Received: 27 April 2022 | Revised: 15 June 2022 | Accepted: 22 June 2022

DOI: 10.1111/tbed.14643

Transboundary and Emerging Diseases

WILEY

ORIGINAL ARTICLE

Wild micromammal host spectrum of zoonotic eukaryotic parasites in Spain. Occurrence and genetic characterisation

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SUMMARY

Micromammals have historically been recognized as highly contentious species in terms of the maintenance and transmission of zoonotic pathogens to humans. Limited information is currently available on the epidemiology and potential public health significance of intestinal eukaryotes in wild micromammals. We examined 490 faecal samples, grouped into 155 pools, obtained from 11 micromammal species captured in 11 Spanish provinces for the presence of DNA from *Cryptosporidium* spp., *Giardia duodenalis*, *Enterocytozoon bieneusi* and *Blastocystis* sp. The presence of *Leishmania* spp. was investigated in individual spleen samples. All micromammal species investigated harboured infections by at least one eukaryotic parasite, except *Apodemus flavicollis*, *Myodes glareolus*, *Sorex coronatus* and *Sciurus vulgaris*, but the sample size for these host species was very low. *Cryptosporidium* spp. was the most prevalent species found (3.7%, 95% confidence interval [CI]: 2.2–5.7), followed by *G. duodenalis* (2.8%, 95% CI: 1.6–4.6) and *E. bieneusi* (2.6%, 95% CI: 1.4–4.3). All pooled faecal samples tested negative for *Blastocystis* sp. *Leishmania infantum* was identified in 0.41% (95% CI: 0.05–1.46) of the 490 individual spleen samples analysed. Sequence analyses allowed the identification of *Cryptosporidium andersoni* (5.9%), *C. ditrichi* (11.7%), *C. muris* (5.9%), *C. parvum* (5.9%), *C. tyzzeri* (5.9%), rat genotypes CR97 (5.9%) and W19 (5.9%), vole genotypes V (11.7%) and VII (5.9%) and *Cryptosproridium* spp. (35.3%) within *Cryptosporidium*

(*n* = 17). Known genotypes C (66.7%) and Peru11 (25.0%) and a novel genotype (named MouseSpEb1, 8.3%) were detected within *E. bieneusi* (*n* = 12). None of the *G. duodenalis*-positive samples could be genotyped at the assemblage level. Molecular data indicate that wild micromammals were primarily infected by rodent-adapted species/genotypes of eukaryotic pathogens and thereby have a limited role as a source of human infections. The presence of ruminant-adapted species *C. andersoni* along with finding *C. parvum* is indicative of an overlap between domestic/peri-domestic and sylvatic transmission cycles of these agents.

KEYWORDS
Blastocystis, *Cryptosporidium*, *Enterocytozoon*, epidemiology, genotyping, *Giardia*, rodents, Spain, transmission, zoonoses

1 | INTRODUCTION

Micromammals have historically been recognized as highly contentious species in terms of the maintenance and transmission of zoonotic pathogens to humans, mainly because they represent a biodiverse and ubiquitous group of pathogen reservoir hosts (Han et al., 2015; Luis et al., 2013; Meerburg et al., 2009). In Europe, more than 20 zoonoses with rodent reservoirs are recognized (Han et al., 2015). Some of the diseases directly transmitted by rodents (e.g., haemorrhagic fever, leptospirosis, salmonellosis, and turalemia) or using rodents as vectors (e.g., babesiosis, cutaneous leishmanisosis, and Lyme disease) have serious human health consequences (Centers for Disease Control and Prevention, 2010). Pathogen transmission frequency at the rodent–human interface is particularly favoured if rodents coexist with humans in agricultural, peri-urban or urban environments and under extremely high rodent densities (i.e., up to 2000 individuals per hectare in the case of the common vole [*Microtus arvalis*]; Bryja et al., 2005). Rodent-borne diseases are, with few exceptions, often neglected in Europe (Tomassone et al., 2018). Little information is currently available on the epidemiology and potential health impact of zoonotic enteric eukaryotes in wild micromammal populations from agricultural and wildlife environments. These include diarrhoea-causing enteric protist species such as *Cryptosporidium* spp. and *Giardia duodenalis* (DuPont, 2016; Kotloff, 2017), the microsporidia *Enterocytozoon bieneusi* (W. Li, Feng, & Santin, 2019), and the stramenopile of uncertain pathogenic significance *Blastocystis* sp. (Ajjampur & Tan, 2016). All these intestinal eukaryotes share the faecal–oral transmission route, either through direct contact with infected individuals or indirectly via ingestion or contaminated water or food. Remarkably, most of the species/genotypes/subtypes (STs) of the above-mentioned eukaryotes can be zoonotically transmitted (Dixon, 2021; Hublin et al., 2021; W. Li, Feng, Zhang, & Xiao, 2019; Ryan, Zahedi, et al., 2021). On the other hand, *Leishmania infantum* is a kinetoplastid protozoon transmitted by phlebotomine sand flies that causes zoonotic leishmaniasis in humans, dogs, and other mammals (Azami-Conesa et al., 2021). This parasite is endemic to southern Europe, where wildlife is suspected to act as a reservoir (Alcover et al., 2020). Anthropophilic rodents such as rats (*Rattus* spp.) and house mice (*Mus musculus*) from endemic areas of the Mediterranean have shown potential as reservoirs (Tsakmakidis et al., 2017). For this reason, assessing their occurrence and genetic diversity in wild small mammal hosts is important to ascertain their transmission dynamics and true zoonotic potential.

Giardia duodenalisa, *Cryptosporidium* spp., *Blastocystis* sp., and *E. bieneusi* exhibit extensive intraspecies genetic diversity. *Giardia duodenalis* comprises eight (A to H) distinct assemblages, of which zoonotic assemblages A and B are commonly reported to infect humans and other mammal species (Dixon, 2021). In addition to assemblages A and B, wild rodents can harbour the rodent-specific *G. duodenalis* assemblage G, *G. muris*, and *G. microti* (Helmy et al., 2018). At least 44 *Cryptosporidium* spp. are considered taxonomically valid (Ryan, Feng, et al., 2021), of which *C. hominis* and *C. parvum* are responsible for most human cryptosporidiosis cases reported globally. The largest diversity of *Cryptosporidium* has been reported in rodents, with 14 species and over 57 genotypes (Ryan, Feng, et al., 2021). In European wild rodents, 35 distinct *Cryptosporidium* spp./genotypes have been described so far (Table 1). For *Blastocystis* sp.,so far 28 genetic variants, called STs, have been identified from mammal and avian hosts (ST1-ST17, ST21, and S23-ST32), of which 12 have been reported in humans (ST1–ST10, ST12, and ST14), with ST4 being the most prevalent in rodents (Higuera et al., 2021; Hublin et al., 2021; Khaled et al., 2020; Maloney et al., 2021a, 2021b; Stensvold & Clark, 2020). STs 18–20 and ST22 are currently considered invalid (Stensvold & Clark, 2020). Finally, more than 600 *E. bieneusi* genotypes have been defined and grouped into 11 phylogenetic groups (W. Li & Xiao, 2020). Groups 1 and 2 include most of the potentially zoonotic genotypes, whereas the rest of the groups display genotypes with strong host specificity (W. Li & Xiao, 2020; Li, Feng, & Santi, 2019).

Genotyping and subtyping methods are central in epidemiological studies to trace the origin of infections, understand the circulation of the pathogen in particular populations and geographical areas and assess the occurrence and directionality of potential zoonotic events. This molecular-based study aims to investigate the occurrence and genetic diversity of eukaryotic parasites in faecal samples of wild micromammals from mainland Spain, with special attention to

TABLE 1 Molecular-based data on the occurrence and genetic diversity of *Giardia duodenalis*, *Cryptosporidium* spp. and *Enterocytozoon bieneusi* in European micromammal species

Host scientific name	Host common name	Parasite genus	Prevalence % (pos./*n*)	Species/genotypes identified	Country	Reference
Alexandromys oeconomus	Root vole	*Cryptosporidium*	36.4 (8/22)	*Cryptosporidiu microti*; *Cryptosporidium* spp.; vole genotype III	Finland	Kivistö et al. (2021)
Apodemus agrarius	Striped field mouse	*Giardia*	68.6 (24/35)	*Giardia. duodenalis* assemblage A; *G. microti*; *G. muris*	Germany	Helmy et al. (2018)
			24.4–41.7 (65–111/266)	Nd	Poland	Perec-Matysiak, Buńkowska-Gawlik, Zaleśny, and Hildebrand (2015)
		Cryptosporidium	61.7–68.3 (164–181/266)	*C. parvum*	Poland	Perec-Matysiak, Buńkowska-Gawlik, Zaleśny, and Hildebrand (2015)
			Nd	*C. ditrichi*; *Apodemus* genotype II	Latvia	Čondlová et al. (2019)
			Nd	*C. apodemi*	Lithuania	Čondlová et al. (2019)
			Nd	*C. apodemi*	Romania	Čondlová et al. (2019)
			Nd	*Apodemus* genotype II	Serbia	Čondlová et al. (2019)
			Nd	*C. apodemi*; *C. ditrichi*; *Apodemus* genotype II	Slovakia	Čondlová et al. (2019)
			31.8 (34/107)	*C. hominis*; *C. parvum*; *C. scrofarum*; *Cryptosporidium* spp.; muskrat genotype II	Slovakia	Danišová et al. (2017)
		Enterocytozoon	42.9 (79/184)[a]	D; gorilla1; WR5; WR7; WR8	Poland	Perec-Matysiak, Buńkowska-Gawlik, Kváč, et al. (2015)
A. flavicollis	Yellow-necked field mouse	*Giardia*	34.2 (13/38)	*G. microti*; *G. muris*	Germany	Helmy et al. (2018)
			48.3 (98/203)	*Giardia* spp.	Poland	Bajer et al. (2002)
			24.4–41.7 (65–111/266)	Nd	Poland	Perec-Matysiak, Buńkowska-Gawlik, Zaleśny, and Hildebrand (2015)
			24.4 (156/638)	Nd	Poland	Bajer (2008)
		Cryptosporidium	Nd	*C. ditrichi*; *C. parvum*	Germany	Čondlová et al. (2019)
			27.8 (58/209)	*C. parvum*	Poland	Bajer et al. (2002)
			61.7–68.3 (164–181/266)	*C. ubiquitum*	Poland	Perec-Matysiak, Buńkowska-Gawlik, Zaleśny, and Hildebrand (2015)
			28.1 (179/638)	Nd	Poland	Bajer (2008)
			27.8 (58/209)	*C. parvum*	Poland	Bajer et al. (2002)
			Nd	*C. apodemi*; *C. ditrichi*; *C. muris*; *Apodemus* genotypes I, II	Poland	Čondlová et al. (2019)
			36.4 (24/66)	*C. ditrichi*; *Apodemus* genotype I; vole genotype II	Finland	Kivistö et al. (2021); Čondlová et al. (2019)

(Continues)

TABLE 1 (Continued)

Host scientific name	Host common name	Parasite genus	Prevalence % (pos./n)	Species/genotypes identified	Country	Reference
			Nd	*C. ditrichi*	Belgium	Čondlová et al. (2019)
			Nd	*C. apodemi*; *C. ditrichi*; *C. microti*; *C. tyzzeri*; *Apodemus* genotypes I, II	Czech Republic	Čondlová et al. (2019)
			Nd	*C. ditrichi*	France	Čondlová et al. (2019)
			Nd	*C. ditrichi*; *Apodemus* genotype II	Serbia	Čondlová et al. (2019)
			Nd	*C. apodemi*; *C. ditrichi*; *C. muris*, *C. parvum*, *Apodemus* genotype I	Slovakia	Čondlová et al. (2019)
			20.5 (15/73)	*C. parvum*; *C. scrofarum*; *C. suis*; *Cryptosporidium* spp.	Slovakia	Danišová et al. (2017)
		Enterocyto-zoon	30.0 (18/60)[a]	D; WR1; WR4; WR6; WR9,	Poland	Perec-Matysiak, Buńkowska-Gawlik, Kváč, et al. (2015)
A. sylvaticus	Long-tailed field mouse	*Giardia*	22.2 (2/9)	*G. muris*	Germany	Helmy et al. (2018)
		Cryptosporidium	Nd	*C. ditrichi*	Czech Republic	Čondlová et al. (2019)
			Nd	*C. ditrichi*	France	Čondlová et al. (2019)
			Nd	*C. ditrichi*	The Nether-lands	Čondlová et al. (2019)
			Nd	*C. tyzzeri*	Serbia	Čondlová et al. (2019)
			Nd	*C. apodemi*	Slovakia	Čondlová et al. (2019)
			44.9 (125/278)	*C. parvum*; *C. muris*[a]	Spain	Torres et al. (2000)
Crocidura russula	White-toothed shrew	*Cryptosporidium*	14.8 (13/88)	*C. parvum*[a]	Spain	Torres et al. (2000)
Microtus agrestis	Field vole	*Giardia*	86.7 (52/60)	*G. microti*; *G. muris*	Germany	Helmy et al. (2018)
		Cryptosporidium	67.6 (44/65)	*C. microti*; *Cryptosporidium* spp.; vole genotypes II, V, VIII, IX	Finland	Kivistö et al. (2021)
M. arvalis	Common vole	*Giardia*	74.2 (311/419)	Nd	Poland	Bajer (2008)
			96.3 (257/267)	Nd	Poland	Bajer et al. (2002)
		Cryptosporidium	62.3 (261/419)	Nd	Poland	Bajer (2008)
			73.0 (200/274)	*C. parvum*	Poland	Bajer et al. (2002)
			14.2 (50/353)	Nd -	Czech Republic and Slovakia	Stenger et al. (2018)
			22.6 (74/328)	*C. microti*, *C. alticolis*, vole genotypes II, III, IV, V, VI, VII	Czech Republic	Horčičková et al. (2019)
M. levis	East European vole	*Cryptosporidium*	1.6 (1/63)	*C. alticolis*	Norway	Myšková et al. (2019)
Micromys minutus	Eurasian harvest mouse	*Cryptosporidium*	50.0 (1/2)	*Apodemus* genotype II	Finland	Kivistö et al. (2021)

(Continues)

TABLE 1 (Continued)

Host scientific name	Host common name	Parasite genus	Prevalence % (pos./*n*)	Species/genotypes identified	Country	Reference
Mus musculus	House mouse	*Giardia*	17.6 (29/165)	*G. duodenalis* assemblage G	Spain	Fernández-Álvarez et al. (2014)
		Cryptosporidium	10.3 (8/78)	*C. muris*; *C. tyzzeri*	Spain	García-Livia et al. (2020)
		Enterocytozoon	28.6 (6/21)[a]	WR3	Poland	Perec-Matysiak, Buńkowska-Gawlik, Kváč, et al. (2015)
			10.7 (31/289)	EpbA; C; CZ3; D; H; Peru8; PigEBITS5; S6	Czech Republic and Germany	Sak et al. (2011)
M. spretus	Western Mediterranean mouse	*Cryptosporidium*	31.8 (7/22)	*C. muris*[a]; *C. parvum*	Spain	Torres et al. (2000)
Myodes glareolus	Bank vole	*Giardia*	63.5 (191/301)	*G. duodenalis* assemblages A and B; *G. microti*; *G. muris*	Germany	Helmy et al. (2018)
			24.4–41.7 (65–111/266)	Nd	Poland	Perec-Matysiak, Buńkowska-Gawlik, Zaleśny, and Hildebrand (2015)
			58.3 (888/1523)	Nd	Poland	Bajer (2008)
			93.9 (418/445)	Nd	Poland	Bajer et al. (2002)
		Cryptosporidium	61.7–68.3 (164–181/266)	Nd	Poland	Perec-Matysiak, Buńkowska-Gawlik, Zaleśny, and Hildebrand (2015)
			53.8 (819/1523)	Nd	Poland	Bajer (2008)
			70.6 (324/459)	*C. parvum*	Poland	Bajer et al. (2002)
			56.5 (104/184)	*C. baileyi*; vole genotypes II, III, IV, VII, IX, shrew genotype II; genotype SW5	Finland	Kivistö et al. (2021)
			7.1 (10/140)	-	Czech Republic and Slovakia	Stenger et al. (2018)
			28.6 (14/49)	*C. muris*[a]; *C. parvum*	Spain	Torres et al. (2000)
		Enterocytozoon	39.1 (18/46)[a]	D; WR2; WR6; WR10	Poland	Perec-Matysiak, Buńkowska-Gawlik, Kváč, et al. (2015)
M. rufocanus	Grey red-backed vole	*Cryptosporidium*	15.4 (2/13)	*C. andersoni*	Finland	Kivistö et al. (2021)
M. rutilus	Red vole	*Cryptosporidium*	11.1 (1/9)	Vole genotype III	Finland	Kivistö et al. (2021)
Myopus schisticolor	Wood lemming	*Cryptosporidium*	100(1/1)	Nd	Finland	Kivistö et al. (2021)
Rattus rattus	Black rat	*Giardia*	36.2(42/116)	*G. duodenalis* assemblages B and G	Spain	Fernández-Álvarez et al. (2014)
		Cryptosporidium	13.9 (14/101)	*C. meleagridis*; *C. muris*; rat genotypes I, II, III	Spain	García-Livia et al. (2020)

(Continues)

TABLE 1 (Continued)

Host scientific name	Host common name	Parasite genus	Prevalence % (pos./*n*)	Species/genotypes identified	Country	Reference
R. norvegicus	Brown rat	*Giardia*	35.0 (35/100)	Nd	Spain	Galván-Puchades et al. (2021)
		Cryptosporidium	34.1 (29/85)	Nd		
			16.0(55/343)	*C. andersoni*; *C. muris*; *C. occultus*; *C. ryanae*; rat genotypes I, IV, V	Czech Republic	Ježková et al. (2021b)
Rattus spp.	Brown and black rats	*Giardia*	14.1 (9/64)	*G. duodenalis* assemblage G	Spain	Köster et al. (2021)
		Cryptosporidium	45.3 (29/64)	*C. muris*; *C. ratti*; rat genotypes IV and V		
Sorex araneus	Common shrew	*Cryptosporidium*	43.8 (35/80)	Shrew genotypes I, II	Finland	Kivistö et al. (2021)
			14.3 (1/7)	*C. scrofarum*	Slovakia	Danišová et al. (2017)
S. minutus	Pygmy shrew	*Cryptosporidium*	25 (1/4)	Genotype SW4	Finland	Kivistö et al. (2021)

Nd: not determined.
[a]In this study, faecal and spleen samples were analysed.

potential human-driven sources of infection and cross-species transmission pathways.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

The capture and handling procedures for sampling used in the present study were approved by the Animal Experimentation Ethics Committee of the University of Castilla-La Mancha (reference number: PR20170201) and were in accordance with the Spanish legislation guidelines (RD 8/2003) and with the International Guiding Principles for Biomedical Research Involving Animals issued by the Council for International Organisation of Medical Sciences and the International Council for Laboratory Animal Science (RD 53/2013).

2.2 | Sampling

Between 2011 and 2014, and in the framework of different ongoing studies (Dominguez et al., 2021; González-Barrio et al., 2021; Jeske et al., 2021), wild micromammals were collected in agricultural and forestry environments from 11 provinces from mainland Spain (Figure 1). This includes a variety of habitats and climates, which can be simplified into five different bio-regions, as defined in the Spanish Wildlife Disease Surveillance Scheme (Spanish Ministry of Agriculture, 2008; Figure 1). The most relevant features of each bio-region are summarized elsewhere (Muñoz et al., 2010). Wild micromammals were captured using LFATDG Sherman Live Traps (7.62 × 8.89 × 22.86 cm, H. B. Sherman Traps, Inc.). The individuals captured were sedated with an intramuscular injection of a solution containing ketamine (10 mg/kg) and medetomidine (1 mg/kg) and then humanely euthanized by cervical dislocation. These animals were transported refrigerated to the laboratory, where a detailed necropsy was performed in cabinets under biosafety 2 containment. Faecal and tissue (small and large intestine, spleen) samples were collected and preserved frozen at −20°C. Additionally, red squirrels (*Sciurus vulgaris*) were surveyed after being accidentally found dead close to trap capture sites or by forestry agents and brought to the lab for necropsy. At sampling processing, pooling of faeces/intestinal content was carried out in those animals for which insufficient faecal matter was available for individual genomic DNA extraction.

2.3 | DNA extraction and purification

Genomic DNA was isolated from about 200 mg of each faecal specimen/intestinal pool using the QIAamp DNA Stool Mini Kit (Qiagen) 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 polymerase chain reaction (PCR)-grade water and kept at 4°C until further molecular analysis. A water extraction control was included in each sample batch processed.

Genomic DNA from the murine spleen was isolated using the Speed Tools DNA Extraction Kit (Biotools). To do so, 10–15 mg of each tissue was homogenized in 100 μl of NET-10 buffer (10 mM NaCl, 10 mM ethylenediamine tetraacetic acid, 10 mM Tris-HCl pH 8) and digested overnight at 56°C with 100 μl of BT1 buffer (Biotools) and 20 μl proteinase K (20 mg/ml). After digestion, genomic DNA was extracted according to the manufacturer's instructions.

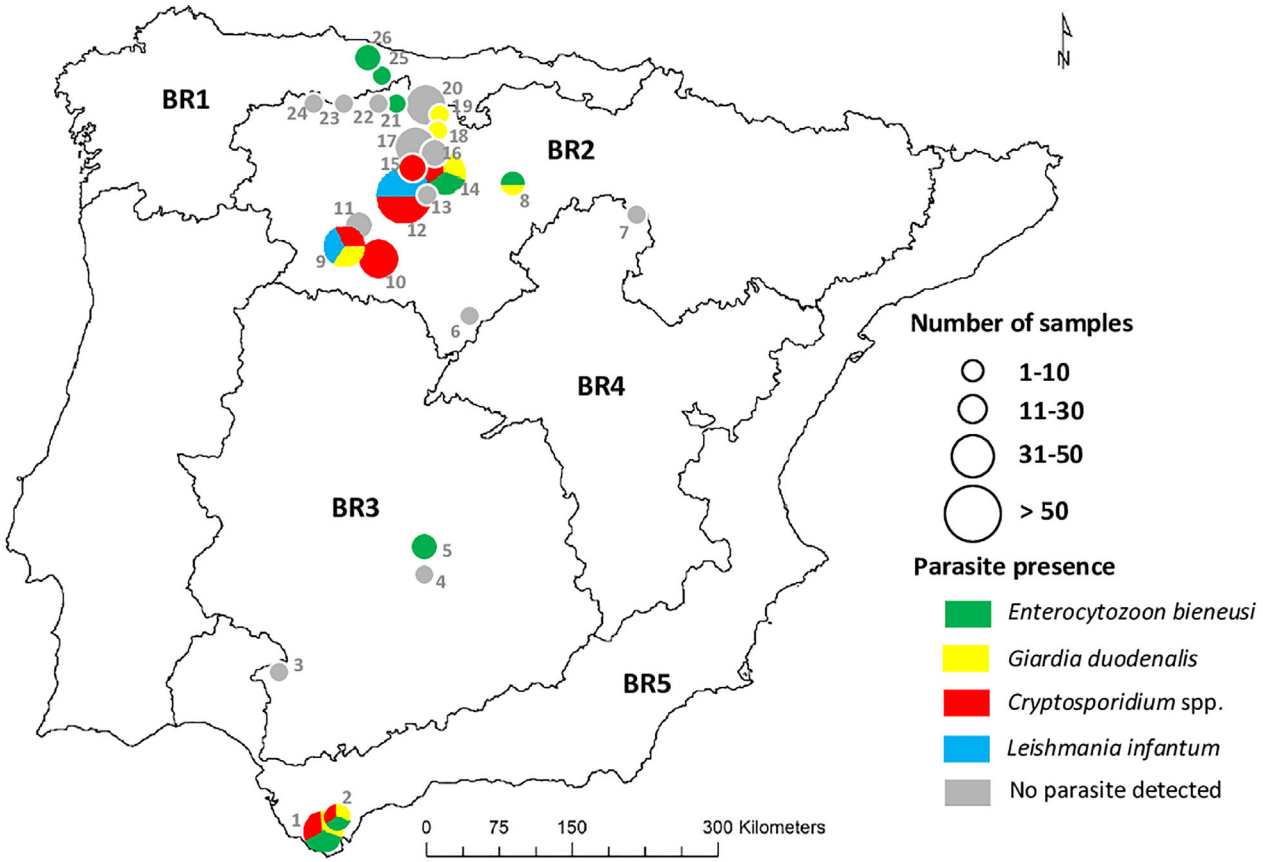

FIGURE 1 Map of Spain showing the sampling areas (numbered consecutively from 1 to 26, see also Table S3) and the geographical distribution of protist DNA detected in wild micromammals according to established bio-regions (BR1-5; Muñoz et al., 2010). Each coloured circle represents the presence of each protist species investigated in a given sampling area

2.4 | Molecular detection and characterisation of *G. duodenalis*, *Cryptosporidium* spp., *Blastocystis* sp., *E. bieneusi* and *Leishmania* spp.

Detailed information on the PCR cycling conditions and oligonucleotides used for the molecular identification and/or characterisation of the protist parasites investigated in the present study is presented in Tables S1 and S2, respectively. Detection of *G. duodenalis* DNA was achieved using a real-time PCR (qPCR) method targeting a 62-bp region of the gene codifying the small subunit ribosomal RNA (*ssu* rRNA) of the parasite (Verweij et al., 2003). *Giardia duodenalis* isolates that tested positive by qPCR and yielded cycle threshold (Ct) values ≤ 32 were subsequently assessed by sequence-based multilocus genotyping of the genes encoding the glutamate dehydrogenase (*gdh*), β-giardin (*bg*), and triose phosphate isomerase (*tpi*) proteins of the parasite. A semi-nested PCR was used to amplify a 432-bp fragment of the *gdh* gene (Read et al., 2004), and nested PCRs were used to amplify 511 and 530 bp fragments of the *bg* and *tpi* genes, respectively (Lalle et al., 2005 Sulaiman, Fayer et al., 2003). The presence of *Cryptosporidium* spp. was assessed using a nested PCR protocol to amplify a 587-bp fragment of the *ssu* rRNA gene (Tiangtip & Jongwutiwes, 2002). Identification of *Blastocystis* sp. was achieved by a direct PCR protocol targeting an ~600 bp fragment of the *ssu* rRNA gene (Scicluna et al., 2006). Detection of *E. bieneusi* was conducted by a nested PCR protocol to amplify the ~243 bp internal transcribed spacer (ITS) region as well as portions of the flanking large and small subunits of the ribosomal RNA gene as previously described (Buckholt et al., 2002).

Initial detection of *Leishmania* spp. was achieved using a real-time PCR (qPCR) method targeting a 358-bp region of the *ssu* rRNA gene of the parasite (Chicharro et al., n.d.). *Leishmania* isolates that tested positive by qPCR were subsequently assessed by *ssu*-PCR for species identification. A nested PCR protocol to amplify a partial fragment (358 bp) of the *ssu* rRNA gene of the parasite was used (Cruz et al., 2006).

All qPCR protocols described above were carried out on a Corbett Rotor Gene 6000 real-time PCR system (Qiagen). Reaction mixes included 2× TaqMan Gene Expression Master Mix (Applied Biosystems) or 2× Quantimix Hotsplit Probes kit (Biotools B&M Labs) for *G. duodenalis* and *Leishmania* spp. initial screening, respectively. All the direct, semi-nested and nested PCR protocols described above were conducted on a 2720 Thermal Cycler (Applied Biosystems). Reaction mixes always included 2.5 units of MyTAQ DNA polymerase (Bioline GmbH) and 5–10 μl MyTAQTM Reaction Buffer containing 5 mM deoxynucleotide triphosphates and 15 mM $MgCl_2$, except for the amplification of *Leishmania* spp., for which 0.7–1.4 units of Tth DNA polymerase (Biotools B&M Laboratories, S.A.) were used.

Laboratory-confirmed positive and negative DNA samples of human and animal origin for each parasitic species investigated were routinely used as controls and included in each round of PCR. PCR amplicons were visualized on 1.5%–2% D5 agarose gels (Conda) stained with Pronasafe (Conda) or Gel Red (Biotium) nucleic acid staining solutions. A 100 bp DNA ladder (Boehringer Mannheim GmbH) was used for the sizing of the obtained amplicons.

2.5 | Sanger sequencing and phylogenetic analyses

Positive PCR products of the expected size were directly sequenced in both directions using appropriate internal primer sets (Table S2). DNA sequencing was conducted by capillary electrophoresis using the BigDye Terminator chemistry (Applied Biosystems) on an ABI PRISM 3130 automated DNA sequencer. Generated DNA consensus sequences were visually inspected and aligned to appropriate reference sequences using MEGA X (Kumar et al., 2018) for species and genotype identification. The sequences obtained in this study have been deposited in GenBank under accession numbers ON306384–ON306394 (*Cryptosporidium* spp.), ON306311–ON306314 (*E. bieneusi*) and ON303966–ON303967 (*L. infantum*).

Enterocytozoon bieneusi nucleotide sequences of the ITS region obtained in this study as well as appropriate nucleotide reference sequences of *E. bieneusi* retrieved from GenBank that included all 11 groups were aligned with the Clustal W algorithm using MEGA X (Kumar et al., 2018). Phylogenetic interference was carried out by the neighbour-joining (NJ) method as previously described (Saitou & Nei, 1987).

2.6 | Statistical analyses

We estimated the prevalence with 95% confidence intervals (95% CI) of eukaryotic parasites in pooled faecal samples. Pooled tests are more accurate than individual tests when the prevalence value is ≤ 5% and the total sample size ≥ 500 (Abel et al., 1999). Calculations were conducted using the publicly available online resource Epitools Epidemiological Calculators (Sergeant, 2018). A 100% test sensitivity and specificity was assumed (Williams & Moffitt, 2001).

We used the Pearson's χ^2 test to assess differences in prevalence data according to seasonality (winter, spring, summer, and autumn) and bio-region (BR1-5) of origin. Analyses were carried out using the R Statistical Package version 2.15.3 (R Core Team, 2012).

3 | RESULTS

3.1 | Study population

Four hundred and ninety faecal samples/intestinal contents from 11 wild micromammal species belonging to the genera *Apodemus* (*Apodemus flavicollis*, *A. sylvaticus*), *Crocidura* (*Crocidura russula*), *Microtus* (*Microtus arvalis*, *M. duodecimcostatus*), *Mus* (*M. musculus*, *M. spretus*), *Myodes* (*Myodes glareolus*), *Rattus* (*Rattus rattus*), *Sciurus* (*S. vulgaris*) and *Sorex* (*Sorex coronatus*) were collected (Table 2). We were able to obtain a sufficient (200 mg) amount of faecal material from 43 micromammals to be individually tested. The faecal material of the remaining 447 micromammals was grouped into pools of 2–7 samples according to species, sampling location, and sampling season. A total of 155 samples, including individual ($n = 43$) and pooled ($n = 112$) faecal samples, were available for analysis.

The common vole (*M. arvalis*) was the most frequent micromammal captured in this study, accounting for 62.6% (307/490) of the trapped individuals, mainly due to targeted trapping of this species as the main objective of the research and taking advantage of its high densities during outbreak years. The next micromammal in numbers was the field mouse (*A. sylvaticus*) with 18.8% (92/490) of the trapped individuals. The black rat (*R. rattus*) was followed by 8.4% (41/490) of the trapped individuals (Table 2). The remaining eight micomammal species accounted for less than 5% of the individuals captured (Table 2).

Castile and Leon was the best represented region of origin (72.3%, 112/155), followed by Andalusia (17.4%, 27/155), Castile-La Mancha (5.2%, 8/155), Asturias (4.5%, 7/155), and Navarre (0.6%, 1/115; Figure 1). According to season, most samples were collected during summer (36.8%, 57/155) and spring (34.2%, 53/155), followed by autumn (15.5%, 24/155) and winter (8.4%, 13/155). This information was unavailable for 5.1% (8/155) of the samples. The full dataset generated in the present study is shown in Table S3.

3.2 | Prevalence of eukaryotic parasites

Among intestinal eukaryotes, the most prevalent species was *Cryptosporidium* spp. (3.7%, 95% CI: 2.2–5.7), followed by *G. duodenalis* (2.8%, 95% CI: 1.6–4.6), and *E. bieneusi* (2.6%, 95% CI: 1.4–4.3). *Blastocystis* sp. was not detected in the investigated rodent population. *Leishmania* spp. was identified in 0.41% (95% CI: 0.05–1.46) of the 490 individual spleen samples analysed by PCR.

According to host, *Cryptosporidium* spp. were identified in members of the genera *Apodemus*, *Microtus*, *Mus*, and *Rattus*; *G. duodenalis* in members of the genera *Microtus* and *Rattus*; and *E. bieneusi* in members of the genera *Apodemus*, *Mus*, and *Rattus*. At least one eukaryotic parasite was found in all micromammal species investigated except for *A. flavicollis*, *M. glareolus*, *S. coronatus*, and *S. vulgaris*, for which no parasitic DNA was detected (Table 2). It should be noted that the sample size for these host species was very low (≤5 specimens each).

Overall, 34 samples tested positive for at least one eukaryotic parasite. Co-infections involving two eukaryotic species were observed in 11.8% (4/34) of these samples, the combinations found being *Cryptosporidium* spp. + *E. bieneusi* (5.9%, 2/34), *G. duodenalis* + *Cryptosporidium* spp. and *G. duodenalis* + *E. bieneusi* (2.9%, 1/34 each). A triple infection *G. duodenalis* + *Cryptosporidium* spp. + *E. bieneusi* was found in two additional samples (5.9%, 2/34; Table 3).

TABLE 2 PCR-based occurrence rates of intestinal eukaryotic parasites in pooled faecal samples of wild micromammal species, Spain 2011–2014

Host species	Individuals (*n*)	Samples (*n*)	*G. duodenalis* *n* (%[a])	*Cryptosporidium* spp. *n* (%[a])	*Blastocystis* sp. *n* (%[a])	*E. bieneusi* *n* (%[a])
A. flavicollis	5	1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
A. sylvaticus	92	32	0 (0.0)	4 (12.5)	0 (0.0)	8 (25.0)
C. russula	14	6	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
M. arvalis	307	79	4 (5.1)	9 (11.4)	0 (0.0)	0 (0.0)
M. duodecimcostatus	3	1	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)
M. musculus	11	4	0 (0.0)	0 (0.0)	0 (0.0)	1 (25.0)
M. spretus	13	8	0 (0.0)	2 (25.0)	0 (0.0)	0 (0.0)
M. glareolus	1	1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
R. rattus	41	21	8 (38.1)	2 (9.5)	0 (0.0)	3 (14.3)
S. vulgaris	1	1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
S. coronatus	2	1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total	490[b]	155[c]	13 (8.4)	17 (11.0)	0 (0.0)	12 (7.7)

[a]Frequencies calculated against the number of (individual and pooled) samples.
[b]Individuals collected for the study
[c]Of them, there were 43 individual samples and 112 pooled samples.

TABLE 3 Single and multiple eukaryotic parasite infections detected in faecal samples of wild micromammal species, Spain 2011–2014

Protist species combination	Faecal samples (*n*)	Relative frequency (%)
Cryptosporidium spp. only	12	35.3
G. duodenalis only	9	26.5
E. bieneusi only	7	20.6
Cryptosporidium spp. + *E. bieneusi*	2	5.9
G. duodenalis + *Cryptosporidium* spp.	1	2.9
G. duodenalis + *E. bieneusi*	1	2.9
G. duodenalis + *Cryptosporidium* spp. + *E. bieneusi*	2	5.9
Total	34	100.0

The distribution of eukaryotic parasites according to the geographical origin of their micromammal hosts is shown in Figure 1. Pooled samples collected from bio-region 5 were more likely to harbour intestinal microeukaryotes (χ^2: 15.0371, *p*-value .0018).

3.3 | Molecular characterisation of *G. duodenalis* isolates

The 13 DNA isolates that yielded a positive result for *G. duodenalis* by qPCR generated Ct values ranging from 29.0 to 41.9 (median: 35.4). Genotyping analyses were only conducted in positive samples with Ct values ≤32 (30.1%, 4/13). None of them could be successfully amplified at the *gdh*, *bg* or *tpi* loci, so the assemblage/subassemblage of the parasite involved in these infections remained unknown. Black rats (*R. rattus*) were the most frequent host for *G. duodenalis*, accounting for eight out of the 13 positive samples, followed by *M. arvalis* (*n* = 4) and *M. duodecimcostatus* (*n* = 1).

3.4 | Molecular characterisation of *Cryptosporidium* spp. isolates

Table 4 shows the diversity, host's distribution and genetic features of the 17 *Cryptosporidium*-positive isolates identified by *ssu*-PCR. Sequence analyses revealed the presence of five *Cryptosporidium* spp. that included murine-adapted species, *C. ditrichi* (11.7%; 2/17) and *C. tyzzeri* (5.9%; 1/17); bovine-adapted species, *C. andersoni* (5.9%, 1/17) and two species with broader host ranges, *C. muris* (5.9%; 1/17) and *C. parvum* (5.9%; 1/17). Six isolates (35.3%; 6/17) were identified at the genus level only due to insufficient sequence quality. Additionally, four rodent-specific *Cryptosporidium* genotypes were identified,

TABLE 4 *Cryptosporidium* spp. sequences at the small subunit ribosomal RNA locus in pooled faecal samples of wild micromammal species, Spain 2011–2014

Species/genotype	No. of isolates	Host species	GenBank ID
C. andersoni	1	*A. sylvaticus*	ON306384
C. ditrichi	1	*M. spretus*	ON306385
	1	*A. sylvaticus*	ON306386
C. muris	1	*A. sylvaticus*	ON306387
C. parvum	1	*A. sylvaticus*	ON306388
C. tyzzeri	1	*M. spretus*	ON306389
Cryptosporidium sp. rat genotype (CR97)	1	*R. rattus*	ON306390
Cryptosporidium sp. rat genotype (W19)	1	*R. rattus*	ON306391
Cryptosporidium sp. vole genotype V	2	*M. arvalis*	ON3063920
Cryptosporidium sp. vole genotype VII	1	*M. arvalis*	ON306393 and ON306394
Cryptosporidium spp.	6	*M. arvalis*	–

Note: GenBank accession numbers are provided.

TABLE 5 Diversity and frequency of *E. bieneusi* genotypes in pooled faecal samples of wild micromammal species, Spain 2011–2014. GenBank accession numbers are provided

Genotype (internal transcribed spacer)	No. of positive samples	Hos species	Genotype frequency (%)	GenBank accession number
C	7	*A. sylvaticus*	58.3	ON306311
	1	*M. musculus*	8.3	ON306312
Peru11	3	*R. rattus*	25.0	ON306313
MouseSpEb1	1	*A. sylvaticus*	8.3	ON306314

Note: GenBank accession numbers are provided.

including *Cryptosporidium* rat genotype CR97 (5.9%; 1/17), rat genotype W19 (5.9%; 1/17), vole genotype V (11.7%; 2/17) and vole genotype VII (5.9%; 1/17). In isolates identified as vole genotype VII, intragenotypic variants were observed, and two different variants were identified. An NJ tree constructed using the *ssu* rRNA sequences of the *Cryptosporidium* isolates identified in the present study formed distinct clusters that were closely related to appropriate reference sequences (Figure S1).

3.5 | Molecular characterisation of *E. bieneusi* isolates

Table 5 shows the diversity and host's distribution of the 12 *E. bieneusi* isolates identified by ITS-PCR. Sequence analyses allowed the identification of three distinct genotypes, including two known genotypes from Groups 1, C (66.7%, 8/12) and Peru11 (25.0%, 3/12) and a novel genotype (named MouseSpEb1) (8.3%, 1/12). The novel genotype MouseSpEb1 was detected in a field mouse (*A. sylvaticus*) trapped in the Palencia province in Castile and Leon (Figure 1). The novel genotype nucleotide sequence is genotype C (AF101199), with the exception that the ITS region comprises 241 base pairs, as it has a two-nucleotide deletion (positions 46 and 47). An NJ tree constructed using the ITS sequences of the *E. bieneusi* isolates identified in the present study as well as nucleotide sequences for reference genotypes for all 11 *E. bieneusi* groups showed that the novel genotype MouseSpEb1 clustered together within the potentially zoonotic Group 1 (Figure 2).

3.6 | Molecular characterisation of *Leishmania* isolates

The two isolates that were found to be positive for *Leishmania* spp. by qPCR yielded Ct values of 25.2 and 33.5, respectively. Both isolates were successfully amplified at the *ssu*-rRNA locus and confirmed as *L. infantum* by Sanger sequencing with 99.7% similarity with the reference sequence (GenBank LR812960). One of the isolates was obtained from an *A. sylvaticus* specimen captured in November 2012 in Palencia Province, and the other isolate was isolated from an *M. arvalis* specimen captured in May 2014 in Zamora Province.

3.7 | Seasonal variation in eukaryotic intestinal parasites

Seasonality was not associated with a higher likelihood of obtaining a positive result for *G. duodenalis* (χ^2: 6.7011, *p*-value: .0821),

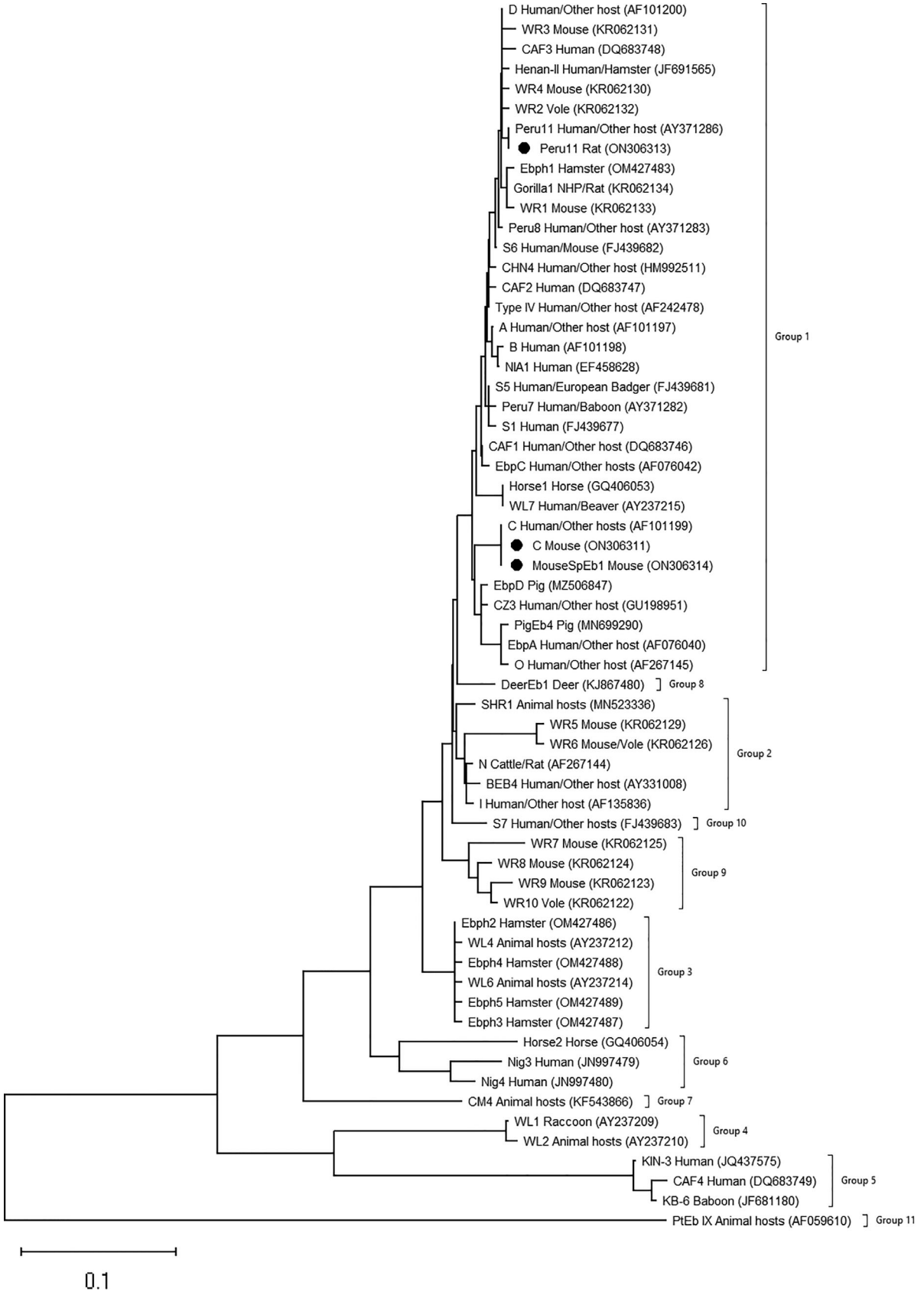

FIGURE 2 Neighbour-joining tree based on internal transcribed spacer ribosomal RNA gene sequences of *Enterocytozoon bieneusi*. Green-filled triangles represent sequences generated in the present study. Purple-filled dots represent sequences from human, animal or environmental origin previously reported in Spain. Genetic distances were calculated using the Kimura two-parameter model. Numbers at the nodes represent the bootstrap values with more than 50% bootstrap support from 1000 pseudoreplicates

Cryptosporidium spp. (χ^2: 2.2367, *p*-value: .5248) or *E. bieneusi* (χ^2: 1.8100, *p*-value: .4045).

4 | DISCUSSION

The epidemiology of intestinal eukaryotes in wild micromammals from non-humanized environments in Spain is poorly understood. To fill this gap in knowledge, this survey provides new molecular-based data on the occurrence, genetic diversity and zoonotic potential of the most relevant eukaryotic species from a public health perspective. Infected micromammals may contribute to the faecal contamination of soil and surface waters of the environment in which they live, normally frequented by other wildlife species and livestock and, occasionally, also by humans. In this regard, we evaluated the potential role of micromammals as a natural reservoir of zoonotic eukaryotic species, including *Leishmania* spp.

Cryptosporidium spp. are major contributors to the global burden of diarrhoeal disease in humans (Kotloff, 2017) and other animals (Ryan, Feng, et al., 2021). In previous European studies, *Cryptosporidium* spp. have been detected in 19 micromammal species from nine different genera from agricultural and forestry environments (see Table 1). According to host genus, *Cryptosporidium* prevalence ranged from 21% to 68% in *Apodemus* spp. (Čondlová et al., 2019; Perec-Matysiak, Buńkowska-Gawlik, Zaleśny, & Hildebrand, 2015) and from 2% to 73% in *Microtus* spp. and *Myodes* spp. (Bajer, 2008; Bajer et al., 2002; Horčičková et al., 2019; Kivistö et al., 2021; Myšková et al., 2019; Perec-Matysiak, Buńkowska-Gawlik, Zaleśny, & Hildebrand, 2015; Stenger et al., 2018). Lower prevalence rates have been documented in *Mus* spp. (10%–32%), *Rattus* spp. (14%–45%) and *Sorex* spp. (14%–44% ; Danišová et al., 2017; García-Livia et al., 2020; Ježková et al., 2021b; Kivistö et al., 2021; Köster et al., 2021; Torres et al., 2000). In the present study, an overall prevalence of 3.7% (17/155) was found in the surveyed micromammal population, well in the lower range described in previous studies (see Table 1). Most infections were identified in *M. arvalis*, in line with reports in other European surveys (Bajer, 2008; Bajer et al., 2002). In Spain, *Cryptosporidium* spp. have been found by conventional microscopy at infection rates of 32% in wild rodents and insectivores in Catalonia (Torres et al., 2000) and of 14%–45% by PCR in black and/or brown rats in Barcelona (Galán-Puchades et al., 2021), Canary Islands (García-Livia et al., 2021) and Córdoba (Köster et al., 2021). As expected, most (81.8%, 9/11) of the *Cryptosporidium* isolates genotyped in the present study corresponded to rodent-adapted species (*C. ditrichi*, *C. muris*, *C. tyzzeri*) or genotypes (rat genotypes CR97 and W19, vole genotypes V and VII). Of these, *C. muris* and *C. tyzzeri* have been previously described in black rats and house mice in the Canary Islands (García-Livia et al., 2020). In that study, they also identified *C. meleagridis* and rat genotype I/II that were not identified in the present survey. It should be noted that, although primarily considered non-zoonotic, *C. ditrichi*, *C. muris*, and *C. tyzzeri* have been sporadically reported in humans (Beser et al., 2020; Xiao & Feng, 2017). Additionally, *C. parvum* and bovine-adapted *C. andersoni* were identified in two pooled samples of *A. sylvaticus*. Natural *C. parvum* infections are relatively rare in rodents, but because of its loose host specificity, *C. parvum* has been previously identified in members of the genera *Apodemus*, *Crocidura*, *Mus*, and *Myodes* in studies conducted in European countries, including Poland (Perec-Matysiak, Buńkowska-Gawlik, Zaleśny, & Hildebrand, 2015), Slovakia (Danišová et al., 2017), and Spain (Torres et al., 2000), among others. These data suggest that free-living small mammals in agricultural or peri-urban habitats can act as a potential source of human cryptosporidiosis by *C. parvum*. On the other hand, *C. andersoni* has been detected in hamsters in China (J. Chen et al., 2021) and in other European surveys (Bajer, 2008), in wild brown rats in the Czech Republic (Ježková et al., 2021), in gray-sided voles in Finland (Kivistö et al., 2021) and in laboratory rats in Nigeria (Ayinmode et al., 2017).

In the present study, the overall pooled prevalence of *G. duodenalis* was estimated at 2.8%. Most (53.8%, 7/13) of the infections were identified in black rats. All *Giardia*-positive samples by qPCR yielded Ct values >29, indicative of moderate-to-low parasite burdens. This fact explains the failure to amplify these isolates at the *gdh*, *bg* and *tpi* markers, all of them single-copy genes with limited sensitivity, compared with the multiple-copy *ssu* rRNA gene used in qPCR for detection purposes. Three previous Spanish studies detected *G. duodenalis* in rodents, 14%–38% of black and/or brown rats in the Canary Islands (Fernández-Álvarez et al., 2014), Catalonia (Galán-Puchades et al., 2021) and Córdoba (Köster et al., 2021) and 18% of house mice in the Canary Islands (Fernández-Álvarez et al., 2014). Although most *G. duodenalis* isolates identified in the latter survey were assigned to rodent-specific assemblage G, a rat isolate was identified as zoonotic sub-assemblage BIV (Fernández-Álvarez et al., 2014). In the European scenario, a *G. duodenalis* occurrence rate of 83% has been described in wild rodents belonging to the genera *Apodemus*, *Microtus* and *Myodes* in Germany (Helmy et al., 2018). Sequence analyses revealed that *Apodemus* spp. were mainly infected with *G. muris*, whereas *G. microti* was predominantly found in *Microtus* spp. and *M. glareolus*. Only a low proportion of samples (1.4%) contained zoonotic *G. duodenalis* assemblages A or B. Taken together, available molecular data from Germany and Spain pointed out a very low potential risk for *G. duodenalis* transmission from wild rodents to humans (Fernández-Álvarez et al., 2014; Helmy et al., 2018).

Blastocystis sp. has been reported in previous studies in captive and wild rodents worldwide (Hublin et al., 2021). Ten different *Blastocystis* STs (ST1-ST5, ST7, ST8, ST10, ST13 and ST17) have been identified in rodents worldwide, with ST4 being the most prevalently found (Hublin et al., 2021). However, *Blastocystis* sp. was not detected in the present study, suggesting that wild micromammals do not seem to play a significant role in the transmission of this stramenopile species.

This study represents the first molecular-based description of *E. bieneusi* in wild micromammals in Spain. Previous surveys in the country have identified these microsporidia at variable occurrence rates in wildlife, including mesocarnivores, pigeons, rabbits and wild boars (Dashti et al., 2020; Galván-Díaz et al., 2014; Haro et al., 2005, 2006; Martínez-Padilla et al., 2020; Santín et al., 2018); farmed animals, including cattle, goats, ostriches, pigs and rabbits (Abarca et al., 2021;

Dashti et al., 2022; Galván-Díaz et al., 2014; Lores et al., 2002) and companion animals, such as cats and dogs (Dashti et al., 2019; Galván-Díaz et al., 2014; Lores et al., 2002). Genotype Peru11 was detected in three black rats from southern Spain. This genotype has been described previously in domestic cats from northern Spain and Korea (Dashti et al., 2019; Kwak and Seo, 2020), which could suggest a predator–prey relationship. Peru11 has also been described in farmed bamboo and pet rats, captive macaques and farmed minks in China (L. Chen et al., 2019; Cong et al., 2018; Karim et al., 2014; Wang et al., 2020; Yang et al., 2017; X. X. Zhang et al., 2018; Zhao, Zhou, Jin, et al., 2020; Zhao, Zhou,Yang, et al., 2020). Additionally, Peru11 has been reported in meadow voles, eastern cottontails and racoons in New York City (Guo et al., 2014), in human immunodeficiency virus (HIV) patients in Lima, Peru (Sulaiman et al., 2003) and in an asymptomatic child in Zambézia, Mozambique (Muadica et al., 2020). Genotype C was detected in seven field mice and a house mouse in our study. This *E. bieneusi* genetic variant has been described in house mice in the border region of Germany and the Czech Republic (Sak et al., 2011), in mountain gorillas in Rwanda (Sak et al., 2014), and in raw wastewaters in China and Spain (Galván-Díaz et al., 2014; N. Li et al., 2012). The zoonotic potential of genotype C is confirmed by its presence in human transplant recipients in France and the Netherlands (Greigert et al., 2018; ten Hove et al., 2009), in HIV patients in Portugal (Lobo et al., 2012), and in foodborne outbreaks of microsporidiosis in Denmark (Michlmayr et al., 2020) and Sweden (Decraene et al., 2012). A novel *E. bieneusi* genotype MouseSpEb1 was found in a field mouse that differed from Castile and Leon. The mouseSpEb1 ITS sequence has a two nucleotide deletion when compared to the genotype C sequence.

Vector-borne *L. infantum*, the causative agent of visceral and cutaneous leishmaniasis, is one of the most important neglected zoonoses in the Mediterranean region. In Spain, and in addition to domestic dogs, leporids such as rabbits and hares have been demonstrated to be competent reservoirs of the infection (Ortega-García et al., 2019). Micromammals have been largely believed to play a limited role in the epidemiology of the parasite (Millán, 2018). This concept has been recently challenged by the finding of *L. infantum* at a high prevalence rate (33%, 28/84) in sewer rats captured in the city of Barcelona (Galán-Puchades et al., 2019). In contrast, no *Leishmania* DNA was found in free-living rats ($n = 64$) trapped in a zoological garden in the city of Córdoba (Köster et al., 2021). To further evaluate the extent of these previous findings in rodent hosts, we investigated the occurrence of *L. infantum* (detected by qPCR and confirmed by ITS-PCR and Sanger sequencing) in individual spleens ($n = 490$) of micromammals from agricultural and forestry environments by PCR. We detected the presence of the parasite at a very low (<0.5%) prevalence rate in two micromammal species including *A. sylvaticus* and *M. arvalis*. To the best of our knowledge, this is the first description of *Leishmania* infection in *M. arvalis*, as *A. sylvaticus* has been reported as a suitable host for *L. infantum* in a previous Spanish study (Risueño et al., 2018). Remarkably, both *Leishmania*-positive micromammals were captured in northwest Spain, a geographical are previously thought not to be the preferred natural habitats of sandflies of the genus *Phlebotomus*, the main vector of leishmaniasis (Ballart et al., 2014; Durán-Martínez et al., 2013). Climate change has already impacted the transmission of a wide range in Europe, and it will continue to do so in the coming decades. Climate change has been implicated in the observed shift of disease vectors to elevated altitudes and latitudes in Europe, including *Aedes albopictus* (which transmits diseases such as Zika, dengue and chikungunya) and *Phlebotomus* sandfly species (Semenza & Suk, 2018). Further studies are warranted to assess the true role of wild rodents on *Leishmania* spp. transmission in these epidemiological scenarios.

5 | CONCLUSION

This is the largest nationwide epidemiological study aiming at investigating the occurrence and genetic diversity of eukaryotic parasites in wild micromammals conducted in Spain to date. We provided molecular evidence indicating that wild micromammals are largely infected by rodent-adapted species/genotypes of the eukaryotic species under investigation and therefore play a limited role as natural sources of human infections. The identification of *C. andersoni* and *C. parvum* is indicative of an overlap between domestic/peri-domestic and sylvatic transmission cycles of these agents. Abundant and widespread species in the Iberian Peninsula (e.g., rats, wood mouse, and white-toothed shrew) as well as species experiencing drastic cyclic demographic outbreaks (e.g., the common vole) might be relevant in the maintenance and spread of these eukaryotic pathogens that can be a matter of concern from a public veterinary health perspective. Micromammal species seem to be sporadically infected by *L. infantum*, suggesting that these hosts play a limited role in the epidemiology of leishmaniasis; the extent of this finding should be confirmed in future field epidemiological surveys.

ACKNOWLEDGEMENTS

This work was supported by the Spanish Ministry for Science and Innovation under projects CGL2011-30274 and CGL2015-71255-P and by the BBVA Foundation under project TOPIGEPLA (2014 call). Additional funding was obtained from the Spanish Ministry for Science and Innovation under projects CGL2017-89866-R and E-RTA-2015-0002-C02-02 and by the Health Institute Carlos III (ISCIII), Spanish Ministry of Economy and Competitiveness under project PI19CIII/00029. David González-Barrio is the recipient of a Sara Borrell Research Contract (CD19CIII/00011) funded by the Spanish Ministry of Science, Innovation and Universities. Alejandro Dashti is the recipient of a PFIS contract (FI20CIII/00002) funded by the Spanish Ministry of Science and Innovation and Universities. The 'Grupo de Rehabilitación de la Fauna Autóctona y su Hábitat' (GREFA) provided partial funding and invaluable logistic and workforce support for samplings in NW Spain, along with many students and staff from the Autonomous University of Madrid (UAM). The authors extend particular thanks to Ana Benítez López, María Calero Riestra, Paqui Talavera, Iván García Egea, Jesús Herranz, Daniel Jareño Gómez, Salvador Luna, Julian Núñez Conde, Juan Oñate, Alfonso Paz Luna, Francisco Díaz Ruiz, Fernando Garcés, Carlos Cuellar, Fernando Blanca Chana, Lorena Hernández and Xurxo Piñeiro Álvarez.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available within the the main body of the manuscript.

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How to cite this article: Vioque, F., Dashti, A., Santín, M., Ruiz-Fons, F., Köster, P. C., Hernández-Castro, C., García, J. T., Bailo, B., Ortega, S., Olea, P. P., Arce, F., Chicharro, C., Nieto, J., González, F., Viñuela, J., Carmena, D., & González-Barrio, D. (2022). Wild micromammal host spectrum of zoonotic eukaryotic parasites in Spain. Occurrence and genetic characterisation. *Transboundary and Emerging Diseases*, *69*, e2926–e2942. https://doi.org/10.1111/tbed.14643