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Last lynxes in Portugal? Molecular approaches in a pre-extinction scenario

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Abstract

The Iberian lynx is the most threatened felid in the world and has suffered a decline throughout its range. Effective monitoring of the species' presence is essential. Fieldwork in previously identified areas of lynx occurrence in Portugal has resulted in the collection of 104 possible lynx scats. Recently, there has been little or no evidence of lynx presence and scats could be confused with others from more abundant carnivores such as wildcat, fox and dog. In order to confirm or not exclude the presence of the species, identification of scats was performed through the amplification of lynx-specific mitochondrial DNA sequences. Two samples collected in Malcata Natural Reserve in 1997 were identified as lynx. This is the most recent and reliable proof of lynx presence in Portugal*. Given the territorial behavior of lynx, stable resident populations would have produced a higher proportion of positively identified scats. Local extinctions might have taken place, and this genetic data supports a suspected national pre-extinction scenario for the species. Genetic analysis using a non-invasive approach has proved to be an informative part of the lynx monitoring program. Technical problems faced and overcome are also presented.

Introduction

The Iberian lynx (*Lynx pardinus*, Themmink 1777) is a Critically Endangered species (IUCN, 2002). It is a carnivore that strongly selects and depends upon European rabbit (*Oryctolagus cuniculus*) (Delibes et al. 2000), whose populations have suffered two severe crashes in the 20th century (Queney et al. 2000). Lynx habitat has been severely modified and reduced by extensive destruction (Delibes et al. 2000). Persecution of this predator has persisted despite legal protection (Ceia et al. 1998). Lastly, the Iberian lynx has an extremely fragmented (Nowell and Jackson 1996) and endemic distribution limited to Portugal and Spain (Delibes et al. 2000), which makes it particularly vulnerable to extinction (Bessa-Gomes et al. 2002). The constant delay in official approval and implementation of effective conservation plans by governments has compounded the critical situation of this species, considered, since the last decade, the most threatened felid in the world (Nowel and Jackson 1996). A decrease of 80% of its former range has occurred mainly since 1960 and therefore effective monitoring of its presence, throughout its range, has become a priority action for this species (Delibes et al. 2000).

Since an elusive and solitary carnivore is difficult to observe directly, scats are the main sign of its presence. Lynx home ranges, exclusive intrasexually, are intensely patrolled and marked with faeces as an effective system of advertising the presence of the territory owners to competitors (Robinson and Delibes 1988; Ferreras et al. 1997). Lynx scats are easy to spot and possible to identify visually by an experienced researcher. However, this identification is always prone to error (Halfpenny 1986 *in* Foran et al. 1997; Davison et al. 2002). Furthermore, confusion

* After acceptance of this paper a lynx scat from the Guadiana area collected in 2001 was identified by molecular analysis (Santos-Reis and Palomares, pers. com.).

is possible with the scats of other carnivores, such as wildcat (*Felis silvestris silvestris*), fox (*Vulpes vulpes*) and dog (*Canis familiaris*), which are generally more abundant.

Portugal has retained small lynx populations (less than 20 animals each) distributed in five areas of occurrence identified in 1997 (Ceia et al. 1998). Local monitoring of the presence of the Iberian lynx has been ongoing, mainly searching for scats, in order to confirm or not exclude the species' presence in those areas. In recent years, there have been several sightings of animals dispersed throughout the country. However, local fieldwork has generated little or no evidence of lynx presence. One hypothesis advanced to describe the situation of the lynx in Portugal is the collapse of its social structure, also reported for other lynx species (Knick 1990). Reduction of rabbit abundance and consequent permanent low availability of prey causes changes in territorial behaviour of resident lynxes. Individuals may become vagrant, thereby incurring higher mortality risks. Probability of extinction strongly increases and resident populations risk disappearance (Bessa-Gomes et al. 2002). It has therefore become extremely important to identify lynx scats beyond doubt to assess the presence of the species.

In recent years an increasing number of studies have used molecular scatology, yielding important information for biology and species conservation, for identification of species and individuals, sex typing, population genetics and estimation of population size (Höss et al. 1992; Foran et al. 1997; Reed et al. 1997; Kohn and Wayne 1997; Hansen and Jacobsen 1999; Kohn et al. 1999; Farrell 2000; Waits et al. 2000; Wasser et al. 2000). In this paper we present a trial work of identification of 104 samples collected in previously identified lynx areas using molecular techniques. Short lynx-specific mitochondrial DNA regions were amplified from scats to provide confirmatory information. Our aim was to supplement ongoing fieldwork by assessing the presence of the species, and assuring a trustworthy monitoring of the species.

Methods

Sampling

Regular fieldwork was carried out in the two most important lynx areas, Algarve and Malcata, but opportunistic sampling was done in other potential lynx areas (see Figure 1), providing a total of 104 samples mainly from 1997 to 2000 (Table 1). Only scats which match morphology or scent described for lynx (Brown et al. 1992; Blanco 1998) were collected.

The source of DNA in scats of wild mammals is thought to be cells exfoliated from the gastrointestinal mucosa as described in Albaugh et al. (1992) for human faeces. The tip of each scat, location of the majority of gastrointestinal cells, was removed and stored in a plastic vial containing silica isolated with porous tissue. The rest of the scat was frozen (–20 °C) in individual plastic bags. Both preservation procedures have previously been used with success (Frantzen et al. 1998). Samples were handled individually to avoid cross contamination.

DNA extraction

The challenge of DNA extraction in samples with poor DNA content is to extract a high enough quantity of DNA for amplification and simultaneously reduce the quantity of PCR inhibitors present in the sample.

Different extraction protocols using GuSCN-silica (Boom et al. 1990; Höss et al. 1992), diatomaceous earth (Gerloff et al. 1995), CTAB (Constable et al. 1995), chelex (Taberlet and Bouvet 1994), "GeneFizz" (Monnier et al. 1996) and commercial extraction kits (Wasser et al. 1997) had previously been tested in potential lynx scats (Castro et al. 1998) showing a variation in quantities and purity of extracted DNA sample for each individual scat. For the present study, in a preliminary comparison using the same samples, extraction kits gave slightly better results (data not shown). Considering that the use of extraction kits makes the probability of cross contamination lower, they were chosen for all the samples.

A small part of the tip and the outside of each dried scat fraction was removed and DNA extracted from it using Qiagen (Qiagen, Hilden, Germany) or IsoQuick (ORCA Research Inc. Bothell, WA) kits with the following adaptations.

During Qiagen extractions, lysis buffer (720 μl) was added to approximately 60 mg of dried faecal material. Samples were vortexed and incubated overnight in a rotary device at 56 °C with 80 μl proteinase K (20 mg/ml). Samples were then centrifuged at 12000 rpm for 1 min in a standard bench-top centrifuge. The supernatant (200 μl) was transferred to a fresh tube, 400 μl of AL buffer added and the mixture vortexed and incubated for 10 min at 70 °C. After adding 600 μl of cold ethanol (–20 °C), lysates

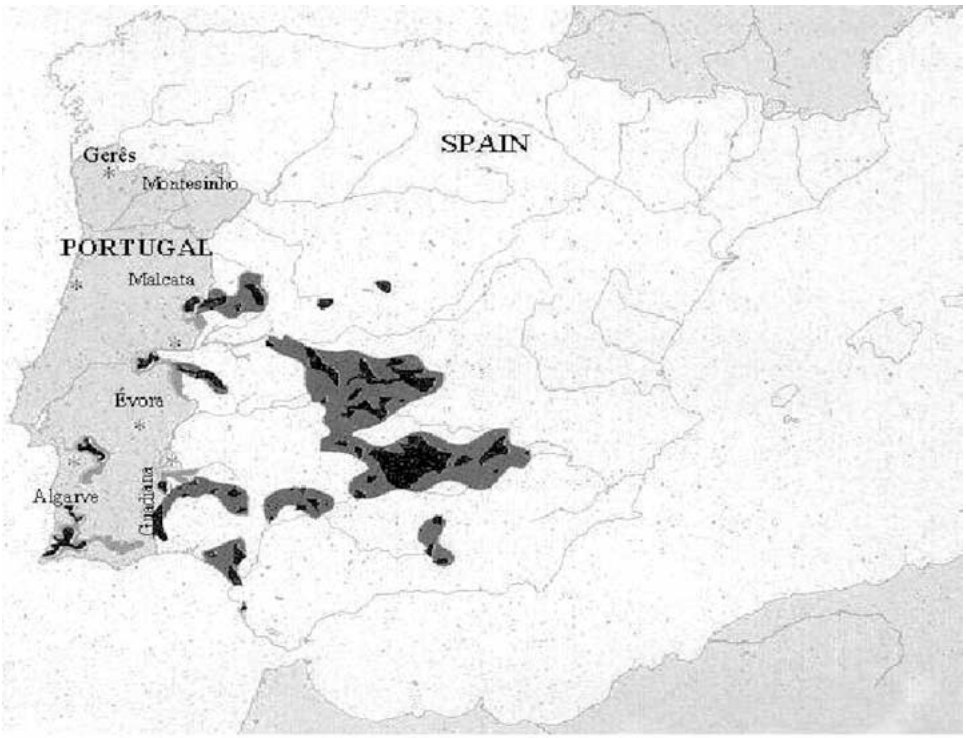

Figure 1. Iberian lynx distribution. Areas in black and grey indicate distribution of populations identified up to 1997, asterisks show areas of unexpected sightings of lynx in the last decade (adapted from Delibes et al. 2000). Location of the areas where scats were collected is named.

Table 1. Geographic distribution and year of collection of samples analysed in this study

Year	Location						Total
	Algarve	Évora	Gerês	Guadiana	Malcata	Montesinho	
1993	—	—	—	—	—	1	**1**
1995	2	—	—	—	—	—	**2**
1996	2	6	—	—	—	—	**8**
1997	4	1	1	1	7	—	**14**
1998	3	—	—	—	17	—	**20**
1999	21	—	—	3	15	—	**39**
2000	9	—	—	10	1	—	**20**
Total	**41**	**7**	**1**	**14**	**40**	**1**	**104**

were centrifuged twice through the spin columns, using two washing buffers. Finally, DNA was eluted in 200 μl of TE buffer in two centrifugation steps and stored at 4 °C.

For Isoquik extractions a 1:1 mixture of sample buffer (200 μl) and lysis buffer was added to approximately 60 mg of each dried scat. After a brief vortex and 90 min at room temperature the extract was centrifuged at 12000 rpm for 10 min and the supernatant (150 μl) purified following the protocol described by the supplier. DNA was eluted in 100 μl of sterile water and stored at 4 °C.

All faecal extractions were performed in a room isolated from PCR products. In all extraction sets, blank controls were included to monitor for contamination.

Table 2. List of Iberian lynx specific primers' sequences, their *loci*, lenght and expected product sizes after Palomares et al. (2002)

Name	Locus	Sequence (5'–3')	Size in bases	Expected product size	Strand[a]
DL1F	Control region	TTGCCCAGTATGTCTTCACC	20	DL1F/DL5R	L
DL5R	Control region	TACATGCTTAATATTCATGGGATT	24	130 bp	H
DL7F	Control region	CTTAATCGTGCATTATACCTTGT	23	DL7F/CR2bR	L
CR2bR	Control region	CCGGAGCGAGAAGAGGTACA	20	130 bp	H
CB4F	Cytochrome b	ACATACATGCCAACGGG	17	CB4F/CB6R	L
CB6R	Cytochrome b	GTGGCTATAACTGTAAATAGTAATAG	26	136 bp	H
CB7R	Cytochrome b	TGGTAGGACATATCCTATGAAG	22	CB4F/CB7R	H
				161 bp	

[a]Sequence is read from L (light) strand or heavy (H) strand.

Sample purification

DNA quantification by spectrophotometer was of limited effectiveness due to low DNA concentrations. Similarly, neither 2% agarose nor 6% polyacrylamide gel electrophoresis were informative.

To reduce individual variation of inhibitor content Microcon columns (YM 50; Millipore Corporation, Bedford, MA) were used before PCR amplification to clean and concentrate DNA. DNA samples of 200 μl volume were centrifuged at 5000 rpm for 2 min at room temperature. The cleaned DNA fraction (100 μl) was collected by a 3 min centrifugation at 10000 rpm.

Amplification

An experiment was designed involving two PCR amplification steps. Firstly, all samples were tested for the presence of DNA only, using the non-specific carnivore primers for mitochondrial DNA gene ATP8 (Johnson et al. 1998). Secondly, all samples were tested for lynx identity, by attempted amplification of known lynx-specific regions of mitochondrial DNA. Palomares et al. (2002) designed four species-specific primers selected to amplify Iberian lynx mitochondrial DNA but no other species (Table 2 lists the primer details). The robustness and specificity of these primers was proved to be very high; i.e., known lynx samples amplified in 99% of the cases and false positives were also never obtained from samples derived from different carnivore species. Furthermore the technique was proved to be efficient for degraded DNA samples such as old faeces exposed to environmental conditions (Palomares et al. 2002). In the present work we attempted amplification of each sample using all four primer pairs independently in order to maximise the probability of diagnostic amplification.

PCR conditions were as follows: 2 to 5 μl of template DNA was added to a PCR reaction mix containing a final concentration of 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.08% Nonidet P40, 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μg/μl of Bovine Serum Albumin (BSA; MBI Fermentas, Vilnius, Lithuania), 1 unit of *Thermus aquaticus* (Taq) DNA polymerase (MBI Fermentas) and 25 pmol of each primer, in a total volume of 25 μl. AmpliTaq Gold polymerase (Perkin Elmer, Foster city, USA) was used for some reactions with the same salt and BSA concentrations. Amplifications were performed as follows: an initial denaturing step of 94 °C for 3–10 min followed by 35–40 cycles of denaturing at 92 °C for 40 sec, annealing at 55 °C (for ATP8 primers) or 60–65 °C (for lynx specific primers) for 1 min and extension at 72 °C for 1 min. Reactions were left at 72 °C for 5–10 min as a final extension step.

All the amplifications were performed in a programmable UNO II Biometra thermocycler and the products detected by UV visualization after electrophoresis on an ethidium bromide-stained 2–3% agarose gel.

A positive control from captive lynx scats (Centro Acebuche, Doñana) collected following desiccation in the open air (and therefore exposed to natural degradation) was used in all amplification reactions as well as negative controls from wildcat and/or domestic dog tissues. PCR blanks were always included to monitor contamination. When a positive result was obtained, sample DNA was re-extracted and an independent amplification reaction performed.

Contamination

To avoid contamination, different rooms were dedicated for PCR reactions and for handling PCR products

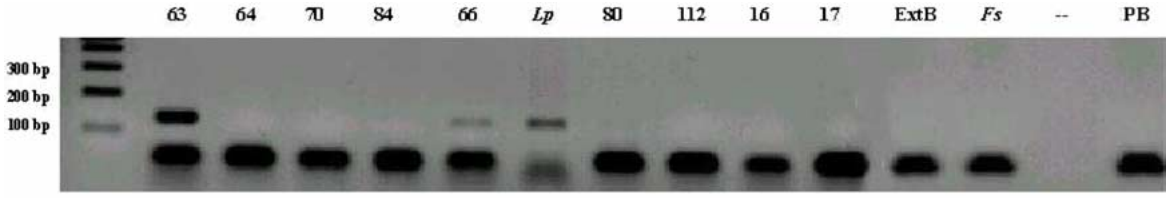

Figure 2. 2% agarose gel image showing amplification of a mitochondrial control region (130 bp) with lynx specific primers (DL7/CR2bR marker) from scats 63 and 66. Lp = *Lynx pardinus* scat, Fs = *Felis silvestris silvestris* muscle sample, ExtB = extraction blank and PB = PCR blank. The other numbers are negative scats. Strong primer dimer indicates absence of inhibition in PCR.

for electrophoresis. A different set of laboratory materials and filter pipette tips was always used in each room. Samples from tissues containing a much higher DNA content than scats (skins, muscle, blood), were handled in a third room.

Results and discussion

Lynx identification

Of the 104 samples, DNA was present in 95 as determined by visualized amplification products of ATP8. Primer dimers were not present after amplification of nine samples, suggesting the presence of polymerase inhibitors.

Of the 95 samples yielding DNA, only two proved to be lynx (see Figure 2). These results were confirmed by two independent replications carried out in Portugal and one in Spain. The samples produced positive results for the DL7F/CR2bR and DL1F/DL5R primer pairs combinations.

These new data confirm the presence of the species (one or two animals) at Malcata Natural Reserve during 1997. The identification of these scats is the most recent and reliable proof of lynx presence in Portugal*, although information on the stability of that presence is lacking. Since 1997, monitoring of the same area including intensive fieldwork has produced no further direct evidence of lynx presence (Sarmento and Cruz 2000). Given just these two positive results we cannot give an estimation of population size, although these could have been the last resident lynxes in Malcata. Another hypothesis, since Malcata is a transborder area, is that the scats were produced by dispersing individuals, but in either case the persistence of the species is called strongly into question.

Since positive controls always amplified, demonstrating that our PCR conditions were adequate for DNA derived from scats, and Palomares et al. (2002) showed that even very old scats can be identified successfully, we consider that the most likely hypothesis is that the other 93 scats were from carnivores other than lynx.

Since the lynx is highly territorial, even eliminating other carnivores from its territory (Palomares et al. 1996), it is expected that the majority of scats found in a lynx area, i.e., with a stable resident population, would belong to lynx. The density of lynx faeces is higher in areas where the home ranges of animals overlap (Robinson and Delibes 1988) but even isolated animals with an established territory keep their territorial marking behaviour using faeces (Francisco Palomares, pers. com.). Therefore, given that regular fieldwork took place for several years to collect samples for the work presented here (Table 1), we would have expected more than two positive lynx samples if resident lynxes were present. Comparatively, in a sample from Andalusia, one of the last redoubts of lynx in Iberia, a higher percentage of positive scats, 26% (n = 252), was found (Palomares et al. 2002). Although resident populations were thought to exist in Portugal, namely in the Algarve and Malcata, in 1997 (Ceia et al. 1998), our genetic results do not support such a scenario. This critical situation is also evidenced by a low number of sightings of live animals in the last years, apparent absence of dead animals since 1990 and no other direct evidence of the species such as footprints or a camera-trap photograph. The hypothesis of a pre-extinction scenario for the lynx in Portugal following rapid collapse of populations (Bessa Gomes et al. 2002) is consistent with our data.

Technical limitations

To explain possible false negatives, i.e., actual lynx samples which may not have been identified by our analyses, we also consider the following: (1) Lynx cells in a scat can be scarce and are probably the first to

suffer DNA degradation, being on the external surface; (2) Lynx cells might therefore not be included in some scat samples at the DNA extraction phase; (3) During the PCR set up, stochastic events when pipetting dilute or degraded DNA template could be a determinant of PCR amplification (Kohn and Wayne 1997); (4) There are PCR limitations related to the size of the amplified fragment. During necrosis of a tissue, cell lysis takes place and all genetic material is thus exposed to the action of environmental agents. Over time the average length of DNA fragments in a dead cell decreases (Hofreiter et al. 2001). Therefore, there is a possibility that some samples in this study no longer contained the complete diagnostic sequences (130 to 161 bp).

Contamination problems

Due to contamination between samples we obtained some false positives. A specific lynx fragment of 130 bp obtained with the DL1F/DL5R primer pair was detected simultaneously for six scat samples and also for a wildcat muscle sample in a reaction with a blank PCR control. To clarify this situation, the products were cloned and sequenced. All sequences were identical and identified as lynx, revealing a cross contamination with the positive control following DNA extraction. New DNA extractions were performed and no bands were detected afterwards. This result highlights the necessity of always replicating the procedure from the initial DNA extraction, as also suggested by Palomares et al. (2002).

Final remarks

A pre-extinction scenario is a unique situation, which can make monitoring of populations in the wild as important as it is difficult. In practical terms vagrant animals produce dispersed signs of presence, sometimes in unexpected areas. Genetic analysis can be a precise tool for the purpose of confirming presence in specific areas, while possibly also pinpointing population core areas.

The results of the genetic analysis of scats described in this paper point to a possible rapid decline and eventual disappearance of resident populations of lynx. This work contributes to the clarification of a suspected pre-extinction scenario in Portugal (Bessa-Gomes et al. 2002). As Palomares et al. (2002) stated for Andalusia, if a reduced quantity of positive scats are thought to reflect lynx status in this region, then the lynx has disappeared from most areas in which it was detected 15 years ago (Rodríguez and Delibes 1992).

Regional and national assessments of the Iberian lynx situation using genetic analysis are currently ongoing. It is predicted that results will confirm local extinctions in most lynx areas of Spain in a scenario similar to Portugal. Local extinctions can be the 'rehearsal' of species extinction (Caughley and Gunn 1996) and this critical situation enhances the urgent need to implement recovery plans for the species. Any such plan with a regional scope presently has to consider reintroduction or restocking. These actions will require many economic and human resources and have little guarantee of success as is known from other cases (IUCN 1998; Kloor 1999).

The Iberian lynx seems to have suffered a spectacular population crash in recent years. The species' numbers in Iberia have decreased from around 1000 individuals in Spain (Rodríguez and Delibes 1992) plus around 40 individuals in Portugal (Ceia et al. 1998) to less than 200 individuals in the wild (Guzmán et al. 2002). The continuous decline of this keystone species has shown the importance of implementing large scale actions in terms of habitat management and how co-ordination has failed to preserve the lynx in its former range. The generalised low density of rabbit populations and the progressive destruction of mediterranean scrubland are still major active threats to Iberian lynx but stochastic factors might also have been determinant for the disappearances of local populations which became too small, isolated and vulnerable to extinction (Bessa-Gomes et al. 2002).

Extinction of the Iberian lynx in Portugal would mean a great loss for the species since its natural variation would be reduced in terms of range, gene pool and ecological adaptations. This would also be the first national extinction of a large carnivore since the bear (*Ursus arctos*) in the XVII century (Baeta Neves 1967) and a great loss of biological diversity for the ecosystem given the top predator role that lynx has had for thousands of years; opportunistic carnivores such as red fox and mongoose would possibly increase their population numbers in the absence of competition with the lynx. Extinction of the lynx in Portugal would furthermore be a great loss of natural heritage and a symbol of failure in terms of nature conservation. Finally, the eventual extinction of Iberian lynx in both countries would be the first documented extinction of a cat species for at least 2000 years.

Some technical limitations faced during this study such as contamination and PCR conditions were thought to be of interest as tips for future studies. The use of cytochrome b gene primers (Janczewski et al. 1995) for identifying scats originating from other carnivore species was tried (data not shown) and revealed itself to be costly and time consuming.

This case study was the first attempt to use molecular scatology for conservation purposes in Portugal. It has proved that monitoring a species using molecular tools is possible and particularly useful in a pre-extinction scenario. It is a non-invasive method that might also be applied to the monitoring of other species such as the wolf (*Canis lupus*), another large carnivore presently threatened in Portugal. This technique is now part of the Portuguese lynx conservation plan.

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