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Cheetah
Acinonyx jubatus (Schreber, 1776)
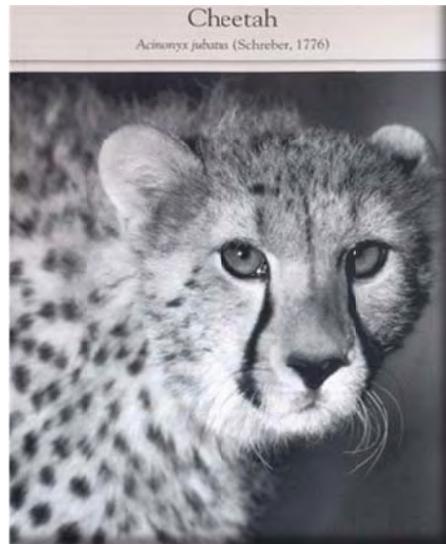

Genetic admixture, inbreeding and heritability estimates in captive African cheetahs

(*Acinonyx jubatus*) including linkage analysis for the *King* cheetah phenotype

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Title Page

GENETIC ADMIXTURE, INBREEDING AND HERITABILITY ESTIMATES IN CAPTIVE AFRICAN CHEETAHS (*ACINONYX JUBATUS*) INCLUDING LINKAGE ANALYSIS FOR THE *KING* CHEETAH PHENOTYPE

By

SOORYAKANTH SASIDHARAN PRIYADERSINI

Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Production Animal Studies in the Faculty of Veterinary Science, University of Pretoria

Date submitted: May 2011

Declaration

I, Sooryakanth Sasidharan Priyadersini, do hereby declare that the research presented in this dissertation, was conceived and executed by myself, and apart from the normal guidance from my supervisors, I have received no assistance.

Neither the substance, nor any part of this dissertation has been submitted in the past, or is to be submitted for a degree at this university or any other university.

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Abbreviations and acronyms

AMOVA	Analyses Of Molecular Variance
ASP	Agouti Signaling Protein
CCF	Cheetah Conservation Fund
CITES	Convention on International Trade in Endangered Species
cM	Centi-Morgan
DNA	Deoxyribonucleic Acid
Dps	Proportion of Shared Alleles
DWCBSRC	De Wildt Cheetah Breeding Station and Research Centre
EDTA	Ethylenediaminetetraacetic Acid
$E\text{-}S$ algorithm	Elston-Stewart Algorithm
f	Individual Inbreeding Coefficients
FCA	Factorial Correspondence Analysis
F_{IS}	Genetic Differentiation Within Populations
F_{ST}	Genetic Differentiation Among Populations
GLM	General Linear Model
GPS	Global Positioning System
G_{ST}	Nei's Measure of Population Genetic Differentiation
h^2	Heritability
H_E	Expected Heterozygosity,
H-F-C	Heterozygosity Fitness Correlations
HMRF	Hidden Markov Random Field
H_O	Observed Heterozygosity,
HWE	Hardy-Weinberg Equilibrium
IR	Internal Relatedness
IUCN	International Union for Conservation Of Nature
K-W ANOVA	Kruskal-Wallis One Way Analysis of Variance on Ranks
LD	Linkage Disequilibrium
LM	Likelihood Method
LOD	Logarithm of Odds
LR	Likelihood Ratio
LRS	Lifetime Reproductive Success
MC1-R	Melanocortin 1 Receptor
MCMC	Markov Chain Monte Carlo
MHC	Major Histocompatibility Complex
MLA	Multilocus Linkage Analysis
MLE	Maximum Likelihood Estimate
NCMP	National Cheetah Metapopulation Program

Ne	Effective Population Size
PCA	Principal Co-Ordinates Analysis
PCR	Polymerase Chain Reaction
PIC	Polymorphic Information Content
RFLP	Restriction Fragment Length Polymorphism
RLBH	Reverse Line Blot Hybridization
R_{ST}	Rst, a Measure of genetic differentiation analogous to F_{ST}
S.E.	Standard Error
S.D.	Standard Deviation
SMM	Stepwise Mutation Model
SSC	Symmetric Similarity Coefficient
Th2	T- Helper 2 Cell
TYRP1	Tyrosinase-Related Protein 1
UTM	Universal Transverse Mercator
V_A	Additive Genetic Variance
V_{Eg}	General Environmental Variance

SUMMARY

GENETIC ADMIXTURE, INBREEDING AND HERITABILITY ESTIMATES IN CAPTIVE AFRICAN CHEETAHS (*ACINONYX JUBATUS*) INCLUDING LINKAGE ANALYSIS FOR THE *KING* CHEETAH PHENOTYPE

BY

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Department: Production Animal Studies

Degree: PhD

This multifaceted study primarily aimed at understanding the genetic make-up of captive versus wild cheetah (*Acinonyx jubatus*) populations in South Africa, with a specific emphasis on a valuable gene pool of a recessive phenotype that is increasingly being maintained in captive population country-wide. The current literature on cheetah genetics has very little information on diversity levels of wild South African cheetahs, and no information on founder dynamics and genetic make-up of South African captive populations. Decisions on cheetah relocations are being made, implementing current conservation policy, from assumptions on origin and relatedness. This research compared population genetic parameters within the largest South African captive cheetah population to free-ranging Namibian and South African conspecifics. The study addressed concerns regarding excessive Namibian genetic introgression into the native captive population and established the extent of genetic variability and Namibian ancestry within the captive population. The study has attempted to address the rising concern among conservation officials with respect to illegal trade of wild-captured cheetahs, wild caught cheetahs that are sold as captive-bred after implanting a microchip. In addition to establishing routine parentage verification using genetic markers that are polymorphic in this species, this study established a technique powerful enough to estimate ancestry in cheetahs of unknown antecedents. The potential of spatial Bayesian clustering to differentiate the point of origin of unknown cheetahs was exploited and in addition, a database for future forensic efforts to address the problem of illegal trade was established. The captive population that was part of this dataset proved to be quite admixed, excepting for the King lineage which was distinct. The second aspect of this study investigated complex conditions such as development of gastritis, renal conditions and/or susceptibility to infections and its relation to pedigree and marker-based inbreeding levels. Heritability values for important breeding traits were estimated from pedigree records of 532 cheetahs and are reported for the first time. Gastritis was weakly correlated to the expression of the *King* trait. Finally, a smaller cohort of the captive pedigree that segregates for a recessive colour variant called the *King* phenotype was tested for the assumption that the variation is a mutation of the *tabby* locus described in domestic cats. Genetic linkage analysis was done by testing microsatellite markers detected linked to *Tabby* for linkage to a conserved region in the cheetah that potentially codes for the *King* coat colour. Genetic linkage analysis was not detected between the *King* locus and the domestic cat microsatellite markers used for this study, with LOD scores remaining non-significant for all the markers.

Chapter 1: Introduction

This thesis brings three different aspects of South African cheetah (*Acinonyx jubatus*) genetics research within one publication. It addresses admixture and genetic diversity in captive populations compared to wild Southern African cheetahs, inbreeding and potential correlations to complex conditions in a single multigenerational pedigree and linkage analysis on a specific lineage that segregates for a unique coat colour pattern. It begins with an introduction, expanding shortly on the work described in the following chapters and includes hypotheses tested and general objectives in mind prior to starting research. Chapter 2 is a summary of the literature studied, with reviews expanding to the life history and genetics of the cheetah, previous research on heterozygosity, inbreeding and loss of fitness, and understanding current evidence in felids and other species. A literature survey on published knowledge of the *King* phenotype follows, with comparative notes on adaptive mutations in wild felids and known biochemistry of skin and coat colour. Chapter 3 reviews materials and methods used in this work, with a summary of the De Wildt Cheetah Breeding Station and other sources of cheetah samples, sample collection, storage, extraction and genotyping. The rationale behind candidate loci selected is explained. The chapter progresses to experimental designs, analytical and statistical procedures for population genetics and heritability estimates. Similar explanations are provided for genetic linkage analysis including testing methodology and computational procedures. The following three chapters are written in journal publication formats, and includes separate introductions, methods, results and discussions, necessitating some overlapping from previous chapters. Accordingly, Chapter 4 is titled 'Spatial Bayesian clustering clarifies admixture and founder origins in a captive cheetah (*Acinonyx jubatus*) population', and describes the first aspect of this work as a standalone publication. Chapter 5 is titled 'Heritability estimates and genetic correlations to inbreeding in a captive cheetah (*Acinonyx jubatus*) population' and describes the second aspect related to inbreeding analysis of the captive population. Chapter 6 is titled 'Linkage analysis of the *King* phenotype in the African cheetah (*Acinonyx jubatus*) to *Tabby*-linked markers', describes the third and final aspect of this work. Chapter 7 concludes with a summary of entire results obtained and explains the findings with relation to hypotheses stated in 'Introduction'. A short discussion follows; bringing together all three aspects of this research project and closes by putting forth some recommendations for future genetic management. The final chapter is a numbered list of references. The system used in this thesis is the Harvard System.

1.1 The captive African cheetah

The future of the African cheetah (*Acinonyx jubatus*) hinges on the sustainable implementation of proactive conservation policies. Habitat loss in the cheetah's former ranges in southern Africa is an undeniable reality and encounters with humans are ever on the increase. The International Union for Conservation of Nature (IUCN) red list for South Africa (*Version 3.1*) lists *A. jubatus* in South Africa as vulnerable (criteria D1). The compilers note that the population is close to the endangered status based on the number of breeding animals (Friedman & Daly 2004). Cheetah populations continue to decline in the wild, lowering overall metapopulation genetic diversity levels. The

conservation management of these highly specialised predators can progress only by closer links between *in situ* and *ex situ* conservation activities (Marker-Kraus 1997).

The cheetahs within the majority of captive breeding programs exhibit poor reproduction rates and develop chronic diseases that cause extensive morbidity and mortality (Munson *et al.* 1999). Southern Africa is home to over half of all wild cheetahs worldwide, with around 550 free-ranging cheetahs in South Africa alone (Purchase *et al.* 2007). A third of the world's captive cheetah population is based in the region (Marker *et al.* 2007) and South Africa alone houses over 500 captive cheetahs in 44 facilities spread over nine provinces (Marnewick *et al.* 2007). Captive breeding programs have, over the years, received a high number of founders having Namibian ancestry. Knowledge of population genetic parameters of current populations and detecting origins of rescued cheetahs for on-going relocation programs are becoming critical for conservation policy implementation. In a species where captivity triggers the onset of multiple complex conditions, there is an urgent need to understand the risks posed by inbreeding and factors affecting mortality rates.

The increasing popularity of the *King* phenotype in zoological collections has led to focused breeding programs in many South African institutions, with increasing numbers of known and suspected carriers of the recessive gene within captive populations. In the absence of a genetic test to determine true carriers, maintaining the trait within pedigrees demands careful selection and attention to maintaining genetic variability.

South African captive propagation programs serve as an important genetic and a demographic reservoir that affords future reintroduction into the wild and reinforcing existing populations. From the conservation point of view, it becomes a logical necessity to understand the population structure, dynamics and genetics of our increasingly captive-bred cheetah populations, especially the *King* phenotype, within our breeding institutions, nature reserves and zoological institutions. From this important perspective, an analysis of the diversity parameters, fitness levels and heritability and genetic linkage analysis for localising the *King* trait within cheetah populations were carried out.

1.2 Hypothesis

- The captive cheetah population has retained genetic variation and population differentiation compared to wild conspecifics.

- The captive cheetah population has Namibian ancestry and levels of ancestry can be determined.

- Unknown ancestry of a cheetah can be described using trained spatial Bayesian clustering.

- There is a correlation between inbreeding and development of pathology and susceptibility to infection

- Heritability of complex conditions like gastritis in the cheetah is measurable.

- There is potential for improved dam selection for litter size in cheetahs.

- In cheetahs, there is a detectable genetic component that contributes to maladaptation, i.e., stress in captivity has a genetic component

- Genetic linkage analysis - the published linkage between *Tabby* locus in domestic cats and microsatellite markers that flank chromosome B1 is similar to and applicable for detecting linkage between *King* locus and similar markers in *Acinonyx jubatus*

1.3 Objectives

- To understand the founder dynamics and genetic make-up within an important captive population and compare genetic parameters to wild conspecifics.

- To establish levels of Namibian ancestry within the current generation of cheetahs in the captive population.

- To devise a technique powerful enough to estimate ancestry in cheetahs of unknown antecedents.

- To establish whether cheetahs in captivity have a heritable component in developing pathological or infectious conditions.

- To detect if gastritis as a complex condition has a heritable component.

- To detect whether *King* phenotypes or known heterozygotes carry significantly more physiological burden from pathological and other data than normal-spotted cheetahs.

- To establish whether selection procedures within a captive cheetah population can be improved.

- To test for genetic linkage between domestic cat microsatellite markers and the *King* phenotype in cheetahs for devising a genetic test for detecting heterozygotes.

Chapter 2 - Literature review

2.1   Distribution of wild cheetahs in South Africa and Namibia

In Africa, cheetahs generally inhabit open plain habitat and savannah woodland. In southern Africa, they inhabit a variety of regions, including grassland, savannah, nama-karoo, succulent-karoo biomes and even desert fringes (Skinner & Chimimba 2005). The current geographic distribution of cheetahs in South Africa include Mpumalanga and Limpopo Eastern lowveld, Limpopo river valley to Marico River, Western North West Province, northern parts of Northern Cape and Northern KwaZulu-Natal. Specific subpopulations exist in Kruger National Park and its adjacent reserves (n = 175), Limpopo Province (n = 125), North West Province and Northern Cape (n = 175), Pilanesburg National Park (n = 25), Madikwe Nature Reserve (n = 25), Hluhluwe-Umfolozi Park (n = 30), Phinda Resource Reserve (n = 15), Mkuzi Falls (n = 8), Shamwari Nature Reserve (n = 6), Kwandwe Game Reserve (n = 6) and Kgalagadi Transfrontier Park (n = 65) (Friedman & Daly 2004). Apart from the natural populations within Kruger National Park, areas in Northern Cape, including the Kgalagadi Transfrontier Park and few farmlands bordering the Kruger National Park, most extant populations in South Africa have been reintroductions. These include the Pilanesburg, Madikwe, Hluhluwe-Umfolozi, Mkuzi, Ndumu and Phinda game reserve populations (Skinner & Chimimba 2005). In Namibia, over 95% of the cheetahs occurs in farmlands north of 24° S, with the protected areas (Etosha and the Namib-Naukluft National Parks) carrying only 1- 4% of the population (Skinner & Chimimba 2005).

2.2   The natural history of cheetahs

The activities of the cheetah are mainly diurnal, with peaks of activity at sunrise and sunset. The female brings up the litter alone (an average number of three to four cubs in the Serengeti) and the cubs remain with the mother until the arrival of the next litter (Kelly *et al.* 1998). Siblings usually stay together, with cheetah males forming coalitions between themselves or with other unrelated males. Coalition formation has also been observed in Namibia (Marker *et al.* 2003). Territories maintained by such coalitions or solitary animals can range up to hundreds of kilometres (Durant *et al.* 2004; Marker & Dickman 2004; Skinner & Chimimba 2005). Female cheetahs in the wild mature around 21 to 24 months and leave their siblings at the onset of oestrus. Females remain with their siblings in coalitions for around 6 months. Feral cheetahs are seasonally polyoestrus. In captivity, there is a notable breeding season in the summer months (November – February) and a shorter season between June and July (Meltzer *et al.* 1998). Cheetahs in the Serengeti produced their first litter at around 27 months, with an average gestation period of 93 days.

2.3   Territorial and dispersal behaviour in wild cheetahs and implications thereof

As noted in the previous section, cheetahs in the wild range and breed within a vast area. Genetic variation in wild cheetah populations is maintained by immigration of males and females. In a Serengeti study population, 46% of all males and 28% of all females observed were immigrants, with only 4% of observed solitary males ever holding a territory, with median tenures being a short 4 months (Durant *et al.* 2004). In comparison, 40% of all observed adult males lived in pairs and 19% lived in trios, with territories being maintained for around 8 months and 22 months

respectively (Caro 2000). From the natural history of cheetahs, it is conceivable that cheetahs captured together in traps form part of a coalition and could potentially be siblings. Farmers interviewed in a Namibian study have reported that it is common to trap a second or third cheetah in adjacent cages in the days following the capture of one animal, indicating that strong social bonds between siblings can cause them to be trapped together (Marker *et al.* 2003). Another factor that promotes larger coalitions and groupings in Namibia is the absence of predators like lions in the fenced farming ranges. In fact, 16% of 102 females were observed in groups of two or more and 28% of all litters were accompanied by more than one adult. This indicates that there is a very high possibility that cheetahs of either sex that are captured together from the wild are siblings or otherwise closely related. The implications are important, due to the fact that the majority of captured animals have traditionally ended up in captive populations and could potentially have been used for breeding.

2.4   Phylogeny of the African cheetah

The 38 cat species in the family Felidae appears to have diversified into the pantherine, domestic cat and ocelot lineages over a period of 10 – 15 million years (Johnson *et al.* 1996). The genomic closeness between the different genera in the cat family Felidae was reported recently, with the evolutionary relationships explored using a panel of six mitochondrial genes and three nuclear genes, leading to a very clear phylogeny among 14 closely related cat species (Yu & Zhang 2005). In concordance with a previous study (Janczewski *et al.* 1995), two monotypic genera, *Acinonyx jubatus* and *Puma concolor* were placed in a sister grouping, indicating a very close relationship between the cheetah and the puma. Similarly, a comprehensive analysis of 37 feline species placed *Acinonyx* along with the jaguarandi and puma in the puma lineage (Johnson *et al.* 2006). Recent Bayesian analysis of cytochrome *b* sequences did not support this relationship, with the cheetah, puma and the extinct American cheetah (*Miracinonyx trumani*), forming a grade rather than a clade in the likelihood tree (Agnarsson *et al.* 2010). Fossil discoveries in China of a Pliocene Chinese cheetah also favour a close relationship between *Puma* and *Acinonyx* lineages based on morphology, with the early puma *Puma pardoides*, described as a possible ancestor of both (Christiansen & Mazák 2009). Charruau *et al*. (2011) estimated the divergence between Asiatic and African cheetahs at 32 000–67 000 years ago (ya) and between *A. j. soemmeringii* and *A. j. jubatus*, at 16 000–72 000 ya.

2.5   Loss of genetic diversity and inbreeding in populations

2.5.1   Inbreeding depression in populations

Inbreeding depression is a proportionate reduction of a fitness trait relative to the level in a non-inbred population (Hartl & Clark 1997). It arises as a consequence of genetic mechanisms that result in decreased heterozygosity during the inbreeding process (Charlesworth & Charlesworth 1987). Observed inbreeding depression usually involves the expression of deleterious alleles (*dominance* hypothesis), though declining heterozygosity among loci exhibiting heterozygote superiority (*overdominance* hypothesis) might also result in inbreeding depression. The fitness levels of the population decreases irrespective of the genetic mechanism. Populations under captive management for long periods are faced with demographic stochasticity, exacerbating environmental conditions that might cause local extinctions and decreasing population fitness due to inbreeding depression (Charlesworth & Charlesworth 1999). Issues peculiar to captive populations that suffer from lack of natural selection include rapid expression of deleterious recessive alleles, allele loss and lowered heterozygosity.

2.5.2 Manifestation of loss of fitness

The eventual fixation of deleterious mutations might lead to subsequent reduction in adaptability and evolutionary potential of a population (Armbruster & Reed 2005; Bowland *et al.* 2001; Higgins & Lynch 2001; Jimenez *et al.* 1994; Lacy 1997; Ralls *et al.* 1988; Wright 1978). There is extensive evidence indicating that inbreeding in captive populations has effects on birth weight, survival rates, reproduction success, disease resistance, exposure to predation and environmental stress (Soule 1986; Thornhill 1993). The severity of manifestation of inbreeding depression has been shown to be increased in stressful conditions at higher levels of inbreeding in the laboratory (Miller 1994) and under natural conditions (Hedrick & Kalinowski 2000). Data collated from studies on inbreeding in butterflies, birds and plants reveal that populations with reduced genetic diversity often experience reduced growth and increased extinction rates (Keller & Waller 2002). Island populations have potentially a greater risk of extinction than mainland populations (Smith *et al.* 1993). Fragmented and fenced in populations have very similar dynamics as island metapopulations, especially if inter-population genetic transfers are negligible or if they are from the same inbred gene pool. Species inhabiting island populations and captive populations that do not add on to its gene pool face similar metapopulation dynamics (Ives & Whitlock 2002)

The effects of inbreeding were reversed in few populations as a result of translocations (the heterosis effect (Treuren *et al.* 2003)). An analogy to the case of the puma, closest genetic relative to the cheetah, is relevant. The introduction of cougars from Texas, USA into the last remaining population of Florida panthers (*Puma concolor coryi*), greatly reduced the incidence of detrimental traits like cowlick, kinked tail and cryptorchidism (Mansfield & Land 2002). Within canids, the addition to the gene pool of a single male immigrant wolf replenished overall heterozygosity levels (increasing from a mean of 0.49 to 0.62) of the endangered Scandinavian grey wolf (*Canis lupus*) population, previously limited in size by lack of genetic diversity and inbreeding depression (Ingvarsson 2002). In an inbred population of captive and wild Scandinavian grey wolves where hereditary blindness was common, there was a significant reduction in the condition after the introduction of an immigrant wolf (Vila *et al.* 2003). Similar genetic restoration of fitness by translocation has been demonstrated in populations of greater prairie chickens (*Tympanuchus cupido pinnatus* (Svedarsky *et al.* 2000)) and adders (*Vipera berus* (Ingvarsson 2002)). In the context of captive populations such exchange of genetic material from differing genetic pools appears to be vital for the long-term viability. In the absence of such exchange, allele frequencies between populations will remain homogenous and could exert considerable effect on any subsequent selection and increase genetic drift.

2.5.3 Exacerbation of fitness loss in harsher environments

Inbred mice released into a semi-natural environment proved less fit than competing outbred controls (Meagher *et al.* 2000). Inbreeding is also correlated to survivorship on reintroduction to a natural habitat, with survivorship significantly reduced for inbred mice (Jimenez *et al.* 1994). Inbred animals continually lost weight while the more outbred ones gained their lost weight, suggesting that inbreeding effects are more severe in stressful environments. Inbreeding depression is typically more severe in harsher environments that include places with unpredictable weather patterns, fluctuating temperatures and limiting resources to feed young (Hoffmann & Parsons 1991; Latter *et al.* 1995). Populations that live under high levels of stress in marginal habitats, show a high degree of variation at

specific stress response loci. In the wild, the rapid adapters survive, as demonstrated in the case of inbred sparrows on Mandarte Island in Canada, where the population had already suffered an intense bottleneck (Keller *et al.* 1994).

The controversy regarding the degree of inbreeding depression in wild populations stems mainly from two reasons: animals in the wild avoid close inbreeding, and therefore do not manifest deleterious fitness effects (Dobson *et al.* 1997). Even if inbreeding does occur, organisms might deal with the deleterious genetic effects either behaviourally or physiologically before they manifest on a phenotypic level (Frankham 1997).

2.5.4   Heterozygosity of a population as an indicator of fitness level

Heterozygosity is a measure that is commonly used to measure genetic variation and its loss (David 1998). It is the proportion of heterozygous individuals at a locus, and is widely preferred since it is proportional to the amount of genetic variance at that locus. The assumptions under which heterozygosity at neural marker loci operate as indicators of general genome-wide heterozygosity have been described earlier (DeWoody & DeWoody 2005; Hirschhorn & Daly 2005; Wang *et al.* 2005). Another advantage of this measure is that it can also be easily adapted for theoretical considerations of the effect of limited population size on genetic variation. The disadvantage is its insensitivity to the actual number of different genotypes at a locus (Allendorf 1986). The proportion of loci known to vary in a population (polymorphism) and that proportion that varies within the individual (heterozygosity) represent the measures upon which genetic diversity is measured (Merola 1994). Heterozygosity estimated from few loci is generally poorly correlated with inbreeding (Slate *et al.* 2004). The power of detecting the correlation is highest in scenarios where systematic consanguineous matings, genetic drift or population bottlenecks have occurred (Szulkin *et al.* 2010). Positive heterozygosity-fitness correlations, using restriction fragment length polymorphism markers (Pogson & Fevolden 1998) and microsatellite markers (Bierne *et al.* 1998; Coltman *et al.* 1998; Coulson *et al.* 1998) have been reported. Various studies have demonstrated that individuals with low allozyme heterozygosity and/or high number of lethal equivalent alleles exhibit higher susceptibility to factors that may not affect more heterozygous individuals (O'Brien *et al.* 1985; Pierce & Mitton 1985; Ralls *et al.* 1988). Ralls *et al.* (1988) calculated the median number of lethal equivalents as 3.14 in a survey of 40 captive species, though for a number of species the lethal equivalent was not significantly greater than zero. Though such effects are suspected to be partly due to genetic associations between the markers and fitness genes (assertive overdominance), the majority of the effects are probably not due to the direct effects of the marker genes on the phenotype (David 1998). Linkage disequilibrium is a probable significant cause of observed physical linkage between fitness genes and marker genes in small populations that have been subjected to genetic drift (Pamilo & Palsson 1998). Charlesworth and Charlesworth (1999) suggest that identity disequilibrium due to variance in inbreeding, that is generated by the correlation between homozygosity of marker loci and low fitness might be a source of association. It is therefore important to test each neutral marker locus for the possibility of such associations and its contribution to overall heterozygosity (Acevedo-Whitehouse *et al.* 2005; Ritland 2005).

2.5.5   Heterozygosity-fitness correlations: methods involved

Attempting to demonstrate inbreeding depression in feral populations can be difficult (Slate *et al.* 2000). If the species is long lived, the study has to extend decades in order to get a good measure of fitness. Unlike captive

populations, in most wild populations, there is usually a lack of pedigree information of the population that extends across multiple generations, which in turn impedes any estimate of relatedness of an individual's parents. One useful method has been where individual inbreeding coefficients (*f*) are estimated and correlated with individual measures of various fitness component or fitness-related traits (Balloux & Lugon-Moulin 2002; Pemberton *et al.* 1999).

An alternative of this method involves analysing individual mean heterozygosity at a sample of codominant molecular markers or individual heterozygosity, which is then inversely correlated with inbreeding coefficient (Hartl & Clark 1997; Roff 1997; Thornhill 1993). Neutral genetic markers like microsatellites, applied to detect levels of shared genes in a population, function on the theory that variations at these neutral loci reflect *genome-wide heterozygosity* at unlinked genes [general effect hypothesis] (Hansson & Westerberg 2002). These unlinked genes might be exhibiting overdominance or segregating for deleterious recessive alleles that influence fitness components. This predicts a '*genome-wide effect*', with the association between heterozygosity and fitness being approximately similar across microsatellite loci. The general effect hypothesis requires identity disequilibria that is created mostly by inbreeding (Lynch & Walsh 1998). This causes correlations in homozygosity through the genome, causing the neutral markers to reflect the fitness effects of homozygosity at genome-wide distributed loci. Only in populations where this hypothesis applies, will the slope of a heterozygosity-fitness regression predict the fitness consequences of matings between close relatives. The general effect hypothesis assumes associative overdominance at genome-wide distributed loci, and is therefore, dependent on the spread of loci over the whole length of the genome. This was achieved by Slate and Pemberton's (2002) study on Scottish red deer (*Cervus elaphus*), where 71 microsatellite loci were used.

Apart from the general effect hypothesis, one or more markers could be physically linked to the loci that affect fitness (termed '*local effect*'), causing the heterozygosity-fitness relationship to be dependent on this locus (Acevedo-Whitehouse *et al.* 2005). This approach might be informative, especially if the detected physical linkage is followed up by further analysis of the area of interest, to detect the actual genes that might be involved with the fitness trait studied. The local effect hypothesis requires non-random associations of alleles at different loci (linkage disequilibrium) and can be expected in recently bottlenecked-and-expanded populations (Reich *et al.* 2001). Strong associations here are mainly restricted to physically linked loci, causing the markers to reflect the fitness effects of loci in the specific area of the chromosome. Markers will be associated with linked fitness loci (physiologically important), and could show associative overdominance as a result of the fitness consequences of recessive deleterious alleles or overdominance at those linked loci (Acevedo-Whitehouse *et al.* 2005). Linkage disequilibria also can arise as a result of selection, genetic drift in small populations, population admixture or after founder events such as bottlenecks (Hansson & Westerberg 2008).

The third hypothesis that explains heterozygosity-fitness correlations is termed '*direct effect*' (Lynch & Walsh 1998). It is considered not important in microsatellite-based research when compared to allozyme-based studies, since the heterozygote advantage that occurs because of functional overdominance at the scored loci is not evident for neutral loci (Hedrick 2003). Overall, the impact of inbreeding on population viability and adaptation potential depends on the correlation of such measurements to negatively impact an organism's physiology. In a heterozygote,

the dominant allele can mask the effects of a deleterious allele or from a general or direct effect on the trait by single or multiple loci (Stirling *et al*. 2002).

2.5.6 Heterozygosity-fitness correlations - results published

Results from studies have revealed positive correlations between heterozygosity and fitness parameters such as longevity, growth rates, fecundity, metabolic efficiency and overall developmental stability (Mitton 1978; Mitton & Grant 1984). Others have documented increased neonatal mortality, decreased litter size, infertility and higher susceptibility to disease (Falconer & Mackay 1996; Ralls *et al.* 1988; Wright 1978). Majority of information that is available are reports from laboratory studies on animals and plants, primarily because of the lack of accurate and deep pedigree information from individuals in feral populations (Amos *et al.* 2001). The natural song sparrow population of Mandarte Island, British Columbia (Keller *et al.* 1994; Keller 1998) and red deer on Rum Island (Pemberton *et al.* 1999) and few metapopulation studies in invertebrates (e.g. butterflies (Saccheri *et al.* 1998) and daphnia (Ebert *et al.* 2002)), are notable exceptions where pedigree information was obtained. The sparrow population studied by Keller (1998) declined to 12 individuals because of a winter storm and this bottleneck resulted in the mean *f* values increasing to levels ranging from 0.06 to 0.09. The authors calculated that the offspring of a full-sib mating was on average 17.5% less likely to survive a year than a noninbred bird. Inbred female sparrows also exhibited a reduced lifetime reproductive success (LRS). Parental similarity was correlated to birth weight and juvenile survival in red deer (Coulson *et al.* 1998)) and harbour seals (Coltman *et al.* 1998). In the Antarctic fur seal (*Arctocephalus gazella*) males with higher heterozygosity, fathered more pups than males with lower heterozygosity (Hoffman *et al.* 2004). The adult reproductive success of male red deer in a wild population was negatively correlated with parental similarity (Slate *et al.* 2000). Inbreeding depression was reported in the Speke's gazelle (*Gazella spekei*) breeding program that was established from four founder animals (Kalinowski *et al.* 2000). Numerous deleterious effects in captive Nordic carnivores were attributed to inbreeding (Laikre 1999) and body sizes in captive inbred Mexican wolves were significantly lower (Fredrickson & Hedrick 2002)

Reproductive parameters have been measured relative to inbreeding, with sperm quality deteriorating in inbred ungulates (*Gazella cuvieri*, *G. dorcas*, and *G. dama*) (Roldan *et al.* 1998; Roldan *et al.* 2006), wild rabbits (*Oryctolagus cumiculus*) (Gage *et al.* 2006), and domestic cats (Pukazhenthi *et al.* 2001). In a captive population of Mexican gray wolves (*Canis lupus baileyi*) previously reported to be highly inbred (Fredrickson & Hedrick 2002), specific semen abnormalities were higher in the lineages with the highest inbreeding coefficients (Asa *et al.* 2007). Reduced genetic diversity in free-ranging felids, especially in lion (*Panthera leo*) populations, has been correlated to poorer sperm quality (Wildt 1994) and abnormal testicular morphology (Munson *et al.* 1996). In conclusion, inbreeding and/or genetic drift that result in reduced genome-wide heterozygosity have been associated with loss of a wide variety of phenotypic attributes related to fitness in wild populations.

2.5.7 Environmental stress and components of fitness in populations

Generally, an environment that reduces fitness or its components relative to more benign conditions is deemed stressful (Hoffmann & Parsons 1991). In a morbillivirus outbreak, higher levels of polychlorinated biphenyls were recorded in the tissues of the more inbred dolphins that were diseased. Stresses for these marine mammals were

three-fold, a novel infective agent, presence of a toxic principle and a form of undefined genetic susceptibility that arose because of inbreeding (Valsecchi *et al.* 2004). A meta-analysis of data from 34 studies reporting inbreeding depression and environmental stress (Armbruster & Reed 2005), report that on average, inbreeding depression is greater in relatively stressful environments. The criteria of manifestation of inbreeding, the median number of lethal equivalents expressed, was 69% greater than under benign environmental conditions. With few notable exceptions (Keller 1998), reports on environmental stressors and inbreeding depression are mostly laboratory and invertebrate based and not under natural conditions. Resistance to artificial stress factors, including chemicals and heavy metals are determined by alleles at a single or few genetic loci in invertebrates (Hoffmann & Parsons 1991) and might not reflect the stress response in placental mammals. Higher organisms might have a more complex response to various physiological and behavioural stressors, and a greater number of genetic loci involved in resistance. However, in the analysis of small population dynamics and addressing issues relating to conservation, it is difficult to separate genetic factors from environmental ones (Mills 1996). Inbreeding depression influences small populations through subtle factors, and might be reported as related to morphology and behaviour, such as parental care and defence against predators. Components of fitness traditionally reported as standard indicators of inbreeding depression include fecundity, survival, growth rates and biomass. Lifetime reproductive success (LRS) is a composite measure expected to give an accurate estimate of overall fitness levels (Merilä & Sheldon 2000). In placental mammals, the distinction between various fitness components, overall fitness levels and its relation to inbreeding is not definable as clearly as in the case of plants and invertebrates. This line of thought is detailed more in chapter 5.

2.5.8 Some inferences from loss of fitness and low diversity

The comparison of average heterozygosity levels, mean numbers of alleles per loci, effective numbers of alleles, mean ranges of microsatellite repeats and microsatellite variance estimates between captive and wild populations are useful to determine loss of variation by genetic drift. In the far eastern leopard, this reflected the historic founder effect that was exacerbated by close inbreeding and manifested as reproductive abnormalities and bone deformities (Uphyrkina & O'Brien 2003). In inbred Soay sheep (*Ovis aries*), allele frequencies at certain loci are distinctly associated with parasite resistance (Gulland *et al.* 1993; Paterson *et al.* 1998). The resistance-associated *S* allele is least frequent in the sheep subpopulation with the highest average faecal egg count and a disease-associated 257-base pair allele of the DRB locus in the major histocompatibility complex was most frequent in the subpopulation where faecal egg count is moderate. In Soay sheep, parasite-mediated selection against inbred animals was reported and parental similarity was correlated to variation in parasite load (Coltman *et al.* 1999). Parasitism, reduced juvenile fitness and extreme climatic conditions have been suggested as being responsible for causing heavier mortality rates among populations proven to be inbred by microsatellite data, when compared to outbred populations (Kalinowski *et al.* 2000). Inbreeding is reported to influence juvenile survival and a component of this is likely to be disease tolerance (Coltman *et al.* 1998; Coulson *et al.* 1998) Genetic variability within wild boars from southern Spain was linked to susceptibility to tuberculosis infection and disease progression (Acevedo-Whitehouse *et al.* 2005). The inbred population of song sparrows (*Melospiza melodia*) inhabiting Mandarte Island was demonstrated to have a deficiency in the cell mediated immune response (Reid *et al.* 2003)

The role of inbreeding in disease dynamics in marine mammals has been an area of interest ever since the outbreak of a morbillivirus epidemic in the early 1990s (Valsecchi *et al.* 2004). Specific correlations have been drawn in

California sea lions (*Zalophus californianus*), with sick animals showing significantly higher than normal parental relatedness (Acevedo-Whitehouse *et al.* 2003). The type of sickness correlates with the degree of relatedness, with the highest mean internal relatedness levels seen in individuals affected with herpesvirus-induced carcinomas followed by helminth infections. The authors conclude that inbreeding could be an important factor in determining susceptibility to complex, long-lived parasitic infections. Infected pups proven homozygous for a microsatellite marker suspected to be near an immunomodulatory gene were shown to be more predisposed to anaemia (Acevedo-Whitehouse *et al.* 2006). The more inbred pups might be unable to expel worm burdens as effectively as the non-inbred, with homozygosity being a key predictor of hookworm-related lesions. In the case of beached dolphins that had succumbed to the morbillivirus disease, mean *internal relatedness* (*IR*) values were higher than would be expected by chance (Valsecchi *et al.* 2004). The infection-related *early mortality* in dolphins was reported as significantly related to higher mean *IR* values than the later mortalities where lower values were detected. On the other hand, an analysis using nine genetic markers in 765 Antarctic fur seals pups (*Arctocephalus gazella*) could find no significant association between heterozygosity, birth weight and neonatal survival (Hoffman *et al.* 2006). More recently, Collins et al. (2011) reported inbred Przewalski's horses exhibiting abnormal reproductive patterns.

2.5.9   African cheetah and inbreeding depression - perceptions from the wild

Field ecologists, drawing from their observations on the natural history of the African cheetah, have questioned the importance of genetic variability for maintaining viable cheetah populations (Caro & Laurenson 1994; Laurenson 1994). This has stemmed primarily from the progress of molecular genetics and application of highly variable microsatellite genetic markers to population genetic studies, resulting in higher levels of variation being reported than previously (Menotti-Raymond & O'Brien 1995). Cheetahs in their natural state apparently do not exhibit reproductive suppression (Kelly 2001). In the Serengeti Plains, most females have the opportunity to reproduce and probably both resident and floater males contribute to the gene pool (Caro 2000). Based on observational data from the Serengeti, Caro (2000) rebuts the correlation between lower genetic diversity in cheetahs and potential for extinction. In natural populations of cheetahs, reproductive success is influenced more directly by ecological factors, with predation and prey availability being the primary influencing parameters. Caro and Laurenson (1994) recorded high levels of fecundity and rapid rates of litter turnover in feral Serengeti cheetahs. They indicate that only 0.5% of observed cub mortalities (n = 48) were potentially due to genetic defects. A review of data from 26 terrestrial carnivores revealed that 30% show genetic diversity levels that are lower than the cheetah, indicating that comparisons of presumptive fitness based on relative levels of heterozygosity can be valid only within species (Merola 1994). The crucial factor limiting cheetah populations in the wild is the high level of cub predation rather than absence of genetic variation as reported earlier (O'Brien *et al.* 1985). In addition, mortality rates in captive cheetahs have been reported to be declining at a steady state in North American zoological institutions due to changes in management practices (Beekman *et al.* 1999).

Reproductive performance of free-roaming cheetah males does not seem to be affected by the levels of abnormal spermatozoa (70.9 – 78.7%) in the ejaculate, with the average male cheetah's sperm normally having high levels of structurally impaired sperm (Lindburg *et al.* 1993). In the wild, 83.3% of such males were capable of producing pregnancies, with 89.5% of the pregnancies arising from a single oestrus. Semen from other species of large cats also presented high percentages of sperm abnormalities. Despite high cub mortality (95%) within the wild cheetah

populations of Serengeti, any obvious indicators of low fitness levels, such as stillborn litters, resorption of litters during gestation and postnatal effects were not observed (Laurenson *et al.* 1995b). The authors conclude from their field observations that the level of mortality attributable to low genetic diversity is of no consequence in natural cheetah populations. From an evolutionary perspective, the high extrinsic mortality rates within natural cheetah populations might have caused, as an adaptive response, the generation of larger litters and faster growth rates when compared to other felids (Promislow & Harvey 1990).

2.5.10   An ancient population reduction in the African cheetah

The African cheetah is thought to have gone through an extreme genetic bottleneck, thus losing most of the genetic variability through the population reduction (Menotti-Raymond & O'Brien 1993). Sustained inbreeding within the species after this founder event is attributed to the problems encountered in captive breeding (Wayne 1986; Yuhki & O'Brien 1990). The indications are that cheetahs underwent one or more episodes of extreme population reduction in the past, around 10,000 to 12,000 years ago (or approximately 200 generations ago). Considering that there has been sufficient time for rare alleles to accumulate, allele frequencies could be expected to be close to neutrality at present. On the other hand, if cheetah populations were always under metapopulation dynamics, the frequent colonisations by few individuals in an area might have been the norm, as supported by field observations (Kelly *et al.* 1998; Marker & Schumann 1998). This would lead to a continually low effective population size and persistence of fewer rare alleles than neutrality expectations (Hedrick 1996). In a mammal like the cheetah, theoretical expectations predict that a high amount of additive genetic variance to be created by spontaneous mutation per generation for a number of inherent qualitative traits (Lande 1988; Lynch 1977). This mutation rate is estimated to be approximately 10^{-3} times the environmental variance. This rate is of the same magnitude as that for microsatellite loci mutations, under the stepwise mutation model (Slatkin 1995). Calculations reveal that under these assumptions the original cheetah metapopulation would initially have been around 200 animals and the average heterozygosity then would be similar to the estimates published using such neutral loci (Hedrick 1994; Hedrick 1996). On the other hand, a concurrent decay in qualitative (fitness) traits is also to be expected in this metapopulation.

2.5.11   Disease susceptibility in the African cheetah

The role of infectious disease as an important ecological factor in determining the selective pressure on the genomes of the surviving species has been studied intensively (Anderson & May 1987; Bellamy & Hill 1998; McClelland *et al.* 2003; O'Brien & Evermann 1988). Even though most reviewers conclude that inbreeding does increase susceptibility to pathogens, consensus on what entails direct evidence regarding these parameters has been very difficult to come by. With the advent of next generation genome-wide association studies, research in human genetics on genetic susceptibility to numerous complex diseases and various infectious diseases have started to identify various candidate genes that are linked with these diseases and inbreeding (Bellamy 2003; Rudan *et al.* 2003).

Association between cellular and humoral immunity, disease and reduced fitness in wild mammalian populations is difficult to prove and there is very little acceptance on what definite proof of such associations should be (Acevedo-Whitehouse *et al.* 2003). It is empirically possible that a captive population lacking genetic diversity, when further

subjected to random selections of small numbers of alleles at each generation, can lead to genetic variation being rapidly lost by drift, leading to development of homozygosity. Though mean allele frequency changes are not induced by genetic drift, it causes the dispersion of allele frequencies around this mean, leading to an increased homozygosity for deleterious alleles (Wang *et al.* 1999). Specific to cheetahs, disease susceptibility and inbreeding has been associated (O'Brien *et al.* 1985; O'Brien *et al.* 1997; O'Brien & Yuhki 1999). Additionally, the low genetic diversity in the African cheetah has been a subject of intense research across many decades. The apparent paucity of genetic variation from allozyme analysis and acceptance of skin grafts without rejection indicated that the cheetah is unique in being uniformly homozygous (O'Brien *et al.* 1981; O'Brien *et al.* 1983; O'Brien *et al.* 1985). The levels of genetic variability in the cheetah were lower than in other species, with the South African subspecies (*Acinonyx jubatus jubatus*; P = 0.02 and H = 0.0004) exhibiting lower levels than the East African subspecies (*Acinonyx jubatus raineyi*; P = 0.04 and H = 0.014). *In vitro* tests to compare the responses between domestic cat and cheetah mononuclear cells to feline herpervirus-1 and *Cryptococcos neoformans* revealed an innate immune system deficiency to the virus in the cheetah (Miller-Edge & Worley 1992). The study population, drawn from various North American institutions and mostly of Namibian origin, also exhibited a high incidence of rhinotracheitis (40%), conjunctivitis/keratitis, and/or herpetic skin lesions

The high levels of juvenile mortality, impaired reproduction and susceptibility to disease in cheetahs have been linked to decreased genetic variation (Marker & O'Brien 1989; Menotti-Raymond & O'Brien 1993; O'Brien *et al.* 1985; O'Brien *et al.* 1987; Wildt *et al.* 1983). The genetic uniformity within the cluster of genes called major histocompatibility complex (MHC) in the cheetahs was demonstrated (O'Brien & Yuhki 1999; Yuhki & O'Brien 1990). MHC variation in African cheetahs (*A .j. raineyi*, n = 13 and *A .j. jubatus*, n = 9) were among the lowest when analysed by restriction fragment length polymorphism (RFLP) for three parameters that indicate variation at the complex. Increased susceptibility to disease has been suspected in cheetahs, especially in the aftermath of an outbreak of feline infectious peritonitis at the Oregon wild animal park in 1982 - 1983 that caused up to 60% mortality in captive cheetahs (O'Brien *et al.* 1985). This disease is caused by a corona virus and mortality rates of 1 – 10% are seen in domestic cats. The high mortality rates in cheetahs were attributed to the lack of variation at the MHC. Demonstrating correlation between MHC variation and resistance or susceptibility to parasites has always been considered a difficult experimental challenge (Garrigan & Hedrick 2003). The fact that the MHC is a multigene family makes it difficult to separate the effects of specific alleles from the background genotype. It is also frequently difficult to determine the MHC sequences that are allelic, and distinguish them from other genes due to the high variability within loci and due to the similarity of alleles within loci (see Hedrick 2002). O'Brien's skin transplant experiments were criticised for a number of reasons, including the lack of controls (Laurenson *et al.* 1995a; May 1995). However, a replication of the skin graft type response study was done with controls, in three long-established populations of the pocket gopher (*Thomomys bottae*) and the use of this technique to prove lack of variation at the histocompatibility complex was later validated (Sanjayan *et al.* 1996). On the other hand, little evidence was found in a study on correlation between low MHC variation and the decline of desert bighorn sheep (*Ovis canadensis*), with the authors reporting extensive polymorphism at the loci analysed (Gutierrez-Espeleta *et al.* 2001). There is little clarity on the relationship between ecological parameters such as population viability or risk of extinction, to diversity within its MHC loci as reflected by the animal's ability to respond to pathogenic challenges (Radwan *et al.* 2010). Free-ranging cheetahs are seropositive for feline calcivirus, feline parvovirus, feline herpesvirus, canine

distemper virus and feline corona virus without any clinical signs (Munson *et al.* 2004; Thalwitzer *et al.* 2010). MHC Class II DRB data from non-pride forming species such as the Eurasian lynx (*L. lynx*; 3 loci, 13 alleles (Wang & Summers 2010), Bengal tiger (*Panthera tigris tigris*; 2 loci, 4 alleles) (Pokorny *et al.* 2010), and the cheetah (2 loci, 4 alleles; (Castro-Prieto *et al.* 2011), might indeed be a reflection of species specific divergent demographic histories, degree of admixture and selective factors (Kelley *et al.* 2005). This might not be a direct measure of low immune adaptability nor indicate increased risks for disease outbreaks and extinction. This concept has been exhaustively reviewed by others (Radwan *et al.* 2010).

Cheetahs have reduced immune responses *in vitro* when compared to cats (Miller-Edge & Worley 1991) and have been recorded to have higher hormonal stress response to captivity (Wells *et al.* 2004). The role that stress plays in captive cheetahs is a field of on-going research. Some captive cheetahs are prone to higher baseline cortisol levels than others due to reasons that might be genetic. The influence of an inherited abnormal T-helper 2 cellular cascade mediating an acute inflammatory response in certain conditions has been proposed as the factor that causes systemic amyloidosis in captive cheetahs (Papendick *et al.* 1997). Cases of *Cryptococcus* infections, an opportunistic fungal pathogen that is known to take advantage of a deficient immune system, was reported from South African cheetahs, with four of the seven cases being *King* cheetahs (Millward & Williams 2005; Picard *et al.* 1998). Captive cheetahs generally have a propensity for developing marked adrenal cortical hyperplasia, indicating abnormal function of the stress-hormone producing glands. They exhibit mean corticosteroid levels four times greater than wild cheetahs, indicative of chronic stress (Munson 1993; Terio *et al.* 1998). An unusually high prevalence of three diseases that occur rarely in other species, including veno-occlusive disease, glomerulosclerosis and *Helicobacter* gastritis, has been reported by the disease surveillance program from captive populations in South Africa and United States (Munson *et al.* 1998). Although a low prevalence of gastritis and helicobacteriosis was reported from a South African study (Lobetti *et al.* 1998), investigations have pointed to a species predilection for the condition in this species, with the evidence that most cheetahs with severe gastritis have parents with severe gastritis (Terio *et al.* 1998). Munson *et al.* (2005) compared the prevalence of diseases in wild cheetahs in Namibia to captive cheetahs in America and South Africa. The data generated from 49 necropsies and 27 gastric biopsies, from adult free ranging cheetahs indicate the relative absence of disease conditions common in their captive cousins.

2.5.12 Evidence for inbreeding depression in wild and captive cheetahs

The results of captive breeding were different for various breeding programs, with 60 – 80% of sexually mature females in the De Wildt facility producing live cubs compared to 9 – 12% in the North American programs. There was an improvement in the North American success rate after the application of novel husbandry practices (Marker-Kraus & Grisham 2005). However, a comparative analysis of juvenile mortality rates from captive North American cheetahs revealed significantly higher mortality in young within related animals than unrelated (Wielebnowski 1996). Captive populations in North America and South Africa have risen from a limited number of founders. There is evidence that a relatively small number of cheetahs have made a large contribution to the North American captive population gene pool (e.g., 21 and 24% of all cubs born until 1996 have been from eight males and 12 females, respectively (Marker 1998)). At the end of 1996, 73% of the captive population (n = 1217) was captive born, with 15% of breeding facilities having mostly Namibian origin cheetahs producing 63% of all cubs born in captivity. Half

of the successful breeding facilities have had only a single breeding pair (Marker 1998). The presence of various *Helicobacter* species in the cheetah's gut is not the sole predisposing factor for the development of gastritis (Terio *et al.* 2005). The negative 'physiological response to local environment' (Munson *et al.* 2005) that contributes to conditions like gastritis, loosely definable as stress in humans, is a condition increasingly seen to have a heritable component (Federenko *et al.* 2006; Imumorin *et al.* 2005; Wurtman 2005). Inbred cheetah offspring in American zoos were significantly more likely to die from intrinsic causes such as stillbirths and congenital defects, factors considered to have a strong genetic basis (Wielebnowski 1996). Available data points to the existence and persistence of deleterious alleles within the captive population in North America, with offspring of related cheetahs in zoos showing higher juvenile mortality than outbred offspring. The mortality caused by the manifestation of intrinsic factors such as stillbirths and congenital defects, might be due to the impact of deleterious loci (Caughley & Gunn 1996).

The majority of phenotypic effects attributed to inbreeding depression, such as infertility, reduced litter sizes, and increased susceptibility to disease are limited to captive individuals (Merola 1994). These 'deviations' from normal can be explained as '*physiological or behavioural artefacts of captivity*'. Others have extensively reviewed 'genetic adaptation to captivity' (Williams & Hoffman 2009) in fish, insect and amphibians, documenting the effects of natural and artificial selective pressures in controlled environments. There is, however, no published documentation of infectious disease outbreaks in wild cheetah populations. There is a view that under natural conditions, cheetahs would not have close contact with conspecifics that tend to aid in disease transmission under captive conditions (Merola 1994). For example, the stocking density within a disease-affected population in Oregon (Heeney *et al.* 1990) was 24 cheetahs within a 3.5 acre (0.14 km^2) area. This has to be contrasted with over 12 – 36 km^2 and 60 – 800 km^2 territory that males and females hold, respectively, in the wild (Skinner & Chimimba 2005). In addition, if a species has not been exposed to a pathogen from an evolutionary context, it is possible that an infection will result in high mortality rates. This susceptibility need not necessarily be linked to the level of intrinsic variation within the MHC. Cheetahs in the wild are exposed to a number of pathogens and parasites, as is evident from blood analyses of wild cheetahs (Caro & Laurenson 1994). Years of observation of Serengeti cheetahs have revealed that mortality due to disease is not a major factor in cheetah population dynamics in the wild. However, as Merola (2004) states, "*disease transmission between cheetahs appears to be exacerbated under conditions of captivity*". The lack of variation within the cheetah MHC might be a liability to the species kept under higher density than normal, as is the case in smaller game reserves and captive-breeding programs.

An exacerbation of fitness levels may not be immediately evident in historically inbred populations where all the individuals have low fitness due to past inbreeding or genetic drift (Kalinowski *et al.* 2000). The genetic load of such populations is considered a mix of highly and weakly deleterious alleles and the eventual inbreeding depression being caused by mildly deleterious alleles. There might be little impact on the heterozygosity levels of populations that have undergone bottlenecks of short duration (Allendorf 1986). Such populations could immediately undergo an extreme reduction in the number of alleles present and consequently exhibit lowered heterozygosity. Populations in mutation drift equilibrium exhibit allelic diversity reductions at a faster level than heterozygosity such that the number of alleles observed is usually less than expected from the observed heterozygosity (Cornuet & Luikart 1996). The fact that Serengeti cheetahs exhibit no apparent reduction in any commonly indexed fitness trait suggests that

the majority of lethal alleles were probably purged from the population. However, in closed populations, recessive alleles with only small but additive negative effects are purged at a much lower rate (Hedrick 1994). There is reason to suspect a greater genetic influence in captive conditions on key fitness components than in free living and natural conditions that are typically the setting of field biology studies. The massive territorial range held by the cheetah and its solitary nature (except when forming coalitions), makes it an unlikely candidate species to succumb to an infectious disease (e.g., feline infectious peritonitis) in the wild. The role of natural selection in the wild (e.g., five cheetahs surviving to 14 months from a total of 125 in the Serengeti (Laurenson 1994)) cannot be underestimated. This type of selection is by itself impossible to replicate in captivity, where conservation priorities are different. It might also mean that in natural populations, the process of selection acts to fix the majority of the 'good genes' that are possessed by few individuals, thus eliminating variance in genetic quality.

Morphological abnormalities in Namibian cheetahs

The wild population in Namibia is considered to be the origin of the majority of all wild caught cheetahs in southern Africa (Marker *et al.* 2003). The overall Namibian wild cheetah population is estimated to have been reduced by 6782 (CITES 1992 report) to 10,000 cheetahs (Cheetah Conservation Fund survey data) over a period of 15 years. There is evidence in the remaining population that indicates the manifestation of deleterious alleles, such as a distinct kink in the tails, dental abnormalities, focal palatine erosions and crowded lower incisors (Marker & Schumann 1998). Cheetah conservation officials in Namibia have noted that over 19% of the cheetahs handled by one institution (Marker-Kraus 1997), exhibited a distinct kink in the posterior tail vertebrae, an attribute reported elsewhere in the inbred Florida panther (Mansfield & Land 2002). The authors reported that over 181 animals had a combination of focal palatine erosions and/or crowded lower incisors, along with a high incidence of deep impressions in the upper palate that could potentially be predisposing to palatine erosion. One hundred and fifty-one cheetahs scored specifically for focal palatine erosion. The incidence of crowded, crooked and maloccluded lower incisors were also high, with 78 of the 123 animals examined exhibiting crowded lower incisors. The initial cases of focal palatine erosion were reported in 1982 in North America, with 86% of the cases originating from a single Namibian cheetah family line (Fitch & Fagan 1982). A recent study, however, reported very low seroprevalence for nine significant felid viruses in 68 free-ranging Namibian cheetahs and generally mirrored human population density in the area of sampling (Thalwitzer *et al.* 2010).

The captive population at De Wildt

The management of breeding within the De Wildt population has been extensively documented elsewhere (Bertschinger *et al.* 2008; Meltzer *et al.* 1998; Meltzer & Van Dyk 1998). The selection of breeders to optimise diversity within the captive population is a challenging task, as cheetahs selected might be incompatible with an optimal partner. Anecdotal evidence indicate that animals of the *King* phenotype are more susceptible to fly strikes, helminthiasis, gastric ulceration, thymus atrophy and bone fractures (Lambrechts 1998; Millward & Williams 2005). In wild populations that are under perfect equilibrium conditions, there is no additive genetic variation to be expected in fitness or traits closely related to fitness (Charlesworth & Charlesworth 1987). However, in captive populations under pressure to maintain certain phenotypic similarity in succeeding generations, there might be unintended selection for fitness related traits. Although the efforts to maintain certain phenotypic similarity in succeeding generations are different from increasing the genotypic similarity (inbreeding), there is perpetuation of certain genes

and a small decline in heterozygosity. The degree of inbreeding is determined by the number of common ancestors on both sides of the pedigree and the number of times a common ancestor is repeated. A third important factor that is most often not revealed is the degree of inbreeding for the common ancestors.

In the specific case of the captive De Wildt population, preliminary investigations revealed that the number of founders in the breeding population might be very low. In a breeding group derived from a subpopulation with few potentially related founders, there might be higher genetic similarity than desired among the founding groups. The relatedness among the wild-captured cheetahs from the former Northern Transvaal and Swaziland-Namibia that formed the original breeding stock (nine females and 20 males) is a critical factor that was unknown (Meltzer & Van Dyk 1998). In breeding the *King* phenotype as a separate lineage, these cheetahs have shared common ancestors (Van Aarde & Van Dyk 1986).

2.5.13   Wild felid diversity levels using microsatellite genetic markers

Microsatellites or short tandem repeats are tandem repetitive stretches of short (2 - 4 base pair) DNA sequences (Ellegren 2000) and differ from most other types of DNA sequences in their unusual degree of polymorphism, making them attractive as genetic markers. They have been widely used in a variety of fields, including conservation genetics, population genetics and forensics (Goldstein & Schötterer 1999) due to their the ease of amplification via PCR, automation of analytical techniques and obtaining high throughputs (Maudet. *et al.* 2002). Microsatellite loci are ideal to investigate fitness consequences of short-term inbreeding (Tsitrone *et al.* 2001). This study assumed that microsatellites will mutate under the stepwise mutation model (SMM), a model considered as standard in statistical evaluation and evolutionary interpretation of microsatellite polymorphisms (Balloux & Goudet 2002). This describes mutation of microsatellite alleles by addition or deletion of one or more repeated motifs or single tandem repeats, and hence alleles may possibly mutate toward allele states already present in the population (Ellegren 2002).

Microsatellites isolated and cloned from one species have been successfully applied to other species, saving time and effort, and allowing rapid progress of genetic studies in several close species (Luikart *et al.* 1998; Slate *et al.* 1998). Microsatellite genetic markers isolated and cloned from the domestic cat genome (*Felis catus*) have been used extensively in other related felid species. The highly endangered Iberian lynx (*Lynx pardinus*) was investigated for levels of genetic diversity using 28 microsatellite markers and mitochondrial DNA markers (Carmichael *et al.* 2000). Allele size variation of 30 microsatellites, isolated from domestic cats, was determined to estimate genetic diversity in various tiger subspecies (Luo *et al.* 2004).The Iberian lynx (subpopulation sample sizes of 10 and 5 animals respectively (Johnson *et al.* 2004)), exhibited lower genetic variation than East African and West African cheetahs (sample sizes of 10 animals each using 11 microsatellite markers (Driscoll *et al.* 2002)). Molecular genetics tools are particularly useful to detect cryptic genetic structure within populations and helpful in understanding the dispersal dynamics in elusive animals like the feral members of the felid family. The Scandinavian lynx (*Lynx lynx*), a solitary-living felid that exploits a territory of several hundreds of kilometres, was investigated using 10 domestic cat microsatellite loci to determine range expansion and the presence of subpopulations within a metapopulation (Rueness *et al.* 2003). The critically endangered far eastern leopard (*Panthera pardus orientalis*), driven close to extinction due to human encroachment of its habitat, illegal poaching and inbreeding, was studied using a similar panel of 24 feline-specific loci (Uphyrkina & O'Brien 2003).

Studies on genetic variation in other mammals, carnivores and felids have revealed that the amount of genetic variation in natural populations vary widely. Heterozygosity from different felid species indicate that most felid populations, except for a distinct few, exhibit moderate to high levels of allelic diversity when examined with microsatellite genetic markers. Lions generally show lower genetic variation than tigers or leopards, (Luo *et al.* 2004; Uphyrkina & O'Brien 2003). Different African lion populations had average heterozygosities from 0.45 to 0.75 (Spong *et al.* 2002). In Asiatic lions, the number of alleles per locus ranged from 1 to 11 per locus with a mean expected heterozygosity of 0.60 (Gaur *et al.* 2006), 0.658 (Singh *et al.* 2002), 0.09 (Buckley-Beason *et al.* 2006) and 0.13 (Luo *et al.* 2004). In Gir lions, diversity values reported were lower for loci derived from the domestic cat. In tigers (*Panthera tigris*), expected heterozygosity ranged from 0.456 - 0.75 (Buckley-Beason *et al.* 2006; Luo *et al.* 2004). In mountain lions (*Puma concolor*), 37 microsatellite loci had mean expected heterozygosity levels of 0.526 (Rodzen *et al.* 2007) and generally ranged from 0.13 to 0.74 (Kurushima *et al.* 2006). Captive snow leopards had average observed and expected heterozygosities for 50 microsatellite loci ranging from 0.52 (0.02 S.E.) and 0.58 (0.03 S.E.), respectively (Waits *et al.* 2007). In clouded leopards, overall expected heterozygosity ranged from 0.32 to 0.652 (Luo *et al.* 2004; Wilting *et al.* 2007). High levels of heterozygosity was detected in a study on 148 captive Neotropical felids, including the ocelot (*Leopardus pardalis*: 0.36 - 0.81), margay (*L. wiedii*; 0.80 - 0.92) and tigrina (*L. tigrinus*; 0.57 - 0.67) using four microsatellite loci (Grisolia *et al.* 2007).

In cheetahs, Marker *et al.* (2007) observed 248 alleles in 89 unrelated animals, with mean expected heterozygosity ranging from 0.64 to 0.70. Other studies have reported heterozygosity values from 0.52 (Luo *et al.* 2004) to 0.46 (Driscoll *et al.* 2002). A report on 147 Serengeti cheetahs revealed observed heterozygosity for 13 loci to vary from 0.51 to 0.84 (Gottelli *et al.* 2007). In 57 southern African cheetahs genotyped at 13 loci, allele numbers ranged from 1-10 (Kotze *et al.* 2008). Charruau *et al*. (2011) estimated the heterozygosity from 60 cheetahs to be 0.76. The pattern of microsatellite marker generated diversity levels indicate that studies succeeding Driscoll *et al.* (2002) have had the opportunity for selecting more polymorphic markers, resulting in more alleles per marker. Succeeding studies on genetics of the African cheetah (Luo *et al.* 2004, Gottelli *et al.* 2007, Marker *et al.* 2007, Kotze *et al.* 2008), have opportunistically selected microsatellites that are more polymorphic. This has resulted in reported heterozygosity levels for the cheetah being at their highest levels by 2011 (*He* = 0.77, Charruau *et al*. (2011)), and also due to repeated validations and eliminating non-amplifying markers from analysis.

2.6   Molecular genetics of the *King* cheetah (*Acinonyx jubatus*) phenotype

2.6.1   History of the King phenotype

The first *King* cheetahs recorded born in captivity were at the De Wildt Cheetah Breeding Station and Research Centre in South Africa, between the 12 and 14th of May 1981 (Brand 1983). Prior to this, cheetahs with the *King* phenotype have been recorded for many decades, mainly in the area around eastern and south-eastern Zimbabwe, the north-eastern region of former Transvaal and eastern Botswana (Bottriell 1987). The founder line at De Wildt has arisen from a normal-type spotted cheetah, captured as a cub in the Messina District of the former Northern

Transvaal in South Africa. The mothers were siblings bred from cheetahs that were rescued from the wild from the former Northern Transvaal in South Africa and Namibia. A study of the inheritance pattern of the phenotype has suggested that the blotched pelage is inherited as a single autosomal recessive allele (Van Aarde & Van Dyk 1986). The mutation that leads to this phenotype has been suggested, based on observations, as similar to the change in the 'striped' to 'blotched' variation in the '*Tabby*' coat colour pattern in the domestic cat (Robinson 1976).

Coat colour genetics is a specialised field developed from the study of fancy mouse coat colour variants and the inbred strains created from them. Recent advances in mouse coat colour genetics have contributed enormously to our understanding of pigmentation genetics and its complexities. Over 100 genes are currently known that affect coat colour and exhibit complex interactions (Barsh 1996). An established breeding population of cheetahs at the De Wildt Cheetah Breeding Station and Research Centre in South Africa that exhibit this phenotype offered an unique opportunity to apply molecular genetic tools to research the genetic mechanisms that govern the expression of the *King* phenotype. The trait appears to be a case of recessive monogenic heredity, with the problem of detecting the heterozygote or carrier. Detection of carriers is, to date, possible only by test mating, by breeding suspected carrier cheetahs to known *King* phenotype or known carriers and checking the resulting litter to confirm if the suspected male or female carrier is a true heterozygote. The availability of a genetic test for detection of true carriers would enable captive breeders to manage genetic diversity levels within their populations and plan for a longer term conservation policy that integrates keeping a unique gene pool of heterozygotes without a hazardous increase of inbreeding levels.

2.6.2   Evolutionary importance of coat and skin colour

Coat colour in mammals is reflective of the evolution of the different species, with colour and pattern controlling loci having to adapt successfully to changing environments (Ortolani 1999, Eizirik *et al.* 2003). This is dramatically evident within Felidae, where speciation has been relatively swift and possibly in response to the differing environments encountered by these specialised predators. Within the last 10 million years, rapid speciation has given rise to 37 known distinct felid species (Nowak 1999). Concurrent adaptations to new environments have conferred in these animals the ability of concealment from prey and predators by cryptic and disruptive methods. These adaptations have also driven the development of display, forming epigamic (display in which a sexual element is involved); aposematic (threat display), and distraction or diversionary patterns. In wild felids, therefore, coat patterns are the result of functional adaptations and are a direct result of evolutionary consequence (Beltran & Delibes 1993, Ragni & Possenti 1996, Werdelin and Olsson 1997). Current understanding of felid coat colour pattern arises from developmental models derived from the *Turing-Type* of pattern-formation models (Turing 1953). It predicts that the size of the animal at the time when pigment pattern-formation is initiated is an important parameter determining the qualitative type of pattern that forms (Figure 2.1). The correlation between adult sizes in felids and patterns is that with increasing sizes, cats should have uniform patterns (e.g., flecks coalescing to form stripes, Werdelin & Olsson 1997).

When compared with insects, mammals have a limited range of coat colouration, ranging from white through grey to black and from black through brown to yellow and orange-red. The actual colour of the pelage might be of lesser

significance than the pattern and shade of the coat, due to the limited range of colour vision in mammals (Searle 1968). In predatory felids, coat patterns have a higher functionality as a concealing colouration than as a disruptive function. In the family Felidae, almost all species have an agouti type of coat colour, sometimes uniform but often marked with stripes, spots or blotches. The pelage pattern differences between different felid subspecies are usually very small and suggestive of multifactorial inheritance rather than the action of individual mutations.
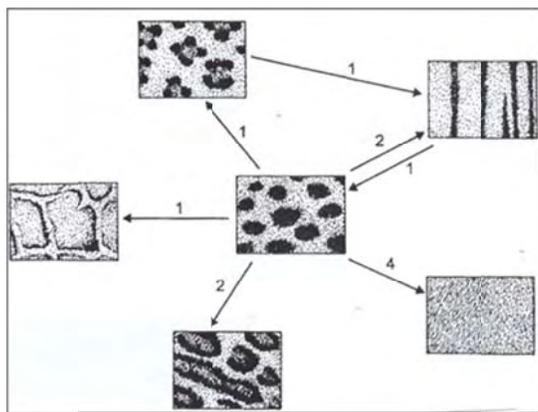
Figure 2.1: Coat pattern transformation in felid evolution (Turing, 1953) the transformation of the primitive character state within Felidae (the centre 'fleck' pattern) giving rise to all other patterns (Werdelin & Olsson 1997). The four possibilities (1-4) of coat pattern transformations from the primitive 'fleck' to other patterns are outlined by arrows.

Werdelin and Olsson (1997) describe six categories of coat pattern expression in Felidae.

    a.   Flecks, with small spots not organised into recognisable patterns

    b.   Rosettes, with small spots organised into patterns of six or fewer spots

    c.   Small blotches, with small irregularly shaped areas of dark in usually lighter background

    d.   Blotches, with large areas of variable colour framed by dark and set on a lighter background

    e.   Vertical stripes, with dark, dorsoventrally or anterodorsally to posteroventrally directed stripes on a lighter background

    f.   Uniform, with no distinguishable pattern. (Figure 2.2)

More recent work on felid coat pattern evolution using reaction–diffusion pattern creation and analysis (Allen *et al*. 2011), the authors concur with the generally supported theory of pattern evolution for background matching camouflage. The existence of unusual sub-optimal morphs such as the *King* cheetah that do not fit in the described diffusion pattern classification tree remain a mystery (W. Allen, personal communication, April 2011). The authors suggest that since very little phylogenetic signal could be correlated to the visual appearance of felid patterning; it is possible that felid camouflage adapts to environment over short time scales.
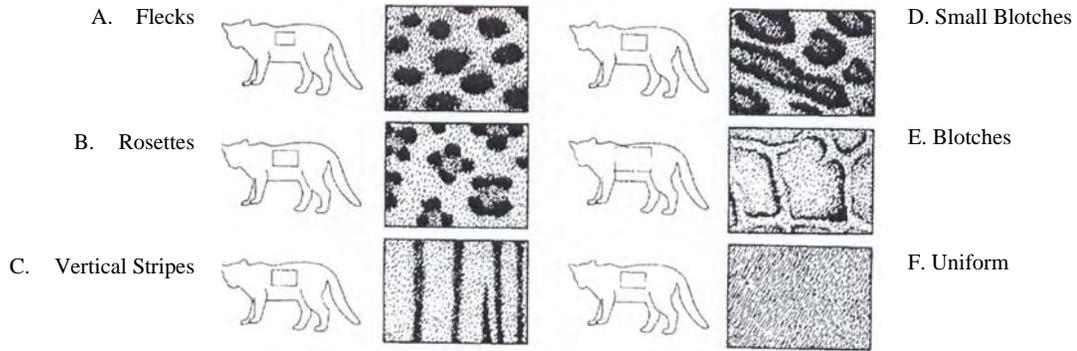
Figure 2.2: The six types of felid coat patterns: from top-left A to C = flecks, rosettes and vertical stripes. From top-right column D to F = small blotches, blotches and uniform (from Werdelin & Olsson 1997)

2.6.3   Coat colour and patterns in *Acinonyx*

The species *Acinonyx* has shown remarkable uniformity in both type and markings throughout its formerly wide distribution in Asia and Africa. The normal coat pattern in the cheetah consists of small close-set black spots on a yellowish ground colour. There may be variation in tail banding of cheetahs attributed to both genetic and environmental effects (Caro & Durant 1991). In the *King* cheetah, the spots merge to form longitudinal stripes and blotches (Figure 2.3). The unique coat colour pattern in the *King* cheetah is thought to represent a variation at a putative *Tabby* colour locus (Van Aarde & Van Dyk 1986). As early as 1968, Searle suggested the possibility that this might be due to a mutation at the *Tabby* locus. Robinson (1976) first stated that the change in the cheetah from the spotted *wild type* pattern to the blotches in the *King* might be similar to the *striped Tabby* to *blotched Tabby* change in the domestic *Tabby* cat (*Felis catus*).

Figure 2.3: The normal cheetah coat colour phenotype (left) and the *King* phenotype (right)

Van Aarde and Van Dyk (1986) provided evidence that the *King* variation is inherited as a single autosomal recessive allele from an analysis of pedigree records. It is, however, unclear how the coat variation in the cheetah occurs and how previous authors have reached conclusions on its similarity to the pattern variations in domestic cat. The domestic *Tabby* exhibits a wide variety of pattern variations, whereas the *King* cheetah exhibits significant uniformity in its pelage. All 38 coat specimens investigated by a previous researcher exhibited uniformity in having a longer mane and embossed stripes and blotches strikingly raised above the longer and silkier hair, a striped and

ringed tail and bold black on cream or ivory colouring (Bottriell 1987). The wild-type (normal) coat pattern in cheetahs appears to be a dominant spotted *Tabby* variation, a pattern that is traditionally considered a variation of the mackerel *Tabby* in domestic cats.

2.6.4   Adaptive mutations recorded in wild carnivores

Genes controlling spotting and striping patterns in *Carnivora* are unique in that they appear to have evolved separately from the basic mammalian genotype for coat colour. The variation of alleles at a putative *Tabby* locus, acting on an agouti or yellow background to give blotched, striped or lined patterns would have an evolutionary advantage for carnivores. Such markings within wild and domestic cats have an evolutionary relevance (Ragni & Possenti 1996). Eizirik *et al.* (2003) reported that populations of jaguarundi (*Herpailurus yaguarondi*) were exhibiting high frequencies of a mutated gene variant of the *MC1-R* gene. The mutated gene is responsible for a gradation in darkening of the pelage. The possibilities of adaptive advantages of such melanistic mutants under certain ecological circumstances in the dense South American jungles are raised by the authors. In case of the *Tabby* pattern, non-*Tabby* felids might actually represent a phenotypic variation and a rarity in the wild state (Kitchener 1991). An earlier study on Iberian lynx (*Lynx pardinus*) reported evidence that *Tabby* pelages in this species might have been actively selected for and become more frequent in feral populations, possibly as an adaptation towards crypsis for hunting. The authors describe three specific pelage types that vary in size, pattern and distribution of spots in the coat (Beltran & Delibes 1993). Kitchener (1991) notes that in the serval (*Felis serval*), there is a servaline phenotype that is recognised as the result of a mutation at the *Tabby* locus (Kitchener 1991). Melanism is the most common morph among wild mammals and black melanistic variants of the common wild tabby phenotype have been reported in at least 20 other felid species (Robinson 1978). Eizirik *et al.* (2003) state that felid melanistic variation has reached high population frequencies in few among the 37 felid species investigated. It has however failed to reach complete fixation in the populations investigated. This might be representative of how adaptive evolution occurs within the felid species.

2.6.5   The *King* phenotype and evaluation of evidence for it being an adaptive mutation

The change in pattern between the cheetah and *King* may be due to a simple shift in a pattern formation parameter, caused by the genetic change that underlies the phenotype. It is debatable whether the area of occurrence of the pelage variant '*King*' phenotype is an adaptation for the particular forest or woodland biome. Most historical sightings of the *King* phenotype have been in the woodland biome area around eastern and south-eastern Zimbabwe, the north-eastern region of former Transvaal and few in eastern Botswana (Rogers 1997). A parallel may be drawn with the finding of high population level frequencies of the mutated jaguarundi (*Herpailurus yaguarondi*) gene variants of the *MC1-R* gene (Eizirik *et al.* 2003). The juvenile cheetah cub is different from other felid juveniles in having on its back, a 'mantle' of long bluish-grey or smoky coloured hair, 70–80 mm in length. The pattern is said to mimic the pelage of the honey badger (*Mellivora capensis*), a particularly aggressive mammal with coloration that is among the best known examples of warning coloration (Skinner & Chimimba 2005). This contrasts with the assumption that the juvenile coat pattern represents the 'primitive' condition of the species (Werdelin & Olsson 1997). The adult felid coat colour pattern, on the other hand, is influenced by adaptation to hunting behaviour and

other characteristics of the species (Ortolani 1999). It is unclear if the blotched pattern of the *King* phenotype confers any particular advantage in the reported habitats. To get a clearer picture on whether the *King* phenotype is a type of adaptive mutation, the biochemistry of the mutation will have to be compared to other reported types of adaptive mutations, as seen in the arctic fox and the jaguarondi. Whether the persistence of such a mutation within a wooded biome in the areas described confers any evolutionary advantage is a possibility that is inherently difficult to rule out. Due to the extreme rarity of the *King* pattern, it might be inferred that the advantage, if any, is minor. Indeed it can be conversely argued that the *King's* rarity makes it likely that it is detrimental for evolutionary success.

2.6.6   Genetics of the *Tabby* locus in Felidae

The difficulty in studying the *Tabby* type of allelomorphism that is common in felids is mainly due to its absence in rodents and other commonly researched laboratory animals. However, the study of melanism in humans and laboratory animals indicate that the different phenotypes of melanocytes are mostly due to differential transcription of melanocyte-specific genes and melanocyte-specific transcription factors. The cause for a pigmentation phenotype is therefore likely due to the mutation in the genes encoding specific transcription factors. The *Tabby* series of alleles is not common in rodents, but in cats, it leads to the formation of darker transverse stripes or blotches on the coat. The standard of comparison is the phenotype seen in the natural, wild state of the species and is the short coated, mackerel striped *Tabby* as found in the European wild cat (*Felis silvestris*). In the wild cat of Europe, the standard pattern is similar to the striped *Tabby*, except that the tail is ringed a deeper black distally. The mackerel *Tabby* has characteristic vertical, gently curving stripes on the sided of the body (Figure 2.4). This may be continuous or broken into bars and spots, especially on the flanks or stomach. The pattern differences in the African bush cat (*Felis lybica*) from the European wild cat include less conspicuous striping and a slender and tapering tail.

A detailed comparative analysis of the coat colour and markings between European, Sardinian (African origin) wildcats (*Felis silvestris*) and domestic cats have revealed clear distinctions between the three groups based on *Tabby* markings than metric measures (Ragni & Possenti 1996). In domestic cats, the *Tabby* pattern consists of multiple components (Lomax & Robinson 1988; Robinson 1978; Vella *et al.* 1999)

1) Areas in which the hair fibres are banded or ticked with alternating black and yellow pigment. This wild type *Tabby* pattern is made of black pigment (eumelanin) against a yellowish ground colour (phaeomelanin).

The first component is akin to the drab yellowish-brown agouti coat that is identical in numerous animal species. The agouti protein production within the melanocyte is inversely proportional to eumelanin. The increased agouti production causes a change over time to the production of phaeomelanin that is then deposited in the hair. Although the agouti protein inhibits phaeomelanin, it does not do so as intensely as with eumelanin.

2) Areas in which all-black hairs predominate and the yellow band is reduced to the very base of the hairs, if it exists at all, resulting in a characteristic ticked, agouti colouration.

Figure 2.4: *Felis silvestris*, the European wild cat (above) and modifications and variations in the tabby phenotype (below, courtesy of Dr Lorimer http://people.ysu.edu/~helorimer/TabPat.html)

Mackerel *Tabby*-dominant to classic

Mackerel *Tabby*-dominant to classic, with unbroken vertical stripes

Mackerel pattern spots

The classic *Tabby*, the most recessive of *Tabby* patterns in domestic cats

The ticked *Tabby* modification-the most dominant modifier: heterozygous ticked with presence of leg and tail barring

The ticked *Tabby* modification-the most dominant modifier: homozygous ticked with lack of leg and tail barring

The second represents the melanistic overlay (bars, spots, rosettes or reticulation), a characteristic feature of the cat family. The *Tabby* pattern possibly consists of two co-existing systems of pigmentation or a background of agouti, with a superimposed system of stronger black pigmentation. The cat has an additional mechanism that causes production of dark stripes interspersed through this agouti coat. This happens when certain areas of the skin lack agouti protein or has few agouti protein receptors within the melanocytes, stopping the eumelanin to phaeomelanin shift. The hairs produced in these areas have eumelanin (black pigments) from tip to base. This is reported as the basic formulation that leads to camouflage colours with the cat family (Lomax and Robinson 1988)

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Traditionally, three alleles of the *Tabby* locus (*T*) have been recognised. The *Tabby* locus (*T*) is considered to have mutated to the Abyssinian (T^a), striped (*T*) and blotched (t^b). The three gene loci that are responsible for *Tabby* pattern in the domestic cat are as follows -

 a. The first determining whether the cat is ticked *Tabby* (*Abyssinian*) or not (T^a versus t^a)

 b. The second determining mackerel type ('wild type' or striped) versus blotched (classic) *Tabby* pattern (*Mc* versus *mc*)

 c. The third determining spotted pattern versus non-spotted (S_p versus s_p)

 d. Possible existence of other modifying factors (causing the wide variation in the striped and spotted patterns)

The Abyssinian or ticked *Tabby* (T^a) is considered incompletely dominant or epistatic to the mackerel striped (*T*) and blotched alleles (t^b). The mackerel or striped (*T*) is completely dominant to the blotched type (Lomax & Robinson 1988). The Abyssinian has a variation where the facial marking is reduced and striping is restricted to the head, lower limbs and distal part of the tail. There is minimal *Tabby* striping, leaving only the underlying agouti colouration. Found in the Abyssinian breed, this has been passed on to the Somali, Singapura, Asian and Oriental breeds (including the Siamese). The blotched allele is expressed as an intensification of the *Tabby* pattern where the stripes are replaced by whorls of eumelanic pigmentation that may join to form blotches on the sides of the body. The bars on the legs and rings on the tail are more prominent. A very dark *Tabby* result when there is more extensive coalescence. The American Shorthair is a classic example for a blotched *Tabby*. An alternative hypothesis of *Tabby* pattern inheritance has been proposed by Dr H Lorimer (http://cc.ysu.edu/~helorime/TabPat.html) with three separate genes controlling *Tabby* pattern inheritance. They include a base pattern of classic or mackerel, then two separate dominant modifying genes. The spotted gene may break the classic pattern into spots and the ticked gene may break the pattern down to a greater scale. This may produce the even gradient of ticking of the ticked *Tabby*.

Figure 2.5: The Spotting Modification in tabby. The spotting modification appears to be a dominant modifier of mackerel and classic patterns. These cats exhibit the classic pattern spots, with spots along spine set in rows parallel to the spine and a circular pattern of spots on sides (pictures courtesy of Dr Lorimer http://people.ysu.edu/~helorimer/TabPat.html)

The spotted *Tabby* pattern has two major forms (Figure 2.5) - Modification of the mackerel or blotched *Tabby* pattern where the vertical stripes are discontinuous, taking the form of spots or bars (causes 'ocicat' type spots)

(Lyons *et al.* 2005a). Litters have been produced with all three above patterns that are inconsistent with a single locus theory. Breeding data suggests a dominant modifying gene is responsible for the spotted *Tabby* type. The change in striping is possibly refined by polygenic factors. A cinnamon Ocicat was found to lack the stop mutation for tyrosinase-related protein 1 (TYRP1) *(*exon 4), suggesting that the Ocicat phenotype could be caused by a different mutation in TYRP1 or by a mutation in a different gene (Lyons *et al.* 2005a).

The second spotting form expresses itself as distinctive, rounded spots at random pattern, possibly due to a separate genetic allele. The Bengal breed is an example, where a unique recessive '*marble*' pattern is seen in offspring of Bengal cats that are heterozygous for the spotting factor.

More recent work on colour patterns in cats by Eizirik *et al.* (2010) has clarified pattern formation to a greater extent, where it is the *Tabby* locus that determines the pattern type. According to this work, the Ta^M (*Mackerel*) allele is dominant to the Ta^b (*Blotched*). A separate locus, called *Ticked* is required for absence of presence of patterns and Ti^A allele is dominant to Ti^+ allele. Figure 2.6 elucidates this theory (Kaelin & Barsh 2010).

Figure 2.6: The various *Tabby* phenotypes and possible genotypes (from Kaelin & Barsh (2010) elucidating recent theory on coat pattern formation by Eizirik *et al.* (2010)). The Ta^M (*Mackerel*) allele is dominant to the Ta^b (*Blotched*). A separate locus, called *Ticked* is required for absence of presence of patterns and Ti^A allele is dominant to Ti^+ allele

The greyish *Tabby* coat colour of the domestic cat is a composite of a background of the common mammalian agouti colouration (with the eumelanic secondary guard hairs subapically banded with phaeomelanin) and a superimposed

and disruptive striped pattern of eumelanin pigmentation that partially obscures the agouti (Vella *et al.* 1999). The wild type of the second component is a striped *Tabby* pattern consisting of gently curving narrow stripes on the sides of the body, barring on the legs, a succession of rings on the tail and dark marking on the cheeks and forehead (e.g., as in *Felis silvestris*). From breeding records, it appears that both the agouti locus (*A*) and its recessive mutation, the nonagouti (*a*), are under the influence of independent gene loci (Lomax & Robinson 1988). The mutation results in hairs to lack the phaeomelanic band, causing the hair to be distally black and becoming a pale slate-blue close to the skin. The *Tabby* and agouti loci are considered independent, inferred from the frequent presence of a shadowy *Tabby* pattern in the juvenile pelage of the nonagouti phenotype. This appears to be confirmed recently, as part of an investigation on the genomic position of the classic X-linked orange (O) locus genotype, where the authors noted that the agouti locus did not influence the presence or absence of tabby pattern in orange-coloured fur, but genotype at agouti signalling protein (ASIP) did (Schmidt-Kuntzel *et al.* 2009).

2.6.7 Melanocyte biochemistry and expression of coat colour

The melanocyte is a neural crest derived cell that migrates via the mesenchyme, into the epidermis, hair follicles, uveal tract of the eye, the leptomeninges and the inner ear during embryogenesis (Jackson 1997; Rees 2003; Slominski *et al.* 2004). The survival and migration of this specialised cell depends on the interactions between specific receptors on the cell surface and their extracellular ligands. Melanocytes reside within the basal layer of the epidermis for the duration of the lifetime.
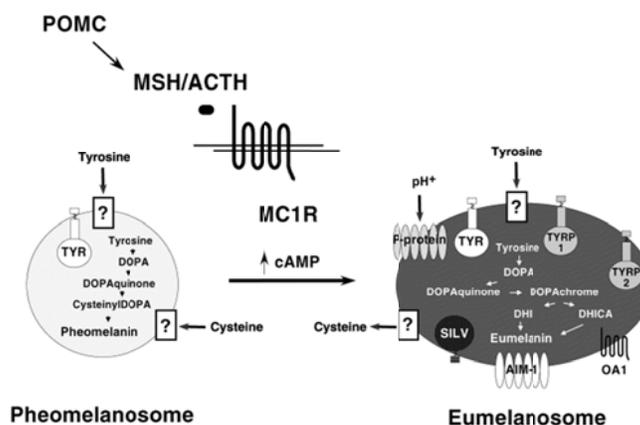
Figure 2.7: Enzymatic pathways in melanin production. The figure illustrates the MC1R control of pigment type switching. POMC cleaves to give MSH/ACTH ligands for the MC1R receptor. It triggers/cAMP pathway and induces phenomelanosome containing the TYR molecule to convert to the eumelanosome containing all the melanogenic enzymes (Sturm *et al.* 2001).

A functional epidermal melanin unit consists of the cell body of the melanocyte in a special layer within basal lamina, with multiple dendrites arising from the body and forming desmosomal connections with up to 30- 40

keratinocytes as far away as the mid stratum spinosum. Embryonic melanoblasts arise from the group of neural crest cells that migrate laterally towards the dorsal portion of the somite, splitting into epidermal melanocytes and dermal melanocytes. In mice, numerous pigmentary effects are produced due to the variations in the cellular environment of the migrating melanoblast

2.6.8 Molecular genetics of skin and coat colour patterns in Felidae

As early as 1976, researchers had predicted that in the domestic cat melanism is inherited as a recessive trait and the possibility that *agouti/ASIP* might be responsible for the variation seen (Robinson 1976). Twenty seven years later, the evolution of melanism in the cat family was reported (Eizirik *et al.* 2003) and the domestic cat homologs of *ASIP* and *MC1-R* were mapped to chromosome A3 and E2 respectively, cloned and sequenced. The rapid increase in publications detailing domestic cat coat colour mutations in the past decade is reflected in the current list for 'Online Mendelian Inheritance in Animals' list (http://omia.angis.org.au/), where agouti, different albinism types, brown, dilute, dominant white, extension, glitter, orange, pink-eyed dilution, silver, tabby, white and white spotting are among the coat colour patterns listed. Most of the genetic modifications that cause change in coat colour arise from base changes in genes within the enzymatic pathways in melanin production (Figure 2.7).

Genetic linkage between the *Tabby* locus and genetic markers located on the domestic cat genome were previously reported (Lyons *et al.* 2006) and more recently modified (Eizirik *et al.* 2010). Lyons *et al.* (2006) used an extended pedigree of 64 cats for linkage analysis with 150 feline microsatellites that gave an average marker spacing of 75cR. The inheritance pattern was assumed by them to be a dominant allelic series. Lyons *et al.* (2006) detected linkage between markers on chromosome B1 and the *Tabby* locus, with the area of interest estimated to span approximately 15.9 Mb or 17 cM on the second generation domestic cat linkage map (Menotti-Raymond *et al.* 2003b). This study investigated the possibility that the *King* variant in cheetah is similarly linked to conserved markers. More recent work by Eizirik *et al.* (2010), published after this study, narrows down the region of interest, with the locus responsible for the Abyssinian (*ticked*) form mapped to a ~3.8 Mb region on chromosome B1 and a putative second locus that controls the *Tabby* phenotype mapped to a ~5 Mb region on chromosome A1, an area this study did not address. The spotted Egyptian Mau breed was used to detect the spotting variant by Eizirik *et al.* (2010) and crossed to the recessive blotched phenotype to yield intermediate patterns, none being spotted in the F1. The non-blotched backcross progenies, however, exhibited a full range from spotted to striped phenotypes, deviating from the phenotype of the true breeding spotted Egyptian Mau. Such epistatic action of modifying genes appears to be absent when the recessive blotched *King* phenotype is crossed with the dominant spotted cheetah. This thought is detailed more in chapters 6 and 7.

Reflecting on the history of human *MC1-R* mutation research, Rees states that in pigment research it is quite possible that if only some loci and alleles are studied, genuine associations may be missed (Rees 2000). Details of the candidate gene/s, the rationale behind their selection, known and potential markers that flank these loci and other cytogenetic data are detailed in the 'materials and methods' chapter.

Chapter 3: Materials and Methods

3.1. Research animals

3.1.1 The De Wildt Cheetah Breeding Station and Research Centre captive cheetahs

The cheetahs at De Wildt Cheetah Breeding Station and Research Centre (DWCBSRC), have had tremendous success in breeding in captivity (Meltzer & Van Dyk 1998). At the South African Mammal Conservation Assessment and Management Plan (CAMP) held in 2002-2003, the total number of cheetahs in captivity was estimated to be around 265 animals, with the De Wildt captive population making up over 38% of the total. Of the total captive births worldwide in 2002 (n = 118), this institution contributed over 28% of cubs born (Marker 2004). Another important captive group in South Africa, the Hoedspruit cheetah breeding population, have many founders originating from the De Wildt population. One estimate points to over 60 animals being direct descendants of De Wildt founders (Marker 1998). The centre is possibly the biggest single contributor to the world's captive cheetah population (Van Dyk 1998). The DWCBSRC also has the largest breeding group of *King* cheetahs, a phenotype with a coat pattern inherited as an autosomal recessive trait (Van Aarde & Van Dyk 1986) and distinct from the normal type (Brand 1983). The central founding premise of this institution is keeping alive the possibility of releasing captive-bred animals into areas where they had previously occurred (Meltzer & Van Dyk 1998). Information on diversity parameters of the founding and current populations of De Wildt and elsewhere, therefore, appears to be crucial to cheetah conservation in South Africa.

3.1.2 The *Africat Foundation* in Namibia

The *Africat Foundation* in Namibia is situated on the farm Okonjima, 50 km south of Otjiwarongo in Namibia. The cheetah population consists of animals rescued from the wild making it an ideal sampling of free-ranging wild cheetahs for genetic comparisons. The cheetahs here cannot be considered for reintroductions into the wild since they were injured and thus unable to hunt or were small cubs that had to be hand-raised and therefore habituated to humans. Many of these cheetahs were rescued as single animals but others were introduced into *Africat Foundation* as siblings. Adult cheetahs and cubs that are old enough are housed in camps ranging from 5 to 3000 hectares. Wild cheetahs sampled were immediately location-tagged and had area of sampling available.

3.1.3 The South African wild cheetahs

Wild cheetahs from South Africa were sampled as part of the National Cheetah Metapopulation Program (NCMP) and obtained on request from the De Wildt Foundation. Cheetahs sampled were immediately geo-tagged and had area of sampling or GPS co-ordinates available.

3.2 Sample collection and storage

All prospective samples were collected by veterinarians employed by the respective organisations during their routine operations. The *Africat* cheetah population in Namibia, where the majority of the animals are wild born, were sampled opportunistically. Blood samples were collected in labelled EDTA tubes and kept chilled until DNA extraction procedures. Tissue samples, where available, were stored frozen till extraction.

3.3 Quantifying diversity and inbreeding: methods

3.3.1 DNA extraction from whole blood

A standard phenyl-chloroform extraction protocol was followed for DNA extraction from blood and tissue (Sambrook *et al.* 1989). Quantification of the DNA yield was determined with a NanoDrop® ND-1000 Spectrofluorimeter (NanoDrop Technologies, DE, USA). DNA was stored in 96-deepwell plates at−20 ∘C until subsequent polymerase chain reaction (PCR).

3.3.2 Microsatellite genetic markers for population genetics

The individual microsatellites that form multiplex panels were selected for their reliability during amplification, annealing temperature and base-pair size. Construction of multiplexes and selection parameters were decided in consultation with other members of the *Genetics subcommittee* of the *National Cheetah Conservation Forum* of South Africa. Thirteen microsatellites that were common with previously published studies were included in the panel for validation and comparison. These were primarily selected from 84 markers proven to amplify well in cheetahs (Driscoll *et al.* 2002) and the domestic cat genome (from in-house test data).

3.3.3 Genotyping error rate

The rate of genotyping error was assessed by selecting 35 samples at random from the database of 274 cheetahs and re-extracting and re-genotyping. Allelic mismatches were counted by comparing these genotypes to the previous ones as described (Bonin *et al.* 2004). Amplification and consistency of PCR products were also checked using MICROSATELLITE TOOLKIT (Park 2001). Estimates of the frequency of null alleles were determined by the maximum likelihood implemented in ML-NULLFREQ (Kalinowski & Taper 2006) and by an iterative algorithm based on the observed and expected frequencies of homozygotes implemented in CERVUS 3 (Kalinowski *et al.* 2007). The dataset included a three generation captive pedigree (N = 89) that was parentage tested for subsequent linkage analysis. This enables us to eliminate and correct any error that might have occurred during data entry and scoring of alleles.

The total number of genotypes analysed in the dataset amounted to 3562 (274 * 13) and the error rate was analysed by re-extracting and analysing 455 of these profiles (35 samples typed for 13 loci each). Failed reactions were noted

in 15 genotypes, all with locus FCA678. No allelic dropouts were evident in the samples reanalysed. This was again confirmed during parentage analysis using the 83 member linkage pedigree. Excluding FCA678, there was not enough data to confirm any allelic dropouts, and therefore the genotyping error rate was determined to be insignificant and would not affect our results. FCA678 was consistently monomorphic in all Namibian origin cheetahs tested.

3.3.4 Candidate loci selected for linkage analysis

Previous work and data from studies on *Tabby* colouration (Lomax & Robinson 1988; Robinson 1978; Vella *et al.* 1999) and molecular genetics of *Tabby* alleles (Lyons *et al.* 2006) indicated that a candidate gene approach, concentrating on the putative location of the *Tabby* locus in the *Acinonyx* might be viable. The *Tabby* locus in domestic cat was previously estimated to be in the B1 chromosome (Lyons *et al.* 2006). The following microsatellite markers, including FCA023, FCA809, FCA811, FCA810, FCA700, FCA254 and FCA813 were chosen for the study. The current known location of these markers on the domestic cat genome is available from http://www.ncbi.nlm.nih.gov/genome/?term=txid9685. Details of the general location of these loci on chromosome B1, details of primer pairs and amplification parameters are listed in Appendix VI. The Genome Annotation Resource Field Felis catus v12.2 (GARField) view of the region (Pontius & O'Brien 2007) spanning 2.918 Mbp between FCA254 (position 77729549) and FCA700 (position 74811696) is in Appendix XVII. These markers have previously been used to demonstrate linkage to the *Tabby* locus in the domestic cat, with the estimate that the putative gene or a tightly linked locus likely resides between markers FCA559 and FCA254.

3.4 Experimental design and analytical /statistical procedures: population genetics and heritability estimates

3.4.1 Database management and pedigree visualisation

The primary database was built using a Microsoft *ACCESS*® masterfile. A Java-based application called ATLAS was utilised for managing genotypes that are available from the pedigree (Perez-Enciso *et al.* 2005). For studies on heritability, the pedigree was recreated using PEDDRAW (ver 3.0) (http://www.pedigree-draw.com/index.html) and JENTI (Falchi & Fuchsberger 2008). A subset of the pedigree was redrawn for linkage analysis and visualised using SUPERLINK online version 1.0 (Silberstein *et al.* 2006b) and CRANEFOOT (Makinen *et al.* 2005).

3.4.2 Analysis of diversity measures

Within-population diversity was estimated using microsatellite genetic markers, and the most commonly taken measure of variability, expected heterozygosity (Caballero & Toro 2000; Petit *et al.* 1998) was used to derive diversity values. The diversity levels were determined as follows: $H_k = 1 - H_{(S/k)} / H_{(S)}$; where $H_{(S)}$ is the average internal heterozygosity of the metapopulation S and $H_{(S/k)}$ is the average internal heterozygosity of the metapopulation excluding the population. H_k is the contribution to within-breed (metapopulation) diversity by the population k. Microsatellite genetic variation measures were estimated using CERVUS (Marshall *et al.* 1998) , FSTAT (Goudet J. 1995) and ARLEQUIN (Schneider *et al.* 2000). These measures included observed and expected heterozygosity, polymorphic information content (PIC), null allele frequencies based on heterozygote deficit and average exclusion probabilities with and without one parent already known. Classical gene diversity parameters

developed by Wright (Wright 1978) and Nei (Nei 1987) were utilised to derive the contribution of the population analysed to the total gene diversity. The F_{ST} parameter of Wright (or the G_{ST} of Nei) represents the proportion of the total genetic variation that is due to differences in allelic frequencies between sub-populations. The aggregate diversity can be stated as F_{ST} (between populations) + (1-F_{ST}) (within populations). A comparison of internal relatedness (Amos *et al.* 2001) and homozygosity by loci (Aparicio *et al.* 2006) was done for all population groupings detected. Internal relatedness is a measure based on allele sharing where the frequency of each allele counts towards the final score, thereby allowing the sharing of rare alleles to be weighted more than the sharing of common. Homozygosity by loci does not underestimate the heterozygosity of individuals carrying rare alleles and weighs the contribution of each locus to a homozygosity index depending on their allelic variability.

Linkage disequilibrium can influence results of population associations and occurs when alleles of different genes are inherited together because they are in close proximity. Tests for genotypic linkage disequilibrium and deviations from Hardy-Weinberg equilibrium for each locus were performed using GENEPOP web version 3.1c. Hardy Weinberg Exact Tests have the null hypothesis, Ho = random union of gametes. For testing genotypic linkage disequilibrium, the null hypothesis Ho is - "*Genotypes at one locus are independent from genotypes at the other locus*". The software makes contingency tables for all pairs of loci in each population, and then performs a probability test or a Fisher exact test for each table using a Markov chain. Deviations from HWE were tested for all locus-population combinations and globally using the Markov chain method of Guo and Thompson in GENEPOP (Guo & Thompson 1992). Significance levels were adjusted using the sequential Bonferroni method to take into account multiple tests on the same data set (Rice 1989). Within the De Wildt population, the extent of differentiation between *King*s and other cheetahs were analysed using analyses of molecular variance (AMOVA) with microsatellite genotypes, implemented in ARLEQUIN. AMOVA computations take into account the number of mutations between molecular haplotypes and a user-defined genetic structure has to be set. This structure is then tested by a hierarchical analysis of variance that partitions total variance into covariance components due to intra and inter-individual differences and/or inter-population differences. The procedure does not require a normal distribution that is common in analysis of variance nor does it need equality of variance among populations or groups of populations. Differentiations between groups were assessed, using analogues of F_{ST} and R_{ST} for microsatellite data (Michalakis & Excoffier 1996). Pairwise F_{ST} values were computed to estimate genetic divergence among groups.

A multivariate ordination of pairwise differentiation statistics (F_{ST}) was done using principal co-ordinates analysis (PCA) implemented in GENALEX 6 (Peakall & Smouse 2006). Here, the relationship between distance matrix elements is plotted based on their first two principal coordinates and when there are distinct groups, the first 2 or 3 axes will typically reveal most of the separation among them. The PCA plots the major patterns that make up the data set with multiple loci and uses the AMOVA distance matrix for plotting the parameters into a scatterplot of the first two coordinates. Visualising pairwise differentiation (F_{ST}) between cheetahs was also done with PCA-GEN 1.2 (Goudet 1999), with 10000 randomizations of genotypes was used to test for significance of axes. In PCA-GEN, a goodness-of-fit statistic called the inertia (I) is used as a measure of how much variance each of the principal components accounts for. The majority of the data should be accounted for in the first two principal components allowing for a qualitative graphical representation of the data in two-dimensional space.

Patterns of differentiation between populations were also visualised using the principle component analysis implemented in GENETIX 4.04 (Belkhir 2000), where a Factorial Correspondence Analysis (FCA) of individual genetic marker scores are used to identify population clusters graphically. FCA draws a synthetic graphical display of similarity among individual samples in a multidimensional space without the limitations of a two-dimensional branching pattern generated by a single tree that tends to oversimplify inter-population relationships. FCA is insensitive to different mutation models for microsatellite marker and gives an unbiased test of population structure. Although the gross placement of individuals to *a priori* identified population groups are possible, identification of admixed animals is not possible as it cannot assign probabilistically different individuals to clusters (Pierpaoli *et al.* 2003).

The pairwise genetic distances among all individuals was detected using the proportion of shared alleles (*Dps*) distance estimator with the (*1-M*) correction in MICROSAT 1.5E (Minch *et al.* 1996). *Dps* was calculated for individuals or population samples (described as taxa), as the mean over loci of the sums of the minima of the relative frequencies of all alleles between compared taxa. Bootstrap values for 1000 bootstrap replicates in MICROSAT 1.5E were calculated with the *Consense* subroutine in *PHYLIP 3.6B* (Felsenstein 2001). The *Consense* subroutine generates trees by the majority-rule consensus tree method, giving branch lengths which are simply the numbers of replicates that support the branch. The optimum method for accurate tree lengths is to use it as input tree in a program that will estimate branch lengths for it. A phylogenetic tree was drawn using the *Neighbour* option and visualised using the program TREEVIEW 1.6.6 (Page 1996).

Effective population size (Ne) estimates are integral to estimate the rate of increase of inbreeding and loss of neutral genetic variation and rate of fixation of alleles in a population (Frankham 1995). We used a modified method for estimating Ne that yields greater accuracy to true population size and also performs well in non-ideal populations with skewed sex ratio or non-random variance in reproductive success (Waples 2006). This single sample linkage disequilibrium (LD) method utilises LD at unlinked gene loci but corrects for a bias when using diallelic loci (England *et al.* 2006). Single sample estimates for N_e were obtained using LDNE (Waples & Do 2008) using the combined cheetah dataset (Namibian and South African born cheetahs), and the Namibian wild and South African wild and captive dataset separately. The jackknife confidence intervals were taken to contain the true N_e (Waples & Do 2008).

A second approach to estimate N_e by using an approximate Bayesian computation (Tallmon *et al.* 2004) from a single dataset was attempted. The procedure simulates 50,000 populations that reproduces following a Wright-Fisher model for somewhere between 2 and 8 generations before being sampled. The populations are generated based on input parameters and draws a sample equal in size to actual population sample. The effective size is assumed to exist between the minimum and maximum effective size specified. The level of population genetic variation is determined by, theta, a product of its historic effective size and the mutation rate (4 N_e*u) and is randomly drawn from a uniform random number between 2 and 12. Estimated mean N_e and confidence intervals were calculated using the software ONESAMP (Tallmon *et al.* 2008) on the basis of the priors on N_e, from 50,000 simulated populations reproducing for two to eight generations following the Wright-Fisher island population model. The priors on N_e for the combined populations were set between two and 10,000, and for Namibian and South African datasets between two and 5,000.

A higher upper prior was preferred since the biologically probable and historical N_e is a best guess for southern African cheetahs and a more conservative estimate chosen than a slightly inflated value in the instance that the user-guessed upper prior on N_e is lower than the actual (Tallmon *et al.* 2008). The method has been found robust in reports on effective population sizes (Johnson *et al.* 2009; Slabbert *et al.* 2009).

Bayesian inference in population genetics takes an unknown state and represents it as a probability distribution over a parameter space (data and input model parameters). The prior distribution is then transformed into a posterior distribution using Bayes' Theorem. An assimilation of the joint posterior distribution narrows down estimates of marginal posterior distributions of individual parameters. Bayesian clustering is better suited for analysing complex genetic data by detecting subtle signals of linkage and Hardy–Weinberg disequilibrium to group individuals into genetically distinct clusters (Beaumont & Rannala 2004; Corander *et al.* 2003; Latch *et al.* 2006; Vaha & Primmer 2006). Initially, the software STRUCTURE 2.2 (Falush *et al.* 2007; Pritchard *et al.* 2000) was used using default values for a total of 10 replicate unsupervised runs for each value of the number of clusters (K) from one to 10. The model in STRUCTURE assumes there are *K* populations (where *K* may be unknown), each having a set of allele frequencies at a locus. As recommended by the authors (Pritchard *et al.* 2000), we did an initial pilot analysis to determine initial estimates of the prior (Pr $(X|K)$). A ''burn-in'' of 100,000 Markov Chain Monte Carlo (MCMC) Gibbs sampler iterations, and 1,000,000 follow-on MCMC iterations provided consistent estimates of the prior (Pr $(X|K)$). We used a model that assumed admixture with a uniform prior on the degree of admixture and alpha (initial value=1.0, max=10.0, SD=0.025). Allele frequencies were set to be correlated among subpopulations (prior mean=0.01, prior SD=0.05, k=1.0) with default *lamda* values, a setting that was reported to perform better for subtle population structure (Falush *et al.* 2003). An additional 10 replicate runs were performed with an admixture model that had a calibrated prior on the degree of admixture (*alpha*) based on the initial results (initial value=0.2, max=10.0, SD=0.025).

We then averaged membership coefficients over several analyses to correctly estimate the likelihood value for runs to override any concerns over plateau formation that estimates cluster numbers (Chen *et al.* 2007a). STRUCTURE has been reported to perform less than optimally at $F_{ST} = 0.01$ or below. To correctly handle results from replicate analysis, we selected 10 replicate runs where levelling of likelihoods was detected and calculated the symmetric similarity coefficient (SSC) using the software CLUMPP (Jakobsson & Rosenberg 2007). CLUMPP estimates the cluster membership coefficient matrices of multiple runs of a clustering program, for any number of clusters, and outputs these same matrices, permuted so that all replicates have as close a match as possible. SSC was estimated as the mean over all individual Q-matrices after the columns have been aligned according to the *Greedy* algorithm with the greatest *H'* value (option M = 3; 1000 repeats specifying the number of random input orders of runs that will be tested). The population *Q*-matrix file with cluster membership coefficients generated from CLUMPP for the *K* values was visualised using DISTRUCT (Rosenberg 2004). DISTRUCT displays results to show each individual as a line segment partitioned into K coloured components representing the individual's estimated membership coefficients in the K clusters.

The number of clusters detected by STRUCTURE might be distorted in hierarchical systems where migration between populations is uneven (Evanno *et al.* 2005). The best estimate of groups is detected by the modal value of

ΔK, a quantity based on the second order rate of change with respect to K of the likelihood function. After plotting the averages across 10 runs, (Pr ($X|K$) estimated by STRUCTURE was plotted for plateau formation for the real K value (Pritchard *et al.* 2007; Pritchard *et al.* 2000) and a determinable peak at a single K identified. The measure ΔK was then derived as the second order rate of change of ln[Pr ($X \mid K$)] with respect to K, obtained by the following calculation on a spread sheet:

$\Delta K = mean \, (|L \, (K + 1) - 2 \, L \, (K) + L \, (K - 1)|)/SD[L \, (K)]$

where $L = \text{Pr} \, (X \mid K)$ and SD = standard deviation (Evanno *et al.* 2005)

In instances where low levels of within population genetic variation (F_{ST} = 0.02 – 0.03) are obtained, a test for significant differences in allele frequencies, as implemented in BAPS 3.2 is recommended (Latch *et al.* 2006). The parameter estimated here is the partition among groups, with all populations considered *a priori* possible, and then using molecular marker data to estimate which clusters are empirically plausible. The method therefore tests for the optimum number of genetically diverged groups, with allele frequencies of the molecular markers being unknown parameters that are the end estimates of the analysis. Ten independent runs were done for K values from 2 – 15, to determine if the results change noticeably, by comparing their corresponding "*logmls*" (Corander *et al.* 2003). Results from the initial clustering of cheetahs were used to perform an admixture analysis using 1000 iterations and analysing 50 reference individuals per population with 20 simulations. The inferred K from MCMC runs was analysed for admixture proportions (q values) for each cheetah, to define group membership. Using BAPS, a value of $q > 0.5$ for individual cheetahs was considered significant, indicating that it is correctly designated to its true cluster of origin (Latch *et al.* 2006). The admixture coefficient for a cheetah in each cluster estimated by BAPS was used as a measure of correct assignment. The number of cheetahs with significant admixture was determined by selecting cheetahs having *p*-values larger than 0.05 (Corander *et al.* 2003). Orozco-Terwengel *et al.* (2011) recently reported concerns on the variability of clustering solutions obtained when using BAPS, where the number of markers used proved critical and up to 105 loci were indicated for getting 95% reliability of results on a human dataset. In general, the error rate in assigning population clusters by Bayesian algorithms increases as loci numbers decrease and population differentiation (*Fst*) decreases to 0.01 or lower. Exacerbation of such cluster assignment errors occur with increasing number of potential populations sampled and the non-availability of private alleles appear to compound the problem (Orozco-Terwengel *et al.* 2011). There is currently no tool to approximate the loci required to have confidence in an obtained solution. The cheetah dataset does not suffer from low levels of genetic differentiation and has above average allele frequencies comparable to other felid datasets, and have strongly differentiated lineages, thus alleviating concerns on clustering solutions obtained.

A third Bayesian clustering technique implemented in TESS 1.1 (Chen *et al.* 2007a), that uses a Hidden Markov Random Field (HMRF) to model spatial dependencies at the cluster membership level were used. The model takes into account that individuals from spatially continuous populations are more likely to share cluster membership with their close neighbours than with distant representatives. It helps to detect significant geographical discontinuities in allele frequencies, thus regulating the number of clusters detected. The prior distribution of population groupings are taken as a HMRF on a spatial individual network (Chen *et al.* 2007a). This is supported by previous reports where the use of spatial coordinates with Bayesian clustering were found better in clarifying the presence of structuring by

the incorporation of prior information about spatial structure, especially with limited number of loci (Francois *et al.* 2006) and in instances when sampled populations do not overlap spatially (Chen *et al.* 2007a).). This study had a dataset of wild-sampled cheetahs with geo-tagging information. Where capture locations were available without GPS co-ordinates, the spatial data was obtained by triangulating the area on Google Earth (Google Inc 2007) and co-ordinates logged by converting latitude and longitudes from 'point of capture/sampling' or 'place of trapping' into Universal Transverse Mercator coordinates using UTMS (National Geographic Society 2002). Details of the admixture model used in TESS 1.1 (Chen *et al.* 2007b) included a total of 100 runs with a burn-in period of length 10,000 followed by 50,000 iterations for the entire dataset. To get a good estimate of K_{max}, runs were done setting K_{max} from a low to higher values. A set of 10 runs each, starting from K_{max} of 5 to 15 and for spatial autocorrelation parameter ψ at intervals of 0.2, increasing from 0.2 to 1 were done. The output was evaluated for estimates generated for the preferred value of potential clusters K, noting that the estimated K should be less than or equal to the user specified maximum value K_{max} (Chen *et al.* 2007b). The value of K_{max} is considered to be not sufficiently high if the estimated K equals K_{max}. The analysis was done for 100 replicates of the dataset, with an initial 10 runs done using settings of no admixture and $\psi = 1$ and no spatial coordinates. Subsequent iterations (n = 90 runs) had incremental increases in ψ as described and included spatial data. Outputs from STRUCTURE and TESS were analysed and used to manually generate cheetah clusters. The dataset of wild-sampled cheetahs with GPS data or geo-tagging information was used as the nucleus around which other cheetahs were assigned membership. Once the spatial assignment was done, the clusters were reanalysed using BAPS to clarify admixture and detect membership at $q > 0.5$. The technique in BAPS utilises individuals with known genetic origins (or a baseline sample) and one without (Corander *et al.* 2008). Selecting 'trained clustering' lets the geo-tagged group to be used for updating information about allele frequencies in the baseline populations. A more informative prior is then derived as a result. Trained clustering uses the specified uniform prior distribution over groups to satisfy the constraint that any sub- set of individuals with an origin in the same local sample population should not be assigned into separate clusters This method has been shown to be informative on the fine scale admixture patterns when complex populations are analysed (Carmichael *et al.* 2007) and helps to detect spatial population genetic structure with clinal variation (Rosenberg *et al.* 2006).

3.4.3 Heritability analysis of fitness components

In populations with recent inbreeding and recent consanguineous matings, the interpretation of heterozygosity-fitness correlations under the general effects hypothesis is extremely difficult (Szulkin *et al*. 2010). In scenarios where pedigree and demographic data are available for analysis, there is greater benefit in using such data for conservation purposes and translocation (Grueber *et al*. 2011). In fact, current literature advises caution when using multilocus heterozygosity from a small number of neutral markers to estimate genome-wide heterozygosity and inbreeding (DeWoody & DeWoody 2005; Ljungqvist *et al*. 2010; Slate *et al*. 2004).

Wright's coefficient of inbreeding (Wright 1922) was calculated over six generations of the pedigree using FSPEED2 (www.tenset.co.uk). For the purposes of statistical analysis, four inbreeding classes were defined, including unknown/wild founder, 0 - 0.09, 0.1 - 0.19 and 0.2 and above. A previously developed model was used for grading gastritis in captive cheetahs (Munson 1993). The methods applied for sampling gastritis data are described in detail in chapter 5, and were part of routine diagnostics done by a South African board certified veterinary

pathologist. Information on other categories including infectious conditions, natural death and cub mortality were obtained from DWCBSRC breeding records and captured into the database. Cheetahs positive for a reverse line blot hybridization (RLBH) assay using a probe specific to the 18S rRNA gene sequences of the intracellular erythrocytic haemoprotozoan parasite *Babesia felis* (Bosman *et al.* 2007) were obtained from the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, Onderstepoort, South Africa. A Kruskal-Wallis One Way Analysis of Variance on Ranks (K-W ANOVA) was used to determine estimates of inbreeding class differences for more than two classes. For comparing two classes, the Mann-Whitney rank sum test was used. Wright's coefficient of inbreeding was the primary fixed effect investigated for significance. Influence of inbreeding on gastritis, infectious conditions and other causes was tested using the GLM procedure of Statistical Analysis System (SAS), version 9.1.3 (SAS Inc., 2005; Cary, NC). Explanatory variables were analysed for significance.

Additive genetic effects were estimated using a univariate linear mixed effects model. The software ASREML2 package (Gilmour *et al.* 2006) was used to estimate (co)variances. The models used had the following conventions -

$$Y_{ijkl} = GT_j + IN_k + GT_j*IN_k + \mathrm{adir}_m + e_{jklm}$$

GT_j = Effect of the ith genotype of the animal with respect to the trait

IN_k = Effect of the kth coefficient of inbreeding class

GT_j*IN_k = Interaction between genotype and inbreeding class

adir_m = The random direct additive genetic effect of animal m

e_{ijklm} = The random residual

Interactions between the other components of the model were investigated but were not included in the final model since none were statistically significant.

The data within the pedigree file included 532 cheetahs from breeding records obtained from 1979 to 2006. Information supplied to the model included 33 sires, 60 dams, litter sizes, genotypes (*King*, carrier or unknown), gender, age at death per cheetah, cheetahs that died under 1 year, gastritis classes, cheetahs that died of infectious conditions, accidents, sibs per cheetah, offspring per cheetah and inbreeding coefficients.

Animal phenotypic variance was divided into additive genetic variance (V_A), partitioned using pedigree data derived from known relationship data and general environmental variance (V_{Eg}) common to all records of an individual cheetah (Falconer & Mackay 1996). Calculation of heritability was carried out as the proportion of the additive genetic variance attributable to phenotypic variance, with the following formula:

$h2 = V_A / (V_A + V_{Eg})$

3.5 Experimental design and analytical /statistical procedures: genetic linkage analysis

In small populations, the amount of disequilibrium between tightly linked markers that occurs due to random drift allows for fine mapping of major genes using a random population sample (Lynch & Walsh 1998). This is linkage disequilibrium or allelic association mapping and is useful for binary traits. The objective is to find markers that are associated with the allele by comparing the distribution of markers in individuals having the trait versus those lacking the trait. The premise is that two homologous chromosomes segregating independently allows for an allele at one locus on one chromosome to segregate together with another allele at a different locus on another chromosome with 50% probability. This indicates that in the absence of chromatid interference, the recombination fraction cannot exceed 50%. However, alleles at loci on the same chromosome co-segregate at a rate that is related to the distance between them on the chromosome. This rate is the probability or *recombination fraction* (θ), of a recombination event occurring between the two loci and is a measure of genetic linkage. The estimation of θ and the tests of the hypothesis of free recombination (H_0; $\theta = \frac{1}{2}$) versus linkage (H_1; $\theta < \frac{1}{2}$) are the primary objectives of linkage analysis (Ott 1999). Haplotypes passed from individual to offspring form the basis of recognition of recombination events and therefore requires the phenotypic information on members of family pedigrees. A linkage group of genetic markers is defined as a set of genetic markers in which each marker is linked with at least one other in the same set. Linkage analysis attempts to correlate the segregation of the trait/disease and random genetic markers with known location in the genome in selected families (Forabosco *et al.* 2005). The standard statistical approaches employed in linkage analysis are those based on likelihood techniques and interpretations of the likelihood function by maximum likelihood and Bayesian methods (Edwards 2005).

The likelihood is a measure of the probability of the observations and depends on the assumptions of the genetic model parameters and value of recombination fraction (Forabosco *et al.* 2005). The degree to which some data or observations confirm to a hypothesis can be measured using likelihood. Likelihood-based methods of drawing inference from a dataset involve treating the observed data as having risen from some random process or *model* having certain unknown aspects, called parameters. The unknown parameter's value is of interest here, and the likelihood, though defined as probability, is nevertheless used as a function of the unknown parameter values (Ott 1999). The aim of the statistical analysis is to use the data to estimate the parameters of the model and to assess the degree of uncertainty. The probability $P(D \mid \psi)$, of observing data D is described if the parameters of the model take the value ψ associated with these estimates. The likelihood $L(\psi)$ is defined to be this quantity considered as a function of ψ:

$$L(\psi) = P(D \mid \psi)$$

The common (decimal) logarithm of the likelihood ratio, *the LOD score* is used as the measure of support for linkage versus absence of linkage. For any value assumed for θ, a corresponding likelihood $L(\theta)$ can be calculated. $L^*(\theta)$ is a ratio obtained by dividing $L(\theta)$ by its value under free recombination. It is called *odds for linkage* as it indicates, for a given value of $\theta < \frac{1}{2}$, how much higher the likelihood of the data is under linkage than under absence of linkage. The logarithm (to the base 10) of the likelihood ratio is easier to manipulate than the ratio itself and is therefore used widely. The description of *LOD score* or *LOD* is therefore:

$$Z(\theta) \quad = \quad \log_{10}L^*(\theta) \quad = \quad \log_{10}[L(\theta)/L(\frac{1}{2})]$$

The Helsinki committee on methods of linkage analysis (Conneally *et al.* 1985) recommended reporting LOD scores with an accuracy of two decimal places, sufficient to represent the maximum LOD score as evidence for linkage.

Others have recommended LOD score reporting with at least three decimal places (Ott 1999). Evidence for linkage is indicated by positive LOD scores, whereas the negative scores indicate absence of linkage. In the presence of linkage the addition of more families tend to increase the LOD score (Ott 1999).

Maximum likelihood is one of the statistical methods for parameter estimation. The maximum likelihood estimate (MLE) of a parameter is a value that maximises the unknown *statistic S* (*q*). Maximisation of *S* may be carried out analytically by taking the first derivative *dS/dq*, of *S* and setting it equal to zero. In linkage analysis, MLEs are usually found numerically by varying the values of the parameters of interest and recompiling the likelihood for many trial values of the parameter until an approximate maximum is found. Linkage analysis uses techniques from the likelihood method (LM) and likelihood ratio (LR) testing. In the general likelihood ratio test, the LR criterion $T = L(H_1)/L(H_0)$ is constructed, where the likelihoods are evaluated at the MLEs of the parameter values assumed.

The *Bayesian method* using Bayes theorem is the other important statistical method for parameter estimation. It involves the calculation of 'inverse' probabilities - e.g., in two non-independent events, *E* (representing genotype) and *F* (representing phenotype). It might be easy to specify the conditional probability $P(F|E)$ and difficult to find the more useful $P(E|F)$. In this case, Bayes theorem can be used to draw an inference on an underlying genotype (given an observed phenotype) -
$P(F|E) = P(F|E) P(E) / P(F) = P(F|E) P(E) / P(F|E) P(E) + P(F|E^c) P(E^c)$, where E^c stands for the complement of *E* (Ott 1999).

While the maximum likelihood technique assumes that the recombination fraction is an unknown parameter, the Bayesian technique assumes that the recombination fraction θ (or the event that the two loci are a distance θ apart) is a random variable with a prior density function, $f(\theta)$. The prior density is then modified by the observations, *F*, leading to a posterior density, $f(\theta|F)$.

3.5.1 Brief history of linkage analysis

Sir Ronald Fisher first introduced a maximum likelihood scheme for linkage analysis of two-generation families in 1935. In the following years, Julia Bell and Haldane pioneered the analysis of multigenerational pedigrees by computing likelihood with respect to gene frequency, recombination fraction between two loci and the mutation rate at the haemophilia locus. Later, Haldane and Smith improved on the likelihood procedures and estimated the MLE of the recombination fraction between haemophilia and colour blindness (Haldane & Smith 1947). *Sequential test procedures* were first applied by Morton between two loci, with null hypothesis $\theta_0 = \frac{1}{2}$ versus fixed alternative $\theta_1 < \frac{1}{2}$ (Morton 1955). He postulated a power of 0.99 and a significance level of 0.001 and proposed to keep sampling families as long as $Z_1 < Z(\theta) < Z_0 | Z_0 = 3, Z_1 = -2$. Here, $Z(\theta)$ is Barnard's (1949) *lod* or *lod score* (Barnard 1949), $\log_{10}[L(\theta_1)/L(\theta_0)]$ summed over all families in the sample. Sampling is terminated when either of the two bounds is reached or exceeded. The value of $Z_0 = 3$ corresponds to an asymptotic p-value of 0.0001 to compensate for the rare probability of linkage between two random loci. The *recursive* method in the *Elston-Stewart (*E-S) *algorithm* (Elston & Stewart 1971) is the analysis of pedigree data by the use of maximum likelihood estimation and likelihood ratio test procedures. The approach allows for the representation of the likelihood, or lod score, for pedigrees with any

number of generations and for quantitative and qualitative data. It can be used for fast and exact calculation of pedigree likelihoods. The above parametric methods are usually applied to a small number of large pedigrees where the trait is under Mendelian inheritance (Dudbridge 2003). The E-S algorithm is implemented in LINKAGE (Lathrop *et al.* 1984) and other popular linkage analysis implementations.

There are nonparametric methods of linkage analysis, where no assumptions are required of the mode of inheritance of one of two loci. The sib pair method of Penrose (Penrose 1935) is one such technique that uses single-sibship data in a 2×2 table. The modern method of sib-pair analysis focuses on affected siblings, based on the hypothesis that a marker locus is closely linked to a disease/trait locus, so that the 'gene of interest' is transmitted to different offspring always with the same marker allele. Pairs of relatives other than siblings may be used in analysis, often relying on identity by descent (IBD (Risch 1990)). Weeks and Lange extended this method from pairs of relatives to sets of affected relatives in the affected relative member method (Weeks & Lange 1988). Haseman and Elston developed an inference method based on the *regression of the squared sib-pair trait difference on the estimated proportion of alleles*, where the trait may be quantitative or qualitative (Haseman & Elston 1972). The sib-pair method for general pedigrees is implemented in MERLIN and GENEHUNTER (with heuristic adjustments for general pedigrees) and other software. Different variations of the two methods have been developed and implemented in various statistical genetics software, enabling the calculation of likelihood of the observed pedigrees as a function of *theta* between the trait/disease and marker loci (Dudbridge 2003).

Multilocus linkage analysis (MLA) utilises more than two loci for finding linkage (Lathrop *et al.* 1984). There is a proportional increase in the number of parameters, haplotypes and genotypes considered simultaneously. In MLA, four measures of statistical support are possible, with *global support* indicating the evidence that a locus belongs to a map of loci, *interval support* measuring the evidence that the locus is in a particular interval of the map and *support for a given order* being the third measure. The fourth support measure indicates the overall evidence that a set of loci form a linkage group and is called *generalised support* or *generalised lod score*. Multipoint linkage analysis requires numerous calculations, with loops in the pedigree exacerbating the computational challenges, especially if they contain untyped individuals. An advantage of MLA is that the probability that a family is informative for linkage at one or more locus increases with the number of loci analysed. Each informative locus has the potential to generate information on the location of a new marker relative to the existing map. Valuable information is obtained if the recombination rate between the flanking markers is previously obtained and no interference assumed. It is useful to set up a genetic map of test markers of sufficient accuracy on a panel of reference families. The locus of interest can then be expressed in terms of one parameter, the location of the disease/trait locus relative to the genetic map of the selected test markers (Lathrop *et al.* 1984).

3.5.2 Testing for linkage

When the maximum of the lod score [$Z(\hat{\theta})$ or Z_{max} or ($\hat{Z}$)] exceeds a critical value of Z_0 (3 for autosomal and 2 for X-linked loci), the data conveys *significant evidence for linkage*. Lander and Kruglyak state that a lod score of 3.3 can provide a genome-wide significance level of 0.05 when multiple tests are performed to detect linkage to a specific locus within a whole genome (Lander & Kruglyak 1995). In a pedigree of size *m*, if x_i denotes the phenotype

and g_i a particular genotype of the ith pedigree member, (where phenotype and genotype may refer to several loci simultaneously), the pedigree (unconditional) likelihood is then expressed as:

$$L = P(\boldsymbol{x}) = \sum_g P(x, g) = \sum_g P(x|g)P(g),$$

where $\boldsymbol{x} = (x_1,\ldots, x_m)$ is the array of phenotypes and $\boldsymbol{g} = (g_1,\ldots, g_m)$ is an array of genotypes. The sum is taken over all sets of genotype assignments to family members. The total number of different multilocus genotypes (haplotype pairs) is equal to $H\,(H+1)/2$. Thus $P\,(\boldsymbol{x}|\boldsymbol{g}) = \Pi P\,(x_i|g_i)$, where $P\,(x_i|g_i)$ refers to an array of multilocus penetrances of the ith family member. The E-S algorithm is particularly suited for simple type of pedigrees. A path exists between two (unrelated) individuals when they are connected by an uninterrupted sequence of lines. A *loop* exists if the path can follow back to the same individual by different lines. A *consanguinity loop* exists if the loop contains at least two individuals who are blood related. In a *marriage loop*, there is no relationship but occurs when in two pairs of siblings, each sib of one pair is mated to one sib in the other pair. In a *simple pedigree* (a family in which no loops occur), where the individuals are ordered such as the parents precede the offspring, the pedigree likelihood can be represented as the telescopic sum:

$$L(\theta) = \sum \ \ P(x_1|g_1)P(g_1|\cdot)\ldots$$
$$\sum \ \ P(x_{m-1}|g_{m-1})\,P(g_{m-1}|\cdot)\sum \ \ P(x_m|g_m)\,P(g_m|\cdot)$$

$P(g_1|\cdot) =$ represents the ith offspring's genotype given the parental genotypes (involves the recombination fraction) or the probability that a founder individual (no parents in pedigree) has genotype g_i. Pedigree likelihood calculation using the E-S algorithm starts with the innermost (rightmost) sum, which is evaluated for each genotype in the next outer sum, with each summation result being 'tagged on' to the outer sum. Once the summations have been carried out for all genotypes in the outerloop, the rightmost sum is not required anymore and is therefore a recursive procedure. This method 'clips off' branches (sibships) off the pedigree one after the other, starting at the bottom and finishing at the top of the pedigree. One such round of calculations yields the pedigree likelihood for a given value of the recombination fraction. To evaluate the $L\,(\theta)$ at other θ values, the procedure is then repeated. The procedure is also usefully applied for non-loop complex pedigrees and general complex pedigrees. The likelihood has to be calculated for times equal to the number of possible genotypes of the ith individual.

In LINKAGE, an intermediate implementation of the E-S algorithm is implemented, where free-ending branches of a pedigree that are not involved in a loop are 'peeled' upward or downward by modifying the above equation and loops are handled by conditioning on an individual's genotypes. The calculations assume an absence of interference in the generation of gamete probabilities given parental genotypes (Ott 1999).

3.5.3 The De Wildt cheetah pedigree: linkage analysis

Multilocus linkage analysis was performed using LINKAGE (Lathrop *et al.* 1984), a technique that has previously been applied to demonstrate different coat colour and disease mutations in the domestic cat (Young *et al.* 2005; Cooper *et al.* 2006; Grahn *et al.* 2005; Imes *et al.* 2006; Lyons *et al.* 2005a; Lyons *et al.* 2005b; Rah *et al.* 2006). Parentage verification was done using a *Felis catus* microsatellite marker panel, developed by the International Society for Animal Genetics as a domestic cat parentage testing and identification panel (Lipinski *et al.* 2007).

PEDCHECK (O'Connell & Weeks 1998) was used to check for inconsistencies within genotyping. The existence of multiple consanguinity loops within the multigenerational pedigree caused the analysis using LINKAGE to be difficult. Loops were broken and reanalysed, however, the computation resources required were too high for successful testing, and caused repeated memory outages and operating system failures

The study therefore used a newer implementation using Bayesian networks and world-wide cluster computing implemented via the online version of SUPERLINK, which optimises and combines the E-S algorithm and Lander-Green approach to multilocus linkage analysis (Fishelson & Geiger 2002; Fishelson & Geiger 2004). SUPERLINK and SUPELINK online (Silberstein *et al.* 2006b) uses multiple pre-processing steps on the Bayesian network to reduce and trim redundant variables and achieve the best time-space trade-off given the memory available for the linkage analysis problem.

Linkage analysis was performed with the *King* phenotype modelled as a fully penetrant recessive trait. The expression of genotype *King* was assumed for the cheetah with the distinct coat phenotype, the genotype has two alleles, the condition is a binary state of *King*/no *King*. These assumptions fit all available data and observations from the pedigree. Changing the penetration model did not give any different Ln (Likelihood) trends. LOD scores were calculated with actual recessive allele observed allele frequency (0.134) from data from the De Wildt population and also with allele frequencies ranging from 0.001 to 0.5, since the real population allele frequency of the *King* phenotype is unknown but likely to be very rare. Version 1.7 of SUPERLINK that was used included a more efficient implementation of the Lander-Green algorithm capable of analysing very large (tens of thousands) number of markers and had improvements in deciding when to run the SUPERLINK-based algorithm and when to run a GENEHUNTER-based algorithm. Examples of the pedigree (.ped) and locus (.dat) input files compiled for analysis are listed in Appendix VIII, IX and X.

Within SUPERLINK, the **SuperGH program (program code 8)** was used to compute likelihood and lod-scores of the disease locus/loci against a fixed map of markers, as obtained from the third generation autosomal genetic linkage map (Menotti-Raymond *et al.* 2009b). Marker order was also varied to fit with a previous linkage and RH map order that detected *Tabby* (Lyons *et al.* 2006). Modelling the *King* phenotype as a one disease locus, SUPERLINK moves it within all intervals included in the specified region of the map and computes and outputs likelihood and lod-scores for each position of the specified locus.

The input lines specified were:
> o **for one iterated disease locus:**

1

< -n num_iter or -s step_iter> start_pos end_pos <-o off_map>
 -n 3 1 7 -o 20.0000 << where *–n num_iter* = the number of equally spaced positions for evaluations between each two successive markers OR
-s 0.5000 1 2 -o 10.0000 << where *–s step_iter* = specifies the size of the increment between each two successive positions of the 'disease' locus

start_pos and *end_pos* specify the indices of the markers that delimit the interval on the map in which the iterated 'disease' locus is moved.

off_map, together with the flag *–o* specifies a distance which extends the scan interval on both sides of the map

The **SuperLinkmap program (program code 4)** was the second option used to estimate the potential location of the *King* locus, assuming it occupies a similar space as detected in the case of the *Tabby* locus in the domestic cat (Lyons *et al.* 2006). The option computes likelihood and lod-scores of one iterated locus against a fixed map of other loci by moving the iterated locus within an exterior interval on one side of the map, or an interior interval on the marker map. This is mostly similar to the LINKMAP program of LINKAGE/FASTLINK with some changes. The interval is divided into segments of equal length according to the number of likelihood evaluations requested. The length of the interval is computed via Haldane's mapping function for interior intervals and set to 0.5 for exterior intervals. Likelihood evaluations are made by moving the iterated locus from left to right in its interval, according to increment calculated by dividing the interval's computed length into the requested number (minus one) of likelihood evaluations. According to Haldane's map function, if A and C are the flanking markers of the interior interval and B is the locus that is being moved, then the length *theta* (AC) is calculated as:

*theta (AC) = theta (AB) + theta (BC) - 2 * theta (AB) * theta (BC)*

In each iteration, *theta* (AB) is incremented by the increment calculated and *theta* (BC) is computed via the above formula keeping theta (AC) constant.

Two options were analysed, with *King* assumed to occupy a described space (Menotti-Raymond *et al.* 2009b) between marker A- FCA023 (83.9 cM) and marker C- FCA700 (108.1) and more narrowly, a space between A- FCA254 (106.7 cM) and C- FCA700 (108.1 cM) (Lyons *et al.* 2006). The second option assumed a theoretical locus equidistant from FCA254 and FCA700 with a recombination rate of 0.1 between each marker.
Therefore -

1. *theta (FCA023- FCA700) = theta (FCA023- FCA811) + theta (FCA811- FCA700) - 2 * theta (FCA023C- FCA811)* theta (FCA811- FCA700)*

From *two-point* linkage analysis performed using CRI-MAP Version 2.4 (University of California-Davis Modification) (Green *et al.* 1990), sex-averaged recombination rates was obtained for the loci used (Appendix VII). *theta (AC)*was calculated as follows:

$$= 0.22 + 0.5 - 2*0.22*0.5$$
$$=0.22 + 0.5 - .22$$
$$=0.22 + 0.28$$
$$=0.5$$

2. *theta (FCA254- FCA700) = theta (FCA254- FCAUNKNOWN) + theta (FCAUNKNOWN - FCA700) - 2 * theta (FCA254- FCAUNKNOWN)* theta (FCAUNKNOWN - FCA700)*

$$= 0.1 + 0.1 - 2*0.1*0.1$$
$$=0.1 + 0.1 - 0.02$$
$$=0.1 + 0.08$$

$$=0.18$$

Likelihood computations were performed for locations 0, 0.17, 0.34 and 0.50 for option 1 and for 0, 0.06, 0.12 and 0.18 for option 2.

The input lines specified were:

locus_varied   finishing_value   number_of_evaluations

where *locus_varied* = the index, in input order, of the iterated locus

finishing_value = the maximal distance allowed, measured in recombination fraction, between the iterated locus and the one following it in case of a left-side interval and between the iterated locus and the one preceding it, in any other interval

number_of_evaluations= the number of points for likelihood evaluations in the interval

    Option 1   5 0.6 0 0.17 0.34 0.5

    Option 2   7 0.2 0 0.6 0.12 0.18

Two point and multipoint linkage analysis were performed with great efficiency using SUPERLINK online version of the software (Silberstein *et al.* 2006b), where parallelization of computation tasks are implemented using a world-wide network of computers that utilise the idle cycles of hundreds of PCs (Silberstein *et al*. 2006a; Silberstein *et al*. 2007). An example of one multipoint analysis using all markers that would have taken 215070 minutes (approximately 150 days) on a single computer running SUPERLINK version 1.7, took 260 minutes after tasks were broken down into 14338 small pieces for parallel runs using the world-wide network. However, for multipoint analysis on the same pedigree with mode of inheritance modelled as dominant (0 – 0.2 – 0.9), calculation for eleven of the 21 possible trait positions were skipped as the computations were determined to be too difficult (Appendix XVI).

3.6   Data analysis

Data analysis on the WINDOWS[®] platform was carried out at the Veterinary Genetics Laboratory, Onderstepoort, using a Core 2 Quad Q6600 – 3.01 GHz (2x4 MB L2 Cache, 1066 MHz FSB) processor with 4 GB RAM running Windows XP[®] . The same machine provided a dual boot platform for LINUX[©]. Analysis requiring Macintosh software was run on an iMac 24" Core 2 Duo 2.4 /1x2G/250GB platform using the OS X 10.4[©] operating system. Online analysis using SUPERLINK is linked via a web front to computers in Technion, Israel and University of Wisconsin-Madison, United States.

Chapter 4:

Spatial Bayesian clustering clarifies admixture and founder origins in a captive cheetah (*Acinonyx jubatus*)

population

Abstract

Southern Africa is host to over 34 % of all captive African cheetahs (*Acinonyx jubatus*) worldwide, with conservative estimates of over 500 captive cheetahs in South Africa alone. The popular assumption of limited founder diversity in captive South African cheetahs was investigated, where a relatively small number of cheetahs assumed to be of Namibian origin is understood to have made a large contribution to the captive gene pool. A better understanding of the founder dynamics and genetic make-up within an important captive cheetah population compared to its wild conspecifics was therefore attempted. This study investigated - 1) genetic diversity and Namibian ancestry within a captive cheetah population, 2) estimated effective population size in captive, Namibian and South African populations and 3) assessed population structure using a variety of clustering methods, with the intention to put in place a method to estimate ancestry in cheetahs of unknown antecedents, given the database at hand. Our results indicate that precise population differentiation was not possible *a priori* but possible after trained Bayesian clustering. Overall, diversity levels were high, with a mean heterozygosity level of 0.64 and an average polymorphism of 6.9 alleles per locus. Significant heterozygote deficit was observed for the captive population, revealed from tests of linkage disequilibria and genetic partitioning using *F*-statistics was very little among population groups. Effective population size was accurate for the captive group but varied between 54 and 330 for wild cheetahs depending on the methods. Spatial Bayesian clustering examining wild cheetahs of known geographical origins enabled us to detect ancestry and assign population memberships to different captive groups. Clear distinction of genetic clusters were possible, and included a Namibian group, De Wildt captive and admixed group, a North West Province and Kalahari group and a Northern areas (including Limpopo Province) group. Contrary to expectations, Namibian genetic introgression into captive population was detected only in 13 % of cheetahs. The methods used have the potential to be considered in cheetah conservation for forensic and translocation-related purposes.

4.1 Introduction

The southern African region is home to over half of all wild cheetahs worldwide, with South Africa itself estimated to have around 550 free-ranging cheetahs (Purchase *et al.* 2007). Less than half of all extant South African cheetahs are in protected areas, with cheetahs found from the south-western part of the Limpopo province towards Bela-Bela, into the north-western part of Mpumalanga province, along the north-eastern part of South Africa in the Kruger National Park and the north-eastern part of Kwa-Zulu Natal province. Trapping and removal of cheetahs from farmlands outside conservation areas has resulted in population decreases throughout Namibia (Marker 2002) and South Africa (Marnewick *et al.* 2007). Approximately 34% of the world's captive population is based in southern Africa, with Namibia and South Africa housing 182 and 294 cheetahs in 21 and 16 facilities respectively (Marker *et al.* 2007). By other estimates, 44 South African facilities in nine provinces, that include 11 breeding facilities, house over 500 captive cheetahs (Marnewick *et al.* 2007). From 1996 to 2005, South African breeders exported over 428 cheetahs worldwide, with 93 % arising from captive populations. Illegal capture and trading of cheetahs from the ranches in the North West, Northern Cape and Limpopo Provinces (Marnewick *et al.* 2007) is a major concern. The captive cheetah is established to be prone to a number of conditions unique to captivity that reduce fitness, including spiral bacteria-associated lymphoplasmacytic gastritis (Eaton *et al.* 1993; Munson 1993; Munson *et al.* 1999) and systemic amyloidosis (type AA) that follows secondary to gastritis resulting in renal failure (Papendick *et al.* 1997). The long-term viability of captive populations is therefore questionable without genetic introgression from the wild.

The captive bred population in South Africa is uniquely important in that it serves as a genetic and a demographic reservoir within protected areas, affording current and future reintroduction into the wild and for reinforcing existing populations. The wild population in Namibia is considered to be the origin of the majority of all wild caught cheetahs in southern Africa (Marker *et al.* 2003), reflecting concerns that the South African captive cheetah population has excessive Namibian ancestry, is inbred and not representative of the South African wild cheetah population. This study therefore focussed on the biggest captive cheetah population in South Africa, the De Wildt Cheetah Breeding Station and Research Centre (DWCBSRC), located near Pretoria, South Africa. This is an institution that has pioneered and established novel *in situ* management practices that have led to a tremendous success in breeding cheetahs (Meltzer & Van Dyk 1998). Possibly the biggest single historical contributor to the world's captive cheetah population (Van Dyk 1998), the DWCBRC accounted for approximately 38% of the total number of mature individual cheetahs in South Africa in 2004 (Friedman & Daly 2004). Of the total captive births worldwide in 2002 (n = 118), over 28% were born in this institution alone (Marker 2004). The DWCBSRC also has the largest breeding group of *King* cheetahs, a phenotype with a coat pattern inherited as an autosomal recessive trait (Van Aarde & Van Dyk 1986) and distinct from the normal spotted phenotype (Brand 1983). On-going relocation programs are increasingly reintroducing captive-born cheetahs to suitable habitats, with varying success rates, all with the intent to establish new breeding populations (Bèga 2007; Hayward *et al.* 2007; Rogers 2007). The DWCBSRC has been instrumental in the relocation into suitable reserves of over 135 wild cheetahs that were removed from livestock and wildlife ranches in Limpopo and North West provinces (Bouwer 2008). There is, however, lack of information on the genetic structure and diversity in South African captive cheetah populations. The relative contributions of

Namibian and South African founders to extant breeding populations are an important but unknown factor, since the captive born cheetahs of Namibian lineage could be potentially be released into South Africa.

Multiple generations of captive breeding could lead to genetic adaptation to captivity (Frankham 2007), especially in the event that there has been insufficient founder contributions from rescued wild cheetahs. Errors in assumptions about relatedness between cheetahs rescued together (Marker *et al.* 2008) impacts decisions on breeding. Gene variants that are identical by descent might be contributing to certain conditions in captivity (Miller-Edge & Worley 1992; Terio *et al.* 2004; Wells *et al.* 2004), making identification of population substructure important for potential genetic association studies. The difficulties with captive management and concerns on potential inbreeding in captive cheetahs are recorded (Beekman *et al.* 1999; Hunter 1997; Merola 1994; Wielebnowski 1996). An apparent loss of genetic variability during the late Pleistocene due to population reductions (Hedrick 1996; Menotti-Raymond & O'Brien 1993) might be predisposing to infectious disease in captivity (O'Brien & Yuhki 1999). The complexity and uniqueness of diseases in captive cheetahs (Munson *et al.* 2005) highlight the challenges facing cheetah breeders. Cheetahs of Namibian origin have been reported to make up the majority of founders within extant South African captive populations, where over a period from 1970 to 1996, 71% of 244 cheetahs rescued from the wild to South Africa were of Namibian descent (Marker *et al.* 2003; Marker 2002). This mirrors concern over the limited founder diversity in captive cheetahs of North America, where cheetahs of Namibian origin have produced 63% of all cubs born in captivity (Marker 1998). Cheetahs of Namibian origin, therefore, appear to have contributed to founder diversity in South African captive populations and worldwide. This study aimed at understanding the founder dynamics and genetic make-up within an important captive population that potentially has extensive Namibian ancestry, is experiencing genetic drift and has a small effective population size as compared to wild Namibian cheetahs.

4.2 Methods

4.2 1   Sample collection, laboratory analysis and validation of data

Captive cheetahs were sampled from DWCBSRC (N = 152) in South Africa. Namibian cheetahs (N = 51) were sampled from Otjiwarongo, Tsumeb, Otavi, Outjo, Omaruru, Okahandja, Windhoek, Dordabis, Gobabis and Grootfontein were sampled by the *Africat Foundation*, which is 50 km south of Otjiwarongo in Namibia. Additional wild cheetahs (N = 71) were from the Kalahari area in Botswana, and Kuruman, Tosca, Bray, Vergelee, Madikwe, Dwaalboom, Thabazimbi, Ellisras, Potgietersrus, Bela-Bela (Warmbaths), Messina, Alldays, and Phalaborwa in South Africa (Figure 4.1). These cheetahs were opportunistically sampled by the conservation team at the National Cheetah Management Program. Samples were collected by approved personnel and according to set guideline and protocols for the different institutions involved. DNA was extracted from whole blood, tissue, and serum using the phenol-chloroform method (Sambrook *et al.* 1989). A panel of 13 domestic cat (*Felis catus*) microsatellites (FCA069, FCA678, FCA649, FCA453, FCA229, FCA075, FCA097, FCA220, FCA224, FCA310, FCA105, FCA149 and FCA293) were selected after testing for amplification and polymorphism on a test run that included 15 random cheetah samples. Selection of markers were decided in consultation with other members of the *Genetics subcommittee* of the *National Cheetah Conservation Forum* of South Africa. The amplification conditions of these markers and primer profiles are described previously (Lipinski *et al.* 2007; Menotti-Raymond *et al.* 1999; Menotti-

Raymond *et al.* 2003b). Following polymerase chain reaction (PCR) amplification by multiplexing the loci in sets of three to five, products were analysed and fragments separated on an ABI 3130 XL DNA analyzer and results visualised using *STRand* (Toonen & Hughes 2001). A selection of random samples (N = 35) were re-extracted and amplified to confirm typing quality. Amplification and consistency of PCR products were checked using *Microsatellite Toolkit* (Park 2001). Estimates of the frequency of null alleles were determined by the maximum likelihood implemented in *ML-NullFreq* (Kalinowski & Taper 2006) and by an iterative algorithm based on the observed and expected frequencies of homozygotes implemented in *Cervus* 3 (Kalinowski *et al.* 2007). Finally, marker performance was tested on a complete three generation captive pedigree (N = 89) to verify allele calls and check for non-amplification.
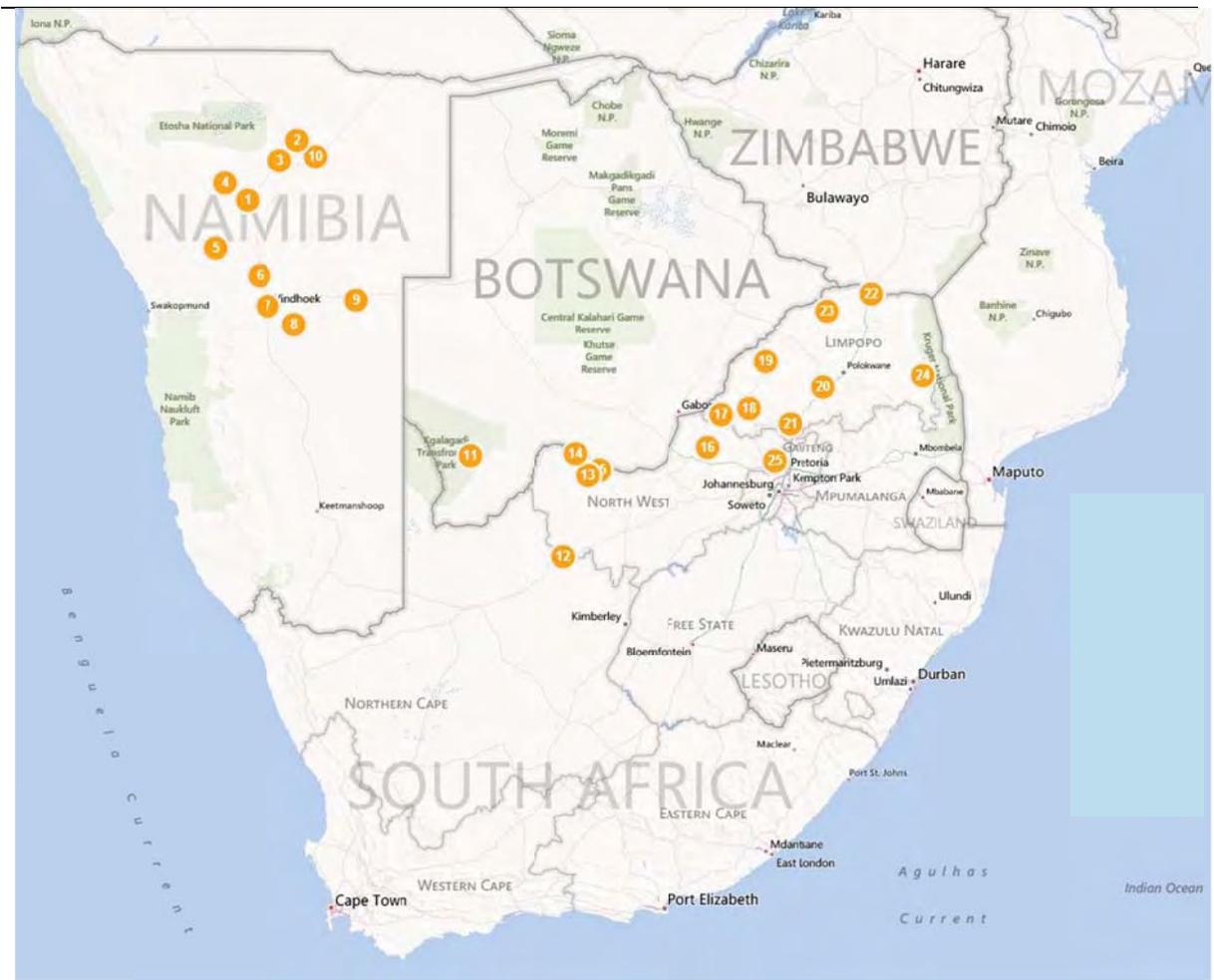
Figure 4.1. Map of Southern Africa showing the areas from where cheetahs were sampled: Namibian cheetahs were sampled extensively from Tsumeb in the North to Dordabis in South (n = 51). The rest of wild samples (N = 71) originated from Kalahari (11) in Botswana, and the areas listed below in South Africa. The captive cheetahs (n = 152) were sampled from DWCBSRC (25 -De Wildt).

1. Otjiwarongo, Namibia
2. Tsumeb, Namibia
3. Otavi, Namibia
4. Outjo, Namibia
5. Omaruru, Namibia
6. Okahanja, Namibia
7. Windhoek, Namibia
8. Dordabis, Namibia
9. Gobabis, Namibia
10. Grootfontein, Namibia
11. Kalahari, Botswana
12. Kuruman, South Africa
13. Tosca, South Africa
14. Bray, South Africa
15. Vergelee, South Africa
16. Madikwe, South Africa
17. Dwaalboom, South Africa
18. Thabazimbi, South Africa
19. Ellisras, Limpopo, South Africa
20. Potgietersrus, Limpopo, South Africa
21. Bela-Bela, South Africa
22. Messina, South Africa
23. Alldays, South Africa
24. Phalaborwa, South Africa
25. De Wildt, South Africa

4.2.2   Genetic diversity and population differentiation

The cheetahs could only be initially classified into Namibian wild origin, South African wild-caught and captive born cheetahs. Combining all available geographical reference data from the sampled cheetahs (Namibian and South African) and historical data on the origins of the captive cheetahs (recorded genealogy) suggested classifying cheetahs into Namibian born, De Wildt Captive (including the *King* pedigree), and two other groups, one broadly representing the North West Province (including cheetahs of Kalahari origin) and another representing Northern areas (including cheetahs of Limpopo origin). This *ad hoc* grouping was supported by known genealogy, locations of NCMP sampled wild cheetahs and extant wild population data in South Africa (Friedman & Daly 2004). Standard indices of genetic diversity, including unbiased heterozygosity (Nei 1978), the effective number of alleles (Hartl & Clark 1997) and counts of allele numbers were calculated from microsatellite genotypes overall, for each population and individual animal. Allelic richness per loci and population were preferred because of the unequal sample sizes among populations and were analysed using *FSTAT 2.9.3.2* (Petit *et al.* 1998). The differences between observed and expected heterozygosities between captive and wild cheetah groupings, where significant differences between the values are due to an excess of observed homozygotes (Cote *et al.* 2002), were tested using paired t-tests. The known groupings were tested for deviations from Hardy–Weinberg equilibrium (HWE) for each locus, using the Markov chain method (Guo & Thompson 1992) implemented in *Genepop 4* (Rousset 2008). Deviations from genotypic linkage equilibrium for all pairwise locus combinations were tested using an exact probability test, with an adjusted P value of 0.000641 corresponding to alpha = 0.05 after Bonferroni correction (Rice 1989). Genetic differentiation between the four *ad hoc* groupings were assessed using F_{ST} and R_{ST} (Michalakis & Excoffier 1996), implemented as Analysis of Molecular Variance (AMOVA) in *Arlequin 3.11* (Excoffier *et al.* 2005). An unbiased estimate of *F*-statistics (Weir & Cockerham 1984; Wright 1951) was used to partition the total genetic variation into different subdivisions, which were tested for overall levels of differentiation with significance levels that were tested with 10000 permutations. A comparison of internal relatedness (Amos *et al.* 2001) and homozygosity by loci (Aparicio *et al.* 2006) was done for all population groupings detected.

A multivariate ordination of pairwise differentiation statistics (F_{ST}) was done using principal co-ordinates analysis (PCA) implemented in *GenAlEx 6* (Peakall & Smouse 2006). The PCA plots the major patterns that make up the data set with multiple loci and uses the AMOVA distance matrix for plotting the parameters into a scatterplot of the first two coordinates. Visualising pairwise differentiation (F_{ST}) between cheetahs was done with *Pca-gen 1.* (Goudet 1999), with 10000 randomizations of genotypes to test for significance of axes. Population differentiation was also visualised using the principle component analysis implemented in *Genetix 4.04* (Belkhir 2000), where a Factorial Correspondence Analysis of individual genetic marker scores is used to identify population clusters graphically. The gross placement of individuals to *a priori* identified population groups is possible, but identification of admixed animals is not possible as it cannot assign probabilistically individuals to clusters (Pierpaoli *et al.* 2003). The pairwise genetic distances among all individuals was detected using the proportion of shared alleles (*Dps*) distance estimator with the (*1-M*) correction in *Microsat 1.5e* (Minch *et al.* 1996) Bootstrap values for 1000 bootstrap replicates in *Microsat 1.5e* were calculated with the *Consense* subroutine in *Phylip 3.6b* (Felsenstein 2001). A phylogenetic tree was drawn using the *Neighbour* option of the *Phylip 3.6b* and visualised using the program *Treeview 1.6* (Page 1996).

4.2.3   Effective population size

Estimates were obtained using the single sample linkage disequilibrium (LD) method that utilised LD at unlinked gene loci but corrected for a bias when using diallelic loci (England *et al.* 2006). Single sample estimates for N_e were obtained using *LDNE* (Waples & Do 2008) using the combined cheetah dataset (Namibian and South African born cheetahs), and the Namibian wild and South African wild and captive (excluding the *King* lineage) dataset separately. The jackknife confidence intervals were taken to contain the true N_e (Waples & Do 2008). A second estimate using an approximate Bayesian computation (Tallmon *et al.* 2004) estimate N_e from a single dataset was also used. Estimated mean N_e and confidence intervals were calculated using *OneSamp* (Tallmon *et al.* 2008) on the basis of the priors on N_e, from 50,000 simulated populations reproducing for two to eight generations following the Wright-Fisher island population model. The priors on N_e for the combined populations were set between two and 10,000, and for Namibian and South African datasets between two and 5,000. A higher upper prior was preferred since the biologically probable and historical N_e is a best guess for southern African cheetahs. Choosing a more conservative estimate is advocated than a slightly inflated value in the instance that the user guessed upper prior on N_e is lower than the actual (Tallmon *et al.* 2008).

4.2.4   Bayesian analysis

Bayesian clustering is better suited for analysing complex genetic data by detecting subtle signals of linkage and Hardy–Weinberg disequilibrium to group individuals into genetically distinct clusters (Beaumont & Rannala 2004; Corander *et al.* 2003; Latch *et al.* 2006; Vaha & Primmer 2006). *Structure 2* (Falush *et al.* 2007; Pritchard *et al.* 2000) was initially used using default values for a total of 10 replicate unsupervised runs for each value of the number of clusters (K) from one to 10. A pilot analysis indicated that a ''burn-in'' of 100,000 Markov Chain Monte Carlo (MCMC) Gibbs sampler iterations, and 1,000,000 follow-on MCMC iterations provided consistent estimates of the prior (Pr $(X|K)$. The model used assumed admixture with a uniform prior on the degree of admixture and alpha (initial value=1.0, max=10.0, SD=0.025). Allele frequencies were set to be correlated among subpopulations (prior mean=0.01, prior SD=0.05, k=1.0) with default *lamda* values, a setting that performs better for subtle population structure (Falush *et al.* 2003). An additional 10 replicate runs were performed with an admixture model that had a calibrated prior on the degree of admixture (*alpha*) based on the initial results (initial value=0.2, max=10.0, SD=0.025). For estimating the correct likelihood value for likelihood runs where plateau formation is a concern, we averaged membership coefficients over several analyses (Chen *et al.* 2007a). From 10 replicate runs where levelling of likelihoods was detected, the symmetric similarity coefficient (SSC) was computed using the software *Clumpp* (Jakobsson & Rosenberg 2007). SSC was estimated as the mean over all individual Q-matrices after the columns have been aligned according to the *Greedy* algorithm (1000 repeats) with the greatest *H'* value. The population *Q*-matrix file with cluster membership coefficients generated from *Clumpp* for the *K* values was visualised using *Distruct* (Rosenberg 2004). After plotting the averages across 10 runs, (Pr $(X|K)$ estimated by *Structure* was plotted for plateau formation for the real *K* value (Pritchard *et al.* 2007; Pritchard *et al.* 2000) and a determinable peak at a single *K* identified. To derive the best estimate for K, the measure Δ*K (Evanno et al. 2005)*, derived as the second order rate of change of ln[Pr $(X | K)$] with respect to *K* was obtained by the following calculation on a spread sheet:

$\Delta K = mean\ (|L\ (K + 1) - 2\ L\ (K) + L\ (K - 1)|)/SD[L\ (K)]$

where $L = \text{Pr}\ (X\ |\ K)$ and SD = standard deviation

In instances where low levels of within population genetic variation ($F_{ST} = 0.02 - 0.03$) are obtained, a test for significant differences in allele frequencies, as implemented in *Baps 3.2* (Corander *et al.* 2003) is recommended (Latch *et al.* 2006). The method tests for the optimum number of genetically diverged groups with allele frequencies of the molecular markers being unknown parameters that are the end estimates of the analysis. Ten independent runs were done for K values from 2 – 15, to determine if the results change noticeably, by comparing their corresponding "*logmls*" (Corander *et al.* 2003). Results from the initial clustering of cheetahs were used to perform an admixture analysis using 1000 iterations and analysing 50 reference individuals per population with 20 simulations. The inferred K from MCMC runs were analysed for admixture proportions (*q* values) for each cheetah, to define group membership. As described by Latch *et al.* (2006), *Baps* was used to detect values of $q > 0.5$ for individual cheetahs and considered to be a correct designation of the true cluster of origin. The estimated admixture coefficient for a cheetah in each cluster was used as a measure of correct assignment. The number of cheetahs with significant admixture was determined by selecting cheetahs having *p*-values larger than 0.05 (Corander *et al.* 2003).

A third Bayesian clustering technique, based on a hierarchical mixture model where the prior distribution of population groupings are categorised as a Hidden Markov Random Field (HMRF) on a spatial individual network (Chen *et al.* 2007a) was applied. The use of spatial coordinates with Bayesian clustering is better in clarifying the presence of structuring by the incorporation of prior information about spatial structure, especially with limited number of loci (Francois *et al.* 2006) and even in instances when sampled populations do not overlap spatially (Chen *et al.* 2007a). Where capture locations were available without GPS co-ordinates, the spatial data was obtained by triangulating the area on Google Earth® (Google Inc 2007) and co-ordinates logged by converting latitude and longitudes from 'point of capture/sampling' or 'place of trapping' into Universal Transverse Mercator coordinates using *UTMS* (National Geographic Society 2002).

The admixture model was used in *TESS* 1.1 (Chen *et al.* 2007b) for a total of 100 runs with a burn-in period of length 10,000 followed by 50,000 iterations for the entire dataset. K_{max} was set at different values for 10 runs each, starting from 5 to 15 and for increasing spatial autocorrelation parameter ψ at intervals of 0.2 from 0.2 to 1. The output was evaluated for the estimate generated for the preferred value of potential clusters K, noting that the estimated K should be less than or equal to the user specified maximum value K_{max} (Chen *et al.* 2007b). The value of K_{max} is considered to be not sufficiently high if the estimated K equals K_{max}. The analysis was done for 100 replicates of the dataset, with an initial 10 runs done using settings of no admixture and $\psi = 1$ and no spatial coordinates. Subsequent iterations (n = 90 runs) had incremental increases in ψ as described and included spatial data. Outputs from *Structure* and *Tess* were analysed and used to manually generate cheetah clusters and reanalysed using *Baps* to clarify admixture and detect membership at $q > 0.5$. The method has been shown to be informative on the fine scale admixture patterns when complex populations are analysed (Carmichael *et al.* 2007) and helps detect spatial population genetic structure with clinal variation (Rosenberg *et al.* 2006).

4.3   Results

4.3.1   Genetic variation and population differentiation

For all cheetahs (N = 274), the mean number of alleles per locus was 6.92, mean expected heterozygosity was 0.642 and the mean polymorphic information content was 0.598 (Table 4.1). There were 10 and 11 private alleles within the Namibian and South African populations respectively, giving a combined non-exclusion probability (first parent = 0.012; second parent = 0.0004), indicating that this marker panel is informative for ancestry analysis. The number of alleles and expected heterozygosity per population group after verification of origin is listed in Table 4.2.

Table. 4 1: Mean parameters of genetic diversity for 13 microsatellite loci in cheetahs

No	Locus[1]	H_E	SD	Allele No	SD	Allele_Size[2]	SD	F_{IS} (Namibian)	F_{IS} (South African)
1	FCA069	0.65	0.09	4.25	0.50	3.75	0.50	0.02	0.05
2	FCA678	0.04	0.06	1.75	0.50	5.25	5.50	0.00	0.17
3	FCA649	0.75	0.05	5.75	0.50	4.75	0.50	-0.10	-0.04
4	FCA453	0.32	0.11	2.5	0.58	2	0.00	-0.11	0.07
5	FCA229	0.63	0.04	4.5	1.29	3.75	1.26	-0.24	-0.08
6	FCA075	0.75	0.08	8	1.41	8.25	0.96	-0.12	0.05
7	FCA097	0.67	0.11	5.75	1.71	5.5	2.08	-0.17	-0.17
8	FCA220	0.77	0.04	6.75	0.96	6	0.82	0.04	0.06
9	FCA224	0.72	0.03	5	0.82	5	0.82	0.00	0.06
10	FCA310	0.67	0.10	6	0.82	7	0.82	-0.02	0.07
11	FCA105	0.65	0.05	5.5	1.73	5.75	1.50	0.13	0.06
12	FCA149	0.60	0.12	5.75	2.36	11.25	1.50	-0.02	0.08
13	FCA293	0.63	0.05	4.5	0.58	3.75	0.96	0.17	-0.05

Parameters of genetic diversity for 13 microsatellite loci in cheetahs. FCA678 was monomorphic in Namibian cheetahs and excluded in subsequent analysis. [2]Allele size implies the number of repeat units identified within the marker. H_E = Expected heterozygosity, F_{IS} = within-population inbreeding coefficient

Table. 4 2: Number of alleles and expected heterozygosity per population group

No	Locus[1]	*King* Pedigree		De Wildt Core		North-West-Kalahari, Northern		Namibian	
		N	H_E	N	H_E	N	H_E	N	H_E
1	FCA069	4	0.71	5	0.55	5	0.72	5	0.69
2	FCA678	2	0.12	2	0.02	2	0.05	1	0.00
3	FCA649	6	0.71	5	0.72	6	0.76	6	0.78
4	FCA453	2	0.23	3	0.36	3	0.33	2	0.46
5	FCA229	3	0.65	5	0.65	4	0.68	6	0.55
6	FCA075	7	0.67	7	0.77	9	0.80	9	0.83
7	FCA097	4	0.56	7	0.72	6	0.68	8	0.79
8	FCA220	6	0.72	6	0.79	8	0.82	5	0.75
9	FCA224	5	0.69	5	0.72	5	0.71	6	0.72
10	FCA310	6	0.55	6	0.68	7	0.78	6	0.71
11	FCA105	5	0.60	4	0.61	5	0.75	8	0.69
12	FCA149	4	0.56	4	0.44	7	0.72	7	0.68
13	FCA293	4	0.58	4	0.60	5	0.65	6	0.66

Parameters of genetic diversity for 13 microsatellite loci in cheetahs per population group. [1]FCA678 was monomorphic in Namibian cheetahs and excluded in subsequent analysis. N = Number of alleles, H_E = Expected heterozygosity

Paired t-tests testing differences between observed and expected heterozygosities revealed significant heterozygote deficit for the captive population versus Namibian wild cheetahs (t = 3.429, 12 df, P = 0.005). Nei's unbiased estimator for gene diversity (Nei 1978) was significantly different (P = 0.002) between DWCBSRC and wild Namibian cheetahs. Global Hardy-Weinberg tests revealed significant heterozygote deficits at 4 loci (FCA649, FCA229, FCA075 and FCA105; $P < 0.05$, Bonferroni-corrected). The South African cheetah grouping had positive average F_{IS} values (F_{IS} = 0.014; $P < 0.05$) whereas the Namibian group had negative values (F_{IS} = -0.031; $P < 0.05$). One microsatellite locus (FCA678), with significantly high null allele frequency by both maximum likelihood and the iterative method (threshold = 0.05 or more) was monomorphic within the Namibian population and excluded from further comparative analysis.

Analysis of Molecular Variance between the four *ad hoc* known cheetah groupings indicated that microsatellite variability was partitioned poorly among these four groups (θ_{ST} = 0.074; R_{ST} = 0.101; P = 0.001), revealing little genetic differentiation among the four populations (8%). The majority of variance (92%) exists within the populations. PCA using AMOVA revealed a lack of clarity in population differentiation, reflecting the AMOVA results where maximal differentiation was within populations (Figure 4.2). After spatial Bayesian clustering and reordering membership, however, PCA using pairwise differentiation between cheetahs had greater usefulness (Figure 4.3). Clear distinction between the groups using factorial correspondence analysis was possible after reordering membership based on Bayesian spatial clustering (Figure 4.4). The phylogenetic tree constructed from the *Dps* distance matrix did not generate an informative tree (Appendix II).
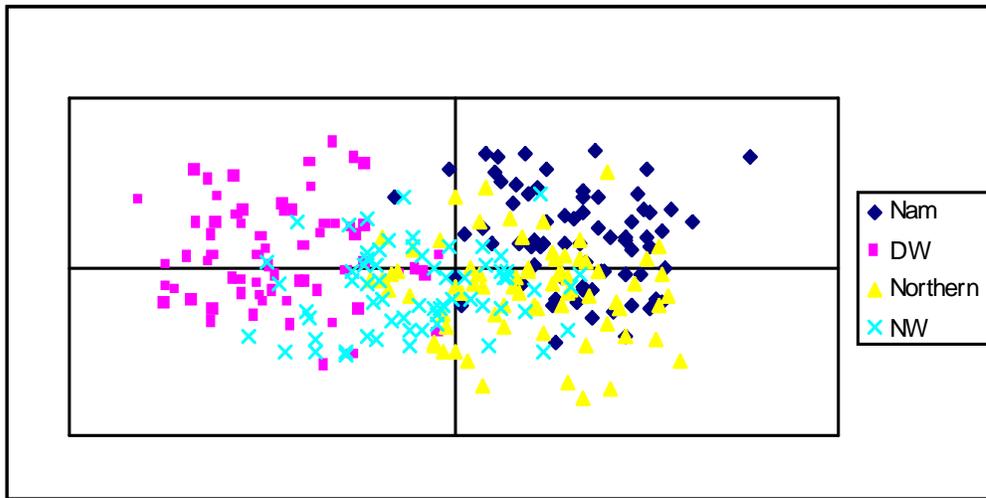
Figure 4.2: Principal Coordinates plotted based on AMOVA distance matrix: Nam = Namibian born and wild caught; DW = De Wildt captive born including the *King* pedigree and admixed; Northern = Cheetahs samples from the Northern areas and Limpopo and NW = North West Province (including cheetahs of Kalahari origin)
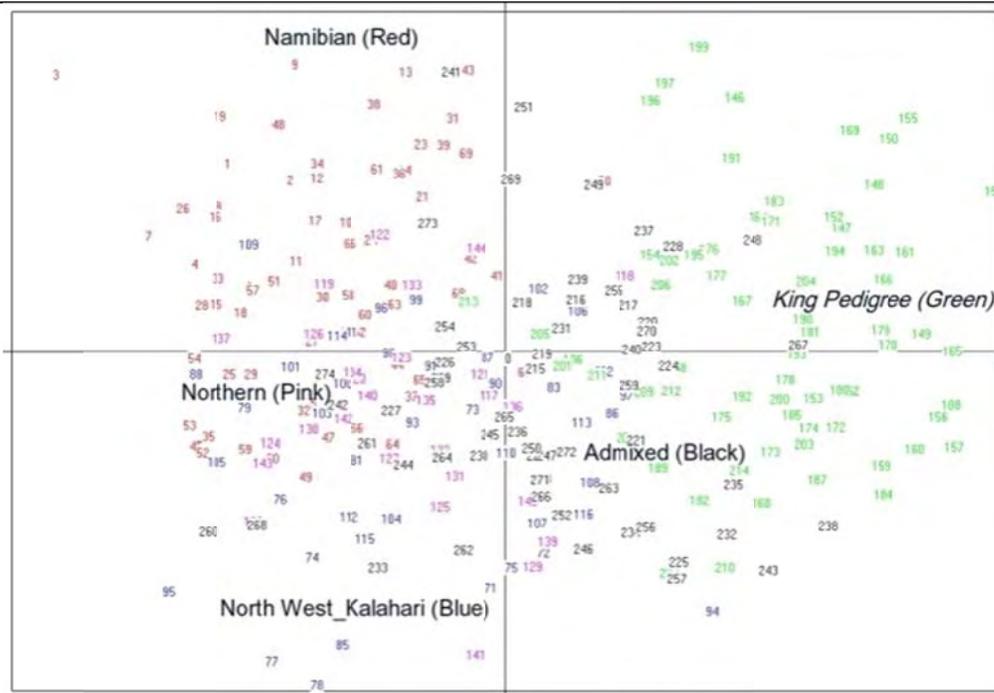
Figure 4.3: A principal component analysis visualising pairwise differentiation among cheetahs into Namibian, Northern, North West/Kalahari, Admixed and *King* using the software PCA-GEN (Goudet 1999).
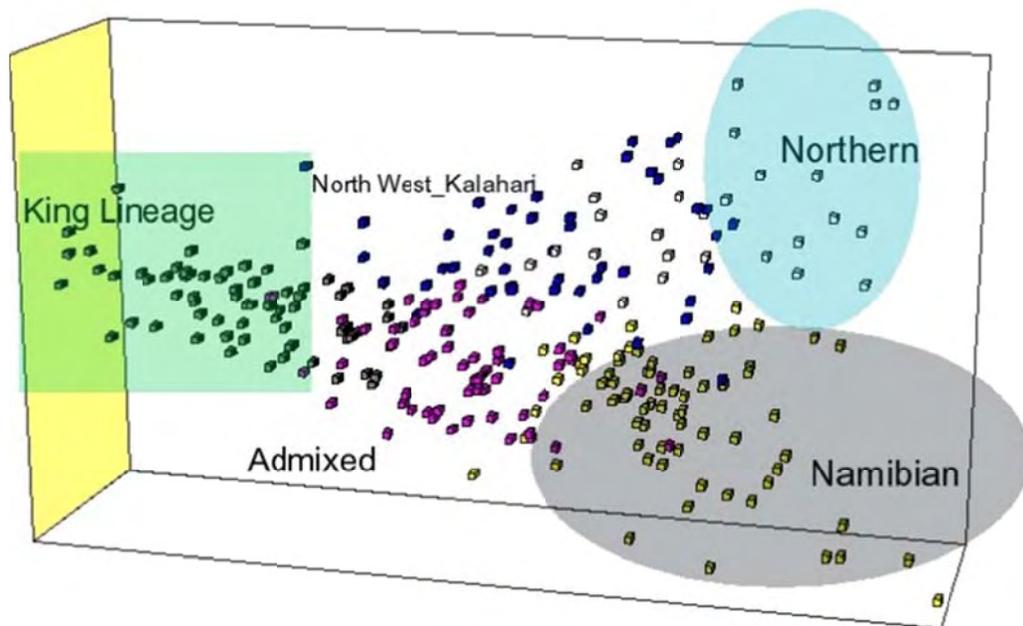
Figure 4.4: Factorial correspondence analysis showing multivariate relationships among microsatellite genotypes of cheetahs - Namibian = Namibian born and wild caught; *King* Lineage = De Wildt captive born within the *King* pedigree; Northern = Cheetahs sampled from the Northern areas and Limpopo Province and North West_Kalahari = Cheetahs sampled from the North West Province (including cheetahs of Kalahari origin)

Significant deviations from linkage equilibrium were detected for 22 locus pair combinations out of a possible 80 locus pairs. However, after Bonferroni corrections, only one locus pair (FCA649-FCA224) remained highly significant. Investigations were carried out to further elucidate the reason for this linkage. Within the parentage-verified three generation pedigree data, Mendelian inheritance patterns for the two loci were checked for anomalies. Evidence for physical linkage was checked using CRI-MAP version 2.4 (Green *et al.* 1990), and the LOD score was found to be not significant for FCA649-FCA224. Of the 22 locus pairs with significant LD that was checked in CRI-MAP, physical linkage was detected only between one pair (FCA097-FCA149; LOD = 3.23) within the three-generation pedigree. All other locus pairs exhibited non-significant LODs, indicating that the loci are physically spaced wide enough to allow independent recombination. Removal of either FCA097 or FCA149 from the analysis did not affect our results.

The high levels of LD were also reflected in our results from the LD-based single sample estimator of N_e (Waples & Do 2008), revealing accurate founder estimates for captive born cheetahs (estimate based on lowest allele frequency = 0.05) (Table 4.3). The DWCBSRC cheetahs had N_e = 27.4 (Jackknife 95% CIs between 22.2 and 33.8), comparing very favourably with the number of founders in the captive population (n = 32). Estimates from the single sample based Bayesian algorithm (Tallmon *et al.* 2008) indicated that the LD based method generated comparably conservative values for the non-captive populations. The South African population that included captive born, had an estimated N_e of 40 (Jackknife 95% CIs between 32.9- 48.5) by the LD method, whereas the estimated mean N_e from the Bayesian approximation was 329.73 (95% CL from the posterior distribution of N_e between 155.25 - 997.06).

The Namibian cheetahs (n = 70), had a higher N_e of 91.2 (Jackknife 95% CIs between 57.3 - 181.1) by the LD method, and 66.34 (95% CL between 53.2 - 122.8) by the Bayesian approximation.

Table. 4 3: Estimates of effective population sizes for different cheetah groups

Lowest Allele Frequency Used	No. of Independent alleles.	Overall Burrows' Delta	Ne	95% CIs for Ne Jackknife on Loci	
Population - southern African Cheetahs (including *King* Lineage)					
0.05000 (CI)	1050	0.01164	**40**	**32.9**	**48.5**
0.02000 (CI)	1482	0.01033	48.3	40.6	57.5
0.01000 (CI)	1871	0.00947	56	47.4	66.3
Population - Namibian Wild Cheetahs					
0.05000 (CI)	1045	0.01858	**91.2**	**57.3**	**181.1**
0.02000 (CI)	1470	0.01883	84.8	57.3	143.6
0.01000 (CI)	1682	0.01927	75.8	50.5	130.8
Population - South African Captive					
0.05000 (CI)	1006	0.01737	**27.4**	**22.2**	**33.8**
0.02000 (CI)	1191	0.01646	30.1	24.8	36.5
0.01000 (CI)	1435	0.01486	36	30.4	42.8
Population - South African Wild Cheetahs					
0.05000 (CI)	1040	0.03355	**39.3**	**24.8**	**73.2**
0.02000 (CI)	1327	0.03153	53	33.5	101.5
0.01000 (CI)	1377	0.03162	52.1	34.3	92.8

Estimates of effective population sizes for different cheetah groups: output generated using the single sample estimator detecting LD, *LDNE* (Waples and Chi, 2007) Version 1.31. The 0.05 level of allele frequency estimate and its 95% CI jackknife on loci are in bold.

4.3.2   Bayesian clustering

After the initial *Structure* analysis, levelling of likelihoods in 10 replicate runs were detected for K=3, 4, 5 and 6. The *Q*-matrix file with cluster membership coefficients for *K = 3 to 6* was visualised (Figure 4.5, A-D). After plotting the averages across 10 runs, (Pr (*X|K*) in *Structure* reached a plateau for K values between 4 and 9, with a peak at K=4 (Figure 4.6.A). The second order rate of change of ln[Pr (*X | K*)] with respect to *K (∆K)* peaked at K = 2, with a second smaller peak at K = 4 (Figure 4.6.B). The estimates obtained from unsupervised *Baps* runs were substantially different, with the best estimate of population clustering at K = 11 (Figure 4.5.E). Admixture analysis in 274 cheetahs using *Baps* revealed 11 cheetahs with admixture levels that were significant (Bayesian *p* value of 0.05). Ten of these cheetahs were verified to be F1 progenies from matings between distinct geographical areas. A reanalysis of the dataset in *Baps* was done after reordering clusters using information from *Structure* and *Tess* outputs (Carmichael *et al.* 2007) resulted in obtaining fine-scale admixtures for each individual (Figure 4.7.A). After addition of known spatial coordinates and trained clustering (10 reference individuals per population/500 iterations), a clear distinction of groups within the captive population was obtained and K = 4 was supported in all the trained

clustering results (Figure 4.7.B). In *Tess* 1.1, the estimated cluster value (K) was always higher than the user input K_{max}.for settings from K = 2 – 30. Once admixture was not set, biologically sensible K_{max} values were generated that were larger than the estimated K (K > 4). Of the 100 replicates, all supported an inference of K values between 4 and 7, with maximum support for K = 6 (92%; average log-likelihood = -8370; *P* = 0.0001) (Figure 4.7.C). Individual assignments for Namibian cheetahs, generated after adding spatial co-ordinates (Figure 4.8), indicated cluster membership to be maximal at K = 2, where the one grouping was determined to be a sibling group. The genetic makeup for the DWCBSRC captive population was estimated based on percentage of membership to a geographic area of origin and the Namibian introgression was estimated to be 13%. (Figure 4.9). The detected ancestry within DWCBRC reflected known breeding and genealogy in the majority of cheetahs, and therefore could be differentiated into three major geographic areas (individual *q* > 0.5), one distinct pedigree and a distinct admixed group (individual *q* < 0.5). Further analysis of the genealogy of the admixed group that formed 27% of the total population reflected the founder group's ancestry to areas that were the former Eastern Transvaal. The North West and Northern areas have an ancestry representation of 16 and 7% respectively. The *King* lineage at DWCBSRC, comprised of cheetahs exhibiting the true phenotype, carriers of the autosomal recessive gene, their progeny and close relatives, make up 37% of the total captive group.

Namibian
Cluster

Northern/L
impopo

North West
&
Kalahari
Cluster

De Wildt
Captive &
King
Cheetahs

Namibian wild
cheetahs and few
cheetahs sampled in
the North West area

DWCBSRC cheetahs
with wild cheetahs
from Northern areas
& Namibia

DWCBSRC cheetahs
with wild cheetahs
from North West &
Kalahari area

DWCBSRC cheetahs
King Lineage & wild
cheetahs from
Northern area

A. *K = 3* **B.** *K = 4* **C.** *K = 5* **D.** *K = 6* **E.** *K = 11*

Figure 4.5: Initial clustering results from multiple unsupervised iterations from *Structure* (K = 3 to 6) and *Baps* (K = 11) analysis from 20 unsupervised runs. Admixture coefficients were calculated using the *Greedy* algorithm in *Clumpp* (1000 iterations) and visualised using *Distruct*.

59

A

B
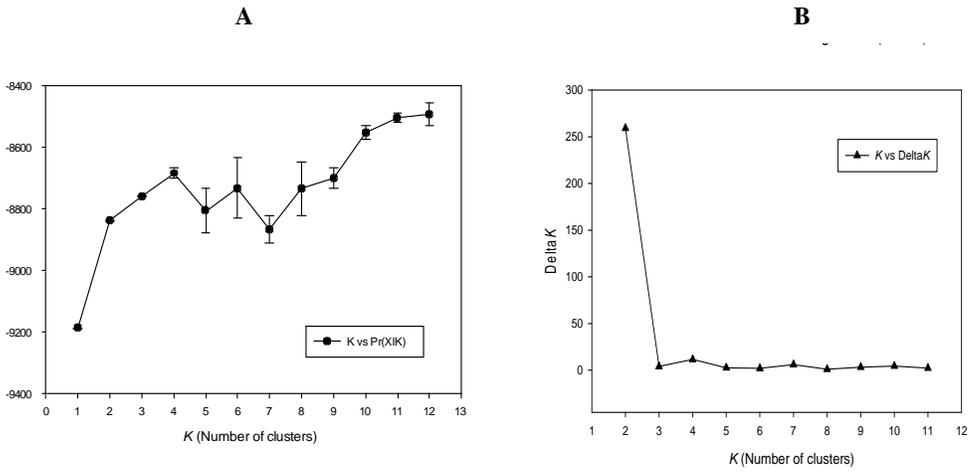
Figure 4.6: The posterior probability of the data given a particular value of K and the second order rate of change for K. A - The posterior probability of the data given a particular value of K (Pr ($X|K$) averaged over ten runs (±SD). Here K = 4 is the best estimate. B - The second order rate of change for K (ΔK) for the complete dataset, indicating a maximum at K=2.

A

Namibian origin   /Okahandja/--North West-Kalahari-Northern/ De Wildt *King* Lineage-——De Wildt Core (admixed)

B

Namibian origin   /Okahandja /North West-Kalahari-Northern/ De Wildt *King* Lineage / De Wildt Core (admixed)

C

Namibian origin   / Okahandja / North-West/Kalahari/Northern / De Wildt *King* Lineage / De Wildt Core (admixed)
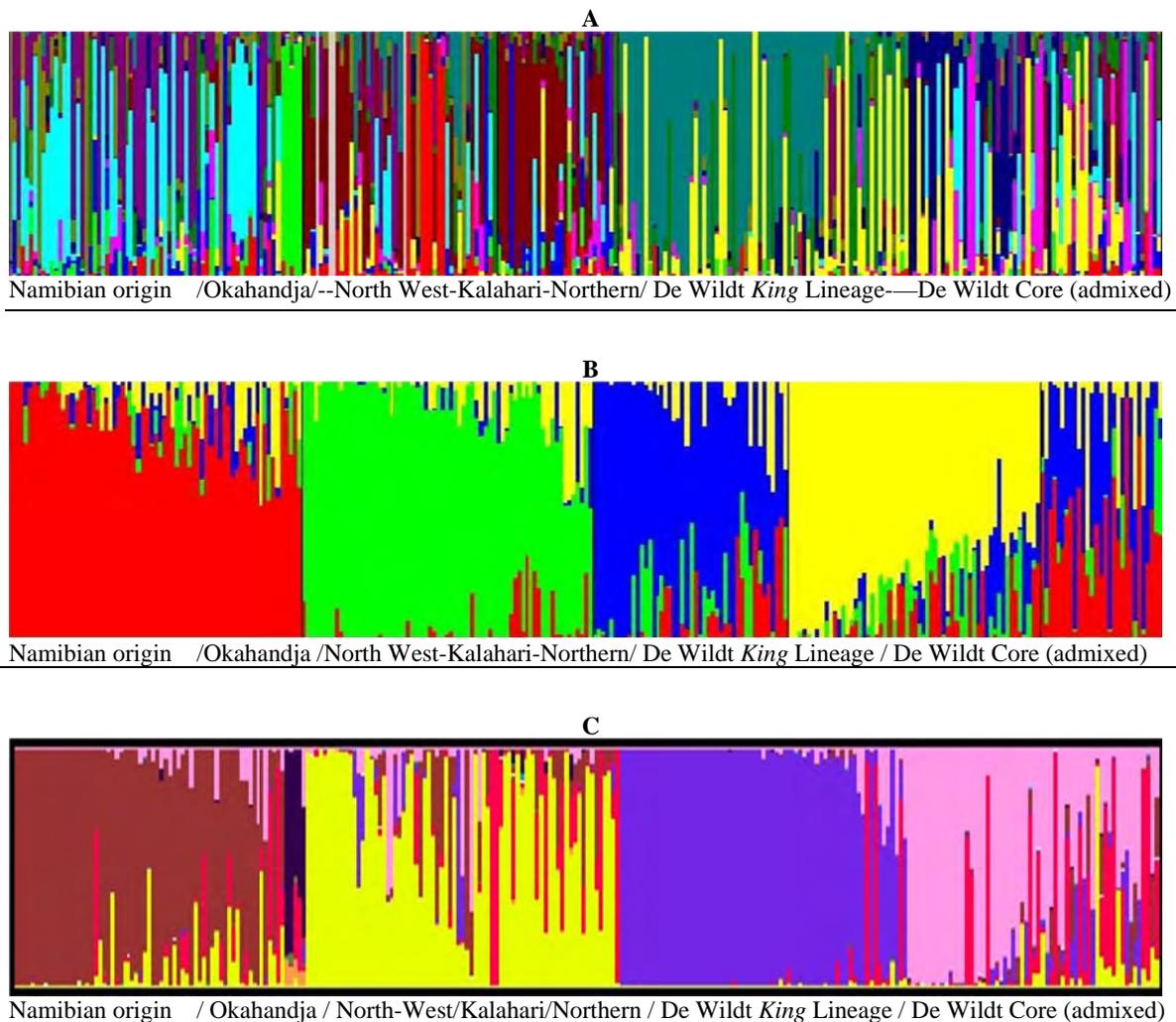
Figure 4.7: *Baps* results before and after addition of spatial co-ordinates vs. *Tess* results

A - *Baps*: prior to addition to spatial co-ordinates. Fine scale resolution is possible for individual cheetahs at this stage, with the full power of ancestry markers being visible.

B. *Baps*: after addition of spatial co-ordinates. *Baps* analysis after reordering samples according to cluster membership suggested by *Structure* and *Tess*. After addition of known spatial coordinates and trained clustering, (10 reference individuals per population/500 iterations), there is a clear distinction of groups within the captive population. K = 4 is supported in all the trained clustering results.

C - *Tess* analysis from 30 runs at K=6. *Tess* analysis from 30 runs at K=6, admixture coefficients calculated using the *Greedy* algorithm in *Clumpp* (1000 iterations) and visualised using *Distruct*.

A   B
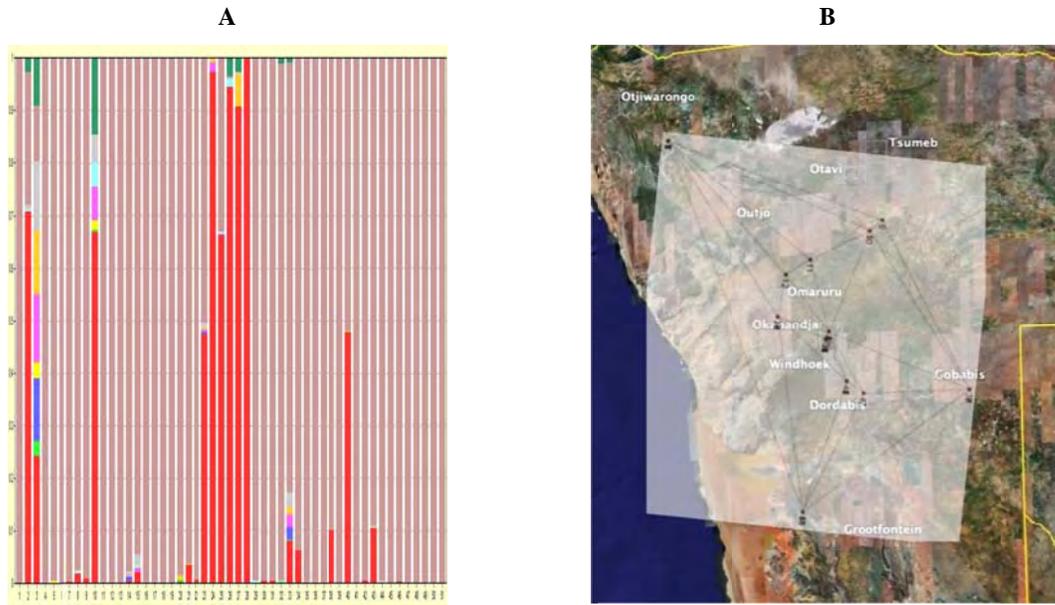
Figure 4.8: Namibian cheetah's individual assignment probabilities and neighbourhood diagram overlaid over Namibia

A - Individual assignment probabilities for 51 Namibian cheetahs at an average log likelihood of -1678.7 (K=2 from 77/100 runs). The red cluster in the middle is from a family group sampled from Okahandja.

B - Neighbourhood diagram from spatial co-ordinates overlaid over the map of Namibia representing 51 Namibian cheetahs across a 2 dimensional scale.
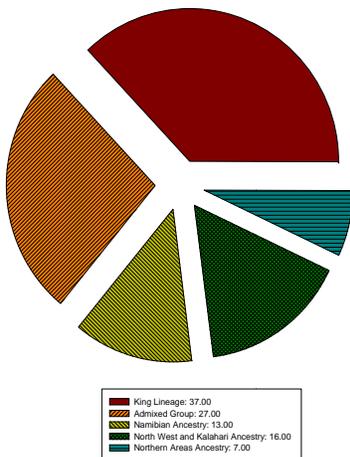
Figure 4.9: Ancestry in percentages within the DWCBRC captive population depicted as percentages and areas of origin. Detected ancestry within the captive population that reflected genetic origins within South Africa and Namibia: Apart from the *King* lineage and the admixed group, 13, 16 and 7% of the captive group have origins placing them within Namibian, North West and Kalahari and Northern Areas respectively.

4.4   Discussion

The lack of comparative genetic data on captive and wild South African cheetahs is beginning to impact current cheetah relocation programs in South Africa. Three distinct cheetah groupings in southern Africa that included the DWCBSRC cheetahs, free-ranging South African cheetahs and their Namibian counterparts were compared in this study. We estimated genetic diversity, effective population size, assessed population structure using a variety of clustering methods and estimated Namibian ancestry within an important captive cheetah population. Diversity levels were high overall, with significant heterozygote deficit observed for a section of the captive population. High levels of LD within the captive group enabled accurate N_e determination for the captive group but had wide margins for wild cheetahs depending on the method used. Distinct groups were detected within cheetahs that reflected genealogy and breeding records, with accurate assignment of animals aided by spatial Bayesian clustering and input of geographic coordinates from wild cohorts. Namibian founder contribution to this specific captive population was lower than expected.

Genetic diversity levels reported for the cheetah have progressed from allozyme variability (O'Brien *et al.* 1985; O'Brien *et al.* 1983) to recent comparisons on the levels of genetic variation among geographic regions in the Namibia, where mean expected heterozygosities ranged from 0.64 to 0.70 in 89 unrelated cheetahs (Marker *et al.* 2008). A study on 147 Serengeti cheetahs reported observed heterozygosity for 13 loci to vary from 0.51 to 0.84 (Gottelli *et al.* 2007) and from 0.46 to 0.52 (Luo *et al.* 2004). Diversity levels within South African cheetahs were comparable to those recently reported for Namibian cheetahs (Marker *et al.* 2008) and southern African cheetah (Charruau *et al*. 2011). The captive group that included the *King* lineage, however, had significantly lower unbiased diversity values compared with Namibian cheetahs. The significant differences between observed (H_O) and expected (H_E) heterozygosities for loci within the captive population (P = 0.005), indicating substantial heterozygote deficiency was expected, as the population includes members that are carriers of a recessive phenotype. On excluding the *King* lineage, high levels of admixture were detected within the captive population, making it difficult to differentiate cheetahs *a priori* into groups. However, ordering of historical mating records and manual classification of origins based on historical institutional records reflected results from Bayesian clustering in most cases where mating history was known. Membership coefficients to a cluster were confirmed after the members of the captive group clustered closely (q > 0.5) with wild cheetah samples that had known geographical co-ordinates.

Deviations from Hardy-Weinberg proportions are to be expected in the event that cheetahs sampled are not from a single breeding population but are in fact derived from geographically distinct and reproductively isolated breeding populations (Hartl & Clark 1997). In this study, all attempts to differentiate between cheetah populations using Wright's F-statistics proved difficult and results to be of little use, as *a priori* differentiation was not possible. The differences between F_{ST} and R_{ST} values indicate that allele size differences are more evident between the four groups than allele types. High levels of gene flow might have caused significant admixture and could be the reason why strong geographical partitioning was not detected. An exhaustive sampling of wild populations of South African cheetahs might be more informative and might be a useful mandate for future studies.

Physical linkage was detected between FCA097 and FCA149 within the three-generation pedigree. Within the domestic cat chromosome B1, FCA097 and FCA149 map closely at positions 1476.2 and 1573.3 respectively (Menotti-Raymond *et al.* 2003b; Murphy *et al.* 2007). Such verifications are impossible for most wild-origin genetic data as multi-generational pedigree information and genetic sampling are usually unobtainable. This is the first report of effective population size for southern African cheetahs derived from genetic markers. Earlier N_e estimates have been based on traditional methods (Cat Specialist Group; Kelly 2001; Marker *et al.* 2007) that consider population size estimates, sex ratios and estimates of breeding males and females. The bias-free method of estimating effective population size that is based the Burrows method to calculate Δ (a measure of linkage disequilibrium) implemented in *LDNE* (Waples & Do 2008) was highly accurate in the captive population. The high levels of LD between loci within relatively closed pedigrees seem to make this option better suited for generating estimates. The estimated mean N_e from South Africa of 330 cheetahs obtained using the Bayesian approximation was lower than expected based on estimated minimum adult population for South Africa (n = 550) reported (Purchase *et al.* 2007). However, it reflects Friedman and Daly's (2004) census estimate of 300 free roaming cheetahs on ranches in the Limpopo and North West provinces. Namibian cheetahs had N_e confidence intervals by both methods estimated to be between 50 and 180 animals, lower than expected based on an estimated minimum population of 2000 wild cheetahs in Namibia (Purchase *et al.* 2007). This might be reflecting the high levels of gene flow within Namibian cheetahs reflecting capture and removal (Marker *et al.* 2008) that lead to lower LD and downwardly bias estimates. Single sample-based estimators might not accurately reflect the genetic behaviour of natural populations, exhibiting asymmetrical gene flow and having unequal (sub)population sizes (Palstra & Ruzzante 2008).

Detection of informative population substructure was not possible by using the second order rate of change for K (Δ*K*) method (Evanno *et al.* 2005), as only gross clustering between Namibian and South African cheetahs (K = 2) was supported. This might be because the method performs poorly in instances where genetic differentiations between populations are weak (Waples & Gaggiotti 2006) and when F_{ST} is less than 0.05 (Latch *et al.* 2006). Therefore, in inbred populations where high levels of LD are suspected, Evanno's method generates superficial gross differentiation and performs poorly. This is supported by reports of Δ*K*=2 from population differentiation attempted in poodles (Bjõrnerfeldt *et al.* 2008) and plains zebras (Lorenzen *et al.* 2008). The algorithm implemented in *Baps* has been shown to estimate more genetically distinct groups than *Structure* (Rowe & Beebee 2007), generating complex admixture-informative groups in our study. Additions of spatial co-ordinates improved clustering, offering a biologically meaningful clustering solution, and clarified ancestry within the captive population. The Namibian cohort did not separate beyond K = 2, indicating significant gene flow within this group. The large home ranges and consistent removal of breeders in this group might make this a feature within wild Namibian cheetahs. Cautious use of BAPS is advised in certain scenarios where population differentiation (F_{ST}) decreases to 0.01 or lower, and the number of putative populations may be high (Orozco-Terwengel *et al.* 2011).

This study focused on an important and prolific captive population that has derived its gene pool of cheetahs from southern African founders. Bayesian clustering was the only useful method to detect partitions within this group, and only when used along with wild cheetah samples from southern Africa of known geographical origins. Charruau *et al*. (2011) used BAPS but the partitioning within southern African cheetahs did not correlate with the geographical origins of cheetahs. In this study, the captive population proved to be quite admixed, excepting for the *King* lineage

which was distinct. This was an expected result, considering that the lineage is inherently managed to carry an autosomal recessive trait within its pedigree. The degree of population differentiation (F_{ST}) was significant only between this group of cheetahs and Namibian wild cheetahs. With respect to Namibian cheetahs, our results were concordant with Marker *et al.* (2008) where no clear population clusters could be inferred. The concern over illegal and undisclosed transportation of cheetahs into South Africa is founded in reality (Marker 2006), with reports indicating that over 60 cheetahs are traded illegally every year (Marnewick *et al.* 2007). The numbers of wild cheetahs ending up illegally in unregulated South African breeding centres is on the rise (Purchase *et al.* 2007). This study has already raised questions on the origin of few of the 'native' cheetahs that were sampled from a private reserve, based on their clustering with over 95 % certainty with the Namibian cohort.

The lack of representation from free roaming cheetahs ranging within Kruger National Park, estimated to be over 100, might be of significance to this study. The *King* phenotype has been recorded for many decades in the area around eastern and south-eastern Zimbabwe, the north-eastern region of former Transvaal and eastern Botswana (Rogers 1997). The addition of samples from this area, including the Kruger National Park, could be helpful in understanding the founder origins of this specific breeding group at DWCBSRC. Our results demonstrate that DWCBSRC cheetahs distinctly cluster into groups that reflect ancestry and historical breeding records and have taken place over three decades. The *King* lineage forms 37% of the total captive group and includes nested half-sib families, as indicated by the significantly different molecular co-ancestry values (P = < 0.001) calculated by the *FT* method using a simulated annealing algorithm with 4 generations and known genealogy (Fernandez & Toro 2006) (Appendix III). The lineage has characteristics that are different from others in captivity, explained in detail in chapter 5, and can be considered as an example of dominance in captivity of rare alleles that are recessive in the wild (Frankham 2007). Contrary to expectations on Namibian introgression into captive populations only 13 % of cheetahs in the DWCBSRC captive population had evidence of Namibian ancestry.

The clarification of hidden ancestry has allowed us to revisit some of the assumed and unknown ancestral origins of few captive born cheetahs. A current breeding male, recorded in the stud book as originating from Swaziland, is now known to closely cluster with others of known Namibian origin ($Q = 0.93$). Two adolescent males rescued from adjacent traps and assumed to be siblings in a male coalition were detected clustering to distinctly separate groups, and could be shown using likelihood-based methods with high significance to be from different families. The breeding implications for such rescued males are that both males might now have the potential to enter into a breeding program on relocation. A more comprehensive sampling of wild cheetahs, over the entirety of its range will help to identify barriers to gene flow and/or migration corridors among cheetahs and help discern the severity of increasing habitat fragmentation in South Africa. The obvious pitfall with an individual sampling scheme and inference development in landscape genetics is that there has to be wide-spread random sampling across the entire study area, and not from *a priori* predefined populations (Manel *et al.* 2003). The number of cheetahs in captivity in South Africa will soon exceed the wild population. The management of fragmented cheetah populations as a metapopulation (Marnewick *et al.* 2007) can be improved by knowledge of their area of origin. The accuracy with which wild-sampled cheetahs can be placed into a spatial genetic cluster will aid assigning unknown cheetahs that enter the captive program as rescued animals into broad geographic areas. Release programs can be tailored to maximise diversity and can include a knowledge-based program for relocation of cubs born of the cheetahs

translocated as part of the program. Current and active re-introduction programs will also benefit in knowing the genetic antecedents of a rescued/trapped cheetah, for legal, forensic and conservation-related reasons.

Chapter 5

Genetic parameters and inbreeding estimates for fitness traits in a captive cheetah (*Acinonyx jubatus*) population

Abstract

The captive cheetah (*Acinonyx jubatus*) is prone to a number of complex pathologies not reported from free-ranging conspecifics. Within a shared captive environment, disease is notable for unexplainable individual variation, indicating complex interactions between host immune system and varying environmental factors that contribute to stress in captivity. The relationship between inbreeding and development of pathology and susceptibility to infection was investigated in an extensive pedigree of 532 cheetahs. Inbreeding coefficients of cheetahs within the entire pedigree ranged from 0 to 0.37 (mean = 0.037, S.D ± 0.0687). The four inbreeding classes (unknown/wild founder, 0-0.09, 0.1-0.19, 0.2 and above) contained 42, 443, 32 and 15 animals, respectively. The development of gastritis was significantly associated with inbreeding levels, whether the diagnosis was made by histopathology ($p = 0.01$) or post-mortem ($p = 0.038$). If the cheetah was a carrier or expressed an autosomal recessive coat colour trait, it was more prone to develop gastritis (r = 0.067, F = 5.08; $p = 0.0074$). Susceptibility to infectious conditions (r = 0 0098; F = 0.47; $p = 0.705$) and to a novel haemoprotozoan parasite of the *Babesia* spp (r = 0.038; F = 1.39; $p = 0.256$) were not significantly correlated to inbreeding. Mortality due to trauma or accidents were also not significantly associated to inbreeding. The pedigree was used to estimate the heritability for litter size (0.55) and the maternal heritability for cub mortality (0.59). The results indicate that female cheetahs can potentially be improved by selection. With approximately 44 South African captive cheetah facilities that include 11 breeding facilities and housing in excess of 500 cheetahs nationwide, there is a growing awareness on the importance of careful selection. The condition gastritis was poorly heritable (0.07), confirming the complex nature of this condition, where individual adaptability to stress is not dependent on any major heritable genetic component. The results indicate that the development of complex conditions in captivity, especially gastritis, is influenced by the general effects of inbreeding depression, but is poorly heritable. The lack of significant association between infectious conditions, susceptibility to a blood-borne parasite and inbreeding levels indicate that the environment in captivity plays a bigger role in the pathogenesis of disease than genetics.

5.1 Introduction

The African cheetah (*Acinonyx jubatus*) in South Africa is close to being classified as endangered in the wild based on the low numbers of breeding adults (Friedman & Daly 2004; Marnewick *et al.* 2007). The species is increasingly being maintained in captivity and bred in captive breeding programs countrywide, with over 11 declared South African breeding facilities currently housing over 500 captive cheetahs (Marnewick *et al.* 2007). The species *Acinonyx* is suspected to have gone through a severe genetic bottleneck (Hedrick 1996; Menotti-Raymond & O'Brien 1993), reducing the overall population size to few hundreds and therefore potentially reducing the adaptive potential of the population.

The established breeding populations in South Africa and North America are generally formed from very limited founder contributions and are small in size (Marker & O'Brien 1989; Marker *et al.* 2007; Marker 1998). Smaller captive populations that are not intensively managed, rapidly lose genetic diversity and might express deleterious recessive alleles, experience allele loss due to drift and exhibit lowered heterozygosity (Frankham 2007). The eventual fixation of deleterious mutations in such populations is suspected to lead to a subsequent reduction in adaptability and evolutionary potential (Armbruster & Reed 2005; Bowland *et al.* 2001; Higgins & Lynch 2001; Jimenez *et al.* 1994; Lacy 1997; Ralls *et al.* 1988; Wright 1978). Higher levels of inbreeding in captive populations have been negatively correlated with birth weight, survival, reproduction and resistance to disease, predation and environmental stress (Soule 1986; Thornhill 1993). In addition, inbred populations are reported to be susceptible to novel infectious diseases (Acevedo-Whitehouse *et al.* 2003; Armstrong & Cassey 2007; Keller & Waller 2002) with significantly higher levels of morbidity (Reid *et al.* 2003; Ross-Gillespie *et al.* 2007; Valsecchi *et al.* 2004).

The cheetah is a non-confrontational predator with extensive home ranges (Caro 1994; Marker *et al.* 2003; Marnewick & Cilliers 2006). Captivity is suspected to trigger an acute and later progressively chronic stress response, demonstrated by significantly higher baseline corticoid concentrations (Terio *et al.* 2004; Wielebnowski *et al.* 2002) and adrenal cortical hyperplasia (Munson 1993; Munson *et al.* 1999) that is significantly larger in captive cheetahs (Terio *et al.* 2004). Similar observations have been made in rats (Ulrich-Lai *et al.* 2006). Measurement of cortisol levels post-movement shows remarkable variability for the individual cheetah (Wells *et al.* 2004). The heritability of basal plasma cortisol levels in humans is reported to be up to 62% (Bartels *et al.* 2003), with the stress response to stimulation also being significantly heritable (Kirschbaum *et al.* 1992). Rats and mice have distinctly different strain–dependent corticosterone responses to stress, where genetic lineage appears to play a significant role in the determination of susceptibility to stress (Jones *et al.* 1998; Marissal-Arvy *et al.* 1999; Redei *et al.* 1994). The role of environmental habituation that contributes towards a 'stress-free' state is potentially limited (Terio *et al.* 2004), pointing more towards an individualistic response to stress stimuli that depends on the animal's disposition (Wielebnowski *et al.* 2002). Such variability in baseline plasma cortisol concentrations have been described in humans as well (Huizenga *et al.* 1998; Van *et al.* 1996).

Captive cheetahs are prone to diseases that have not been reported in free-ranging conspecifics, including spiral bacteria-associated lymphoplasmacytic gastritis (Eaton *et al.* 1993; Munson 1993; Munson *et al.* 1999) and systemic amyloidosis (type AA) that follows secondary to gastritis resulting in renal failure (Papendick *et al.* 1997). Prospective (Munson 1993) and retrospective (Munson *et al.* 1999) surveys have documented the high prevalence of

Helicobacter-associated chronic gastritis in captive cheetahs worldwide, where unknown host factors are suspected to play a role in the pathogenesis of this condition (Terio *et al.* 2005). In contrast, free ranging cheetahs with high levels of *Helicobacter* colonisation on gastric mucosa have been demonstrably free of clinical gastritis (Munson *et al.* 2005). The lack of evidence directly linking *Helicobacter* colonization and gastritis in captive cheetahs suggests an immuno-modulatory effect of glucocorticoids, potentially altering local and systemic immune response to commensal flora in the gastric mucosa (Citino & Munson 2005; Terio *et al.* 2005). The differences noted between individual cheetahs in the pathogenesis of gastritis are suspected to be due to the animal's genetic makeup or temperament (Citino & Munson 2005). Complex interactions between host alleles that are predisposing to gastric disease and environment variations that define pathogenesis might be critical, especially where host genetic makeup influences the local mucosal immune response to antigens present in normal gastric commensals (Bleich & Mahler 2005).

Susceptibility genes have been described to blood-borne pathogens in mouse models (Lipoldova & Demant 2006) and a genetic basis for susceptibility to infections in humans is reported (Clementi & Gianantonio 2005). A potentially novel tick-borne intracellular erythrocytic haemoprotozoan parasite of mammals, closely related to *Babesia felis* (Bosman *et al.* 2005), appears to be endemic in captive and wild cheetahs (Bosman *et al.* 2007). Currently, there is no published evidence to suggest a role played by this pathogen clinically or subclinically in the pathogenesis of captivity-induced conditions.

This study hypothesised that the susceptibility to developing gastritis and by inference, maladaptation to captivity in the African cheetah might be heritable. The rapid increase in breeding institutions is occurring against a background where there is no published information on pedigree-based genetic parameters and heritability estimates for life-history traits in captive cheetahs. The potential for improvement for any trait is dependent on its heritability (Lynch & Walsh 1998) and current cheetah breeding programs have the potential for improved selection methods. Litter size heritability and cub mortality as an indicator of mothering ability was therefore investigated. Sires are currently selected from a group that are chosen for superior semen quality and prior breeding success (Bertschinger *et al.* 2008). Data on breeding, mortality and clinic-pathological investigations, including postmortem, histopathology and restriction fragment length analysis of *Babesia* data were used to determine the effect of inbreeding on the reported pathology.

The aim of the study was to estimate the heritability for litter size, mothering ability and gastritis, and to estimate the effects of level of inbreeding on these three traits.

5.2   Materials and Methods

5.2.1   Study site

The De Wildt Cheetah Breeding Station and Research Centre in the Republic of South Africa, established in 1971, has pioneered the establishment of novel management practices for the successful breeding of captive cheetahs (Meltzer 1998; Meltzer & Van Dyk 1998; Van Dyk 1998). The centre has a breeding group of a coat colour variant

of the normal spotted cheetah in captivity, the *King* phenotype (Brand 1983; Meltzer 1998). Pedigree and breeding data for 532 progeny from 33 sires and 60 dams from 1971 to 2007 were analysed for this study.

5.2.2   Pedigree information and inbreeding estimation

The pedigree was recreated using PEDDRAW (ver 3.0) (http://www.pedigree-draw.com/index.html) and JENTI (Falchi & Fuchsberger 2008). The entire pedigree is attached as appendix IV. Wright's coefficient of inbreeding (Wright 1922) was calculated over six generations of the pedigree using FSpeed2 (www.tenset.co.uk). For the purposes of statistical analysis, four inbreeding classes were defined, including unknown/wild founder, 0 - 0.09, 0.1 - 0.19 and 0.2 and above. Cheetahs from wild founders belonging to the first generation or F1 (n = 42) were not assigned an inbreeding value and were included in the class wild/outbred. Founders that did not contribute to the pedigree were not included in any inbreeding classes and were excluded from this study.

5.2.3   Gastritis diagnosis and classes

Histological scoring, based on lymphocyte and plasma cell infiltration of the gastric mucosa, was based on the grading system previously developed (Munson 1993). The scoring system had four classes, with grades 0 (no infiltration present), 1 (mild, multifocal or widespread infiltration), 2 (moderate widespread or severe multifocal infiltration) and 3 (severe, widespread with epithelial necrosis rarely noted), indicating increasing levels of gastritis. All examinations were done by a one South African veterinary board-certified specialist pathologist (EL). The methodology involved obtaining at least 3 gastric biopsies each, from the gastric cardia, fundus and pylorus at each sampling period. An average score was allocated for each anatomical site, and the highest of these three scores was taken as an overall gastritis score for each sampling period. Only the first biopsy sample scores for a cheetah were included in this study, ensuring that there is no confounding caused by subsequent treatment protocols that were part of another study (Lane *et al.* 2004). Animals with a biopsy score of 2 and above were included in the class 'affected with gastritis in order to eliminate the possibility of a grade 1 score not being representative of the cheetah's overall gastric health. A separate class of gastritis-affected animals was obtained from post-mortem records, where specific gross lesions including mild thickening of gastric rugae, a mucosa with a cobblestone appearance and increased tendency to bleed following endoscopic biopsy were taken as indicative of gastric inflammation.

5.2.4   Infectious conditions, natural death and cub mortality

Cheetahs that were diagnosed with infectious conditions of specific bacterial or fungal etiology, or had died of uterine infection, colibacillosis, feline panleukopenia, meningitis or were euthanised as a result of cancerous growths, were included in the class: *infections and cancer*. A second category *Babesia+* included cheetahs that tested positive using a reverse line blot hybridization (RLBH) assay using a probe specific to the 18S rRNA gene sequences of intracellular erythrocytic haemoprotozoan *Babesia felis* (Bosman *et al.* 2007). The positives indicate presence of the antigen in the blood at the point of sampling and were analysed separately and in combination with class *infections and cancer* for subsequent analysis. The category *trauma and natural* included animals that died of old age or after trauma due to accidents or fights with conspecifics. The category *cub mortality* included records of new-

born cubs that died within 365 days after birth, determined from records of each female. Cubs that died of infectious disease and trauma, as determined by a post-mortem examination but were not diagnostic were excluded.

5.2.5 Genotypic classes

Three genotypic classes were constructed based on expression of the autosomal recessive *King* trait and were full phenotype, known carriers and unknown.

5.2.6 Statistical analysis

Initial estimates of inbreeding class differences were carried out using Kruskal-Wallis One Way Analysis of Variance on Ranks (K-W ANOVA) when comparing more than two classes and the Mann-Whitney rank sum test for two classes. The primary fixed effect investigated for significance was Wright's coefficient of inbreeding. The effect of inbreeding on gastritis, infectious conditions and other causes was tested using the GLM procedure of Statistical Analysis System (SAS), version 9.1.3 (SAS Inc., 2005; Cary, NC) and to interrogate significant explanatory variables. Analysis of variance was done to investigate the influence of the afore-mentioned parameters and their two-way interactions on the prevalence of conditions. A least-squares approach was used, to determine the fixed effects in the model using the stepwise exclusion procedure. A univariate linear mixed effects model was used to estimate additive genetic effects. The ASREML2 package (Gilmour *et al.* 2006) was used to estimate the (co)variances. The models used were as follows:

$$Y_{ijkl} = GT_j + IN_k + GT_j*IN_k + \text{adir}_m + e_{jklm}$$

GT_j = Effect of the ith genotype of the animal with respect to the trait
IN_k = Effect of the kth coefficient of inbreeding class
GT_j*IN_k = Interaction between genotype and inbreeding class
adir_m = The random direct additive genetic effect of animal m
e_{ijklm} = The random residual

Interactions between the other components of the model were investigated but were not included in the final model since none were statistically significant. Phenotypic variance was divided into additive genetic variance (V_A), partitioned using pedigree data derived from known relationship data and general environmental variance (V_{Eg}) common to all records of an individual cheetah (Falconer & Mackay 1996). Calculation of heritability was carried out as the proportion of the additive genetic variance attributable to phenotypic variance, with the following formula:
$h2 = V_A / (V_A + V_{Eg})$

5.3   Results

5.3.1   Inbreeding classes and differences detected

The cheetahs analysed in the dataset had coefficients of inbreeding, ranging from 0 to 0.37 (mean ± S.D.: 0.037 ± 0.0687). The four predefined inbreeding classes contained 42, 443, 32, 15 individuals, .respectively. Proportions of gastritis and infectious condition-affected cheetahs, differentiated based on inbreeding classes are listed in Figures 5.1-3. The absolute numbers of positively scored biopsy slides for gastritis and cheetahs tested positive for *Babesia*, differentiated based on inbreeding classes are depicted in Figure 5.4 and Figure 5.5, respectively. Within the post-mortem diagnosed with gastric inflammation at post-mortem, the K-W ANOVA detected no significant differences between the inbreeding classes (H = 4.596, 3 DF, *P* (exact)= 0.210). In the combined histology and gross gastric inflammation positives, the K-W ANOVA test revealed significant differences between the inbreeding classes (H = 6.167, 3 DF; P (exact)= 0.038). Similar differences were noted in the combined class of gastritis and *Babesia* positives (H = 6.667, 3 DF P (exact)= 0.010). Proportions of animals that died under one year are depicted in Figure 5.6. Litter size was lower for the class with highest inbreeding (Figure 5.7) but was not significant (K-W ANOVA; P = 0.211). There were no significant differences between the litter sizes of cheetahs that were known carriers (mean = 3.125; SD ± 1.708), the *King* phenotype (mean = 3.30; SD ± 1.28) and assumed non-carriers (mean = 3.58; SD ± 1.08). Wright's inbreeding coefficients between the *King* gene carriers and cheetahs expressing the trait (n = 91; mean = 0.0225) were not significantly different from that of assumed non-carriers (n = 441; mean = 0.023; Mann-Whitney rank sum test, *P* = 0.989) A larger proportion of the known carriers of the recessive gene were diagnosed with gastric inflammation at post-mortem when compared to the unknown genotype (25.23 vs. 17.23 %), but this difference was not statistically significant. The majority of cheetahs (83.27 %) had low coefficients of inbreeding and were within the category 0 – 0.09.
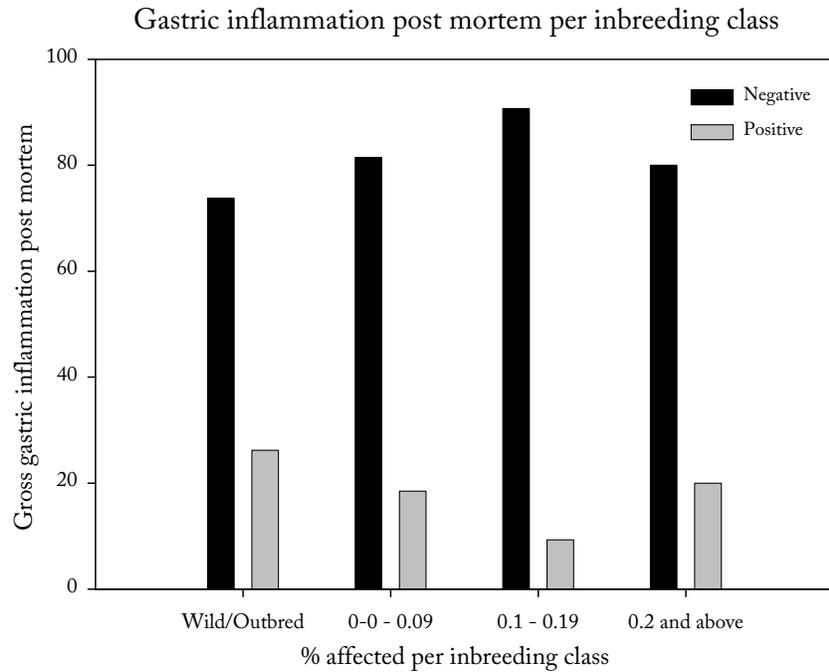
Figure 5.1: Proportions of affected cheetahs based on inbreeding classes: gastric inflammation described on post mortem examination. Comparison of gross gastritis diagnosed post mortem (Y axis) and percentage positive or negative per inbreeding classes on X axis.
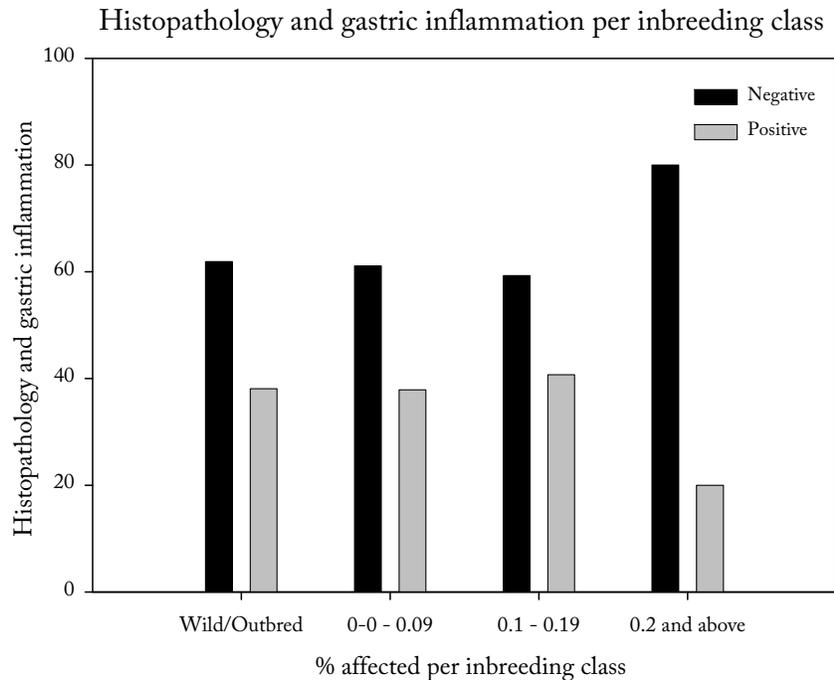
Figure 5.2: Proportions of affected cheetahs based on inbreeding classes: Histopathology and gastric inflammation on post mortem examination. Comparison of histopathology based gastritis diagnosed post mortem (Y axis) and percentage positive or negative per inbreeding classes on X axis.

Infection/cancer and histopathology per inbreeding class
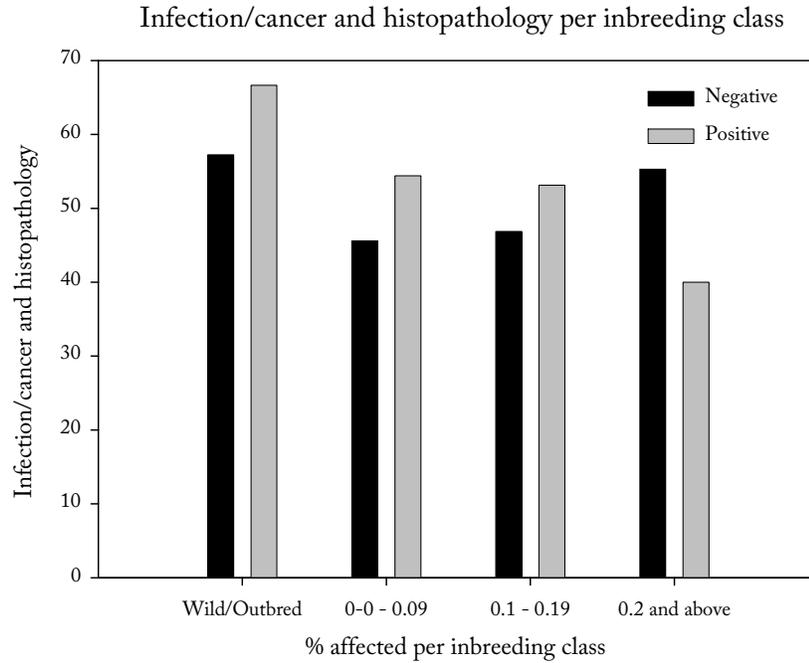
Figure 5.3: Proportions of affected cheetahs based on inbreeding classes: Infection/cancer and histopathology per inbreeding class. Comparison of infection/cancer that is laboratory diagnosed (Y axis) and percentage positive or negative per inbreeding classes on X axis.

Gastritis: Histological grading per inbreeding class
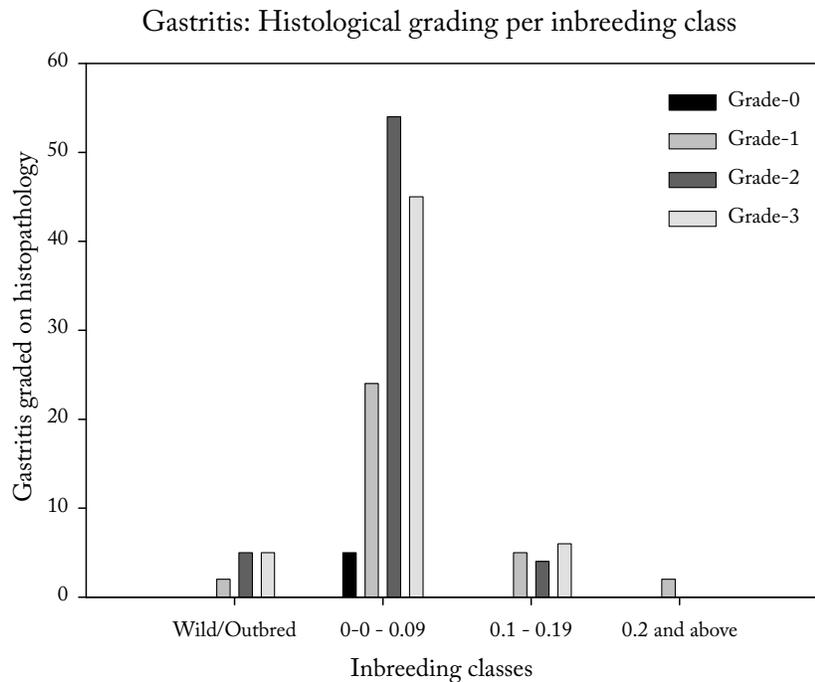
Figure 5.4: Proportions of affected cheetahs based on inbreeding classes: Histological grading per inbreeding class. Comparison of gastritis graded on histopathology (Y axis) and percentage of cheetahs per inbreeding class and gastritis grades 0 - 3 on X axis.
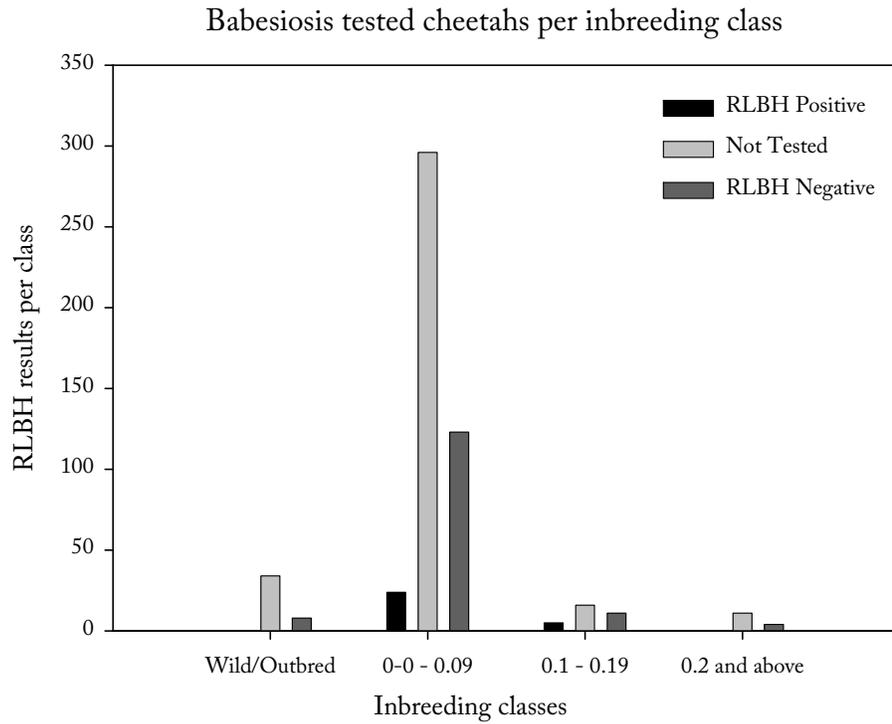
Figure 5.5: Proportions of affected cheetahs based on inbreeding classes Babesiosis tested cheetahs per inbreeding class. Comparison of babesia positive on laboratory diagnosed (Y axis) and percentage positive/negative/not tested per inbreeding classes on X axis.

Mortality under one year and inbreeding classes
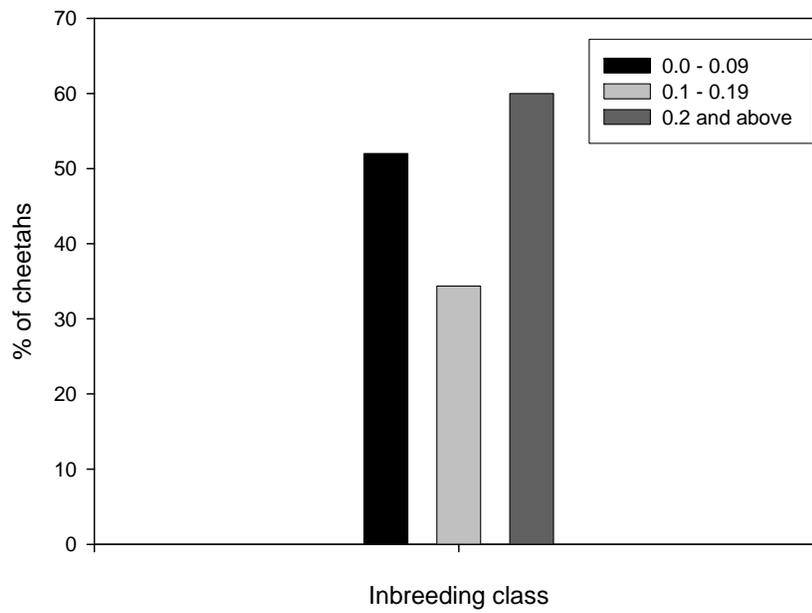
Figure 5.6: Proportions of affected cheetahs based on inbreeding classes: Mortality under one year on Y axis and the three inbreeding classes on X axis.

Litter size between inbreeding classes (± SD)
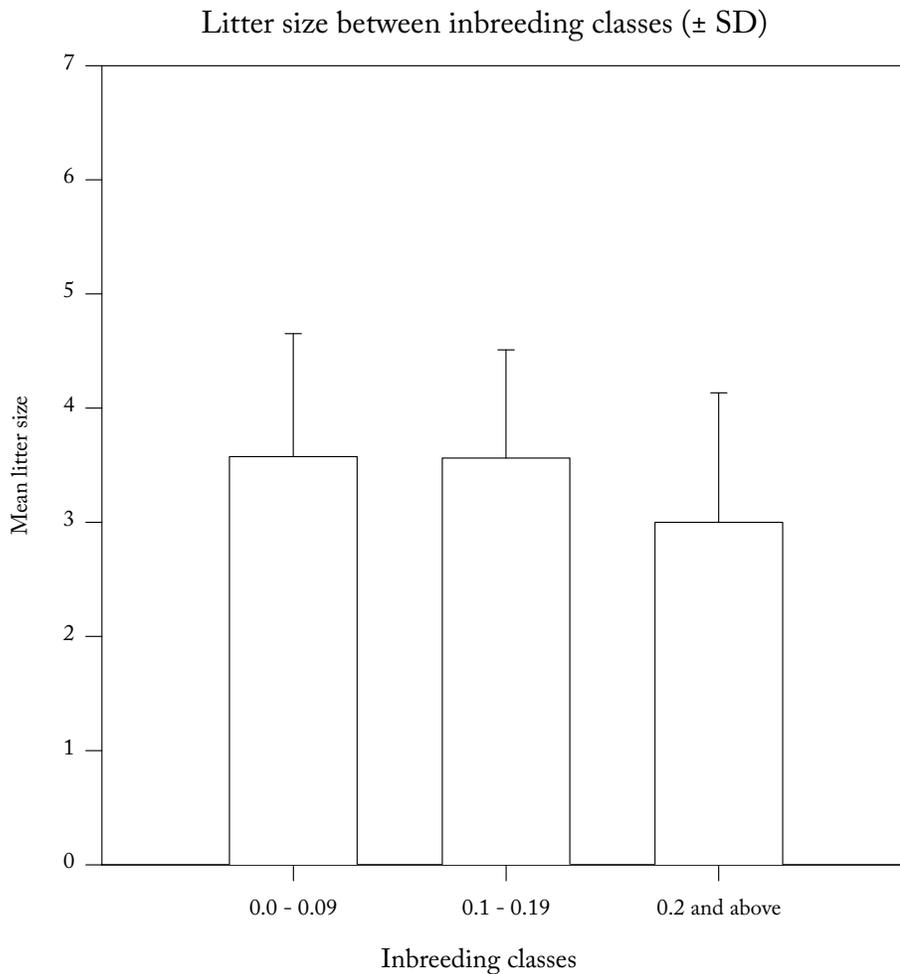
Figure 5.7: Proportions of affected cheetahs based on inbreeding classes: Mean litter size between inbreeding classes (± SD) (Y axis) per inbreeding classes (X axis).

The condition gastritis diagnosed histologically (grade 2 and above) and at post-mortem was correlated to Wright's coefficient of inbreeding (Table 5.1). Gastritis was correlated to whether the animal expressed the *King* trait or was a carrier. Cheetahs with infectious conditions alone were not correlated to inbreeding as was the case with *Babesia* positive cheetahs. There was no correlation with the expression of the *King* trait and other infectious conditions, including babesiosis. Cub mortality under one year was weakly correlated with inbreeding ($r = 0.057$; $F = 4.08$; $P = 0.006$) but not correlated to whether the animal was a carrier of the recessive trait.

Table 5.1: Trait / condition correlations to inbreeding

Trait / condition vs.	Correlation	Significance Levels
Gastritis- histological diagnosis (grade 2 and above)	r = 0.068, F = 3.45	$P = 0.01$*
Gastritis- post-mortem diagnosis	r = 0.12, F = 3.34	$P = 0.038$*
Gastritis- in *King* or carrier	r = 0.067, F = 5.08	$P = 0.0074$*
Gastritis- histological diagnosis (grade 2 and above) and infectious/cancer conditions	r = 0.077, F = 3.97	$P = 0.0094$*
Infectious/cancer conditions	r = 0 0098; F = 0.47	$P = 0.705$
Infectious/cancer conditions and *Babesia* positive	r = 0.038; F = 1.39	$P = 0.256$
Cub mortality under one year	r = 0.057; F = 4.08	$P = 0.006$*

5.3.2 Heritability of traits

The direct heritability for litter size was 0.548 (S.E = 0.078) and was lower for gastritis at 0.0768 (S.E = 0.058). The maternal heritability for cub mortality was estimated to be 0.5968 (S.E = 0.131).

5.4 Discussion

This study used pedigree-based Wright's coefficient of inbreeding to evaluate associations between inbreeding and pathology. A direct correlation between quantifiable traits and the coefficient of inbreeding is more efficient than marker-based genome-wide heterozygosity correlates with life-history traits (Pemberton 2004). The dataset analysed is relatively small compared to breeding records that are routinely used to calculate heritability estimates for important traits in domestic animals. This constraint is directly related to the endangered status of the species and poor breeding success in captivity.

The effects of increased levels of inbreeding and subsequently greater levels of disease is evident within the cohort of cheetahs exhibiting the recessive *King* trait or being a carrier for the trait. Generalised inbreeding effects appear to be greater for carriers of the *King* trait, possibly because selection for a trait generally leads to decreases in genome-wide heterozygosity and expression of deleterious alleles (Charlesworth & Charlesworth 1999). Animals expressing the *King* trait have been reported with fungal and bacterial conditions more often than non-carriers, are more susceptible to fly strikes, helminthosis, gastric ulceration, thymus atrophy and bone fractures (Lambrechts 1998; Millward & Williams 2005; Picard *et al.* 1998). Our study did not include any measurable record of human interaction by the individual cheetah over its lifetime nor whether potentially aggressive cheetahs might have been housed in nearby pens, both potential triggers of stress response (Wielebnowski *et al.* 2002). In this captive population, it is however typical for cheetahs to be moved randomly between pens to facilitate routine management, and contact to human activity is uniform for most animals, except for females near term where minimal contact is the norm.

There was lack of significant correlation between inbreeding levels and presence of *Babesia* in the blood of cheetahs. Infected cheetahs appear to be coping with the parasite in the wild, although recent reports have not tested specifically for this antigen (Munson *et al.* 2005). The RLBH test results are predominantly based on testing the current generation of captive cheetahs. A program of testing succeeding generations, similar to that currently in place for gastric pathology, will generate enough data for a more comprehensive study on genetic correlations to *Babesia* susceptibility. A more specific test on a wider cohort of cheetahs is currently underway.

The heritability estimated for life history traits had large standard errors, reflecting the amount of data available. The heritability estimates for litter-size and mothering ability might be considered high, but the lack of similar estimates from other wild felid populations makes meaningful comparison and evaluation of data difficult. In the captive population of Sumatra tiger (*Panthera tigris sumatrae*), an analysis of stud book data using an animal (mixed) model revealed significant heritability (0.281 ± 0.063) and additive genetic variance (0.232 ± 0.066; $\chi^2 = 37.05$, $P < 0.001$) (Pelletier *et al.* 2009). One direct application of this result could be more diligent selection of dams in future breeding programs. Heritability estimates have the potential to indicate evolutionary direction and reproductive strategies employed by a species (Axenovich *et al.* 2006). The ability of dams to nurture their litter appears to be highly heritable, reflecting the natural history of the cheetah, where a very small minority of dams contribute disproportionably to the entire population (Kelly *et al.* 1998; Kelly 2001).

These results pose an interesting paradox for institutions successfully breeding cheetahs. The high levels of heritability for mothering ability, a trait that has a substantial behavioural component, indicates that this component has the potential to be fixed by selective breeding (Maynard-Smith 1989). This trait appears to be part of social behaviour that has a history of domestication (Mignon-Grasteau *et al.* 2005). In wild cheetahs, this trait is closely related to risk-taking and predator-avoidance behaviour, and is rapidly punished by poor reproductive success if injudicious in the wild (Durant 2000). Evidence from the Serengeti points to a very high rate of cub mortality, due to perturbation of the mother's immediate environment (Laurenson *et al.* 1995b). Approximately 8% of the lineages in the Serengeti survived and reproduced to give rise to over 50% of the total population between 1970 and 1994 (Kelly 2001). This trait is heavily relied upon by mothers with new-born litter at kills, to protect them from inter-specific predation-related disturbance, the biggest cause of mortality (Hunter *et al.* 2007b). Therefore, from an evolutionary perspective, the high levels of stress-induced glucocorticoids produced in cheetahs would be considered an adaptive advantage based on the natural history of this predator, where flight is favoured, caution is paramount, and more so in females with cubs (Durant *et al.* 2004; Hunter *et al.* 2007a). The fact that a very low percentage of cheetahs born in the wild survive to maturity, most falling prey to inter-specific predation (Kelly 2001), indicate the extreme penalty paid by careless mothers. It is therefore paradoxical that in captivity, the apparent selective advantage normally granted to females with greater threat or interspecific predation-awareness, is an unwanted trait that potentially leads to exclusion from breeding and possibly manifests as a stress-related disease. The best breeders in captivity, potentially the more socially-adapted females, have greater success rearing young, and by the above understanding of the natural behaviour of the female in the wild, would probably not pass its genes on in the natural state. Current literature states that type of selection is the foremost and predominant mechanism causing rapid reproductive (genetic) isolation of populations, rather than divergence due to drift or bottlenecks (Rolshausen *et al*. 2009; Sobel *et al*. 2010; Templeton 2008; Thorpe *et al*. 2010). Institutions will unavoidably select for the best female

by selecting from those with prior history of above-average litter size and nurturing cubs to maturity, potentially contributing to fixing for 'social adaptation'. On the other hand, this 'selection under captivity' might be advantageous for a species that looks increasingly institution-bound for the foreseeable future.

Faced with a novel or chronic insult, the innate and adaptive immune system in vertebrates intervenes at multiple levels with a cascade of genes being activated sequentially (Tizard 2004). In vertebrates, there appears to be a more complex response to various physiological and behavioural stressors, involving a greater number of genetic loci (Trowsdale & Parham 2004). Variations in human and animal stress-responsive adrenocortical function is largely under polygenic inheritance and influenced by the environment (Redei 2008). Genetic marker-based variability is a poor indicator of adaptive potential of populations (Leinonen *et al.* 2008) and gene-expression data might more accurately reflect the underlying genetic variance of such quantitative traits. A population level study might therefore detect the generalised inbreeding effects, but heritability estimates for a trait under polygenic control and influenced by environment would be biased downwards.

The difficulty in an analysis of small population dynamics and addressing issues relating to conservation is that it is often difficult to separate genetic factors from environmental ones (Mills 1996). In a complex condition like spiral-bacteria induced gastritis, the genetic mechanisms that result in decreased heterozygosity might exert a generalised effect at the population level by the expression of deleterious alleles (Charlesworth & Charlesworth 1987). In this study, a proportionate reduction of a fitness trait relative to the level in a non-inbred group , definable as inbreeding depression (Hartl & Clark 1997), is reported for a specific lineage of captive cheetahs that carry a recessive gene. Stress in captivity that leads to a higher glucocorticoid response or 'environmental stress', is increasingly being redefined in the case of higher mammals with sophisticated immune responses (Valsecchi *et al.* 2004). Generally, it is any response to an environment that impairs normal function relative to more benign conditions (Hoffmann & Parsons 1991) and tends to exacerbate inbreeding depression (Armbruster & Reed 2005). This study indicates that in higher mammals, there is added usefulness in considering quantifiable pathology as an estimator for overall fitness, in addition to other composite measures such as lifetime reproductive success.

Chapter 6

Linkage analysis of the *King* phenotype in the African cheetah (*Acinonyx jubatus*) to *Tabby*-linked markers

Abstract

The African cheetah (*Acinonyx jubatus*) exhibits a unique coat colour pattern traditionally called 'King' that appears to be inherited as an autosomal recessive trait. Strikingly different from the 'wild-type' spotted phenotype, the *King* pattern has embossed stripes and blotches raised above the hair, with a striped and ringed tail and bold black on cream or ivory colouring. Cheetahs with the colour variation are a much sought-after attraction in game farms and zoological collections in South Africa, Eastern Europe and Asia, bring increased revenue for captive breeders. We investigated a long standing assumption that the coat colour variation is a modification of the *Tabby* allele that dominates coat patterns in most carnivores, especially among Felidae. The *Tabby* locus was previously shown to be linked to markers within a 17-cM region in the B1 chromosome of the domestic cat (*Felis catus*). The single largest breeding group of cheetahs that segregates for this trait was investigated for linkage to a putative conserved *Tabby* allele within Felidae. Seven microsatellite genetic markers that flank *Tabby* in the domestic cat chromosome B1 were selected and linkage analysis was performed on an extensive pedigree consisting of 89 captive cheetahs. There was no evidence for linkage between the *King* locus and the markers selected, with LOD scores remaining non-significant for all the markers. Despite the largely conserved karyotype between different felids, the possibility of paracentric inversions within cheetah B1 cannot be ruled out. More recent research has shown promising linkage to *F. catus* chromosome A1 that could not be undertaken in this study. The pedigree also had the significant handicap in that it exhibited a lack of phase-known informative meiosis for three of the seven markers used. Compared to *Tabby* in the domestic cat, the *King* phenotype in cheetah appears to be evolutionarily conserved and under tight regulation, with gene modifiers possibly playing an insignificant role. A full genome scan on a smaller pedigree with a panel of moderately spaced markers will possibly narrow down the unique genomic location that harbours *King*.

6.1 Introduction

The African cheetah (*Acinonyx jubatus*) has a colour variant of the normal spotted phenotype called the '*King* Cheetah' on account of its unique pattern of striped markings, slightly longer and distinctly silkier hair. The *King* coat colour pattern, as the variation is called, derived its name from the originally erroneous description of the pelage variant as a distinct species called *Acinonyx rex* (Pocock 1927). It was originally described as a unique African species from Zimbabwe. The phenotype is rarely reported in the wild, with the first sighting recorded in 1928 and sporadically later through the decades, mainly from a restricted area in and around eastern and south-eastern Zimbabwe, Limpopo Province and Mpumalanga in South Africa and eastern Botswana (Bottriell 1987; Hills *et al.* 1980). In South Africa, sightings have taken place from Tshokwane in the Kruger National Park (1989) and Sabi-Sand Game Reserve (1992) where a *King* female and three cubs (two *Kings*) were reported (Skinner & Chimimba 2005). *King* cheetahs were first born in captivity on the 12 and 14th of May 1981, at the De Wildt Cheetah Breeding Station and Research Centre in South Africa (Brand 1983). The parents were a normal-type spotted male cheetah originating from the Messina District of the former Northern Transvaal in South Africa and two normal spotted females, sisters bred from cheetahs that were wild-rescued from South Africa and Namibia (Van Aarde & Van Dyk 1986). Two *King* cubs rescued in the Limpopo Province in 1989 were later transferred to the Kapama Cheetah Project, near Hoedspruit, where another *King* litter was born in 1998 (Skinner & Chimimba 2005). The observed inheritance pattern of the phenotype within the De Wildt pedigree suggests that the blotched pelage is inherited as a single autosomal recessive allele (Van Aarde & Van Dyk 1986). The mutation that leads to this phenotype has been suggested, based on observations, as similar to the change in the 'striped' to 'blotched' variation in the '*Tabby*' coat colour pattern in the domestic cat (Robinson 1976).

The *King* phenotype is popular within zoological collections worldwide, increasing pressure on captive breeding programs in South Africa to maintain the recessive allele without dilution, potentially increasing the numbers of known and suspected carriers. As is the case with breeding for any recessive trait, animals of the *King* phenotype might be more susceptible to fly strikes, helminthiasis, gastric ulceration, thymus atrophy and bone fractures (Lambrechts 1998; Millward & Williams 2005). *Cryptococcus* infection, by an opportunistic fungal pathogen known to flourish within a deficient immune system, was reported with four of the seven cases being *Kings* (Millward & Williams 2005; Picard *et al.* 1998).

Investigations into the molecular genetics of the *Tabby* locus allelomorphism have been hampered by the lack of a potential candidate gene that could be inferred from human or commonly studied laboratory animals. The *Tabby* alleles lead to the formation of darker transverse stripes or blotches on the coat of the domestic cat. Various domestic cat reference families around the world have been contributing to research on coat colour mutations (Ishida *et al.* 2006; Lyons *et al.* 2005a; Lyons *et al.* 2005b; Menotti-Raymond *et al.* 2007; Menotti-Raymond *et al.* 2009a; Schmidt-Kuntzel *et al.* 2005; Schmidt-Kuntzel *et al.* 2009), reporting mainly from differential transcription of melanocyte-specific genes and melanocyte-specific transcription factors. The inherent difficulty in getting pedigrees to segregate for patterns that could be truly considered for the phenotypic determination of *Tabby* have been a challenge. Problems arise when determining homozygotes and heterozygotes for this allele, due to the multiple variations seen within *Abyssinian*, *Mackerel* and *Blotched* patterns that may be taken for phenotypes. These

phenotypes apparently do not segregate in pedigrees and the *spotted* pattern is suspected to be affected by modifiers (Lyons *et al.* 2006). Compared to the domestic *Tabby* that exhibits a wide variety of pattern variations, the *King* cheetah exhibits significant uniformity in its pelage, with minor shape and size changes in blotches that make individual identification possible. Within 38 coat specimens of the *King* type investigated, all had uniformity in having a longer mane and embossed stripes and blotches strikingly raised above the longer and silkier hair, a striped and ringed tail and bold black on cream or ivory colouring (Bottriell 1987). Cheetahs are normally spotted on the torso with numerous jet black, round or slightly oval shaped spots, with spots on the distal parts of the tail tending to coalesce into black rings (Skinner & Chimimba 2005). The spotting on each cheetah is an uniquely identifiable arrangement on face and body (Caro 1994). The only reported variant to the normal spotting has been the tail banding, attributed to genetic and environmental effects (Caro & Durant 1991). Spots merge to form longitudinal stripes and blotches in the *King* cheetah, a result of a possible mutation at a putative *Tabby* colour locus (Van Aarde & Van Dyk 1986). The possible mutation at the *Tabby* locus was suggested earlier (Searle 1968) and reported similar to the *striped Tabby* to *blotched Tabby* change in the domestic Tabby cat (*Felis catus*) (Robinson 1976). The occurrence of the trait and the genetic variation of tabby pattern in the domestic cat has been extensively documented elsewhere (Lomax & Robinson 1988; Robinson 1978; Vella *et al.* 1999).

Using an extended pedigree of 64 cats, genetic linkage between the *Tabby* locus and markers was previously reported within a 17-cM region in B1 chromosome (Lyons *et al.* 2006). Initial genome scans were done using a panel of 150 feline microsatellites that gave an average marker spacing of 75cR. Linkage was detected between markers on chromosome B1 and the *Tabby* locus, with the area of interest estimated to span approximately 15.9 Mb or 17 cM on the second generation domestic cat linkage map (Menotti-Raymond *et al.* 2003b). A remarkable degree of karyotypic conservation is typical across the various subspecies of Felidae, in contrast to the high proportion of interchromosomal rearrangements seen within the dog genome (Yang *et al.* 2000). Consequently, this study used the potential gene-linked microsatellite markers reported by Lyons *et al.* (2006), to do a two-point and multipoint linkage analysis on a pedigree of cheetahs segregating for the *King* phenotype.

6.2   Materials and methods

6.2.1   Pedigree structure and sampling

Blood samples were collected from 89 captive cheetahs within the extensive breeding group of cheetahs maintained at the De Wildt Cheetah Breeding Station and Research Centre in South Africa. The centre has a breeding group, the characteristics of which are reported elsewhere (Bertschinger *et al.* 2008; Brand 1983; Meltzer 1998). Pedigree and breeding data for 532 progeny from 33 sires and 60 dams from 1971 to 2007 were analysed for this study. The dataset included for this study was determined by the availability of samples and included 42 females and 47 males, with 11 cheetahs exhibiting the *King* phenotype (Figure 6.1). The full pedigree (Appendix V) segregating for the trait was drawn using CRANEFOOT V3.2 (Makinen *et al.* 2005) and the subset (Figure 6.2) drawn using SUPERLINK online version 1.0 (Silberstein *et al.* 2006b).

6.2.2   Marker selection and DNA extraction

Genomic DNA from the ethylenediaminetetraacetic acid (EDTA) blood samples was extracted by using the phenol-chloroform method (Sambrook *et al.* 1989). Parentage was verified for all cheetahs using a previously published international parentage and identification panel for the domestic cat (Lipinski *et al.* 2007). PEDCHECK (O'Connell & Weeks 1998) was used to check for inconsistencies within genotyping. Seven microsatellite markers, FCA023, FCA809, FCA811, FCA810, FCA700, FCA254 and FCA813, previously reported to be linked to *Tabby* in domestic cat, were used for linkage analysis. The positions of these markers are on 15.9 cM region on chromosome B1 based on the available autosomal linkage map for the domestic cat (Menotti-Raymond *et al.* 2009b).

Figure 6.1: A representative of the *King* phenotype with the pattern magnified (right)

The locations of FCA810 and FCA813 appear to be unresolved as per the 2009 autosomal linkage map (Menotti-Raymond *et al.* 2009b) but is reported on a previous generation radiation-hybrid map (Menotti-Raymond *et al.* 2003a) The genotyping methods for the markers used have been reported previously (Young *et al.* 2005). The primer pair sequences and amplification conditions are described in appendix VI. Briefly, all forward primers were labelled with the fluorescent dyes (Applied Biosystems) at the 5' terminus, with reverse primers remaining unlabelled. Cycling conditions included an initial denaturation at 95 ℃ for 5 min followed by 35 cycles of 95 ℃ for 45 s, 58 ℃ for 45 s and 72 ℃ for 30 s. Following polymerase chain reaction (PCR) amplification by multiplexing the loci in two sets, products were analysed and fragments separated on an ABI 3130 xl Genetic Analyzer with the GeneScan-500 ROX size standard (Applied Biosystems), and results visualised using *STRand* software (Toonen & Hughes 2001). A selection of random samples (N = 15) were re-extracted and amplified to confirm typing quality. Amplification and consistency of PCR products were checked using MICROSATELLITE TOOLKIT (Park 2001).

6.2.3   Genetic Linkage Analysis

The inheritance pattern of *Tabby* in domestic cat previously reported was assumed to be a dominant allelic series (Lyons *et al.* 2006). This study performed two-point and multipoint linkage analysis on the *King* coat colour pattern modelled as a binary trait and inheritance of the phenotype to be fully penetrant and autosomal recessive. Analysis

was initially performed using LINKAGE (Lathrop *et al.* 1984). Although the software has been applied to demonstrate different coat colour and disease mutations in the domestic cat (Young *et al.* 2005; Cooper *et al.* 2006; Grahn *et al.* 2005; Imes *et al.* 2006; Lyons *et al.* 2005a; Lyons *et al.* 2005b; Rah *et al.* 2006), the existence of multiple loops within our pedigree caused the analysis using LINKAGE difficult and failed due to memory outage. A newer implementation using Bayesian networks named SUPERLINK, that optimises and combines the E-S algorithm and the Lander-Green approach to multilocus linkage analysis was used for this study (Fishelson & Geiger 2002; Fishelson & Geiger 2004). SUPERLINK uses multiple pre-processing steps on the Bayesian network to reduce and trim redundant variables and achieve the best time-space trade-off given the memory available for the linkage analysis problem. Two-point and multipoint linkage analysis and log of the odds (LOD) scores were computed using SUPERLINK online version 1.0 (Silberstein *et al.* 2006b). The actual gene frequencies of a putative *King* gene were generated from observed phenotypes within the study population. On the assumption that this population might be having an artificially higher allele frequency for this particular locus, based on the rarity of the phenotype in the wild, we also generated LOD scores with the allele associated with coat colour pattern assumed to have a frequency of 0.01 and all other markers of equal allele frequencies (Appendix XII). Identical LOD score trends were generated in each scenario. Linkage was calculated using all seven markers and leaving out FCA810 and/or FCA813 that were not described in available maps (Davis *et al.* 2009; Menotti-Raymond *et al.* 2009b). Therefore, multipoint analysis involved the following map orders:

- FCA023, FCA809, FCA811, FCA810, FCA254, FCA700 and FCA813. This combines the information from the new autosomal linkage map and the older combined map (Menotti-Raymond *et al.* 2003b; Menotti-Raymond *et al.* 2009a).

- FCA023, FCA809, FCA810, FCA811, FCA700 and FCA254, as described in the *Tabby* study (Lyons *et al.* 2006) and

- FCA023, FCA809, FCA811, FCA254 and FCA700, that excludes the unknown positions of FCA810 and FCA813 as reported in the newer maps available at the time of analysis (Davis *et al.* 2009; Menotti-Raymond *et al.* 2009a).

Sex-averaged recombination was calculated and integrity of data were checked by running a two-point linkage analysis of each marker against itself using CRI-MAP Version 2.4 (University of California-Davis modified version) (Green *et al.* 1990). The analysis of the generalised LOD score is a measure that indicates the overall evidence that a set of loci form a linkage group (Lathrop *et al.* 1984).
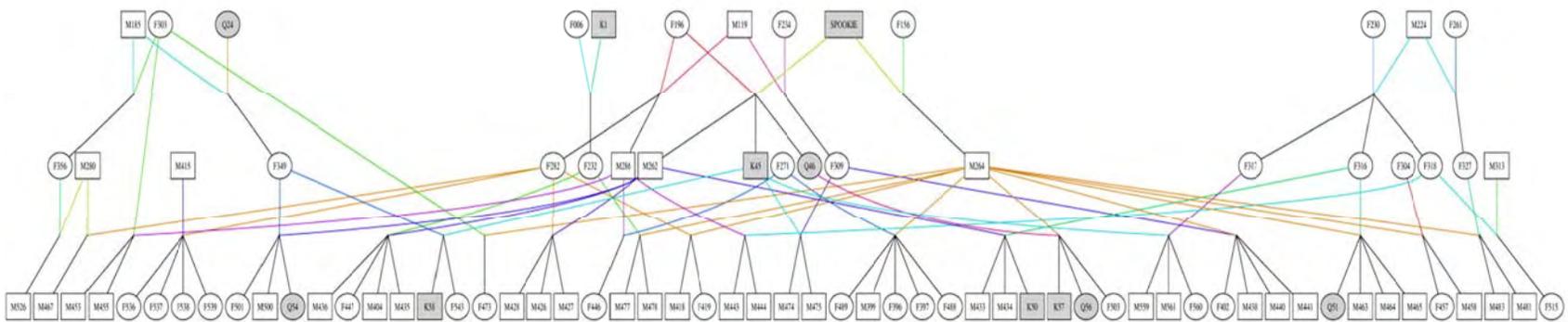
Figure 6.2: Pedigree segregating for the *Acinonyx King* locus used for linkage analysis. Circles represent females, squares represent males, shaded symbols indicate *King* cheetahs, un-shaded represent the unknown phenotype or carrier.

6.3   Results

All the loci were heterozygous and had observed heterozygosities ranging from 0.09 to 0.77, with polymorphic information content (PIC) values ranging from 0.083 to 0.56. Allele frequencies were calculated from 98 unrelated or wild cheetahs from the populations described in chapter 4. Informative meiosis was lacking for three of the seven loci used (Table 6.1). Sex-averaged recombination fractions from two-point linkage analysis are listed in Appendix VII.

Table 6.1: Information content of markers used

Locus	Observed Heterozygosity	PIC	Informative Meiosis	Phase Known Informative Meiosis	theta* (Lyons *et al*, 2006)
FCA023	0.091	0.083	14	0	0.038
FCA254	0.778	0.561	103	0	0.106
FCA700	0.263	0.269	28	1	0.03
FCA813	0.722	0.537	90	15	0.05
FCA809	0.356	0.278	39	0	0.081
FCA810	0.579	0.389	81	1	0
FCA811	0.696	0.658	87	9	0.048

*theta (Lyons *et al.* 2006)

Markers appeared linked to each other on checking for data integrity with a tolerance level set at 0.01 in CRI-MAP ver. 2.4 (Table 6.2).

Table 6.2: LOD Scores from two-point linkage analysis with TOL = 0.01 (CRI-MAP Ver. 2.4 UCD Mod)

	FCA023	FCA809	FCA811	FCA810	FCA700	FCA254	FCA813
FCA023	**3.31**	-0.00	0.27	0.00	-0.00	-0.00	-0.00
FCA809	-0.00	**8.43**	0.25	0.23	0.12	0.42	0.1
FCA811	0.27	0.25	**21.67**	0.00	0.00	1.86	0.63
FCA810	0.00	0.23	0.00	**17.76**	0.45	0.45	0.00
FCA700	-0.00	0.12	0.00	0.45	**6.62**	-0.00	0.00
FCA254	-0.00	0.42	1.86	-0.00	-0.00	**24.99**	0.63
FCA813	-0.00	0.10	0.63	0.00	0.00	0.63	**21.67**

Two-point LOD scores for the markers and the *King* trait ranged from non-significant to 0.32 (Table 6.3); therefore, no significant linkage was demonstrated for the markers used on the candidate-gene location that might harbour a *Tabby* analogue. The LOD score for the *King* trait segregating pedigree at theta=0 is negative infinity, indicating that the log likelihood is negative infinity, equivalent to a likelihood of zero. The hypothesis that there is no recombination between the trait and loci is inconsistent with this data. The scores showed little change, irrespective of the gene frequencies used. The LOD scores for each recombination fraction tested (0.00, 0.01, 0.05, 0.10, 0.20, 0.30 and 0.40) are reported in appendix XI, XII and XIII.

Graph of lod curve
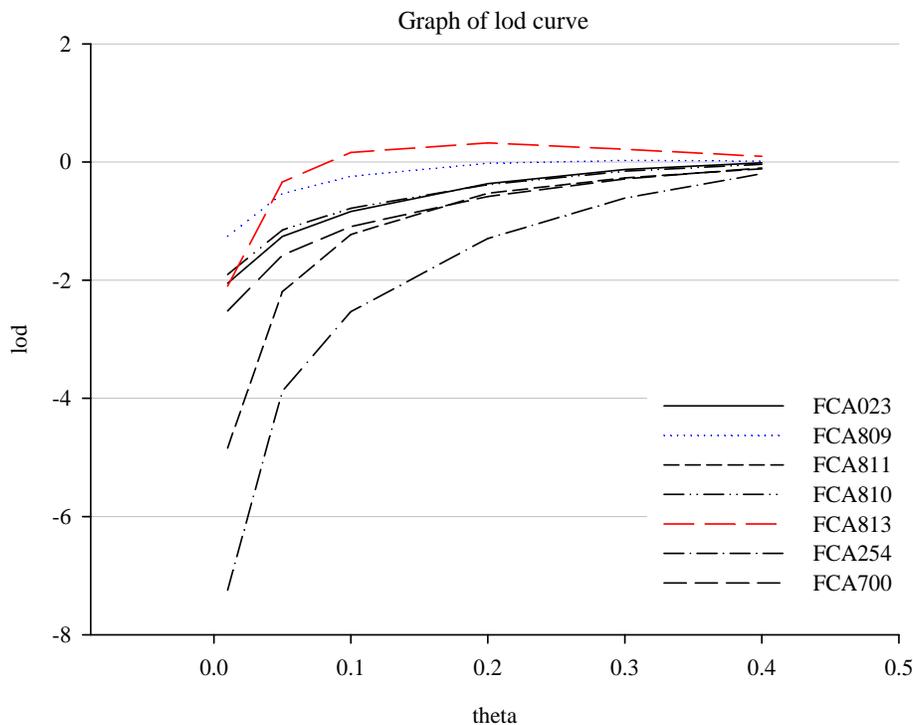
Figure 6.3: Graph of two-point LOD score (y axis) versus recombination fraction (x axis) using values for markers assuming equal allele frequencies.

The trend did not change irrespective of whether actual population allele frequencies or equal frequencies were used. Multipoint LOD scores (Ott 1999) using the three different fixed map of markers were non-significant irrespective of the map order used.

Table 6.3: LOD scores for pairwise comparisons between *King* locus and *Felis catus* chromosome B1 microsatellite markers.

Marker	LOD	Theta	Cat chrom.	Linkage map position	RH map pos. 5c	Human chr.	Hsa36 position	Dog chr.	Canine2f position
FCA023	-1.2607	0.05	B1	83.6	463.3	8	31307618	16	32706503
FCA809	0.0259	0.3	B1	84.5	476.7	8	27646753	25	32866052
FCA811	-1.2284	0.1	B1	84.5	488.6	8	27433306	25	33063420
FCA810	-0.0381	0.4	B1	Unknown	510.2*	No RH	Unknown	Unknown	Unknown
FCA254	-0.2013	0.4	B1	106.7	551.1	4	175869632	25	28175855
FCA700	-0.1021	0.4	B1	108.1	571.9	4	174967845	25	27356441
FCA813	0.3208	0.2	B1	Unknown	701.2*	No RH	Unknown	Unknown	Unknown

* Radiation hybrid (RH) position previously published (Menotti-Raymond *et al.* 2003a). FCA810 and FCA813 had not been mapped/described in the autosomal linkage or RH maps available (Davis *et al.* 2009; Menotti-Raymond *et al.* 2009b)

The graph (Figure 6.3) plotting the LOD scores over a 15 cM region on either side of the first marker (values attached in appendix XV) was non-significant. A similar trend was noted when plotting the Ln (Likelihood) values for each LOD score against the trait position (XIV).
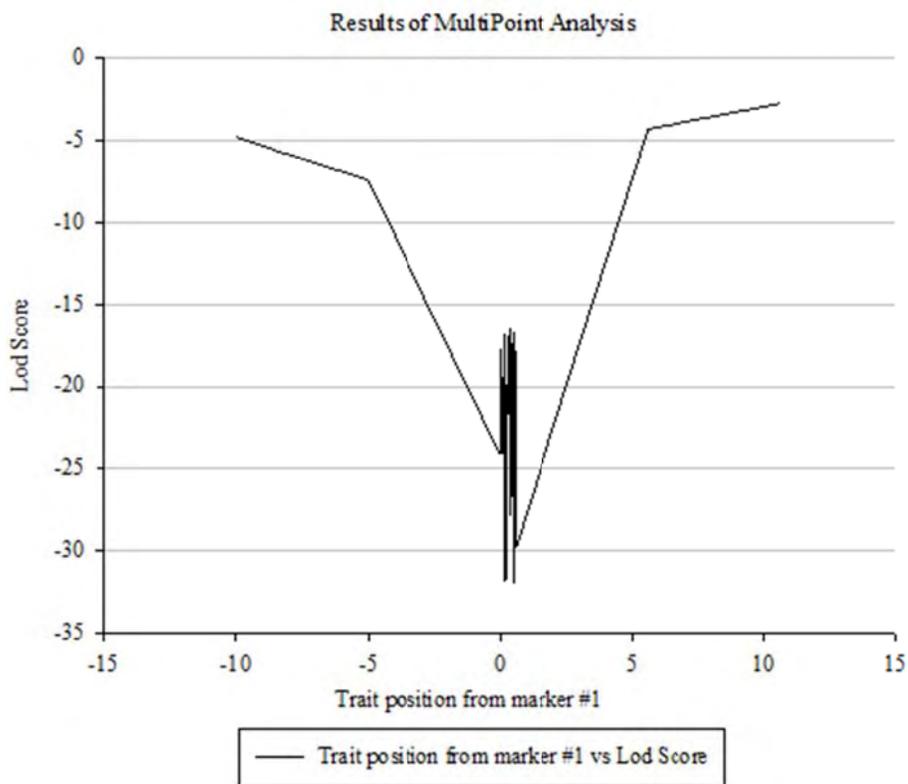
Figure 6.4: Graph plotting LOD scores over a 15 cM region on either side of the first marker

6.4 Discussion

All available date at the time, including observations and data from studies on *Tabby* colouration (Lomax & Robinson 1988; Robinson 1978; Vella *et al.* 1999) and molecular genetics of *Tabby* alleles (Lyons *et al.* 2006) indicated that a candidate gene approach, concentrating on the putative location of the *Tabby* locus in the *Acinonyx* might be a viable proposition. The microsatellites chosen have previously been used to demonstrate linkage to the *Tabby* locus in the domestic cat, where the locus was estimated to reside between markers FCA559 and FCA254 on chromosome B1. This area spans a length of approximately 68.2 cM according to the 2009 autosomal linkage map (Menotti-Raymond *et al.* 2009b). This distance, between markers FCA559 (38.5 cM) and FCA254 (106.7 cM), were previously estimated to be 17 cM based on the second generation linkage map (Menotti-Raymond *et al.* 2003b). A similar increase was seen within the RH map, where the area spanned an estimated 170 cR compared to a 113.6 cR region described earlier (Lyons *et al.* 2006). The GARField: Genome Annotation Resource Field *Felis catus* v12.2 resource (Pontius & O'Brien 2007) listed the region between FCA254 (position 77729549) and FCA700 (position 74811696) as spanning 2.918 Mbp (Appendix XVII). The region involved is therefore quite extensive and does not code for any known genes that are linked to coat colour in other mammals.

Pericentric inversions within the different subspecies of Felidae were suspected to be common (Yang *et al.* 2000). However, more recent work using domestic cat BAC clones hybridising on related species such as the serval (*Profelis serval*) and snow leopard (*Panthera uncial*) suggest that there is a higher degree of karyotypic conservation than suspected and reveals very little G-banding differences (Davis *et al.* 2009). The authors suggest that structural (pericentric) inversions might not be common but smaller paracentric inversions or smaller sequence changes that do not affect gene order might be the norm within Felidae. All available data points to the assumption that the markers isolated from chromosome B1 of the domestic cat does indeed map to a similar locations on the cheetah. However, this needs to be verified by a pilot study using fluorescence in situ hybridization or other techniques in the cheetah before cat markers can be used with confidence.

Another significant challenge faced was that the number of phase-known meioses (Table 6.1) pointed to a modest number of recombinants. The inclusion of more numbers of meiosis in the pedigree will improve the accuracy of multipoint mapping of the region. The penetrance model used was autosomal recessive, and trying different levels of penetrance or various dominant model combinations did not improve LOD scores. Confirmation of the marker order was obtained from running a two-point linkage analysis of each marker against itself and noting the peaking of LOD scores.

The *King* phenotype itself is explained by a *Turing-type* model, with the change in pattern between the cheetah and *King* cheetah explainable due to a simple shift in a pattern formation parameter, that is triggered by the genetic change that underlies the phenotype (Werdelin & Olsson 1997). Whether the phenotype is an adaptation for a particular forest or woodland biome that has evolved for cryptic or aposematic benefit is a matter of debate. Most historical sightings of the *King* phenotype have been in the woodland biome area around eastern and south-eastern Zimbabwe, the north-eastern region of former Transvaal and few in eastern Botswana (Rogers 1997). Crypsis can arise through either pattern matching a background or disruptive patterns like large spots that break up the prey outline (Sinervo & Calsbeek 2006). The jaguarundi (*Herpailurus yaguarondi*) that have the gene variants of the *MC1-R* gene and are therefore darker than the ancestral reddish form (Eizirik *et al.* 2003) may hold an evolutionary advantage in thick foliage. Genes controlling the spotting and striping patterns in *Carnivora* appear to have evolved separately from the basic mammalian genotype for coat colour. The *Tabby* locus, acting on an agouti or yellow background to give blotched, striped or lined in wild and domestic cats appear to confer an evolutionary advantage (Ragni & Possenti 1996). Within the Iberian lynx (*Lynx pardinus*), the tabby pelage appear to have been actively selected for and have become more frequent in feral populations, possibly as an adaptation towards crypsis for hunting (Beltran & Delibes 1993). The complexity inherent within *Tabby* was recently demonstrated in an investigation on the genomic position of the classic X-linked orange (O) locus genotype, where the authors noted that the agouti locus did not influence the presence or absence of tabby pattern in orange-coloured fur, but genotype at the agouti-signalling-protein did (Schmidt-Kuntzel *et al.* 2009). There might also be a cofounding factor played by genetic drift, founder effect and most importantly, human selection, when investigating gene frequency patterns of coat colour and length in domestic cat. Unlike wild felid species, few founders and intensive selection have laid the foundation of many extant domestic cat phenotypes (Wagner 1996). Although the *King* phenotype is suspected to be a fully penetrant autosomal recessive, it is unclear if there might be more than one mutation, among tightly linked

genes. Kaelin & Barsh (2010), commenting on recent developments in cat coat colour genetics state that the "conserved, adaptable, and largely unexplored mechanism"(s) that underlie tabby patterns arise from a mechanism that is programmed to be spatially constrained. The *King* pattern expressed in the African cheetah might well be a type of 'pigmentation-oriented' mechanism that regulates expression of pigmentary genes in response to an ancient pre-pattern (Eizirik *et al.* 2010) The evolution of specific pigmentary genes that interpret the pre-pattern could have occurred independently in an animal like the cheetah and a variation of it in the *King* lineage.

This study did not detect linkage between the *King* phenotype in cheetahs and seven microsatellite markers that have previously been demonstrated to be linked to the *Tabby* phenotype in the domestic cat. More recent work has since reported significant linkage to the *Tabby* locus in the region of chromosome A1 (markers FCA566 (LOD=31.4, θ=0.01), FCA90 (LOD = 10.7, θ = 0.05), and FCA1331 (LOD=42.61, θ=0), a genomic region of approximately 5 Mb based on conserved synteny (Eizirik *et al.* 2010). The domestic cat breed 'spotted Egyptian Mau' was used to detect the spotting variant by Eizirik *et al.* (2010) and crossed to the recessive blotched phenotype to yield intermediate patterns. The F1 non-blotched backcross progenies exhibited a full range of phenotype from spotted to striped, deviating from the phenotype of the true breeding spotted Egyptian Mau and thereby indicating epistatic action of modifying genes. Similar crosses in De Wildt, however, have failed to reveal the presence of any modifying genes in the F1 cheetah progeny when recessive blotched *King* phenotypes are crossed with dominant spotted cheetahs. Further investigations using markers flanking this genomic region within A1 or a wider genome scan might be required to detect and localize the area of interest. The population continues to thrive and generate additional samples every breeding season, potentially adding to more informative meiosis for future studies. Captive breeders in South Africa are faced with the challenge of detecting the heterozygote, possible now only by test mating suspected carrier cheetahs to a known *King* phenotype or known carriers and checking the resultant litter for the birth of the variant. Considering the difficulties in captive breeding this vulnerable predator, the availability of a genetic test for detection of true carriers would facilitate institutions to better manage genetic diversity levels within their populations. Conservation efforts can then be integrated with a longer term breeding policy that keeps a unique gene pool of heterozygotes within the pedigree without hazardously increasing inbreeding levels.

Chapter 7

Conclusions and Discussion

This study expands our knowledge about the genetics of captive cheetahs in South Africa, with the largest and most successful breeding group, the De Wildt Cheetah Breeding Station and Research Centre, being the focus of this work. Anecdotal information and extrapolations from studbook data had previously described the captive population in South Africa as having little genetic variability and higher levels of Namibian ancestry. The study looked at addressing some of the concerns among conservation officials involved in the relocation of captured cheetahs on how best to manage the population as to retain diversity levels. Illegal trade of wild-captured cheetahs that are later sold as captive-bred after implanting a microchip is an increasing problem.

The study looked at the potential to use spatial Bayesian clustering to differentiate the point of origin of unknown cheetahs and therefore potentially set a database for future forensic efforts to address the problem of illegal trade. The focus of the second part of the study was to investigate whether complex conditions such as development of gastritis, renal conditions and/or susceptibility to infections was related to inbreeding levels. The availability of breeding records from over 500 cheetahs also made it possible to calculate heritability values for important breeding traits. Finally, the study looked at a cohort of pelage variants called *King* cheetahs and tested the assumption that the variation is a mutation of the *tabby* locus by testing genetic markers detected linked to *Tabby* in the domestic cat for linkage to a conserved region in the cheetah that potentially codes for *King*.

7.1   Hypothesis 1: The captive cheetah population has retained genetic variation and population differentiation compared to wild conspecifics

Diversity levels overall (for 274 cheetahs) matched or exceeded those reported for the species elsewhere. Overall, the mean number of alleles per locus was 6.92, mean expected heterozygosity was 0.642 and the mean polymorphic information content was 0.598, with significant heterozygote deficit and gene diversity for the captive population versus Namibian wild cheetahs. This was expected as the captive population includes a lineage of cheetahs that segregates for a recessive allele. The differences between diversity levels within South African wild cheetahs and captive cheetahs were not significant. Inbreeding levels were the least for Namibian cheetahs, reflecting the high levels of gene flow that has been reported by others.

Using a single sample Bayesian approximation, the estimated mean effective population size of free-ranging cheetahs in South Africa was 330 cheetahs, lower than estimated by Purchase *et al.* (2007). Within the captive population, a distinctly unique admixed group of cheetahs exist, that have been born of parents of diverse geographical origins. Population differentiation was possible after spatial Bayesian clustering, with a distinct Northern Group and a North-West_Kalahari group remaining within South African cheetahs after assigning individual genetic membership coefficients of $q > 0.5$. The results are valuable in the context of the captive population at De Wildt and elsewhere, in that such captive populations are a genetic and a demographic reservoir that affords planning for future reintroduction into the wild.

7.2 Hypothesis 2: The captive cheetah population has Namibian ancestry and levels of ancestry can be quantified

Previous concerns on Namibian genetic introgression into the South African captive cheetah gene pool were not evidenced from our results. After assigning individual membership coefficients of $q > 0.5$, only 13% of all captive cheetahs showed significant Namibian ancestry. It might be that wild-rescued Namibian cheetahs do not breed well in captivity but it is more likely that within the sampled cheetahs there are potentially more animals of Namibian descent. However, due to the fact that the current generations sampled would have had their Namibian ancestry diluted over the years (and forming part of the admixed group), none of these cheetahs would exceed the set individual membership coefficients of $q > 0.5$ to be assigned to the Namibian wild cohort.

7.3 Hypothesis 3: Unknown ancestry of a cheetah can be described using trained spatial Bayesian clustering

Prior to the initiation of this study, we could only discern three distinct cheetah groupings in our southern African cheetah sampling, and included the DWCBSRC cheetahs, free-ranging South African cheetahs and their Namibian counterparts. In a wide-ranging species like the cheetahs, it is difficult to *a priori* differentiate population groupings and our results confirm this. Spatial Bayesian clustering using known co-ordinates from a wild sampling of cheetahs has enabled us to differentiate individual cheetahs based on their membership to different geographic clusters. The captive population that was part of this dataset proved to be quite admixed, excepting for the *King* lineage which was distinct. The results have allowed us to relook at the ancestry of few cheetahs that were sampled in game farms, where there was doubt on the origins. One such breeding male, recorded in the stud book as originating from Swaziland, is now known to closely cluster with others of known Namibian origin ($q = 0.93$). Within the relocation program, two adolescent males rescued from adjacent traps and assumed to be siblings in a male coalition have distinctly different genotypes. These males probably joined together to form a coalition when they were trapped and rescued. The breeding implications for these two males are that now both have the potential to enter into a breeding program together in a suitable area. The database this study has generated will hopefully be utilised in the future for similar investigations on unknown ancestry.

7.4 Hypothesis 4: There is a correlation between inbreeding and development of pathology and susceptibility to infection

This study investigated the relationship between inbreeding and development of pathology and susceptibility to infection in an extensive pedigree of 532 cheetahs. Inbreeding levels directly influenced the development of gastritis, but what was more significant was that if the cheetah was a carrier or expressed the autosomal recessive *King* coat colour trait, it was more prone to develop gastritis. This finding has implications on the management and breeding of cheetahs that form part of the *King* lineage within the captive population. The effect noted in the lineage appears to be due to generalised inbreeding. Current literature supports the hypothesis that selection for this trait would lead to a decrease in genome-wide heterozygosity and expression of deleterious alleles. Inbreeding levels did not influence the

development of infectious conditions or being tested positive for a novel haemoprotozoan parasite of the *Babesia* species or mortality due to trauma and accidents. It remains to be seen if the weak but significant correlations detected are signs of genetic erosion that should serve as a "warning signal" (Szulkin *et al.* 2010) to population managers

7.5   Hypothesis 5: Heritability of complex conditions like gastritis in the cheetah is low

Heritability of the complex condition gastritis, that is developed as a result of a cascade of reactions to an unknown stressor and potentially acted on by a multitude of genes, was determined to be poorly heritable. Abnormalities within complex physiological pathways that are most often under the influence of multiple gene regulatory systems are more influenced by environmental factors than genetic. In the era of depleted monogenic susceptibilities, current investigations into the genetics and heritability of complex conditions in humans and animals have detected multitudes of susceptibility loci. The application of newer methods for large-scale genome-wide association scans appears to be narrowing the knowledge gap that exists in our understanding of such conditions.

7.6   Hypothesis 6: Selection can be improved by dam selection for litter size in cheetahs

One of the important estimates this study generated was the heritability of litter size in cheetahs. The value for this common life history trait has not been known previously for cheetahs and is generally scarcely found in the literature for conserved wildlife. Our estimate of 0.55 had large standard errors, reflecting the amount of data available. This is a direct reflection of the difficulty in getting estimates from pedigree records of endangered animals, where there usually is not enough data. The estimate, along with the maternal heritability for cub mortality (0.59) indicates that female cheetahs can potentially be improved by selection. Current selection methods are focussed on the male, with the analysis of semen quality and prior breeding records of the male being the important priorities. There is potential to select cheetahs that have come from a larger than normal sibship. This data are applicable to any of the 44 institutions and breeding facilities nationwide, and would improve awareness on the importance of careful selection. These results reflect observations on the natural history of cheetahs, where the ability of dams to nurture their litter appears to be highly heritable and where a very small minority of dams contribute disproportionably to the entire population.

7.7   Hypothesis 7: In cheetahs, there is a detectable genetic component that contributes to maladaptation or stress in captivity has a genetic component

Our results did not detect a single genetic component that contributed to maladaptation or stress in captivity. We detected a generalised inbreeding effect that was more pronounced within a particular lineage. This effect did manifest as apparent maladaptation to captivity in that the captive population had higher incidence of complex conditions such as gastritis. The lack of significant association between infectious conditions, susceptibility to a blood-borne parasite and inbreeding levels indicate that the environment in captivity plays a bigger role in the pathogenesis of infectious disease than genetics. The population that was investigated is housed in camps of a hectare each, in semi-wild habitat, with a regular daily routine that the cheetahs are used to. Institutions world-wide have

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development of infectious conditions or being tested positive for a novel haemoprotozoan parasite of the *Babesia* species or mortality due to trauma and accidents. It remains to be seen if the weak but significant correlations detected are signs of genetic erosion that should serve as a "warning signal" (Szulkin *et al.* 2010) to population managers

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learned from the observations first reported from De Wildt on optimal breeding management and reducing litter loss, especially in primiparous females. The lessons learnt over the decades from research done in zoological institutions world-wide have led to the understanding that it is crucial to get the environment optimal, that the cheetahs are housed under conditions that reduce stress. Nevertheless, there is an unexplainable individual susceptibility to complex conditions that appears to be predisposed by stress. Future genome scanning studies on the extensive pedigree within this population might reveal susceptibility loci that contribute to stress in captivity

7.8   Hypothesis 8: Genetic linkage analysis: the described linkage between *Tabby* locus in domestic cats and microsatellite markers that flank chromosome B1 is replicable for detecting linkage between *King* locus and markers in *Acinonyx jubatus*

Genetic linkage analysis did not detect linkage between the *King* locus and the domestic cat microsatellite markers used for this study, with LOD scores remaining non-significant for all the markers. All available literature points to a generally conserved karyotype within Felidae. This assumption would need to be tested using fluorescent mapping techniques to confirm that the markers used here do indeed map within the area of interest on chromosome B1. This would rule out any paracentric inversions as the cause of our inability to detect linkage. More recent work done after this study point to a similar area of interest on chromosome B1, with the locus responsible for the Abyssinian (*ticked*) form mapped to a ~3.8 Mb region on chromosome B1. However, a putative second locus that controls the *Tabby* phenotype was mapped to a ~5 Mb region on chromosome A1 (Eizirik *et al.* 2010). The spotted Egyptian Mau breed was crossed to the recessive blotched tabby phenotype to yield a range of intermediate patterns in the F1 progeny. Such epistatic action of modifying genes appears to be absent when the recessive blotched *King* phenotype is crossed with the dominant spotted cheetah. Despite all available literature indicating that the *King* phenotype is a type of tabby mutation, where spots have changed to blotches, there are significant differences between the two phenotypes. The action of modifying genes causing the different spotting and banding modifications in the domestic cat tabby appears to be non-existent in the cheetah. There is comparably remarkable similarity within the *King* phenotype, indicating that the phenotype in cheetah is evolutionarily conserved and under tight regulation without gene modifiers playing any significant role. A full genome scan on a smaller pedigree with a panel of moderately spaced markers or a selected panel that saturates chromosomes B1 and A1 is recommended to narrow down the unique genomic location that harbours *King*.

7.9 Discussion

Prior to this study, very little information was available on the genetic diversity and admixture within captive South African cheetah populations, with over 500 cheetahs estimated to be in captivity in South Africa alone. In fact, approximately 34% of the world's captive cheetah population is based in southern Africa and South Africa in itself hosting the majority of these (Marker *et al.* 2007). This captive pool gave rise to 93% of the over 428 cheetahs exported worldwide and are the origin of cheetahs being reintroduced to suitable habitats (Bèga 2007; Hayward *et al.* 2007; Rogers 2007). This is an important genetic pool, and considering the current meta-population status, of underestimated genetic value. These captive cheetahs afford conservation practitioners and policy-makers considerable breathing space while waiting for policy and politics to slowly work its way down to grass-roots action plans formulated to maintain and revive cheetah populations in South Africa.

Captive cheetahs at DWCBSRC have traditionally been reproducing successfully, (Bertschinger *et al.* 2008; Meltzer and Van Dyk 1998 ;Meltzer *et al.* 1998) with 60 – 80% of sexually mature females in the De Wildt facility producing live cubs compared to 9 – 12% in the North American programs (Marker-Kraus & Grisham 2005). This success has been tempered by the concern that the De Wildt cheetah population has risen from a limited number of founders. The concern was that the 'natural' selection of breeders using the successful template at De Wildt has unwittingly decreased genetic diversity within the captive population. According to Meltzer & Van Dyk (1998), the genetic relatedness among the wild-origin founder cheetahs from the former Northern Transvaal and Swaziland-Namibia, including nine females and 20 males, were not known. In addition, the effort to keep the *King* phenotype as a separate lineage and maintain the phenotype within the captive population (Van Aarde & Van Dyk 1986) might have increased overall inbreeding levels. Additionally, it was reported that over a period from 1970 to 1996, 71% of 244 cheetahs imported from the wild to South Africa were of Namibian descent (Marker *et al.* 2003; Marker 2002). This study has clarified genetic diversity, effective population size, population structure using a variety of clustering methods and estimated Namibian ancestry within this important captive cheetah population.

This work has proven beyond doubt that overall genetic diversity within the captive population matches or exceeds those reported for cheetahs elsewhere (Driscoll *et al.* 2002; Luo *et al.* 2004; Gottelli *et al.* 2007; Marker *et al.* 2007; Kotze *et al.* 2008). The general pattern reflects the selection of polymorphic markers for studies after Driscoll *et al.* (2002). Significant heterozygote deficit was observed for the captive population but of the for 22 locus pair combinations out of a possible 80 locus pairs. Sufficient due diligence was given prior to initiating this study to select only those markers spaced sufficiently far apart based on available maps and polymorphic enough to merit use, and was done after wide-spread consultation within the Cheetah genetics subcommittee of the National Cheetah Conservation Forum. The availability of parentage-verified three generation pedigree data also enabled checking for actual physical linkage. Of the 22 locus pairs, only one locus pair exhibited a significant LOD score. The removal of this pair from subsequent analysis did not affect the results. The fact that 37% of the total captive group cluster with the *King* lineage, the level of significant linkage disequilibrium detected was probably predictable. Estimated effective population sizes for captive and wild South cheetahs either reflected previously published estimates or could be explained as a result of high levels of gene flow within Namibian cheetahs. Admixture results allayed

concerns of extreme Namibian genetic introgression. Apart from the *King* lineage, genetic admixture data reveals that over the years, gene flow within the captive group has been augmented by the diversity of male and female cheetahs willing and able to breed in captivity. The presence of a well definable Northern/Limpopo Group and a North-West_Kalahari is potentially a genetic and demographic reservoir available for future reintroduction into the wild. The ability to detect inconsistencies in genetic signatures of few cheetahs ostensibly sampled as captive born from a private game reserve but clustered with high certainty with Namibian cohort, points to the potential forensic use of this database and technique.

Gastritis continues to be actively managed in the De Wildt captive population through a number of managemental, nutritional and veterinary interventions. The poor heritability of gastritis (0.07) confirmed the complex nature of this condition, where it fits with the understanding that individual adaptability to stress is not dependent on major heritable genetic components. Data available to this study reflected the reality of getting pedigree information from wild animals and in turn, caused large standard errors. Nevertheless, the results indicate that more diligent selection of dams in future breeding programs might be fruitful. This mirrors a similar selection in the wild, where a very small minority of dams contribute disproportionably to the entire population (Kelly *et al.* 1998; Kelly 2001). Inbreeding effects were more pronounced for carriers of the *King* trait, potentially due to the selection for the recessive feature that decreased genome-wide heterozygosity and facilitated expression of deleterious alleles (Charlesworth & Charlesworth 1999). The results indicate the difficulties in separating genetic factors from environmental ones (Mills 1996), considering the polygenic nature of the traits investigated (Trowsdale & Parham 2004). Stress in captivity and its influence on individual genetic susceptibility to complex conditions might be more fruitfully investigated within this pedigree using genome-wide scanning methods currently being developed for domestic cats

The study failed to establish linkage between the *King* locus and microsatellite markers determined to be *tabby* – linked in the domestic cat. It is easy to rule out common reasons for lack of linkage such as environmental modification of phenotype that is undetected, researcher incorrectly assigning the phenotype (a common mistake in coat colour studies) or that the phenotype is a result of a mutation of low penetrance. The obvious remaining factor is either incorrect or incomplete genome coverage or both.

The presumption that this linkage analysis was based on was that the colour variation in the *King* cheetah is a modification or mutation of the *tabby* locus that predominates coat patterns in most carnivores, especially among Felidae (Robinson 1976) and was reported as due to a mutation at a putative *tabby* colour locus (Van Aarde & Van Dyk 1986). At odds with the variation seen in the domestic *Tabby*, the *King* cheetah exhibits significant uniformity in its pelage (Bottriell 1987). More recent work by Eizirik *et al.* (2010) have reported significant linkage to the *Tabby* locus in a region of chromosome A1, a genomic region of approximately 5 Mb based on conserved synteny. One basic assumption with the *King-Tabby* model still needs to be explained, where Eizirik *et al.* (2010) demonstrated epistatic action of modifying genes. This was seen in the F1 non-blotched backcross progenies exhibiting a full range of phenotype from spotted to striped, however, no such action of modifying genes could be noted in F1 cheetah progeny when recessive blotched *King* phenotypes were crossed with dominant spotted cheetahs in breeding similar to that described. The *King* lineage continues to grow in De Wildt and elsewhere, where detecting the heterozygote is

currently done by test mating suspected carrier cheetahs to a known *King* phenotype or known carriers. Future developments and availability of more multigenerational pedigrees with more informative meiosis might make it possible for the development of a genetic test for detection of true carriers. The future availability of a larger cohort and genome-wide SNP association studies using domestic cat resources may prove successful in understanding complex conditions in captive cheetahs. Cheetah conservation can then be integrated with a longer term breeding policy that keeps a unique gene pool of heterozygotes within the pedigree without hazardously increasing inbreeding levels.

7.10 Recommendations for future genetic management

This study has generated a sustainable database of southern African cheetahs that can be applied to or added to other databases to improve our conservation efforts with respect to the African cheetah. The management of the cheetah in South Africa as a metapopulation with known genetic links is a sustainable policy that is already in place and functioning. The methods outlined in this study will add to the efforts to infer the genetic ancestry of any cheetah entering the breeding or relocation program. The aid of genetic tools will greatly improve implementation of the metapopulation policy when it comes to transfer and relocation of the second generation of cheetahs that have been born from their rescued and relocated parents. This work captured a snapshot of the genetic profile of the largest breeding group of cheetahs and compared it with their wild counterparts. The different genetic groupings detected in the captive group indicate that genetic heterogeneity is high. Wild caught cheetahs are not allowed to join the breeding group unless re-homing them is impossible, most often due to them being problem animals or having a chronic disability or injury. Therefore there is very limited number of potential founder animals added every generation, making selection of future breeding pairs important. The *King* lineage forms a substantial part of the captive group investigated and is destined to remain so. Known male heterozygotes are recommended to be mated with females from families having a history of above average litter size. Breeding management should include a list of potential males and females as a backup in the scenario that the pairing selected does not mate naturally. The captive cheetah breeders group in South Africa can go a long way in improving the overall genetic management of their respective populations by having an exchange program, with potential breeding females and males being exchanged between the populations. Any unknown entering this program might be tested for its genetic ancestry. The cheetah in southern Africa and especially South Africa is increasingly being maintained in captivity, with populations that are institution-bound exceeding their wild counterparts in the near future. This study has helped to increase our knowledge on the genetics of this vulnerable predator in captivity.

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I. Appendix

A comparison of observed heterozygosity, homozygosity and internal relatedness in cheetahs between different population clusters

II.   Appendix

Phylogenetic relationships between individual cheetahs from composite microsatellite genotypes from 13 loci, based on proportion of shared alleles (1000 bootstrap iterations).
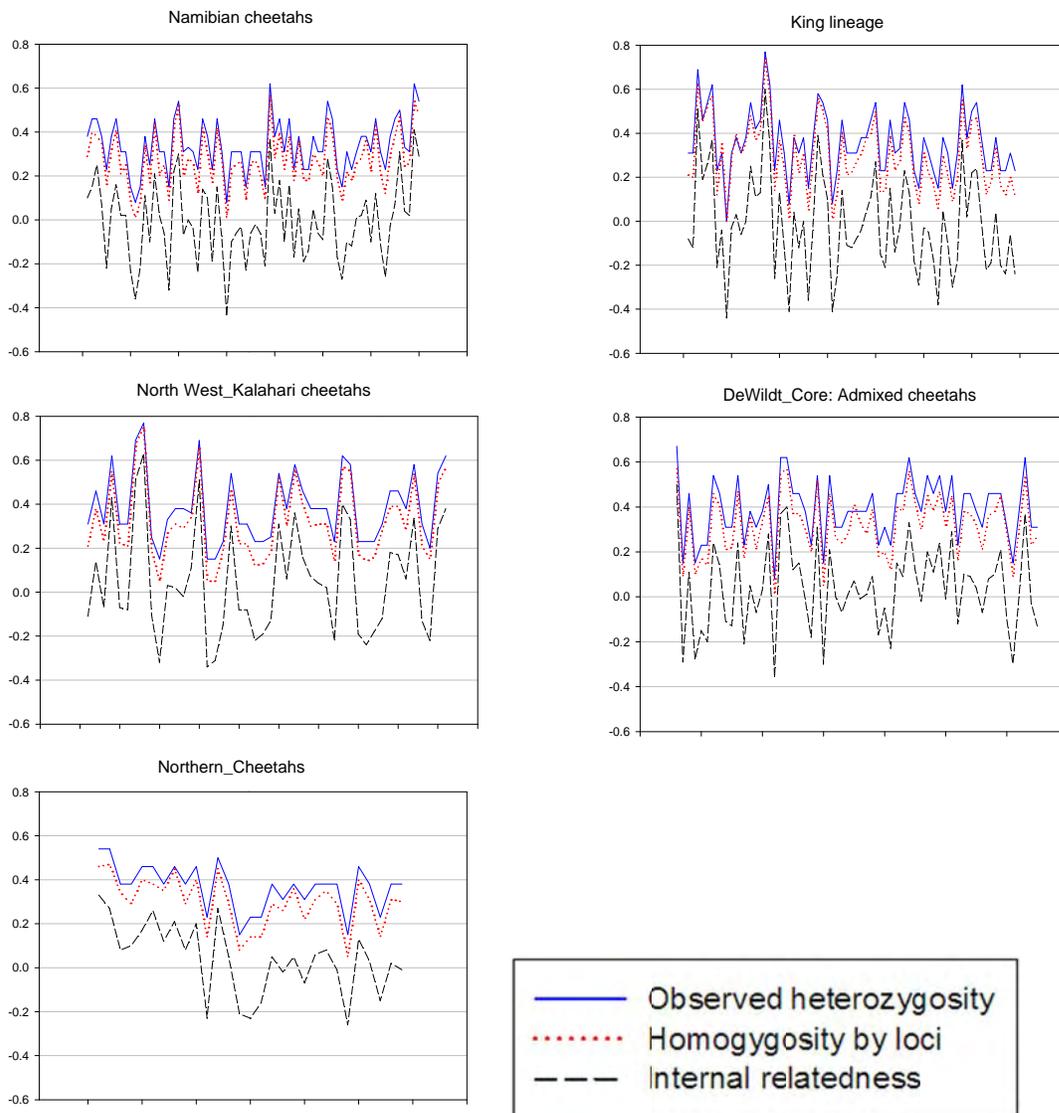
III.   Appendix

Molecular co-ancestry values (below) were significantly different for the two major groups in the captive population, (Standard errors shown, P = <0.001)

Molecular Co-ancestry (*FT* Method)

t = 3.402 with 164 degrees of freedom. (P = <0.001)
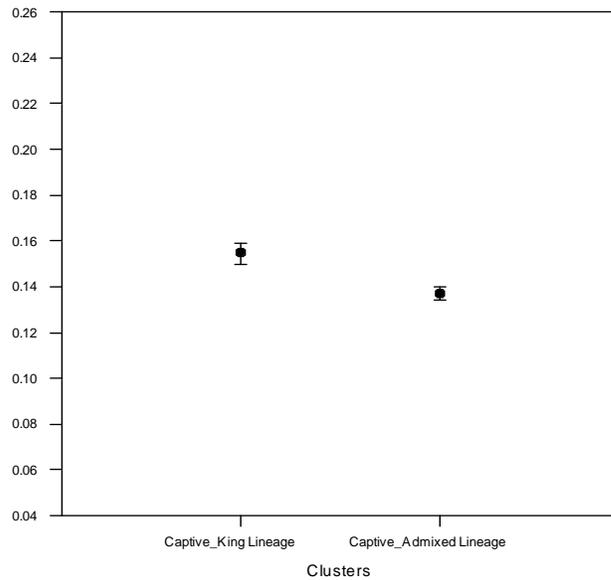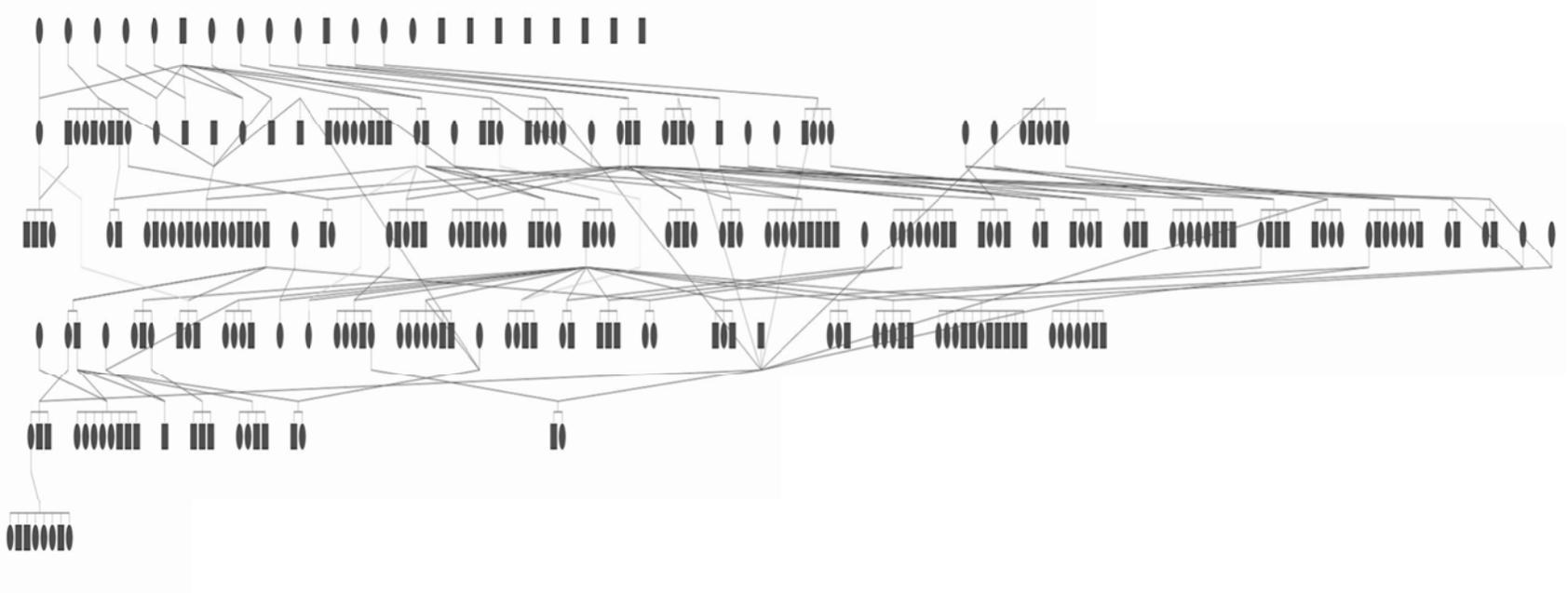
The six generation pedigree visualised using *Jenti* (Falchi & Fuchsberger 2008). This is the complete pedigree consisting of 532 cheetah and includes those segregating for the *Acinonyx King* locus. Symbols: Ovals represent females, rectangles represent males
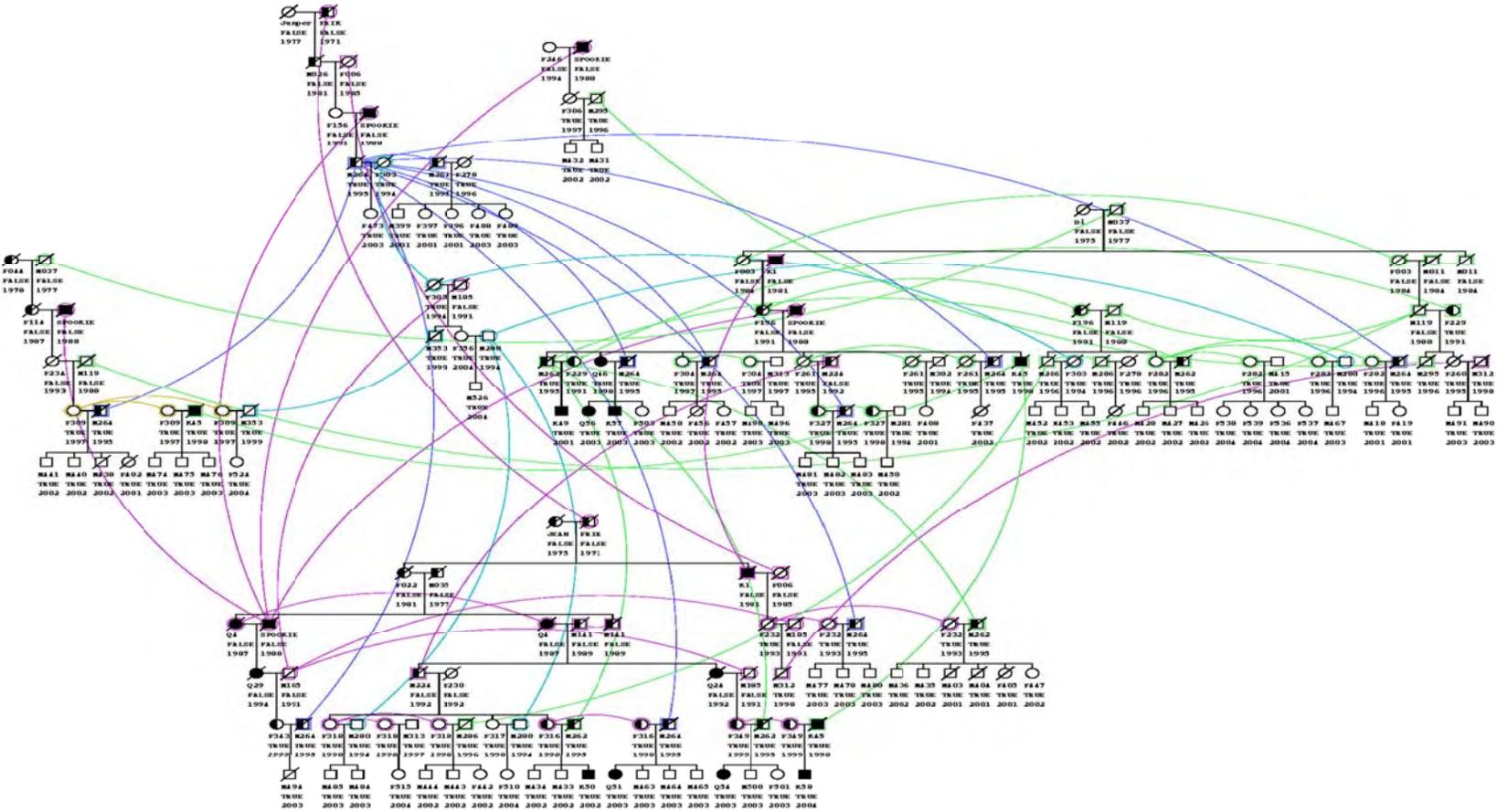
Complete pedigree segregating for the *Acinonyx King* locus. A smaller pedigree was used for linkage analysis and depended on the availability of samples. Symbols: Circles represent females, squares represent males, half-shaded symbols indicate *King* pattern cheetahs, full-shaded symbols are the true phenotypes and un-shaded represent the unknown phenotype. Dead cheetahs are represented by a stripe across the symbol

VI. Appendix

Marker locations on Chr B1, primer pair sequences and amplification conditions for seven microsatellite markers
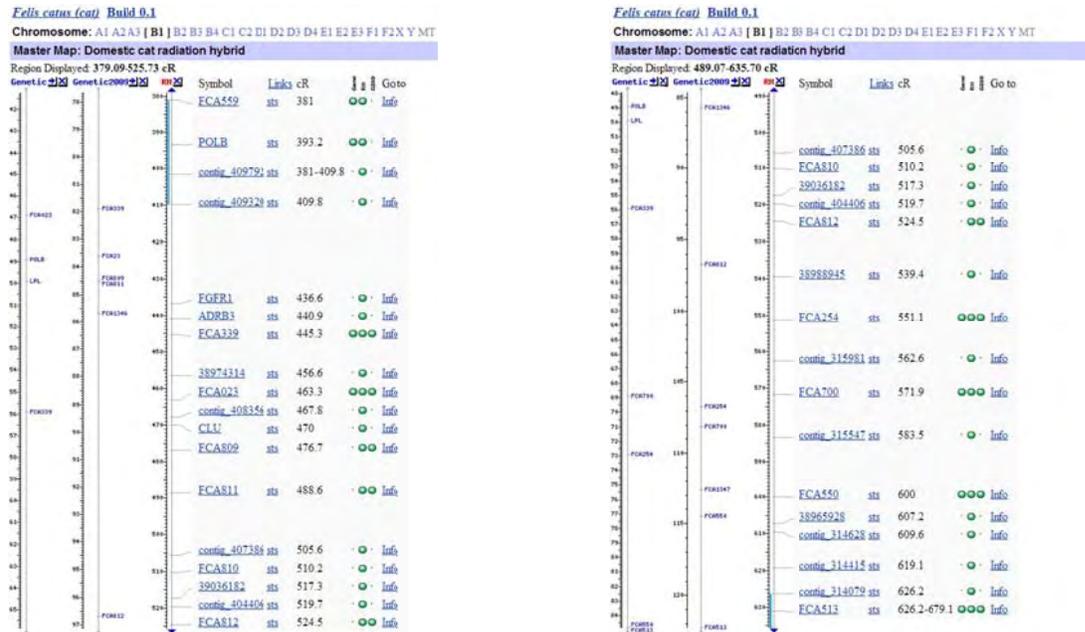
Master Map: Domestic cat radiation hybrid of B1, indicating locations of the 7 markers used

Primer Name	Primer Sequence and Labelling	MgCl2 (mM)	TM (ºC)	Primer Conc (µM)
Multiplex A				
FCA023-FAM-F	AAGAATGGACTTGGGAAATGG	2.0 mM	58	.2
FCA023R	AAACCACAACAGGCAAAAGG			.2
FCA254-VIC-F	VIC-CAGATGCTCAACCGACTAAGC	2.0 mM	58	.2
FCA254-R	GCTTTGGCAACCCCTCTAC			.2
FCA700-NED-F	NED-CCCTTAAAATCGCAGCTCTG	2.0 mM	58	.2
FCA700-R	AATCCAAGGAAAACAGGCCT			.2
FCA813-FAM-F	6Fam-AGGAAGACCCCACTGATGTG	2.0 mM	58	.2
FCA813-R	ATGTGCTAGGACATTGTGTTGG			.2
Multiplex B				
FCA809-NED-F	TAAAACGACGGCCAGTGCGATCAGGACACTGATGGGCT	2.0 mM	58	.02
FCA809-R	CAGAATGGACCCTCTGGTGT			.2
FCA810-FAM-F	TTTCCCAGTCACGACGTTGCAGCAGCAAGGAAATGACAA	2.0 mM	58	.02
FCA810-R	AATGCCATAGCCAGTGAGCT			.2
FCA811-VIC-F	GCGGATAACAATTCACACAGGTTTCTGCCAAGCTACGGAGT	2.0 mM	58	.02
FCA811-R	TCACCGTAAACAGTCACCTCC			.2

VII.   Appendix

Sex-averaged recombination fractions from two-point linkage analysis (CRI-MAP Ver. 2.4 UCD Mod)

	FCA023	FCA809	FCA811	FCA810	FCA700	FCA254	FCA813
FCA023	0.00	0.37	0.22	0.00	0.37	0.49	0.47
FCA809	0.37	0.00	0.25	0.29	0.21	0.22	0.32
FCA811	0.22	0.25	0.00	0.5	0.5	0.25	0.32
FCA810	0.00	0.29	0.5	0.00	0.24	0.44	0.5
FCA700	0.37	0.21	0.5	0.24	0.00	0.46	0.5
FCA254	0.49	0.22	0.25	0.44	0.46	0.00	0.3
FCA813	0.47	0.32	0.32	0.50	0.50	0.30	0.00

VIII. Appendix

Pedigree (.ped) Input file for linkage analysis in SUPERLINK format (COL = Column)

Column 1: Pedigree number
Column 2: Individual number
Column 3: Number of father
Column 4: Number of mother
Column 5: Number of first child
Column 6: Number of next sibling with same father
Column 7: Number of next sibling with same mother
Column 8: Sex - (1 = Male, 2 = female)
Column 9: ignored (value = 0)
Column 10: Phenotypic data (1=unaffected, 2=affected, 0=unknown)
Column 11 - 17: Allele data (numbered)

Translation for ID number in SUPERLINK input format

Animal ID=1 name=M264
Animal ID=2 name=SPOOKIE
Animal ID=3 name=F156
Animal ID=75 name=Q51
Animal ID=81 name=K45
Animal ID=4 name=F278
Animal ID=36 name=F446
Animal ID=5 name=F488
Animal ID=6 name=F489
Animal ID=7 name=M399
Animal ID=8 name=F396
Animal ID=9 name=F397
Animal ID=10 name=F232
Animal ID=11 name=K1
Animal ID=12 name=F006
Animal ID=67 name=F447
Animal ID=13 name=M477
Animal ID=14 name=M478
Animal ID=15 name=Q46
Animal ID=16 name=F196
Animal ID=19 name=F503
Animal ID=17 name=Q56
Animal ID=18 name=K57
Animal ID=20 name=F327
Animal ID=21 name=M224
Animal ID=22 name=F261
Animal ID=24 name=M483
Animal ID=86 name=F317
Animal ID=23 name=M481
Animal ID=25 name=F304
Animal ID=27 name=M458
Animal ID=26 name=F457
Animal ID=28 name=M313
Animal ID=31 name=F515
Animal ID=29 name=F318
Animal ID=30 name=F230
Animal ID=35 name=M444
Animal ID=32 name=M286
Animal ID=33 name=M119
Animal ID=39 name=M455
Animal ID=47 name=F282
Animal ID=34 name=M443
Animal ID=37 name=F303
Animal ID=57 name=F356
Animal ID=38 name=M453
Animal ID=40 name=F473
Animal ID=41 name=F309
Animal ID=42 name=F234
Animal ID=85 name=M475
Animal ID=43 name=F402
Animal ID=44 name=M438
Animal ID=45 name=M440
Animal ID=46 name=M441
Animal ID=63 name=M428
Animal ID=48 name=F419
Animal ID=49 name=M418
Animal ID=50 name=M415
Animal ID=54 name=F539
Animal ID=51 name=F536
Animal ID=52 name=F537

Animal ID=53 name=F538
Animal ID=55 name=M280
Animal ID=59 name=M526
Animal ID=56 name=M467
Animal ID=58 name=M185
Animal ID=76 name=F349
Animal ID=60 name=M262
Animal ID=80 name=F501
Animal ID=61 name=M426
Animal ID=62 name=M427
Animal ID=64 name=M404
Animal ID=65 name=M435
Animal ID=66 name=M436
Animal ID=68 name=F316
Animal ID=69 name=M433
Animal ID=70 name=M434
Animal ID=71 name=K50
Animal ID=72 name=M463
Animal ID=73 name=M464
Animal ID=74 name=M465
Animal ID=77 name=Q24
Animal ID=83 name=K58
Animal ID=78 name=Q54
Animal ID=79 name=M500
Animal ID=89 name=M561
Animal ID=82 name=F543
Animal ID=84 name=M474
Animal ID=87 name=F560
Animal ID=88 name=M559

Pedigree file:

Col 1	Col 2	Col 3	Col 4	Col 5	Col 6	Col 7	Col 8	Col 9	Col 10	Col 11	Col 12	Col 13	Col 14	Col 15	Col 16	Col 17
1	1	2	3	75	0	0	1	0	1	1 1	1 1	1 2	1 2	1 1	1 2	1 2
1	2	0	0	81	0	0	1	0	2	0 0	0 0	0 0	0 0	0 0	0 0	0 0
1	3	0	0	1	0	0	2	0	1	0 0	0 0	0 0	0 0	0 0	0 0	0 0
1	75	1	68	0	74	74	2	0	2	1 1	1 1	1 1	1 4	1 1	3 2	2 2
1	81	2	16	89	60	60	1	0	2	1 1	2 1	1 2	1 3	1 1	1 2	2 2
1	4	0	0	36	0	0	2	0	1	1 1	1 1	1 3	1 3	2 2	3 2	3 4
1	36	32	4	0	35	9	2	0	1	1 1	1 1	1 1	3 3	1 2	3 2	0 0
1	5	1	4	0	0	0	2	0	1	1 1	1 1	1 2	1 2	1 2	1 2	2 4
1	6	1	4	0	5	5	2	0	1	1 1	1 1	1 3	1 1	1 2	2 2	2 3
1	7	1	4	0	6	6	1	0	1	1 1	1 1	1 2	1 3	1 2	3 2	2 3
1	8	1	4	0	7	7	2	0	1	1 1	0 0	0 0	0 0	1 2	2 2	0 0
1	9	1	4	0	8	8	2	0	1	1 1	1 1	1 2	1 3	1 2	3 2	2 3
1	10	11	12	67	0	0	2	0	1	1 1	1 1	1 1	3 4	1 1	3 2	1 2
1	11	0	0	10	0	0	1	0	2	0 0	0 0	0 0	0 0	0 0	0 0	0 0
1	12	0	0	10	0	0	2	0	1	0 0	0 0	0 0	0 0	0 0	0 0	0 0
1	67	60	10	0	66	66	2	0	1	1 1	1 1	1 2	3 4	1 1	3 2	2 4
1	13	1	10	0	9	0	1	0	1	1 1	1 1	1 2	2 4	1 1	1 3	2 2
1	14	1	10	0	13	13	1	0	1	1 1	1 1	1 1	1 4	1 1	3 2	2 2
1	15	2	16	19	1	0	2	0	2	1 1	1 1	1 2	1 3	1 3	1 2	4 4
1	16	0	0	81	0	0	2	0	1	0 0	0 0	0 0	0 0	0 0	0 0	0 0
1	19	1	15	0	18	18	2	0	1	1 1	1 1	1 1	1 1	1 1	1 2	0 0
1	17	1	15	0	14	0	2	0	2	1 1	1 1	1 2	1 2	1 1	1 1	0 0
1	18	1	15	0	17	17	1	0	2	1 1	1 1	1 2	1 2	1 1	1 1	2 4
1	20	21	22	24	0	0	2	0	1	1 1	1 1	1 1	1 4	1 1	1 3	2 4
1	21	0	0	86	0	0	1	0	1	0 0	0 0	0 0	0 0	0 0	0 0	0 0
1	22	0	0	20	0	0	2	0	1	0 0	0 0	0 0	0 0	0 0	0 0	0 0
1	24	1	20	0	23	23	1	0	1	1 1	1 1	1 1	1 4	1 1	3 2	2 2
1	86	21	30	89	68	68	2	0	1	1 1	2 1	1 2	0 0	1 1	1 3	2 2
1	23	1	20	0	19	0	1	0	1	1 1	1 1	1 1	1 1	1 1	1 2	2 4
1	25	0	0	27	0	0	2	0	1	1 1	1 1	1 1	3 4	1 1	1 3	2 4
1	27	1	25	0	26	26	1	0	1	1 1	1 1	1 2	2 4	1 1	1 2	1 2
1	26	1	25	0	24	0	2	0	1	1 1	1 1	1 2	1 3	1 1	1 2	2 4
1	28	0	0	31	0	0	1	0	1	1 1	2 2	1 1	3 3	1 2	3 2	2 2
1	31	28	29	0	0	0	2	0	1	1 2	0 0	1 2	3 3	1 1	1 3	1 2
1	29	21	30	35	20	0	2	0	1	1 2	1 1	2 2	3 3	1 1	1 1	1 2
1	30	0	0	86	0	0	2	0	1	0 0	0 0	0 0	0 0	0 0	0 0	0 0
1	35	32	29	0	34	34	1	0	1	1 2	1 1	2 2	3 3	1 1	1 2	2 4
1	32	33	16	39	0	15	1	0	1	1 1	2 1	1 2	3 3	1 1	2 2	4 4
1	33	0	0	47	0	0	1	0	1	0 0	0 0	0 0	0 0	0 0	0 0	0 0
1	39	32	37	0	38	38	1	0	1	1 1	2 1	1 1	1 3	1 2	1 2	4 4
1	47	33	16	63	41	32	2	0	1	1 1	2 1	1 2	3 3	1 3	3 2	2 4
1	34	32	29	0	0	31	1	0	1	1 2	1 1	2 2	3 3	1 1	1 2	2 4
1	37	0	0	57	0	0	2	0	1	1 1	1 1	1 1	1 1	1 2	1 2	3 4
1	57	58	37	59	0	40	2	0	1	1 1	1 1	1 2	0 0	2 3	1 2	2 4
1	38	32	37	0	36	0	1	0	1	1 1	1 1	1 2	1 3	1 1	1 2	4 4
1	40	1	37	0	27	39	2	0	1	1 1	1 1	1 2	1 1	1 2	2 2	2 3
1	41	33	42	85	32	0	2	0	1	1 1	2 1	1 2	1 3	1 1	1 3	2 4
1	42	0	0	41	0	0	2	0	1	0 0	0 0	0 0	0 0	0 0	0 0	0 0
1	85	81	41	0	84	84	1	0	1	1 1	1 1	1 2	1 3	1 1	1 1	0 0
1	43	1	41	0	40	0	2	0	1	1 1	2 1	1 2	3 2	1 1	1 3	2 2

1	44	1	41	0	43	43	1	0	1	1 1	1 1	1 2	1 2	1 1	1 1	2 4
1	45	1	41	0	44	44	1	0	1	1 1	2 1	1 2	1 1	1 1	3 2	2 2
1	46	1	41	0	45	45	1	0	1	1 1	1 1	1 2	1 1	1 1	1 2	2 4
1	63	60	47	0	62	62	1	0	1	1 1	1 1	2 2	1 3	1 3	1 2	2 4
1	48	1	47	0	46	0	2	0	1	1 1	2 1	1 2	1 3	1 1	2 2	2 4
1	49	1	47	0	48	48	1	0	1	1 1	0 0	1 2	1 3	1 1	2 2	0 0
1	50	0	0	54	0	0	1	0	1	1 1	1 1	1 2	2 4	1 2	3 3	2 2
1	54	50	47	0	53	53	2	0	1	1 1	2 1	1 1	3 4	1 1	3 2	2 4
1	51	50	47	0	0	49	2	0	1	1 1	2 1	1 1	3 4	1 3	3 2	2 4
1	52	50	47	0	51	51	2	0	1	1 1	0 0	1 2	3 4	2 3	3 2	2 4
1	53	50	47	0	52	52	2	0	1	1 1	0 0	2 2	0 0	1 3	3 2	2 4
1	55	0	0	59	0	0	1	0	1	1 1	1 1	2 2	3 3	1 2	1 2	2 4
1	59	55	57	0	56	0	1	0	1	1 1	1 1	1 2	3 3	1 2	1 1	2 2
1	56	55	47	0	0	54	1	0	1	1 1	1 1	2 2	3 3	2 3	3 2	4 4
1	58	0	0	76	0	0	1	0	1	0 0	0 0	0 0	0 0	0 0	0 0	0 0
1	76	58	77	83	57	0	2	0	1	1 2	1 1	2 2	3 2	1 3	1 2	2 4
1	60	2	16	80	15	47	1	0	1	1 1	2 1	1 2	1 4	1 1	1 2	2 4
1	80	60	76	0	79	79	2	0	1	1 2	1 1	1 2	2 4	1 1	1 2	2 2
1	61	60	47	0	0	56	1	0	1	1 1	2 1	1 1	1 3	1 1	2 2	2 4
1	62	60	47	0	61	61	1	0	1	1 1	2 1	1 1	1 3	1 1	2 2	2 4
1	64	60	10	0	63	14	1	0	1	1 1	2 1	1 1	4 4	1 1	1 2	2 2
1	65	60	10	0	64	64	1	0	1	1 1	2 1	1 1	3 4	1 1	3 2	2 4
1	66	60	10	0	65	65	1	0	1	1 1	2 1	1 1	4 4	1 1	3 2	2 4
1	68	21	30	75	29	29	2	0	1	1 2	2 1	1 1	2 4	1 1	1 3	1 2
1	69	60	68	0	67	0	1	0	1	1 1	2 1	1 1	1 4	1 1	1 3	2 4
1	70	60	68	0	69	69	1	0	1	1 1	0 0	1 1	4 4	1 1	1 3	1 2
1	71	60	68	0	70	70	1	0	2	1 1	2 1	1 1	4 4	1 1	1 2	1 2
1	72	1	68	0	49	71	1	0	1	1 1	2 1	1 2	0 0	1 1	1 2	2 2
1	73	1	68	0	72	72	1	0	1	1 1	2 1	1 2	1 2	1 1	1 1	2 2
1	74	1	68	0	73	73	1	0	1	1 1	2 1	1 2	1 2	1 1	1 3	2 2
1	77	0	0	76	0	0	2	0	2	0 0	0 0	0 0	0 0	0 0	0 0	0 0
1	83	81	76	0	82	82	1	0	2	1 2	1 1	2 2	3 2	1 1	1 1	2 4
1	78	60	76	0	71	0	2	0	2	1 1	1 1	1 2	1 3	1 3	2 2	2 4
1	79	60	76	0	78	78	1	0	1	0 0	0 0	0 0	0 0	0 0	0 0	0 0
1	89	81	86	0	88	88	1	0	1	1 1	0 0	0 0	0 0	1 1	1 1	0 0
1	82	81	76	0	0	80	2	0	1	1 1	2 1	2 2	1 3	1 1	1 2	0 0
1	84	81	41	0	83	46	1	0	1	1 1	2 1	1 1	1 3	1 1	3 2	2 2
1	87	81	86	0	85	0	2	0	1	1 1	0 0	0 0	0 0	1 1	3 2	0 0
1	88	81	86	0	87	87	1	0	1	1 1	0 0	0 0	0 0	1 1	1 3	0 0

IX.   Appendix

Locus file 1 (population-wide gene frequencies from marker data):

0 0.0 0.0 0 << MUT LOCUS, MUT RATE, HAPLOTYPE FREQUENCIES (IF 1)

1 2 3 5 4 8 7 6 <<ORDER OF LOCI FROM 2009 AUTOSOMAL LINKAGE MAP, FCA810 and FCA813 disputed

1 2#aff# << AFFECTION, NO. OF ALLELES

0.7889 0.2111 << GENE FREQUENCIES (population-wide gene frequencies from marker data):

1 << NO. OF LIABILITY CLASSES

0 0 1 << PENETRANCES

3 2#FCA023# << ALLELE NUMBERS, NO. OF ALLELES

0.9545 0.0455 << GENE FREQUENCIES

3 2#FCA809# << ALLELE NUMBERS, NO. OF ALLELES

0.2111 0.7889 << GENE FREQUENCIES

3 3#FCA810# << ALLELE NUMBERS, NO. OF ALLELES

0.5842 0.4 0.0158   << GENE FREQUENCIES

3 4#FCA811# << ALLELE NUMBERS, NO. OF ALLELES

0.3478 0.3478 0.1359 0.1685 << GENE FREQUENCIES

3 3#FCA700# << ALLELE NUMBERS, NO. OF ALLELES

0.8333 0.1111 0.0556 << GENE FREQUENCIES

3 3#FCA254# << ALLELE NUMBERS, NO. OF ALLELES

0.3737 0.197 0.4293 << GENE FREQUENCIES

3 4#FCA813# << ALLELE NUMBERS, NO. OF ALLELES

0.1056 0.5278 0.0444 0.3222 << GENE FREQUENCIES

0 0 << SEX DIFFERENCE, INTERFERENCE (IF 1 OR 2)-NO DIFFERENCE, NO INTERFERENCE

0.37 0.22 0.1 0.49 0.37 0.22 0.47 << RECOMBINATION VALUES AS PER CRI-MAP VER.2.4 UCD MOD

1

-n 3 1 7 -o 10.0000

X.   Appendix

Locus file 2 (gene frequencies equal):

8 0 0 8 0 << NO. OF LOCI, RISK LOCUS, SEXLINKED (IF 1) PROGRAM, # COMPLEX LOCI

0 0.0 0.0 0 << MUT LOCUS, MUT RATE, HAPLOTYPE FREQUENCIES (IF 1)

1 2 3 5 4 8 7 6

1 2#aff# << AFFECTION, NO. OF ALLELES

0.999 0.001 << GENE FREQUENCIES

1 << NO. OF LIABILITY CLASSES

0 0 1 << PENETRANCES

3 2#FCA023# << ALLELE NUMBERS, NO. OF ALLELES

0.5 0.5 << GENE FREQUENCIES

3 2#FCA809# << ALLELE NUMBERS, NO. OF ALLELES

0.5 0.5 << GENE FREQUENCIES

3 3#FCA810# << ALLELE NUMBERS, NO. OF ALLELES

0.333333 0.333333 0.333333   << GENE FREQUENCIES

3 4#FCA811# << ALLELE NUMBERS, NO. OF ALLELES

0.25 0.25 0.25 0.25 << GENE FREQUENCIES

3 3#FCA700# << ALLELE NUMBERS, NO. OF ALLELES

0.333333 0.333333 0.333333 << GENE FREQUENCIES

3 3#FCA254# << ALLELE NUMBERS, NO. OF ALLELES

0.333333 0.333333 0.333333 << GENE FREQUENCIES

3 4#FCA813# << ALLELE NUMBERS, NO. OF ALLELES

0.25 0.25 0.25 0.25 << GENE FREQUENCIES

0 0 << SEX DIFFERENCE, INTERFERENCE (IF 1 OR 2)

0.1 0.1 0.1 0.1 0.1 0.1 0.1 << RECOMBINATION VALUES

1

-n 3 1 7 -o 10.0000

XI.   Appendix

Trait vs. markers using actual population-wide allele frequency

Marker information	Recombination fraction						
Test linkage	**0.00**	**0.01**	**0.05**	**0.10**	**0.20**	**0.30**	**0.40**
1 *King* vs. FCA023	-Infinity	-2.0004	-1.2286	-0.8273	-0.3761	-0.1364	-0.0223
2 *King* vs. FCA809	-Infinity	-1.1566	-0.4855	-0.2256	-0.0324	**0.0112**	**0.0041**
3 *King* vs. FCA811	-Infinity	-4.8894	-2.2140	-1.2218	-0.5089	-0.2627	-0.1161
4 *King* vs. FCA810	-Infinity	-1.8265	-1.0884	-0.7375	-0.3603	-0.1524	-0.0405
5 *King* vs. FCA813	-Infinity	-2.2806	-0.5125	-0.0016	**0.1914**	**0.1281**	**0.0501**
6 *King* vs. FCA254	-Infinity	-7.1784	-3.8340	-2.5011	-1.2551	-0.5710	-0.1759
7 *King* vs. FCA700	-Infinity	-3.5664	-2.2753	-1.5874	-0.8417	-0.4136	-0.1486

XII.   Appendix

Trait vs. markers assuming all alleles have equal frequency

| Marker information	Recombination fraction						
Test linkage	**0.00**	**0.01**	**0.05**	**0.10**	**0.20**	**0.30**	**0.40**
1 *King* vs. FCA023	-Infinity	-2.0512	-1.2607	-0.8382	-0.3679	-0.1254	-0.0147
2 *King* vs. FCA809	-Infinity	-1.2567	-0.5368	-0.2418	-0.0213	**0.0259**	**0.0139**
3 *King* vs. FCA811	-Infinity	-4.8389	-2.1970	-1.2284	-0.5295	-0.2710	-0.1146
4 *King* vs. FCA810	-Infinity	-1.9045	-1.1509	-0.7843	-0.3828	-0.1582	-0.0381
5 *King* vs. FCA813	-Infinity	-2.1010	-0.3383	**0.1617**	**0.3208**	**0.2152**	**0.0935**
6 *King* vs. FCA254	-Infinity	-7.2449	-3.8778	-2.5346	-1.2958	-0.6128	-0.2013
7 *King* vs. FCA700	-Infinity	-2.5214	-1.5768	-1.0948	-0.5826	-0.2873	-0.1021

XIII.   Appendix

Trait vs. markers using actual population-wide allele frequency (SUPERLINKMAP program code4)

Marker information	Recombination fraction						
Test linkage	**0.00**	**0.01**	**0.05**	**0.10**	**0.20**	**0.30**	**0.40**
1 *King* vs. FCA023	-Infinity	-1.9866	-1.2145	-0.8131	-0.3656	-0.1313	-0.0209
2 *King* vs. FCA809	-Infinity	-1.1700	-0.4893	-0.2232	-0.0282	**0.0129**	**0.0041**
3 *King* vs. FCA811	-Infinity	-4.7693	-2.1291	-1.1637	-0.4776	-0.2397	-0.1026
4 *King* vs. FCA810	-Infinity	-1.9367	-1.1804	-0.8093	-0.3997	-0.1692	-0.0440
5 *King* vs. FCA813	-Infinity	-2.1419	-0.3774	**0.1242**	**0.2867**	**0.1874**	**0.0767**
6 *King* vs. FCA254	-Infinity	-7.2404	-3.8821	-2.5452	-1.3062	-0.6159	-0.2006
7 *King* vs. FCA700	-Infinity	-3.4758	-2.1613	-1.4986	-0.7947	-0.3911	-0.1402

XIV. Appendix

Graph plotting Ln (Likelihood) values over a 15 cM region on either side of the first marker

Results of MultiPoint Analysis
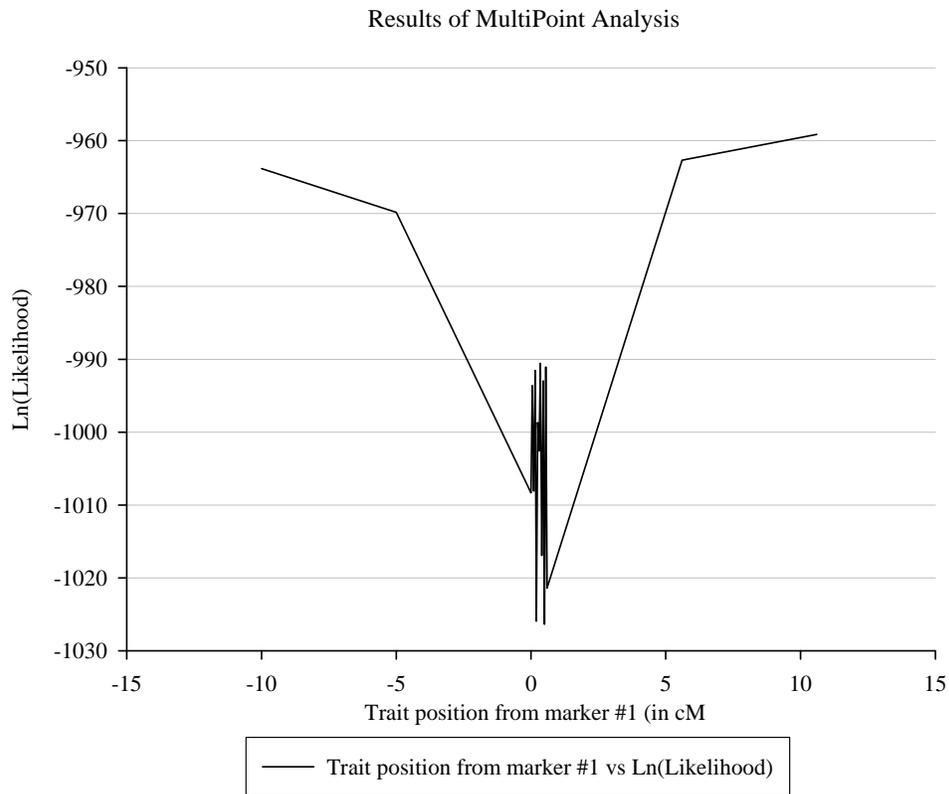
XV.   Appendix

Ln (Likelihood) and LOD scores over a 15 cM region on either side of the first marker

Trait position from marker #1 (in cM)	Ln (Likelihood)	LOD-SCORE
-10.0000	-963.8269	-4.8137
-5.0000	-969.8305	-7.4210
0.0000	-1008.3285	-24.1405
0.0500	-993.6198	-17.7526
0.1000	-1008.0865	-24.0354
0.1500	-991.5352	-16.8473
0.2000	-1025.9508	-31.7938
0.2500	-998.6695	-19.9456
0.3000	-1002.5851	-21.6462
0.3500	-990.5688	-16.4276
0.4000	-1016.9340	-27.8778
0.4500	-992.9511	-17.4622
0.5000	-1026.3441	-31.9646
0.5500	-991.0712	-16.6458
0.6000	-1021.3892	-29.8127
5.6000	-962.6800	-4.3156
10.6000	-959.1331	-2.7752

The input used by Superlink to generate these results:
Mode of inheritance (MOI) = Recessive 0 0 0.99
Number of markers 7
Distances between markers = 0.1, 0.1, 0.1, 0.1, 0.1, 0.1
Disease mutant gene frequency = 0.01
Marker Names = FCA023,FCA809,FCA811,FCA810,FCA700,FCA254,FCA813
Points to calculate LOD score = -n 2 1 7 -o 10.0000

XVI. Appendix
Computational difficulties when mode of inheritance is set as dominant

Trait position from marker #1 (in cM)	Ln (Likelihood)	LOD-SCORE
-10.0000	-946.1034	-2.3391
-9.5000	-946.2451	-2.4007
-9.0000	skipped: too hard	skipped: too hard
-8.5000	skipped: too hard	skipped: too hard
-8.0000	skipped: too hard	skipped: too hard
-7.5000	-946.8864	-2.6792
-7.0000	skipped: too hard	skipped: too hard
-6.5000	-947.2634	-2.8429
-6.0000	skipped: too hard	skipped: too hard
-5.5000	-947.6912	-3.0287
-5.0000	-947.9290	-3.1319
-4.5000	-948.1860	-3.2436
-4.0000	-948.4662	-3.3653
-3.5000	skipped: too hard	skipped: too hard
-3.0000	skipped: too hard	skipped: too hard
-2.5000	-949.5041	-3.8160
-2.0000	-949.9510	-4.0101
-1.5000	skipped: too hard	skipped: too hard
-1.0000	skipped: too hard	skipped: too hard
-0.5000	skipped: too hard	skipped: too hard
0.0000	skipped: too hard	skipped: too hard

The input used by Superlink to generate these results:
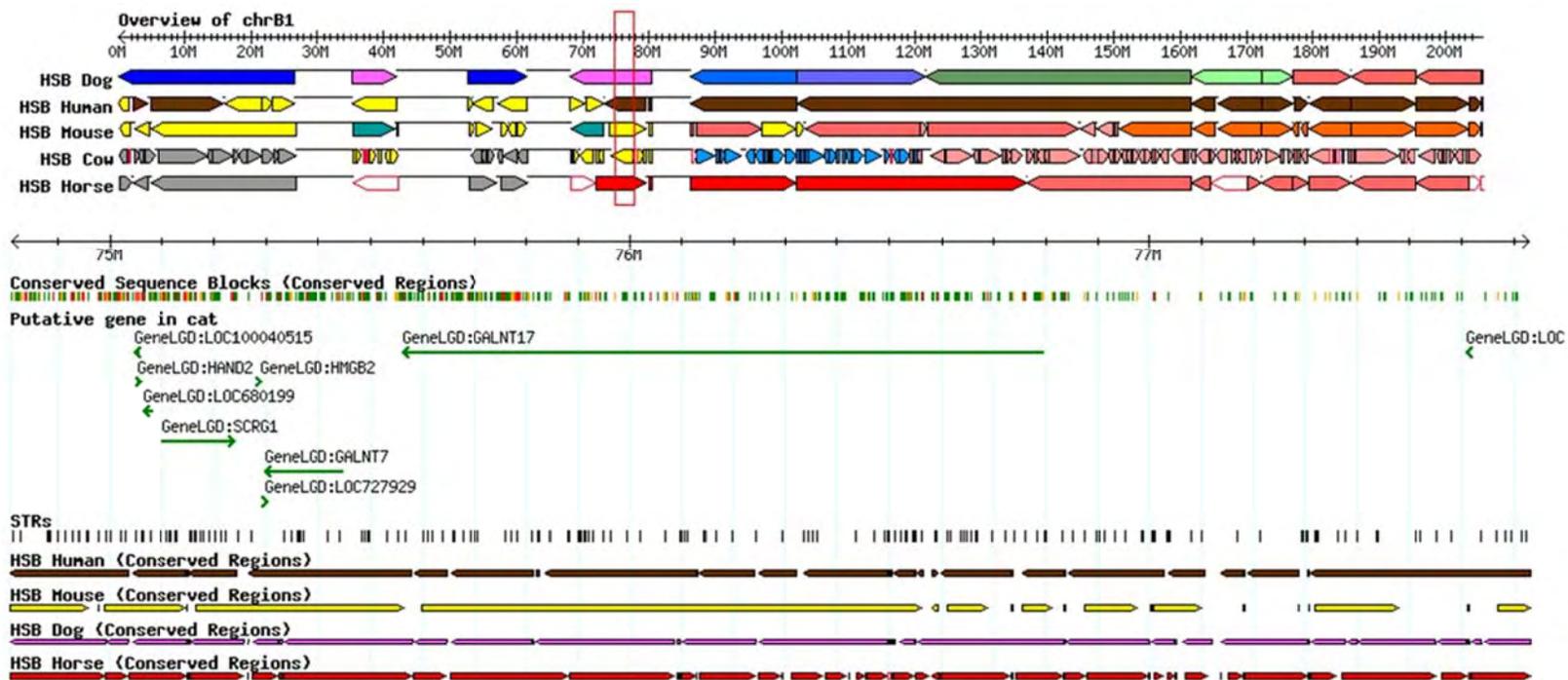Mode of inheritance (MOI) = Dominant 0 0.2 0.9
Number of markers 7
Distances between markers = 0.1, 0.1, 0.1, 0.1, 0.1, 0.1
Disease mutant gene frequency = 0.01
Marker Names = FCA023, FCA809, FCA811, FCA810, FCA700, FCA254, FCA813
Points to calculate LOD score = -s 0.5000 1 2 -o 10.0000

GARField: Genome Annotation Resource Field *Felis catus* v12.2 view of the region (Pontius & O'Brien 2007) spanning 2.918 Mbp between FCA254 (position 77729549) and FCA700 (position 74811696)

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