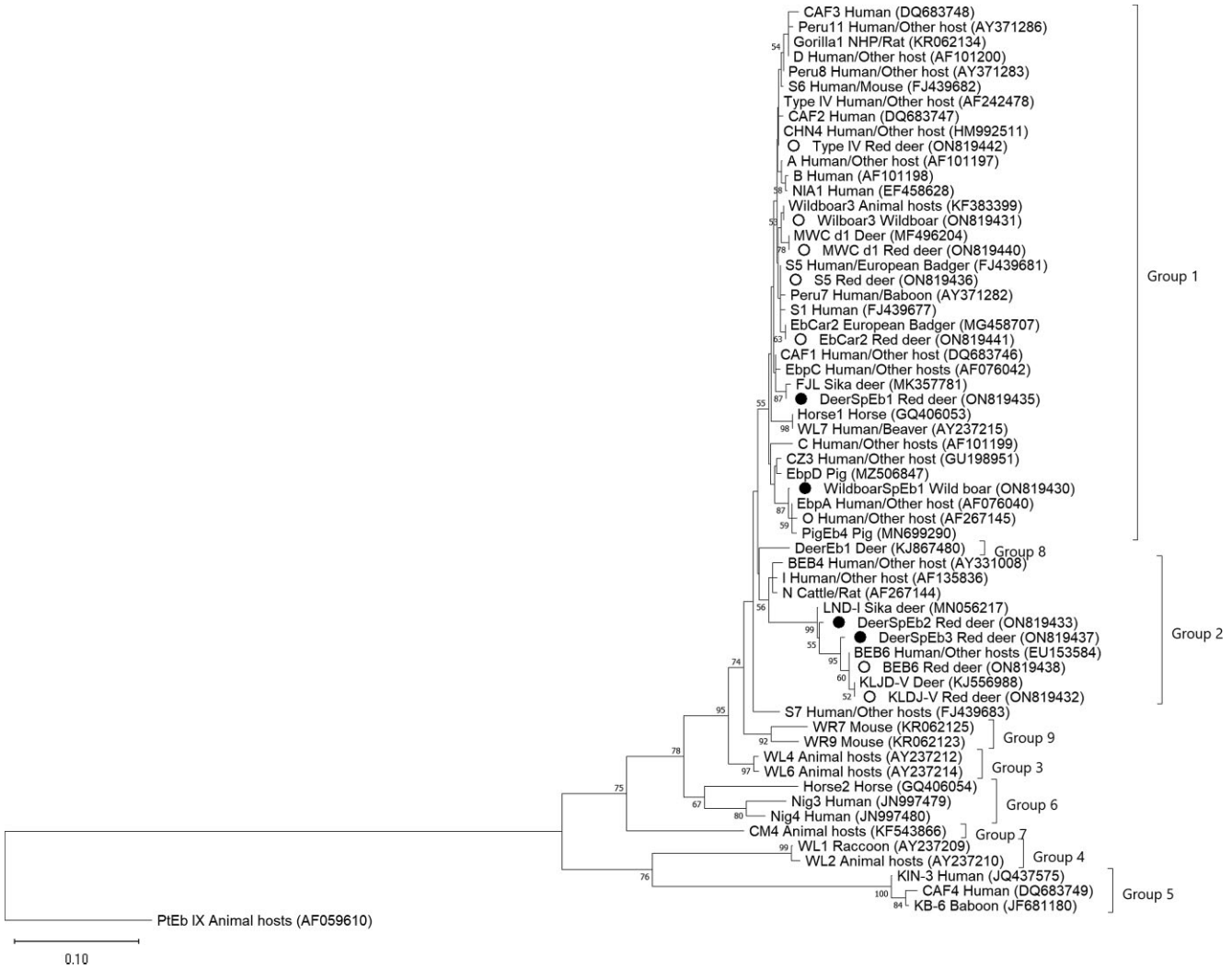

Figure 2. Phylogenetic relationships among *E. bieneusi* complete ITS sequences (243 bp) generated in the present study (novel subtypes are represented with a black filled circle and other subtypes with an unfilled circle) and representative reference sequences for all *E. bieneusi* groups. PtEb XI genotype was used as outgroup to root the tree. Analysis was conducted by a neighbor-joining method and genetic distances calculated using the Kimura two-parameter model. Analysis involved 62 nucleotide sequences. Numbers at the nodes represent the bootstrap values with more than 50% bootstrap support from 1000 pseudoreplicates.

semi-captive animals due to increased contact among animals or to cross-species transmission from synathropic hosts infected by the pathogen. This finding may also have unforeseen public health consequences, as farm workers may be more exposed to *E. bieneusi* infections during the handling of these animals or their manure. In this farm, management interventions (sanitary issues, weaning, reposition, and artificial insemination) were limited to two to four times per year to minimise the risk of animal stress.[39]

Genotype HLJD-V was the most prevalent *E. bieneusi* genotype identified in red deer. To date, this genetic variant had only been detected in cervids including red deer, fallow deer, sika deer, Chinese water deer, and Père David's deer in China.[18,24,27] In addition, genotype Wildboar3 has been previously reported in wild foxes and badgers in Spain,[55] farmed foxes and raccoons in China,[64–66] wild boars in Central Europe,[34] and wild raccoons in Poland.[67] Genotype BEB17 has been only reported in cattle in Brazil.[68] Therefore, this is the second report of this *E. bieneusi* genotype worldwide. In Spain, genotype BEB6 was previously detected in domestic dogs from the northern area of the country.[56] This genotype is commonly seen in cervids (see Table 1) and other animals including human and non-human primates, alpacas, horses, cattle, cats, sheep, goats, and birds, suggesting that BEB6 has loose host specificity and, therefore, has zoonotic potential.[11] Genotype MWC_d1 was first reported in wild Sambar deer in Australia[20] and subsequently described in wild Père David's deer in China.[24] In the present study, a single red deer was found infected with genotype S5. This *E. bieneusi* genetic variant has been reported in wild badgers in Spain[55] and in four HIV-positive adults in Malawi,[69] suggesting that this genotype has zoonotic potential and cross-transmission between humans and animals is possible. Additionally, this is the second report of genotype EbCar2, a variant previously found infecting badgers in Spain and Poland.[55,70] Finally, Type IV was observed in a single sample from free-ranging wild red deer. Although this is the first description of this genotype in this host, Type IV has been commonly reported in humans and numerous hosts including non-human primates, bovids, other cervid species, rodents, cats, birds, and domestic dogs.[11]

Two *E. bieneusi* genotypes were identified in wild boars including known Wildboar3 genotype and novel Wildboar-

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SpEb1 genotype. Only genotypes EbpA and PigSpEb1 had been previously described in Spanish wild boars,[38] so this is the first description of Wildboar3 in this host in Spain. Of note Wildboar3 genotype was first described in wild boars from Czech Republic and Poland[34] and subsequently identified in other European wildlife species including introduced raccoon dogs in Poland and Germany,[67] and badgers and red foxes in Spain.[55]

In conclusion, this large molecular-based epidemiological survey provides first-time nationwide data on the presence and genetic diversity of *E. bieneusi* in wild ungulate populations in Spain. Major contributions of the survey include (i) first report of *E. bieneusi* in wild red deer worldwide, (ii) first description of this pathogen in farmed red deer in Spain, (iii) confirmation that all known and novel *E. bieneusi* genotypes described belonged to the zoonotic Groups 1 and 2, and (iv) expansion of the known host range for certain *E. bieneusi* genotypes. The relatively common finding of zoonotic *E. bieneusi* genetic variants in wild boars and red deer—the two more abundant and widely distributed wild ungulate species—pose a public health risk for individuals (e.g., veterinarians, farmers, hunters) in close contact with these animals or their manure that should not be underestimated. Overall, these results expand our current knowledge on the epidemiology and public veterinary health relevance of *E. bieneusi*.

Supplementary material

Supplementary material is available at *Medical Mycology* online.

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Declaration of interest

The authors have declared no conflict of interest.

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