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Looking for the Iberian lynx in central Spain: a needle in a haystack?

F. Alda[1], J. Inogés[2], L. Alcaraz[1], J. Oria[3], A. Aranda[4] & I. Doadrio[1]

1 Department Biodiversidad y Biología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC, Madrid, Spain
2 Fundación CBD-Habitat, Madrid, Spain
3 C/San Agustín 22, Segovia, Spain
4 Consejería de Medio Ambiente y Desarrollo Rural, Junta Comunidades de Castilla-La Mancha, Toledo, Spain

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Correspondence
Fernando Alda, Department Biodiversidad y Biología Evolutiva, Museo Nacional de Ciencias Naturales, CSIC, José Gutiérrez Abascal 2, 28006 Madrid, Spain. Tel: 34 91 411 1328; Fax: 34 91 564 50 78
Email: alda@mncn.csic.es

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Abstract

The Iberian lynx *Lynx pardinus* has suffered a dramatic reduction in its range throughout the Iberian Peninsula and at present is one of the most endangered mammals in the world. The latest studies report that, out of the 48 breeding areas that existed in 1990, only two populations are left in southern Spain. As a consequence, some of the formerly largest populations, such as Montes de Toledo (central Spain), are to all intents and purposes regarded as extinct. To determine the current distribution of Iberian lynx outside the two recognized populations, we surveyed five different areas where the species is considered extinct and collected 581 faeces for the genetic identification of the species. We identified 18 samples as belonging to Iberian lynx in four out of the five areas studied, providing clear evidence for the presence of lynx in central Spain. In some areas the species was detected repeatedly at different localities and on different dates, indicating a regular occurrence of an unknown number of individuals. The conservation implications of these results are discussed in terms of the genetic importance of the individuals found and future reintroductions of the species from an ongoing captive-breeding programme.

Introduction

The Iberian lynx *Lynx pardinus* (Temminck, 1824) was once widespread throughout the Iberian Peninsula and southern France (Rodríguez & Delibes, 2002). However, in the 1960s, it was already described as a rare species, being present only in central and southern parts of the Iberian Peninsula (Valverde, 1963). The Iberian lynx has since experienced more than an 80% reduction throughout its range (Delibes, Rodríguez & Ferreras, 2000), and today it is one of the most endangered mammals in the world, having been classified as critically endangered by the IUCN since 2002 (IUCN, 2006).

One of the main reasons for its decline has been the reduction of its main prey: the wild rabbit, which constitutes over 85% of Iberian lynx's diet (Delibes, 1980; Aymerich, 1982; Aldama, Beltrán & Delibes, 1991; Calzada & Palomares, 1996). The introduction of myxomatosis to Spain produced a severe crash in rabbit numbers during the 1950s and 1960s (Rodríguez & Delibes, 1992; Villafuerte *et al*., 1993). This was further compounded by the introduction of rabbit haemorrhagic disease in 1988 (Argüello, Llano & Pérez-Ordoyo García, 1998). Other factors affecting the Iberian lynx include the loss or fragmentation of its habitat due to changes in land use and the construction of roads or dams (Rodríguez & Delibes, 1992, 2002). In addition, non-natural mortality represents an important issue in the conservation of this species. Until the 1980s, death caused by traps was the cause of over 60% of non-natural deaths (García-Perea, 2000; Rodríguez & Delibes, 2004). However, since the prohibition of snares for hunting in 1989, these deaths have progressively diminished and in the 1990s they represented $<10\%$ of non-natural deaths (Guzmán *et al*., 2004). On the other hand, road kill has increased dramatically in some areas in southern Spain in recent times from 0.14% before 1980 to 62.5% between 2000 and 2003 (Guzmán *et al*., 2004).

In 1990, the Iberian lynx population in Spain was estimated to hold 880–1150 individuals, distributed across 48 breeding areas corresponding to nine genetically isolated populations, two of which (Eastern Sierra Morena and Montes de Toledo-Villuercas) held 70% of the total population and were considered to be the only viable populations (Rodríguez & Delibes, 1992). Furthermore, the Montes de Toledo-Villuercas population was one of three populations with the highest densities (relative to other populations) of lynx, with an estimated 272 individuals (Rodríguez & Delibes, 1992). However, more recent studies suggest that there are only two populations left in Spain: Doñana in southwestern Spain and Andújar-Cardeña within Eastern Sierra Morena (Fig. 1), with 40–50 and 150 individuals, respectively, with the Montes de Toledo population

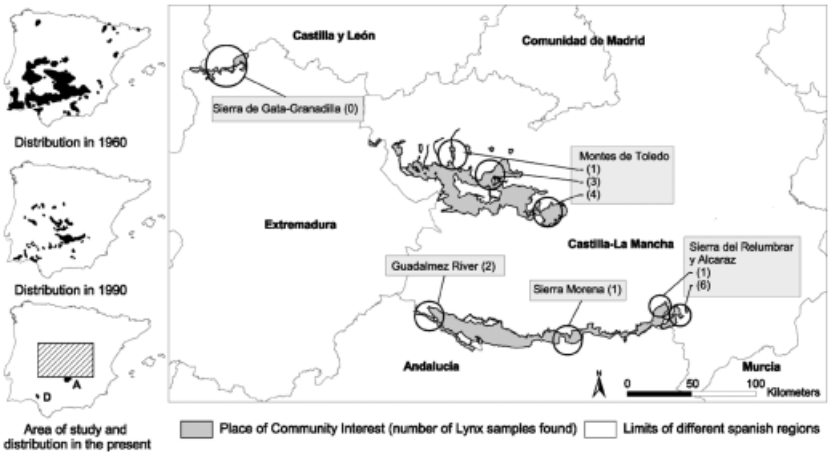

Figure 1 Maps representing the historical distribution of Iberian lynx *Lynx pardinus* in the Iberian Peninsula (modified from Rodríguez & Delibes, 1992), the area of study and the location of samples identified as Iberian lynx for the different areas. The populations of Doñana and Andújar-Cardeña are indicated in the bottom-left map by D and A, respectively.

regarded as 'probably extinct' (Pertoldi *et al*., 2006). The last national survey across most of the lynx's historical distribution was carried out between 1999 and 2002, and the only evidence for the presence of lynx outside the Doñana and Andújar-Cardeña populations was a single excrement collected in Montes de Toledo (Guzmán *et al*., 2004). Consequently, the latter is not considered to be a stable population but rather a group of 'isolated individuals difficult to detect' (Guzmán *et al*., 2004).

Some of the actions proposed within the conservation plan for the Iberian lynx include the estimation of the presence and abundance of lynx, and the identification of habitat requirements and landscape ecology, especially outside the Doñana area (Delibes *et al*., 2000), where the status of the lynx is largely unknown. Thus, we consider that any information (e.g. sightings, faeces, footprints) indicating a possible presence of lynx in this area should be taken into account and subject to further investigation, especially when evidence is found across years in the same area, because the frequency of the indicators of occurrence is positively correlated with species abundance (Tellería, 1986).

Thus, it is important to accurately corroborate the species' presence, a key step in conservation biology and the basis for any conservation or management plan (Frankham *et al*., 2002; Prugh *et al*., 2005). For carnivores, which are generally secretive, difficult to capture or rare, the analysis of scats is one of the best non-invasive methods for monitoring populations, offering information on animal distribution, abundance, movements or diet (Foran, Crooks & Minta, 1997). However, unequivocal assignment of faeces to the species of interest may be difficult based on scat morphology alone, especially if several species of similar body sizes and dietary habits occur in sympatry (Kohn & Wayne, 1997; Davison *et al*., 2002; Piggott & Taylor, 2003). Such unequivocal assignment requires the use of species-specific molecular DNA markers, many of which have been recently developed (Foran *et al*., 1997; Davison *et al*., 2002; Palomares *et al*., 2002; Piggott & Taylor, 2003; Dalén, Gotherstrom & Angerbjorn, 2004; Gómez-Moliner *et al*., 2004; Ruiz-González *et al*., 2007).

In this paper, we present the results of an intensive survey of the presence of Iberian lynx in areas of central Spain where the species could be regarded as extinct (Pertoldi *et al*., 2006). The study is based on the genetic identification of field-collected faeces by the amplification and sequencing of a fragment of the cytochrome *b* gene and/or RFLP analysis of the 16S rDNA mitochondrial genes. Our aim was to unequivocally determine areas where the Iberian lynx is present and to improve our understanding of the species' distributional limits.

Materials and methods

Sampling

One hundred and ten 5×5 km quadrats were systematically surveyed by foot. Sampling effort for each quadrat was 4 h per person. We chose quadrats located in areas of central Spain with a historical presence of Iberian lynx and that had a high probability of containing lynx based on habitat type and prey abundance. The study area comprised five regions with a historical presence of lynx, which are also classified as 'Places of Community Interest by the European Union'. These were Sierra de Gata-Granadilla, Montes de Toledo, Sierra del Relumbrar-Alcaraz, Guadalmez River and Sierra Morena (Fig. 1). All the sampled areas, except Sierra de Gata-Granadilla, were within the Autonomous Region of Castilla-La Mancha. Part of this survey was carried out within the Autonomous Region's framework for the conservation of the Iberian lynx and the LIFE project for the Conservation of the Iberian lynx in Montes de Toledo-Guadalmena 2002–2006 (Fundación CBD-Habitat, 2006*a*).

In addition, areas for which we had reliable information of recent sightings (within the last 5 years) were sampled by

two to five people who walked through tracks and firebreaks collecting scats that matched the morphology and scent described for lynx. Lynx faeces are usually found in visible areas, such as tracks, or in latrines. They have a diameter of about 22 mm and contain an abundance of rabbit hair. When the excrements are fresh they have a glossy dark colour that turns to light grey as they become dry (Brown, Lawrence & Pope, 1992; Blanco, 1998). A total of 581 faeces were collected that ranged from 2 to 3 weeks to <1 day old. All the samples were handled individually, changing gloves between samples so as to avoid cross-contamination, placed into separate paper envelopes that were then placed into separate tubes containing silica gel. Samples were stored in a dry and dark place until DNA extraction.

DNA extraction and amplification

Two commonly used extraction protocols were tested: (1) Guanidine thiocyanate and silica gel (Boom *et al.*, 1990) and (2) QIAamp DNA Stool Mini Kit (Qiagen, Crawley, UK). However, both methods yielded poor amplification results (data not shown). As an alternative, we used an extraction method based on the QIAquick PCR Purification Kit (Qiagen), which had previously been tested on degraded DNA samples from lynx and other species (Alda, Rey & Doadrio, 2007). Briefly, faeces were surface washed with phosphate-buffered saline (PBS), 1 mL of the recovered PBS was transferred to a 2 mL tube and incubated overnight in a shaking water bath at 55 °C with 1 mL of extraction buffer (0.5 M EDTA pH 8.0, 0.5% SDS and 50 μg mL^{-1} proteinase K). The sample was centrifuged for 10 min at 2000 $\times$ ***g*** and the supernatant was transferred to a 15 mL Falcon tube and mixed with an equal volume of binding buffer (4.5 M guanidine thiocyanate, 0.5 M potassium acetate pH 5.0). Volumes of 750 μL of the supernatant/binding buffer mixture were passed consecutively through a QIAquick spin column by centrifugation for 1 min at 12 800 $\times$ ***g*** until the entire mixture had been processed. Two washes with 700 and 500 μL of PE (Qiagen) were performed. Finally, the DNA was eluted in 100 μL of EB buffer (Qiagen) after a 10-min incubation period at room temperature and 5 min of centrifugation at 12 800 $\times$ ***g***. Eluted DNA was stored at -20 °C.

Species identification was initially performed by amplification and direct sequencing of a fragment of the cytochrome *b* mitochondrial gene. This method served to identify the most common species occurring in the surveyed areas and to design a more straightforward method based on the amplification and posterior digestion of a fragment of the 16S rDNA mitochondrial gene. The primers used for the amplification of cytochrome *b* were CBLF: 5′-ATG ACCAACATTCGAAAATCACACCCCC-3′ and CBLR: 5′-CCCGTTGGCATGTATGTACCGGATG-3′, which amplify a 257 bp region corresponding to positions 15 038 and 15 294 of the cat mitochondrial genome (U20753; Lopez, Cevario & O'Brien, 1996). For the 16S rDNA, we amplified a 350 bp region corresponding to positions 2923 and 3274 of the cat mitochondrial genome with the primers: LP16S2: 5′-CTTGTATGAAYGGCCACACG-3′ and LP16S3: 5′-TATTGTCGATATGGACTCTG-3′. All reactions were performed in a final volume of 25 μL containing 1X HotMaster *Taq* Buffer (Eppendorf, Hamburg, Germany), 2.5 mM Mg^{2+}, 0.1 μg μL^{-1} of bovine serum albumin (Promega, Madison, WI, USA), 0.2 mM of each dNTP, 1 U of HotMaster *Taq* DNA polymerase (Eppendorf), 0.4 μM of each primer and 0.5–2 μL of the DNA extraction (depending on amplification success). Thermocycling conditions consisted of a denaturing step at 94 °C for 2 min, followed by 40 cycles at 94 °C for 30 s, 50 °C for 15 s, 65 °C for 30 s and a final extension step at 65 °C for 5 min. All extractions and PCR reactions were prepared in separate rooms, free from modern DNA and PCR products, and included negative controls to check for contamination.

Cytochrome *b* fragments were purified with Exo-SAP-IT (UBS, Cleveland, OH, USA) following the manufacturer's instructions and sequenced on an automated DNA sequencer (ABI-3700) using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA).

PCR products of 16S rDNA were digested with *Hae*III following the manufacturer's instructions (Promega) and fragments were separated by electrophoresis on 2% agarose gels and visualized with ethidium bromide under UV light. Domestic and wild cats (*Felis* sp.) have a restriction site for *Hae*III in the amplified 16S rDNA fragment that is absent in the genus *Lynx* and canids. Digestion with *Hae*III produces two fragments of *c.* 160 and 190 bp in cat samples, whereas in lynx and canid samples the 350 bp amplified fragment appears intact after digestion. Thus, only fragments that were not cleaved by *Hae*III were sequenced to distinguish between lynx and canids.

Species identification and sequence analyses

The sequences obtained for both mitochondrial genes were initially compared with entries in Genbank using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). To infer the species of origin for our samples, we constructed Neighbour–Joining trees in PAUP* 4.0b10 (Swofford, 1998) using the HKY mutation model using reference sequences of carnivores and possible lynx prey species, in which DNA could be present in the faeces (e.g. *Oryctolagus cuniculus*), obtained from Genbank or by us (Supplementary Material Appendix S1). Reference samples included two sequences obtained from two lynx faeces collected from the Andújar population (Genbank accession numbers: EU588715, EU588716). As the faecal samples were identified, their sequences were included in the following analyses for comparison with the next samples analysed.

All the samples that were identified as *L. pardinus* were double checked. Firstly they were re-amplified and sequenced again by us, and secondly they were extracted, amplified and sequenced again by different personnel in a completely independent laboratory of the DNA and Tissue Collection, Museo Nacional de Ciencias Naturales, CSIC.

Table 1 Number of samples analyzed, number of samples successfully analyzed and number of samples identified as *Lynx pardinus* are given for region and year

	Year					
Region	2003	2004	2005	2006	2007	Total
Sierra de Gata-Granadilla	–	–	69/27/-	–	15/9/-	84/36/-
Montes de Toledo	13/5/-	61/36/3	178/98/5	97/54/-	46/22/-	395/215/8
Sierra del Relumbrar-Alcaraz	13/8/6	40/18/-	13/6/1	8/4/-	–	74/36/7
Guadalmez River	2/1/-	3/2/2	5/2/-			10/5/2
Sierra Morena	1/1/1	2/1/-	14/6/-	1/1/-	–	18/9/1
Total	29/15/7	106/57/5	279/139/6	106/59/-	61/31/-	581/301/18

To represent the genetic relationships among the cytochrome *b* haplotypes found in central Spain, Andújar and those previously published from Doñana (Palomares *et al*., 2002; Johnson *et al*., 2004), we calculated a Median Joining Network (Bandelt, Forster & Rohl, 1999) using the software NETWORK 4.5 (www.fluxus-engineering.com).

Results

A total of 581 samples were collected from 2003 to 2007 (Table 1, Fig. 1). Species were identified for 301 samples by at least one of the two methods described. Of these, 263 belonged to cats (*Felis* sp.), 18 to Iberian lynx *L. pardinus*, 11 to dogs (*Canis* sp.) and nine to foxes *Vulpes vulpes*.

From the 18 Iberian lynx samples detected, 14 were identified through the sequencing of cytochrome *b*, and four using the 16S rDNA digestion and sequencing method (Fig. 2). Both markers showed enough variation to discriminate between species. For instance, the most similar sequences available in Genbank for the wild cat *Felis silvestris*, which is the only wild felid co-occurring with the Iberian lynx in the Iberian Peninsula (Palomo & Gisbert, 2002), showed 25 nucleotide differences for the cytochrome *b* fragment (EF689045) and 14 nucleotide differences for the 16S rDNA fragment (*Felis catus*: U20753 and *F. silvestris*: DQ334822) with respect to the Iberian lynx sequences obtained in this study. The molecular markers used do not differentiate between wild and domestic cats, or between dogs, wolves and their hybrids. Therefore, they will be referred as *Felis* sp. and *Canis* sp.

Unexpectedly, the cytochrome *b* fragment revealed six distinct lynx haplotypes differing by 1–3 bp differences (Table 2, Fig. 3). The most common haplotype was found in 10 samples and was the same as that described for the population of Doñana (Palomares *et al*., 2002; Johnson *et al*., 2004). This haplotype was found in all the study areas, except in Andújar, where the two reference samples had different haplotypes, one of them shared with Montes de Toledo (Fig. 3). Only one 16S rDNA haplotype was detected in the lynx samples (DQ655652), which was identical to sequences published elsewhere (Johnson *et al*., 2004).

Iberian lynx were identified in four of the five areas surveyed. In Montes de Toledo, eight samples were ascribed to lynx at three different localities (Fig. 1). In Eastern Montes de Toledo, we found two samples in June and July of 2004 and another two in February and March of 2005. In Central Montes de Toledo, one sample was found in August of 2004 and two in March and July of 2005. A single sample was found in March 2005 in Western Montes de Toledo. Five of these samples were identified by their cytochrome *b* sequence, which revealed four haplotypes (Table 2, Fig. 3). In Eastern Montes de Toledo, one individual had a haplotype (Hap_3) that differed by one base from another two that had the same haplotype described for the Doñana population. In Central Montes de Toledo, one individual had a haplotype (Hap_4) that differed from all the others found in Eastern Montes de Toledo (Table 2, Fig. 3). The sample identified in Western Montes de Toledo had the same haplotype (Hap_1) as one of the reference samples from Andújar, which differed by two bases from the Doñana haplotype (Table 2, Fig. 3).

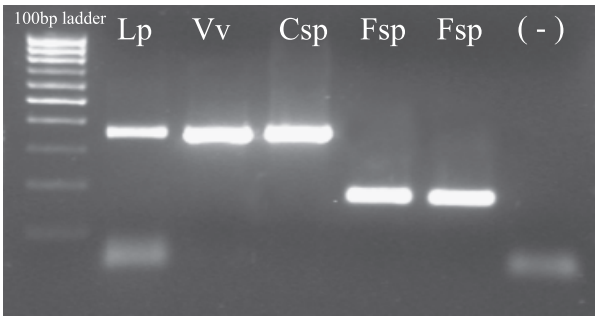

Figure 2 Photograph of a 2% agarose gel showing the 16S rDNA restriction enzyme patterns generated using *Hae*III for *Lynx pardinus* (Lp), *Vulpes vulpes* (Vv), *Canis* sp. (Csp) and *Felis* sp. (Fsp). The brightest band in the molecular weight marker (100 bp ladder Biotools) corresponds to the 500 bp band. The right lane is the PCR negative control (–).

In Sierra Morena, one sample found in August 2003 proved to be lynx. In the Guadalmez River, we found two samples at the same site in September 2004, with haplotypes (Hap_Doñana and Hap_5) that differed by two bases (Table 2, Fig. 3).

In Sierra del Relumbrar-Alcaraz, seven samples were identified as Iberian lynx. At one locality, six samples were collected in October 2003 and all of them had the haplotype described for the Doñana population (Table 2, Fig. 3).

Table 2 Differences in the Iberian lynx *Lynx pardinus* cytochrome *b* haplotypes obtained at each of the areas surveyed in central Spain and Iberian lynx reference samples

Haplotype	129	138	140	167	194	211	250	Doñana	Andújar	Montes de Toledo	Guadalmez River	Sierra Morena	S. Relumbrar -Alcaraz	All	GenBank Accession no.
Hap_Doñana	G	T	G	T	A	T	T	6		2	1	1	6	16	
Hap_1	T	C	·	·	·	·	·		1	1				2	EU588715
Hap_2	·	·	C	·	·	·	·		1					1	EU588716
Hap_3	·	·	·	·	·	·	C			1				1	EU588717
Hap_4	·	·	·	C	·	·	·			1				1	EU588718
Hap_5	·	·	·	·	G	C	·				1			1	EU588719
All								6	2	5	2	1	6	22	

Polymorphic sites are shown with reference to the Doñana haplotype AJ441331.

Figure 3 Median-Joining Network constructed from the cytochrome *b* haplotypes obtained from Iberian lynx *Lynx pardinus* in central Spain and reference samples in Doñana and Andújar. Circles are proportional to haplotype frequency. Black dots represent mutational steps or missing haplotypes.

Another sample was found at a different locality in July 2005.

We did not find evidence of lynx in the area of Sierra de Gata-Granadilla in any of the samples collected during 2006 and 2007.

Discussion

The results reported in this study constitute the strongest evidence in recent years for the presence of Iberian lynx outside the two recognized populations of Doñana and Andújar-Cardeña (Guzmán *et al.*, 2004). We demonstrate that the Iberian lynx is not extinct in central Spain. Furthermore, in some of the areas where we detected the species, evidence of its presence was found repeatedly (Table 1).

Method evaluation

Two different methods for species identification were used in this study. The first method was a direct approach, based on the direct sequencing and comparison of 257 bp of the cytochrome *b* gene with Genbank entries or other reference samples. This method is unequivocal and straightforward (Höss *et al.*, 1992; Farrell, Roman & Sunquist, 2000), but it is also time consuming and expensive (Dalén *et al.*, 2004). However, this technique might be a useful starting point when working in poorly known areas, as it permits the identification of all the samples collected and not just the detection of a single species. Based on the results obtained from this first analysis, a second identification method was tested based on the amplification and restriction enzyme digestion of a 351 bp fragment of the 16S rDNA gene. This PCR–RFLP method proved to be useful to identify *Felis* sp. samples, which represented most of the faeces collected. However, canids (*Canis* sp. and *V. vulpes*) and *Lynx* species present the same restriction pattern, and therefore their identification requires direct sequencing.

Overall, we were able to determine the species of origin for 52% of the samples collected. These results are lower than other recent studies that positively identified over 77% (Dalén *et al.*, 2004) or even 88% (Ruiz-González *et al.*, 2007) of the samples collected. However, success rates are difficult to compare among studies as they vary considerably depending on the species and area of study (Waits & Paetkau, 2005). In addition to these, many other factors influence the final success, such as (1) the age of the sample on collection (Murphy *et al.*, 2007); (2) the weather (Murphy *et al.*, 2007); (3) the species' diet (Murphy, Waits & Kendall, 2003; Nsubuga *et al.*, 2004); (4) the preservation method and storage time (Frantzen *et al.*, 1998; Murphy, Waits & Kendall, 2000; Murphy *et al.*, 2002); (5) the extraction method used (Kohn & Wayne, 1997; Piggott & Taylor, 2003; Waits & Paetkau, 2005; Alda *et al.*, 2007 and references therein).

In our study, these variables were difficult to control in the field, and the only constant factors among samples were the preservation method and laboratory processing. Despite the fact that we did not test for the effect of these variables on the success of the analyses, we did observe that samples that had been stored for a longer period of time between collection and extraction had a lower success rate (data not shown). To overcome this problem in the future, we might evaluate a different preservation method, such as ethanol and freezing (Murphy *et al.*, 2002; Ruiz-González *et al.*, 2007), substituting the silica desiccation to the detriment of ease in the field.

Similar studies have successfully used a different identification approach, based on the PCR amplification of a species-specific fragment for the Iberian lynx (Palomares *et al.*, 2002; Pires & Fernandes, 2003). However, this method

has been criticized as it is prone to producing false negatives due to amplification failure (Dalén *et al*., 2004). Conversely, the method described here is unlikely to render false negatives, as non-amplification is not considered to be a result of the test, but rather missing data. Furthermore, our method can account for digestion failure due to mutation in the restriction site (Dalén *et al*., 2004) because a non-digested fragment would be considered an indication of either lynx or canid and consequently be sequenced, detecting the error.

One drawback of the method described here is that it needs a longer fragment to be amplified, which is a limiting factor for PCR success, especially when dealing with samples with degraded DNA (Pääbo, 1989; Höss *et al*., 1996; Waits & Paetkau, 2005; Deagle, Eveson & Jarman, 2006). Designing new primers to amplify a smaller fragment could, in part, solve this problem. Nonetheless, we consider that our method could be a conservative alternative for studies in areas where the presence of the species in question is uncertain and there is an interest to identify the species of origin for all the scats.

In 2007, video footage was published showing lynx in the wild in an area close to the Guadalmez River where we had previously identified the presence of lynx in 2004. The lynx observed are thought to belong to a small breeding population holding tens of individuals and several cubs (Supplementary Material Appendix S2). This finding not only corroborates both the validity and the accuracy of our results but also demonstrates the utility of molecular tools as a rapid means to obtain presence/absence data for the management and conservation of species relative to other more laborious methods (Schwartz, Luikart & Waples, 2007).

Occurrence of Iberian lynx

Only 6% of the successfully analysed samples were Iberian lynx, whereas over 87% were identified as cats (*Felis* sp.). This small proportion demonstrates the difficulty of identifying faeces in the field (Palomares *et al*., 2002), especially in areas of low species occurrence and an abundance of other carnivores with similar size and habits (Paxinos *et al*., 1997; Davison *et al*., 2002).

A total of 18 samples were ascribed to Iberian lynx in four of the five areas surveyed. In all of them, except in Sierra Morena, more than one lynx sample was detected. This might indicate a regular occurrence of the species in some of these areas, such as Montes de Toledo, where lynx samples were identified from 2004 to 2005 in three different localities along the mountain range and, therefore, could be suggestive of small populations. The six lynx faeces collected in a single day in October 2003 in Sierra del Relumbrar-Alcaraz following reports of a sighting probably belong to the same individual. Two years later, another sample was collected in the same region.

In other cases, the presence of lynx might be sporadic, such as in Sierra Morena, where the individual identified could be dispersing from the neighbouring, and largest, population of Andújar-Cardeña. However, it seems unlikely that the lynx detected in Montes de Toledo were dispersants from the southern Iberian lynx populations. Firstly, the distance between these areas, over 200 km, is considerably larger than the average home range for the Iberian lynx (10.3 km^2 for males and 5.3 km^2 for females), even if we consider the largest possible home ranges (e.g. 19.6 km^2) that might occur when rabbit abundance is low (Beltrán & Delibes, 1994; Ferreras *et al*., 1997). Secondly, the landscape in central Spain is highly fragmented by estates, which are mostly dedicated to hunting, and by human infrastructures such as roads and railways (San Miguel, 2006). Thus, dispersal across this habitat would be difficult and would involve a significant risk of mortality from trapping, shooting or traffic collision (Rodríguez & Delibes, 2004). Therefore, the most plausible explanation is that these lynx are survivors of the historically large population of Montes de Toledo (Rodríguez & Delibes, 1992), and that they have remained undetected due to the elusive habits of the species and the difficulties of tracking lynx in its natural habitat (Palomares *et al*., 2002). Nevertheless, further studies with appropriate molecular tools (e.g. microsatellites) and the inclusion of putative source populations could confirm the origin and genetic status of these individuals (Ernest *et al*., 2000; Piggott & Taylor, 2003; Waits & Paetkau, 2005; Schwartz *et al*., 2007).

Based on our findings, we could not support the presence of lynx in Sierra de Gata-Granadilla. In this area, the reproduction of the species was documented in 1992 and 1995 (González Oreja & González Vázquez, 1996), but today rabbits are scarce in the area.

It is well known that determining the presence and abundance of endangered carnivores is particularly difficult (Palomares *et al*., 2002). In addition, comparison of our study with previous ones (Guzmán *et al*., 2004) suggests that the results obtained when working with a scarce species distributed over a large area largely depend on the intensity and scale of the sampling. For instance, the identification of lynx in areas where previous studies failed to detect lynx may be due to the different sampling strategies applied (Guzmán *et al*., 2004). In the present study, and for the same areas as Guzmán *et al*. (2004), we collected more samples (e.g. 395 faeces collected against 175, in Montes de Toledo) in more, but smaller, quadrats (5×5 km). This intensive strategy is thought to be more appropriate and accurate for species with an unequal distribution across sampling units (Tellería, 1986), as would be expected for the scarce Iberian lynx in central Spain. Thus, it would be risky to rule out the possibility that some lynx still occur in the area of Sierra de Gata-Granadilla without making a more intensive sampling effort.

Implications for conservation and management

In addition to the empirical information obtained by the molecular identification of lynx in central Spain, these results provide important data for conservation efforts.

Firstly, in our study area, we found five new haplotypes together with the haplotype described for the Doñana population (Johnson *et al*., 2004). This could mean that the Doñana haplotype was a common and widespread haplotype that today is fixed in the genetically impoverished population of Doñana (Beltrán & Delibes, 1993; Johnson *et al*., 2004). However, other haplotypes could have remained at lower frequencies in other isolated populations, such as Montes de Toledo. Further research is needed to determine whether these isolated individuals have also retained nuclear genetic variability that could have an important 'genetic rescue effect' (Vilà *et al*., 2002; Tallmon, Luikart & Waples, 2004) over the inbred populations of Iberian lynx (Johnson *et al*., 2004).

Another aspect of our data that has important implications for the development of conservation strategies is the identification of areas of suitable habitat to maintain populations of lynx. At present, there is a captive-breeding programme for the Iberian lynx in Spain that aims to release individuals into the wild in 2010 (Lacy & Vargas, 2004). It has been recommended that reintroduction sites should be mainly chosen from localities with the best habitat for rabbits (Rodríguez & Delibes, 2004), some of which occur in Montes de Toledo and eastern Sierra Morena (Blanco & Villafuerte, 1993). Other criteria recommended for the reintroduction of species (IUCN, 1998) and the *in situ* conservation of Iberian lynx (Guzmán *et al*., 2004) could support Montes de Toledo's candidacy as an area for lynx reintroduction. Firstly, rabbit populations are abundant. During the period of this study, rabbit numbers ranged from 0.10 to 2.66 rabbits ha^{-1} in low rabbit density estates, and from 1.22 to 14.0 rabbits ha^{-1} in high rabbit density estates (Fundación CBD-Habitat, 2006*b*,*c*, Supplementary Material Appendix S3). Secondly, the current land management carried out on many private estates in this region improves habitat for rabbit and lynx, and recognizes the importance and the benefits of protecting lynx, and other species, through collaborations with land owners and hunters (Fundación CBD-Habitat, 2006*a*). However, other factors still need to be evaluated, such as non-natural mortality, which, in the 1990s, was mainly attributed to illegal trapping and shooting in this area (García-Perea, 2000). Today these causes may have decreased but others have become more important, such as traffic collisions or habitat fragmentation by highways and railroads (San Miguel, 2006).

Furthermore, the possibility that the reintroduced individuals could interbreed with the few remaining lynx in the area is an almost unique feature of Montes de Toledo, compared with other potential areas for reintroduction. Therefore, in the same way as the Iberian lynx captive-breeding programme is favouring gene flow between the isolated populations of Doñana and Andújar-Cardeña (Vargas *et al*., 2007), the genetic contact in the wild between individuals descended from the southern populations and those from central Spain could increase the genetic diversity, with positive evolutionary consequences (Vilà *et al*., 2002; Tallmon *et al*., 2004).

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Supplementary material

The following material is available for this article online:

Appendix S1. List of taxa and their Genbank Accession numbers used for comparison with the sequences obtained in this study.

Appendix S2. Internet links for the newspaper article and video footage recently published accounting for the presence of a small breeding population of Iberian lynx in the Autonomous Region of Castilla-La Mancha.

Appendix S3. Abundance of rabbits (rabbits/ha) at three estates of Montes de Toledo during the period of study.This material is available as part of the online article from http://www.blackwell-synergy.com/doi/abs/10.1111/j.1469-1795.2008.00185.x

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