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, 1998), 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...
North America. Florida panthers may react to \textit{C. felis} in a way similar to bobcats (Forrester, 1992). A fatal case of cytauxzoonosis was reported in a captive white tiger (\textit{Panthera tigris}) in the United States (Garner et al. 1996). A closely related piroplasm, \textit{C. manul}, was reported parasitizing Pallas cats (\textit{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 \textit{C. felis}.

The relevance of \textit{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 \textit{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 \textit{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 \textit{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 \textit{Cytauxzoon} sp. described by Luaces et al. (2005) showed maximum homology with the 18S rRNA gene sequence of \textit{C. manul} obtained from Pallas cat (Ketz-Riely et al. 2003) and with a \textit{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 \textit{Cytauxzoon} genetic diversity in Iberian lynx. This information may be relevant to correlate genotypes with pathogenicity and evaluate possible impact of \textit{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 \textit{Cytauxzoon} spp. in Iberian lynx and to characterize the genetic diversity of this pathogen in the Iberian lynx.

\section*{Materials and Methods}

\textbf{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.

\textbf{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.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
& \textbf{Sierra Morena} & & \textbf{Doñana} & \\
\hline
\textbf{Male} & \textbf{Female} & \hline
\textbf{Age} & \textbf{Subadult} & \textbf{Juvenile} & \textbf{Adult} & \textbf{Subadult} & \textbf{Juvenile} \\
\hline
\textbf{Adult} & 0 & 3 (1) & 3 (2) & 3 (0) & 1 (0) & 1 (0) \\
\textbf{Subadult} & 0 & 1 (0) & 2 (0) & 5 (0) & 1 (0) & 0 \\
\textbf{Juvenile} & & & & & & \\
\hline
\end{tabular}
\caption{Age and sex-classes of the Iberian lynxes sampled for the detection of \textit{Cytauxzoon} sp. (Parasitized animals are given in parentheses.)}
\end{table}
with a combination of ketamine (Imalgène®, Merial, France) and medetomidine (Domitor®, 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 Piroplasmoridia was amplified by PCR using primers 7549 (5’-GTCAGGATCTGGG-TTGATCTTGCCAG-3’) and 7548 (5’-GACTGAATTCCGACTT-CTCCCTTCCTTTAAG-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 MgSO₄, 0.2 mM dNTP, 1X AMV/Tfl reaction buffer, 5μ AMV 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 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 determination 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.
**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* sp. 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 obey 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.
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* 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 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* (*Loríscola*) *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 *Cytauxzoons* 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
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 cytauxzoosis 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 cytauxzoosis 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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