Conservation Genetics (2006)
DOI 10.1007/s10592-005-9062-0

Lost populations and preserving genetic diversity in the lion *Panthera leo*: Implications for its *ex situ* conservation

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Received 2 March 2005; accepted 21 September 2005

Key words: barbary lion, cape lion, mane, mitochondrial DNA, North Africa, *Panthera leo leo*, *Panthera leo melanochaita*, South Africa

Abstract

Two of the eight recognized lion subspecies, North African Barbary lion (*Panthera leo leo*) and South African Cape lion (*Panthera leo melanochaita*), have become extinct in the wild in the last 150 years. Based on sequences of mitochondrial DNA (mtDNA) control region (HVR1) extracted from museum specimens of four Barbary and one Cape lion, the former was probably a distinct population characterized by an invariable, unique mtDNA haplotype, whilst the latter was likely a part of the extant southern African lion population. Extinction of the Barbary line, which may still be found in "generic" zoo lions, would further erode lion genetic diversity. Therefore, appropriate management of such animals is important for maintaining the overall genetic diversity of the species. The mtDNA haplotype unique to the Barbary lion, in combination with the small size of the HVR1 analyzed (c. 130 bp), makes it possible and cost-effective to identify unlabelled Barbary specimens kept in museums and "generic" captive lions that may carry the Barbary line. An initial study of five samples from the lion collection of the King of Morocco, tested using this method, shows that they are not maternally Barbary.

Introduction

Conservation of species' genetic diversity is one of the major challenges that need to be tackled for preserving endangered wildlife (Uphyrkina and O'Brien 2003; Chen et al. 2004). The lion (*Panthera leo*) is a charismatic large felid, which has become increasingly endangered in the wild (Jackson 1999). In addition to the critical condition of the Asiatic lion (*P. l. persica*), African lions may now be facing rapid population decrease and local extinctions caused by human activities (Nowell and Jackson 1996; Bauer and van der Merwe 2002; Chardonnet 2002). Anthropogenic factors causing lion population contraction have likely increased with technological sophistication (Nowell and Jackson 1996) causing gradual genetic erosion in the lion. In the last 150 years this process has resulted in two famous lion populations, the North African Barbary lion (*P. l. leo*) and the South African Cape lion (*P. l. melanochaita*), becoming extinct in the wild (Harper 1945; Nowell and Jackson 1996; Yamaguchi and Haddane 2002). These populations represent two of the eight customarily recognized subspecies of lion (Hemmer 1974; Nowell and Jackson 1996), and their extinction may have further reduced the genetic diversity of the species. Extinct populations are crucial for assessing the natural genetic diversity of the lion, and yet, to date, no research has

investigated the genetic characteristics of the lost populations.

Although they have become extinct in the wild, both Barbary and Cape lions had previously been exhibited in European zoos (especially the former, apparently being the most commonly exhibited lion), and hence, their genes may still be present in captive lions (Guggisberg 1963; Hemmer 1978). Survival of the Barbary line is especially likely in the royal lion collection of the King of Morocco (Hemmer 1978; Yamaguchi and Haddane 2002). Therefore, preservation of the lion genetic diversity that is believed to have been lost may be still possible through *ex situ* conservation (Yamaguchi and Haddane 2002). As a first step, conservationists need to know which lions carry the genotypes lost in the wild. Early authors believed that the seemingly fixed external morphology of both Barbary and Cape lions (male's huge mane extending behind shoulders and covering belly) would justify their "distinct" subspecific status and be used to identify them (Harper 1945; Mazák 1970, 1975). However, it is now known that the color and size of a lion's mane are influenced by various extrinsic factors, including the ambient temperature (Kays and Patterson 2002; West and Packer 2002). Therefore, a heavy mane developed in a cooler place (e.g. European or North American zoos) is an inappropriate marker for identifying Barbary or Cape lines, which need to be identified by molecular markers (Yamaguchi and Haddane 2002). Although analyzing the genetic characteristics of extinct populations has not previously been practical, recent advances in ancient DNA (aDNA) techniques are enabling researchers to investigate them (Wayne et al. 1999; Cooper et al. 2001).

In this paper, we report the first genetic study into the extinct Barbary and Cape lions, using an intraspecific network of mitochondrial DNA (mtDNA) obtained with aDNA techniques, from museum specimens. On the basis of the results obtained, we discuss possible implications for *ex situ* conservation of the genetic diversity of the lion.

Materials and Methods

Sampling and laboratory procedures

Samples of 32 lion individuals were obtained from across most of the species' range. Small pieces of bone (up to c. 5×5×5 mm block: cortical part of the bone if possible), dried tissues (up to a few grams) or skins (up to c. 10×10 mm area) were sampled from specimens of known origins kept in natural history museums in the UK, Europe, and southern Africa. In most cases, sampling did not leave noticeable damage to the specimens. Phenol chloroform extractions with multiple negative controls were performed according to strict aDNA criteria (Cooper and Poinar 2000; Hofreiter et al. 2001) in a facility dedicated to aDNA extractions at the Henry Wellcome Ancient Biomolecules Centre (ABC), which is geographically separated from any molecular biology work including polymerase chain reaction (PCR). A set of generic primers, CR_2F 5′GTGCTTGCCCAGTATGTC and CR_4R 5′ATATAAACTACTGTACATGC were designed to amplify the HVR1 of the mtDNA control region using published mtDNA sequences of the genus *Panthera* (Cracraft et al. 1998; Jae-Heup et al. 2001). PCR reactions were performed using High-Fidelity Platinum® *Taq* (Invitrogen, UK) with a 2 min activation step at 94 °C followed by 45 cycles at 94 °C for 45s, 46 °C for 45s and 68 °C for 1.30 min. PCR products were purified using the Qiaquick system (Qiagen Ltd., UK), then directly sequenced with ABI BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA), and imaged on ABI Prism® capillary DNA 377 and 310 automated sequencers (Applied Biosystems). As the CR_4R primer is able to bind multiple RS2 repeats, a double-banded product was produced with well-preserved samples. The unique nested primer RS2_Seq2 5′ GCACGATATACATAGTATGCC was designed for direct sequencing from the reverse strand.

Cloning of PCR products was performed with the TOPO® system (Invitrogen, UK).

Data authenticity and analysis

In addition to the multiple extractions and PCR and extraction controls at Oxford, five samples (sample ID numbers: 8, 9, 20, 24, and 32 in Table 1) were sent to University College London, and the entire procedure including extraction was independently replicated there. All intra- and inter-laboratory replications produced identical sequences.

The Felidae are known to contain macrosatellites resulting from nuclear translocation of the

Table 1. Lion samples analyzed in this study. Sample sources are National Museums of Scotland (I); National Museum of Natural History, Paris, France (II); National Natural History Museum, Leiden, Holland (III); Swedish Museum of Natural History, Stockholm, Sweden (IV); Amathole Museum, King William's Town, RSA (V); Natural History Museum of Zimbabwe, Bulawayo, Zimbabwe (VI); Transvaal Museum, Pretoria, RSA (VII); National Zoological Park, Rabat, Morocco (VIII); Port Lympne Wild Animal Park, Kent, UK (IX); and Zoological Museum, University of Amsterdam, Amsterdam, Holland (X). Abbreviations are as follows: CAR (Central African Republic), DRC (Democratic Republic of Congo), and RSA (Republic of South Africa)

ID	Sample type	Origin	Source	Haplotype: museum ID, (date) and [comments]
Origin-known specimens				
1	frozen tissue	India	I	9: [location: Gir Forest]
2	pelvis	India	II	9: 1838–890 (31/12/1838)
3	skull	Iran	II	10: 1962–2847
4	skull	Iran	II	10: 1962–2854
5	dried skin	Tunisia	III	11: Specimen-c (1823)
6	vertebra	Algeria	II	11: 1862–54
7	vertebra	North Africa	II	11: A-7912 (died 1839)
8	mandible	Barbary	IV	11: A58:5287 (1831)
9	skull	Senegal	II	4: A-1892 (1841)
10	skull	Senegal	II	4: 1890–490
11	skull	Burkina	II	3: 1926–248 (died 23/12/1927)
12	skull	CAR	II	6: 1996–2516
13	skull	CAR	II	6: 1996–2517
14	vertebra	Sudan	II	8: 1995–164 [location: Nubia]
15	skull	Ethiopia	II	6: A-12942
16	skull	Kenya	II	2: 1962–2853 (1921)
17	skull	DRC	IV	2: A59:5062 (1921) [location: s. of L. Edward]
18	mandible	DRC	IV	2: A59:5066 (1921) [location: s. of L. Edward]
19	N/A	Tanzania	GenBank	2: Ple180-CL1 [location: Serengeti]
20	skull	Tanzania	V	1: 107.1 [location: s. Tanganyika]
21	skull	Zambia	VI	2: 5728 (1957) [location: Kafue National Park]
22	dried tissue	Zambia	V	2: 15908 (1927)
23	skull	Zimbabwe	VI	5: 29119 (1967) [location: Tsholotsho, s.w. Zimbabwe]
24	skull	RSA(Cape)	V	5: 15904 [location: King William's Town]
25	dried tissue	RSA	VI	2: 1072 (1939) [location: s. Kalahari]
26	dried tissue	RSA	V	2: 38248 [location: Kalahari Gemsbok National Park]
27	skull	Botswana	VI	5: 63589 (1964)
28	dried tissue	Namibia	IV	2: A58:1971 (1856) [location: Walvis Bay]
29	skull	Botswana	VI	5: 63591 (1965) [location: Moremi, n. Botswana]
30	dried tissue	Namibia	V	5: 18319 [location: Etosha Pan, n. Namibia]
31	skull	DRC	II	7: 1961–2849
32	phalange	Gabon	II	5: 1960–3680 (1959)
Origin-unknown specimens				
Possible Barbary lion				
33.	pelvis	N/A	II	11: 1882–502 [described in Cuvier's Animal Kingdom?]
34.	hair	N/A	VIII	7: [Moroccan King's lion]
35.	skull	N/A	VIII	6: [Moroccan King's lion]
36.	skull	N/A	VIII	6: [Moroccan King's lion]
37.	blood	N/A	IX	6: 82007 [Moroccan King's lion]
38.	blood	N/A	IX	6: 88016 [Moroccan King's lion]
Possible Cape lion				
39.	dried tissue	N/A	X	9: ZMA710 (before 1810) [owned by Luis Napoleon]

ID numbers correspond to those in Figure 1, and haplotype numbers to those in Figure 2. All DNA sequences were deposited in Genbank (accession numbers DQ248045-DQ248055).

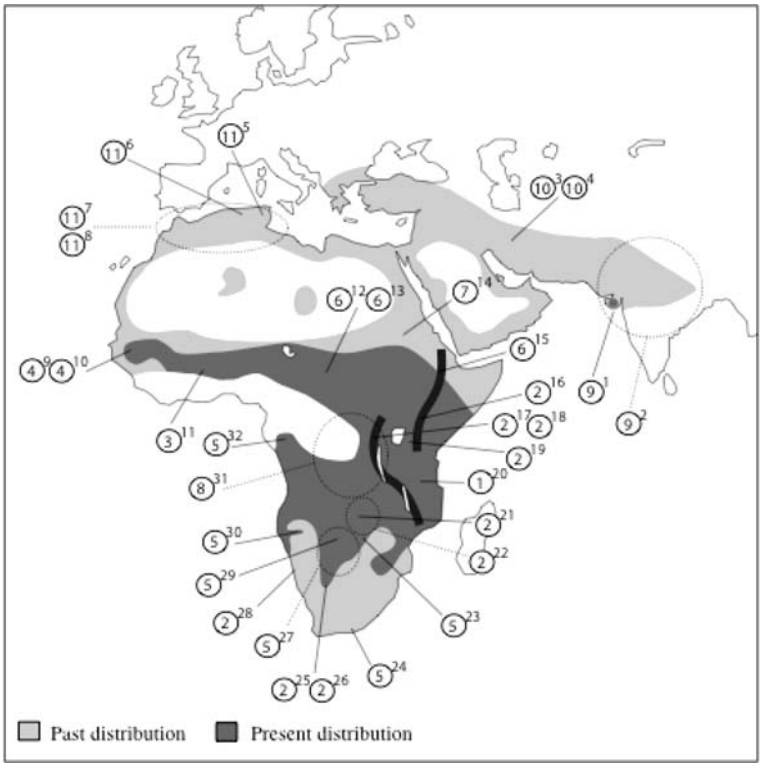

Figure 1. Map showing approximate sample collection sites with mtDNA haplotype numbers (inside the circle) and individual ID numbers (upper right corner of the circle). In cases where more than one sample originated from the same country (or region) and the exact sampling location was not known for every animal, a dashed line and a circle indicates the approximate area of the country. The Great Rift Valley is shown as thick dark lines. Historical and current geographical distributions of lion (based on Nowell and Jackson 1996) are also shown.

Table 2. Mitochondrial DNA control region (HVR1) haplotypes identified for origin-known lions analyzed in this study. Nucleotide positions showing variation amongst lion haplotypes are depicted

Haplotype	Nucleotide position												
	0	0	0	0	0	0	1	1	1	1	1	1	1
	2	3	5	7	9	9	1	1	1	2	2	2	3
	9	4	0	9	2	3	4	8	9	2	4	5	6
1	C	–	A	C	T	–	G	G	C	T	C	–	A
2	T	.	.	.	.	.	.	.	.	.	.	.	.
3	T	.	G	.	.	.	.	.	T	C	–	.	.
4	T	.	G	.	.	C	.	.	T	C	–	.	.
5	T	.	.	.	–	.	.	A	.	.	.	C	.
6	.	C	G	.	.	.	.	.	.	.	.	C	.
7	.	C	G	.	.	.	A	.	.	.	.	C	.
8	.	C	G	T	.	.	.	.	.	.	.	C	.
9	T	.	G	.	.	C	.	.	.	.	.	C	.
10	T	.	G	.	.	C	.	.	.	.	.	C	G
11	T	.	G	.	.	.	.	.	.	.	.	C	G

mtDNA (Numts) (Lopez et al. 1997; Cracraft et al. 1998). Two extracts that were cloned produced a minority sequence similar to published Numts, significantly different from the mitochondrial sequence. All other extracts generated sequences that grouped closely with those obtained using purified mitochondria from modern tissue of the genus *Panthera* (Cracraft et al. 1998; Jae-Heup et al. 2001). Subsequent cloning of amplifications from 21 other extracts (at least one sample from every haplotype), clarified that the two extracts had produced a probable Numt (Cracraft et al. 1998), and only the mitochondrial sequence was used in the analysis.

The mtDNA sequences obtained were aligned using Se-Al v2.0a11 (Rambaut 1996) and visually checked. A cytosine repeat sequence in three African lions (sample ID numbers: 12, 13 and 15 in Table 1) could not be unambiguously scored, due to slippage during the initial PCR, and this region

was excluded from the analysis. A median-joining network was constructed using Network v4.1.0.3 (Bandelt et al. 1999) to depict phylogeographic patterns amongst the identified sequences. Maximum parsimony bootstrap analysis (100 replicates, gaps as fifth state, TBR model) was implemented in *PAUP*4.0b10* (Swofford 2001) to show degree of statistical support for intraspecific groupings.

Results

Approximately 130 bp of the mitochondrial HVR1 was amplified from 32 origin-known samples, including four Barbary lions and one Cape lion (Table 1, Figure 1). Sequences from those 32 lion individuals comprised 11 different haplotypes defined by 13 variable sites (Table 2). The haplotypes-10 and −11 were detected only in extinct populations (North African and Iranian), suggesting that these haplotypes have been lost in the wild with the extinction of the corresponding populations (Figures 1 and 2). All four origin-known Barbary lions had an identical haplotype (one homoplasious indel separate from nearest haplotype, see Table 2), unique to the population, suggesting that mtDNA (HVR1) provides a good molecular marker to identify Barbary lions. The median-joining network suggests that a relatively simple mtDNA structure existed amongst the closely-related North African–Asian lion populations in comparison to that amongst the sub-Saharan populations (Figure 2). In contrast, the origin-known Cape lion had haplotype-5, one of the two haplotypes (haplotypes-2 and −5) that were widespread in eastern-southern Africa.

Using the geographic framework produced by the median-joining network we were able to identify two specimens with uncertain provenance. An old specimen kept at the National Museum of Natural History, Paris (Sample 33 in Table 1), was assignable to the Barbary lion. An old specimen in the Zoological Museum, University of Amsterdam (Sample 39 in Table 1), tentatively identified as a Cape lion (van Bree 1998) contained the Indian haplotype (Haplotype-9). Amongst the Moroccan King's lions tested, one produced a sequence identical to haplotype-7 (characterizing the lion from northeast Sudan), and the other four to haplotype-6 (characterizing lions from the Central African Republic and Ethiopia).

Discussion

The Barbary lion

Our results suggest that a closely related group of lion populations was formerly distributed from North Africa through the Middle East to India. The critically endangered c. 300 Asiatic lions found around the Gir Forest, northwest India (Singh 1997), appear to be the only survivors of this group. All North African–Asian haplotypes found are unique to this group, suggesting that they form a distinct phylogeographic cluster within the species, although this is not strongly supported by Bootstrap analysis. However, intraspecific grouping amongst the modern lions appear to be recent as they share a last common ancestor only c. 70–200 thousand years ago (Burger et al. 2004).

The results suggest that haplotype-11 is a good molecular marker for identifying Barbary lions. A few European museums keep old lion specimens, which are suspected to be of Barbary origin (Yamaguchi, unpublished). The small size (c. 130 bp) of the HVR1 would make DNA recovery from (and origin identification of) old museum specimens not only realistic, but also quick and cost-effective. Newly discovered Barbary lion specimens could contribute to increasing our understanding of the North African lion population, both morphologically and anatomically.

The Cape lion

Unlike the Barbary lion, our results do not support the "distinctness" of the Cape lion. This is consistent with a geography-based idea, upon which the continuous lion distribution in Southern Africa challenges the "distinctness" of the Cape lion (Yamaguchi 2000). Admittedly, only one sample that yielded a sequence originated from the southern parts of the former Cape Province and Orange Free State, Republic of South Africa. However, we found only two haplotypes (haplotypes-2 and −5) over the entire lion range in southern Africa (see Figure 1) surrounding the region where the Cape lion was formerly found (Mazák 1975), and the sample from the southern Cape itself possesses one of these two haplotypes. Considering these findings, it seems quite probable that the Cape lion was not a phylogenetically

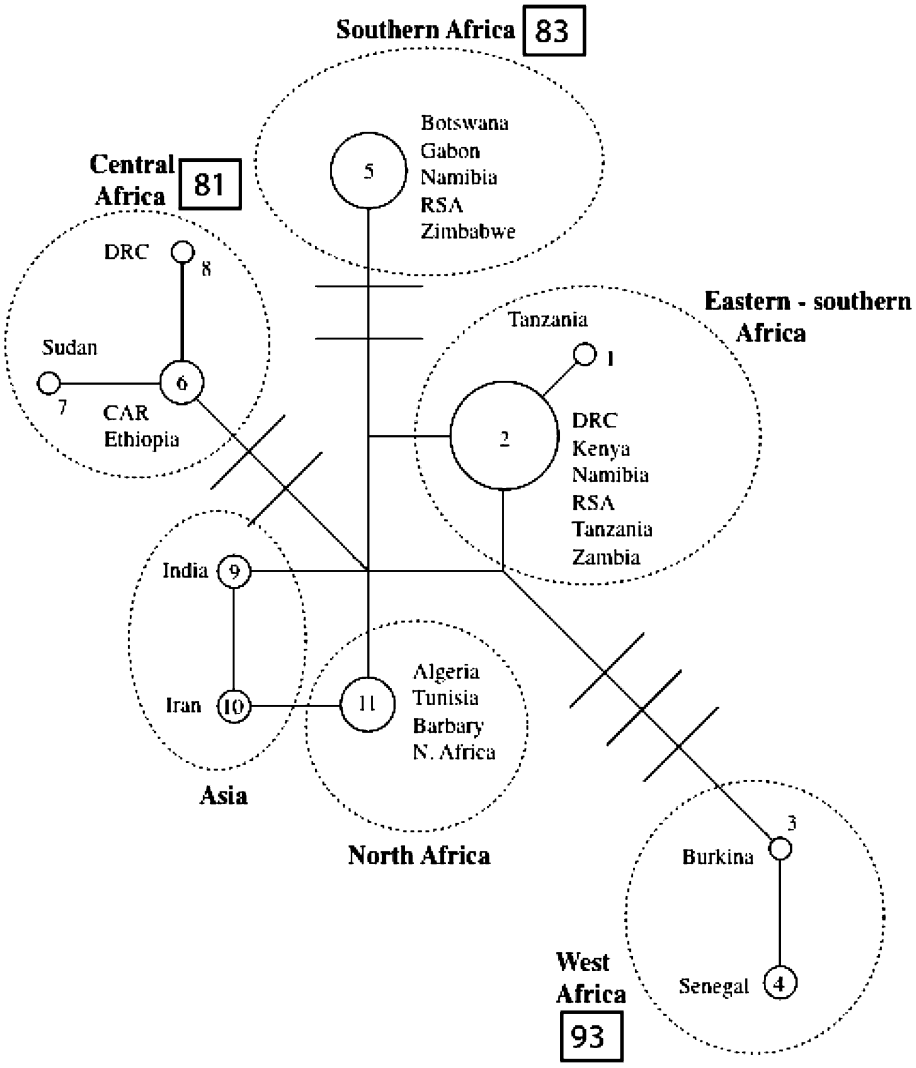

Figure 2. Median-joining network depicting phylogenetic and geographical relationships amongst lion mtDNA haplotypes based on c.130 bp of control region sequence. Different numbers denote different haplotypes, and the area of the associated circle is proportional to the haplotype frequency. The length of each connecting line is proportional to the distance between haplotypes (defined as the number of mutations). A point of intersection without an associated circle denotes a hypothetical common ancestor. Maximum parsimony bootstrap (100 replicates, gaps as fifth state) support for groups is indicated within squares.

distinct population, but the southernmost population of the extant southern African lion. In this context, we suggest that the Cape lion may be restored *in situ* by using the extant southern African lion, although defining a population based only on neutral genetic markers would need careful evaluation (Wayne and Brown 2001). Unless further evidence suggests otherwise, the southern African lions possessing haplotype-5 should be used for the restoration of the Cape lion.

Implications for ex situ lion conservation

Resource-effective strategy is necessary for *ex situ* conservation as available space and other resources are limited (Ryder 1986). It is suggested that taxon identification, at both subspecies and species levels, is a necessary step for a resource-effective *ex situ* conservation (Uphyrkina and O'Brien 2003; Chen et al. 2004). Although this may justify animals of known-origin receiving

higher priority for breeding compared to generic zoo animals, ironically, doing so may decrease the overall genetic diversity of some species (Nowell and Jackson 1996; Hendrickson et al. 2000; Uphyrkina and O'Brien 2003). If generic animals contain the genes of extinct populations, they should prove crucial for preserving the overall genetic diversity of the species. According to the International Species Information System (http://www.isis.org/abstracts/abs.asp), approximately 77% of c. 1,300 lions registered have uncertain origins. Admittedly, our results show only maternal lines, and further studies are necessary before deciding which animals should be given breeding priorities. However, the apparent lack of haplotypes-10 and −11 within the current geographic range of the lion may suggest the importance of keeping any animal possessing either haplotype. Also, saving wild lion populations in the Sahel (steppe/savannah areas immediately south of the Sahara) (see Figure 2) is a conservation priority (Nowell and Jackson 1996; Bauer and van der Merwe 2002). Therefore, although the Moroccan King's lions are unlikely to be maternally Barbary, it would be worthwhile maintaining the collection for the purpose of preserving overall lion genetic diversity.

Acknowledgements

We thank C. Claude, W. Cotteril, J. Cuisin, B. Fernholm, O. Grönwall, B. Haddane, A. Harland, K. Hill, F. Kigozi, A. Kitchener, G. Lenglet, M. Lockyer, D. MacFadyen, S. Pitteri, F. Renoud, D. Robineau, C. Smeenk, M. Tranier, P.J.H. Van Bree, L. Werdelin, R. Ziegler and V. Ziswiler for their support for sampling. We also thank Simon Y. W. Ho for insightful comments on the manuscript. Financial support for RB, IB, and AC was provided by NERC, BBSRC, and the Leverhulme and Wellcome Trusts.

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