Journal of Zoology

Journal of Zoology. Print ISSN 0952-8369

Historical DNA reveals the phylogenetic position of the extinct Alpine lynx

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Keywords
Felidae; large carnivores; reintroduction; conservation genetics; nucleotide misincorporations.

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Editor: Jean-Nicolas Volff

Received 25 October 2007; revised 28 January 2008; accepted 1 February 2008

doi:10.1111/j.1469-7998.2008.00428.x

Abstract

During the last two centuries, lynx populations have undergone severe declines and extinctions in Europe. The Alpine lynx, once distributed across the whole Alpine arc, became extinct due to direct human prosecution and deprivation of its main prey in the 1930s. Similar to the Iberian lynx *Lynx pardinus*, its taxonomy has been subject to several controversies. Moreover, knowing the taxonomic status of the Alpine lynx will help to define conservation units of extant lynx populations in Europe. In this study, we investigated two mitochondrial DNA regions in museum specimens ($n = 15$) representing the autochthonous Alpine population and in samples from extant Eurasian lynx *Lynx lynx* populations in Europe and Asia ($n = 17$). Phylogenetic analysis (cytochrome *b*, 345 bp) placed the Alpine lynx within the Eurasian lynx lineage. Among all individuals examined, seven different haplotypes (control region, 300 bp) were observed but no unique Alpine haplotype was discovered. Haplotypes of the extinct Alpine population were identical to previously described haplotypes in Scandinavian lynx signifying a recent genetic ancestry with current European populations. Moreover, our genetic data suggest two distinct glacial refugia for the Carpathian and Balkan population. Overall this study demonstrates that historical DNA from extinct populations can help to disentangle the phylogenetic relationships and historical biogeography of taxa with only a limited number of extant populations remaining.

Introduction

The evolution of carnivores has been marked by periods of extinction and speciation during climatic changes in the Pleistocene. Yet these prehistoric effects on large carnivore diversity and distribution are thought to be less important than the consequences of landscape alterations by modern humans (Purvis, Mace & Gittleman, 2001; Sunquist & Sunquist, 2001). Habitat degradation and fragmentation with direct human persecution resulted in population declines of all 37 modern felid species (McDonald, 2001).

During the 19th and the early 20th century, both Eurasian lynx species (Iberian lynx *Lynx pardinus*, and Eurasian lynx *Lynx lynx*) experienced severe population decline and loss, bringing them to the edge of extinction in Europe (Breitenmoser & Baettig, 1992; Breitenmoser, 1998; Arx *et al*., 2004). Lynx populations were first eradicated in the densely settled lowlands of Western and Central Europe and subsequently in the Alps and Pyrenees. By the 1850s, lynx populations disappeared from the Eastern Alps and only persisted in the Italian and French Alps to the 1930s (Breitenmoser, 1998, and references herein). Around the 1950s, the decline of lynx populations in most European countries slowed down because large-scale deforestations ended, natural prey returned and either legal protection for large carnivores or controlled hunting had been established (Arx *et al*., 2004). Nonetheless, the Iberian lynx survived only in very small and isolated populations and is nowadays one of the world's most threatened carnivores. In contrast, native but often small and highly fragmented populations of the Eurasian lynx survived in the Balkans, Carpathian Mountains, the Baltic region, Scandinavia and Finland (Fig. 1). In addition, several reintroduction attempts of the Eurasian lynx (official and unofficial) from the Carpathian Mountains into the Alps and into secondary mountain chains in Western and Central Europe were successful since the 1970s (Breitenmoser *et al*., 2001). Resolving the taxonomic status of the Alpine lynx population in central Europe will be helpful to define conservation units of the remaining and extant European lynx populations.

Although the monophyly of the known four recent species including the North American bobcat *Lynx rufus* and the Canada lynx *Lynx canadensis* is undisputed, their recognition as unique species and the evolutionary relationship among them has been debated (Hemmer, 1993; Janczewski *et al*., 1995; Johnson *et al*., 2004). In particular, the taxonomy of the Iberian lynx remained controversial until molecular data confirmed its species status (Beltran, Rice & Honeycutt, 1996). Several molecular studies revealed the

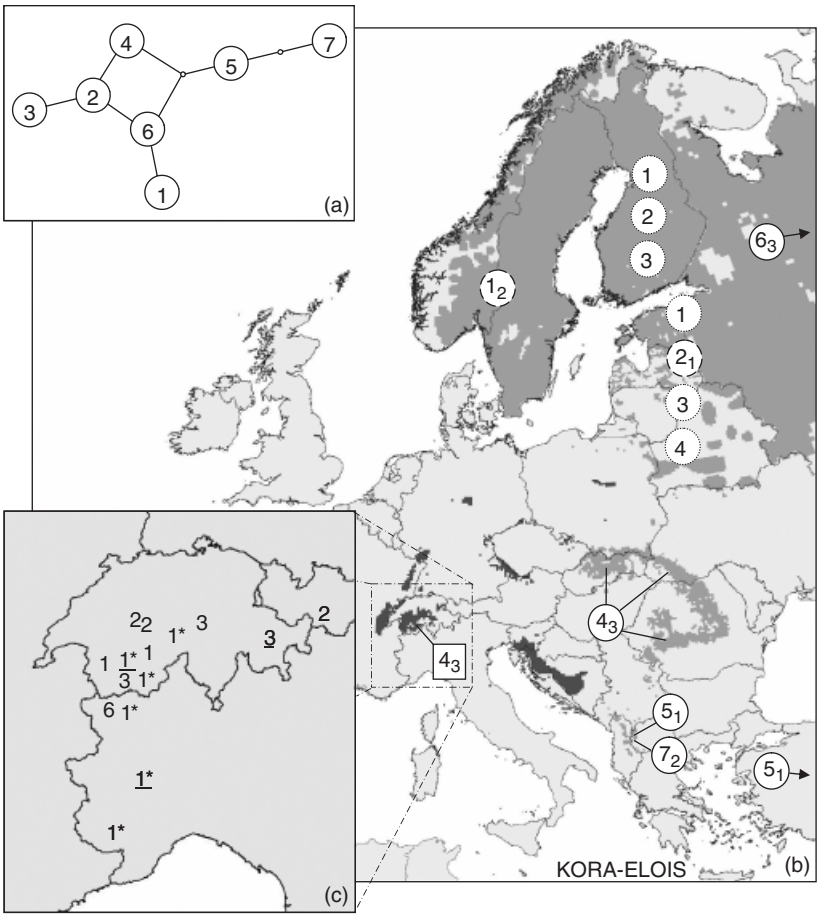

Figure 1 Eurasian lynx *Lynx lynx* haplotype diversity of autochthonous and reintroduced populations in Europe and of the extinct Alpine population for two non-overlapping fragments (157 and 143 bp) of the mitochondrial control region. (a) Minimum spanning tree illustrates the relationship among haplotypes. Small circles indicate missing haplotypes. (b) Modern lynx populations (autochthonous: grey areas; reintroduced: dark grey areas) and mitochondrial haplotypes (autochthonous: circles; reintroduced: squares) in Europe. Subscript digits stand for sample size in this study and arrows refer to the Siberian–Mongolian and the Caucasus populations (for details, see Table 1). Dotted and dashed circles designate haplotypes described previously in northern European populations (Hellborg *et al.*, 2002) while the latter are haplotypes also found in this study. (c) Spatial distribution of 15 historical Alpine lynx samples. Digits refer to haplotypes. Underlined haplotypes represent samples with only approximate information of sampling sites and the asterisk indicates samples where only one fragment (157 bp) could be amplified. bp, base pairs.

bobcat to be the first species to diverge whereas the evolutionary history of the Canada lynx and the two European species was less clear (Werdelin, 1981; Beltran *et al.*, 1996; Johnson *et al.*, 2004). More recently, a highly resolved molecular phylogeny defined the two European lynx species as sister taxa (Johnson *et al.*, 2006).

Based on morphological measurements, the extinct Alpine lynx has been associated with the cave lynx *Lynx spelaeus* (Bonifay, 1978), *Lynx pardinus spelaea* (Werdelin, 1981) or *Lynx lynx spela* (Hemmer, 1993), a late Pleistocene form with an unknown evolutionary history. Moreover, the prehistoric distribution of this lynx was thought to have ranged between Italy, the Pyrenees and south-eastern Spain, therefore covering the known historical range of both the Eurasian lynx and the Iberian lynx. As a consequence, the Alpine lynx itself has been linked to the *pardinus* lineage, the *lynx* lineage or even to the more distinct *spelaeus* lineage (reviewed in Hemmer, 1993, 2001).

Mitochondrial DNA (mtDNA) has been widely used to identify evolutionary divergent populations, that is evolutionary significant units (Ryder, 1986) and to assess the conservation status of populations from a phylogenetic perspective (Moritz, 1994). Moreover, DNA extracted from historical material is pivotal to assessing phylogenetic positions of extinct species (e.g. Parham *et al.*, 2004) and further has the potential to infer demographic changes and micro-evolutionary processes (Wandeler, Hoeck & Keller, 2007). The objectives of this study were to assess the phylogenetic position of the extinct autochthonous Alpine lynx within the lynx tree as well as to clarify the biogeographic history of the Alpine lynx population and extant European populations.

Materials and methods

Samples and DNA extraction

The DNA of 15 museum specimens representing the extinct Alpine lynx population was extracted. All samples had precise records of sampling locations and sampling date (Table 1). All but one specimen were tissue samples (*ca.* 0.5 cm^2) from mounted animals or dried skins. To reduce potential PCR inhibitors, hairs were removed and samples were pre-washed with NTE buffer (pH 9.0, NaCl 10 mM, Tris 50 mM, EDTA 20 mM, Johnson *et al.*, 2004) overnight. DNA was extracted applying a silica spin column (QIAamp® DNA Micro, Qiagen, Hombrechtikon, Switzerland) following the manufacturer's protocol. In brief, samples were digested with proteinase K and DNA was bound to the silica matrix with the addition of 1 μL of supplied

carrier RNA. Subsequently, extracted DNA was washed twice and eluted in 30 μL of AE buffer. The DNA of a broken molar tooth was extracted from one juvenile sample (specimen: 1031025, Table 1) applying the same extraction procedure, with the exception that the whole tooth was added to the digestion buffer, allowing the digestion of the soft tissue within its pulp cavity. Historical samples were complemented with 16 modern samples from the Eurasian lynx representing different European and Asian populations (Balkans, Baltic region, Scandinavia, Carpathian Mountains, Caucasus, Mongolia and Siberia) and from the reintroduced population in Switzerland (Table 1). In

Table 1 Skin (S), teeth (T), blood (B) and tissue (M) samples used in this study from historical Alpine lynx specimens and modern Eurasian lynx *Lynx lynx*

Accession number	Origin	Year of collection	Sample	Amplification success for CB and CR		Haplotype for CR
				238/262 bp	199/182 bp	
Historical Alpine lynx samples						
1031023[A]	Grimsel, Bern, Switzerland	1804	S	–/–	+/–	H1[a]
1031024[A]	Wimmis, Bern, Switzerland	1804	S	+/+	+/+	H2
10.098[B]	Axenberg, Schwyz, Switzerland	1813	S	+/+	+/+	H3
11429[C]	Valle Santa, Aosta, Italy	1824	S	–/–	+/+	H6
94-63[D]	Zermatt, Valais, Switzerland	1835	S	–/–	+/–	H1[a]
10.099[B]	Graubünden, Switzerland	<1837	S	+/+	+/+	H3
1031030[A]	Lötschental, Valais, Switzerland	1850	S	–/–	+/+	H1
15711[E]	Piemont, Italy	<1865	S	–/–	+/–	H1[a]
268[D]	Mayens, Valais, Switzerland	1867	S	–/–	+/–	H1[a]
4705[F]	Nauders, Tirol, Austria	1872	S	+/–	+/+	H2
11430[C]	Valle d' Aosta, Italy	1872	S	–/–	+/–	H1[a]
11428[C]	Cuneo, Piemont, Italy	1881	S	–/–	+/–	H1[a]
1045[D]	Val d'Hérens, Valais, Switzerland	1899	S	–/–	+/+	H3
28749[G]	Bex, Vaud, Switzerland	<1850	S	+/+	+/+	H1
1031025[A]	Wimmis, Bern, Switzerland	<1923	T	+/+	+/+	H2
Eurasian lynx *Lynx lynx* sample						
10.100[B]	Asia[b]	1865	S	+/+	+/+	H5
LX0887[H]	Caucasus[b]	2000	S			H5
LX6517[H]	Balkans, Macedonia	2000	S			H5
LX0880[H]	Balkans, Macedonia	?	S			H7
LX0883[H]	Balkans, Macedonia	?	S			H7
LX6755[H]	Carpathian Mountains, Poland	2000	M			H4
LX6467[H]	Carpathian Mountains, Rumania	2000	S			H4
LX7314[H]	Carpathian Mountains, Slovakia	2001	M			H4
LX0221[H]	Alps, Switzerland (reintroduced)	1999	B			H4
LX0237[H]	Alps, Switzerland (reintroduced)	1999	B			H4
LX0238[H]	Alps, Switzerland (reintroduced)	1999	B			H4
LX6725[H]	Scandinavia, Norway	2000	M			H1
LX6726[H]	Scandinavia, Norway	2000	M			H1
LX6888[H]	Baltic region, Latvia	2001	M			H2
LX0819[H]	Mongolia[a]	2004	S			H6
LX0820[H]	Mongolia[a]	2004	S			H6
LX0842[H]	Siberia, Russia (zoo)	2004	B			H6

Amplification success (+ or –) in historical samples for two overlapping fragments of the cytochrome *b* (CB) and two non-overlapping fragments of the control region (CR) are indicated. Labelling of haplotypes followed Hellborg *et al.* (2002).

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[B]Zoological Museum, University of Zurich, Switzerland.

[C]Zoological Museum *La Specola*, University of Florence, Italy.

[D]Natural History Museum, Sion, Switzerland.

[E]Research Institute and Natural Museum Senckenberg, Germany.

[F]Museum of nature Chur, Switzerland.

[G]Natural History Museum, Lausanne, Switzerland.

[H]Coordinated Research Projects for the Conservation of Carnivores, Switzerland.

[a]based on fragment CR-L16298/CR-H16456 only.

[b]No details of sampling origin are available.

addition, DNA of one historical skin sample from Asia was extracted as described above.

PCR amplification and sequencing

mtDNA sequences for the cytochrome *b* (CB) and control region (CR) were collected for historical and modern samples. To account for the degraded nature of the DNA recovered from museum samples and the likely potential of human DNA contamination (Wandeler *et al.*, 2003), new primers for CB and CR were developed (Table 2). Primers were designed based on previously published human *Homo sapiens*, domestic cat *Felis catus*, Iberian lynx and Eurasian lynx sequence data. For the CB, a total of 392 base pairs (bp) were amplified in two overlapping fragments of 238 bp (CB-L15046/CB-H15240) and 262 bp (CB-L15174/CB-15392), respectively. To reduce the potential of co-amplification of human DNA contaminants, all CB primers contained at the 3′ end between one and three nucleotide mismatches to the homologous human sequence. Sequence data for CR of two non-overlapping fragments (CR-L16298/CR-H16456, 199 bp, and CR-L16782/CR-H16922, 182 bp) were collected, flanking an *ca.* 280 bp long tandem repetitive element (Hellborg *et al.*, 2002).

PCR amplifications for historical samples were performed in a total volume of 10 μL containing 5 μL Multiplex PCR Kit (Qiagen), 0.5 μM of each primer and 3 μL of template DNA. Amplifications were carried out at an initial denaturation step of 94 °C for 12 min, followed by 35–40 cycles at 94 °C for 30 s, annealing at 46 °C (both CB fragments, CR-L16782/CR-H16922) and 51 °C (CR-L16298/CR-H16456) for 30 s, extension at 72 °C for 45 s and a final extension step of 72 °C for 7 min. PCR conditions for modern samples were identical, except that 1 μL of template DNA was used, the initial denaturation step was increased to 15 min and the number of PCR cycles was reduced to 28. Alternatively, PCR amplifications for CB and CR were performed by combining the two outer primers of the CB (CB-L15046/CB-15392; annealing at 58 °C) and the CR (CR-L16298/CR-H16922; annealing at 63 °C) fragment. PCR products were verified for the correct fragment size and amplification intensity on an agarose gel (1%) and purified subsequently (QIAprep®, Qiagen). Forward and reverse sequencing was performed using BigDye™ Terminator v3.1 (ABI, Crewe, UK) chemistry and Better Buffer (Web Scientific, Rotkreuz, Switzerland) on a 3100-Avant Genetic Analyzer (ABI). Sequences were aligned and edited in BIOEDIT (v.7.0.2) and deposited in the GenBank (Accession numbers: EU036218, EU107389–EU107391).

Table 2 Mitochondrial DNA primer sequences of two overlapping fragments for the partial gene of the cytochrome *b* (CB) and two non-overlapping fragments of the control region (CR) in lynx

Primer	Sequence (5′–3′)
CB-L15046	AACTATAAGAACTTAATGACCAAC
CB-H15240	GTTAACGTCGCGGCAGATAT
CB-L15174	TTTGCCTAATCCTACAGATCCT
CB-H15392	GTGGCTATAACTGTGAATAGTAA
CR-L16298	TCCCAAAGCTGAAATTCTTT
CR-H16456	CAGTGGTTGGTAGGTTAATTTT
CR-L16782	TAGTGCTTAATCGTGCATT
CR-H16922	CAGATGCCAGGTATAGTTCC

Primer names identify the gene, the light (L) and heavy (H) strand and the position of the 3′ end of the primer in the domestic cat (*Felis catus*, Lopez *et al.*, 1996).

Precautions and authenticity

Owing to the expected degraded nature and the low copy number of historical DNA samples, precautions were taken to avoid cross-contamination among historical samples, contamination of historical samples with modern DNA and contamination with PCR products (Wandeler *et al.*, 2007). DNA extraction and pre-PCR procedures of all historical samples were conducted in an isolated laboratory with positive pressure with respect to the outside air especially dedicated to working with DNA extracted from museum samples. Equipment and working surfaces were UV irradiated and treated with DNA–off™ (Axon Lab AG, Baden, Switzerland). No more than six samples were extracted simultaneously. Each extraction step included a negative extraction control and negative PCR controls were used in all PCR amplifications. Controls were surveyed throughout the laboratory work and no evidence of contamination was observed. Unknown haplotypes were independently re-amplified and sequenced to account for the potential of misincorporated nucleotides. Single nucleotide misincorporations are thought to be frequent when the PCR amplification started with only a few copies of template DNA (Nyström, Angerbjorn & Dalen, 2006; Sefc, Payne & Sorenson, 2007).

Phylogenetic and population genetic analyses

Phylogenetic reconstruction of the CB partial gene was accomplished using maximum parsimony (MP), neighbour-joining (NJ) and maximum likelihood (ML) in PAUP*4.0b10 (Swofford, 2002). CB sequences obtained were complemented with homologous sequence data from GenBank for all four extant lynx species (*L. pardinus*, AY499320; *L. lynx*, AY499324, AY499325, AY773083; *L. rufus*, AY499328; and *L. canadensis*, AY319506–12, AY499333) and three outgroup species (clouded leopard *Neofelis nebulosa*, AY499337; snow leopard *Uncia uncia*, DQ097339, and tiger *Panthera tigris*, AF053051). The evolutionary model of nucleotide substitution that fit the data best (HKY + G, Hasegawa, Kishino & Yano, 1985) was obtained using Modeltest 3.5 (Posada & Crandall, 1998). The base frequencies were estimated at 0.298 (A), 0.325 (C), 0.124 (G) and 0.253 (T), with a transition/transversion ratio of 19.99. The rate of heterogeneity among variable sites was estimated to follow a gamma distribution ($\alpha = 0.138$). Parameters derived from the evolutionary model were applied to the NJ and the ML analyses. MP analyses were computed

using a heuristic search with a stepwise addition option, tree bisection reconnection, branch swapping and 100 additional replicates. The reliability of internal branches was assessed by bootstrapping with 1000 (MP and NJ) and 100 (ML) pseudo-replicates.

The two non-overlapping sequences of CR were combined and will be treated as a single sequence hereafter. Assignment of the CR haplotypes was carried out following Hellborg *et al*. (2002). The evolutionary relationship between CR haplotypes was visualized in a parsimony network using the software TCS (v.1.21; Clement, Posada & Crandall, 2000).

Results

CB

Consensus sequences of 345 bp for CB were obtained for five Alpine and 17 Eurasian lynx samples. In addition, partial sequence data of 194 bp were collected for one additional Alpine specimen (specimen: 4705). All sequence data recovered for the Alpine lynx and 15 Eurasian lynx were homologous to the previously published sequence AY499325 (Johnson *et al*., 2004). Consequently, all Alpine samples clustered within the *L. lynx* lineage with a high bootstrap support of 87, 97 and 92% for the ML, MP and NJ analyses, respectively (Fig. 2). Two samples from Central Asia (specimen: 10.100) and from the Caucasus (specimen: LX0887) revealed a silent mutation (C to T) at position 15049 (position refers to the mtDNA of *F. catus*, Lopez, Cevario & O'Brien, 1996).

Among all lynx species, 52 nucleotide sites in CB were variable (15.1%), 24 of which (7.0%) were parsimony informative. ML and MP analyses defined *L. lynx* and *L. pardinus* as sister taxa, yet with low bootstrap values (51 and 58%, respectively). In addition, the 50% majority rule NJ bootstrap consensus tree indicates an unresolved polytomy among *L. lynx*, *L. pardinus* and *L. canadensis*. In contrast, all three phylogenetic analyses support *L. rufus* as the sister species to the remaining lynx species (99, 89 and 87% bootstrap values for ML, MP and NJ, respectively).

CR

Consensus sequences of 157 and 143 bp for two non-overlapping fragments were obtained for nine out of 15 Alpine lynx samples and for all 16 modern samples. In addition, sequence data for the remaining historical samples were collected for the longer fragment (CR-L16298/CR-H16456). Seven different haplotypes were detected across all samples, four of which had been described previously in northern European populations (Table 1, Hellborg *et al*., 2002). A low level of nucleotide polymorphism of only eight polymorphic sites (2.7%, excluding the repetitive region) was observed and all but one substitution were transitions (Table 3). The statistical parsimony network illustrates the close relationship between the different haplotypes (Fig. 1a).

Haplotype H4 was found in all three modern samples from three regions of the Carpathian Mountains and in all samples from the reintroduced Alpine populations. In

Table 3 Variable sites of two non-overlapping fragments (157 and 143 bp) of the control region in the Eurasian lynx *Lynx lynx*

Haplotype	1 0 4	1 5 0	1 5 6	1 5 7	0 0 9	0 1 6	0 8 9
H1	A	T	G	T	C	A	C
H2	·	C	·	C	·	·	·
H3	·	C	·	C	·	G	·
H4	·	C	·	C	·	·	T
H5	·	·	·	C	T	·	T
H6	·	·	·	C	·	·	·
H7	C	·	A	C	T	·	T

Haplotypes H1–H4 have been described previously (Hellborg *et al*., 2002).

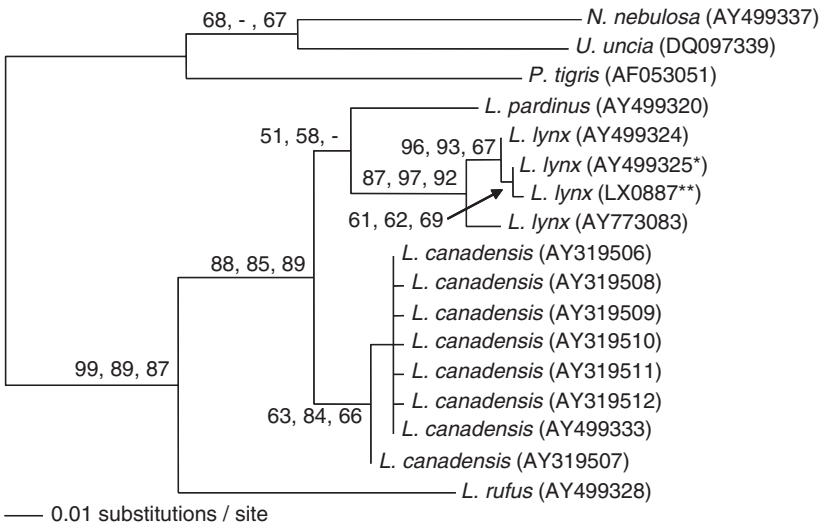

Figure 2 Phylogeny of the extinct Alpine lynx (bold), the four extant lynx species and three outgroup Felid species for 345 bp of sequence for the cytochrome *b*. The maximum likelihood (ML) tree inferred using the HKY + G model is shown. Numbers at nodes indicate bootstrap support values higher than 50% for ML, maximum parsimony and neighbour joining, respectively. All historical sequences of the Alpine lynx were identical to the Eurasian lynx sequence AY499325. *1031024, 10.098, 10.099, 4705, MZL28749, 1031025, LX6517, LX0880, LX0883, LX6755, LX6467, LX7314, LX0221, LX0237, LX0238, LX6725, LX6726, LX6888, LX0819, LX0820, LX0842; **10.100; for sample details, see Table 1. bp, base pairs.

contrast, four different haplotypes (H1, H2, H3 and H6) were detected in the 15 historical Alpine samples (Table 1). H1 was the most common (53.3%), followed by haplotypes H2 and H3 (each 20%) and one sample with haplotype H6 (6.7%). Two previously unknown haplotypes (H5 and H7) were found in the Balkan population. Haplotype H5 was further described in the samples from the Caucasus. Finally, the two samples from Mongolia shared the same haplotype (H6) with a sample from Siberian and an Alpine sample (Table 1). Misincorporated nucleotides (both C to T transitions) were found in sequences for two (12.5%) historical samples (specimens: 11428 and 11430) as revealed by additional and independent PCR amplifications.

Discussion

This study provides strong molecular evidence that the extinct Alpine lynx was a Eurasian lynx. All examined historical sequences of the CB gene clustered within the *L. lynx* linage and were identical to a homologous sequence described previously for the Eurasian lynx. Moreover, no haplotype unique to the Alpine lynx could be observed of the faster evolving CR in 15 historical specimens, suggesting a shared evolutionary history of the Alpine lynx population with other European populations until relatively recent times. Based on the haplotype diversity observed among the examined lynx populations, the following postglacial recolonization scenario for the Eurasian lynx in Europe after the last glacial maximum (LGM) can be postulated.

All three haplotypes H1, H2 and H3 found in historical Alpine specimens have been described previously in the Baltic, Finnish and Scandinavian populations (Hellborg *et al.*, 2002). This finding signifies a recent and common evolutionary history of the Alpine and northern European populations and revises the scenario of their independent origin (Hemmer, 1993). A northwards expansion from a southern refugium, most likely in the Italian peninsula or in western France, can thus be postulated through the Alps, Central Europe and subsequently via a land bridge from Denmark to Sweden, Norway and Finland. An alternative colonization route could have further split in Central Europe, colonizing Finland along the south-eastern shore of the Baltic Sea. Note that the observed fixation of haplotype H1 in the current Scandinavian lynx population has been explained by strong genetic drift following bottlenecks during the last two centuries rather than by reflecting a genetic footprint of a postglacial colonization event via Finland (Hellborg *et al.*, 2002). The occurrence of a southern refugium is also supported by a number of fossil records from the late Pleistocene found in Italy, Croatia and Southern France (Sommer & Benecke, 2006).

We discovered haplotype H6 in one Alpine specimen, a haplotype otherwise observed in the distant and by the Ural Mountains-separated Mongolian and Siberian populations. Haplotype H6 is closely related to all three other haplotypes found in the Alps and in northern Europe, therefore indicating the possibility of a recent common evolutionary history. Alternatively, the records of the nearly 200-year-old specimen could simply be incorrect. Given the still poor knowledge of the phylogeography of the Eurasian lynx currently available and the fact that haplotype H6 has only been observed once in our Alpine sample, we consider this to be the more likely explanation. After excluding haplotype H6, three different CR sequences were found among the remaining 14 Alpine specimens. Moreover, sequence data for six samples could only be obtained for one fragment (CR-L16298/CR-H16456), and as a consequence, the genetic diversity in the historical sample might be underestimated. The higher genetic diversity observed in the extinct Alpine lynx population in comparison with that found among 276 examined lynx samples from northern Europe (Hellborg *et al.*, 2002) could well indicate a reduction of genetic diversity from southern to northern Europe and supports the likely scenario of a northwards population expansion in Central Europe following the LGM.

Our molecular data support an independent evolutionary history of the Eurasian lynx population in the Balkans. Although only three samples have been analysed, two new haplotypes were described, one of them unique to this area. Both haplotypes were found to be genetically more differentiated compared with all the other haplotypes observed. Moreover, by sharing the same haplotype with the Caucasian sample and a historical sample with an unknown origin from Asia, a southern and independent eastwards recolonization route from the proximate Carpathian population can be postulated. The Balkan population is the smallest and the most threatened autochthonous Eurasian lynx population in Europe, with an estimated current population size of <100 adult individuals (Arx *et al.*, 2004). Although the Balkan lynx has already been described as a subspecies *Lynx lynx martinoi* (Miric, 1978), this taxonomic status is currently not recognized (Arx *et al.*, 2004). Given the vulnerability of the Balkan population, it is important to follow up the initial findings of this study and to test in-depth the likely independent evolutionary history of the Balkan population and, if necessary, to revise the taxonomic status of the Balkan lynx.

We observed haplotype H4 only in the Carpathian population and the reintroduced Alpine population, which derives from animals reintroduced from the Carpathian Mountains. Haplotype H4 has been further described at a low frequency in the Baltic region (Hellborg *et al.*, 2002). These findings suggest an isolated Carpathian lynx population during the last glacial periods. The existence of a glacial refugium in the Carpathians has been supported by the discovery of plant pollen and macrofossiles from coniferous and broad-leaf trees (Willis & van Andel, 2004) and by records of several mammals from various deposits dating back to the last glaciations (Sommer & Nadachowski, 2006). Moreover, recent phylogeographic studies on vertebrate species confirmed the occurrence of a Carpathian refugium and demonstrated its contribution to the recolonization of Europe (Babik *et al.*, 2005; Kotlik *et al.*, 2006). By postulating a Carpathian refugium for the Eurasian lynx, the low frequency of H4 observed in the Baltic region could therefore indicate a secondary contact with the Baltic population.

Both investigated mitochondrial regions revealed a low level of genetic variation in the Eurasian lynx. This low level of sequence divergence between all observed CR haplotypes made it impossible to apply advanced statistical analyses (e.g. nested clade analyses) to support our findings of numerous glacial refugia of the Eurasian lynx in Europe. As a result, data on additional nuclear markers with a higher resolution (e.g. microsatellites) and larger sample sizes from extant populations will be needed to support the conclusions of this initial assessment. Moreover, additional genetic data from historical specimens representing the three extinct populations of the Pyrenees, the Italian peninsula and the Dinaric Mountains will be invaluable to shed further light on the evolutionary history of the Eurasian lynx in Europe and to define the conservation status of European lynx populations.

Acknowledgements

We are very grateful to Urs Breitenmoser, Lukas Keller, Sébastien Regnaut and Manuela von Arx for discussions and two anonymous referees for their detailed and helpful comments. Many thanks are due to Gabriela Obexer-Ruff for extracting the DNA of recent samples. For their help with obtaining recent and historical lynx samples, we are very grateful to Paolo Agnelli (Florence, Italy), Zanete Andersone-Lilley (Latvia), Beatrice Blöchlinger and Peter Lüps (Bern, Switzerland), Laurence Colas (France), Olivier Glaizot (Lausanne, Switzerland), Eva Gregorova (Slovakia), Miso Hristovski and Svetozar Petkovski (Macedonia), Ilpo Kojola and Seija-Sisko Kilpelä (Finland), Petra Kaczensky and D. Ochino (Mongolia), Katrin Krohmann (Senckenberg, Germany), Tor Kvam and Terje Bo (Norway), V. Lukarevsky (Caucasus), Jürg-Paul Müller (Chur, Switzerland), Henryk Okarma (Poland), Jean-Claude Praz (Sion, Switzerland), Barbara Promberger (Romania) and Dominik Steinmann and Marianne Haffner (Zürich, Switzerland).

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