

Faecal genetic analysis to determine the presence and distribution of elusive carnivores: design and feasibility for the Iberian lynx

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Abstract

Noninvasive methods using genetic markers have been suggested as ways to overcome difficulties associated with documenting the presence of elusive species. We present and assess a novel, reliable and effective molecular genetic technique for the unequivocal genetic identification of faeces from the endangered Iberian lynx (*Lynx pardinus*). From mitochondrial DNA (mtDNA) cytochrome *b* and D-loop region sequences, we designed four species-specific primers (for products 130–161 bp long) that were considered to be likely to amplify degraded DNA. We compared two DNA extraction methods, various DNA amplification conditions and the robustness and specificity of the primer pairs with 87 lynx samples from 5 potentially different lynx populations and with 328 samples of other carnivore species. The utility of the identification technique was tested with faeces of different ages, with faeces from controlled field experiments, and with faeces collected from locales with possible lynx populations from throughout the state of Andalusia, Spain (8052 km²). Faecal mtDNA extraction was more efficient using PBS wash of the faeces instead of a faeces homogenate. Our assay increased from 92.6 to 99% efficiency with a second amplification and a reduction in template concentration to overcome polymerase chain reaction (PCR) inhibition. Our assay never produced false positives, and correctly identified all lynx faeces. Of 252 faeces samples of unknown species collected throughout Andalusia, 26.6% (from three different areas) were classified as Iberian lynx, 1.4% showed evidence of PCR inhibition and 1.2% were of uncertain origin. This method has proven to be a reliable technique that can be incorporated into large-scale surveys of Iberian lynx populations and exemplifies an approach that can easily be extended to other species.

Keywords: cytochrome *b*, D-loop, faecal DNA, genetic analyses, Iberian lynx, mitochondrial DNA markers, presence and distribution

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Introduction

Documenting the presence and abundance of species is the first step in designing conservation plans for endangered species and in understanding population ecology. However, in many cases this is not an easy task because individuals are difficult to detect and methods requiring the capture and handling of animals are not feasible owing to logistical and ethical problems. This problem is particularly

acute for carnivores and endangered species, which are usually scarce, live in low densities, and have elusive and secretive habits.

Methods of noninvasive sampling of animal populations using genetic markers are being developed to overcome these problems (Morin & Woodruff 1996; Kohn & Wayne 1997). However, the emphasis of most studies has been the development and presentation of the molecular technique (e.g. Höss *et al.* 1992; Tikel *et al.* 1996; Foran *et al.* 1997a,b; Paxinos *et al.* 1997). Little attention has been given to the analytical and statistical methods that can validate the data obtained (Parker *et al.* 1998; Mills *et al.* 2000a) or

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the reliability and utility of these laboratory techniques for field studies (Schwartz *et al.* 1998; Taberlet *et al.* 1999). Only limited attempts to determine the cost, technical difficulty, appropriateness of resulting data and sophistication of existing statistical methods have been carried out (e.g. Kohn *et al.* 1995, 1999; Taberlet *et al.* 1997; Wasser *et al.* 1997; Ernest *et al.* 2000; Farrell *et al.* 2000; Mills *et al.* 2000b; Mowat & Strobeck 2000). The allure of utilizing noninvasive molecular genetic techniques needs to be tempered by a thorough consideration of the trade-offs associated with different approaches, their value for large-scale field studies, and the reliability of the resulting data. Therefore, comprehensive studies addressing the complexities of the laboratory and field methods are needed before the widespread use of any of these techniques.

Here, we present a novel, reliable and highly effective molecular genetic technique for the unequivocal identification of DNA from material derived from a scarce and elusive felid species, the Iberian lynx (*Lynx pardinus*). We focus on the general feasibility and reliability of the technique in determining the distribution of this species through the analysis of faeces, the only sign of lynx that can normally be found in the field. Conceptually, this study had three main goals, consisting of the development, laboratory testing and field implementation of a method to identify Iberian lynx.

The main requirement for a molecular technique or assay for the identification of Iberian lynx was that it could be applied to degraded and scarce DNA, such as that obtained from faeces. The ideal assay would be reliable and unambiguous, but also simple and economical enough for routine application with a large number of samples. We chose an approach based on the species-specific amplification of lynx mitochondrial sequences. We tested and optimized the technique in the laboratory, and determined the effect of age and the condition of the faecal material had on the efficiency and accuracy of the technique. Finally, to assess the general utility of the technique we conducted a pilot study to determine the distribution of Iberian lynx in Andalusia, an area of 8052 km² in the south of the Iberian Peninsula where the highest number of Iberian lynx are thought to exist (Rodríguez & Delibes 1992).

The Iberian lynx is the most endangered felid species in the world (Nowell & Jackson 1996). It is estimated that there are fewer than 1000 individuals distributed in fragmented areas of the southwestern portion of the Iberian Peninsula (Rodríguez & Delibes 1992; Castro & Palma 1996). However, geographical limits and actual population sizes are not well known because they are nocturnal animals that inhabit areas of low human density and grounds that do not allow for tracking them (Beltrán & Delibes 1994; Palomares *et al.* 2000). This situation imposes several logistical and legal impediments for implementing and monitoring lynx conservation plans.

Precise estimates of lynx distribution, metapopulation structure and persistence have only been possible in the

Doñana National Park area, where radiotracking studies have been conducted for more than 15 years (e.g. Ferreras *et al.* 1997; Palomares *et al.* 2001) and sandy soils allow for easy identification of the species by tracks (Palomares *et al.* 1991; Gaona *et al.* 1998). However, in the rest of its potential distribution area, the most common substrates are not amenable to track identification, and faeces are the most visible sign of the animals. Unfortunately, faeces identification is not easy and lynx faeces may be confused with other species such as wild cats (*Felis silvestris*), domestic cats (*Felis catus*), red foxes (*Vulpes vulpes*) and domestic dogs (*Canis familiaris*).

Materials and methods

Molecular methods

Sample preservation. Lynx DNA was initially extracted from blood and hair samples from wild animals, and muscle, kidney, skin, ear, nose and foot pads of specimens from scientific collections. Blood samples were immediately mixed after collection with 4 vols of lysis buffer (0.1 M Tris-HCl pH 8.0; 0.1 M Na-EDTA; 0.01 M NaCl, 0.5% SDS). Organ (kidney or muscle) samples were kept frozen or at room temperature in lysis buffer (see above). Approximately 1 cm² of skin of museum specimens or smaller pieces of ear were cut, and internal tissue from nose or the foot pad were scraped with a sterile scalpel after superficial cleaning with 10% commercial bleach, distilled water and 70% ethanol. These samples were kept in dry and cool conditions. Hairs were stored in clean paper envelopes at room temperature in silica gel.

DNA isolation. DNA extractions were carried out in a dedicated room. Extraction blanks were included to monitor for contamination. DNA from blood, muscle or kidney samples was extracted using a proteinase K/phenol protocol (Sambrook *et al.* 1989). DNA from lynx skin pieces from scientific collections was extracted using the same protocol, but was first washed extensively in excess NTE (0.05 M Tris-HCl, 0.01 M NaCl, 0.02 M EDTA, pH 9.0; Goetz *et al.* 1985) and GTE (0.1 M glycine, 0.01 M Tris-HCl, pH 8.0, 1 mM EDTA; Shedlock *et al.* 1997) to remove enzyme inhibitors (Hall *et al.* 1997). DNA from hairs was extracted using a Chelex-100 protocol (Walsh *et al.* 1991) as described by Gagneux *et al.* (1997). Faecal DNA extracts were also prepared with Chelex-100, using two alternative strategies (see below).

Polymerase chain reaction (PCR). DNA amplification reactions contained 67 mM Tris-HCl pH 8.0, 16 mM (NH₄)₂SO₄, 2.5 mM MgCl₂, 0.01% Tween-20, 0.2 mM dNTPs, 1 µM of each primer, 0.5 U of *Taq* DNA polymerase and 50–100 ng of total DNA from blood, 5 µL of museum (not organs)

DNA extracts, or hair DNA extracts as template. Bovine serum albumin (BSA) was included at a concentration of 0.1 µg/µL for amplification of blood DNA and 0.8 µg/µL for museum, hair and faecal DNA (see below). Amplification reactions were performed in a MJ Research thermocycler, Model PTC-100 programmed for an initial denaturation cycle of 94 °C for 2 min, followed by 35 cycles of denaturation at 92 °C for 30 s, annealing at 55–67.5 °C (depending on primers and tests; see below) for 30 s, and extension at 72 °C for 30 s. All reactions were finished with a final extension at 72 °C for 5 min. To control for performance of the process and contamination, positive (diluted blood DNA) and negative DNA controls, respectively, were included with each set of PCR. An additional negative extraction control was included in every amplification of hair, museum or faecal extracts.

Amplification products were separated by electrophoresis in 2–3% agarose gels in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) in the presence of 0.5 mg/L EtBr. Gels were visualized under UV and photographed with a digital image system (Eastman Kodak Company).

DNA sequencing. PCR products were cleaned by ultrafiltration through Centricon-100 (Millipore Corp.) and sequenced on an automated DNA sequencer (ABI-377) using the BigDye Terminator Cycle Sequencing Kit following the manufacturer's instructions (Applied Biosystems, Inc.). Sequences were edited, assembled and aligned using the program SEQUENCHER (Gene Codes Corporation).

Design and evaluation of PCR-based lynx-diagnostic assay

In order to design species-specific primers mitochondrial DNA (mtDNA) sequences were obtained. Both the highly variable domain 1 of the control region (CR) and a fragment of the cytochrome *b* (*Cytb*) gene were targeted. Control region hypervariable domain 1 was amplified and sequenced from two domestic cats, two wild cats and three Iberian lynx (GenBank Accession nos. AJ441317–441320, AJ456977–456979) using primers CR1 (5'-CCACTATCAGCACCCAAAGC-3') and CR2R (5'-CCCGGAGCGAGAAGAGG-3'), designed to match conserved flanking sequences at the tRNA-Pro and the central conserved region, respectively. Sequences were aligned to the corresponding region of the cat mitochondrial genome (GenBank Accession no. NC_001700; López *et al.* 1996). Cytochrome *b* sequences were obtained from different Iberian carnivore species (Iberian lynx, domestic cat, wild cat, red fox, wolf, *Canis lupus*, domestic dog, European genet, *Genetta genetta*, stone marten, *Martes foina*, and Egyptian mongoose, *Herpestes ichneumon*), European rabbits (*Oryctolagus cuniculus*; the main prey of lynx;

Delibes 1980) and human, using primers Cytb-1 and Cytb-2 (Janczewski *et al.* 1995) for both PCR and sequencing. Sequences were deposited in GenBank (Accession nos. AJ441328–AJ441340).

Interspecific sequence differences in control region or cytochrome *b* were used to design lynx-specific primers that could be used in a diagnostic PCR of faecal material. Six lynx specific primers were designed so that they matched several lynx specific bases at its 3'-end (Kwok *et al.* 1990). Four primer pairs were consequently selected to amplify Iberian lynx mtDNA but not other species. To maximize the probability of amplification from degraded DNA primer pairs were designed to amplify fragments < 200 bp. The optimal annealing temperatures for the exclusive amplification of lynx DNA was selected for each primer pair after testing temperatures ranging from 55 to 67.5 °C in 2.5 °C increments on a set of carnivores species (four Iberian lynx, two domestic cats, two wild cats, two red foxes, one wolf, one domestic dog, two Eurasian badgers, *Meles meles*, two European genets, one stone marten, one pine marten, *Martes martes*, one Egyptian mongoose, one polecat, *Mustela putorius*, and one weasel, *Mustela nivalis*, and one Eurasian otter, *Lutra lutra*), and one rabbit and one human samples. The usefulness of the diagnostic amplification assay might be compromised by the occurrence of intraspecific polymorphisms that could prevent the amplification of the diagnostic product in some individuals, resulting in false negatives. We tested the robustness of the assays with 87 lynx from 5 potentially different lynx populations (Appendix I). However, polymorphisms in other species, especially in closely related felid species, could result in false positives. In addition, the specificity of these PCR-based lynx diagnostic assays was further evaluated by applying them to good quality samples of a large and diverse set of individuals of different carnivore species from the Iberian Peninsula coming from as broad a distribution as possible, so as other three lynx species (Eurasian lynx, *Lynx lynx*, Canadian lynx, *Lynx canadensis* and bobcats, *Lynx rufus*; Appendix I).

Testing the suitability of the molecular markers for faecal samples

Optimization of faecal molecular analysis. A small portion (3–5 cm long) of the faeces was removed and immediately stored at ambient temperature in a paper envelope surrounded by excess silica gel with moisture indicator (Wasser *et al.* 1997), which was replaced as needed.

We compared two different Chelex-100 faecal DNA extraction methods (Walsh *et al.* 1991). In the first, ≈70 mg of the external part of dried faeces was washed with 1 mL of 70% ethanol, followed by two washes with 1 mL of double-distilled water. In each wash faecal material was

dispersed with a pipette tip, vortexed, centrifuged at 9000 g for 5 min, and the supernatant discarded. Following the last wash, the faecal sediment was resuspended in an equal volume of 10% Chelex-100 solution, incubated at 56 °C overnight and boiled for 5 min. After centrifugation at 16 000 g for 5 min, the supernatant was transferred to new tube and stored at -20 °C until further use. With the second method, the sample was superficially washed by gentle incubation in 350 µL of phosphate-buffered saline (PBS) once to isolate exfoliated colonic epithelial cells (Flagstad *et al.* 1999); sample disruption and homogenization was avoided. Subsequently, 200 µL of supernatant was transferred to a new tube, 200 µL of 10% Chelex-100 was added and from this point onward the sample was processed as in the first method. The efficiency of each method was compared with 37 lynx faeces. PCR were carried out with 35 cycles, 1 µM of primer, 5 µL of template DNA, and 0.8 µg/µL of BSA.

The primer pair DL7F/CR2bR was used to assess different aspects of the faecal identification technique as it consistently amplified well across both good and poor quality samples (see Results). As the marker DL7F/CR2bR was designed using DNA extracted from blood, we first tested whether the results were the same for DNA extracted from blood and faecal samples. We compared blood and faeces of eight individual lynx which we had both types of samples. The size of the amplified PCR products was identical for blood and faeces samples, although as expected, yields were greater for blood samples (data not shown). The optimal conditions for faecal DNA amplification were determined by varying several reaction parameters. From a baseline of 35 cycles, 1 µM of primer, 10 µL of template DNA and 0.8 µg/µL of BSA, we varied the number of cycles (35, 40 and 45), quantity of primers (0.2, 0.5 or 1 µM), amount of extract (5, 10 or 15 µL of template DNA), and BSA concentration (0.1, 0.5 or 0.8 µg/µL), one at a time in PCR of 22 different faeces samples, except for BSA tests that were conducted with 8 different samples.

Effect of age of faeces and technique efficacy. Two experiments were conducted to test whether age of faeces affected the efficacy of the technique. First, we examined the type of faeces that would commonly be found in field surveys. During three periods, winter (February), summer (July) and autumn (November), 25 lynx faeces were collected in a 700-ha area of the Doñana National Park (southwestern Spain, 37°9' N, 6°26' W), where we have been intensively monitoring Iberian lynx for more than 7 years (Palomares *et al.* 2001). We are confident that these faeces samples were from lynx as there are no domestic or wild cats in the area. Summer is usually very dry and hot, autumn is cooler and wet, and winter is the coldest period. Each collected faeces sample was assigned one of three possible age categories: fresh, medium and old. We classified as

fresh, faeces samples that were of dark colour, with intense odour and no insect degradation. Medium samples had some odour and limited insect degradation. Old faeces samples had no smell and had clear insect degradation. We estimated that faeces in these categories were < 4–5 days old, 5–15 days old, and older than 15 days, respectively.

In the second experiment we periodically collected small portions of 11 different faeces samples from 7 different lynx captured during a trapping season carried out in February 1999 in Valquemado (Sierra Morena, 38°15' N, 4°9' W), and one free-ranging lynx which we saw to defecate in February 1999 while collecting faeces in Doñana. For each faeces sample, we collected and conserved, as described above, a portion within the first 24 h, and new portions at the end of 1, 2, 3 and 4 weeks and at 2 and 3 months. After the first sampling at < 24 h old, all faeces was kept at -70 °C until set in outdoor conditions to continue with the experiment. An extraction and one amplification were undertaken for the faeces collected from the field, and two extractions and two amplifications per extraction were undertaken in the controlled experiment.

Estimates of the frequency of false negatives were obtained from the same series of extraction/amplification experiments testing for the effect of faeces age. There was a total of 7 such extraction/amplification experiments: 3 with 69 samples and 1 with 67 faeces or faeces portions from the controlled experiment, and 3 with 25 faeces from the field samples collected in Doñana (see above). From these data we were also able to estimate the frequency of PCR inhibition. Inhibition of PCR was indicated by the absence of primer dimer in negative amplifications. When PCRs were inhibited, we tried to solve the problem of inhibition by using less template DNA in the PCR (5 and 2 µL when needed).

Specificity of the DL7F/CR2bR marker for faeces identification. Reliability of the marker DL7/CR2bR (i.e. possibility of false positives) was also tested by analysing nonlynx carnivore faeces of two origins: captive or domestic individuals and faeces collected in the field far away from the any potential area of lynx presence. In the first case, we collected a total of 90 faeces samples of 9 species of carnivores (Appendix I). In the second case, we collected 10 faeces samples in each of 10 sites in northern Spain, the closest of which was situated at least to 300 km from the estimated 1988 distribution area of lynx (Rodríguez & Delibes 1992). The most common carnivores in these areas were wild cats, red foxes, stone marten, wolves, polecats, stoats, *Mustela erminea* and domestic dogs.

Pilot field study of Iberian lynx in Andalusia

The utility of the faecal identification technique was tested with carnivore faeces collected from throughout the state

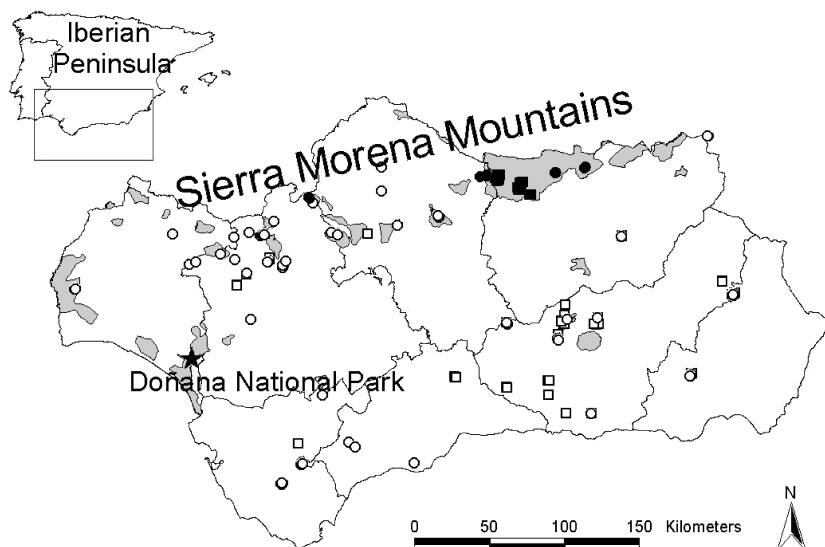


Fig. 1 Location of Andalusia within the Iberian Peninsula. The location of faeces diagnosed as positive (black) and negatives (open) during systematic (circles) and non-systematic (squares) surveys. Star indicates the location of the Doñana National Park. Shaded areas are the patches identified by Rodríguez & Delibes (1992) as probable lynx areas. Lines indicate provincial limits.

of Andalusia (south Spain; Fig. 1) between February and August 1999. Our aim was not to exhaustively survey the region for lynx, but instead to apply the technique through sampling a wide area in which lynx should be present only in some parts (Fig. 1; Rodríguez & Delibes 1992). For that, we planned systematic surveys for 73 natural areas with potential lynx habitats (see Palomares *et al.* 2000). In these areas 1–3 people walked looking for faeces for at least 4 h. From a total of 193 patches > 400 ha of potential lynx habitat identified in Andalusia using a geographical information system (F. Palomares, unpublished data), 25 were randomly selected and 48 were chosen because they were the most likely to have lynx (Rodríguez & Delibes 1992; see Fig. 1). Faeces were found in only 28 of the 73 patches during these systematic surveys. Additional samples from these and other patches were collected during nonsystematic surveys.

Faeces samples collected in the field to test for the presence of lynx were analysed as outlined in Fig. 2 using the primer pair DL7F/CR2bR.

Using a conservative strategy (i.e. trying to minimize false positive), we considered as clearly positive only samples that yielded PCR products that were notably more intense than those that usually were obtained from PCR contamination (Fig. 3).

Results

Design and evaluation of a PCR-based diagnostic assay for the Iberian lynx

Four primer pairs (DL7F/CR2bR [CTTAATCGTGCATTAT-ACCTTGT/CCGGAGCGAGAAGAGGTACA], DL1F/

DL5R [TTGCCAGTATGTCTTCACC/TACATGCTTA-ATATTCATGGGATT], CB4F/CB6R [ACATACATGCCA-ACGGG/GTGGCTATAACTGTAAATAGTAATAG] and CB4F/CB7R [ACATACATGCCAACGGG/TGGTAGGACAT-ATCCTATGAAG]), which yielded product amplification sizes of 130, 130, 136 and 161 pb, respectively, were designed to amplify Iberian lynx mtDNA but not other species from the Iberian Peninsula. The optimal annealing temperatures were 62.5 °C for DL7F/CR2bR and 65 °C for the other three primer pairs.

Testing primer pairs on lynx of as diverse origin as possible, in a first set of good quality samples ($n = 44$) from Doñana and Central population all were positively identified as lynx with all four diagnostic primers. To extend the test to populations for which good quality samples were not available, a total of 43 lynx samples of poor quality from museum specimens and hairs from wild animals were tested (Appendix I). The four lynx-specific primer pairs differed in their ability to amplify this suboptimal material. Whereas DL7F/CR2bR detected as positives all poor quality extracts (see Appendix I for localities), the rest of the primer pairs failed to amplify some samples (successful amplifications of 16.3% for CB4F/6R, 20.9% for CB4F/7R, and 51.2% for DL1F/5R).

In contrast, the diagnostic amplification product was never seen in specimens of the other sympatric species analysed, including 8 domestic and 35 wild cats (Appendix I), indicating a high specificity for all these makers. Products of the expected length were obtained from the other lynx species analysed (Eurasian lynx, Canadian lynx and bobcat) with primer pairs DL1F/DL5R and DL7F/CR2bR, but not with primers CB4F/CB6R and CB4F/CB7R.

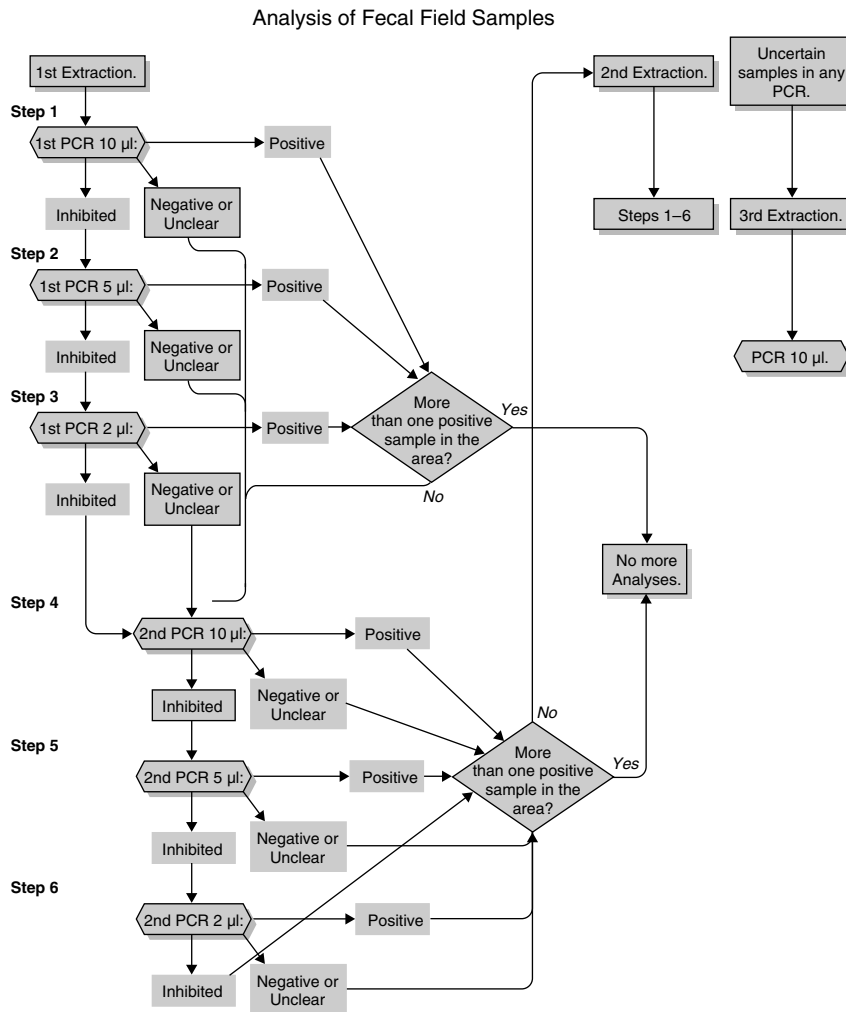


Fig. 2 Summary of the steps used for the analysis for faecal samples collected during the field survey for Iberian lynx in Andalusia.

Identification of Iberian lynx faeces

Extraction protocol and PCR optimization. Successful amplification from faecal DNA was observed from significantly more ($z = 2.829$, $P = 0.005$) of the DNA extracts obtained from the PBS wash (36 of 37 faeces) than from a homogenate of the faeces (26 of 37 faeces).

Amplification yields were highest using $1 \mu\text{M}$ of primer, but specific products were consistently amplified (100% of cases) with $0.5 \mu\text{M}$ of primer. Rates of successful amplification dropped significantly (72.7% of cases; $z = 2.198$, $P = 0.028$) using $0.2 \mu\text{M}$ of primers. There were problems of inhibition (18.2% of cases) when we used $15 \mu\text{L}$ of template DNA; however, inhibition disappeared when we used 10 or $5 \mu\text{L}$ of template DNA. Nevertheless, product yields were higher and more homogeneous with $10 \mu\text{L}$. With 45 cycles nonspecific bands and small traces of contamination appeared that were not evident with both 40 and 35 cycles. Band intensity was most consistent using 35 cycles. Finally, although with all 0.8, 0.5 and $0.1 \mu\text{g}/\mu\text{L}$

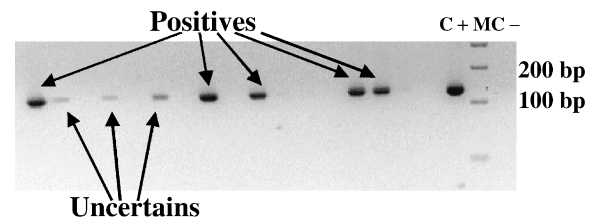


Fig. 3 Results of a PCR with lynx yields of different quantity. C+ = positive controls; C- = negative controls; M = size marker.

of BSA we obtained amplification of lynx DNA, products were highest using $0.8 \mu\text{g}/\mu\text{L}$ (data not shown). Therefore, we found that the optimal PCR condition for lynx faecal samples was 35 cycles, $10 \mu\text{L}$ of template DNA, $1 \mu\text{M}$ of primer and $0.8 \mu\text{g}/\mu\text{L}$ of BSA.

Effect of faeces age. Seventy-four of 75 putative lynx faeces samples collected from the field (36 old, 17 medium and 22 fresh) yielded the expected product with diagnostic

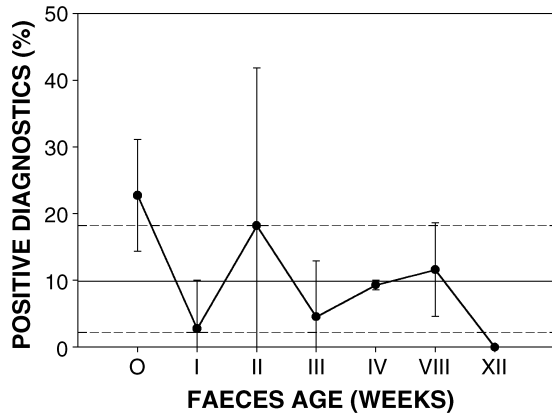


Fig. 4 Percentage (mean \pm 95% CI) of positive diagnostics for four series of amplifications of different-aged lynx faeces. The reference lines are the mean (continuous line) and the superior and inferior lines (broken lines) the 95% confidence interval around the mean.

primers DL7F/CR2bR. The only failed sample (medium-aged) was inhibited and was collected during July. In the controlled age-experiment, the average percentage of positive identifications ranged from 75% (SE = 2.27, $n = 4$ series of amplifications) for faeces collected within a few hours after defecation, to 100% for faeces collected 3 months later, however, the pattern was not clearly related with age of faeces (Fig. 4). Differences were due to problems of inhibition as the average percentages of inhibited samples inversely mirrored percentages of positive

diagnostics ($r = -0.994$, $P < 0.0001$, $n = 7$; Pearson product moment correlation; percentages arcsin transformed).

Specificity of the Iberian lynx specific marker. The specificity of primers DL7F/CR2bR as lynx-specific primers was high for carnivores found in the Iberian peninsula. We never obtained amplification product from faeces from known individuals of several species of carnivores ($n = 90$) or from field collected faeces ($n = 100$) from nonlynx areas. In 17% of cases there was evidence of PCR inhibition. Therefore, the actual number of nonIberian lynx faeces tested was 158.

Robustness of the technique. On average 92.6% (SE = 2.35, $n = 7$ series of extractions/amplifications) of the lynx samples were identified as positive, ranging from 84.1 to 100% (Table 1). The rest of the samples were mostly inhibited 6.8% (SE = 2.10) or were false negatives (0.6%, SE = 0.43; Table 1).

When we reamplified the inhibited samples using lower quantities of DNA template, the average percentage of accumulated positives (i.e. considering the sum of all positives), increased to 98.1% (SE = 0.44) when we used 5 μ L of template DNA and to 99.0% (SE = 0.70) when on the samples still inhibited with 5 μ L of template DNA we used 2 μ L of template DNA (Table 1). On average, the problem of inhibition persisted only in the 0.4% (SE = 0.43) of the samples, and the rest (mean = 0.6%, SE = 0.43) were false negatives (Table 1).

Two separate PCR using the same template DNA from only one extraction were enough to avoid false negatives.

Table 1 Accumulated positive diagnostics, false negatives and inhibited reactions during series of PCR for lynx faecal samples using 10 μ L of template DNA, 5 μ L of template DNA on samples that were inhibited with 10 μ L, and 2 μ L of template DNA on samples that remained inhibited using 5 μ L. n refers to number of faeces or faeces portions

Extraction	Accumulated positives-negatives-inhibited (%)			Total accumulated
	10 μ L	5 μ L	2 μ L	
Extraction A.1* ($n = 69$)	88.4-0-11.6¶	97.1-0-2.9	100-0-0	100-0-0
Extraction A.2* ($n = 69$)	84.1-2.9-13.0	94.2-2.9-2.9	97.1-2.9-0	97.1-2.9-0
Extraction B.1† ($n = 69$)	88.4-0-11.6	100-0-0	—	100-0-0
Extraction B.2† ($n = 67$)	91-1.5-7.5	95.5-1.5-3.0	95.5-1.5-3.0	95.5-1.5-3.0
Extraction C ($n = 25$)	96.0-0-4.0	100-0-0	—	100-0-0
Extraction D ($n = 25$)	100-0-0	—	—	100-0-0
Extraction F ($n = 25$)	100-0-0	—	—	100-0-0
Average‡ ($n = 7$ §)	92.6-0.6-6.8	98.1-0.6-1.3	99.0-0.6-0.4	99.0-0.6-0.4
	2.35-0.43-2.10	0.94-0.43-0.59	0.70-0.43-0.43	0.70-0.43-0.43

*These are two series of amplifications from the same extraction.
 †These are two series of amplifications from the same extraction.
 ‡Averages in the first row and standard error in the second.
 §It refers to number of series of extractions and/or amplifications.
 ¶In order, data refer to positives:negatives:inhibited.

In extractions A.1 and B.1 of Table 1, the percentage of positives was 100%. This result indicates that we were always able to extract DNA from the lynx faecal samples ($n = 213$ faeces or faeces portions) using our protocols.

Faecal field survey for lynx

We collected 252 faeces samples from throughout Andalusia, of which, after applying our protocol, 26.6% were classified as Iberian lynx, 72.2% as negative, 1.4% as PCR inhibited and 1.2% of uncertain diagnostics. Of the three faeces samples of uncertain diagnostics, two were only tested from one extraction, and one was PCR inhibited in all cases except one.

Comparing the results from each series of analysis, most positives were found in the first amplification of the first extraction (24.6%). Only a few of the remaining faeces samples were diagnosed as positives from a second amplification of the first extraction (3.7%), whereas no new positive was found from additional extractions/amplifications.

In addition to faeces from Doñana, positive faeces were found in only two other habitat patches of Andalusia (Fig. 1). Two of these areas are in the Sierra Morena Mountains, separated by > 100 km. These populations are 150 and 250 km from Doñana (Fig. 1). We found lynx faeces in only three of the areas that Rodríguez & Delibes (1992) considered to have the Iberian lynx in 1988 (Fig. 1).

Discussion

The molecular genetic technique

Methods for species identifications are generally based on the detection of species-specific DNA sequence patterns, either through sequencing followed by phylogenetic analysis (Bartlett & Davidson 1992; Farrell *et al.* 2000), or by indirect methods, like single-strand conformation polymorphism analysis (SSCP), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) or diagnostic amplification, that circumvent the cost of sequencing (several chapters in Clapp 1996). To identify carnivore species, Farrell *et al.* (2000) used an approach consisting in amplifying a portion of the cytochrome *b* gene of the mitochondrial genome followed by sequencing to differentiate between four carnivore species in Venezuela. However, simpler approaches involving only PCR amplification and agarose gel electrophoresis are more appropriate for medium- and large-scale projects because are the simplest and most economic assays. Some authors have adopted an approach involving amplification of part of cytochrome *b* or D-loop region of mtDNA with conserved universal primers, followed by digestion with diagnostic restriction enzymes to distinguish among

alternative species (Paxinos *et al.* 1997; Pilgrim *et al.* 1998; Hansen & Jacobsen 1999; Mills *et al.* 2000b; Foran *et al.* 1997a,b). The method that we adopted to identify Iberian lynx faecal samples was based on the specific diagnostic amplification of lynx DNA. In comparison to the restriction-based assays used to identify carnivores, our approach involves fewer steps (namely DNA isolation, PCR amplification and agarose gel electrophoresis), minimizing the chances of contamination, reducing costs and facilitating the screening of large numbers of samples across broad geographical areas. Furthermore, whereas the digestion-based approach requires the amplification of relatively long fragments, diagnostic amplification products can be designed to be short to facilitate the application of the method to fairly degraded material, such as faeces or hairs. Finally, the use of specific primers permits the application of the assay to multispecific samples, i.e. the lynx DNA will be detected even in the presence of excess nonlynx DNA. This feature is especially useful for the analysis of faecal samples, in which DNA from the donor species, its prey, microorganisms and often parasites and arthropods occur together. In the restriction-based approach, these diverse DNAs could be coamplified by conserved universal primers, confounding or even totally preventing the results. One limitation of our approach is that it yields a yes/no answer, so, if negative, the identity of the sample remains unknown. In cases in which it is important to identify the species, whichever it is, several alternative species-specific primers might be designed and used in parallel amplifications or even in the same tube if designed to amplify slightly different size products. Finally, even though the high success of the amplification made it unnecessary in our case, false negatives arising from failed amplifications might be identified by the same-tube coamplification of a slightly larger nonspecific product that serves as an internal amplification control.

The assay was validated by robustness and reliability tests. For the primer pair DL7F/CR2bR the assays were highly robust when applied to lynx of diverse geographical origin. It is therefore unlikely that there are polymorphisms at the annealing sites, or at least they do not prevent amplification of the diagnostic fragments. However, failed amplifications from museum skins with other primer pairs are most likely due to the lower sensitivity of these primer pairs when compared with DL7F/CR2bR in conditions of limiting template. This is particularly clear when comparing results for each primer pair from hair samples from the Doñana population. The assay based on DL7F/CR2bR was the most robust, detecting all lynxes tested independent of their origin and performing well even from traces of degraded DNA. Although we cannot totally discard the possibility that they were due to intraspecific polymorphisms, which would limit the usefulness of these

primers, this is unlikely in view of the limited mtDNA diversity found in Iberian lynx (Johnson *et al.* unpublished). Furthermore, our assay was shown to be highly reliable, as false positives were never obtained from a wide screening of domestic and wild carnivores species that may be sympatric to Iberian lynx.

The efficiency of our technique in identifying Iberian lynx faecal samples is high compared with similar methods (e.g. Kohn *et al.* 1995; Wasser *et al.* 1997; Farrell *et al.* 2000). Several features of our methodology appeared to be important to our high success rate. First, the use of PBS washes for faecal extractions was much more efficient than the use of faecal homogenates, and it was also simpler as it required fewer steps for the extraction. Second, although our extraction success was very high (we were able to extract lynx DNA in all of 213 attempts), 0–11.6% of the times we obtained results only after conducting other PCR with the same extract. The PCR inhibition was easily solved by decreasing the quantity of DNA template in the reaction mix.

Finally, the high success rate of the technique was due in part to the small mtDNA fragment that was amplified. Frantzen *et al.* (1998) also reported high success rates of amplified mtDNA in faecal samples of baboons (*Papio cynocephalus ursinus*) when the expected products were short (190 bp). For medium- and long products, success rates decreased slightly (Frantzen *et al.* 1998).

Two of the primer pairs designed to be specific for Iberian lynx did amplify the other three lynx species, providing a potential marker for the genus and for the species in areas where they do not overlap. However, their use as diagnostic tools for these species should be further evaluated, testing both for specificity and robustness as we did for the Iberian lynx.

Applicability of the technique to estimate the distribution area of elusive carnivores

The results of this study show that the method can be reliably applied to field surveys of Iberian lynx. The efficacy of the technique is very high, even for old faeces samples collected in a variety of weather conditions. The technique is also robust, with only a very low probability of false positives. Reliable use in old faeces is particularly important in studies on the distribution of species such as the Iberian lynx that are scarce and present in low densities.

The results of the field survey for Iberian lynx in Andalusia confirmed the high efficacy of the technique, as all positive lynx samples were detected from one first extraction of faecal samples. Nevertheless, additional DNA extractions and PCR amplifications with samples that gave uncertain or unconvinced results in a given area proved to be important. Two of the initial positive results

could not be confirmed in subsequent PCRs, demonstrating that weak results should be confirmed to avoid false positives.

Faeces age did not greatly affect the efficacy of the technique. That the molecular technique works well for old faeces samples is of particular importance because medium-aged and old faeces samples are the most commonly found in the field for species such as the Iberian lynx that live at low population densities. If fresh, well-conserved faeces samples were necessary for this technique, its applicability for field sampling would have restricted to those few circumstances of abnormally high local density or to very common species.

In designing the field collection of faecal samples, several factors should be considered. Farrell *et al.* (2000) found that amplification success was higher for faecal samples collected in the dry season (66%) than for samples collected in the wet season (28%). Although this will likely be a consideration for some studies, we were able to successfully amplify lynx DNA from samples collected both in dry and wet seasons. Some faeces collected in the wet season (November 1999) had even been under water for several days.

Another important consideration in the timing of field samples is whether the target species exhibits any seasonal pattern of marking behaviour with faeces, or if there are times of the year when encountering faeces is more likely. For instance, Eurasian otters, defecate more frequently in conspicuous sites in winter and early spring (Macdonald & Mason 1987), defecation rates of coyotes, *Canis latrans*, are higher in summer when they consumed a higher proportion of fruit (Andelt & Andelt 1984), and in southwestern Spain coprophagous beetles are more abundant in spring and summer (Revilla 1998), and can destroy faeces in minutes (personal observations). Nevertheless, most carnivores defecate in visible and predictable sites (Macdonald 1980; Roper *et al.* 1986; Robinson & Delibes 1988; Vila *et al.* 1994), increasing the potential of the proposed technique. Perhaps the timing of our sampling reduced the number of patches where faeces were found during our field survey in Andalusia (28 of 73 visited).

Distribution area of Iberian lynx

Our field survey was not specifically designed to accurately determine the distribution of the Iberian lynx in Andalusia. However, samples were collected from a broad area including places where it seemed clear that there were lynx 15 years ago (Rodríguez & Delibes 1992). Although many areas suspected to have lynx were sampled, lynx faeces were collected in only two of these areas. Both areas are in Sierra Morena mountains, but are probably isolated from each other. The one furthest east was predicted to be the largest remaining lynx habitat and to have the highest

number of individuals (Rodríguez & Delibes 1992). If our results reflect the actual status of lynx in Andalusia, they may almost exclusively survive in the largest patch detected 15 years ago. Unfortunately, this would be in agreement with previous predictions about extinction patterns for vertebrates (Diamond 1984; Pimm *et al.* 1988; Burkey 1995), and with the finding that, by 1988, Iberian lynx had disappeared from many of the smaller patches they had occupied in 1960 (Rodríguez & Delibes 1990). In addition to these localities in Sierra Morena, lynx also survive in the Doñana area, where intensive radiotracking of the population has taken place during the last 17 years (e.g. Ferreras *et al.* 1997; Palomares *et al.* 2001). Although the Doñana population consists of fewer than 50 individuals (Palomares *et al.* 1991), it represents the efforts by both national and local governments to conserve the lynx (Aymerich 1990).

Urgent studies on the accurate distribution of lynx are needed. Lynx usually defecate along the borders and crossing of trails (Robinson & Delibes 1988) and prominent places (personal observation), making it feasible for trained observers to survey for faeces. However, other studies to determine the best time of year to conduct field surveys should also be carried out to maximize the efficiency of survey efforts. Accurate determination of the presence of lynx in Spain's many isolated habitats is crucial for designing future management and conservation plans. Because, Iberian lynx live in isolated metapopulations, where dispersal is primordial for the survival of the species in most patches (Gaona *et al.* 1998), large-scale field studies focusing on rates of patch occupancy as a function of size, shape and isolation will also be important in developing a landscape ecology basis for the better conservation of the species. There is now hope that the needed information on lynx distribution and presence can be confidently obtained by sampling for faeces.

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Appendix I

Species, type and geographical origin of samples used in this study

Species	Faecal samples		Other type of samples	
	Number	Origin	Number†	Origin
Iberian lynx, <i>Lynx pardinus</i>	10	Central Population	47 ^{1,2,3,4,5}	Doñana
	1	Doñana	52 ^{1,3,4,6,9}	Central Population
	—		84 ^{6,7,8,9}	Sierra Gata
	—		34 ⁶	Unknown
	—		36 ⁹	Western Sierra Morena
Wild cat, <i>Felis silvestris</i>	—		1 ⁹	Mira
	20	Cádiz*, Madrid*, Albacete*, Jaén	35 ^{1,3}	Lerida*, Navarra, Badajoz, Palencia, Cádiz*, Madrid*, Albacete*, Jaén
Domestic cat, <i>Felis catus</i>	10	Sevilla, Huelva	8 ¹	Córdoba
European lynx, <i>Lynx lynx</i>			6 ¹	Switzerland, unknown
Canadian lynx, <i>Lynx canadensis</i>			6 ¹	Maine, unknown
Bobcat, <i>Lynx rufus</i>	8	Cádiz*	6 ¹	Florida
European genet, <i>Genetta genetta</i>	10	Madrid*, Albacete*	71 ^{2,3}	Doñana, Huelva, Madrid*, unknown
Eurasian otter, <i>Lutra lutra</i>	—		62 ³	Cádiz, Sevilla, Extremadura, Huelva
Pine marten, <i>Martes martes</i>	—		14	Navarra
Weasel, <i>Mustela nivalis</i>	—		13	Sevilla
Stone marten, <i>Martes foina</i>	—		22 ³	Huelva, unknown
Wolf, <i>Canis lupus</i>	6	Albacete*	4 ¹	Madrid*, Cádiz*, Albacete*
Egyptian mongoose, <i>Herpestes ichneumon</i>	7	Cádiz*	11 ^{1,2,3}	Doñana, Jaén, Cádiz*
Domestic dog, <i>Canis familiaris</i>	10	Madrid, Sevilla	13 ¹	Córdoba, Madrid
European badger, <i>Meles meles</i>	9	Cádiz*, Doñana	16 ¹	Doñana, Jaén
Red fox, <i>Vulpes vulpes</i>	10	Madrid*, Albacete*, Jaén	16 ¹	Doñana, Navarra, Jaén, Madrid*

*Samples from captive individuals.

†Superscript numbers refer to type of sample: 1, blood; 2, muscle; 3, kidney; 4, dry skin; 5, hair; 6, foot pad; 7, nose; 8, ear; 9, museum skin.