CONSERVATION GENETICS OF *PANTHERA PARDUS* IN SOUTH AFRICA: PHYLOGEOGRAPHY OF MITOCHONDRIAL LINEAGES

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: ……..

Date: ……..

ABSTRACT

Leopards (*Panthera pardus*) are one of the most adaptable cats, having a wider distribution than any other large felid. In South Africa some populations are currently threatened, existing as heavily fragmented or isolated entities due to human expansion, habitat loss and direct persecution. Using 309 base pairs of the mitochondrial DNA control region (mtDNA CR), the population structure, population history and genetic diversity of leopards in South Africa was investigated. Segments revealed 7 variable sites, resulting in 7 mtDNA haplotypes. Analyses by AMOVA revealed two distinct mtDNA genetic assemblages, the first corresponding to the Western Cape; and the second comprising the Eastern Cape, Kwazulu Natal, Limpopo and Mpumalanga regions. Clades were estimated to have diverged during the Pleistocene, between 66,500 and 112,000 years ago. The leopard population in South Africa appears to have been stable for a long period of time and overall displays high levels of mtDNA genetic diversity. Genetic diversity estimates for leopards in the Western Cape, however, were exceptionally low (π = 0.16%), comparable to that found in inbred cheetah populations. This suggests that Western Cape clade may have suffered genetic impoverishment due to having undergone a recent demographic change. Although our data only reflects maternal phylogeography and rely on small sample sizes, it can nonetheless be used as a framework for developing future management strategies for leopards in South Africa.

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GENERAL INTRODUCTION

In Africa, the leopard's (*Panthera pardus*) historic range spanned the entire continent. Today, although its' geographic range is still extensive, many leopard populations now exist as heavily fragmented or isolated entities. This has largely been due to anthropogenic factors such as, human population expansion, habitat reduction, hunting, poaching and 'problem-animal' control (Norton, 1986; Uphyrkina *et al.*, 2001). In South Africa, a severe threat to the leopards' continued survival is 'leopard-farmer' conflict. In the former Cape Province, bounty systems were implemented as early as 1658, as leopards' and other predators, posed a serious threat to the livelihood of the Dutch settlers (Skead, 1980; Norton, 1986). Three hundred years of persecution followed with bounty systems abolished in the 1960's. By the time leopards were declared a "protected wild animal" in 1974, population numbers and distributional range had been severely reduced (Norton, 1986). In the Cape, dwindling leopard populations cling to survival within key refuges in the Cape Fold Mountains.

An important component of current management strategies to conserve threatened or endangered species is not only to maintain viable population sizes, but also to preserve genetic diversity. Populations in the southern part of South Africa (Western and Eastern Cape) have long been recognized as morphologically different due to their smaller stature, being only half the size of their northern counterparts (Skinner and Smithers, 1990). Recent taxonomic and phylogeographic research identified only one subspecies, *P. p. pardus*, occurring throughout Africa (Miththapala *et al.* 1996; Uphyrkina *et al.* 2001). This study, however, was able to collect only 5 samples from one population in the Kruger National Park, South Africa. It is generally assumed that leopards in sub-Saharan Africa comprise one continuous population with little or no population structuring, as they are able to disperse over large distances and are highly adaptable. This, however may not be true for South African populations, as leopards appear to be absent from the central region (Fig. 1), thereby making any north-south migration unlikely. Uphyrkina's (2001) study therefore can not be used as an accurate representation of the phylogeographic partitioning of leopards in South Africa. This study therefore is an attempt to elucidate the genetic diversity and population structuring of leopards in South Africa. It will also determine whether the smaller leopards of the 'Cape' should be recognized as a separate management unit. This in turn will have important implications for the

conservation status of these leopards as well as for future management and translocation policies.

Felidae Phylogeny

The Felidae is represented by 37 extant species, which diverged from a common ancestor during the Miocene, 10-15 million years ago (O'Brien & Johnson, 2005). Recent phylogenetic research reveals the separation of this family into eight distinct lineages (Appendix 1), the domestic cat, the leopard cat (Asian), the pumas, the *Lynx*, the ocelot, the *Pantherine* group, the caracal and the bay cat lineage (Johnson & O'Brien, 1997; Johnson, *et al*. 2005, as cited in O'Brien & Johnson, 2005).

Origin and Systematics of *Panthera pardus*

The origin of modern leopards

Based on fossil records, the lion and the leopard occurred simultaneously in Tanzania, Africa, approximately 3.5 million years ago while fossils of a jaguar-like leopard, approximately 2 million years old, were found in the Indian Siwaliks (Hemmer, 1976; Turner & Anton, 1997). Divergence between lion and leopards should therefore pre-date these fossil remains.

Genetic markers, together with the fossil record, argue for an African origin for modern leopard subspecies dating between 470,000 and 825,000 years ago, with a more recent migration into Asia approximately 169,000 to 400,000 years ago (Uphyrkina *et al.* 2001, O'Brien & Johnson, 2005). Using DNA sequences from the control region (CR) and NADH-5 of mitochondrial DNA (mtDNA), and 25 polymorphic microsatellite loci, Uphyrkina *et al.* (2001), established that African leopards, *Panthera pardus pardus* (Linneaus, 1758), possessed the highest genetic diversity in both mtDNA and microsatellite loci than any other population (Appendix 2). African leopards were also shown to have more mtDNA sites in common with outgroups, *P. leo* (lion)*; P. tigris* (tiger)*; P. onca* (jaguar)*; and P. uncia* (snow leopard), than other populations sampled.

Taxonomy of Panthera pardus

Pocock (1932) described 27 subspecies of the leopard, based on global variation in morphology, pelage colour and patterning (Appendix 3). Thirteen subspecies were recognised in Africa alone with *P. p. melanotica* and *P. p. shortridgei* occurring in the sub-Saharan region (Smithers, 1971; Skinner and Smithers, 1990). *P. p. melanotica* was, however, later regarded as a melanistic form rather than a separate subspecies (Dobroruka, 1966). Melanism in leopards can be found throughout their range and is inherited as a recessive trait (Eizirik *et al*., 2003). New research employing molecular genetic tools has led to the taxonomic revision of the leopard into nine distinct subspecies worldwide (Miththapala *et al.,* 1996; Uphyrkina *et al.,* 2001), with only one, *P. p. pardus*, occurring throughout Africa. Uphyrkina *et al.* (2001), however, states that "this may be an underestimate of modern phylogeographic population structure" due to inadequate sampling of populations in Africa: only 17 leopards from 7 populations throughout Africa were included in these analyses, presumably due to the logistical difficulties of obtaining DNA samples.

Life history characteristics of *Panthera pardus*

Leopards are generalist predators, preferring to hunt at night. They will prey on anything from large antelope (Le Roux & Skinner, 1989), to rodents (Norton *et al*., 1986), fish or even dung beetles (Fey, 1964). Their adaptable diet has allowed leopard populations to extend their range, occupy diverse habitats, and so become one of the most widespread cat species in the world. Where leopards co-habit agricultural regions, they are occasionally responsible for depredation of livestock such as sheep, goats or cattle. Such 'leopard-farmer' conflict often results in the local extermination of the species (Stuart, 1981).

Leopards are secretive, solitary cats, making demographic studies of populations virtually impossible. Current studies utilizing camera traps or GPS collars are costly and generally only assess one particular population. Populations, however, can vary drastically in ecological characteristics, depending on food availability and terrain. In South Africa, leopards were found to have home range sizes of approximately 400 km^2 in the Kgalagadi National Park (Bothma & le Riche, 1984), between 388 to 487 km^2 in the Jonkershoek mountains (Norton &

Lawson, 1985), but an order of magnitude smaller (40 to 69 km^2) in the Cederberg mountains (Norton & Henley, 1987). This variation between populations often makes demographic studies less revealing as the information is not always generalizable.

Both male and female leopards are territorial, scent marking by spraying urine and protecting their territories against individuals of the same sex (Skinner and Smithers, 1990). Territories of males are generally larger than those of females, while the territories of several females may overlap that of one male. Sub-adults disperse at roughly 13-18 months of age (Skinner and Smithers, 1990). Young males will usually disperse further in search of territories as they require larger home ranges, and this could affect the genetic structuring of a population.
Leopards also have short generation times, reaching reproductive maturity between 2-4 years of age (Skinner and Smithers, 1990).

Morphological differences in leopards from various habitats in South Africa have been observed (Pocock, 1932; Norton, 1984; Skinner & Smithers, 1990), the most apparent difference being the significantly smaller size (mass) of leopards occurring in the Cape region. "Bushveld" leopards of the savanna regions have an average weight of 50-60 kg for males, and 30-40 kg for females, while leopards in the Cape average 30-40 kg for males and 20-28 kg for females (Norton, 1984; Skinner & Smithers, 1990). Differences in size have long been attributed to variation in nutrition. In the Cederberg mountains of the Western Cape, South Africa and the Matopo hills of Zimbabwe, leopards were found to prey predominantly on rock hyrax (*Procavia capensis*) and smaller antelope species, such as klipspringer (*Oreotragus oreatragus*) (Norton et al., 1986; Grobler & Wilson, 1972), while feeding mainly on medium sized mammals and antelope species in the Kalahari (Bothma & le Riche, 1984). This factor, however, has never been studied or shown to cause the variation in size of leopards.

Distribution of *Panthera pardus*

Leopards are one of the most adaptable cats, having a wider distribution than any other large carnivore. Globally their range spans both hemispheres, where they can be found in at least 80 countries (Turnbull-Kemp, 1967). Leopards can occupy mountainous, forested, or semi-desert

areas (Skinner & Smithers, 1990), occurring at elevations ranging from sea level to 5700m – recorded on Mount Kilimanjaro (Guggisberg, 1975). Their ability to inhabit such a diverse range of habitats is attributed primarily to their flexible diet. In South Africa, their distribution includes the mountains of the Cape Fold Belt, the Magaliesberg, Waterberg, Soutpansberg, Drakensberg, lowveld areas of Mpumalanga, northern KwazuluNatal as well as along the Orange river (Fig. 1). The fact that leopards are so ubiquitous throughout Africa, has led to a degree of complacency amongst conservation bodies to better manage and protect the species.

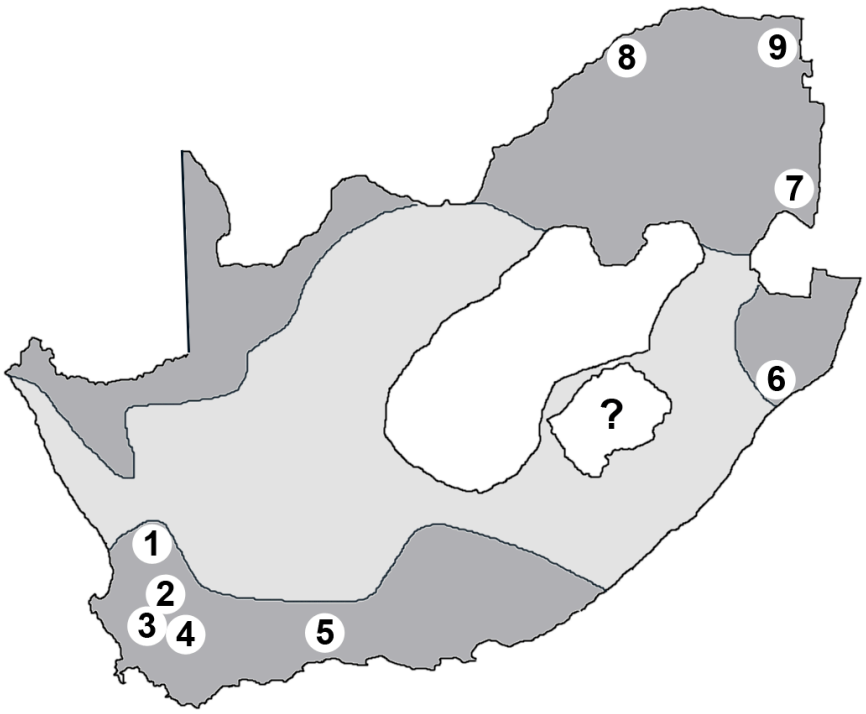


Fig. 1 Map showing current (light and dark grey) and core (dark grey) distribution of leopards in South Africa, along with sampling localities used in this study. Populations were sampled in the Western Cape at 1 = Cederberg mountains (n = 6), 2 = Ceres (n = 1), 3 = Hottentots Holland mountains (n = 2), 4 = Worcester (n = 1); in the Eastern Cape at 5 = Baviaanskloof mountains (n = 5); in Kwazulu Natal at 6 = KZN National Parks (n = 1); in Mpumalanga at 7 = lowveld (n = 3); in Limpopo at 8 = Tuli (n = 2) and 9 = Tzaneen (n = 8). (redrawn from Norton, 1984).

Aims

The aims of this project were to:

1. Collect as many genetic samples from leopards, throughout their South African range, as possible. These will be available for planned future genetic studies undertaken, as an important component of conservation strategies for South African leopards.
2. To attain preliminary estimates of gene flow and genetic relatedness amongst South African populations by analyzing a segment of the mtDNA CR.
3. To test the hypothesis that the Cape population is a unique genetic unit.

MATERIALS AND METHODS

Samples

Samples of 82 individual leopards were obtained from various localities within South Africa (Appendix 4). Nature conservation officials and private researchers provided most of the specimens used to construct the phylogeography of leopards in South Africa. It should be noted that none of the individuals sampled, were from captive populations or from breeding projects. Hair, tissue and pelt samples were preserved by collectors in ethanol, saline solutions or by drying. Tissue samples received in solution were dehydrated with the use of salts before DNA extraction. Due to the poor quality of DNA in some of the samples, not all successfully amplified the mtDNA CR. A total of 29 samples were eventually used in this investigation (Table 1).

Table 1: Leopard sample collection used in this study, including region of origin, sampling code, assigned mtDNA haplotype and source of specimens.

Geographical Area	Number of Individuals	Sample Code	mtDNA Haplotype	Sample Sources
Western Cape:				
Cederberg	6	CED001	1	Cape Nature – Rika du Plessis
		CED002	1	Cape Nature – Rika du Plessis
		CED009	1	Amathole museum
		CED060	1	The Cape Leopard Trust - Quinton Martins
		CED067	1	The Cape Leopard Trust - Quinton Martins
		CED068	1	The Cape Leopard Trust - Quinton Martins

Ceres	1	CER019	1	Amathole museum
Worcester	1	WOR020	1	Amathole museum
Hottentots Holland mountains	2	HOT066	1	Cape Nature – Peter Lloyd
		HOT063	2	Cape Nature – Guy Palmer
Eastern Cape:				
Baviaanskloof mountains	5	BAV003	3	Eastern Cape Nature Conservation - Hennie Swanevelder
		BAV004	3	Eastern Cape Nature Conservation - Hennie Swanevelder
		BAV037	3	University of Port Elizabeth - Graham Kearley
		BAV038	3	University of Port Elizabeth - Graham Kearley
		BAV039	3	University of Port Elizabeth - Graham Kearley
Kwazulu Natal (KZN):				
KZN Parks	1	KZN052	4	KZN Parks - Dr. Adrian Armstrong
Mpumalanga:				
Lowveld	3	MPU040	6	Mpumalanga Parks Board - Gerrie Camacho

		MPU043	6	Mpumalanga Parks Board - Gerrie Camacho
		MPU048	5	Mpumalanga Parks Board - Gerrie Camacho
Limpopo:				
Tuli Block	2	TUL061	6	Tuli Leopard Project - Villiers Steyn
		TUL062	6	Tuli Leopard Project - Villiers Steyn
Tzaneen	8	TZA069	5	K.E.R.I Research - Cailey Owen
		TZA070	5	K.E.R.I Research - Cailey Owen
		TZA071	5	K.E.R.I Research - Cailey Owen
		TZA073	5	K.E.R.I Research - Cailey Owen
		TZA074	7	K.E.R.I Research - Cailey Owen
		TZA075	5	K.E.R.I Research - Cailey Owen
		TZA076	5	K.E.R.I Research - Cailey Owen
		TZA077	5	K.E.R.I Research - Cailey Owen

Molecular Analysis

DNA extraction, amplification and sequencing

Approximately 0.5 g of tissue or skin was digested using proteinase K-mediated lysis for at least 24 hours at 45°C. Total genomic DNA was extracted following the standard phenol-chloroform method and precipitated with cold ethanol in the presence of salt at -70°C overnight (Sambrook *et al*. 1989). Samples were centrifuged at 13,000 rpm for 20 minutes and supernatant discarded. The DNA pellet was left to dry completely on a heat block at 45°C until all traces of alcohol were removed. DNA pellets were then reconstituted in TE buffer (10mM Tris-Cl [pH 7.6], 0.1 mM EDTA) and stored at -20 °C.

DNA from bone and teeth was extracted following a nondestructive protocol developed by Rohland *et al*. (2004), which utilizes a guanidinium-thiocyanate (GuSCN) buffer.

DNA was extracted from hair and skin samples, for which very little starting material was available, using QIAGEN DNeasy® or QIAamp® DNA Micro kits (QIAGEN Ltd.).

Polymerase chain reaction (PCR) was used to amplify the mtDNA CR using universal primers N777 (5' – TACACTGGTCTTGTAAACC - 3') and DLH1 (5' - CTTGAAGTAGGAACCAGAT - 3') as described by Kocher *et al*. (1989). Where samples failed to amplify with universal primers, designed primers LeoCRF (5'-GTGCATTAACTGCTTGTC-3') and LeoCRR (5'-CAGGTGATCAAGCTCTTT-3') were used in an attempt to improve amplification success.

The 25µl PCR reaction mixture contained between 5 and 60 ng target DNA, 2,5 µl (10x) buffer, 4 µl $MgCl_2$ (25 mM), 0.5 µl of forward and reverse primer (10 µM), 0,5 µl dNTPs (10mM), 0.5 U Taq and distilled water to make up total volume. PCR amplifications were performed in a GeneAmp PCR system 2700 (Perkin-Elmer) under the following conditions: an initial denaturation step for 3 minutes at 94°C; followed by 30 - 35 cycles of denaturation for 30 seconds at 94°C, annealing for 45 seconds at 48°C, an extension for 1 minute 30 seconds at 72°C; followed by a one cycle extension step for 10 minutes at 72°C. PCR products were separated and checked on a 0.8 % agarose gel. DNA that successfully amplified was excised and subsequently purified using the QIAquick® gel extraction kit (QIAGEN Ltd.). Purified

products were cycle sequenced using BigDye sequencing kit (Applied Biosystems) and analysed on an ABI 3100 automated sequencer.

A long repetitive G-C rich region, similar to that found in jaguars and other felid species (Eizirik *et al*. 2001) was identified within the mtDNA control region of leopards. Only partial DNA fragments flanking these repeats could be sequenced. Sequences obtained using both forward and reverse primers showed no or very little overlap and therefore only reverse sequencing was performed as it produced the largest usable nucleotide fragment.

Some problematic samples only amplified once 3µl bovine serum albumin (BSA) was added to the reaction mixture. DNA amplification may be improved by BSA as it acts to bind potential polymerase inhibitors during PCR reactions (Sensabaugh, 1994; Satoh *et al*., 1998).

Sequence alignment and data analysis

A homologous region of 309 nucleotides of the mtDNA CR, was obtained for most individuals sequenced (Appendix 5). DNA sequences were edited and aligned using CLUSTALX (Thompson *et al*., 1997). Some individuals had ambiguous base calls (double peaks on the chromatogram) at specific positions along the mtDNA sequence. Given that mtDNA is uniparentally inherited, the presence of multiple bases at a specific site is unexpected. The ambiguities were attributed to having co-amplified and sequenced a numt (mt copy present in the nuclear genome) (Appendix 6). Numts have been reported in many vertebrates including, primates, birds and cats (Schmitz *et al.*, 2005, Grosso *et al*., 2006, Kim *et al.*, 2006). Pertinent to the focus of the present study, a large nuclear mitochondrial pseudogene was found in at least five Panthera species, the tiger, jaguar, leopard, lion and the snow leopard (Kim *et al*., 2006). This transposed element consisted of at least 12,536 base pairs (bp), representing 74% of the mitochondrial genome and is one of the largest numts found in eukaryotes.

A consensus mtDNA sequence was created in CLUSTALX from samples which showed no ambiguities. Ambiguities were then scored based on this consensus sequence and extra peaks which were attributable to the numt were disregarded. No additional changes were made to sequences, i.e. if a site showed ambiguities which were not present in the mtDNA consensus sequence, it was not scored.

The program TCS version 1.06 (Clement *et al*., 2000) was used to identify mtDNA haplotypes (Table 1) and to construct an unrooted haplotype network (Fig.2).

Measures of genetic variability within populations (haplotype diversity, *h* and nucleotide diversity, π) were calculated from the mtDNA data set using ARLEQUIN 3.01 (Excoffier *et al*., 2006). Haplotype diversity is described as the probability that two mtDNA sequences randomly selected from a sample will be different, while nucleotide diversity is the probability that two homologous nucleotides randomly selected from a sample will be different (Nei, 1987).

An Analysis of Molecular Variance (AMOVA) (Excoffier *et al*., 2006) was used to investigate population structuring of leopards within South Africa. F_{ST} and Φ_{ST} values were calculated in order to assess genetic divergence between populations. F_{ST} estimates the amount of diversity between populations based on observed haplotype frequencies, while Φ_{ST} utilizes both haplotype frequencies and nucleotide diversity. The statistical significance of these values were tested in ARLEQUIN using 10 000 permutations. The Tamura-Nei model (Tamura and Nei, 1993) was used to construct a distance matrix, while the gamma shape distribution parameter was calculated using maximum likelihood in PAUP* version 4.

The divergence time between genetically distinct clades was estimated by applying the equation:

$$\mathbf{T = \tau/2u\ldots} \qquad \text{(Rogers and Harpending, 1992)}$$

Where: T = time since divergence

τ = mutational time, measured in 1/2u generations

u = mutation rate of sequenced DNA segment.

If the substitution rate (μ) for a region of DNA is known, the mutation rate (u) can be calculated, as u = μ x the number of bases sequenced x generation time

To investigate whether populations showed signs of expansion, the Harpending's raggedness statistic (Harpending, 1994) was calculated and a mismatch distribution analysis performed. Mismatch distribution analysis compares the distribution between the observed and expected

pairwise nucleotide site differences among haplotypes (Rogers and Harpending, 1992). Populations which have been expanding show nearly unimodal (Poisson) distributions, whereas populations which have been stable for longer periods generally show multi-modal distributions.

Tajima's D and Fu's F were calculated to test for deviations from Hardy-Weinberg equilibrium. In populations where an excess of rare alleles and a decline in common alleles is detected, both Tajima's D and Fu's F will have statistically significant negative values (Fu, 1997, Excoffier *et al*., 2006). This departure from equilibrium is indicative of a recent demographic change such as a population expansion or contraction or, alternatively, selection (van Hooft *et al*., 2002). Both the raggedness statistic and neutrality tests were performed using ARLEQUIN.

RESULTS

A 309 bp region of the mtDNA CR, representing 1.87% of the 16,500 bp found in feline mtDNA (Menotti-Raymond & O' Brien, 1993) was successfully sequenced in 29 *P. pardus* individuals from various localities within South Africa. DNA from tooth and hair samples proved to be extremely difficult to amplify having a success rate of only 15% and 20%, respectively (Appendix 4). DNA from salted skin samples amplified moderately well (54% success rate), while fresh tissue, either dried with the use of salt or preserved in saline solution, had the greatest amplification success (71%). Table 2 lists the segregation sites, the nucleotide transition and the nucleotide position along the segment at which the change occurred. These variable sites defined 7 haplotypes (Table 1). The Western Cape contained two mtDNA haplotypes which were unique to the area, while the Eastern Cape, Kwazulu Natal and Limpopo each had one unique mtDNA haplotype (Table 3). The province of Mpumalanga was found to share 2 haplotypes with Limpopo. Three haplotypes (Haplotypes 2, 4 and 7) were represented by single individuals.

Table 2: Variable sites of derived haplotypes in an analysis of the mtDNA CR (309 bp) in *Panthera pardus*. Nucleotides identical to haplotype 1 are marked with a dash, with the position of the variable site indicated above.

	Segregation sites						
Haplotype	108	120	121	148	230	241	291
1	**G**	**A**	**G**	**C**	**C**	**C**	**C**
2	A	-	-	-	-	-	T
3	A	-	A	-	-	T	T
4	A	-	A	-	T	T	T
5	A	G	A	-	-	T	T
6	A	G	A	T	-	T	T
7	A	-	A	T	-	T	T

Table 3: Frequency of occurrence of *Panthera pardus* haplotypes in the five sampled provinces in South Africa. The number of sampled individuals (n) differed at each site.

	n				
Haplotype	Western Cape	Eastern Cape	Kwazulu Natal	Mpumalanga	Limpopo
1	9	0	0	0	0
2	1	0	0	0	0
3	0	5	0	0	0
4	0	0	1	0	0
5	0	0	0	1	7
6	0	0	0	2	2
7	0	0	0	0	1

The haplotype network constructed using TCS revealed significant phylogeographic structuring of the leopard population in South Africa and clustered the individuals into two assemblages/clades. Clade A corresponds to the Western Cape haplotypes (1 and 2), while Clade B is composed of the Eastern Cape, Kwazulu Natal, Limpopo and Mpumalanga haplotypes (3, 4, 5, 6 and 7) (Fig. 2). The network also indicates that haplotypes belonging to these two clades are 2 - 4 mutational steps apart.

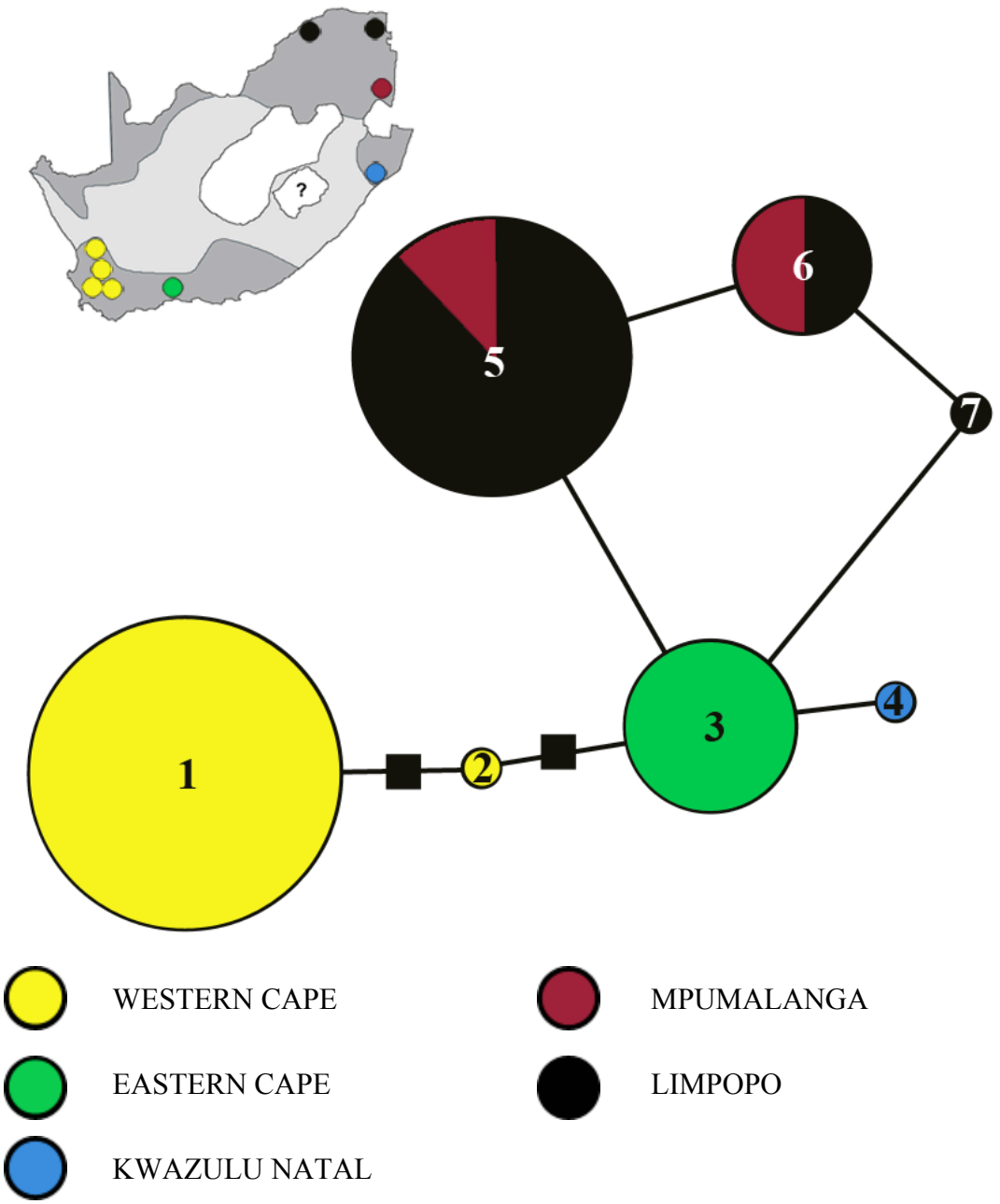


Fig. 2: Network of CR mtDNA haplotypes of *Panthera pardus*. Haplotypes are represented by circles, the area of which is proportional to the haplotype frequency. Colours represent different geographic regions (provinces) sampled, while subdivision of haplotypes represents the proportion of haplotypes found in each region. Connecting lines indicate a single nucleotide substitution, with a ≥95% probability of being correct. A solid square indicates an internal node absent from the sample. Network drawn to depict the approximate geographic origin of haplotypes (North, South, East, West).

Samples were assigned to two phylogeographic groups, clades A and B. Samples from the Western Cape, corresponding to haplotypes 1 and 2, were assigned to Clade A. Samples from the Eastern Cape, Kwazulu Natal, Mpumalanga and Limpopo, corresponding to haplotypes 3, 4, 5, 6 and 7, were assigned to Clade B. The division of samples into these 2 clades was based on haplotype clustering in the minimum spanning network (Fig. 2) as well as a neighbour-joining tree (not shown) which indicated 78% bootstrap support for the separation of haplotypes into these two clades. Samples from these two clades were pooled to obtain indications of genetic variation and population structuring and also to establish whether populations were in Hardy-Weinberg equilibrium. AMOVA analysis based on this separation revealed significant structuring among haplotypes, with an F_{ST} value of 0.478 and Φ_{ST} value of 0.826 ($p < 0$). The significant separation among haplotypes was also confirmed when the two clades were compared and separated into southern (Western Cape and Eastern Cape) and northern (Kwazulu Natal, Mpumalanga and Limpopo) clades, as F_{ST} values decreased to 0.406, while Φ_{ST} decreased to 0.614 ($p < 0$).

Haplotype diversity and nucleotide diversity was considerably low in Clade A, while Clade B showed a moderate level of haplotype diversity, but a low level of nucleotide diversity (Table 4). As expected when overall levels were determined within the species there was an increase in both haplotype and nucleotide diversity.

Table 4: Genetic variation within the study's designated Clade A, Clade B and combined populations. Indices measured include the number of haplotypes (H); haplotype diversity (h); nucleotide diversity (π); and number of variable sites (S).

Group	n	H	h	π	S
Clade A	10	3	0.200	0.0016	55
			+/- 0.154	+/-0.0017	
Clade B	19	4	0.743	0.0035	67
			+/- 0.064	+/-0.0027	
Combined	29	7	0.803	0.0091	71
			+/- 0.039	+/-0.0055	

The divergence time between genetically distinct clades was estimated by applying the Rogers and Harpending (1992) equation, as stated in methodology. The substitution rate for a similar segment of the mtDNA CR of leopards was previously estimated to range between 0.0142/site/Myr (+/- 1.4% per Myr) and 0.024/site/Myr (+/- 2.5% per Myr) (Uphyrkina *et al*., 2001). By applying these substitution rates, a generation time of 2 years and a τ value of 5.911 obtained from ARLEQUIN, the estimated coalescence date of southern African leopard mtDNA haplotypes is between 199,265 and 336,786 years ago. Clades A and B were estimated to have diverged between 66,500 and 112,000 years ago.

The mismatch distribution for the combined leopard dataset resulted in a bi-modal histogram, characteristic of a stable population (Fig. 3), however, the sum of squared differences (SSD) statistic, revealed that the observed mismatch distribution did not depart from the estimated model of population expansion (SSD = 0.04; p = 0.35). Separate mismatch distribution plots for Clade A and Clade B were not undertaken as the number of haplotypes in each group, were too low to generate a meaningful distribution of haplotypes.

Further evidence that leopard populations have not undergone recent demographic change was indicated by Harpending's raggedness statistic for the combined dataset (r = 0.065; p = 0.530, Table 5). Tajima's D and Fu's F values for combined leopard populations were not significant, indicating that populations are in Hardy-Weinberg equilibrium (Table 5).

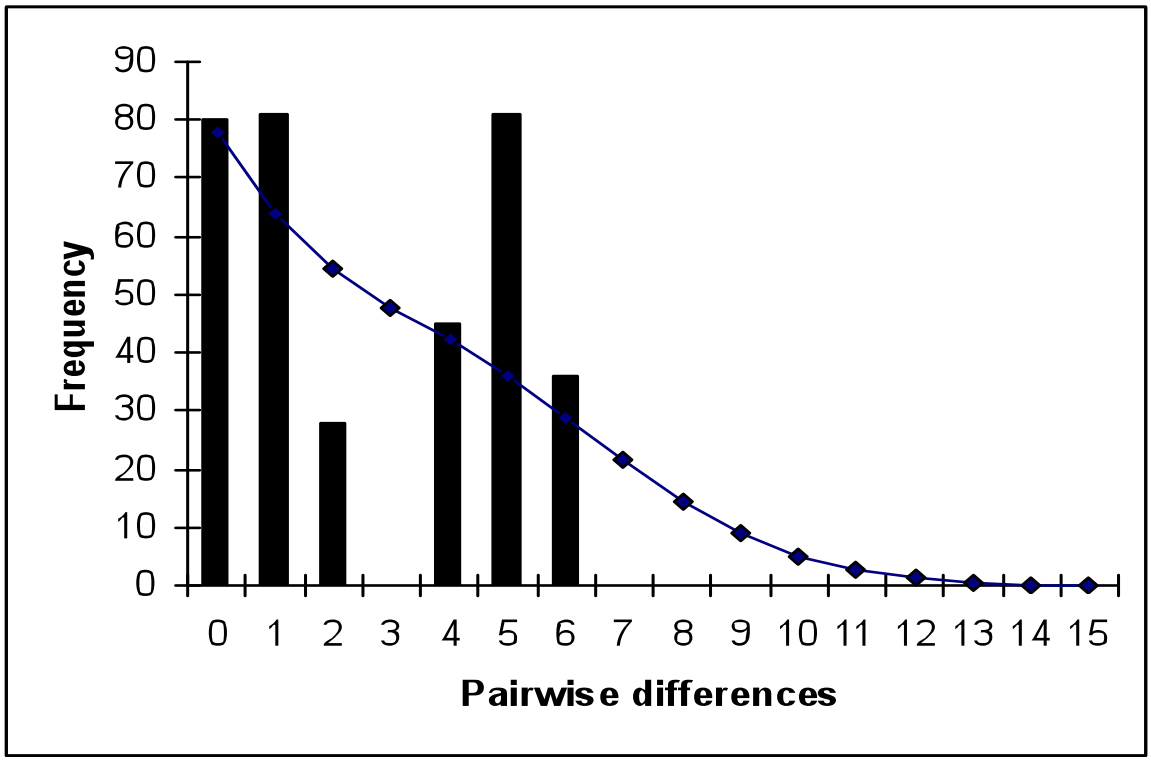


Fig. 3: Mismatch distribution plot of the combined leopard dataset. Columns indicate the observed frequency distribution for the number of pair-wise differences, while the line indicates the expected distribution under a model of population expansion.

Table 5: Results of tests for raggedness and selective neutrality (Tajima's D and Fu's F) for Clade A, Clade B and the combined leopard dataset.

Group	Harpending's Raggedness (p-value)	Tajima's D (p-value)	Fu's F (p-value)
Clade A	0.720 (0.960)	-1.401 (0.080)	12.032 (1.00)
Clade B	0.172 (0.460)	0.467 (0.704)	6.123 (0.990)
Combined	0.065 (0.530)	1.516 (0.940)	7.625 (0.989)

DISCUSSION

Population structure and gene flow

Two distinct mtDNA genetic assemblages were present among south African *P. pardus* individuals. One is located in the Eastern Cape, Kwazulu Natal, Mpumalanga and Limpopo and these locations contained mtDNA haplotypes 3, 4, 5, 6 and 7. The second mtDNA clade is confined to the Western Cape and contained two unique haplotypes (haplotypes 1 and 2; Table 3). Given the limited sampling it is possible that some haplotypes were not represented by the data set, however, if the results hold up against more intensive sampling it is important to realize that there are no or low levels of current female gene flow and major geographical partitioning within the South African leopard population. This conclusion is based on both the haplotype network (Fig. 2) and a neighbour-joining tree (not shown). The network indicates an absence of female migration between the western and eastern Cape regions, and between the southern and northern regions of South Africa. It should be noted, however, that a previous study on jaguar (*P. onca*) found significant structure in mtDNA phylogenies, yet higher levels of gene flow when examining microsatellite data (Eizirik *et al.*, 2001). This is most likely due to differences in dispersal patterns between females and males, where young males usually disperse further in search of territories, while females are generally philopatric. Alternatively, it could be that the separation among jaguar populations was too recent for the multilocus nuclear markers to detect the isolation.

My data shows some level of gene flow via female migration between Mpumalanga and Limpopo. Kwazulu Natal was represented by only a single individual and therefore no inferences could be made with regards to female migration into or out of this population.

Phylogeographic partitioning among the two clades was further supported by AMOVA analysis, as both F_{ST} and Φ_{ST} estimates were significant when samples were separated into clades A and B. This genetic clustering, however, does not agree with the distribution pattern observed in leopard populations in South Africa (Fig. 1), as one would expect leopards to be able to migrate the entire length of the Cape Fold mountains, which extend all along the southern and eastern coast. It also appears incongruent with previously recorded

morphological data, as leopards in the southern parts of South Africa (Western and Eastern Cape) exhibit smaller body sizes and mass when compared to their northern counterparts (Pocock, 1932; Norton, 1984; Skinner & Smithers, 1990). These results could imply that variation in body size is possibly due to selection caused by external factors such as the environment (terrain, prey availability or prey size), and that the small body size is not a synapomorphic characteristic. The smaller stature of leopards in the southern region may also be due to the lack of interspecific competition with other predators. In the north, leopards are sympatric with lion (*Panthera leo*), hyena (*Crocuta crocuta*), and wild dog (*Lycaon pictus*), whereas in the southern regions leopards occupy the role of apex predator.

The Mantel Test (Mantel, 1967) was not used to investigate isolation by distance as a possible cause for population structuring. This was due to sample sizes for each province being too small to calculate accurate F_{ST} values. The overall pattern of mtDNA genetic structuring, however, does not appear to be due to isolation by distance as the Western-Eastern Cape divergence appears more defined than between any two other populations (Fig. 2).

Population History

Observed levels of mtDNA genetic diversity within leopard populations in South Africa ranged from 0.200 (± 0.154) to 0.803 (±0.039) (Table 4). In Clade A (Western Cape), both π and *h* diversity estimates were low, indicating that this population could recently have undergone a demographic change which may have led to genetic impoverishment. Genetic diversity estimates for this study can not be directly compared with data from other studies, as homologous segments for other species are not available. However, when compared to mtDNA diversity estimated using RFLP's, the π diversity within this clade (π = 0.16%) was comparable to that estimated for inbred cheetah populations (π = 0.182%, Menotti-Raymond & O' Brien, 1993). It should be noted, however, that diversity estimates for Clade A were based on a small sample size and may be an underestimate for this population.

Clade B shows moderate levels of π diversity and high levels of *h* diversity, indicative of a more stable population.

When clades were combined, both π and h diversity estimates were higher (π = 0.91%; h = 0.803). Compared to results obtained for similar regions in the mtDNA CR, or from composite CR and NADH-5 segments (Appendix 2), genetic variation of leopards within South Africa is comparable to, or higher than, that found in jaguar populations or other leopard subspecies (Eizirik *et al*., 2001; Uphyrkina *et al.,* 2001).

The intraspecific mtDNA haplotype network (Fig. 2), generated by TCS showed very little divergence between haplotypes. All haplotypes were 1 – 6 mutational steps apart. This pattern is suggestive of a fairly common history, however, enough time has passed to allow for regional differentiation within populations.

Mismatch distribution analysis for combined populations showed a bi-modal distribution (Fig. 3), suggesting that in South Africa as a whole, the leopard population has not undergone recent demographic change. Sample sizes for each province, or separate clades, were too small to interpret using mismatch distribution. The sum of squares deviation indicated that the distribution did not diverge significantly from a model of population expansion. This test, however, may not be sensitive enough as sample size was low.

The inference of a stable population was supported by Fu's F and Tajima's D (Table 5) which suggests that the South African population is in Hardy-Weinberg equilibrium and has been stable for some time. Fu's F has been shown to be a more sensitive test than mismatch distribution analysis, in testing for demographic change under a variety of different circumstances (Ramos- Onsins & Rozas, 2002, Excoffier *et al*., 2006).

Based on previously reported estimates of mtDNA CR substitution rates, the estimated coalescence date of leopard mtDNA haplotypes in South Africa, is between 199,000 and 337,000 years ago (Uphyrkina *et al.,* 2001). This is in agreement with the findings above, which indicate that the South African leopard population has been relatively stable for a long period of time. The Western Cape population is estimated to have diverged from Clade B between 66,500 and 112,000 years ago. Divergence dates coupled with the absence of shared haplotypes between clades, suggests long-standing restriction of maternal gene flow.

Separation of these two clades may have been caused by the expansion and contraction of suitable habitat due to temperature fluctuations during the Pleistocene (Brain, 1985). The last 1 Myr have been marked by a chain of major glacial periods which led to a series of climatic and habitat fluctuations on the African continent (DeMenocal, 1995). Changes within the western region of the continent appear to have been more severe, while the eastern region remained more stable (Deacon & Lancaster, 1988; Matthee & Flemming, 2002). These climatic fluctuations and its associated habitat changes may have resulted in the west-east population structuring of leopards in South Africa. Recently other studies on *Mesamphisopus* (freshwater isopods), *Pedioplanus* (sand lizards), *Agama atra* (Rock agama) and *Myosorex* (shrews) have uncovered similar west-east population fragmentation within the Cape Fold Mountain range (Gouws *et al*., 2005, Makokha, 2006, Swart, 2006, Willows-Munrow *pers. comm*.).

Implications for the conservation of leopards in South Africa

Anthropogenic factors (hunting, poaching, human expansion, habitat loss and 'problem-animal' control) have led to marked declines in population numbers and range distribution of southern African leopards (Norton, 1986; Uphyrkina *et al.*, 2001) Populations in the Cape were the most hard-hit because this was the first region to be colonized by Dutch settlers. In the Cederberg mountains of the Western Cape, current research indicates that previous estimates of leopard population densities were vastly overestimated and that leopards occur in very low densities within the Cape Fold Belt (Martins, *pers comm*.). Their continued survival as small populations consequently relies on the ability of conservation bodies to make informed management decisions. Results presented in this study therefore have important implications for future conservation and management strategies of leopards in South Africa.

Our mtDNA data suggests the presence of two distinct mtDNA genetic assemblages, one comprising the Western Cape, while the other comprising the Eastern Cape, Kwazulu Natal, Limpopo and Mpumalanga provinces. Although the structuring of the leopard population is based on female genetic separation only, we follow Ryder and Moritz (Ryder, 1986, Moritz, 1994) and recommend that these clades be viewed as separate Management Units.

Translocation of leopards between these two clades should therefore be avoided. Within the larger genetic assemblage (Clade B) it would be advisable for translocation to occur between neighbouring groups or to mimic naturally occurring gene flow, as leopards may be adapted to local environments.

On the whole, the South African leopard population appears not to have undergone any recent demographic change and to have been stable for a long period of time. The Western Cape population alternatively, may have suffered recent genetic impoverishment. This small, remnant population shows genetic diversity comparative to that of inbred cheetah populations and may require special conservation strategies to ensure its survival.

In closing, we advise that future genetic testing include nuclear markers in order to assess the effects of male dispersal on the population structuring of leopards in South Africa as well as more extensive sampling in order to improve the accuracy of genetic testing.

REFERENCES

Applied Biosystems. Inc. 1994. Sequence Navigator DNA and Protein sequence comparison Software, Perkin-Elmer Corp., Forster City, CA.

Bothma J. D. P. & le Riche, E. A. N., 1984. Aspects of the ecology and the behaviour of the leopard, *Panthera pardus*, in the Kalahari desert. *Koedoe* **27**: 259 – 279.

Brain, C. K., 1985. Temperature-induced environmental changes as biological isolating mechanisms in southern Africa. *Species and Speciation* (ed. E. S. Vrba). Transvaal Museum, Pretoria, South Africa, pp 45 – 52.

Clement, M., Posada, D. & Crandall, K. A., 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**: 1657 – 1660.

Deacon, H. J. & Lancaster, N., 1988. *Late Quaternary paleoenvironments of Southern Africa.* Claredon Press, Oxford.

DeMenocal, P. B., 1995. Plio-pleistocene African climate. *Science* **270**: 53 – 59.

Eizirik, E., Kim, J., Menotti-Raymond, M., Crawshaw, P. G., O'Brien, S. J. & Johnson, W. E., 2001. Phylogeography, population history and conservation genetics of jaguars (*Panthera onca*, Mammalia, Felidae). *Molecular Ecology* **10**: 65 – 79.

Eizirik, E., Yuhki, N., Johnson, W. E., Menotti-Raymond, M., Hannah, S. S., O' Brien, S. J., 2003. Molecular genetics and evolution of melanism in the cat family. *Current Biology* **13**: 448 – 453.

Excoffier, L., Laval, G. & Schneider, S., 2006. *Arlequin version 3.01: An integrated software package for population genetics data analysis*. Computational and Molecular Population Genetics Lab, Switzerland.

Fey, V., 1964. The diet of leopards. *African Wildlife* **18**: 105 – 109.

Fu, Y. X. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* **147**: 915 – 925.

Gouws, G., Stewart, B. A., Matthee, C. A., 2005. Lack of taxonomic differentiation in an apparently widespread freshwater isopod morphotype (Phreatoicidea: Mesamphisopidae: *Mesamphisopus*) from South Africa. Molecular Phylogenetics and Evolution **37**: 289 – 305.

Grobler, J. H. & Wilson, V. J., 1972. Food of the leopard *Panthera pardus* (Linn.) in the Rhodes Matopos National Park, Rhodesia, as determined by faecal analysis. *Arnoldia* **5**(35): 1 – 10.

Grosso, A. R., Bastos-Silveira, C., Coelho, M. M., Dias, D., 2006. *Columba palumbus* Cyt b-like Numt sequence: comparison with functional homologue and the use of universal primers. *Folia Zoologica* **55**(2): 131 – 144.

Harpending, H. C., 1994. Signature of ancient population growth in a low-resolution mitochondrial DNA mismatch distribution. *Human Biology* **66**: 591 – 600.

Hemmer, H., 1976. Fossil history of living Felidae. *The World's Cat* (ed. Eaton R. L.). Carnivore Research Institute, Burke Museum, Seattle, pp. 1- 14.

Hunt, M. H., 1996. Biogeography of the order Carnivora. *Carnivore behaviour, ecology, and evolution, vol. 2* (ed. Gittleman J. L). Cornell University Press, Ithaca, NY, pp. 485 – 541.

Johnson, W. E., Eizirik, E., Murphy W. J., Pecon-Slattery J., Antunes A., O' Brien, S. J., 2005. *The explosive late Miocene radiation of the Felidae*. *In press*.

Johnson W. E. & O' Brien S. J., 1997. Phylogenetic reconstruction of the Felidae using 16S rRNA and NADH-5 mitochondrial genes. *Journal of Molecular Evolution* **44**(Suppl 1): S98 – S116.

Kim, J. H., Antunes, A., Luo, S. J., Menninger, J., Nash, W. G., O' Brien, S. J., Johnson, W. E., 2006. Evolutionary analysis of a large mtDNA translocation (numt) into the nuclear genome of the Panthera genus species. *Gene* **366**(2): 292 – 302.

Kocher, T. D., Thomas, W. K., Meyer, A. *et al*., 1989. Dynamics of mitochondrial DNA evolution in animals: Amplification with conserved primers. *Proceedings of the National Academy of Sciences* **86**: 6196 – 6200.

Le Roux, P. G. & Skinner, J. D., 1989. A note on the ecology of the leopard (*Panthera pardus* Linneaus) in the Londolozi Game Reserve, South Africa. *African Journal of Ecology* **27**: 167 – 171.

Linneaus, C., 1758. *System Naturae* (1), 10th Edition. British Museum of Natural History, London.

Makokha, J. S., 2006. Molecular phylogenetics and phylogeography of sand lizards, *Pedioplanis* (Sauria: Lacertidae) in southern Africa. *MSc. Thesis*, University of Stellenbosch.

Mantel, N., 1967. The detection of disease clustering and a generalized regression approach. *Cancer Research* **27**: 209 – 220.

Martin, R. B. & de Meulenaer, T., 1988. *Survey of the status of the leopard (Panthera pardus) in sub-Saharan Africa*. CITES Secretariat, Lausanne.

Matthee, C. A. & Flemming, A. F., 2002. Population fragmentation in the southern rock

agama, *Agama atra*: more evidence for vicariance in Southern Africa. *Molecular Ecology* **11**: 465 – 471.

Menotti-Raymond, M. & O' Brien, S. J., 1993. Dating the genetic bottleneck of the African cheetah. *Proceedings of the National Academy of Sciences USA* **90**: 3172 – 3176.

Miththapala, S., Seidensticker, J. and O' Brien, S. J., 1996. Phylogeographic subspecies recognition in leopards (*Panthera pardus*): Molecular genetic variation. *Conservation Biology* **10** (4): 1115 – 1132.

Moritz, C., 1994. Defining 'Evolutionary Significant Units' for conservation. *Trends in Ecology and Evolution* **9**: 373 – 375.

Nei, M., 1987. *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Norton, P. M., 1984. Leopard conservation in South Africa. *African Wildlife* **38**: 191 – 196.

Norton, P. M., 1986. Historical changes in the distribution of leopards in the Cape Province, South Africa. *Bontebok* **5**: 1 – 9.

Norton, P. M. & Henley, S. R., 1987. Home range and movements of male leopards in the Cedarberg Wilderness Area, Cape Province. *S. Afri. J. Wildl. Res.* **17**: 41 – 48.

Norton, P. M. & Lawson, A. B., 1985. Radio tracking of leopards and caracal in the Stellenbosch area, Cape Province. *South African Journal of Wildlife Research* **15**: 17 – 24.

Norton, P. M., Lawson, A. B., Henley, S. R. & Avery, G., 1986. Prey of leopards in. four

mountainous areas of the south-western Cape Province. *South African Journal of Wildlife Research* **16** (2): 47 – 52.

O'Brien, S. J., 1994. A role of molecular genetics in biological conservation. *Proceedings of the National Academy of Sciences USA* **91**: 5748 – 5755.

O' Brien, S. J., Johnson, W. E., 2005. Big cat genomics. *Annu. Rev. Genomics Hum. Genet*. **6**: 407 – 429.

Pocock, R. I., 1932. The leopards of Africa. *Proceedings of the Zoological Society, London* , pp. 543 – 591.

Ramos-Onsins, S. E. & Rozas, J., 2002. Statistical properties of new neutrality tests against population growth. *Molecular Biology and Evolution* **19**(12): 2092 – 2100.

Rogers, A. R. & Harpending, H., 1992. Population growth makes waves in the distribution of pairwise genetic differences. *Molecular Biology and Evolution* **9**: 552 – 569.

Rohland, N., Siedel, H., Hofreiter, M., 2004. Nondestructive DNA extraction method for mitochondrial DNA analyses of museum specimens. *BioTechniques* **36**: 814 – 821.

Ryder, O. A., 1986. Species conservation and systematics: The dilemma of subspecies. *Trends in Ecology and Evolution* **1**: 9 – 10.

Satoh, Y., Takasaka, N., Hoshikawa, Y., *et. al*.,1998. Pretreatment with restriction enzyme or bovine serum albumin for effective PCR amplification of Epstein-Barr virus DNA in DNA extracted from paraffin-embedded gastric carcinoma tissue. *Journal of clinical Microbiology* **36**(11): 3423 – 3425.

Sambrook, J., Fritsch, E. F., Maniatis, T., 1989. *Molecular cloning: A laboratory*

manual. 2nd Edition. Cold Habour Laboratory Press, New York.

Schmitz, J., Piskurek, O., Zischler, H., 2005. Forty million years of independent evolution: A mitochondrial gene and its corresponding nuclear pseudogene in primates. *Journal of Molecular Evolution* **61**(1): 1 – 11.

Sensabaugh, G. F., 1994. DNA typing of biological evidence material. *Ancient DNA*, (eds) B. Herrman & S. Hummel. Springer Verlag, New York, pp. 141 – 148.

Skead, C.J., 1980. *Historical Mammal incidence in the Cape Province, vol.1 : The Western and Northern Cape*. Cape Dept. of Nature & Environmental Conservation**,** Cape Town.

Skinner, J. D. & Smithers, R. H. N., 1990. *The mammals of the Southern African Subregion*. University of Pretoria, Pretoria.

Smithers, R. H. N., 1971. The mammals of Botswana. *Mus. mem. Natl Mus. Monum. Rhod.* **4**: 1- 340.

Stuart, C.T., 1981. Notes on the mammalian carnivores of the Cape Province, South Africa. *Bontebok* **1**: 1 – 58.

Swart, B.L., 2006. The phylogeography of Southern rock Agama (*Agama atra)* in the Cape Fold Mountains South Africa. *MSc. Thesis*, University of Stellenbosch.

Tamura, K. & Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10**: 512 – 526.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G., 1997. The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **24**: 4876 – 4882.

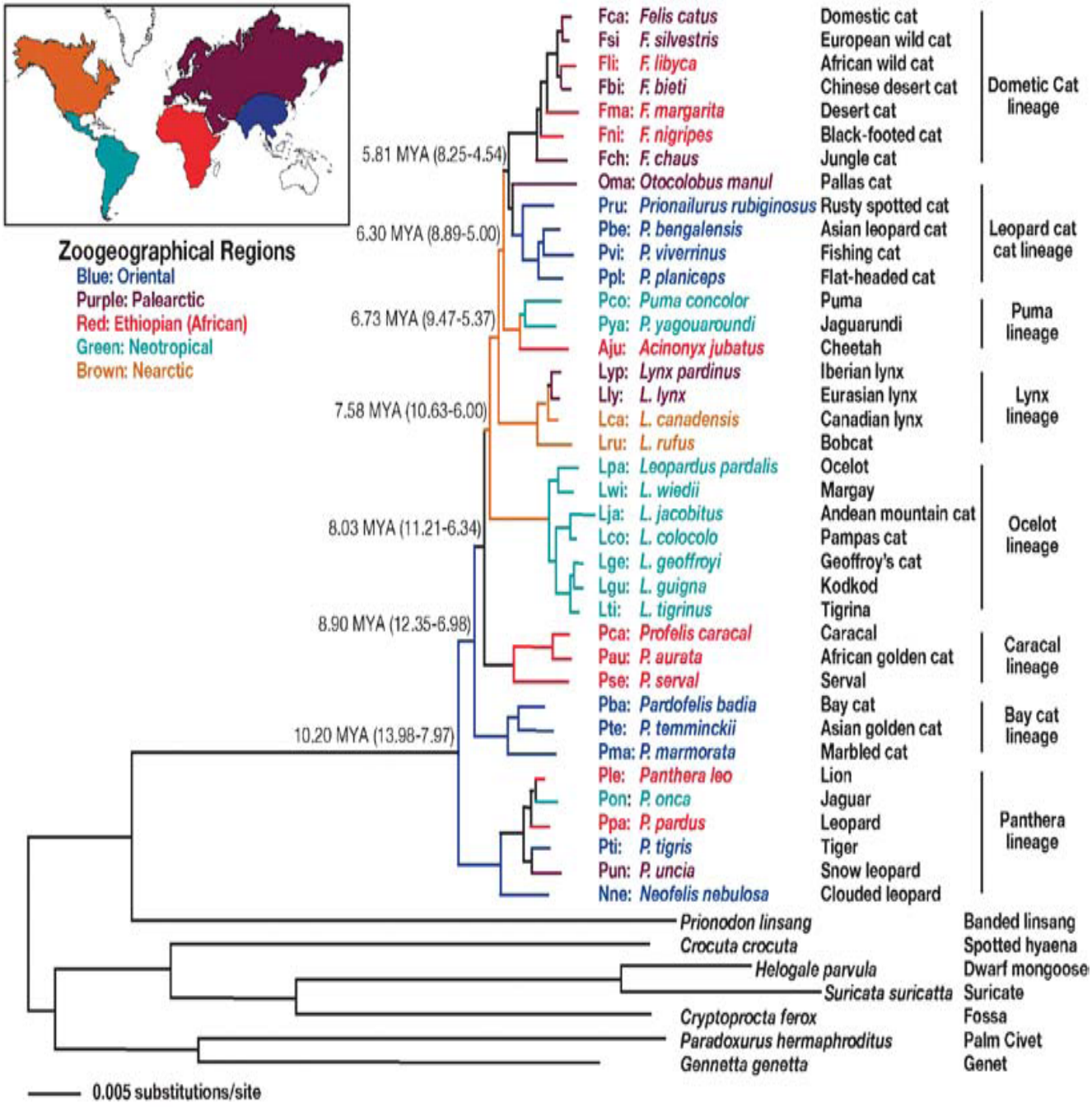
Turnbull-Kemp, P., 1967. *The Leopard*. Timmins, Cape Town.

Turner, A. & Anton, M., 1997. *The Big Cats and Their Fossil Relatives*. Columbia University Press, New York.

Uphyrkina, O., Johnson, W., Quigley, H., Miquelle, D., O' Brien, S. J., 2001. Phylogenetics, genome diversity and the origin of modern leopard. *Panthera pardus*. *Mol. Ecol*. **10**: 2617 – 33.

Van Hooft, W. F., Groen, A. F. & Prins, H. H. T., 2002. Phylogeography of the African buffalo based on mitochondrial and Y-chromosomal loci: Pleistocene origin and population expansion of the Cape Buffalo subspecies. *Molecular Ecology* **11**: 267 – 279.

APPENDIX 1



Appendix 1: Showing the phylogenetic relationships among 37 Felidae species and 7 outgroup taxa based on a maximum likelihood tree. Species were grouped into eight major felid lineages. Colour coding of scientific names is used to depict recent and historic associations with biogeographical regions (taken from O' Brien & Johnson, 2005).

APPENDIX 2:

Genetic variation across NADH-5 and CR of mtDNA and 25 microsatellite loci (as cited in Uphyrkina *et al.* 2001)

	mtDNA					Microsatellites			
Subspecies	Number of leopards mtDNA/μsat	Number variable sites	Mean number of pairwise differences (SE)	Π x 10^2 (SE)	% Polymorphic loci	Average H_E (SE)	Average number alleles/locus	Average range repeat/locus	Microsat variance
***P.p. pardus* (I+II)**	**15/17**	**21**	**8.77 (4.29)**	**1.22 (0.67)**	**100**	**0.803 (0.076)**	**8.52**	**9.72**	**7.28**
P. p. saxicolor	8/10	2	0.50 (0.47)	0.07 (0.07)	100	0.616 (0.083)	4.24	5.12	4.28
P. p. fusca	9/9	8	2.61 (1.54)	0.36 (0.24)	100	0.696 (0.144)	5.52	6.2	5.38
P. p. kotiya	10/11	2	0.56 (0.50)	0.08 (0.08)	96	0.485 (0.202)	3.52	4.58	4.25
P. p. japonensis	3/4	5	3.41 (2.37)	0.48 (0.41)	100	0.674 (0.126)	4.20	5.56	5.70
P. p. orientalis	9/11	1	0.95 (0.71)	0.21 (0.15)	100	0.549 (0.171)	3.76	4.44	2.70
	12/12	1	0.17(0.24)	0.02 (0.04)	92	0.356 (0.222)	2.60	2.84	1.71

APPENDIX 3:

Classical and revised subspecies of *Panther pardus* (revised from Miththapala *et al.* 1996)

Common name	**Classic subspecies**	**Revised Subspecies** Uphyrkina *et al.* (N = 9; 2001)	**Conservation Status**
Zanzibar Leopard	*P. p. adersi* (Pocock 1932)	*P. p. pardus*	Extinct
Cape Leopard	*P. p. melanotica* (Gunther 1885)	*P. p. pardus*	Threatened
Eritrean leopard	*P. p. antinorii* (de Beaux 1923)	*P. p. pardus*	Endangered
Ugandan leopard	*P. p. chui* (Heller 1913)	*P. p. pardus*	Threatened
East African leopard	*P. p. suahelicus* (Neumann 1900)	*P. p. pardus*	Threatened
North African leopard	*P. p. pardus* (Linneaus 1758)	*P. p. pardus*	Endangered
Congo leopard	*P. p. iturensis* (Allen 1924)	*P. p. pardus*	Threatened
Central African leopard	*P. p. shortridgei* (Pocock 1932)	*P. p. pardus*	Threatened
West African leopard	*P. p. reichenowi* (Cabrera 1918)	*P. p. pardus*	Threatened
West African forest leopard	*P. p. leopardus* (Schreber 1777)	*P. p. pardus*	Endangered
Somalian leopard	*P. p. nanopardus* (Thomas 1904)	*P. p. pardus*	Endangered
Barbary leopard	*P. p. panthera* (Schreber 1777)	*P. p. pardus*	Critically endangered (IUCN)
North Persian leopard	*P. p. saxicolor* (Pocock 1927)	*P. p. saxicolor*	Endangered (IUCN)
Caucasus leopard	*P. p. ciscaucasicus* (Satunin 1914)	*P. p. saxicolor*	Endangered, may be extinct
Asia Minor leopard	*P. p. tulliana* (Valenciennes 1856)	*P. p. saxicolor*	Nearly extinct; Critically Endangered (IUCN).
Sinai leopard	*P. p. jarvis*i (Pocock	*P. p. saxicolor*	Endangered, may be

	1932)		extinct
Central Persian leopard	*P. p. dathei* (Zukowsky 1964)	*P. p. saxicolor*	Endangered
Baluchistan leopard	*P. p. sindica* (Pocock 1930a)	*P. p. saxicolor*	Endangered
South Arabian leopard	*P. p. nimr* (Ehrenberg & Hemprich 1833)	*P. p. nimr*	Critically endangered (IUCN)
Indian leopard	*P. p. fusca* (Meyer 1794)	*P. p. fusca*	Endangered
Kashmir leopard	*P. p. millardi* (Pocock 1930)	*P. p. fusca*	Endangered
Nepal leopard	*P. p. pernigra* (Hodgson 1863)	*P. p. fusca*	Endangered
Javan leopard	*P. p. melas* (Cuvier 1809)	*P. p. melas*	Endangered (IUCN)
Sri Lankan leopard	*P. p. kotiya* (Deraniyagala 1956)	*P. p. kotiya*	Endangered and almost extinct; Endangered (IUCN)
South Chinese leopard	*P. p. delacouri* (Pocock 1930)	*P. p. delacouri*	Endangered
North Chinese leopard	*P. p. japonensis* (Gray 1862)	*P. p. japonensis*	Endangered (IUCN)
Amur leopard	*P. p. orientalis* (Schlegel 1857)	*P. p. orientalis*	Critically endangered (IUCN)

APPENDIX 4:

Geographic location of specimens used in this study, along with collector or institution which provided samples, collector's reference number, sample description and preservation method.

Sample number	Collector	Collectors reference number	Locality data	Sample Type	Amplified mtDNA CR
leo 001	Cape Nature (CN), Rika du Plesis		Cederberg, Western Cape	Skin (salted)	Yes
leo 002	CN, Rika du Plesis		Cederberg, Western Cape	Skin (salted)	Yes
leo 003	Eastern Cape Nature Conservation (ECNC), Hennie Swanevelder		Baviaanskloof District, Eastern Cape	Skin (salted)	Yes
leo 004	ECNC, Hennie Swanevelder		Baviaanskloof District, Eastern Cape	Skin (salted)	Yes
leo 005	Amathole museum	KM14422	Kakamas Upington District, Northern Cape	Skin (salted)	
leo 006	Amathole museum	KM14423	East London, Eastern Cape	Tooth	
leo 007	Amathole museum	KM18876	Uitenhage District, Eastern Cape	Skin (salted)	
leo 008	Amathole museum	KM18978	Phalaborwa District, Northern Province	Tooth	
leo 009	Amathole museum	KM24203	Clanwilliam District, Western Cape	Skin (salted)	Yes
leo 010	Amathole museum	KM24204	Ladismith District, Western Cape	Tooth	Numt
leo 011	Amathole museum	KM24206	Worcester, Western Cape	Skin (salted)	
leo 012	Amathole museum	KM24211	Gordonia, Northern Cape	Tooth	
leo 013	Amathole museum	KM24214	Ceres, Western Cape	Tooth	
leo 014	Amathole museum	KM24228	Caledon, Western Cape	Tooth	Numt
leo 015	Amathole museum	KM24229	Clanwilliam, Western	Tooth	

			Cape		
leo 016	Amathole museum	KM24233	Tulbagh, Western Cape	Tooth	
leo 017	Amathole museum	KM24234	Caledon, Western Cape	Tooth	
leo 018	Amathole museum	KM24236	Van Rynsdorp, Western Cape	Tooth	
leo 019	Amathole museum	KM24237	Ceres, Western Cape	Tooth	Yes + Numt
leo 020	Amathole museum	KM24238	Worcester, Western Cape	Skin (salted)	Yes + Numt
leo 021	Amathole museum	KM24245	Ceres, Western Cape	Tooth	
leo 022	Amathole museum	KM24247	Worcester, Western Cape	Tooth (canine)	
leo 023	Amathole museum	KM24250	Caledon, Western Cape	Tooth	
leo 024	Amathole museum	KM24252	Ceres, Western Cape	Tooth	
leo 025	Amathole museum	KM24253	Ceres, Western Cape	Tooth	
leo 026	Amathole museum	KM24257	Ceres, Western Cape	Tooth	
leo 027	Amathole museum	KM24260	Tulbagh, Western Cape	Tooth	
leo 028	Amathole museum	KM24263	Robertson, Western Cape	Tooth	
leo 029	Amathole museum	KM24269	Kimberley, Northern Cape	Tooth	
leo 030	Amathole museum	KM28025	Humansdorp, Eastern Cape	Tooth	
leo 031	Amathole museum	KM28026	Knysna, Eastern Cape	Tooth	
leo 032	Amathole museum	KM28027	Knysna, Eastern Cape	Tooth	
leo 033	Amathole museum	OAM 5813	Alexandria, Eastern Cape	Tooth	
leo 034	Amathole museum	OAM 6228	Kowie Bush - Wolf's Crag, Eastern Cape	Tooth	
leo 035	Amathole museum	OAM 6590 (1057)	Albany District, Eastern Cape	Tooth	
leo 036	Amathole museum	OAM 6590 (1060)	Albany District, Eastern Cape	Tooth	
leo 037	University of Port Elizabeth (U.P.E.), Graham Kearley		Baviaanskloof District, Eastern Cape	Tissue (salted)	Yes

leo 038	U.P.E., Graham Kearley		Baviaanskloof District, Eastern Cape	Tissue (salted)	Yes + Numt
leo 039	U.P.E., Graham Kearley		Baviaanskloof District, Eastern Cape	Tissue (salted)	Yes
leo 040	Mpumalanga Parks Board (M.P.B), Gerrie Camacho		Marlof Park, Mpumalanga	Tissue (salted)	Yes
leo 041	M.P.B., Gerrie Camacho	MPT 2	Loskop Dam, Mpumalanga	Hair	
leo 042	M.P.B., Gerrie Camacho	MPT 3	Delmas Town, Mpumalanga	Hair	
leo 043	M.P.B., Gerrie Camacho	MPT 4	Malalane, Southern lowveld, Mpumalanga	Hair	Yes
leo 044	M.P.B., Gerrie Camacho	FS 2 / 385 KT	Waterval, Mpumalanga	Hair	
leo 045	M.P.B., Gerrie Camacho	341 KT	Burgersfort, Mpumalanga	Hair	
leo 046	M.P.B., Gerrie Camacho	FST 3 "Bella"	Lydenburg, Mpumalanga	Hair	
leo 047	Thys de Wet		Broederstroom, Mpumalanga	Skin (salted)	
leo 048	M.P.B., Gerrie Camacho		Lydenberg, Mpumalanga	Bone	Yes + Numt
leo 049	The Cape Leopard Trust (C.L.T), Quinton Martins	Mxabene female	Londolozi, Mpumalanga	Hair	
leo 050	C.L.T., Quinton Martins	Sunset bend female	Londolozi, Mpumalanga	Hair	
leo 051	C.L.T., Quinton Martins	Rockdrift male	Londolozi, Mpumalanga	Scat	
leo 052	Dr. Adrian Armstrong		Pietermaritzburg, Kwazulu Natal	Skin (salted)	Yes
leo 053	Iziko Museum	ZM41400	Western Cape	Tissue (saline solution)	
leo 054	Iziko Museum	ZM41404	Western Cape	Tissue (saline)	

				solution)	
leo 055	Iziko Museum	ZM41405	Western Cape	Tissue (saline solution)	
leo 056	Iziko Museum	ZM41523	Western Cape	Tissue (saline solution)	
leo 057	Iziko Museum	Gavin Ritchie	still awaiting locality data from museum	Tissue (saline solution)	
leo 058	Amathole museum	KM14416	Kaokoland, Namibia	Skin (salted)	
leo 059	Amathole museum	KM14418	Western Caprivi Namibia	Skin (salted)	
leo 060	C.L.T., Quinton Martins	lekkerlag	Cederberg, Western Cape	Tissue (salted) and blood	Yes
leo 061	Tuli Leopard Project, Villiers Steyn	F2	Tuli Block, Limpopo	Tissue (saline solution)	Yes
leo 062	Tuli Leopard Project, Villiers Steyn	F3	Tuli Block, Limpopo	Tissue (saline solution)	Yes
leo 063	C.N., Guy Palmer	NGP2324	Houw Hoek Pass, Western Cape	Tissue (EtOH)	Yes
leo 064	C.N., Guy Palmer	NGP2345	Van Rhynsdorp, Western Cape	Tissue (EtOH)	
leo 065	C.N., Guy Palmer	NGP2346 (LKCS/M/001)	Helderberg District, Western Cape	Tissue (EtOH)	
leo 066	C.N., Peter Lloyd		Hottentots Holland Mountains, Western Cape	Hair	Yes
leo 067	C.L.T., Quinton Martins	Houdini	Cederberg, Western Cape	Tissue (salted)	Yes
leo 068	C.L.T., Quinton Martins	Tom	Cederberg, Western Cape	Tissue (salted)	Yes
leo 069	K.E.R.I Research,		Tzaneen, Limpopo	Tissue	Yes

	Cailey Owen			(EtOH)	
leo 070	K.E.R.I Research, Cailey Owen		Tzaneen, Limpopo	Tissue (EtOH)	Yes
leo 071	K.E.R.I Research, Cailey Owen		Tzaneen, Limpopo	Tissue (EtOH)	Yes
leo 072	K.E.R.I Research, Cailey Owen		Tzaneen, Limpopo	Tissue (EtOH)	Numt
leo 073	K.E.R.I Research, Cailey Owen		Tzaneen, Limpopo	Tissue (EtOH)	Yes
leo 074	K.E.R.I Research, Cailey Owen		Tzaneen, Limpopo	Tissue (EtOH)	Yes
leo 075	K.E.R.I Research, Cailey Owen		Tzaneen, Limpopo	Tissue (EtOH)	Yes
leo 076	K.E.R.I Research, Cailey Owen		Tzaneen, Limpopo	Tissue (EtOH)	Yes
leo 077	K.E.R.I Research, Cailey Owen		Tzaneen, Limpopo	Tissue (EtOH)	Yes
leo 078	C.N., Jaco van Deventer		Du Toits Kloof, Western Cape	Tissue (salted)	Numt
leo 079	C.N., Peter Lloyd		Caledon, Western Cape	Tissue (salted)	Numt
leo 080	C.N., Jaco van Deventer		Porterville, Western Cape	Tooth	Numt
leo 081	C.L.T., Quinton Martins	Max	Cederberg, Western Cape	Tissue (salted)	Numt
leo 082	C.N., Jaco van Deventer		Porterville, Western Cape	Hair	

APPENDIX 5

Panthera pardus sequences used in this study with corresponding haplotype number:

Haplotype 1:
>CCCCACATTAAAATGGGCCCGGAGCGAGAAGAGGTACACGCTCAGGCAAGGATT
GCTGGTTTCTCGAGGCCAGGTGATCAAGCTCTTTCGGACAGTTGAGGTCCATAGGA
GACTGGTATAGATCATGAATATGCACGATAAAGCACTCATATGTCTTATGTAATAT
ATATAAACTACTGTACATGCTTAATATTCATGGGGACAAGCAGTTAATGCACGATA
TACATAACATGTCTTATGCAATATATATAAACTACTGTACATGCTTAATATTCATG
GGGACAAGCAGTCAATGCACGACGTACATAG>

Haplotype 2:
>----------
TGCTGGTTTCTCGAGGCCAGGTGATCAAGCTCTTTCGGACAGTTGAGGTCCATAAG
AGACTGGTATAGATCATGAATATGCACGATAAAGCACTCATATGTCTTATGTAATA
TATATAAACTACTGTACATGCTTAATATTCATGGGGACAAGCAGTTAATGCACGAT
ATACATAACATGTCTTATGCAATATATATAAACTACTGTACATGCTTAATATTCAT
GGGGACAAGCAGTTAATGCACGACGTACATAG>

Haplotype 3:
>CCCCACATTAAAATGGGCCCGGAGCGAGAAGAGGTACACGCTCAGGCAAGGATT
GCTGGTTTCTCGAGGCCAGGTGATCAAGCTCTTTCGGACAGTTGAGGTCCATAAGA
GACTGGTATAAATCATGAATATGCACGATAAAGCACTCATATGTCTTATGTAATAT
ATATAAACTACTGTACATGCTTAATATTCATGGGGACAAGCAGTTAATGCACGATA
TACATAACATGTCTTATGTAATATATATAAACTACTGTACATGCTTAATATTCATG
GGGACAAGCAGTTAATGCACGACGTACATAG>

Haplotype 4:
>----------
CGAGGCCAGGTGATCAAGCTCTTTCGGACAGTTGAGGTCCATAAGAGACTGGTAT
AAATCATGAATATGCACGATAAAGCACTCATATGTCTTATGTAATATATATAAACT
ACTGTACATGCTTAATATTCATGGGGACAAGCAGTTAATGCACGATATACATAATA
TGTCTTATGTAATATATATAAACTACTGTACATGCTTAATATTCATGGGGACAAGC
AGTTAATGCACGACGTACATAG>

Haplotype 5:
>CCCCACATTAAAATGGGCCCGGAGCGAGAAGAGGTACACGCTCAGGCAAGGATT
GCTGGTTTCTCGAGGCCAGGTGATCAAGCTCTTTCGGACAGTTGAGGTCCATAAGA
GACTGGTATGAATCATGAATATGCACGATAAAGCACTCATATGTCTTATGTAATAT
ATATAAACTACTGTACATGCTTAATATTCATGGGGACAAGCAGTTAATGCACGATA
TACATAACATGTCTTATGTAATATATATAAACTACTGTACATGCTTAATATTCATG
GGGACAAGCAGTTAATGCACGACGTACATAG>

Haplotype 6:
>CCCCACATTAAAATGGGCCCGGAGCGAGAAGAGGTACACGCTCAGGCAAGGATT
GCTGGTTTCTCGAGGCCAGGTGATCAAGCTCTTTCGGACAGTTGAGGTCCATAAGA
GACTGGTATGAATCATGAATATGCACGATAAAGCACTTATATGTCTTATGTAATAT
ATATAAACTACTGTACATGCTTAATATTCATGGGGACAAGCAGTTAATGCACGATA
TACATAACATGTCTTATGTAATATATATAAACTACTGTACATGCTTAATATTCATG
GGGACAAGCAGTTAATGCACGACGTACATAG>

Haplotype 7:
>CCCCACATTAAAATGGGCCCGGAGCGAGAAGAGGTACACGCTCAGGCAAGGATT
GCTGGTTTCTCGAGGCCAGGTGATCAAGCTCTTTCGGACAGTTGAGGTCCATAAGA
GACTGGTATAAATCATGAATATGCACGATAAAGCACTTATATGTCTTATGTAATAT
ATATAAACTACTGTACATGCTTAATATTCATGGGGACAAGCAGTTAATGCACGATA
TACATAACATGTCTTATGTAATATATATAAACTACTGTACATGCTTAATATTCATG
GGGACAAGCAGTTAATGCACGACGTACATAG>

APPENDIX 6

Consensus Numt sequence:

>CCCCACGTTAGAATGGGCCCGGAGCGAGAAGAGGTACACGCTCAGGCAAG
GGTTGCTGGTTTCTCGAGGCCAGGTGATTAAGCTCTTTCGGACAGTTGAG
GTCCATAGAGGACTGTTATAGATCATGGATATGCACGATTAAGCACTATT
ATGTCTTATGTAATATATATAAACTACTGTACATGCTTAATATTCATGGG
GACAAGCAATTAATGCACGATATACATAGTATGTCTTATGTAATATATAT
AAACTATTGTACATGCTTAATATTCATGGGGACAAGCAGTTAATGCACGA
TATACATAGTATGTCTGGGGGGGG>