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50,000 years of genetic uniformity in the critically endangered Iberian lynx

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Abstract

Low genetic diversity in the endangered Iberian lynx, including lack of mitochondrial control region variation, is thought to result from historical or Pleistocene/Holocene population bottlenecks, and to indicate poor long-term viability. We find no variability in control region sequences from 19 Iberian lynx remains from across the Iberian Peninsula and spanning the last 50 000 years. This is best explained by continuously small female effective population size through time. We conclude that low genetic variability in the Iberian lynx is not in itself a threat to long-term viability, and so should not preclude conservation efforts.

Keywords: coalescence, female effective population size, *Lynx pardinus*, mitochondrial DNA, mutation rate

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Introduction

The Iberian lynx, *Lynx pardinus* (Temminck 1827), classified as a Critically Endangered species (IUCN 2010), is the most endangered carnivore in Europe (Mallinson 1978) as well as the most threatened of the 36 extant species of the family Felidae (Nowell & Jackson 1996). The genus *Lynx* comprises three additional species: the bobcat (*L. rufus*), the Canadian lynx, (*L. canadensis*) and the Eurasian lynx (*L. lynx*). All major felid lineages were established within a relatively short time period spanning 10.8–6.2 Myr before present (Ma BP), and the split between the genus *Lynx*, the ocelot (*Leopardus pardalis*) and puma (*Puma concolor*) is estimated to have occurred approximately 8.0–6.7 Ma BP (Johnson *et al.* 2006). The geographical origin of the lynx lineage is uncertain. Palaeontological evidence points towards an African origin according to Werdelin (1981) whereas other authors suggest a North American origin (MacFadden & Galiano 1981; Martin 1989). Moreover, phylogeographic evidence has been interpreted as supporting a North American origin for the lynx lineage based on the fact that the most basal members of this group (*L. rufus* and *L. canadensis*) have an American distribution (Johnson & O'Brien 1997; Johnson *et al.* 2006). It has been suggested that at approximately 1.6–1.2 Ma the ancestors of the Eurasian and Iberian lynxes expanded across the Bering land bridge to Eurasia (Johnson *et al.* 2006).

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The Iberian lynx is thought to have been endemic to the Iberian Peninsula during most of the late Quaternary, where it has co-existed with its preferred prey species, the European rabbit (*Oryctolagus cuniculus*) (Branco *et al.* 2002; López-Martínez 2008). Although there are records of Iberian lynxes (the subspecies *Lynx pardinus spelaeus*, Boule 1910) in southern France during the Late Pleistocene (Kurtén & Granqvist 1987), there is no evidence of *Lynx pardinus* outside the Iberian Peninsula during the last glacial maximum (LGM) (Sommer & Benecke 2005). The postglacial occurrence of *Lynx pardinus* in France is not fully clear, although some Iberian lynx remains have been reported in France from the Holocene until the Iron Age (Vigne 1986, 1996; Vigne & Pascal 2003).

During the Late Glacial (16 950–11 550 BP), and perhaps even during the Holocene, the Iberian and Eurasian lynxes were sympatric in northern Iberia and France (Altuna 1980, 1981; Vigne & Pascal 2003; Sommer & Benecke 2005). One possible scenario that is consistent with the fossil record is that during the coldest periods, such as the LGM, the Eurasian lynx occupied the north of Iberia whereas the Iberian lynx would contract in range towards the south as a consequence of cold and unfavourable conditions, as well as interspecific competition from the Eurasian lynx. Conversely, during warm periods, the Iberian lynx would expand its distribution towards western, southern and central France.

The Iberian lynx was widely distributed throughout the Iberian Peninsula until about 100 years ago (Graells 1897). However, at the beginning of the twentieth century, it became rare in the north of the Iberian Peninsula (Cabrera 1914). Furthermore, although the main reduction in range and population size began 100 years ago, the Iberian lynx has gone through an even more dramatic decrease in population size during the last 50 years (Rodríguez & Delibes 1992, 2002). The decline of its main prey species, the European rabbit, and the loss and fragmentation of its habitat are thought to be the main reasons for this severe recent reduction (Rodríguez & Delibes 1992, 2002). During the 1980s the population size was estimated at 1000–1200 lynxes distributed in 48 patches and 9 populations in the southwest of the Iberian Peninsula (Rodríguez & Delibes 1992). According to Guzmán *et al.* 2004; the Iberian lynx currently persists in only two isolated populations, Andújar-Cardeña in Eastern Sierra Morena with an estimated population size of 60–110 individuals and Doñana with 24–33 individuals. However, its presence has also been reported in other areas of central Spain (Alda *et al.* 2008) and the latest census in 2010 reports a total of 73 individuals in Doñana, with 20 territorial females and 172 in Sierra Morena, with 43 territorial females (http://www.lifelince.org).

The mitochondrial DNA (mtDNA) control region is the most variable region of the mitochondrial genome (Aquadro & Greenberg 1983; Hoelzel & Dover 1991; Brown *et al.* 1993; Lopez *et al.* 1997). However, genetic analyses have shown that extant Iberian lynx populations display very low control region variability, with only one haplotype observed throughout its current distribution (Johnson *et al.* 2004). One additional control region haplotype, differing by one mutation from the major haplotype, has been observed in museum specimens from Huelva in the Western Sierra Morena (Johnson *et al.* 2004), but appears to have been lost when the lynx became locally extinct in the Western Sierra Morena. Low levels of genetic variation have been observed in many other endangered carnivores that have gone through recent or historical bottlenecks in population size, e.g. brown bears (*Ursus arctos*; Valdiosera *et al.* 2008), African cheetah (*Acinonyx jubatus*; O'Brien *et al.* 1983), the lions of the Ngorongoro crater (*Panthera leo*; Packer *et al.* 1991) and the Florida panther (*Puma concolor coryi*; Roelke *et al.* 1993). From a conservation perspective, the amount of genetic diversity in a population is expected to have a strong impact on its future survival (Frankham 2002, 2005). When isolated populations become critically small they may become prone to inbreeding depression. A population with low genetic variability may also be at risk of having a reduced capacity to adapt to changing environments, thereby reducing the long-term fitness of the population (Keller *et al.* 1994; Frankham *et al.* 2002; Frankham & Kingslover 2004). All of these factors apply to some extent to the populations of Iberian lynx. During the last decades, considerable conservation efforts have been conducted to avoid further loss of genetic diversity in the Iberian lynx populations from Doñana and Sierra Morena. The two existing populations of Iberian lynx are managed as a single unit. In 2007/2008, the first translocation of a male from Sierra Morena to Doñana was made in order to alleviate its low genetic diversity and to avoid a decrease in population size within the Doñana population (Ruíz *et al.* 2009).

In order to investigate when the decline in mtDNA control region diversity originated in the Iberian lynx, we have analysed control region sequences retrieved from a series of subfossil remains obtained from different geographic locations within the Iberian Peninsula, spanning a time range of approximately the last 50 000 years. Three hypotheses were tested: (i) mtDNA control region variability decreased as a consequence of the severe bottleneck that occurred 50 years ago, (ii) variation was lost much earlier, for example during a bottleneck coinciding with the Pleistocene/Holocene transition, or (iii) mtDNA variability has been low throughout the last 50 000 years.

Materials and methods

Samples and DNA extraction

A total of 51 Iberian lynx (bone and teeth) remains were collected from different localities in Spain, spanning a time range from the Late Pleistocene to the twentieth century (see Fig. 1 and Table 1). Specimens were sampled by grinding into powder using a multitool drill (DREMEL), and 150 mg of this bone/tooth powder was used for DNA extraction using both solvent and silica binding approaches (Yang *et al.* 1998; Leonard *et al.* 2000). A 183 bp fragment of the mitochondrial control region was targeted using two non-overlapping primer pairs for samples 4–19 and for samples 1–3 the same fragment was targeted using four non-overlapping primer pairs (Table 2). All samples that were successfully sequenced were radiocarbon dated using accelerator mass spectrometry (BETA Analytic Limited, London), except for those from recent specimens, where approximate sampling date was available, and samples from Valdegoba, whose age was determined from radiocarbon dates at the University of Oxford obtained from associated remains (Table 1). For consistency, all radiocarbon dates were recalibrated using the 'Bchron' library (Haslett & Parnell 2008; Parnell *et al.* 2008) within the statistical package ''R'' (URL: http://www.R-project.org/) and the IntCal09 calibration curve (Reimer *et al.* 2009).

In order to minimise the risk of contamination, the samples were divided in three different laboratories for extraction and amplification. Individuals 1–3 were extracted and sequenced at the Institute of Evolutionary Biology (CSIC-UPF), Barcelona (Spain), Individuals 4–15 at the Centro Mixto (ISCIII-UCM) de Evolución y Comportamiento Humanos in Madrid (Spain), and individuals 16–19 at the Center for Geogenetics in Copenhagen (Denmark). DNA amplification was performed in a 25 μL reaction consisting of 1× PCR buffer (Naxo /Qiagen), 1 mM $MgCl_2$ (Naxo/Qiagen), 0.8 μg/μL of bovine serum albumin (BSA) (Sigma), 0.2 mM dNTPs (Sigma), 0.2 μM of each primer and 4 Units of HotStarTaq DNA Polymerase (Naxo/Qiagen) and 3 μL of DNA extract. Cycling conditions were as follows: a 15 min activation step at 95 °C, followed by 54 cycles of 30 s at 94 °C, 30 s at 54 °C, 30 s at 72 °C, with a final extension of

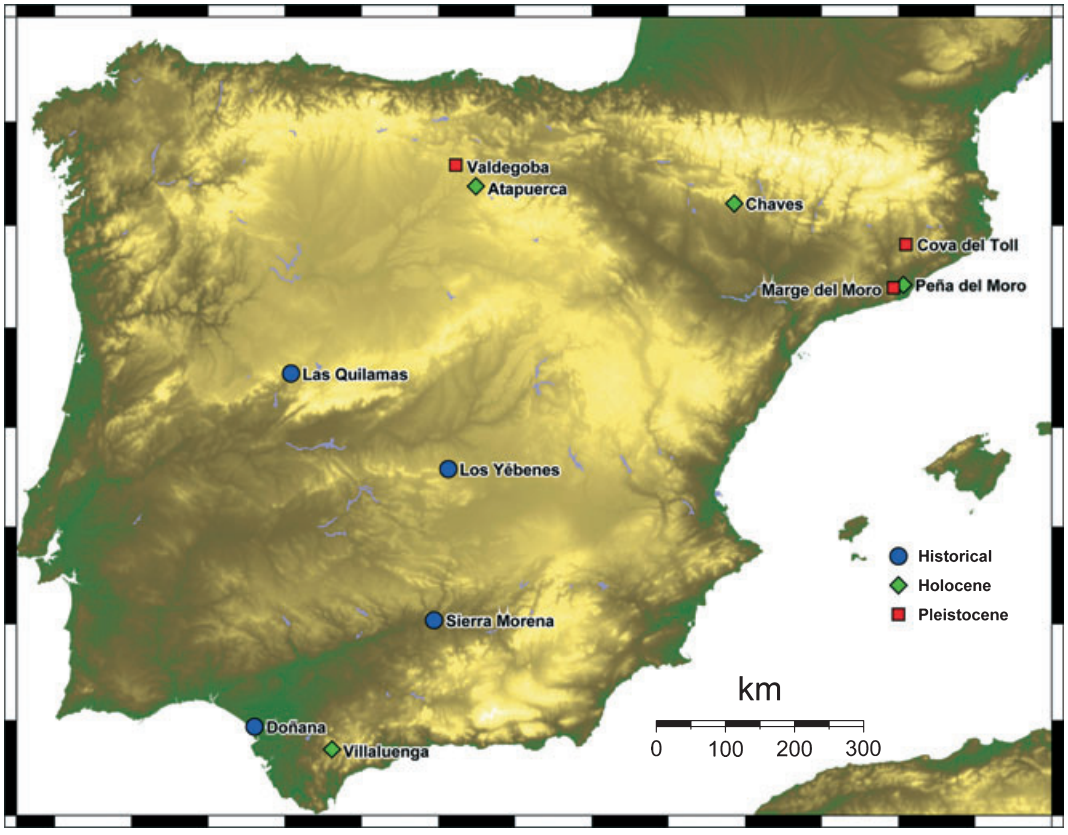

Fig. 1 Geographic locations of sites where samples were successfully amplified and sequenced. The map was generated using GMT software (http://gmt.soest.hawaii.edu/) and the ETOPO1 one arc-minute global relief model (Amante & Eakins 2009).

Table 1 Sample number, code, site and museum collection number for each sample

Nº	Code	Site	Collection no.	Age (uncal. BP)	Calibrated age (95% CI)	C14 lab numbers	Year of specimen collection	Exact age of the samples (until 2011)	Generations before 2011)
1	Peña del Moro V1374	Peña del Moro (Barcelona)	Peña del Moro V1374	2 210 ± 40	2 125–2 327	262440		2 186–2 388	547–597
2	Cova del Toll 9276	Cova del Toll (Barcelona)	Cova del Toll 9276	1 1420 ± 70	13 146–13 425	2 62 442		13 207–13 486	3 302–3 372
3	Marge del Moro 4201	Marge del Moro (Barcelona)	Marge del Moro 4201	1 7660 ± 90	20 551–21 413	2 62 441		20 612–21 474	5 153–5 369
4	Doñana22	Doñana (Sevilla)	A595864 (NRM)	NA	NA	NA	1 856	155	39
5	Quilamas26	Las Quilamas (Salamanca)	A585291 (NRM)	NA	NA	NA	1 955	50–155	13–39
6	Portalon46	Portalón (Atapuerca)	Portalon46	4 680 ± 40	5321–5569	2 62 443		5 382–5 630	1 346–1 408
7	Yebenes53	Los Yébenes (Toledo)	Yebenes53	NA	NA	NA	1 860–1 950	61–151	15–38
8	Sierra Morena 54	Sierra Morena (Jaén)	1842.2.26.1 (NHM)	NA	NA	NA	1 842	169	42
9	Doñana55	Doñana (Sevilla)	1872.10.26.1 (NHM)	NA	NA	NA	1 872	139	35
10	Sierra Morena56	Sierra Morena (Jaén)	1889.8.27.1 (NHM)	NA	NA	NA	1 889	122	31
11	Yebenes57	Los Yébenes (Toledo)	1894.6.11.1 (NHM)	NA	NA	NA	1 894	117	29
12	Doñana58	Doñana (Sevilla)	1895.9.4.1 (NHM)	NA	NA	NA	1 895	116	29
13	Yebenes3AR	Los Yébenes (Toledo)	Yebenes3AR	NA	NA	NA	1 860–2 000	11–151	3–38
14	Doñana4AR	Doñana (Sevilla)	Doñana4AR	NA	NA	NA	1 860–2 000	11–151	3–38
15	Doñana5AR	Doñana (Sevilla)	Doñana5AR	NA	NA	NA	1 860–2 000	11–151	3–38
16	Valdegoba87	Valdegoba (Burgos)	Valdegoba87	4 8400 ± 3300*	44 560 to infinite	OxA-21970		44 621–90 000	11 155–22 500
17	Valdegoba89	Valdegoba (Burgos)	Valdegoba89	4 8400 ± 3300*	44 560–infinite	OxA-21970		44 621–90 000	11 155–22 500
18	Chaves	Chaves (Chaves)	Chaves	6 020 ± 40	6 756–6 885	2 61 610		6 817–7 030	1 704–1 758
19	Villaluenga	Villaluenga (Cádiz)	Villaluenga	5 950 ± 40	6 679–6 885	2 61 609		6 740–6 946	1 685–1 737
20	EBD-1373	Western Sierra Morena	From Johnson *et al.* (2004)	NA	NA	NA	1 960–1 990	21–51	5–13
21	EBD-1376	Sierra de Gata	From Johnson *et al.* (2004)	NA	NA	NA	1 977	34	9
22	EBD-1377	Western Sierra Morena	From Johnson *et al.* (2004)	NA	NA	NA	1 960–1 990	21–51	5–13
23	EBD-1387	Eastern Sierra Morena	From Johnson *et al.* (2004)	NA	NA	NA	1 963	48	12
24	EBD-19299	Eastern Sierra Morena	From Johnson *et al.* (2004)	NA	NA	NA	1 990	21	5
25	EBD-22650	Eastern Sierra Morena	From Johnson *et al.* (2004)	NA	NA	NA	1 994	17	4
26	EBD-23122	Doñana	From Johnson *et al.* (2004)	NA	NA	NA	1 995	16	4
27	EBD-23127	Doñana	From Johnson *et al.* (2004)	NA	NA	NA	1 996	15	4
28	EBD-23223	Eastern Sierra Morena	From Johnson *et al.* (2004)	NA	NA	NA	1 997	14	4
29	EBD-23531	?	From Johnson *et al.* (2004)	NA	NA	NA	1 950–2 000	11–61	3–15
30	EBD-23731	Doñana	From Johnson *et al.* (2004)	NA	NA	NA	1 997	14	4
31	EBD-846	Gredos	From Johnson *et al.* (2004)	NA	NA	NA	1 968	43	11
32	MNCN-5446	Montes de Toledo	From Johnson *et al.* (2004)	NA	NA	NA	1 950–2 000	11–61	3–15
33	MNCN-5449	Montes de Toledo	From Johnson *et al.* (2004)	NA	NA	NA	1 950–2 000	11–61	3–15
34	MNCN-5450	Montes de Toledo	From Johnson *et al.* (2004)	NA	NA	NA	1 989	22	6
35	MNCN-5451	Montes de Toledo	From Johnson *et al.* (2004)	NA	NA	NA	1 974	37	9
36	MNCN-5452	Eastern Sierra Morena	From Johnson *et al.* (2004)	NA	NA	NA	1 979	32	8
37	MNCN-5454	Montes de Toledo	From Johnson *et al.* (2004)	NA	NA	NA	1 975	36	9
38	MNCN-5456	Montes de Toledo	From Johnson *et al.* (2004)	NA	NA	NA	1 950–2 000	11–61	3–15
39	MNCN-5462	Montes de Toledo	From Johnson *et al.* (2004)	NA	NA	NA	1 950–2 000	11–61	3–15

Table 1 (Continued)

Nº	Code	Site	Collection no.	Age (uncal. BP)	Calibrated age (95% CI)	C14 lab numbers	Year of specimen collection	Exact age of the samples (until 2011)	Generations before 2011)
40	MNCN-5463	Eastern Sierra Morena	From Johnson *et al.* (2004)	NA	NA	NA	1 976	35	9
41	MNCN-5466	Montes de Toledo	From Johnson *et al.* (2004)	NA	NA	NA	1 975	36	9
42	MNCN-5467	Montes de Toledo	From Johnson *et al.* (2004)	NA	NA	NA	1 970–1 974	37–41	9–10
43	MNCN 5468	Montes de Toledo	From Johnson *et al.* (2004)	NA	NA	NA	1 950–2 000	11–61	3–15
44	MNCN 5469	Montes de Toledo	From Johnson *et al.* (2004)	NA	NA	NA	1 950–2 000	11–61	3–15
45	MNCN 5470	Montes de Toledo	From Johnson *et al.* (2004)	NA	NA	NA	1 950–2 000	11–61	3–15

Sample ages are given as uncalibrated radiocarbon years and calibrated years before present, as well as the exact age of the samples in both years and number of generations (taking into account that 'present' is defined as the year 1950 for radiocarbon dates). * Samples from Valdegoba cave was determined from radiocarbon dates at University of Oxford obtained from an associated bone sample

10 min at 72 °C. Sequences were edited with Sequencher v.4.7 (Gene Codes Corporation, Ann Arbor, MI, USA) and manually aligned using the software MEGA version 4 (Tamura *et al.* 2007). Pleistocene and Holocene samples were sequenced at least three times from three different PCR products in order to obtain a consensus sequence, the historical ones were sequenced two times from different PCR products (Krause *et al.* 2006). From the 19 samples analysed, only Peña del Moro V1374, Cova del Toll 9276 and Villaluenga showed sequence inconsistencies likely caused by PCR misincorporation (C/T changes). However, these errors could be resolved by applying a majority rule consensus approach on the three replicates. The damage does not appear to correlate with specimen age since several of the oldest specimens showed no evidence of damage after three replicates. However, no historical samples (<200 years old) showed any evidence of damage. Generally, high presence of damage in a sequence data set would tend to overestimate diversity levels (Axelsson *et al.* 2008), which is something we did not detect in our study.

Mutation rate estimates and coalescence simulations

A mutation rate was estimated using the program BEAST (Drummond & Rambaut 2007) for the fragment of the control region analysed in this study. This was done using as calibrating point the date of split between the Eurasian lynx and the Iberian lynx; estimated to be 1.18 (95% CI = 0.7–1.98) (Ma BP) (Johnson *et al.* 2006). Two different analyses were performed, one assuming a demographic model of constant population size and the other a Bayesian Skyline Plot. For both analyses 10^8 permutations were performed with 25% burn in and using a strict clock model. Estimates of the mean rate and 95% highest posterior densities (HPD) were done using two approaches, one with a prior uniform distribution (interval 0.7–1.98 Ma) and the other with the initial value of 1.18 Ma and with a normal distribution adjusting the values so the 95% intervals of the distribution fit with the divergence time intervals.

To examine which combinations of mutation rate and long-term population size are compatible with the observed diversity in the ancient and modern mtDNA control region sequences generated, serial coalescent simulations were performed using the software Bayesian Serial SimCoal (Excoffier *et al.* 2000; Anderson *et al.* 2005) and assuming a constant female effective population size. We considered two datasets; the first consisting of the 19 sequences reported here and the second consisting of those 19 sequences plus the 26 sequences reported by Johnson *et al.* (2004). Consequently, two sets of coalescent simulations were performed to generate homologous datasets consisting of either 19 or 45

Table 2 Primer sequences and product sizes for control region amplicons obtained from samples analysed in this study

Primer Name	Sequence (5′–3′)	Sample	Product size (bp)	Region	Reference
CR2F/DL5R	GTGCTTGCCCAGTATGTC/ TACATGCTTAATATTCATGGGATT	4–19	134	Control region	This paper/(Palomares *et al.* 2002)
CR2F/LynxR1	GTGCTTGCCCAGTATGTC/ GTGGGTTGGTAGTTGATAGG	1–3	114	Control region	This paper/This paper
LynxF2/DL5R	TAAAAACCCCCTATCACCATG/ TACATGCTTAATATTCATGGGATT	1–3	92	Control region	This paper/(Palomares *et al.* 2002)
LI2F/CR2b_R	TAGTGCTTAATCGTGCATTATA/ CCGGAGCGAGAAGAGGTACA	4–19	133	Control region	(Hellborg *et al.* 2002)/ (Palomares *et al.* 2002)
LI2F/LynxR168434	TAGTGCTTAATCGTGCATTATA/ TTTCTCGAGACCAGGTGACT	1–3	95	Control region	(Hellborg *et al.* 2002)/ This paper
Lynx16831F/CR2b_R	GGACCTCAATTATCCAGAGGAAG/ CCGGAGCGAGAAGAGGTACA	1–3	86	Control region	(Hellborg *et al.* 2002)/ (Palomares *et al.* 2002)

Sample numbers are indicated in Table 1

sequences. For each dataset, 10 000 iterations were performed for each combination of 41 assumed effective female population sizes (N_{ef}) ranging from 10–10 000, and 64 assumed mutation rates (μ) ranging from 0.1 to 30% per million years, giving a total of 26 240 000 simulations for each dataset. Sequences of 183 bp were evolved on coalescent trees assuming a transition bias of 0.96296 and a continuous gamma distribution of mutation rates among sites, of parameter 0.35. Sequences were sampled (either $N = 19$ or $N = 45$) from each coalescent simulation according to the ages or age ranges (in generations, rounded to the nearest generation) presented in Table 1. A generation time of 4 years was assumed (O'Grady *et al.* 2008) and when the age of a sample was provided as a range, or a 95% confidence interval for calibrated radiocarbon dates, sampling times from the coalescent simulation were picked from a uniform distribution bracketed by that range. All ages or age ranges (both historical and radiocarbon) were calculated as before 2011 AD. For the two lynx samples from Valdegoba (Valdegoba87 and Valdegoba89) the radiocarbon dates were out of range for reliable calibration. However, the palaeontological age for these specimens has been estimated to be between 75 000 and 90 000 years (Quam *et al.* 2001). As a conservative measure, we therefore assumed an age range for these samples of between 44 621 (the lower bound of the calibrated radiocarbon date in years before 2011 AD) and 90 000 years (or 11 155–22 500 generations). The proportion of iterations for each combination of N_{ef} and μ that generated diversities equal to, or less than those observed were then collated and plotted as an image using the statistical package '*R*' (http://www.r-project.org). For simulations considering only the 19 sequences presented here, we calculated the proportion of iterations for each combination of N_{ef} and μ that generated a mean pairwise difference (MPD) equal to zero. For simulations considering the 19 sequences presented here plus the 26 sequences from Johnson *et al.* (2004), we calculated the proportion of iterations for each combination of N_{ef} and μ that generated a MPD equal to or less than 0.0869. This is the MPD of the combined sample of the 19 sequences presented here plus the 26 sequences from Johnson *et al.* (2004), and is the expected MPD for a sample of 45 sequences where only two are variant, both at the same single site. Execution of Bayesian Serial SimCoal was controlled and simulation results were harvested using scripts written in the programming language Python (http://www.python.org).

Results and discussion

We successfully amplified and sequenced DNA from 19 individuals (Table 1). A total of 183 bp was recovered

from the control region, corresponding to position numbers 16 392–16 483 and 16 783–16 873 (nucleotide numbers from the reference domestic cat sequence; Lopez *et al.* 1996). Only one haplotype was detected for all 19 samples. This haplotype corresponds to the one present in the contemporary populations.

At equilibrium the amount of genetic variation in a population is determined by the mutation rate and the effective population size. Low levels of genetic variation are often attributed to historical declines in population size due to past climatic and environmental changes, or human-related activities such as over-exploitation or habitat fragmentation (Bouzat *et al.* 1998; Leonard *et al.* 2005; Nyström *et al.* 2006). However, the analysis of modern and historical museum samples from the Morro Bay Kangaroo (*Dipodomys heermanni morroensis*) showed that low levels of genetic diversity were present in this species prior to its recent decline in population size (Matocq & Villablanca 2001). Similarly, a lack of mitochondrial control region diversity pre-dating a twentieth century bottleneck samples has been observed in Scandinavian wolverines (*Gulo gulo*), and it has been suggested that such a long-term low mtDNA diversity could be the result of postglacial founder events (Walker *et al.* 2001).

Our results indicate a lack of mtDNA control region diversity through time, with the same haplotype being observed in our sample over at least the last 50,000 years. This result is in stark contrast to previous ancient DNA studies on other extant mammals, which generally seem to have had higher levels of genetic variation during the Late Pleistocene than at present (e.g. Barnes *et al.* 2002; Shapiro *et al.* 2004; Dalén *et al.* 2007; Leonard *et al.* 2007; Valdiosera *et al.* 2007, 2008; Campos *et al.* 2010; Hofreiter & Barnes 2010). However, our results are based on only four samples of Pleistocene age. Thus, a more extensive sampling would be needed to further confirm the lack of genetic variability found in Pleistocene populations of Iberian lynx. Moreover, future studies of ancient DNA could be focused on other parts of the mitochondrial genome (e.g. the ATP-8 and cytochrome *b* genes), where some variability has been observed in the contemporary population (Johnson *et al.* 2004; Alda *et al.* 2008). Nevertheless, the fact that Pleistocene and living lynxes share the same control region haplotype supports the hypothesis that the ancient genetic diversity of the Iberian lynx was extremely low.

The Iberian lynx displays some of the lowest levels of mtDNA diversity among felids (Johnson *et al.* 2004). The results presented here strongly indicate that the low mtDNA diversity in the extant populations was not caused by the observed demographic decline in the last 50 years or the hypothesised bottleneck at the Pleistocene/Holocene transition. Instead, the lack of diversity over the last 50 000 years suggests an unusually low mutation rate in the mtDNA control region or a contin-

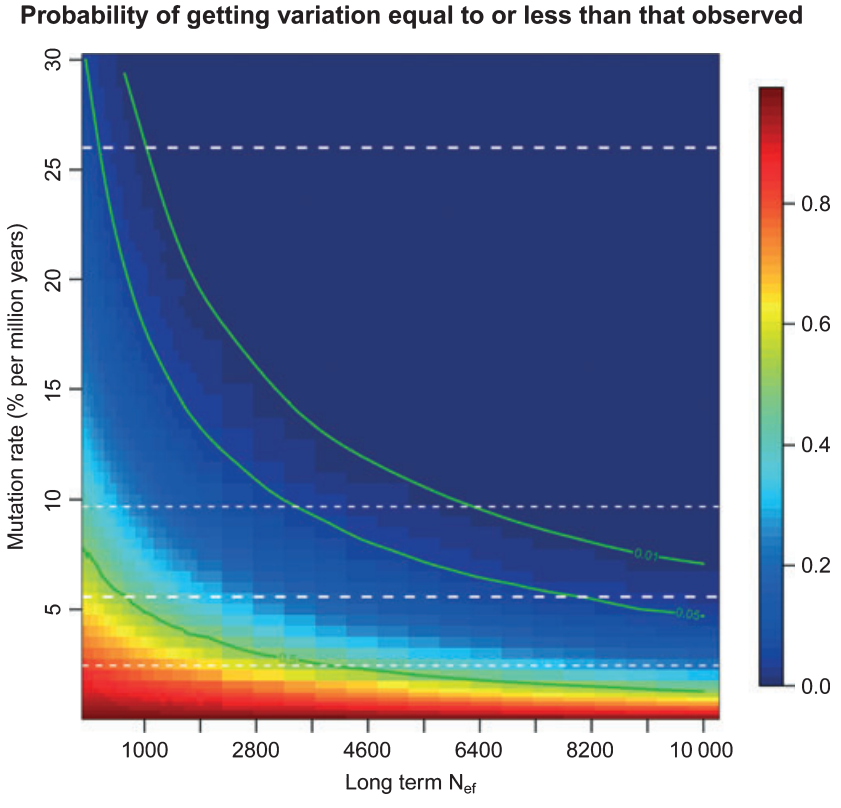

Fig. 2 Probabilities of observing a mean pairwise difference equal to or less than that detected in the 183 bp of Iberian lynx sequences reported in this study (GenBank accession numbers JN122007–JN122008) and Johnson *et al.*(2004), under different combinations of long-term constant female population size (N_{ef}) and mutation rate (μ, in % per million years). The *lower thick dashed line* indicates the estimated mutation rate for *Iberian lynx* (*thin dashed lines* are 95% HPDs), based on the split between *L. lynx* and *L. pardinus* and assuming a constant molecular clock. The *upper dashed line* shows a mutation rate estimated from a serially sampled cave lion dataset, assuming a time-dependency of the molecular clock (Ersmark E, Orlando L, Barnett R, Barnes I, Stuart A, Lister A, Dalén L (unpublished).

uously low N_{ef} through time (Fig. 2; Fig. S1, Supporting information). The different approaches to estimate the control region mutation rate using fossil calibration based on the split between the Eurasian and Iberian lynx gave similar results, with an average rate estimate of 5.57% (95% HPD = 2.44–9.68%) per million years. This rate seems to be lower than that reported for other mammals (Shapiro *et al.* 2004; Saarma *et al.* 2007). However, mutation rates of other lynx species do not seem lower than in other felids (e.g. Johnson & O'Brien 1997). It should be noted that the rate reported here was estimated using fossil calibration, and thus might not accurately reflect the intra-specific mutation rate, which may be much higher (Ho *et al.* 2011). Obtaining a correct estimate of the intra-specific mutation rate is important to evaluate the outcome of the simulation presented in this study. The lack of variation found in the Iberian lynx precludes the possibility to estimate the intra-specific mutation rate using internal calibration of the molecular clock (Drummond *et al.* 2002). However, a recent mutation rate estimate for cave lions (*Panthera spelaea*) obtained through internal tip-calibration yielded an estimate of 26% per million years for the control region (Ersmark E, Orlando L, Barnett R, Barnes I, Stuart A, Lister A, Dalén L (unpublished).). Some caution needs to be exercised when comparing mutation rates of lions and lynxes, given the differences in body size and generation time between the two taxa (Bromham & Penny 2003). Nonetheless, the cave lion study provides an indication that the mutation rate in felids may be higher than previously thought (Barnett *et al.* 2009; Ho *et al.* 2011).

Taking these mutation rate estimates into consideration, the results from the simulations suggest that the long-term N_{ef} has been relatively low in the Iberian lynx throughout the last 50 000 years. With a conservative estimate of the mutation rate, without controlling for time dependency of the molecular clock (Ho *et al.* 2005), the simulations suggest a long-term N_{ef} of less than 8100 females (Fig. 2). The higher mutation rate estimate, based on the cave lion internally-calibrated rate, would on the other hand correspond to a maximum long-term N_{ef} of 278 females (Fig. 2). Given the uncertainties in the current data set, both due to the small Pleistocene sample size and the difficulty in estimating a reliable mutation rate, it is difficult to provide a more exact assessment of the past effective population size. However, assuming that our sequence data is representative of the lynx's past diversity and taking into account the mutation rates estimated in other mammals, it seems likely that the effective population size has been comparatively small for several tens of thousands of years.

The Iberian lynx is endemic to the Iberian Peninsula, and seems to have been so throughout most of the species' history. The distribution and abundance of the Iberian lynx depends on the presence of its main prey, the European rabbit, which was also present in Iberia during the last glacial maximum (Branco *et al.* 2002; Rodríguez & Delibes 2002). The inferred small N_{ef} may thus be a consequence of both the species' endemism as well as its staple prey species dependence. The lack of observed mtDNA genetic diversity over at least 50 000 years indicates that it is not in itself a threat, nor is it a proxy for a threat to the long-term viability of the Iberian lynx. However, the geographic range and the population size have collapsed in recent decades, and today the Iberian lynx only persists in some small areas in central and southern Spain. While it is difficult to assess at what point loss of autosomal genetic variation will preclude long-term viability, we argue that the lack of modern mtDNA variation should not impede conservation activities.

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References

Alda F, Inogés J, Alcaraz L, Oria J, Aranda A, Doadrio I (2008) Looking for the Iberian lynx in central Spain: a needle in a haystack? *Animal Conservation*, **1**, 297–305.

Altuna J (1980) Hallazago de un Lince Nordico (*Lynx lynx* L. Mammalia) en la Sima de Pagolusieta, Gorbea (Vizcaya). *Munibe. Sociedad de Ciencias Naturales Aranzadi (San Sebastian)*, **3/4**, 317–322.

Altuna J (1981) Restos oseos de yacimiento prehistorico del Rascaño. *Centro de Investigacion y Museo de Altamira Monografias*, **3**, 223–269.

Amante C, Eakins BW (2009) ETOPO1 1 Arc-Minute Global Relief Model: procedures, data sources and analysis. *NOAA Technical Memorandum NESDIS NGDC-24*, 19.

Anderson CNK, Ramakrishnan U, Chan YL, Hadly EA (2005) Serial SimCoal: A population genetic model for data from multiple populations and points in time. *Bioinformatics*, **21**, 1733–1734.

Aquadro CF, Greenberg BD (1983) Human mitochondrial DNA variation and evolution: analysis of nucleotide sequences from seven individuals. *Genetics*, **103**, 287–312.

Axelsson E, Willerslev E, Gilbert MTP, Nielsen R (2008) The effect of ancient DNA damage on inferences of demographic histories. *Molecular Biology Evolution*, **25**, 2181–2187.

Barnes I, Matheus P, Shapiro B, Jensen D, Cooper A (2002) Dynamics of Pleistocene population extinctions in Beringian brown bears. *Science*, **295**, 2267–2270.

Barnett R, Shapiro B, Barnes I *et al.* (2009) Phylogeography of lions (*Panthera leo* ssp.) reveals three distinct taxa and a Late Pleistocene reduction in diversity. *Molecular Ecology*, **18**, 1668–1677.

Bouzat JL, Lewin HA, Paige KN (1998) The ghost of genetic diversity past: historical DNA analysis of the greater prairie chicken. *The American Naturalist*, **152**, 1–6.

Branco M, Monnerot M, Ferrand N, Templeton AR (2002) Postglacial dispersal of the European rabbit (*Oryctolagus cuniculus*) on the Iberian Peninsula reconstructed from the nested glade and mismatch analyses. *Evolution*, **56**, 792–803.

Bromham L, Penny D (2003) The modern molecular clock. *Nature Reviews Genetics*, **4**, 216–224.

Brown JR, Beckenbach AT, Smith MJ (1993) Intraspecific DNA sequence variation of the mitochondrial control region of white sturgeon (*Acipenser transmontanus*). *Molecular Biology and Evolution*, **10**, 326–341.

Cabrera A (1914) *Lynx pardellus* Miller. In: *Fauna Ibérica. Mamíferos*, pp. 207–210. Museo Nacional de Ciencias Naturales, Madrid.

Campos PF, Willerslev E, Sher A *et al.* (2010) Ancient DNA analyses exclude humans as the driving force behind late Pleistocene musk ox (*Ovibos moschatus*) population dynamics. *Proceedings of the National Academy of Sciences, USA*, **107**, 5675–5680.

Dalén L, Nyström V, Valdiosera C *et al.* (2007) Ancient DNA reveals lack of postglacial habitat trackingin the arctic fox. *Proceedings of the National Academy of Sciences, USA*, **104**, 6726–6729.

Drummond AJ, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology*, **7**, 214.

Drummond AJ, Nicholls GK, Rodrigo AG, Solomon W (2002) Estimating mutation parameters, population history and genealogy simultaneously from temporally spaced sequence data. *Genetics*, **161**, 1307–1320.

Excoffier L, Novembre J, Schneider S (2000) SIMCOAL: a general coalescent program for the simulation of molecular data in interconnected populations with arbitrarydemography. *Journal of Heredity*, **91**, 506–509.

Frankham R (2005) Genetics and extinction (Review). *Biological Conservation*, **126**, 131–140.

Frankham R, Kingslover JG (2004) Response to environmental change: adaptation or extinction. In: (edsFerrier R, Dieckman U, Couvet D), pp. 85–100. *Evolutionary Conservation Biology*. Cambridge University Press, Cambridge.

Frankham R, Ballou JD, Briscoe DA (2002) *Introduction to Conservation Genetics*. Cambridge University Press, Cambridge.

Graells MP (1897) *Felis pardina* (Temminick). Fauna mastodológica ibérica. *Memorias Real Academia Ciencias*, **XVII**, 224–229.

Guzmán JN, García F, Garrote G, Ayala R, Iglesias C (2004) *El lince ibérico (Lynx pardinus) en España y Portugal. Censo-diagnóstico de sus poblaciones*. Dirección General para la Biodiversidad, Madrid.

Haslett J, Parnell A (2008) A simple monotone process with application to radiocarbon-dated depth chronologies. *Journal of The Royal Statistical Society Series C-Applied Statistics*, **57**, 399–418.

Hellborg L, Walker CW, Rueness EK *et al.* (2002) Differentiation and levels of genetic variation in northern European lynx (*Lynx lynx*) populations revealed by microsatellites and mitochondrial DNA analysis. *Conservation Genetics*, **3**, 97–111.

Ho SY, Phillips MJ, Cooper A, Drummond AJ (2005) Time dependency of molecular rate estimates and systematic overestimation of recent divergence times. *Molecular Biology and Evolution*, **22**, 1561–1568.

Ho SY, Lanfear R, Phillips MJ *et al.* (2011) Bayesian estimation of substitution rates from ancient DNA sequences with low information content. *Systematic Biology*, **60**, 366–375.

Hoelzel AR, Dover GA (1991) Evolution of the cetacean mitochondrial D-loop region. *Molecular Biology and Evolution*, **8**, 475–493.

Hofreiter M, Barnes I (2010) Diversity lost: are all Holarctic large mammal species just relict population? *BMC Biology*, **8**, 46.

IUCN (2010). IUCN Red List of Threatened Species. Version 2010.3. <http://www.iucnredlist.org>. Downloaded on 12 September 2010.

Johnson WE, O'Brien SJ (1997) Phylogenetic reconstruction of the Felidae using 16S rRNA and NADH-5 mitochondrial genes. *Journal of Molecular Evolution*, **44**, 98–116.

Johnson WE, Godoy JA, Palomares F *et al.* (2004) Phylogenetic and phylogeographic analysis of Iberian lynx populations. *Journal of Heredity*, **95**, 19–28.

Johnson WE, Eizirik E, Pecon-Slattery J *et al* (2006) The Late Miocene radiation of modern Felidae: a genetic assessment. *Science*, **311**, 73–77.

Keller LF, Arcese P, Smith JNM, Hochachka WM, Stearns SC (1994) Selection against inbred song sparrows during a natural population bottleneck. *Nature*, **372**, 356–357.

Krause J, Dear PH, Pollack JL *et al.* (2006) Multiplex amplification of the mammoth mitochondrial genome and the evolution of Elephantidae. *Nature*, **439**, 724–727.

Kurtén B, Granqvist E (1987) Fossil pardel lynx (*Lynx pardina spelaea* Boule) from a cave in southern France. *Annales Zoologici Fennici*, **24**, 39–43.

Leonard JA, Wayne RK, Cooper A (2000) Population genetics of Ice Age brown bears. *Proceedings of the National Academy of Sciences, USA*, **97**, 1651–1654.

Leonard JA, Vilà C, Wayne RK (2005) Legacy lost: genetic variability and population size of extirpated US gray wolves (*Canis lupus*). *Molecular Ecology*, **14**, 9–17.

Leonard J, Vilà C, Fox-Dobbs K, Koch LP, Wayne RK, Valkenburgh BV (2007) Megafaunal extinctions and the disappearance of a specialized wolf ecomorph. *Current Biology*, **17**, 1146–1150.

Lopez JV, Cevario S, O'Brien SJ (1996) Complete nucleotide sequences of the domestic cat (*Felis catus*) mitochondrial genome and a transposed mtDNA tandem repeat (Numt) in the nuclear genome. *Genomics*, **33**, 229–246.

Lopez JV, Culver M, Stephens JC, Johnson WE, O'Brien SJ (1997) Rates of nuclear and cytoplasmic mitochondrial DNA sequence divergence in mammals. *Molecular Biology and Evolution*, **14**, 277–286.

López-Martínez N (2008) The lagomorph fossil record and the origin of the European rabbit. In: *Lagomorph biology evolution,*

ecology and conservatio (eds Alves PC, Ferrand N, Hackländer K). pp. 27–46, Springer, Berlin.

MacFadden BJ, Galiano H (1981) Late Hemphillian cat (Mammalia, Felidae) from the Bone Valley Formation of central Florida. *Journal of Paleontology*, **55**, 218–226.

Mallinson J (1978) Lynxes. European lynx (*Lynx lynx*) and pardel lynx (*Lynx pardina*). In: *The shadow of extinction: Europe's Threatened Wild Mammals*. pp. 141–148, MacMillan, London.

Martin LD (1989) Fossil history of the terrestrial Carnivora. In: *Carnivore Behaviour, Ecology, and Evolutio* (ed Gittleman JL). pp. 536–568, Cornell University Press Ithaca, New York.

Matocq MD, Villablanca FX (2001) Low genetic diversity in an endangered species: recent or historic pattern? *Biological Conservation*, **98**, 61–68.

Nowell K, Jackson P (1996) *Wild Cats: Status Survey and Conservation Action Plan*, IUCN publications. Burlington Press, Cambridge.

Nyström V, Angerbjörn A, Dalén L (2006) Genetic consequences of a demographic bottleneck in the Scandinavian arctic fox. *Oikos*, **114**, 84–94.

O'Brien SJ, Wildt DE, Goldman D, Merril CR, Bush M (1983) The cheetah is depauperate in genetic variation. *Science*, **221**, 459–462.

O'Grady JJ, Reed DH, Brook BW, Frankham R (2008) Extinction risk scales better to generations than to years. *Animal Conservation*, **11**, 442.

Packer C, Pusey AE, Rowley H, Gilbert DA, Martenson J, O'Brien SJ (1991) Case study of a population bottleneck – lions of the Ngorongoro Crater. *Conservation Biology*, **5**, 219–230.

Palomares F, Godoy JA, Piriz A, O'Brien SJ, Johnson WE (2002) Faecal genetic analysis to determine the presence and distribution of elusive carnivores: design and feasibility for the Iberian lynx. *Molecular Ecology*, **11**, 2171–2182.

Parnell AC, Haslett J, Allen JRM, Buck CE, Huntley B (2008) A flexible approach to assessing synchroneity of past events using Bayesian reconstructions of sedimentation history. *Quaternary Science Reviews*, **27**, 1872–1885.

Quam RM, Arsuaga JL, de Castro JMB *et al.* (2001) Human remains from Valdegoba Cave (Huermeces, Burgos, Spain). *Journal of Human Evolution*, **41**, 385–435.

Reimer PJ, Baillie MGL, Bard E *et al.* (2009) Intcal09 and Marine09 Radiocarbon Age calibration curves, 0–50,000 years Cal Bp. *Radiocarbon*, **51**, 1111–1150.

Rodríguez A, Delibes M (1992) Current range and status of the Iberian lynx *Felis pardina* Temminck, 1824 in Spain. *Biological Conservation*, **61**, 89–196.

Rodríguez A, Delibes M (2002) Internal structure and patterns of contraction in the geographic range of the Iberian lynx. *Ecography*, **25**, 314–328.

Roelke ME, Martenson JS, O'Brien SJ (1993) The consequences of demographic reduction and genetic depletion in the endangered Florida Panther. *Current Biology*, **3**, 340–350.

Ruíz G, López M, Fernández L, Franco JA, López G, Simón MA (2009) Short communication on the first Iberian lynx translocation from Sierra Morena to the Doñana population. In: *Iberian Lynx ex situ Conservation: An Interdisciplinary Approac* (eds Vargas A, Breitenmoser C, Breitenmoser U). pp. 445–447, Fundación Biodiversidad, Madrid, Spain.

Saarma U, Ho SYW, Pybus OG *et al.* (2007) Mitogenetic structure of brown bears (*Ursus arctos* L.) in north-eastern Europe and a new timeframe for the formation of brown bear lineages. *Molecular Ecology*, **16**, 401–413.

Shapiro B, Drummond AJ, Rambaut A *et al.* (2004) Rise and fall of the Beringian steppe bison. *Science*, **306**, 1561–1565.

Sommer RS, Benecke N (2005) Late Pleistocene and Holocene development of the felid fauna (Felidae) of Europe: a review. *Journal of Zoology*, **216**, 7–19.

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, **24**, 1596–1599. (Publication PDF at http://www.kumarlab.net/publications).

Valdiosera CE, García N, Anderung C *et al.* (2007) Staying out in the cold: glacial refugia and mitochondrial DNA phylogeography in ancient European brown bears. *Molecular Ecology*, **16**, 5140–5148.

Valdiosera CE, Garcia-Garitagoitia JL, Garcia N *et al.* (2008) Surprising migration and population size dynamics in ancient Iberian brown bears (*Ursus arctos*). *Proceedings of the National Academy of Sciences, USA*, **105**, 5123–5128.

Vigne JD (1986) *Le problème de l'interpretation des restes de repas en milieu funéraire vu au travers des ensembles fauniques de deux sites stratifiés: Can Pey (Montferrer, Pyrénées Orientales) et Capula (Lévie, Corse) Acted du Colloque Anthropologie Physique et Archéologie*. Editions du Centre National de la Recherche Scientifique, Paris.

Vigne JD (1996) La faune des vertébrés. In: *Le Laouret et la Montagne d'Alaric à la fin de l'age du Bronze. Un hameau abandonné entre Floure et Monze (Aude)* (ed Gasco J), pp. 197–239. Centre d' Anthropologie, Toulouse.

Vigne JD, Pascal M (2003) Le Lynx pardelle *Lynx pardinus* (Temnick, 1827). In: *Evolution de la Faune de Vertebres en France Metropolitaine depuis le debut de Holocene. Invasions et Extinction* (eds Pascal M, Lorvelec O, Vigne JD, Keith P, Clergeau P). pp. 87–89, National de la Recherche Agronomique, Museum National d'Histoire Naturelle de Paris.

Walker CW, Vila C, Landa A, Linden M, Ellegren H (2001) Genetic variation and population structure in Scandinavian wolverine (*Gulo gulo*) populations. *Molecular Ecology*, **10**, 53–63.

Werdelin L (1981) The evolution of lynxes. *Annales Zoologici Fennici*, **18**, 37–71.

Yang D, Eng B, Waye J, Dudar J, Saunders S (1998) Technical note: improved DNA extraction from ancient bones using silicabased spin columns. *American Journal of Physical Anthropology*, **105**, 539–543.

R.R.: Ancient DNA, phylogeography and conservation genetics. O.R.: Ancient DNA and conservation genetics. C.E.V.: Phylogeography, ancient DNA, paleogenomics and conservation genetics. N.G.: Palaeobiology, evolution and ecology of carnivores. F.A.: Rabbit hemorrhagic disease virus, molecular epidemiology and molecular evolution. J.M-M.: Paleontology and evolution. J.M.: Paleontology and evolution. I.D.: Biodiversity and evolutionary biology. E.W.: Ancient DNA and paleogenomics. A.G.: Ancient DNA techniques development and cattle domestication. J.L.A.: Evolutionary history of hominids. M.G.T.: Genetic anthropology. C.L-F: Ancient DNA, paleogenomics and human evolution. L.D: Ancient DNA, evolution and ecology of mammoths.

Data accessibility

DNA sequences for the two non-overlapping fragments have been deposited in GenBank under accession numbers JN122007–JN122008. Details regarding individual samples are available in Table 1.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Probabilities of observing no variation in the nineteen date-stamped samples of 183bp sequence reported in this study under different combinations of long-term constant female population size (N_{ef}) and mutation rate (in % per million years). The lower thick dashed line indicates the estimated mutation rate for Iberian lynx (thin dashed lines are 95% HPDs), based on the split between *L. lynx* and *L. pardinus* and assuming a constant molecular clock. The upper dashed line shows a mutation rate estimated from a serially sampled cave lion dataset, assuming a timedependency of the molecular clock (Ersmark E, Orlando L, Barnett R, Barnes I, Stuart A, Lister A, Dalén L, unpublished).

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