# Molecular survey of Besnoitia spp. (Apicomplexa) in faeces from European wild mesocarnivores in Spain. Short running title: Molecular survey of Besnoitia spp in faeces from wild carnivores.

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**Abstract**

Numerous studies have unsuccessfully tried to unravel the definitive host of the coccidian parasite *Besnoitia besnoiti*. Cattle infections by *B. besnoiti* cause a chronic and debilitating condition called bovine besnoitiosis that has emerged in Europe during the last two decades, mainly due to limitations in its control associated to the absence of vaccines and therapeutical tools. Although the exact transmission pathway of *B. besnoiti* is currently unknown, it is assumed that the parasite might have an indirect life cycle with a carnivore as definitive host. Current lack of studies in wildlife might underestimate the importance of free-living species in the epidemiology of *B. besnoiti*. Thus, the aim of the present study is to assess the presence of *Besnoitia* spp. in free-ranging mesocarnivores in Spain. DNA was searched by PCR on faeces collected from wild carnivores as a first approach to determine which species could be considered as potential definitive host candidates in further research. For this purpose, a total of 352 faecal samples from 12 free-living wild carnivore species belonging to the Canidae, Felidae, Herpestidae, Mustelidae, Procyonidae, and Viverridae families were collected in seven Spanish regions. PCR testing showed that *Besnoitia* spp. DNA was present in four faecal samples from red foxes collected in western Spain, an area with the greatest density of extensively reared cattle and associated to high incidence of bovine besnoitiosis in the country. To date, this is the first report of a *Besnoitia besnoiti*-like sequence (99.57% homology) from carnivore faeces in a worldwide context. Red foxes might contribute to the epidemiology of *B. besnoiti*, although further studies, mostly based on bioassay, would be needed to elucidate the accuracy and extent of these interesting findings.

## Introduction

*Besnoitia besnoiti* (Apicomplexa) is the aetiological agent of a chronic and debilitating disease of cattle called bovine besnoitiosis characterized by a low body score, non-specific systemic clinical signs and skin lesions. Reproductive failure is a major concern since males may develop infertility, sterility or even may die

during the acute phase of the infection (González-Barrio et al., 2020). Bovine besnoitiosis causes considerable economic losses in many countries in Africa, the Middle East and Europe. In these regions, the disease has been increasingly spreading over the last two decades (EFSA, 2010; Álvarez-García et al., 2013), mainly due to control drawbacks including the absence of vaccines and therapeutical tools, also pre-movement of cattle testing may be insufficient and contribute to the spread of the disease. Several surveys confirmed the increased prevalence and geographical expansion of this disease in cattle, in areas of Western and Northern Europe (Álvarez-García et al., 2016). A few studies carried out in Spain reported high seroprevalence rates in beef cattle herds, ranging from 36 to 87% in Urbasa Andía mountains and the Pyrenees in North Spain (Cortes et al., 2014; Álvarez-García et al., 2014; Gutiérrez-Expósito et al., 2014).

Despite the fact that its biological cycle is not fully known, it is assumed that *B. besnoiti* might have an indirect life cycle with a carnivore as definitive host able to shed oocysts after ingestion of tissues harbouring mature cysts (Jaquiet et al., 2010; Cortes et al., 2014). Although domestic cattle act as intermediate hosts of the parasite, specific antibodies against *B. besnoiti* were also described in roe deer (*Capreolus capreolus* ) (Arnal et al., 2017) and red deer (*Cervus elaphus* ) in North Easter Spain, regions where bovine besnoitiosis is endemic, showing that these wild ruminant species are also intermediate host (Gutiérrez-Expósito et al., 2013, 2016). Despite this fact, their role in the epidemiology of bovine besnoitiosis seems to be of scarce importance (Gutiérrez-Expósito et al., 2016). In contrast, specific antibodies were not detected in small ruminants (Gutiérrez-Expósito et al., 2017). Similarly, a serosurvey on*Besnoitia* spp. in wild carnivore species from Spain provided no evidence to support that wild carnivores are implicated in the epidemiology of *B. besnoiti* within the geographical regions covered by the analysis (Millán et al., 2012).

Domestic and wild felines have been suggested as definitive hosts for *B. besnoiti* , however, experimental infections in several felid species failed to confirm their suitability as potential definitive hosts (Diesing et al., 1988; Basso et al., 2011). The putative role of a sylvatic life cycle, involving other Carnivora species, in the epidemiology of the disease has not been fully elucidated (see Table 1).

The aim of the present survey is to assess the presence of *Besnoitia* spp. DNA in faeces from wild mesocarnivores in Spain as a first step to determine which species might be considered as potential definitive host candidates in further investigations.

## Material and Methods

### Sample collection

A total of 352 faecal samples from 12 free-living carnivore species belonging to the Canidae ($n$= 193), Felidae ($n = 15$), Herpestidae ($n = 1$), Mustelidae ($n = 131$), Procyonidae ($n = 1$), and Viverridae ($n = 11$) families were collected in seven Spanish regions between December 2013 to October 2017 (Table 2, Figure 1). The sampling was mainly focused on areas with higher densities of extensively reared cattle and where positive cases of bovine besnoitiosis had been previously reported (Nieto-Rodríguez et al., 2016), as is the case of Central and Western Spain (Figure 1). Samples were obtained from road- and hunter-killed animals, from accidentally found carcasses, camera-trap surveys, or animals entering rescue shelters (see Calero-Bernal et al., 2020). Faeces were collected directly from the rectum of each animal and placed in individual plastic cups with records of the date, location and species, and frozen within 12 h after collection.

### *DNA extraction and molecular detection of* **Besnoitia*spp.***

Genomic DNA was isolated from about 200 mg of faecal material by using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, except that samples mixed with ASL lysis buffer were incubated for 10 min at 95 °C for improving oocyst wall rupture. Purified DNA samples (200 µl) were stored at –20 °C until downstream PCR-based diagnostic and subtyping analyses were conducted. A water extraction control was routinely included in each sample batch processed for DNA extraction. The products of the DNA extraction process were tested for the specific detection of *Besnoitia* spp. by ITS-1 rDNA PCR (Cortes et al., 2007). The forward primer ITS1F (50-TGACATTTAATAACAATCAACCCTT-30) and the reverse primer ITS1R (50-GGTTTGTATTAACCAATCCGTGA-30) were added at a concentra-

tion of 10 µM, and the rest of reagents were incorporated in the mixture (final volume: 25 µl), as indicated by Frey et al. (2013). The amplified products were visualized after electrophoresis on a 1.5% agarose gel containing 0.1 µl/ml GelRed Nucleic Acid Gel Stain (Biotium, Fremont, USA). DNA extraction and PCR were performed in separate laboratories under biosafety level II conditions (BIO II A Cabinet, TELSTAR, Madrid, Spain) to avoid cross contamination. The positive control was DNA extracted from *in vitro* cultured tachyzoites of *B. besnoiti* , and PCR grade water was used as the negative control.

Sequence and phylogenetic analyses

In order to confirm the specificity of the amplicons yielded, positive PCR products were subjected to direct sequencing at the Center for Genomic Technologies of the Complutense University of Madrid (Spain). Briefly, amplicons were sequenced in both directions with the same internal primer pair used for PCR amplification, Big Dye chemistries and an ABI 3730xl sequencer analyzer (Applied Biosystems, Foster City, CA). Raw sequencing data in both forward and reverse directions were manually edited by Bioedit Software, and BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare resulting nucleotide sequences with sequences retrieved from the National Center for Biotechnology Information (NCBI) database.

The evolutionary relationships among the identified *Besnoitia*sp.-positive samples were inferred by a phylogenetic analysis using the Neighbor-Joining method (Saitou & Nei, 1987) in MEGA 6. The evolutionary distances were computed using the Kimura 2-parameter method and modelled with a gamma distribution. The reliability of the phylogenetic analyses at each branch node was estimated by the bootstrap method using 1000 replications. Representative sequences of different*Besnoitia* spp. isolates were retrieved from the NCBI database and included in the phylogenetic analysis for reference and comparative purposes. Representative sequences obtained in the present study were deposited in GenBank under the accession numbers MW035607 to MW035610.

**Results and discussion**

PCR and sequencing results showed that *Besnoitiabesnoiti* -like DNA was present in four faecal samples (1.13%) analysed from 352 wild carnivores. Those positive samples corresponded to four red foxes from Castilla y León and Extremadura in western Spain. To date, this is the first finding of a *B. besnoiti* -like sequence from a carnivore in Europe, and from any carnivore species in a worldwide context.

To the best of our knowledge, this is the first large-scale molecular survey for *Besnoitia* spp. DNA in free-living carnivores in Europe. The survey benefits from the inclusion of 12 different species of free-living carnivores and a national coverage, paying special attention to regions where bovine besnoitiosis is present (except in North West Spain) in conjunction with greater densities of extensive cattle production.

Serological, molecular and parasitological techniques have been used in an attempt to elucidate the role of several animal species as potential definitive host of *B. besnoiti* , but they failed to find it in wild and domestic carnivores, in addition to mammals, reptiles and birds (see Table 1). Several studies on felines, including the domestic cat (*Felis catus* ), have attempted to clarify its role as a definitive host (see Table 1). Rommel (1975) and Peteshev et al. (1974) reported inconclusive results to confirm domestic cats as definitive hosts of*B. besnoiti* in experimental studies. Despite detecting oocysts in the faeces, authors could not achieve further characterization for fully confirmation of their identity as *B. besnoiti.* Other authors did not find *B. besnoiti* oocysts in the faeces of *per os*challenged cats over a 3 to 20 weeks observation period (Diesing et al., 1988; Basso et al., 2011). Several serological studies have been also carried out to detect antibodies against *B. besnoiti* in felines (Table 1). Millán et al. (2012) found antibodies by IFAT (indirect fluorescent antibody test) in eight feral cats (*Felis silvestris catus* ). However, no individual tested positive by WBs. These animals originated from areas where no cases of bovine besnoitiosis had been detected until year 2010. The results suggested their unlikely implication in the parasite transmission. In a recent study in Namibian wildlife, antibodies have been detected in two lions, *Panthera leo* (Seltmann et al., 2020). On the other hand, two studies have managed to detect by molecular techniques *Besnoitia* spp. DNA in faeces from pond bat (*Myotis dasycneme* ) in the Netherlands (Hornok et al., 2015) and in faecal matter from cheetahs (*Acinonyx jubatus* ) in Namibia (Schares et al., 2021); in the first report, authors hypothesized that

*B. besnoiti* -like sequences might have originated from French cattle via bloodsucking dipterans (*Stomoxys calcitrans, Tabanus* spp.). In this regard, bats frequently use cattle stables for roosting, where they can prey on the mechanical vectors of*B. besnoiti* . In addition, the later study (Schares et al., 2021) suggests that a so far unknown *Besnoitia* species closely related to *B. darlingi* , *B. neotomofelis* , *B. oryctofelisi* ,*B. akodoni* or *B. jellisoni* is cycling in Namibian wildlife.

In the present survey, *B. besnoiti*- like DNA has been demonstrated in four individual faecal samples from red foxes from Ávila, Badajoz and Salamanca provinces (Table 2) in western Spain. All four fox-derived *Besnoitia* spp. sequences were equivalent to positions 527–737 of reference sequence KX013107 (a bovine isolate of the parasite previously reported in Spain), differing from it by a single di-nucleotide site (a G/C double peak) at position 706. An additional ambiguous position (an A/G double peak) was also detected at position 711 of reference sequence KX013107 in one (GenBank accession number MW035609) of the four generated sequences. The topology of the produced phylogenetic tree clearly clustered all these sequences with other *Besnoitia* species in large mammals (*B. bennetti* ,*B. caprae* and *B. tarandi* ) but particularly with *B. besnoiti* , from European countries (Belgium, Finland, Italy, Germany, Portugal and Spain), Israel and Iran. The *ITS1* rDNA sequence of*B. besnoiti* -like from red foxes suggest a closer relationship to*B. besnoiti* , which infects cattle in the Old World. In a separate phylogenetic cluster and with large evolutionary divergence, other species of *Besnotia* genus (*B. neotomofelis* , *B. oryctofelisi* , *B. akodoni* and *B. darlingi* ) infecting small mammals from Argentina, Brazil and USA (Figure 2) are placed. These results are in agreement with those described by Olias et al. (2011), in which *ITS-1* region shows the most informative nucleotide variances and phylogenetically clearly splits small mammalian from large mammalian *Besnoitia* species. Of note, all foxes with*Besnotia* sp. PCR-positive faecal samples were caught within Western Spain (Figure 1), where the highest number of bovine besnoitiosis clinical cases were found in a previous survey (Nieto-Rodríguez et al., 2016).

This is the first molecular evidence of the occurrence of *B. besnoiti* -like DNA in a European mesocarnivore gut. The red fox is present in a wide range of habitats in the Iberian Peninsula (Macdonald & Reynolds 2004) with densities of 0.7–2.5 foxes/Km$^2$, depending on environmental conditions (Sarmento et al., 2009). In addition, this wild canid is a highly adaptable omnivorous mammal distributed across all continents on the northern hemisphere. Numerous studies on the red fox diet show it as a generalist predator, feeding mainly on prey which are abundant and easily accessible. Red foxes feed most frequently on small mammals as rodents and wild rabbits, but utilize also other food items such as carrion, birds, reptiles, amphibians, invertebrates, fruit and vegetables (Díaz-Ruiz et al., 2013).

The prevalence rate found in red fox (2.1%; 4/187) is in agreement with worldwide reported data for the excretion of oocysts of the closely related Toxoplasmatinae parasite Toxoplasma *gondii* by Felidae (Hatam-Nahavandi et al., 2021). With this in mind the presence of bovine besnoitiosis could be explained in regions where the disease is endemic not only by animal trade and mechanical transmission but also by the possible high prevalence of oocysts shedding and wide abundance of foxes.

Taking into account that in our study the faecal samples were collected from regions where beef cattle are usually raised in extensive production systems (Figure 1) and bovine besnoitiosis is widespread (Nieto-Rodríguez et al., 2016), there are three possible explanations for such interesting findings; i) our first hypothesis is that red foxes may have a role in the transmission of the parasite as definitive host: the red fox is considered to be one of the most widespread generalist vertebrate predators in the world (Macdonald & Reynolds 2004). Therefore, predation on small mammals as rodents and wild rabbit, intermediate hosts of *B. darlingi* , *B. neotomofelis* and*B. oryctofelisi* respectively, suggests that it could make us think that red fox might have a role as a definitive host in other species of *Besnoitia.* However, the sequences found in fox faeces are genetically not so closely related to small mammalian*Besnoitia* spp. as to *B. besnoiti* . In addition, other small mammalian prey of the fox may be unknown intermediate hosts of *B. besnoiti* , and the fox may act as a definitive host for *B. besnoiti* ; ii) our second hypothesis is that there has been consumption of carrion infected with *B. besnoiti,* and foxes are acting as passive carriers without developing the infection; iii) and the third and last hypothesis is that the red fox could act as carrier, after the accidental ingestion of

the hypothetical *B. besnoitia* oocysts from the contaminated soil (and foodstuff) and we would find parasite DNA in the fox faeces.

Although we have found *Besnoitia* spp. DNA in red fox faeces and subsequently confirmed it by Sanger sequencing, present survey has several limitations. First, no serological analysis has been performed on these species, sampling was carried out in most cases on road- and hunter-killed animals, from accidentally found carcasses, and camera-trap surveys. Thus, fresh, good quality blood samples were unavailable for serological testing, in addition to the difficulty of finding validated techniques in wildlife for detecting this parasite (González-Barrio & Ruiz-Fons, 2019). Second, no additional parasitological techniques (e.g., floatation) were used due to the retrospective nature of this study and the insufficient amount of remaining faecal material for performing complementary techniques. Finally, identification of *Besnoitia*spp. was accomplished on a single locus. Low quantity and quality of genomic DNA from faeces prevented us of conducting multilocus microsatellite analyses. However, on the other hand, foxes were the only species among the twelve studies, where *B. besnoiti* -like DNA was identified, suggesting that this/such species might play a role in the epidemiology of the disease.

To conclude, Sanger sequencing analysis of four of the 129 faecal samples revealed that the presence of *B. Besnoiti*- like species in red fox ( *Vulpes vulpes* ) faeces has been confirmed in Spain. Further epidemiological and experimental studies with a similar approach may help in the search for the definitive host of this parasite. In addition, future studies are needed to identify its natural intermediate host in small mammalian prey of foxes.

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**Conflict of interest**

The authors have no conflict of interest to declare.

**Data availability statement**

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

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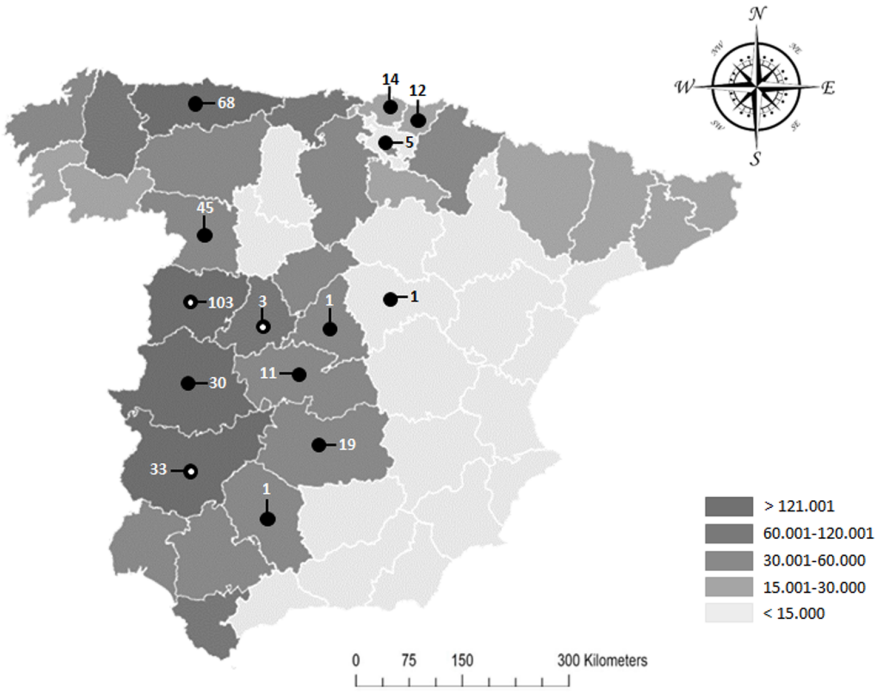
**Figure caption.**

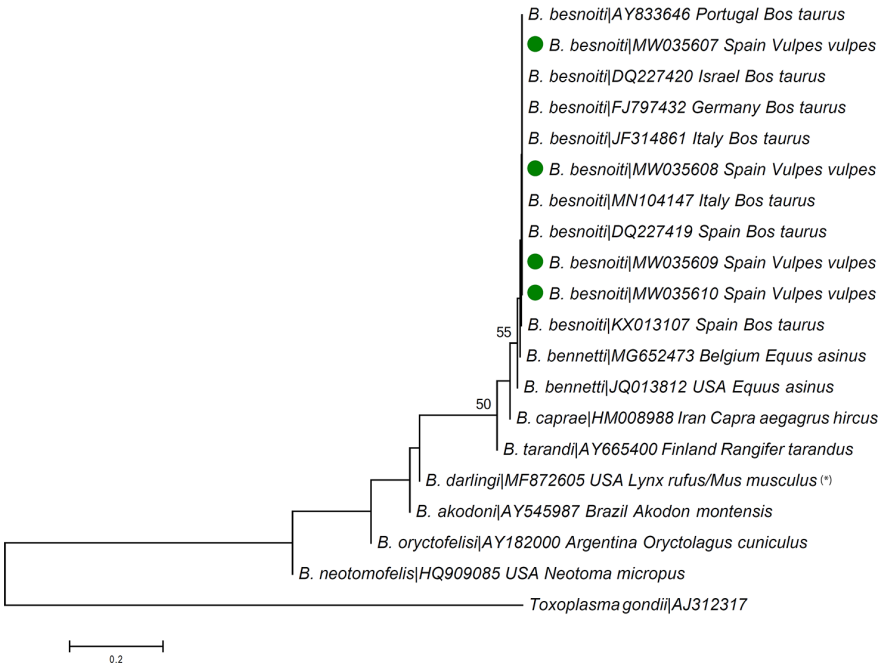
**Figure 1** . Spatial distribution of sample collection. Each black dot represents the province of sampling. The current geographic distribution and census of beef cattle raised under extensive husbandry conditions for each province is represented by grayscale. The number of fecal samples per province is shown. The white dot indicates that*Besnoitia* spp. DNA has been found.

**Figure 2** . Phylogenetic relationships among *Besnoitia*spp. sequences identified in this study and known *Besnoitia* spp. isolates, as inferred by a neighbour-joining analysis of ITS ribosomal RNA gene partial sequences, based on genetic distances calculated by the Kimura two-parameter model. Nucleotide sequences determined in this study are identified with dark green filled circles. Bootstrap values lower than 50% are not displayed. *Toxoplasma gondii* was used as outgroup taxa.

**Table 1** . Summary of the available studies reporting investigations to elucidate possible definitive host of *Besnoitia besnoiti* .

**Table 2** . Wild carnivore species examined, region, number of samples tested and number of samples in which DNA from *Besnoitia*spp. has been detected in Spain. In bold the species and number of faeces samples in which DNA from *Besnoitia* spp. has been found and confirmed by sequencing.



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