Prevalence of infection and 18S rRNA gene sequences of *Cytauxzoon* species in Iberian lynx (*Lynx pardinus*) in Spain

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SUMMARY

The Iberian lynx (*Lynx pardinus*) is the most endangered felid in the world. Only about 160 individuals remain in 2 separate metapopulations in Southern Spain (Sierra Morena and Doñana). We obtained blood samples of 20 lynxes captured from 2004 to 2006, and determined the prevalence of infection and genetic diversity of *Cytauxzoon* spp. using 18S rRNA PCR and sequence analysis. Prevalence of infection was 15% (3 of 20). *Cytauxzoon* sp. was only detected in Sierra Morena. For phylogenetic analysis, we used the sequences reported in the present study and those characterized in different domestic and wild felids and ticks from North and South America, Asia and Europe. Three different *Cytauxzoon* sp. sequences were obtained. They were closely related to that obtained from a Spanish cat, but diverged in up to 1·0% with respect to the only previously reported sequence from an Iberian lynx. Conversely, the latter sequence clustered together with *C. manul* sequences obtained from Pallas cats (*Otocolobus manul*) in Mongolia. Our analysis yields a separate cluster of *C. felis* sequences from cats, wild felids and ticks in the United States and Brazil. These results suggest that at least 2 different *Cytauxzoon* spp. may be present in Iberian lynx. The apparent absence in one of the areas, together with the possibility of fatal cytauxzoonosis in lynxes makes necessary disease risks to be taken into account in management conservation strategies, such as translocations and re-introductions.

Key words: conservation, *Cytauxzoon felis*, cytauxzoonosis, endangered species, *Lynx pardinus*, molecular characterization, piroplasm.

INTRODUCTION

According to the World Conservation Union, the Iberian lynx (*Lynx pardinus*) is the most endangered felid in the world (Nowell and Jackson, 1996) with approximately 160 individuals inhabiting 2 separate areas of Southern Spain, namely Sierra Morena and Doñana (Guzmán *et al*. 2004). Among mammalian predators, felids are especially vulnerable to human action (habitat transformation, road-killing, illegal hunting) and 44% of the species in this taxon experience serious threats (Nowell and Jackson, 1996). An increasing concern exists about the role of diseases as a threat for conservation of endangered species (Deem *et al*. 2001; Haydon *et al*. 2002; Smith *et al*. 2006). It has been shown that diseases could induce extinction of wildlife species (e.g. Thorne and Williams, 1988), especially when their population size is small and reservoir hosts are present in the area (de Castro and Bolker, 2005).

Cytauxzoonosis is a tick-borne disease caused by *Cytauxzoon felis*, a piroplasm belonging to the family Theileriidae. Its life-cycle in the vertebrate host includes an intra-erythrocytic phase and a tissue phase consisting of large schizonts that develop in macrophages or monocytes. The tissue phase is necessary for the disease to be fatal (Kier and Greene, 1998). The bobcat (*Lynx rufus*) is considered the natural host of *C. felis* in North America. In this species, *C. felis* causes a persistent, subclinical infection. In contrast, *C. felis* infections in domestic cats are characterized by an acute, highly fatal febrile disease (Kier and Greene, 1998). *Cytauxzoon felis* was also reported in free-living cougars and Florida panthers (*Puma concolor*; Rotstein *et al*. 1999) in

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Table 1. Age and sex-classes of the Iberian lynxes sampled for the detection of *Cytauxzoon* sp.

(Parasitized animals are given in parentheses.)

	Sierra Morena			Doñana		
	Adult	Subadult	Juvenile	Adult	Subadult	Juvenile
Male	0	3 (1)	3 (2)	3 (0)	1 (0)	1 (0)
Female	0	1 (0)	2 (0)	5 (0)	1 (0)	0

North America. Florida panthers may react to *C. felis* in a way similar to bobcats (Forrester, 1992). A fatal case of cytauxzoonosis was reported in a captive white tiger (*Panthera tigris*) in the United States (Garner *et al*. 1996). A closely related piroplasm, *C. manul*, was reported parasitizing Pallas cats (*Otocolobus manul*) in Mongolia (Ketz-Riely *et al*. 2003; Reichard *et al*. 2005). In Iberian lynx, Luaces *et al*. (2005) reported the finding of a small intra-erythrocytic piroplasm in a blood film of a juvenile animal from Sierra Morena. 18S rRNA PCR amplification and sequencing revealed similarity to *C. felis*.

The relevance of *Cytauxzoon* spp. as a threat for Iberian lynx conservation is unknown. The infected lynx reported by Luaces *et al*. (2005) did not show any sign of disease and haematological and biochemical values were normal. However, although bobcats can develop the tissue phase of the pathogen and may die of experimental cytauxzoonosis (Kier *et al*. 1982; Blouin *et al*. 1987), it was considered that *C. felis* could not cause the death of wild bobcats. This view changed when Nietfeld and Pollock (2002) reported a free-living bobcat cub that died of acute cytauxzoonosis. These authors suggested that some bobcats may die each year due to cytauxzoonosis, but these cases remain undetected by current surveillance protocols. It is unknown whether the Iberian lynx experiences a similar situation.

Several questions regarding the aetiology and epidemiology of piroplasmosis in the Iberian lynx emerged from the findings of Luaces *et al*. (2005). As discussed by Nietfeld and Pollock (2002), factors such as host age and sex, and *C. felis* strain may play a role in the epidemiology of the disease. Surveillance for monitoring prevalence of a disease in different areas and age classes is the first step for preventing major disease problems in animal populations (Scott, 1988). The retrospective analysis of 50 lynx blood and organ samples (47 from Doñana and 3 from Sierra Morena) revealed no additional positive animal in Doñana (Luaces *et al*. 2005). Thus, it is currently unknown whether *Cytauxzoon* is distributed in both populations or is currently confined to Sierra Morena. If the latter is true, disease risks may arise if lynx translocations from Sierra Morena to Doñana are carried out (Mathews *et al*. 2006). The *Cytauxzoon* sp. described by Luaces *et al*. (2005) showed maximum homology with the 18S rRNA gene sequence of *C. manul* obtained from a Pallas cat (Ketz-Riely *et al*. 2003) and with a *Cytauxzoon* sp. from a Spanish domestic cat (Criado-Fornelio *et al*. 2004). However, Luaces *et al*. (2005) reported only 1 infected animal, which precluded the analysis of *Cytauxzoon* genetic diversity in Iberian lynx. This information may be relevant to correlate genotypes with pathogenicity and evaluate the possible impact of *Cytauxzoon* infection on wild Iberian lynx endangered populations.

The aim of the present work was to determine by 18S rRNA PCR and sequence analysis the observed prevalence of *Cytauxzoon* spp. in Iberian lynx and to characterize the genetic diversity of this pathogen in the Iberian lynx.

MATERIALS AND METHODS

Study areas

The last metapopulations of Iberian lynx persist in Sierra Morena and Doñana, two localities 230 km apart (Guzmán *et al*. 2004). Doñana (37°0′N, 6°30′W) is a protected, coastal area with sandy soils of marine origin. It is isolated by marshland and farmland from other forest blocks. Sierra Morena (38°13′N, 4°10′W) is a hilly area with heights up to 1300 m. In both areas Mediterranean scrubland dominates, and climate is Mediterranean subhumid with mild, wet winters and hot, dry summers.

Animals and sample preparation

Twenty different free-living Iberian lynxes were surveyed, 11 in Doñana and 9 in Sierra Morena (see Table 1 for detailed sex and age-classes), from November 2004 to June 2006. Lynxes were separated into 3 age classes according to Ferreras *et al*. (2004): juveniles living in the natal area (<1 year old); subadults during the modal natal dispersal period (1–2 years old); and adults (>2 years old). Animals were sampled in 3 seasons, summer (July–August), autumn (November–December) and winter (January–March). Lynxes had to be sampled during captures for incorporations into the Captive Breeding Program, or for radio-collaring, and immobilized

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Table 2. *Cytauxzoon* species sequences included in the analysis

Sequence[a] (Reference)	Host	Country	Abbreviation
AF399930 (Meinkoth *et al*. 2000; Birkenheuer *et al*. 2006*a*)	Domestic cat (*Felis catus*)	USA	DC1
AY531524 (Birkenheuer *et al*. 2006*b*)	Domestic cat (*Felis catus*)	USA	DC2
AY309956 (Criado-Fornelio *et al*. 2004)	Domestic cat (*Felis catus*)	Spain	DC3
Identical to AF399930 (Yabsley *et al*. 2006)	Florida panther (*Puma concolor coryi*)	USA	—[b]
Identical to AF399930 (Yabsley *et al*. 2006)	Texas cougar (*P. c. stanleyana*)	USA	—[b]
Identical to AF399930 (Bondy *et al*. 2005)	Tick (*Amblyomma americanum*)	USA	—[b]
DQ382277 (Unpublished)	Spotted cat (*Leopardus tigrinus*)	Brazil	LE
AY496273 (Luaces *et al*. 2005)	Iberian lynx (*Lynx pardinus*)	Spain	IL1
EF094469 (This work)	Iberian lynx (*Lynx pardinus*)	Spain	IL2
EF094470 (This work)	Iberian lynx (*Lynx pardinus*)	Spain	IL3
EF094468 (This work)	Iberian lynx (*Lynx pardinus*)	Spain	IL4
AF531418 (Ketz-Riley *et al*. 2003)	Pallas cat (*Otocolobus manul*)	Mongolia	PC1
AY485690 (Reichard *et al*. 2005)	Pallas cat (*Otocolobus manul*)	Mongolia	PC2
AY485691 (Reichard *et al*. 2005)	Pallas cat (*Otocolobus manul*)	Mongolia	PC3
L19080 (Allsopp *et al*. 1994)	Not reported	USA	SA

[a] GenBank Accession number.
[b] Sequences identical to AF399930 (DC1).

with a combination of ketamine (Imalgène®, Merial, France) and medetomidine (Domtor®, Pfizer, Spain). Blood was collected from the brachial vein in tubes with lithium heparin as anticoagulant and stored at −20 °C.

DNA was extracted from whole blood (400 μl) using the ReadyAmp Genomic DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Polymerase chain reaction (*PCR*) *and sequence analysis*

A 1726-bp region of the 18S rRNA gene from members of Piroplasmorida was amplified by PCR using primers 7549 (5′-GTCAGGATCCTGGGTTGATCCTGCCAG-3′) and 7548 (5′-GACTGAATTCGACTTCTCCTTCCTTTAAG-3′) (Reichard *et al*. 2005). One μl (1–10 ng) DNA was used with 10 pmol of each primer in a 50 μl volume PCR (1·5 mM $MgSO_4$, 0·2 mM dNTP, 1X AMV/*Tfl* reaction buffer, 5u *Tfl* DNA polymerase) employing the Access RT-PCR system (Promega). Reactions were performed in an automated DNA thermal cycler (Techne model TC-512, Cambridge, England, UK) for 35 cycles. After an initial denaturation step of 1 min at 94 °C, each cycle consisted of a denaturing step of 30 sec at 94 °C, an annealing step for 30 sec at 65 °C and an extension step of 2 min at 68 °C. The programme ended by storing the reactions at 10 °C. Negative control reactions were performed with the same procedures, but adding nuclease-free distilled water (Promega) instead of DNA to monitor contamination of the PCR. Positive control reactions were done with *C. felis* DNA, kindly provided by Drs A. Alan Kocan and Mason V. Reichard (Oklahoma State University, Stillwater, OK, USA; Reichard *et al*. 2005). PCR products were electrophoresed on 1% agarose gels to check the size of amplified fragments by comparison to a DNA molecular weight marker (1 Kb DNA Ladder, Promega). Amplified fragments were resin purified (Wizard, Promega) and cloned into the pGEM-T vector (Promega) for sequencing both strands by double-stranded dye-termination cycle sequencing (Secugen SL, Madrid, Spain). At least 2 independent clones were sequenced for each PCR.

Multiple sequence alignment was performed using the programme AlignX (Vector NTI Suite V 5.5, InforMax, North Bethesda, MD, USA) with an engine based on the Clustal W algorithm (Thompson *et al*. 1994). BLAST (Altschul *et al*. 1990) was used to search the NCBI databases to identify previously reported sequences with identity to those obtained in the study described here.

For phylogenetic analysis, nucleotides were coded as unordered, discrete characters with 5 possible character-states: A, C, G, T, or N and gaps were coded as missing data. The phylogenetic analysis was conducted using Mega version 3.1 (Kumar *et al*. 2004) and the sequence distance method using the Neighbor-Joining (NJ) algorithm of Saitou and Nei (1987) with Kimura 2 parameters correction. Stability or accuracy of inferred topology(ies) were assessed via bootstrap analysis (Felsenstein, 1985) of 1000 iterations. *Cytauxzoon* sp. sequences discovered in this study and those reported previously were included in the analysis (Table 2). Character-state changes for *C. felis* 18S rRNA sequences were polarized by designating *Theileria equi* (GenBank Accession number AY534882) and *Babesia gibsoni* (AF158702) as outgroups.

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Table 3. Percentage identity among 18S rRNA nucleotide sequences between *Cytauxzoon* species

(Sequences were aligned and percentage identity/similarity was determined using the program AlignX. Abbreviations are described in Table 1.)

	Percentage sequence identity											
	DC1	DC2	DC3	LE	IL1	IL2	IL3	IL4	PC1	PC2	PC3	SA
DC1	100											
DC2	99·59	100										
DC3	95·62	95·62	100									
LE	99·42	99·50	96·19	100								
IL1	95·49	95·37	99·11	95·77	100							
IL2	95·85	95·79	99·13	96·68	99·40	100						
IL3	95·68	95·68	99·02	96·44	98·99	99·53	100					
IL4	95·56	95·56	98·90	96·44	98·99	99·42	99·76	100				
PC1	96·33	96·33	98·71	96·35	99·16	99·48	99·74	99·48	100			
PC2	95·76	95·76	98·91	96·35	99·21	99·51	99·75	99·51	100	100		
PC3	95·76	95·70	98·79	96·27	99·21	99·51	99·75	99·51	100	100	100	
SA	99·76	99·71	95·79	99·50	95·79	96·02	95·79	95·74	96·20	96	95·94	100

Sequence Accession numbers

The GenBank Accession numbers for Iberian lynx *C. felis* 18S rRNA sequences are EF094468-EF094470.

RESULTS

Prevalence of Cytauxzoon *spp.*

Only 3 lynxes were found to be infected (Table 1). The overall observed prevalence of *Cytauxzoon* spp. in Iberian lynx was 15% (95% CI: 4–37%). All the infected lynxes were young males (1 subadult, 2 juveniles) sampled in the early summer of 2006 in Sierra Morena. Thus, the observed prevalence in Sierra Morena was 33% (95% CI: 10–68%). No positive results were obtained in samples from Doñana. Although all the infected individuals were young males (Table 1), we did not find sex, or age-related differences in the observed prevalence of *Cytauxzoon* spp.

Molecular characterization of Cytauxzoon *spp.*

To characterize the genetic diversity of *Cytauxzoon* spp. globally, we used the sequences reported in this study and those from *Cytauxzoon* spp. that have been genetically characterized in different domestic and wild felids and ticks from North and South America, Asia and Europe (Table 2). Three different *Cytauxzoon* sp. 18S rRNA sequences were obtained from the Iberian lynx studied in this work. These sequences differed from each other in a maximum of 0·6% nucleotides but diverged in up to 1·0% with respect to the previously reported sequence of a *Cytauxzoon* sp. from an Iberian lynx (Luaces *et al.* 2005) (Table 3). Highly identical (>99·4%) 18S rRNA sequences were found in *C. felis* from cats, wild felids and ticks in North and South America (Table 3). However, these sequences diverged in 3–5% from Asian and European *Cytauxzoon* spp. sequences, which showed a higher degree of sequence divergence (up to 1·3%) (Table 3).

The phylogenetic analysis of *Cytauxzoon* spp. using the 18S rRNA sequences resulted in 2 well-defined clusters. The *Cytauxzoon* spp. from Iberian lynx clustered together with organisms obtained from a cat in Spain and Pallas cats in Mongolia (Fig. 1). The second cluster contained *C. felis* obtained from cats, wild felids and ticks in the United States and Brazil (Fig. 1). The *Cytauxzoon* sp. from Iberian lynx described here was more closely related to that obtained from the Spanish cat than to the organism previously described in an Iberian lynx, which clustered together with Mongolian Pallas cat *C. manul* organisms (Fig. 1).

DISCUSSION

Prevalence of Cytauxzoon *spp.*

The primacy of ecological and conservation criteria caused our sample to be age- and sex-biased. Given its conservation status, every capture of a wild Iberian lynx obeyed a very specific purpose. Juvenile lynxes caught in the summer dominated the Sierra Morena sample because this age class was selected to supply the Captive Breeding Program. Adult lynxes were caught in autumn-winter in Doñana because this season was most suitable for concurrent ecological studies.

The prevalence observed in the present study is similar to that reported in bobcats from Oklahoma (United States; Glenn *et al.* 1982; Kocan *et al.* 1985). The absence of positive results in samples from Doñana agrees with the previous study of Luaces *et al.* (2005), who did not detect the piroplasm in any of the 47 samples from this area.

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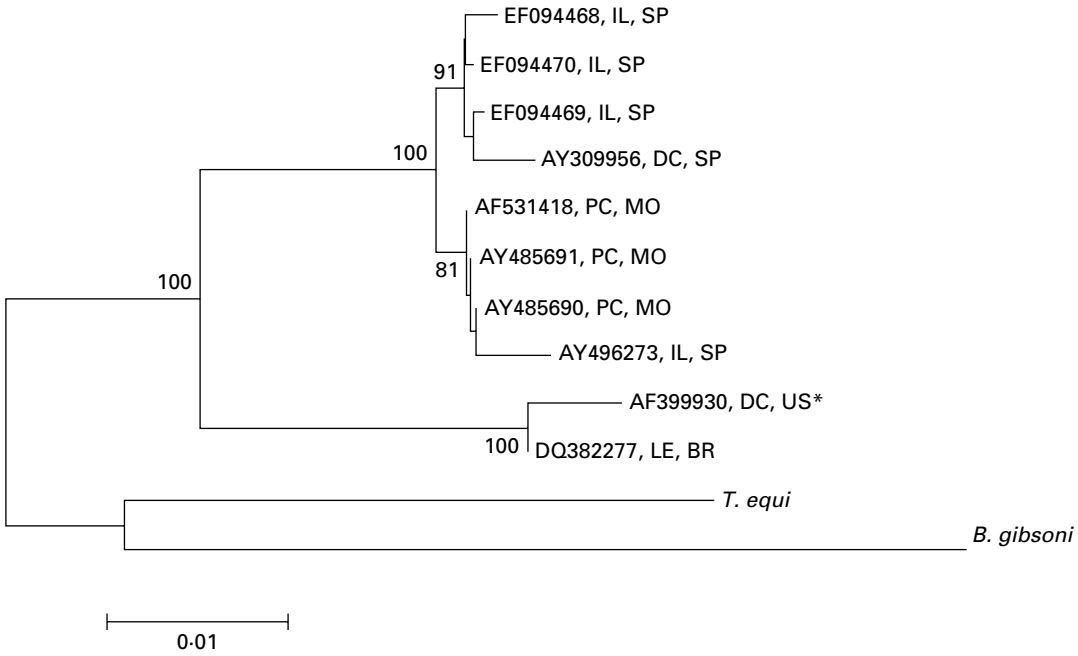

Fig. 1. Phylogenetic analysis of *Cytauxzoon* spp. 18S rRNA sequences based on NJ sequence distance method and 1000 bootstrap iterations. Numbers on branches indicate >70% support for each clade. *Cytauxzoon* spp. are described in Table 1 and are represented as GenBank sequence Accession number, host, location. Abbreviations: IL, Iberian lynx; DC, domestic cat; PC, Pallas cat; LE, leopard; SP, Spain; MO, Mongolia; USA, United States; BR, Brazil. * Sequences identical to AF399930 have been reported in organisms from Florida panther (USA), Texas cougar (USA) and *Amblyomma americanum* ticks (USA).

The 3 parasitized lynxes were sampled in summer. The lynx analysed by Luaces *et al*. (2005) was caught in March. In agreement with these findings, Kier and Greene (1998) reported that most cases of cytauxzoonosis in domestic cats in the United States were observed between May and September. Although impossible to analyse in this study, the sampling season may affect the prevalence of *Cytauxzoon* spp. in Iberian lynx due, among other factors, to the life cycle of the currently unknown tick vector in Spain.

Our results did not determine to what extent the parasite is absent from the Doñana area or whether the infection is only detectable during summer months. Sierra Morena and Doñana Iberian lynx populations could have been functionally connected in the past (Rodríguez and Delibes, 2002). Therefore, the hypothetical absence of *Cytauxzoon* infections in Doñana would be only possible if the vector tick species is absent from this area or the pathogen has become extinct due to the small population size of the vertebrate host (<50 lynxes; Palomares *et al*. 1991; Guzmán *et al*. 2004). The possibility of Iberian lynx parasite species becoming extinct together with their host was already suggested for the host-specific louse, *Felicola* (*Lorisicola*) *isidoroi* (Pérez and Palma, 2001).

Molecular characterization of Cytauxzoon *spp*.

Based on sequence analysis, Reichard *et al*. (2005) proposed a new name, *C. manul*, for *Cytauxzoon* sp. found in Pallas cat from Mongolia. The analysis reported here supports the distinction between American and Eurasian *Cytauxzoon* spp. and suggests that different species or strains may exist in different geographical locations. The results described here also suggest that at least 2 different *Cytauxzoon* species or strains may infect Iberian lynx in Spain, 1 closely related to *C. manul*, and a new species described here and different from *C. felis* and *C. manul*. However, further analyses with more *Cytauxzoon* strains will be required to fully address this question.

On the basis of 18S rRNA gene sequences analysed in the present study, the *C. felis* strains responsible for deaths among cats (Meinkoth and Kocan, 2005) and presumably in bobcats, the natural reservoir host (Nietfeld and Pollock, 2002) in the United States are not genetically distinct from the other American *C. felis* strains that have been obtained and sequenced from non-fatal cases. However, in some instances rRNA sequence analyses cannot differentiate closely related species, subspecies or strains (Fox *et al*. 1992). Therefore, it is possible that *Cytauxzoon* spp. strains with different virulence exist but their discrimination may require the use of different gene sequences for analysis.

In conclusion, the present study showed that (i) infections with *Cytauxzoon* spp. occur in wild Iberian lynx, (ii) the pathogen could be absent from one of the last two lynx metapopulations, and iii) the sequences detected in Iberian lynx are genetically

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variable and may represent 2 different *Cytauxzoon* species or strains.

Cytauxzoon felis causes fatal infections in wild bobcat (Nietfeld and Pollock, 2002) and at least 1 exotic felid, a white tiger, died of cytauxzoonosis in the United States (Garner *et al*. 1996). These facts reinforce the threat for fatal *Cytauxzoon* infections in Iberian lynx. Disease risks must be taken into account in the Iberian lynx management strategies, e.g. if translocations or re-introductions are carried out. Coexistence with domestic or feral cats might be an additional source of infection.

The characterization of the genetic diversity in *Cytauxzoon* spp. isolated from fatal and non-fatal cases of cytauxzoonosis in different feline species and regions of the world may contribute to the understanding of the phylogeny and pathogenicity of different species/strains of the organism and the potential risk for endangered species.

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