

Phylogenetic and Phylogeographic Analysis of Iberian Lynx Populations

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Abstract

The Iberian lynx (*Lynx pardinus*), one of the world's most endangered cat species, is vulnerable due to habitat loss, increased fragmentation of populations, and precipitous demographic reductions. An understanding of Iberian lynx evolutionary history is necessary to develop rational management plans for the species. Our objectives were to assess Iberian lynx genetic diversity at three evolutionary timescales. First we analyzed mitochondrial DNA (mtDNA) sequence variation to position the Iberian lynx relative to other species of the genus *Lynx*. We then assessed the pattern of mtDNA variation of isolated populations across the Iberian Peninsula. Finally we estimated levels of gene flow between two of the most important remaining lynx populations (Doñana National Park and the Sierra Morena Mountains) and characterized the extent of microsatellite locus variation in these populations. Phylogenetic analyses of 1613 bp of mtDNA sequence variation supports the hypothesis that the Iberian lynx, Eurasian lynx, and Canadian lynx diverged within a short time period around 1.53–1.68 million years ago, and that the Iberian lynx and Eurasian lynx are sister taxa. Relative to most other felid species, genetic variation in mtDNA genes and nuclear microsatellites were reduced in Iberian lynx, suggesting that they experienced a fairly severe demographic bottleneck. In addition, the effects of more recent reductions in gene flow and population size are being manifested in local patterns of molecular genetic variation. These data, combined with recent studies modeling the viability of Iberian lynx populations, should provide greater urgency for the development and implementation of rational in situ and ex situ conservation plans.

The Iberian lynx (*Lynx pardinus*), the largest remaining cat species in southwestern Europe, is one of the world's most endangered felids (Nowell and Jackson 1996). The genus *Lynx*, including the Iberian lynx, bobcat (*L. rufus*), Canadian lynx (*L. canadensis*), and Eurasian lynx (*L. lynx*) descend from a common ancestor that diverged from other cat species more than 6 million years ago (Johnson and O'Brien 1997). These four *Lynx* species have almost completely disjunctive distributions and occupy different habitats. However, they share numerous physical charac-

teristics and are distinguished primarily by their relative sizes (Nowell and Jackson 1996) and a few cranial or postcranial skeletal measurements (García-Perea 1992). There has been consistent support for a monophyletic ancestry for the four species from morphological (Nowak 1999) and DNA-based analyses (Janczewski et al. 1995; Johnson et al. 1996; Johnson and O'Brien 1997; Pecon-Slattery and O'Brien 1998). However, the hierarchical relationships among lynx species and their recognition as unique species have been debated.

Fossil records suggest that lynx species originated in North America (MacFadden and Galiano 1981; Martin 1989). The bobcat appears to represent the earliest lineage to diverge (Johnson and O'Brien 1997), but subsequent evolutionary history is less clear. The Iberian lynx and Eurasian lynx may be derived from an ancestral lynx species (*Lynx issiodorensis*) whose remains have been found in China (Wederlin 1981). The Canadian and Eurasian lynx have been hypothesized to be sister taxa (Wederlin 1981). The Iberian lynx and Eurasian lynx were both found in central Europe during the Pleistocene (Kurten 1968; Kurten and Grandqvist 1987), but may never have had significantly overlapping geographic ranges.

The distribution and population sizes of the Eurasian and Iberian lynx have been reduced significantly during the last two centuries. The Eurasian lynx was extirpated from most of central and southern Europe during the 19th century (Breitenmoser and Breitenmoser-Wursten 1990). Similarly, by the beginning of the 20th century the Iberian lynx was rare in northern Spain and by the 1960s its range was essentially limited to isolated populations in the southwestern portion of the peninsula (Rodriguez and Delibes 1990). By the 1990s, it was estimated that no more than 1000 lynx were restricted to fewer than 50 disjunct breeding areas that could be grouped into less than 10 distinct subpopulations (Castro and Palma 1996; Rodriguez and Delibes 1992). Only two of these subpopulations, Sierra Morena and Montes de Toledo (Figure 1) inhabit areas larger than 2000 km², which is of major concern since between 1960 and 1988 Iberian lynx are presumed to have disappeared from most of the remaining small habitat patches (from 91% of the areas smaller than 1000 km²). This corresponds to an 80% reduction in occupied range (Rodriguez and Delibes 1990). However, current geographical limits and population sizes are not well known and continued loss of habitat and the disappearance of lynx from previously occupied areas suggests that fewer than 500 individuals may now remain (Beltrán and Delibes 1994; Palomares et al. 2000; Pires and Fernandes 2003).

The best known of the Iberian lynx populations inhabits Doñana National Park, Andalusia, where field data on the species have been collected since the 1950s and radio-tracking studies have been conducted for more than 15 years (e.g., Ferreras et al. 1997; Gaona et al. 1998; Palomares et al. 1991, 2001). The Doñana metapopulation (1500 km²) of approximately 40–50 individuals currently is distributed in at least four distinct areas that were last completely connected during the middle of the last century. Within the Doñana metapopulation, the population occupying the protected parkland areas is relatively stable, while mortality in the less-protected areas is higher, especially among dispersing animals (70–85% mortality rate) (Ferreras et al. 1992; Gaona et al. 1998). Because of its small numbers and isolation, the Doñana metapopulation would be susceptible to low levels of genetic variation (Beltrán and Delibes 1993).

The Doñana metapopulation has been isolated from other Iberian lynx populations for at least 50 years by an expanse of more than 50 km of croplands to the north and almost 30 km of comparatively dense human settlements to

the west (Rodriguez and Delibes 1992). The nearest lynx populations are found in the Sierra Morena Mountains (Figure 1), where several of the largest remaining lynx metapopulations are suspected to still exist. This mountain range, perhaps one of the keys to the future preservation of the Iberian lynx, has received increasing attention by conservationist and resource managers.

The objectives of this study were to quantify the evolutionary relationship of the Iberian lynx with the other *Lynx* species using mtDNA sequence variation, describe patterns of lynx mtDNA variation across the Iberian peninsula, and compare patterns of microsatellite size variation and estimate gene flow between the important lynx populations of Doñana and Sierra Morena. An improved understanding of patterns of Iberian lynx molecular genetic variation and recent evolutionary history is necessary to develop rational management plans for the species (Vargas 2000) and provide further insights into the biogeographical history of the Iberian peninsula.

Methods

DNA Extraction

Lynx DNA was extracted from blood and tissue samples from 20 wild animals captured or found dead during capturing and radio-tracking of lynx from 1985 to 2000, and from 35 tissue and skin samples in scientific collections from seven metapopulations throughout the range of the Iberian lynx (Table 1). Blood samples were stored in four volumes of lysis buffer (0.1 M Tris-HCl pH 8.0; 0.1 M Na-EDTA; 0.01 M NaCl, 0.5% SDS) and tissue samples were kept frozen or at room temperature in a dimethyl sulfoxide (DMSO)-salt solution (20% DMSO, 0.25M Na-EDTA, and NaCl to saturation, pH 8.0). From museum specimens, approximately 1 cm² of skin was cut with a sterile scalpel after superficial cleaning with 10% commercial bleach, distilled water, and 70% ethanol. DNA extractions of museum materials were conducted in a dedicated room along with extraction blanks to monitor for contamination. DNA was extracted following standard proteinase K/phenol chloroform protocols (Sambrook et al. 1989); for museum skin extractions, several prewashes with NTE pH 9.0 (NaCl 10 mM; Tris base 50 mM; EDTA 20 mM) were included to remove possible enzyme inhibitors. For comparative purposes we used DNA extracted using similar techniques in previous studies from three European lynx (Lly 12, 15, and 16), two Canadian lynx (Lca 3 and 7), five bobcat (Lru 6, 26, 48, 68, and 73), two marbled cats (*Pardofelis marmorata*) (Pma 4 and 5), and one clouded leopard (*Neofelis nebulosa*) (Nne 80) (Johnson and O'Brien 1997).

Mitochondrial DNA Markers

Sequence variation in portions of five mtDNA genes (ATPase-8, 16S rRNA, 12S rRNA, NADH-5, and cytochrome *b*) was assessed in four Iberian lynx, three European lynx, two Canadian lynx, and five bobcat, along with two

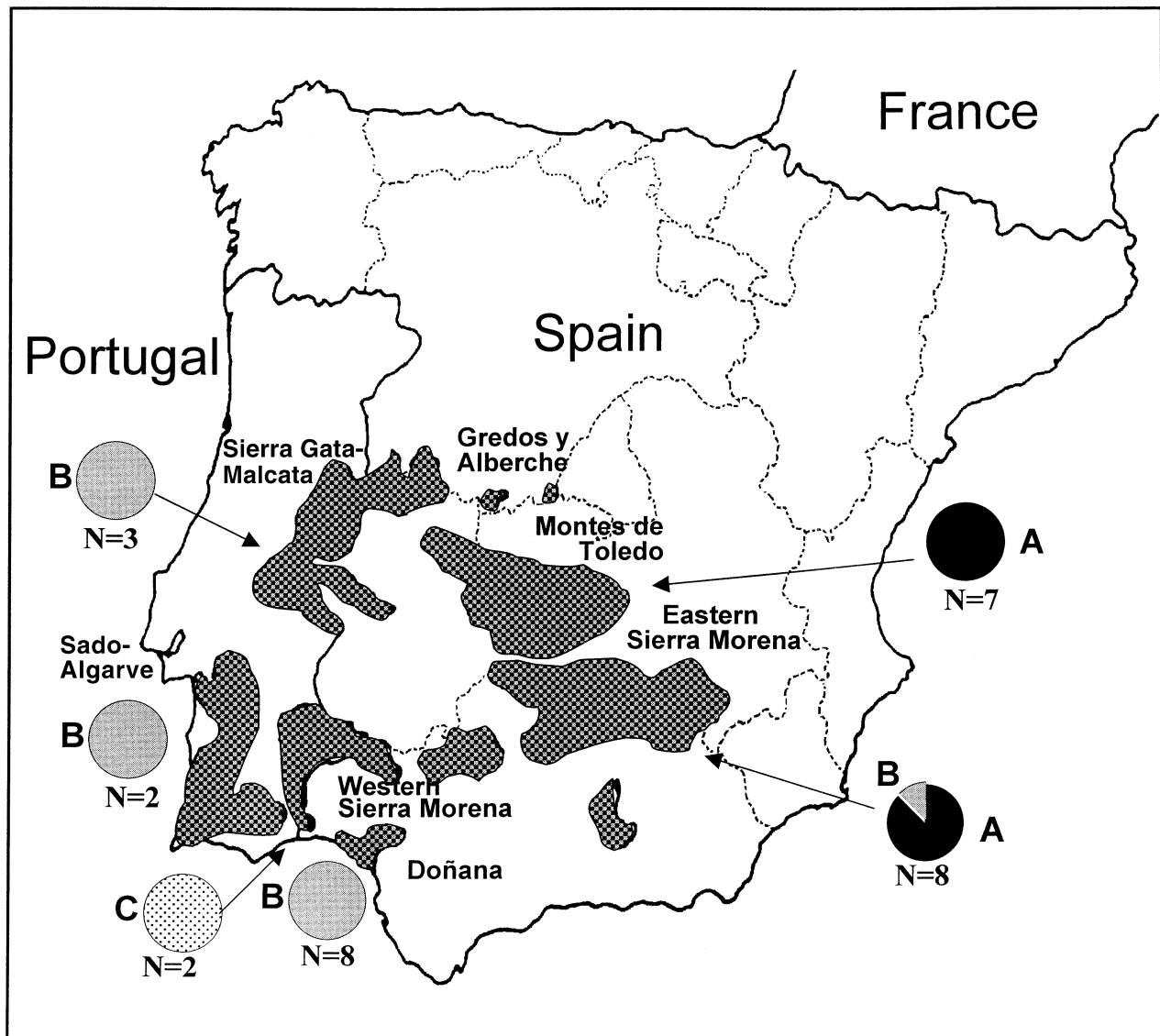


Figure 1. Geographic distribution of the Iberian lynx populations sampled in the study modified after Rodríguez and Delibes (1992) and Castro and Palma (1996). In Spain, the data represent estimated distributions from the 1980s and in Portugal the data are from 1987–1996. The distribution of mtDNA haplotypes in each of the major populations (which are labeled following Table 1) are represented in pie charts, along with the number of individuals sampled from each population.

marbled cats and a clouded leopard as outgroups, to assess the uniqueness of the Iberian lynx and to determine the evolutionary relationships among *Lynx* species. A larger set of Iberian lynx from across the Iberian peninsula (43 individuals from seven metapopulations; Table 1) was used to characterize patterns of mtDNA sequence variation in three mtDNA fragments: 191 of ATPase-8 gene, and 195 and 89 bp of two noncontiguous fragments of the hyper-variable segment 1 of the control region (Kim et al. 2001).

DNA amplification reactions contained 67 mM Tris-HCl pH 8.0, 16 mM $(\text{NH}_4)_2\text{SO}_4$, 2.5 mM MgCl_2 , 0.01% Tween-20, 0.2 mM dNTPs, 1 μM of each primer, 0.5 U of *Taq* polymerase and 50–100 ng of total DNA for blood samples or 5 μl of museum skin extracts as template. Polymerase

chain reaction (PCR) primers and primer conditions have been published previously (Janczewski et al. 1995; Johnson and O'Brien 1997; Johnson et al. 1998; Palomares et al. 2002). Bovine serum albumin (BSA) was included at a concentration of 0.1 $\mu\text{g}/\mu\text{l}$ for amplification of blood DNA and 0.8 $\mu\text{g}/\mu\text{l}$ for museum and skin samples. Amplification reactions were performed in an MJ Research (Boston) thermocycler, model PTC-100, at 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) for 30 s, extension at 72°C for 30 s, and were completed with a final extension at 72°C for 5 min. Positive and negative DNA controls were included with each set of PCRs. Amplification products were separated by

Table 1. Sample identifier, sample type, population and metapopulation of origin, nucleotide residue at positions 8672 and 16,804 (domestic cat sequence, López et al. 1996) and mtDNA haplotype for gene segments of ATP-8, control region 1 (CR1), and control region 2 (CR2) combined

Sample identification	Tissue	Population	Metapopulation	8672	16,804	mtDNA haplotype	Source
Barro*	Blood	Coto del Rey	Doñana	C	T	B	Authors
Borja*	Blood	Coto del Rey	Doñana				Authors
Escarlata*	Blood	Coto del Rey	Doñana				Authors
Gloria*	Blood	Coto del Rey	Doñana	C	T	B	Authors
Maki*	Blood	Coto del Rey	Doñana				Authors
Nuria*	Blood	Coto del Rey	Doñana	C	T	B	Authors
Vampi*	Blood	Coto del Rey	Doñana				Authors
23122	Skin	Coto del Rey	Doñana	C	T	B	EBD
Anibal*	Blood	Vera	Doñana				Authors
Celia*	Blood	Vera	Doñana				Authors
Cova*	Muscle	Vera	Doñana	C	T	B	PND
Isabel*	Blood	Vera	Doñana				PND
Jabata*	Tissue	Vera	Doñana				PND
Juanito*	Muscle	Vera	Doñana	C	T	B	Authors
Navidad*	Blood	Vera	Doñana				Authors
Understand*	Tissue	Vera	Doñana				PND
23127	Skin	Unknown	Doñana	C	T	B	EBD
23731	Skin	Unknown	Doñana	C	T	B	EBD
Ibiza*	Blood	Sierra Morena	Eastern Sierra Morena				Authors
Morena*	Blood	Sierra Morena	Eastern Sierra Morena	T	T	A	PND
Piriz*	Blood	Sierra Morena	Eastern Sierra Morena	T	T	A	Authors
Sierra*	Blood	Sierra Morena	Eastern Sierra Morena				Authors
Sofía*	Blood	Sierra Morena	Eastern Sierra Morena	T	T	A	Authors
22650	Skin	Sierra Morena	Eastern Sierra Morena	C	T	B	EBD
5452	Skin	Jaen	Eastern Sierra Morena	T	T	A	MCNM
5463	Skin	Jaen	Eastern Sierra Morena		T		MCNM
1387	Skin	Andujar Jaen	Eastern Sierra Morena	T	T	A	EBD
1732	Skin	Andujar Jaen	Eastern Sierra Morena	T			EBD
19299	Skin	Andujar Jaen	Eastern Sierra Morena	T	T	A	EBD
23223	Skin	Andujar Jaen	Eastern Sierra Morena	T	T	A	EBD
c28	Skin	Malcata	Sierra Gata Malcata	C	T	B	ICN
1376	Skin	Salamanca	Sierra Gata	C	T	B	EBD
c21	Skin	Penamacor	Sierra Gata Malcata	C	T	B	ICN
c22	Skin	Penamacor	Sierra Gata Malcata		T		ICN
5456	Skin	Montes de Toledo	Montes de Toledo		T		MCNM
5458	Skin	Montes de Toledo	Montes de Toledo	T			MCNM
5446	Skin	Los Yebenes To	Montes de Toledo	T	T	A	MCNM
5450	Skin	Los Yebenes To	Montes de Toledo	T	T	A	MCNM
5451	Skin	Los Yebenes To	Montes de Toledo		T		MCNM
5462	Skin	Los Yebenes To	Montes de Toledo	T	T	A	MCNM
5465	Skin	Los Yebenes To	Montes de Toledo	T			MCNM
5467	Skin	Los Yebenes To	Montes de Toledo	T	T	A	MCNM
5470	Skin	Los Yebenes To	Montes de Toledo	T	T	A	MCNM
5449	Skin	Toledo	Montes de Toledo	T	T	A	MCNM
5454	Skin	Toledo	Montes de Toledo	T	T	A	MCNM
5466	Skin	Toledo	Montes de Toledo		T		MCNM
5468	Skin	Toledo	Montes de Toledo		T		MCNM
5469	Skin	Toledo	Montes de Toledo		T		MCNM
1373	Skin	Huelva	Western Sierra Morena	T	C	C	EBD
1377	Skin	Huelva	Western Sierra Morena	T	C	C	EBD
c14	Skin	Alcáçovas	Sado Valley Algarve	C	T	B	ICN
C32	Skin	Alcácer	Sado Valley Algarve	C	T	B	ICN
846	Skin	Avila	Gredos o Alto Alberche	T	T	A	EBD
23531	Skin	Unknown	Unknown	T	T	A	EBD
C3	Skin	Unknown	Portugal	C	T	B	ICN

Metapopulations are also depicted on the map in Figure 1. Samples used in microsatellite analyses are marked with an asterisk (*) and samples from the Museo de Ciencias Naturales de Madrid (MCNM), Estacion Biologica de Doñana (EBD), Parque Nacional de Doñana (PND), and the Instituto da Conservação da Natureza (ICN) are noted. The additional samples were collected by several of the authors during field studies.

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 ultraviolet light and photographed with a digital image system (Eastman Kodak).

Polymerase chain reaction 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). Sequences were edited, assembled, and aligned using the program Sequencher (Gene Codes Corp.) and submitted to GenBank (accession numbers AY499248–AY499337).

Microsatellite Markers

Twenty-eight microsatellite loci (*Fca43*, 71, 75, 80, 82, 90, 96, 97, 98, 102*S*, 117, 161, 132, 193, 232, 272, 369, 391, 424, 441, 453, 476, 493, 519, 547, 566, 571, 698) from 11 of the 19 domestic cat chromosomes were characterized in 20 presumably unrelated lynx from two populations (Vera and Coto del Rey) in Doñana and in Valquemado from Sierra Morena (Table 1) following previously described PCR amplification conditions (Menotti-Raymond et al. 1997, 1999). All microsatellites were dinucleotide repeats except FCA391, FCA441, and FCA453, which had tetranucleotide repeats. Of the 28 loci, 22 were unlinked or at least 20 cM apart in the domestic cat and are presumed to be unlinked in Iberian lynx (Menotti-Raymond et al. 1999; submitted). Three pairs of loci were linked at distances of 9 cM (*Fca75* and *Fca96*), 8 cM (*Fca90* and *Fca566*), and 1 cM (*Fca132* and *Fca369*). The dye-labeled PCR products of the microsatellite primer sets were pooled and diluted together based on size range and fluorescent dye so that three to six loci could be multiplexed and electrophoresed and subsequently analyzed in an ABI 377 automated sequencer. Microsatellite allele sizes were estimated by comparison with a GS350 TAMRA (ABI) internal size standard. Data were collected and analyzed using the ABI programs GENESCAN (version 1.2.2-1) and GENOTYPER (version 1.1). PCR product length was used as a surrogate for actual repeat length (Ellegren et al. 1995).

Phylogenetic and Population Analyses

Phylogenetic comparisons among lynx species were conducted with sequence variation from five mtDNA gene fragments (ATPase-8, cytochrome *b*, 12S rRNA, 16S rRNA, and NADH-5). Marbled cat and clouded leopard sequences were included for outgroup comparisons. Sequences from each of the mtDNA gene fragments were combined into a contig of 1613 bp after separate analysis of each gene fragment (Huelsenbeck et al. 1996). Phylogenetic relationships among the haplotypes were estimated using minimum evolution (ME), maximum likelihood (ML), and maximum parsimony (MP) methods using PAUP* (Swofford 2001). An MP analysis was conducted using a heuristic search, with random addition of taxa and tree-bisection reconnection

branch swapping. The ME approach employed a neighbor-joining tree (Saitou and Nei 1987) constructed from Kimura two-parameter distances, with the proportion of invariable sites estimated to be 0.3687 from the empirical data and the rate for variable sites assumed to follow a gamma distribution. After testing and comparing several models, ML analysis was done using the HKY85 model (Hasegawa et al. 1985) with parameters estimated from the dataset. The reliability of the nodes in each of the analyses was assessed by 100 bootstrap iterations (Hillis and Bull 1993).

Mitochondrial DNA sequence variation across Iberian lynx was assessed in 452 bp from three mitochondrial gene fragments (ATP-8 and two control region segments). Measures of mtDNA sequence variation were estimated using MEGA 2.1 (Kumar et al. 2001). The divergence date among European lynx, Iberian lynx, and Canadian lynx was estimated by averaging all pairwise (*p*) distances among haplotypes. Feline-specific mtDNA divergence rates of 1.39% (ATPase-8), 0.97% (cytochrome *b*), 1.22% (NADH-5), 0.88% (12S rRNA), and 0.97% (16S rRNA), as developed by López et al. (1997), were weighted based on the number of base pairs used for each gene to obtain a composite divergence rate of 1.04% per million years.

Estimates of microsatellite size variation, such as average expected heterozygosity, average variance, number of unique alleles, and average number of repeats, were derived from the program MICROSAT (version 1.5) (Minch et al. 1995). Deviations from Hardy-Weinberg equilibrium, following the procedure of Guo and Thompson (1992), and estimates of population subdivision, F_{ST} , and R_{ST} analogs (Michalakis and Excoffier 1996; Slatkin 1995; Weir and Cockerham 1984) were derived using ARLEQUIN (Schneider et al. 2000).

Pairwise genetic distances among individuals using the composite microsatellite genotypes were estimated using the proportion of shared alleles (Dps) algorithm with a $(1 - M)$ correction as implemented in the program MICROSAT (version 1.5) (Minch et al. 1995). A phylogenetic tree was constructed from the Dps distance matrix using the Neighbor option of the program PHYLIP (version 3.572) (Felsenstein 1993) and was drawn using the program TREEVIEW (version 1.5) (Page 1996).

Results and Discussion

Phylogenetic Analyses

Analysis of the evolutionary relationships among the four *Lynx* species from 1613 bp of sequence from five mtDNA genes (ATPase-8, 16S rRNA, 12S rRNA, NADH-5, and cytochrome *b*) confirmed the taxonomic status of the Iberian lynx as a unique species with a relatively long evolutionary history (Beltrán et al. 1996). Among the four *Lynx* species, there were 158 variable sites, of which 141 were parsimoniously informative (Figure 2). There were two Iberian lynx haplotypes from four individuals, two haplotypes from three European lynx, two haplotypes from two Canadian lynx, and three haplotypes from five bobcats, along with two marbled

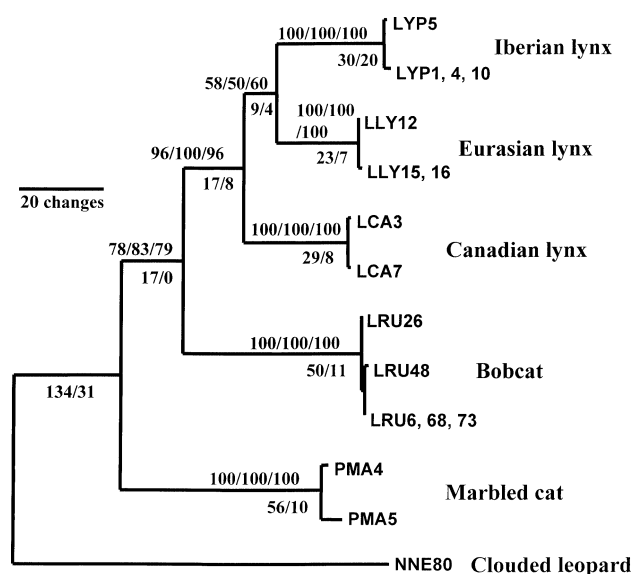


Figure 3. Phylogenetic relationships among *Lynx* species and outgroup Felid species from 1639 bp of sequence from six combined mtDNA gene fragments. Depicted is a maximum likelihood phylogenetic tree constructed with the HKY85 model using empirical nucleotide frequencies, a transition/transversion ratio of 12.6, an assumed proportion of invariable sites of 0.295, and a shape parameter (α) of 0.206. Above the branches are bootstrap values (100 iterations) for maximum parsimony/minimum evolution/maximum likelihood analyses and below the branches are the number of base substitutions/number of homoplasies from the maximum parsimony analyses. Maximum parsimony trees were obtained via a tree-bisection reconnection algorithm with starting trees obtained by stepwise addition. Minimum evolution trees were depicted using the neighbor-joining algorithm using Kimura two-parameter distances.

These results confirm that the Iberian lynx is a unique species and that the Eurasian lynx, Canadian lynx, and Iberian lynx all speciated or diverged into monophyletic lineages around the same time. Assuming an mtDNA divergence rate of 1.04% per million years, based on a feline gene-specific mtDNA mutation rate (Culver et al. 2000; Johnson and

O'Brien 1997), the rapid divergence among the three lynx species occurred around 1.53–1.68 million years ago.

Mitochondrial DNA Diversity

Mitochondrial DNA sequence variation across 46 Iberian lynx from throughout most of their distribution on the Iberian Peninsula (from seven metapopulations) was assessed in 452 bp from three mitochondrial gene fragments (positions 8657 to 8818, as numbered in the complete *Felis catus* mtDNA sequence of López et al. [1996] and two control region segments). Because many of these samples were hides from museums, not all individuals amplified for each of the three gene segments. MtDNA diversity among Iberian lynx was low (Figure 2 and Table 2). Among the 46 Iberian lynx sequenced, there were two variable sites in 452 bp from three mtDNA fragments that defined three haplotypes (A, B, and C). At position number 8672 (nucleotide numbers from the reference domestic cat sequence; López et al. 1996) in the ATP-8 gene, there was either a T (haplotypes A and C) or a C (haplotype B). At position number 16,804 of the control region, two individuals from the Sado-Algarve metapopulation had a C (haplotype C), compared with T for the other lynx (haplotypes A and B). All eight Iberian lynx from the southernmost metapopulation of the Doñana National Park area had haplotype B, as did one lynx from eastern Sierra Morena, two lynx from Sado-Algarve, Portugal, and four lynx from Sierra Gata Malcata, Portugal. Haplotype A was found in the easternmost metapopulations of eastern Sierra Morena and Montes de Toledo. Haplotype C was restricted to the two samples from the western Sierra Morena metapopulation (Figure 1).

The Iberian lynx displayed among the lowest levels of mtDNA diversity that have been documented for a felid species, as is apparent from a comparison of the sequence variation across the roughly 880 bp from the mtDNA gene fragments of NADH-5, 16S, and ATP-8 among several cat species (Table 2). This overall pattern suggests that Iberian lynx is descended from a recent founder effect or a population bottleneck. In addition, the disjunct distribution of the haplotypes (Figure 1) suggests that recent isolation of populations and reduced population sizes may have led to haplotype fixation. All three haplotypes were found between

Table 2. Measures of mtDNA sequence variation in the same combined fragments of NADH-5 (positions 12,647 to 12,946 from the complete *Felis catus* mtDNA sequence of López et al. 1996), 16S (positions 2904 to 3285), and ATP-8 (positions 8657 to 8818) (a total of about 880 bp) among eight felid species

Species	Sample size	Number of variable sites	Number of haplotypes	$\pi \times 100$	Reference
Puma (<i>Puma concolor</i>)	286	15	13	0.32	Culver et al. (2000)
Andean mountain cat (<i>Oreailurus jacobita</i>)	9	6	9	0.45	Johnson et al. (1998)
Tigrina (<i>Leopardus tigrinus</i>)	32	44	11	0.97	Johnson et al. (1999)
Pampas cat (<i>Lynchaillus colocolo</i>)	22	44	14	1.12	Johnson et al. (1999)
Geoffroy's cat (<i>Oncifelis geoffroyi</i>)	38	48	32	1.25	Johnson et al. (1999)
Kodkod (<i>Oncifelis guigna</i>)	6	7	3	0.25	Johnson et al. (1999)
Iberian lynx (<i>Lynx pardinus</i>)	20	3	3	0.05	This study

Table 3. Measures of variation in 28 microsatellites in three Iberian lynx populations

	Sample size	Percent polymorphic	Percent observed heterozygosity	Microsatellite variance	Average allele size range	Mean number alleles per locus	Unique alleles
Sierra Morena	5	75.0	36.4	1.655	2.57	2.30	26
Doñana	15	71.4	29.4	0.825	1.68	1.88	19 ^a
Coto del Rey	7	71.4	26.7	0.773	1.57	1.86	1b, 3c
Vera	8	67.9	29.4	0.825	1.57	1.89	1b, 3c
Total	20	85.7	32.9	1.240	2.12	2.05	

^a Relative to Sierra Morena.

^b Relative to Sierra Morena and Vera population (or Coto del Rey).

^c Between Coto del Rey and Vera.

the eastern and western Sierra Morena metapopulations, in south-central Spain, in the middle of the historic distribution of lynx.

Microsatellite Diversity

The amount of microsatellite allele variation among Iberian lynx from the two Doñana populations was very similar (Table 3). Average observed heterozygosity was 26.7% in Coto del Rey and 29.4% in Vera and the average range in allele sizes in both populations was 1.57. Estimates of microsatellite size variation were slightly higher in Sierra Morena than in Doñana (Table 3), including a larger percentage of polymorphic microsatellite loci (75% versus 71.4%). Overall, this amount of microsatellite variation is less than or comparable to that seen in felids such as cheetahs and North American pumas (Table 4), which experienced demographic bottlenecks around the time of the Pleistocene ice ages (Culver et al. 2000).

The populations of Coto del Rey and Vera each had three unique alleles that were not observed in the other. In contrast, the metapopulation of Doñana had 19 unique alleles not observed in Sierra Morena, and Sierra Morena had 26 that were not seen in Doñana (Table 3). These differences were reflected in the analyses of population structure. F_{ST} values among all three populations were significant ($P < .05$), but were highest between Sierra Morena and the two Doñana populations (0.378 with Coto del Rey and 0.227 with Vera; 0.132 between Coto del Rey and Vera). R_{ST} values were only significant between Sierra Morena and Coto de Rey ($R_{ST} = 0.576$). However, several loci were significantly out of Hardy-Weinberg equilibrium in the Doñana metapopulation and in

the Vera population when analyzed separately. In each case there was a deficiency of heterozygotes that may reflect inbreeding, disproportionate reproductive success of some individuals, or some degree of allele dropout.

The pattern of differentiation among populations can be visualized in the dendrograms resulting from the phylogenetic analyses of individual composite genotypes (Figure 4). The six lynx from Sierra Morena are separated from Doñana individuals with high bootstrap support (93%). In comparison, lynx from the two Doñana populations were intermixed, although animals from the same population tended to be most closely linked with another individual from the same population. During the last 15 years there has been only one documented instance of a lynx that moved from Vera to Coto del Rey and another three cases of lynx moving from Coto del Rey to Vera (Ferrerias 2001). Of these, only the lynx that emigrated to Coto del Rey established a territory and successfully bred.

Evolutionary Implications

Our estimation, based on mtDNA sequence variation and a felid-specific divergence rate for these genes, that *L. pardinus* diverged as an unique species 1.53–1.68 million years ago is compatible with paleontological evidence that the *Lynx* species inhabiting Europe during the late Pliocene and early Pleistocene was probably a common ancestor (frequently called *L. issiodorensis*) of three current *Lynx* species, *L. lynx*, *L. pardinus*, and *L. canadensis* (Kurten 1968; Werderlin 1981). The earliest paleontological evidence of *L. pardinus* has been found in France from around several hundred thousand years ago, in the Middle Pleistocene and later (Kurten and

Table 4. Measures of microsatellite size variation at 12 loci among populations of four felid species

Population	Sample size	Loci typed	Observed heterozygosity	Mean microsatellite variance	Number of alleles/locus	Reference
Asian lion	10	12	16.7	0.314	1.67	Driscoll et al. (2002)
Lion, Crater	10	12	37.8	3.223	2.92	Driscoll et al. (2002)
Lion, Serengeti	10	12	48.2	3.732	3.50	Driscoll et al. (2002)
Cheetah, East Africa	10	11	53.0	5.067	4.09	Driscoll et al. (2002)
Cheetah, West Africa	10	11	51.0	3.935	4.00	Driscoll et al. (2002)
Puma, Big Cypress Swamp	10	11	26.0	3.908	2.09	Driscoll et al. (2002)
Puma, Idaho	10	11	61.7	11.651	3.91	Driscoll et al. (2002)
Puma, South America	10	11	81.3	31.667	7.82	Driscoll et al. (2002)
Iberian lynx, Doñana	10	12	25.4	0.484	1.75	This study
Iberian lynx, Sierra Morena	5	11	38.0	1.540	2.27	This study

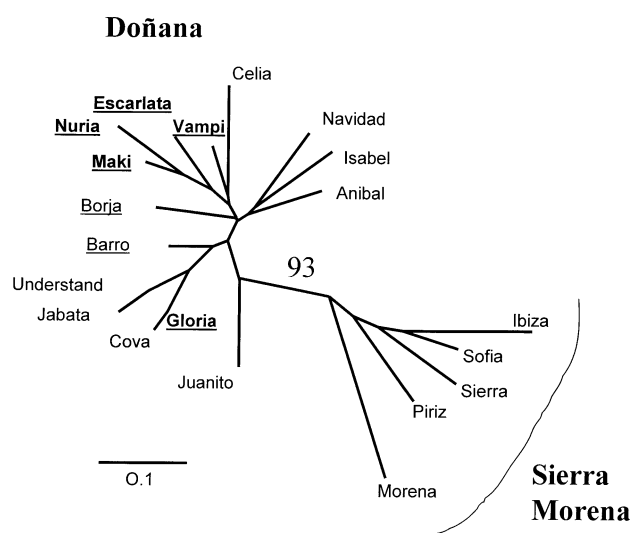


Figure 4. Phylogenetic relationships among Iberian lynx individuals from three lynx populations based on the proportion of shared alleles among the combined genotypes of 28 microsatellite loci. Among the individuals from the Doñana metapopulation, those from the Coto del Rey population are underlined and those from the Vera population are not. Bootstrap support (100 iterations) between populations is listed.

Granqvist 1987; Werderlin 1981). These lynx, often referred to as cave lynx (*L. p. speleus*), were larger than current *L. pardinus*.

Ecological and biogeographical data suggest that the Iberian lynx was restricted to a glacial refugia in southern Iberia during one or more of the frequent ice periods, as occurred with numerous other species (Bennet et al. 1991; Hewitt 1996), such as the grasshopper *Chorthippus parallelus* (Cooper et al. 1995) and some shrews of the *Sorex araneus* group (Taberlet et al. 1994). The relatively low levels of mtDNA sequence and microsatellite size variation relative to other cat species in evidence today may have resulted from at least one demographic bottleneck during this time period.

Conservation Implications

There currently are no recognized subspecies of Iberian lynx. Most of the conservation programs that have been envisaged for the Iberian lynx have been based on metapopulations. These molecular genetic results suggest that there is modest genetic differentiation among microsatellites between Doñana versus Sierra Morena metapopulations. However, the differences are slight and inapparent with mtDNA. These results suggest that the overriding genetic concern of Iberian lynx populations may be their small effective population sizes at risk for extinction and further genetic reduction. If current trends continue, it is very likely that much more active management of some populations will be necessary in order to maintain sufficient population sizes and existing levels of genetic variation (Johnson et al. 2001).

We recommend that any future movement of animals in the wild or the establishment of captive programs be accompanied by more thorough analysis of the population sizes and levels of microsatellite variation in the other lynx metapopulations in addition to those of Doñana and Sierra Morena. This will necessitate an increased emphasis on coordinated conservation action plans, both in situ and ex situ, among the numerous jurisdictions encompassing lynx distributions in Portugal and Spain (Vargas 2000).

References

- Beltrán JF and Delibes M, 1993. Physical characteristics of Iberian lynxes (*Lynx pardinus*) from Doñana, southwestern Spain. *J Mamm* 74:852–862.
- Beltrán JF and Delibes M, 1994. Environmental determinants of circadian activity of free-ranging Iberian lynxes. *J Mamm* 75:382–393.
- Beltrán JF, Rice JE, and Honeycutt RL, 1996. Taxonomy of the Iberian lynx. *Nature* 379:407–408.
- Bennett KD, Tzedakis PC, and Willis KJ, 1991. Quaternary refugia of north European trees. *J Biogeog* 18:103–115.
- Breitenmoser U and Breitenmoser-Wursten C, 1990. Status, conservation needs and reintroduction of the lynx (*Lynx lynx*) in Europe. Strasbourg, Council of Europe, Nature and Environment Series, no. 45.
- Castro LR and Palma L, 1996. The current status, distribution and conservation of Iberian lynx in Portugal. *J Wildl Res* 2:179–181.
- Cooper SJ, Ibrahim KM, and Hewitt GM, 1995. Postglacial expansion and genome subdivision in the European grasshopper *Chorthippus parallelus*. *Mol Ecol* 4:49–60.
- Culver M, Johnson WE, Pecon-Slattery J, and O'Brien SJ, 2000. Genomic ancestry of the American puma (*Puma concolor*). *J Hered* 91:186–197.
- Driscoll CA, Menotti-Raymond M, Nelson G, Goldstein D, and O'Brien SJ, 2002. Genomic microsatellites as evolutionary chronometers: a test in wild cats. *Genome Res* 12:414–423.
- Ellegren H, Primmer CR, and Sheldon BC, 1995. Microsatellite “evolution”: directionality or bias? *Nat Genet* 11:360–362.
- Felsenstein J, 1993. Phylogeny inference package (PHYLIP) version 3.5c. Seattle: University of Washington.
- Ferreras P, 2001. Landscape structure and asymmetrical inter-patch connectivity in a metapopulation of the endangered Iberian lynx. *Biol Conserv* 100:125–136.
- Ferreras P, Aldama JJ, Beltrán JF, and Delibes M, 1992. Rates and causes of mortality in a fragmented population of Iberian lynx *Felis pardina* (Temminck). *Biol Conserv* 61:197–202.
- Ferreras P, Beltrán JF, Aldama JJ, and Delibes M, 1997. Spatial organization and land tenure system of the endangered Iberian lynx (*Lynx pardinus*, Temminck, 1824). *J Zool Lond* 243:163–189.
- Gaona P, Ferreras P, and Delibes M, 1998. Dynamics and viability of a metapopulation of the endangered Iberian lynx (*Lynx pardinus*). *Ecol Monogr* 68:349–370.
- García-Perea R, 1992. New data on the systematics of lynxes. *Cat News* 16:15–16.
- Guo SW and Thompson EA, 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics* 48:361–372.
- Hasegawa M, Kishino H, and Yano TA, 1985. Dating of the human ape splitting by a molecular clock of mitochondrial-DNA. *J Mol Evol* 22: 160–174.
- Hewitt GM, 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. *Biol J Linn Soc* 58:247–276.
- Hillis DM and Bull JJ, 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst Biol* 42:182–192.

- Huelsbeck JP, Bull JJ, and Cunningham CW, 1996. Combining data in phylogenetic analysis. *Trends Ecol Evol* 11:152–157.
- Janczewski DN, Modi WS, Stephens JC, and O'Brien SJ, 1995. Molecular evolution of mitochondrial 12S rRNA and cytochrome *b* sequences in the pantherine lineage of Felidae. *Mol Biol Evol* 12:690–707.
- Johnson WE, Culver M, Iriarte JA, Eizirik E, Seymour K, and O'Brien SJ, 1998. Tracking the elusive Andean mountain cat (*Oreailurus jacobita*) from mitochondrial DNA. *J Hered* 89:227–232.
- Johnson WE, Dratch PA, Martenson JS, and O'Brien JS, 1996. Resolution of recent radiations within three evolutionary lineages of Felidae using mitochondrial restriction fragment length polymorphism variation. *J Mamm Evol* 3:97–120.
- Johnson WE, Eizirik E, Roelke-Parker M, and O'Brien SJ, 2001. Applications of genetic concepts and molecular methods to carnivore conservation. In: *Carnivore conservation* (Gittleman JL, Funk SM, Macdonald O, and Wayne RK, eds). Cambridge: Cambridge University Press; 196–219.
- Johnson WE and O'Brien SJ, 1997. Phylogenetic reconstruction of the Felidae using 16S rRNA and NADH-5 mitochondrial genes. *J Mol Evol* 44(suppl 1):S98–S116.
- Johnson WE, Pecon-Slatery J, Eizirik E, Kim J, Menotti-Raymond M, Bonacic C, Cambre R, Crawshaw P, Nunes A, Seuanez H, Moreira MA, Seymour KL, Simon F, Swanson W, and O'Brien SJ, 1999a. Disparate phylogeographic patterns of mitochondrial DNA variation in four closely related South American small cat species. *Mol Ecol* 8:S79–S94.
- Johnson WE, Shinyashiku F, Menotti-Raymond M, Driscoll C, Leh C, Sunquist M, Johnston L, Bush M, Wildt D, Yuhki N, and O'Brien SJ, 1999b. Molecular genetic characterization of two insular Asian cat species, Bornean bay cat and Iriomote cat. In: *Evolutionary theory and processes: modern perspectives* (Wasser SP, ed). Dordrecht: Kluwer Academic; 223–248.
- Kim J, Eizirik E, O'Brien SJ, and Johnson WE, 2001. Structure and evolution of the mitochondrial DNA control region in the great cats (Panthera). *Mitochondrion* 14:279–292.
- Kumar S, Tamura K, Jakobsen IB, and Nei M, 2001. MEGA2: molecular evolutionary genetics analysis software. Tempe, AZ: Arizona State University.
- Kurten B, 1968. Pleistocene mammals of Europe. Chicago: Aldine Press.
- Kurten B and Granqvist E, 1987. Fossil pardel lynx (*Lynx pardina spelaea* Boule) from a cave in southern France. *Ann Zool Fennici* 24:39–43.
- López JV, Cevario S, and 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.
- López JV, Culver M, Stephens JC, Johnson WE, and O'Brien SJ, 1997. Rates of nuclear and cytoplasmic mitochondrial DNA sequence divergence in mammals. *Mol Biol Evol* 14:277–286.
- MacFadden BJ and Galiano H, 1981. Late Hemphillian cat (Mammalia: Felidae) from the Bone Valley formation of central Florida. *J Paleontol* 55:218–226.
- Martin LD, 1989. Fossil history of the terrestrial Carnivora. In: *Carnivore behavior, ecology, and evolution* (Gittleman JL, ed). Ithaca, NY: Cornell University Press; 536–568.
- Menotti-Raymond M, David VA, Chen ZQ, Menotti KA, Sun S, Schaffer AA, Agarwala R, Tomlin JF, O'Brien SJ, and Murphy WJ, 2003. Second generation integrated genetic linkage/radiation hybrid maps of the domestic cat (*Felis catus*). *J Hered* 94:95–106.
- Menotti-Raymond M, David VA, Lyons LA, Schaffer AA, Tomlin JF, Hutton MK, and O'Brien SJ, 1999. A genetic linkage map of microsatellites in the domestic cat (*Felis catus*). *Genomics* 57:9–23.
- Menotti-Raymond M, David VA, Stephens JC, and O'Brien SJ, 1997. Genetic individualization of domestic cats using feline STR loci for forensic analysis. *J Forensic Sci* 42:1039–1051.
- Michalakis Y and Excoffier L, 1996. A generic estimation of population subdivision using distances between alleles with special reference to microsatellite loci. *Genetics* 142:1061–1064.
- Minch E, Ruiz-Linares A, Goldstein DB, Feldman MW, and Cavalli-Sforza LL, 1995. MICROSAT. Available at <http://lotka.stanford.edu/microsat.html>.
- Nowak RM, 1999. Walker's mammals of the world, 6th ed. Baltimore: Johns Hopkins University Press.
- Nowell K and Jackson P, 1996. Wild cats: status survey and conservation action plan. IUCN publications. Cambridge: Burlington Press.
- Page RD, 1996. Tree view: An application to display phylogenetic trees on personal computers. *Comp Appl Biosci* 12:357–358.
- Palomares F, Delibes M, Ferreras P, Fedriani JM, Calzada J, and Revilla E, 2000. Iberian lynx in a fragmented landscape: predispersal, dispersal and postdispersal habitats. *Conserv Biol* 14:809–818.
- Palomares F, Delibes M, Revilla E, Calzada J, and Fedriani JM, 2001. Spatial ecology of the Iberian lynx and abundance of its primary prey, the European rabbit, in a heterogeneous environment. *Wildl Monogr* 148:1–36.
- Palomares F, Godoy JA, Piriz A, O'Brien SJ, and Johnson WE, 2002. Fecal genetic analysis to determine the presence and distribution of elusive carnivores: design and feasibility for the Iberian lynx. *Mol Ecol*.
- Palomares F, Rodríguez A, Laffitte R, and Delibes M, 1991. The status and distribution of the Iberian lynx *Felis pardina* (Temminck) in Coto Doñana area, SW Spain. *Biol Conserv* 57:159–169.
- Pecon-Slatery J and O'Brien SJ, 1998. Patterns of Y and X chromosome DNA sequence divergence during the Felidae radiation. *Genetics* 148:1245–1255.
- Piris AE and Fernandes ML, 2003. Last lynxes in Portugal? Molecular approaches in a pre-extinction scenario. *Con Gen* 4:525–532.
- Rodríguez A and Delibes M, 1990. El lince ibérico en España: distribución y problemas de conservación. Madrid: ICONA-CSIC.
- Rodríguez A and Delibes M, 1992. Current range and status of the Iberian lynx *Felis pardina* Temminck, 1824 in Spain. *Biol Conserv* 61:189–196.
- Rodríguez A and Delibes M, in press. Internal structure and contraction patterns in the geographic range of the Iberian lynx. *Ecography*.
- Saitou N and Nei M, 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425.
- Sambrook J, Fritsch E, and Maniatis T, 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Schneider S, Roessli D, and Excoffier L, 2000. ARLEQUIN version 2000: a software for population genetic data analysis. Geneva, Switzerland: University of Geneva.
- Slatkin M, 1995. A measure of population subdivision based on microsatellite allele frequencies. *Genetics* 139:457–462.
- Swofford DL, 2001. PAUP* phylogenetic analysis using parsimony and other methods. Sunderland, MA: Sinauer Associates.
- Taberlet P, Fumagalli L, and Hausser J, 1994. Chromosomal versus mitochondrial DNA evolution: tracking the evolutionary history of the southwestern European populations of the *Sorex araneus* group (Mammalia, Insectivora). *Evolution* 48:623–636.
- Vargas A, 2000. Plan de acción para la cría en cautividad del lince ibérico. Madrid, Spain: Ministerio de Medio Ambiente.
- Weir BS and Cockerham CC, 1984. Estimating F-statistics for the analysis of population structure. *Evolution* 38:1358–1370.
- Werderlin L, 1981. The evolution of lynxes. *Ann Zool Fenn* 18:37–71.

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