IDENTIFICATION OF INDIVIDUAL CHEETAHS (*Acinonyx jubatus*) REPRESENTED IN A SAMPLE COLLECTION, COMBINING NON-INVASIVE GENETIC AND CAMERA-TRAPPING TECHNIQUES.

A THESIS SUBMITTED IN FULFILMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

OF

THE UNIVERSITY OF NAMIBIA

BY

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200611411

July 2015

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Abstract

Namibia holds the largest free-ranging population of cheetah (*Acinonyx jubatus jubatus*) in the world, a population estimated to be about 3,000 adult individuals. The cheetah is an elusive species living at low densities, making it hard to assess their abundance and density. Traditional detection methods such as direct observation, physical mark recapture, and invasive collection of tissue samples (e.g. blood) requires the physical handling of individuals. Non-invasive techniques such as remote camera traps and genetic analysis of scat samples do not require handling of individuals as these rely on the presence and detection (as well as collection) of animal signs. The study aim was to demonstrate that a complete individual profile can be obtained using non-invasive techniques. This was achieved by obtaining genetic profiles from 56 scat samples collected around remote camera trap stations in the study area between August 2008 and January 2014. These samples were genotyped with three microsatellite markers for individual identification, confirming the presence 10 individual cheetahs. Each individual was genotyped with another 13 microsatellite loci, which were then used for relatedness assessment, identifying three related groups. Individuals inferred from the genetic profiles were successfully assigned to physical profiles by comparison to a database of physical identities obtained from camera trap pictures during the same time period. In conclusion, the study shows that it is possible to obtain complete genetic and physical profiles, as well as pedigree information for an individual by using scat DNA data and photographs from remote camera traps.

ACRONYMS

CITES: Convention on International Trade in Endangered Species of wild Fauna and Flora

MHC: Major Histocompatibility Complex

DNA: Deoxyribonucleic Acid

nDNA: nuclear Deoxyribonucleic Acid

mDNA: mitochondrial Deoxyribonucleic Acid

PCR: Polymerase chain reaction

ATP: Adenosine Triphosphate

IUCN: International Union for Conservation of Nature

IBD: Identical By Descent

SNP: Single Nucleotide Polymorphism

AFLP: Amplified Fragment Length Polymorphism

RAPD: Random Amplification of Polymorphic DNA

RFLP: Restriction Fragment Length Polymorphisms

MER: Moment Estimate of Relatedness

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Acknowledgement

I would like to express my special appreciation and thanks to my supervisors (advisor) Dr Schmidt-Küntzel, Dr Naomab and Dr Kahaka, you have been tremendous mentors for me. I would like to thank you for encouraging and allowing me to grow as a research scientist. I would like to thank you for all your guidance and for going an extra mile, believing in me and give me the opportunity to learn, I will forever remain grateful to you.

I would like to thank the Cheetah Conservation Fund (CCF), in particular Dr Marker for giving me the opportunity to do my masters with you and for all forms of support. I would also like to thank all of my friends for support. I would like to thank everyone who helped me with writing and any type of support.

Dedication

This piece of work is dedicated to my dear loving parents (Rauha Namulo and Titus Amugulu) for all the love, support and guidance that helped mould me into the person I am today. This thesis is also dedicated to my brothers for all the love that you have given me.

Declaration

I, Lusia Mhuulu, hereby declare that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

…… Date ……

Chapter 1: Introduction

1.1 Orientation of the study

The level of biodiversity on the Earth is rapidly declining mainly due to anthropogenic factors. Large numbers of species have already become extinct, while many others have been reduced to population sizes that put them at risk of extinction (Frankham *et al.,* 2002). The cheetah (*Acinonyx jubatus*) is classified as an endangered species under IUCN guidelines by fulfilling the criteria of reduced population sizes. Within their global range, Namibia represents a particular stronghold for the species with one of the largest remaining cheetah populations (Marker-Kraus *et al.*, 1996; Purchase, Marker, Marnewick, Klein, & Williams, 2007). As is the case in other parts of Africa, this population has decreased up to 50% in the 1980s as over 6700 individuals were trapped and killed as vermin (CITES 1992).

The situation of the cheetah is further aggravated by the species' low genetic variation relative to other carnivores, even between cheetah populations (Menotti-Raymond & O'Brien, 1995; O'Brien *et al.*, 1987; Driscoll *et al.*, 2002). Without sufficient genetic variation, a population may not be able to respond fast enough to changing environmental variables and as a result may face an increased risk of extinction. For instance, if a population is exposed to a new disease, positive selection pressures will act on those individuals with genes for resistance to the disease if they exist in the population. In contrast, if sufficient genetic variation is not present, any reduction in biological fitness incurred by a particular gene is likely to affect the majority of the population, potentially resulting in high levels of mortality within the population and so it will not evolve and could be wiped out by the disease. There is one documented case where six of

seven wild cheetahs in Etosha national park, Namibia died after eating prey contaminated with anthrax (Ray, Justina, Luke, and Joanna, 2005).

1.2 Statement of the problem

Currently there is limited information on the genetic variation of the Namibian cheetah population (Marker *et al*., 2008a; Driscoll *et al*., 2002; Yuhki and O'Brien, 1990; Castro-Prieto, Wachter and Sommer, 2011). Marker *et al*., (2008) looked at the geographic patterns of molecular genetic diversity of wild cheetah population throughout Namibia. In addition, the basis of social behaviour of Namibian cheetahs was investigated by assessing relatedness within known social groups including; male coalitions, females with cubs, and sibling groups, using microsatellite variation. Driscoll *et al*., (2002) assessed/evaluated the patterns of genomic diversity using 90 feline-specific microsatellite loci among well-defined populations of three species of felidae (cheetahs, pumas, and lions), to determine the ability of a large sampling of microsatellites to detect historic population reductions. Castro-Prieto *et al*., (2011) examined the genetic diversity in major histocompatibility complex class I (MHC I) and MHC class II loci on free-ranging Namibian cheetahs. The MHC genes are responsible for the adaptive immune response in vertebrates and are thereby involved in modulating host resistance to emerging pathogens (Eizaguirre, Lenz, Kalbe, and Milinski, 2012). Immunocompetence is influenced by environmental factors as well as genetic factors such as those underpinning the MHC because they govern the nature of cell surface molecules which are vital attachment points for the body's natural defences to disease (Frankham *et al*., 2002; Castro-Prieto *et al*., 2011).

The cheetah is an elusive species, which means there is often only a low probability of detecting it; this in turn can make collecting the data needed to inform effective conservation management decisions difficult. In such cases, gathering data can often require at least some degree of

invasive methodology such as capture prior to fitting a collar for instance. However non-invasive methods can be valuable in studying this species (Kohn *et al*., 1999; Banks *et al*., 2003; Frantz *et al*., 2003, 2004). There are three main non-invasive methodologies which are commonly used, the first of which is camera traps which allow identification of individuals based on their unique pattern morphology. Camera traps are passive detectors with automatic cameras attached to heat-motion sensors that trigger a photograph when an animal pass through the (infrared) sensor beams (Boitani, and Powell, 2012). Camera trapping provides information on abundance or density (population estimates), gender, behaviour and health (physical) (Kelly, 2008). The second non-invasive technique is the collection of samples for genetic analyses i.e., the extraction of DNA and determination of DNA profiles for individual identification from shed material such as excretion (scat), hair, feathers, urine, eggshells, and saliva from chewed materials (Beja-Pereira *et al*., 2009). Scat samples provide a spectrum of information including genetic profile as well as general fitness and condition of the individuals. For instance scat samples provide information on gender (sex), relatedness, paternity/maternity (parentage), presence of inheritable disease, parasite load, hormone levels, variation within and between groups and estimates of numbers of animals (if study design and samples number allows for this). Finally, tracking an animal's movements using footprints on the ground (spoor) can also be an effective means of data collection; however this can be very intensive in terms of resource requirements.

Combining any of these methods permits a more comprehensive identity including physical and genetic profile, as well as health information, of individuals without using any invasive technique and therefore having any effect on the study animal. The present study is the first to combine two non-invasive approaches: non-invasive genetic analysis of scat samples together

with camera trapping to obtain complete information regarding the identity of individuals. In this study, non-invasive genetic analysis of scat samples collected in the wild, around camera trap stations was used. This interdisciplinary approach was made possible using existing camera trap data which was collected as part of a predator census survey being conducted at CCF which could then be supplemented with the genetic profiles developed for this study.

1.3 Study aim and objectives

The study aim is the use of non-invasive techniques to obtain more comprehensive individual profiles than is possible using either one technique alone.

Specific Objectives

1. To determine number of individual's cheetah present in a sample collection from the study area.
2. To assign genetic profile of each cheetah to available visual ID obtained from remote camera traps.
3. To assess the relatedness of cheetahs within the study area.

1.4 Hypothesis of the study

Non-invasive techniques can provide complete individual profiles for elusive endangered species (cheetah).

1.5 Significance of the study

Long-term wildlife monitoring programs (such as relatedness, population estimates, home range and territory) require methods that can efficiently produce reliable data, and genetic monitoring has also been proven to be a useful tool (Schwartz *et al.,* 2006, Stenglein *et al.,* 2010). Data collected in this study will help researchers to study the effect of potential translocation or

reintroduction of cheetah to the study area. Additionally, non-invasive methods are important because they provide reliable data to answer key conservation, such as abundance and density, population structure, demographic history, behavioural ecology and wildlife management questions without the need of invasive methods which could alter the behaviour of the subject animal(s). To our knowledge, no published study has combined the two non-invasive techniques (genetic and camera traps) to obtain a complete (genetic and physical) profile of an animal/individual under study. Gopalaswamy, *et al*., 2012 combined the two non-invasive approaches to estimate tiger density and could not associate scat samples to corresponding photographs, due to no overlap in the data collection between the two approaches.

1.6 Limitation of the study

Ideally, this study would include cheetahs from all 13 regions of Namibia. However, scat samples are not easy to find due to the elusiveness of the species. Moreover, it would be time consuming and costly to cover the entire country, and since this study is limited to two years and a set budget, this will not be possible. Therefore this study focused on a subset of the population located in the study area. Another limitation of the study is the fact that it is not possible to collect scat from every single individual from the study area, therefore a census will not be possible; however it was possible to compare the number of individuals identified using a genetic approach to the number of individuals identified using camera trapping.

CHAPTER 2: LITERATURE REVIEW

2.1 Cheetah background

The cheetah (*Acinonyx jubatus*) is a specialized member of the Felidae and in the order Carnivora. It is the only existing member of the genus *Acinonyx*. There are five subspecies of cheetah, based on geographical range, includes the southern Africa (*Acinonyx jubatus jubatus*) is found in Namibia, Botswana, Zimbabwe and South Africa; the eastern Africa cheetah (*A. j. raineyii*) found in Kenya, Uganda, and Tanzania; the western Africa (*A. j. hecki*) found in Senegal, Southern Mauritania, Burkina Faso, southern Mali, and northern Benin; the central Africa (*A. j. soemmeringii*) found in Sudan, Chad, Ethiopia, central Africa republic and Niger; and the Asiatic cheetah (*A. j. venaticus*) (O`Brien *et al*., 1986; Freeman *et al*., 2001). The cheetah is well-known as the fastest land animal that can reach the speed of 110km/h (Freeman *et al*., 2001).

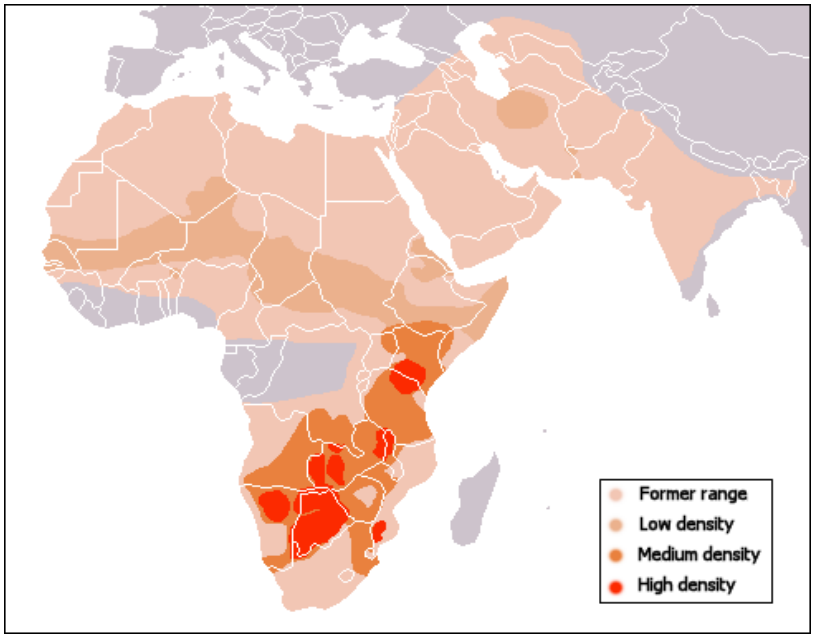

Figure 1: Geographic distribution of cheetahs (www.marcoevolution.net)

Cheetahs are phenotypically characterized by their pale yellow coats that are covered in small round black spots and white underbellies. Their black tear-lines are also distinctive. Female cheetahs are often solitary and males usually form coalitions (sibling or non related males) of four to five individuals. Cheetahs have large overlapping home ranges. They use scat and urine to maintain their territories (Caro, 1994; Marker, 2002).

Formerly cheetahs were found across Africa, Asia and the Middle East (Marker, 1998; Marker *et al*., 2008). However, their range has declined severely during the past century (Marker *et al*., 2008). Currently, a small population remains in Iran while eastern and southern Africa hold the largest wild populations (Marker, 1998; Purchase, Marker, Marnewick, Klein, & Williams, 2007; Marker *et al.* 2008).

Today, the northern central region of Namibia hosts the largest free-ranging cheetah population in the world, with an adult population size estimate of about 3000 individuals (this is about a third of cheetah population in the world) (Marker *et al*., 2008; Purchase *et al*., 2007), with most of them roaming around in commercial farmland, not in protected areas (Marker-Kraus *et al*., 1996). The majority of these populations are threatened due to various factors such as competition from other large carnivores (lion *Panthera leo* and spotted hyena *Crocuta crocuta*), habitat loss-due to urbanization, poaching, and extensive killing of cheetah by farmers to protect livestock outside protected areas (human-wildlife conflict) (Nowell and Jackson, 1996; Marker and Schumann, 1998; Marker *et al*., 2003; Marker *et al.*, 2008).

Cheetah is classified as vulnerable or endangered by the world conservation union red list 2014 (IUCN) and regulated by Convention for International Trade in Endangered Species of Wild Flora and Fauna (CITES) as appendix I. The state of the cheetah is become progressively worse due to the species' low genetic diversity, as compared to other carnivores (O'Brien *et al*., 1983,

1985, 1987; Driscoll *et al*., 2002). Despite their low genetic diversity, this seems not affecting the wild population yet (Ray, *et al*., 2005).

2.2 Cheetah genetic diversity

Genetic studies on cheetahs have found the species to have lower levels of genetic diversity compared to other species (O'Brien *et al*., 1983, 1985, 1987). Menotti-Raymond and O'Brien 1995; Dalton *et al*., 2013, report genetic diversity indexes that are 10 to 100 times less in comparison to other felids such as tiger, puma, jaguar, leopard. Lack of genetic diversity is thought to be associated with a variety of deleterious effects such as increased susceptibility to pathogens, reduced reproductive success, and poor ability to respond to environmental perturbations (O'Brien & Evermann, 1988). Although some possible effects of genetic homogeneity such as poor sperm quality in males (O'Brien *et al*., 1983; 1985) have been demonstrated, there is scant evidence that it has impacted wild populations (Caughley, 1994). Evidence of epidemics in wild populations, however, is limited to one incident in Etosha National Park, Namibia in which anthrax killed six of seven radio-collared cheetahs that were presumed to have fed on anthrax infected prey (Lindeque *et al*., 1998; Ray *et al*., 2005).

2.3 Genetic diversity

Genetic diversity is the variation of heritable characteristics present in a population of the same species (Vellend and Geber, 2005; Frankham, Ballou, and Briscoe, 2010). Genetic diversity can be measured in terms of the number of genetic variants (alleles) present in a gene pool (allelic diversity) or in terms of homo and heterozygosity of individuals (Hughes *et al*., 2008). The importance of genetic diversity is based on two aspects: 1) resistance and adaptability; and 2) the expression of deleterious recessive alleles (Reed and Frankham, 2003). Genetic diversity provides the basis for adaptability (through natural selection) as environmental condition change

(Reed and Frankham, 2003). Environments do not stay constant- variables that may change would include new diseases and new food sources. If a population lacks genetic diversity, it is in great danger of not having the resources to survive environmental change (Frankham, 2005; Allendorf, and Luikart, 2007). Many deleterious conditions are based on recessive alleles. The condition is therefore only expressed when an individual is homozygous at the locus (Reed and Frankham, 2003). In a population with a low level of genetic diversity, the probability of individuals having two deleterious recessive alleles at any particular locus is greatly increased (Reed and Frankham, 2003; Frankham, 2005).

Among the prime causes of loss of genetic diversity are genetic bottlenecks and founder effectss. A genetic bottleneck occurs when a population (or species) is reduced to a few reproductive individuals whose offspring then increase in numbers to re-establish the population (Frankham *et al*., 2002; Allendorf, and Luikart, 2007). While the number of individuals in the population may be restored, genetic diversity or part of it may be permanently lost (Allendorf, and Luikart, 2007). The founder effect is based in the fact that a small sample may differ substantially in composition from the larger population it is draw from, purely random "sampling error". When a population is started or found using a small number of individuals, there is a risk of losing genetic diversity, i.e. the new population may not contain the full range of variation that occurred in the ancestral population (Allendorf and Leary, 1986; Frankham *et al*., 2002; Frankham, Ballou, and Briscoe, 2010).

Loss of genetic diversity is often associated with a reduction in fitness or inbreeding depression- when related individuals mate. The maintenance of representative levels of genetic diversity is therefore a primary objective in the management of wild and captive populations Frankham, Ballou, and Briscoe, 2010.

Loss of genetic diversity may reduce the potential of a population to respond to selective pressure (Allendorf & Leary, 1986; Frankham *et al*., 2002), and increased consanguinity may lead to a reduction in population viability (Madsen *et al*., 1996; Newman & Pilson, 1997; Ralls *et al*., 1988). Low genetic diversity represents a population or a species with high levels of shared alleles across their genome. Endangered species usually have lower levels of genetic diversity than related, non-endangered species (Frankham *et al*., 2002; Hoglund, 2009). High genetic diversity is high extent or frequency of genetic variation within a population (Hoglund, 2009).

2.3.1 Conservation genetics

Conservation genetics deals with the effects of loss of genetic diversity and recent changes in genetic structuring in the long-term survival of endangered species (Frankham *et al*., 2002; Frankham, Ballou, and Briscoe, 2010). Because endangered species have small and/ or declining populations, this will lead to inbreeding and loss of genetic diversity resulting in elevated extinction risks (Hoglund, 2009; Allendorf, Luikart, and Aitken, 2012).

Conservation genetics deals with the genetic factors that affect extinction risk and genetic management regimes required to minimize these risks (minimize the risk of extinction from genetic factors) (Frankham, Ballou, and Briscoe, 2010). There are 11 major genetic issues in conservation biology:

- The deleterious effects of inbreeding on reproduction and survival (inbreeding depression)
- Loss of genetic diversity and ability to evolve in response to environmental change
- Fragmentation of populations and reduction in gene flow

- Random process (genetic drift) overriding natural selection as the main evolutionary process
- Accumulation and loss (purging) of deleterious mutations
- Genetic adaptation to captivity and its adverse effects in reintroduction success
- Resolving taxonomic uncertainties
- Defining management units within species
- Use of molecular genetics analyses to understand aspects of species biology important to conservation
- Deleterious effects on fitness that sometimes occur as a result of out-crossing (out breeding depression)

The effects of small population size are of major concern in conservation biology, since endangered species have small and/ or declining populations (Frankham, Ballou, and Briscoe, 2010). Small populations suffer from inbreeding and loss of genetic diversity. Inbreeding reduces fecundity and survival and so directly increases extinction risk (Hoglund, 2009; Allendorf, Luikart, and Aitken, 2012). Genetic diversity is the extent of heritable variation in a population, or species, or across a group of species e.g. heterozygosity or number of alleles, or heritability. Reduced genetic diversity compromises the ability of populations to evolve to cope with environmental change and reduces their chances of long term persistence (Frankham, Ballou, and Briscoe, 2010). For example, the endangered Florida panther suffers from genetic problems as evidenced by low genetic diversity and inbreeding-related effects (poor sperm quality and quantity and morphological abnormalities) (Frankham *et al.*, 2002; Frankham, Ballou, and Briscoe, 2010).

Information regarding the extent of gene flow among populations is critical to determine whether a species requires translocation of individuals to prevent inbreeding and loss of genetic diversity Frankham *et al*., 2002; Frankham, Ballou, and Briscoe, 2010).

Conservation genetics highlight the effects of contemporary genetic structuring on preserving endangered species as dynamic entities. This requires sensitive molecular makers in order to collect abundant and appropriate data from small populations Frankham *et al*., 2002; Frankham, Ballou, and Briscoe, 2010).

2.3.2 Genetic Markers

Genetic maker is a segment of DNA whose characteristics can be measured and infer the ecology and evolution of individuals, population and species. Genetic markers' main characteristics are: polymorphism, expression stability during environmental, ontogeny, and morphologic changes, well identifiable and amplifiable, expression co-dominance, Mendelian heredity and many species application. The following are commonly used or have been commonly used molecular markers in conservation genetics (Frankham, Ballou, and Briscoe, 2010).

- Microsatellite
- DNA sequences
- SNPs (Single Nucleotide Polymorphisms)
- AFLPs (Amplified Fragment Length Polymorphisms)
- RAPDs (Random Amplified Polymorphic DNA)
- RFLPs (Restriction Fragment Length Polymorphisms)

Table 1: Types of DNA makers, their characteristic and application (Liua and Cordes, 2004)

Marker	Genome	Cost	Inheritance	Typical application
Microsatellites	Nuclear	medium	Co-dominant	Individual identification, kinship, population structure, demographic history
DNA fingerprints	Nuclear	medium	Dominant	Individual identification
RAPDs	Nuclear	low	Dominant	Population structure, demographic history
AFLPs	Nuclear	medium	Dominant	Population structure, demographic history
RFLPs	mtDNA	medium	Co-dominant	Population structure, phylogeography
DNA sequences	Nuclear, mtDNA	high	Co-dominant	Species ID, population structure, phylogeography, phylogenetics
SNPs	Nuclear	medium	Co-dominant	Individual identification, kinship, population structure, demographic history

2.3.2.1 Microsatellite

Microsatellites are stretches of short DNA sequence in which a motif of 1-6 bases are tandemly repeated. They are also known as simple sequence repeats (SSR) or short tandem repeats (STR) (Selkoe and Toonen, 2006). Microsatellites are randomly found at high frequency in the nuclear genome of most taxa (Ellegren2004); however dinucleotides, trinucleotides and tetranucleotides are most commonly used (Selkoe and Toonen, 2006).

Microsatellites have become the most frequently used DNA marker in conservation genetics (Frankham *et al*., 2010). Microsatellites have emerged as the most popular and versatile type of

molecular marker for ecological studies. Microsatellite loci vary in repeat length from 5 to 40 at each locus. Microsatellites used as genetic markers are embedded in the noncoding DNA either in the intergenic sequence or in the introns and are generally assumed to evolve neutrally. They are surrounded by flanking regions which do not participate in coding process during protein synthesis.

Microsatellites are single locus co-dominant markers and a number of loci can be combined in the genotyping process thus saving on time and the costs involved in the process. During degradation, DNA breaks in shorter strands and chances of finding microsatellites intact are high even after high levels of degradation which commonly occurs with non-invasively collected samples (Selkoe and Toonen, 2006; Frankham, Ballou, and Briscoe, 2010).

Most of these molecular markers are species specific or can be used with very closely related species and therefore there are lower chances of cross contamination by non target species that could be present in the extract compared to other techniques that involve the use of universal primers (Selkoe and Toonen, 2006). Unlike other markers with low mutation rates, microsatellites mutate fast resulting into a very high allelic diversity in the population. This makes them very informative in the analysis of small fragmented and recently bottlenecked populations as compared to allozymes where only loci with highest mutation rates will be informative (Hedrick, 1999; Selkoe and Toonen, 2006). The selection of a marker depends on the question to be addressed. However in the case of paternity analysis, identity analysis, relatedness between individuals, population structure or migration that employ allele frequency estimates (Wilson & Rannala, 2003), microsatellites are the best choice since only a few loci can provide a lot of information on a number of questions and unique genotypes (Queller *et al.,* 1993).

Despite the numerous advantages, the use of microsatellites has its own limitations among which are; unclear mutation mechanisms, the need to isolate species specific markers, hidden allelic diversity (homoplasy) and amplification problems. The mode of mutation is not very clearly understood, though it is thought that microsatellites evolve by step-wise mutation, this overshadows point mutations that do not result into fragment length change (Selkoe and Toonen, 2006).

The cheetah is an elusive species which makes it hard to study them. In such cases, non-invasive methods can be valuable in estimating population size without the need to capture animals (Kohn *et al*., 1999; Banks *et al*. 2003; Frantz *et al*., 2003, 2004).

2.4 Non-invasive methods

Carnivore populations are declining globally at a higher rate due to mainly anthropogenic related activities (Sawaya *et al*., 2012). The majority of felid species are secretive and solitary, which often makes research based on visual observation impossible (Nowell and Jackson, 1996; Macdonald and Loveridge, 2010; Sollmann *et al*., 2013). Carnivores such as the cheetah are elusive, a behavioural trait that makes their monitoring and study difficult (Nowell and Jackson, 1996; Rodgers and Janecka, 2013) resulting often on insufficient data to guide appropriate conservation action. Invasive methods such as radio-collar or blood/tissue collection require physical detention which can be costly and labour intensive (invasive). Traditional methods such as telemetry studies provide adequate information on animal behaviour and ecology (Piggott and Taylor, 2003; Kelly *et al*., 2012). Blood and tissue collection provide information on species or population genetic diversity, gene flow, population structure and relationships between individuals. However these methods require live capture that may cause stress and disturbance, or potential injury, which rise to ethical concern and permit requirements (Greenwood, 1996;

Piggott and Taylor, 2003; Kelly *et al*., 2012). Hence, traditional methods may not be the most suitable approach for monitoring carnivores.

Conservation and management of wildlife populations require information on parameters such as population size, demography, gene flow, and population structure but these parameters are difficult to obtain for species that are rare or elusive such as carnivores, most of which are endangered (Creel *et al*., 2003; Williams *et al*., 2002; Schipper *et al*., 2008; Mondol *et al*., 2009). The solution to these challenges is the use of non-invasive techniques. Nowadays, researchers can explore various aspects of carnivore biology. These aspects include demography (e.g. estimate population size, population structure, survival, inter-birth interval), estimate historic and current rates of movement across fragmented landscape, genetic identification of individual and measure carnivore stress loads without ever catching, handling or even seeing a single animal (DeSalle and Amato, 2004; Boitani and Powell, 2012). A number of non-invasive methods are proposed including remote camera trapping, genetic analysis of scat, hair and chewed food material, and spoor tracking (Karanth and Nichols, 1998; Perez *et al*., 2006; Boitani and Powell, 2012).

Camera trapping (Karanth, 1995), and non-invasive genetics methods has become more commonly used in carnivore research (Kitano *et al.*, 2007; Chaves *et al.*, 2010). These two methods do not require capture, handle or directed observation of animals under study. Non-invasive sampling is advantageous for a number of reasons including cost effectiveness and can lead to large sample sizes, which increases the amount of data that can be collected, increasing parameter estimation precision especially for elusive and low density animals. Furthermore, this technique eliminates stress to both human and animals and is more logistically feasible (e.g. no need to obtain permits, etc) (Boitani and Powell, 2012).

2.4.1 Non-invasive genetics analysis

Non-invasive sampling for genetic analysis involves collection of samples for analysis without distracting the normal daily activities of the target species, this allows biologists to study individuals and populations without handling, capturing or even observing organisms (Waits and Paetkau, 2005). Samples collected include scat, urine, hair, saliva from food remains and feathers. DNA extracted from scat or hair collected noninvasively can be used to identify species, individuals, and an individual's sex (Sawaya *et al.,* 2012). Furthermore, Beja-Pereira *et al*., 2009, explained that the largest contributions of non-invasive methods are studies that focus on identification of individuals for studies of population size and individual movement, wildlife forensic cases, population genetic parameters (such as structure, gene flow and demographic history such as bottleneck detection), assessment of mating systems and behavioural ecology.

The approach has been successfully used for a range of mammalian species over the last decade, including ungulates (Flagstad *et al*., 2000; Maudet *et al*., 2004), and carnivores such as wolves (*Canis lupus*) (Lucchini *et al*., 2002; Creel *et al*., 2003), wolverines (*Gulo gulo*) (Flagstad *et al*., 2004), and brown bears (*Ursus arctos*) (Taberlet *et al*., 1997; Bellemain *et al*., 2005).

The molecular tools used in non-invasive genetics are microsatellites, multilocus genotyping and mitochondrial DNA (mtDNA) sequencing (Farrell *et al*., 2000). This method allows characterizing the genetic identity of individuals and their molecular sex.

It has been recently shown that non-invasive genetic analysis methods can provide reliable and accurate information on a carnivore population (Sawaya, *et al*., 2012). For instance, non invasive genetic analysis methods have been used to address a wide range of research questions in wildlife biology. Such as, classical conservation genetic evaluations of genetic diversity and population structure have been performed using non invasive sources of DNA from brown bears

(Taberlet, and Bouvet, 1992; Kohn, *et al*., 1995; Taberlet, *et al*., 1997; Waits in editor William L. Thompson, p. 212). Non invasive genetic sampling has been used to evaluate migration and gene flow in humpback whales (*Megaptera novaeangliae*). Genetic analysis of scat DNA has provided important information about paternity and social structure in gibbons (*Hylobates muelleri*) (Oka and Takenaka, 2001).

Borthakur *et al*., (2011), demonstrated the effectiveness of this approach on tigers. In this study, DNA based techniques were used for identifying individual tigers present in the study area, this was performed from scat samples. Fresh scat samples were collected in plastic vials containing silica gel. For individual identification, 19 microsatellites derived from Sumatran tiger, Amur tiger, Asiatic lion and domestic cat, were used to screen samples. Genotypes were determined on a capillary electrophoresis based ABI 3130 genetic analyzer and analyzed using the software GENEMAPPER V3.7 (Applied Biosystems, USA). Seventeen individual tigers were obtained based on available genotype data from scat samples. Karmacharya *et al*., 2011, uses this technique to determine the presence of snow leopards in study areas, by analyzing putative scat samples collected from the two study areas.

2.4.1.1 Type of non-invasive samples for genetic analysis

Carnivore scat and hair samples are the two most commonly used sources of DNA for non-invasive genetic analysis studies (Waits and Paetkau, 2005). In this study scat DNA was used. Faeces are commonly used as non-invasive samples because, for many species, it is the easiest to find in the wild and it provides more information (for instance, genetic fingerprint, diet, stress hormone status, reproductive hormone levels and parasite infection) than other sample types (e.g. hair) (Schwartz and Monfort, 2008; Beja-Pereira *et al*., 2009). Scat DNA originates from cells sloughed from the intestinal lining and extracted from scat samples, collected from elusive carnivores, which often deposit scat at prominent sites for intra and interspecific communication

(Gorman and Trowbridge, 1989; Barja *et al.*, 2005; Boitani and Powell, 2012). Several studies have shown that storage methods could potentially have a great effect on DNA quality and yield (Murphy *et al.,* 2002; Roeder *et al.,* 2004; Kelly *et al.,* 2012). Once collected, samples must be properly stored to inhibit enzymes and inactivate bacteria that degrade DNA. Freezing samples at -20˚C is recommended for better DNA yield (Boitani and Powell, 2012).

2.4.1.2 Individual identification

There are two main types of DNA in animal cells: mitochondrial DNA (mtDNA) and nuclear DNA (nDNA). Mitochondrial DNA is a double-stranded, circular molecule contained in the mitochondria, which are maternally inherited (Avise *et al.*, 1987; Hurst and Jiggins, 2005). Mitochondrial DNA is present in hundreds to thousands of copies per cell and in non-invasive genetic analysis is generally used for species identification (Waits and Paetkau, 2005). On the other hand, nuclear DNA chromosomes are found in the nucleus of the cell and have a biparental mode of inheritance. Two copies of nDNA are found in most animal cells and are used for individual identification and gender identification from non-invasive genetic samples (Waits and Paetkau, 2005; Boitani and Powell, 2012).

Individual identification (DNA fingerprinting) is one of the mostly widely used applications of molecular markers in conservation genetics, forensics and molecular ecology (Allendorf, Luikart, and Aitken, 2013). For example, in conservation genetics, researchers have to individualize scat samples to know the number of different individuals sampled. In addition individual identification is important in wildlife forensics, for instance wildlife officers might need to match a tissue sample at the scene of a wildlife crime to a trophy animal being exported (Allendorf *et al.,* 2013), or match illegally killed animals to samples obtained from poachers (Wait *et al.,* 2001). Individual identification of non-invasive genetic samples is generally

achieved by using PCR to amplify nDNA microsatellite markers and by collecting genotype data at four to twelve microsatellite loci, depending on population variation (Paetkau, 2003).

Waits, and Paetkau, 2005 pointed out that, there are two main challenges to collecting accurate data on individual identity using non-invasive genetic samples. Researches must (1) analyze enough loci to have adequate resolution to distinguish individual and (2) minimize genotyping errors. If data are not collected for an adequate number of loci, then the number of individuals in the dataset will be underestimated (Waits *et al.*, 2001; Waits, and Paetkau, 2005). In contrast, genotyping errors (allelic drop out and false alleles) will inflate the number of individuals in a dataset.

2.4.1.3 Genotyping error

Multiple studies have documented that errors in multilocus genotypes can lead to overestimates in population size. There are three main types of errors that may be present in multilocus genotypes: (1) laboratory or recording errors; (2) contamination and (3) PCR amplification errors. The two main types of PCR amplification errors are false homozygotes (allele dropout) and false alleles (Miller *et al.*, 2002). Allelic dropout is the amplification of only 1 of 2 alleles in a heterozygote pair producing a false homozygote, and amplification of false alleles occurs in non-invasively collected samples because DNA is often present in very low quantity, compare to tissue samples (McKelvey *et al.*, 2004). Secondly, genotyping errors can cause samples that came from the same individual to appear to have different genotypes, and therefore appear to have come from different individuals. Genotyping error is a substantial concern in wildlife studies using noninvasive samples because hair and scat contain small amounts of DNA, and this DNA degrades in the field (Taberlet *et al.*, 1999). These genotyping errors may lead to over or under estimate of the population under study. A number of different methods have been

proposed for minimizing the impact of genotyping errors; the multiple-tubes approach was the first standardized technique (Taberlet *et al.* 1996). This conservative approach involves repeating amplification of homozygous loci, an average of eight times and heterozygous loci an average of three times to achieve 99% confidence in the accuracy of a genotype (Taberlet *et al.* 1996, Paetkau 2003). This approach produces accurate genotypes but can be extremely expensive and time consuming to implement (McKelcey *et al*., 2004). A more efficient maximum likelihood-based approached has been recommended that assesses genotype reliability and strategically re-amplifies loci that are most likely to contain errors (Miller *et al*., 2002).

2.4.2 Camera trapping

Camera trapping refers to the use of remotely triggered cameras that automatically take images of whatever walks in front of them. Most camera trap models are triggered by a passive infrared sensor detecting a moving object warmer than the ambient temperature such as animals, people, or vehicles passing in front of them. Camera trapping is most often used to capture images of medium to large sized terrestrial mammals and birds, but has also been recently used for arboreal mammals (Oliveira-Santos *et al.,* 2008). Camera traps (motion-sensitive cameras) have been used to record fauna in a wide range of habitats, from snow leopard in the Himalayas (Jackson *et al.,* 2006) and bobcat in northern California (Larrucea *et al.,* 2007) to a wealth of studies in the humid tropics (*e.g.* Karanth & Nichols, 1998; Rovero & De Luca, 2007; Tobler *et al.,* 2008a).

In studies that try to estimate abundance or density of a single species, camera traps must yield high quality pictures that permit identification of individual animals. Naturally occurring marks on animals, such as the shape, arrangement, and patterns of stripes (tigers *Panthera tigris*), spots (cheetah *Acinonyx jubatus*), or rosettes (jaguars *Panthera onca*, leopards *Panthera pardus* and ocelots *Felis pardalis*) (Figure 2, A, B, C, D and E, respectively); the shape and configuration of

body parts such as head, tusks and ears (elephants *Elephas maximus*, *Loxodonta africana*,) skin folds (Javan rhinos *Rhinoceros sondaicus*), and even injuries and scars (manatees *Trichechus* spp.) can be used to identify individuals. Because natural markings on animals are asymmetric, unambiguous individual identifications may necessitate photographs of both flanks, requiring the deployment of two or more cameras with each trap (Thompson, 2006, p. 235).

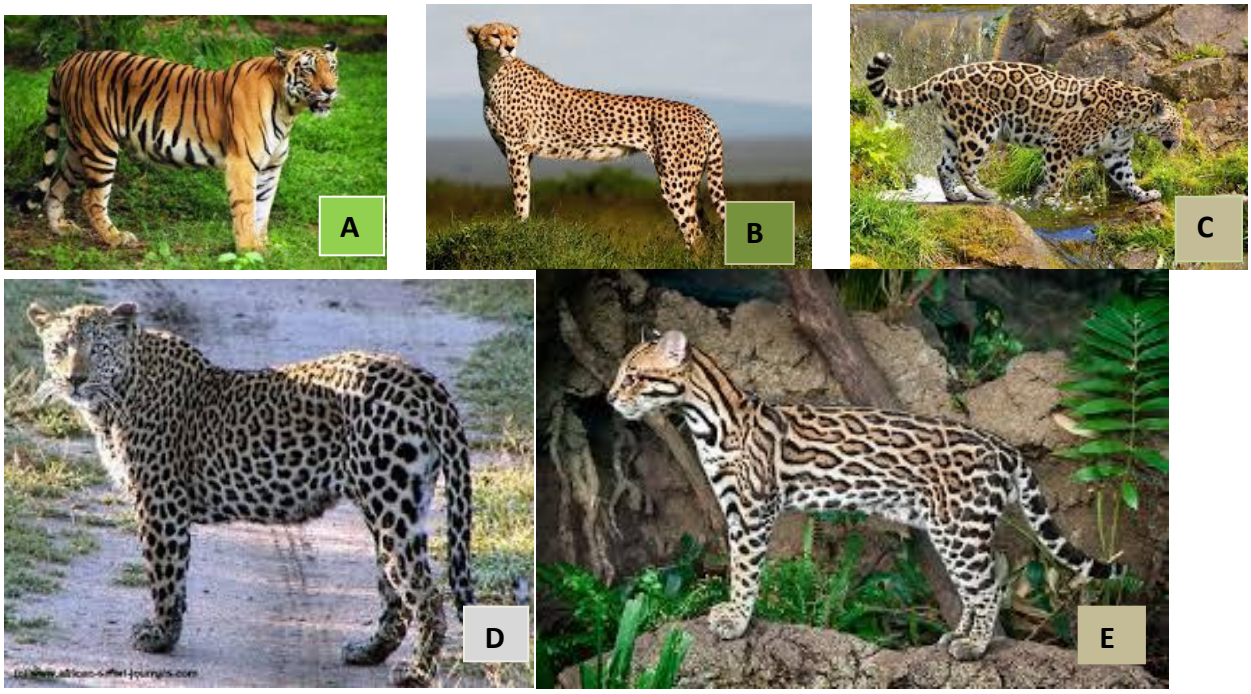

Figure 2: Natural occurring pattern morphology on carnivore: (A) tiger, (B) cheetah, (C) jaguar, (D) leopard and (E) ocelot.

Marker, *et al*., 2008a, used camera trap technique to census free-ranging cheetah in northern-central Namibia. Cheetah individual identification from photographs was done manually (Kelly, *et al*., 1998), based on an individual's spot pattern (Figure 3). Thirteen individual cheetahs (10 males including a coalition of two brothers and female with two cubs) were identified in this study.

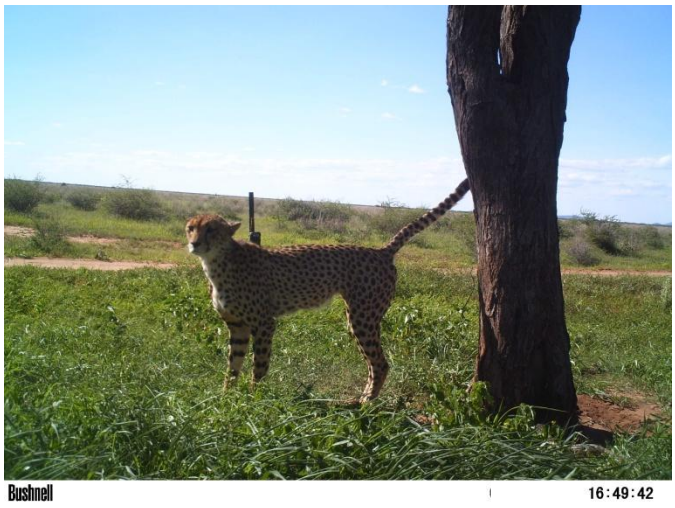

Figure 3: Example of camera-trap images; cheetah at a "playtree" (Marker, *et al*., 2008a)

2.5 Probability of identity

The probability of identity is the probability of two individuals drawn randomly from a population having the same genotype at multiple microsatellite loci (Wait *et al*., 2001). This can be valuable information in a study where individual identification is needed (Mishra *et al*., 2014). According to Mills *et al*., 2000 a probability of identity value of <0.01 (1 in 100) is considered essential for genetic studies in which population size estimation is required. However, a sufficiently low probability of identity value of 0.001-0.0001 has been recommended in wildlife forensic applications for law enforcement (Wait *et al*., 2001, Lorenzini *et al*., 2014).

2.6 Relatedness estimation

It's of great importance for conservation management to have knowledge on genetic relatedness between individuals in natural populations, this is essential in many research areas, such as conservation genetics, quantitative genetics and ecology (Wang, 2002; Wang, 2004). In addition, knowledge of relatedness between individuals is valuable in minimizing inbreeding and loss of genetic diversity in conserved populations. MER is a program that can be used to estimate relatedness coefficients between individuals from co-dominant or dominant genetic markers.

Relatedness is estimated based on the probability of identity-by-descent, as individuals share alleles by descent. MER reports three coefficients of relatedness; Phi (ϕ), relatedness (r), and delta (Δ). Where phi is the probability that two individuals share one allele identical by descent (IBD) under a given relationship, delta is the probability that two individuals share two alleles identical by descent under a given relationship and relatedness is the expected fraction of alleles shared that are IBD (Wang, 2002; Wang, 2004).

Cervus is a computer program for assignment of parents to their offspring using genetic markers. This program uses likelihood, a well-established statistical method for parentage analysis, to assign parentage (Kalinowski *et al*., 2007). Cervus analyses genetic data from co-dominant genetic markers such as microsatellites.

CHAPTER 3: METHODOLOGY

3.1 Research design

A total of 56 putative cheetah scat (faeces) samples were collected in the wild from north-central Namibia (figure 4). Upon encountering samples, researchers collected as much of the scat as possible. Samples were placed in labelled sterile Ziploc plastic bags and frozen immediately after returning to the laboratory. To avoid contamination, samples were handled with care; ensuring not to touch the samples or the inside of the container. Samples were scooped into collection bags using sticks which had not been previously used. Samples were stored in a freezer at -20˚C. DNA extraction, genomic DNA amplification using thermocyclers (Gene Amp® PCR System 9700) and microsatellite markers, followed by genotyping using the 310 Genetic Analyser (Applied Biosystems) were performed in the Life Technologies Conservation Genetics Laboratory, at the Cheetah Conservation Fund (CCF), Namibia.

In addition analysed photographs obtained with remote camera traps were made available, and were used to match the scat with an individual that visited the playtree that day. The freshness of the scat samples was used to estimate the approximate date when the cheetah dropped the scat. This date was then used to identify all cheetahs that were seen visiting the playtree in the period of the dropping. For scat samples from different dates with the same genotype, the cheetah present at all times is assumed to be the individual matching the genetic genotype.

3.2 Study area

The study was conducted at the Cheetah Conservation Fund (CCF), which is about 45,000 ha and located in the northern-central region of Namibia (20˚28’46.8” S and 17˚03’03.0”E). CCF is

part of the Waterberg Conservancy, an area classified as semi-arid. The vegetation consists of thorn bush savannah, and bush encroachment has been reported to be common (Barnard 1998, Marker *et al.*, 2008).

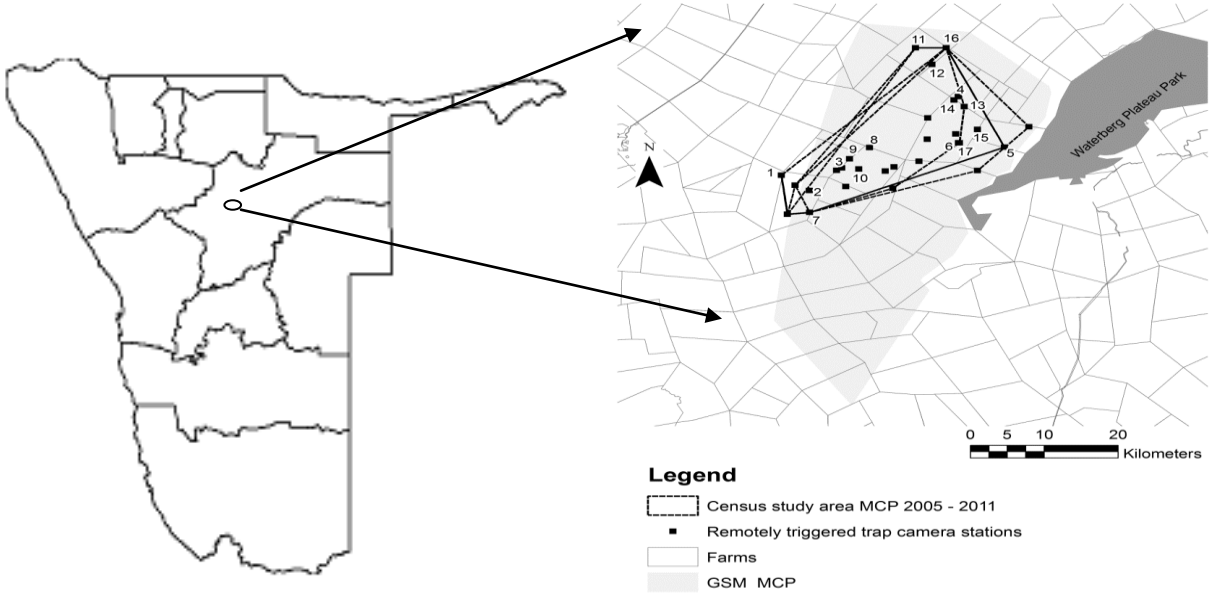

Figure 4: Shows study area were scat samples were collected and camera trap stations where cameras were placed.

3.3 Sample collection and storage

Putative wild cheetah scat (faeces) samples have been opportunistically collected from August 2008 to January 2014 next to camera trap stations located next to 'playtrees'. This study is part of long term CCF cheetah monitoring study. The latter are trees used by cheetahs for marking their territory with urine and faeces. Fifteen playtrees are included in the study and were equipped with remotely triggered camera-traps.

Upon encountering samples, researchers collected as much as possible of the scat. Samples were placed in Ziploc bags (13cm X 6cm) with a white labelling surface. The sample location was recorded with a South and East GPS coordinate, the date, location, a field sample name and

quality/freshness of the scat (e.g. <24hrs, dry, moist, colour). The unique sample description consists of a combination of the collection date and the given sample name from that day (e.g. Cheetah A for the first sample collected that day); a unique sample ID was then assigned once the sample information was entered in the laboratory database. Samples were stored in a constant temperature freezer, at -20˚C, immediately after returning to the laboratory until the extraction date.

3.4 DNA extraction

There are many different methods available for the isolation of DNA (nuclear and mitochondrial). In general, all methods involve disruption and lysis of the starting material followed by the removal of proteins and other contaminants and finally recovery of the DNA.

Faecal DNA extracts can contain high concentrations of PCR inhibitors, and extraction methods are designed to minimize inhibitors while maximizing DNA yield (Waits and Paetkau, 2005). Currently, the most commonly used method of extracting DNA from faecal samples is silica-binding extraction kits (Qiagen) (Waits and Paetkau, 2005). Preservation methods of hair and faecal samples collected in the field can have an important impact on DNA amplification success rates (Roon *et al.*, 2003). Waits and Paetkau, 2005 suggested that for optimal results on non-invasive samples, one has to combine silica desiccation extraction method and freezing (store samples at -20˚C).

DNA was extracted for all 56 scat samples collected using QIAamp DNA Stool Mini Kit, (QIAGEN). During extraction, all types of DNA (mitochondrial DNA and nuclear DNA) from all source of DNA (i.e. cheetah, prey and bacteria) in the scat are extracted. The Qiagen extraction kit uses a silica-membrane (spin-column) technique to separate the DNA, whereby

nucleic acids bind to the spin-column membrane and the rest of cell particles go through. This process of scat extraction is achieved by collection or isolation of cells from scat using a special buffer and a centrifugation step to pellet out all other scat components. Scat samples contain high levels of PCR inhibitors, those PCR inhibitors are removed using a tablet (inhibitorEX), which binds the inhibitors, and can then be separated out with a centrifugation step. Once the unwanted scat components and inhibitors are removed, the cells are lysed with an enzymatic and chemical lysis step, with the help of a lysis buffer and proteinase K; this aids breaking up the cell membrane to release the nucleus and the DNA. DNA is then bound to the column membrane using the hydrophilic properties of nucleic acids, the rest of the cell debris and lipids, which are hydrophobic, go through the membrane. To remove all salt and impurities from the membrane washing buffers are used, after which DNA is released from the membrane and collected in an elution step.

This is an efficient and non toxic method. In addition, no alcohol precipitation and re-suspension step is required. In other words, no risk of losing DNA during precipitation or experiencing difficulties in re-suspending over-dried DNA.

The extraction was done following the manufacturer's protocol with the following modifications: in the first step 100 or 200mg of scat was added to the 2ml tube containing the lysis buffer, instead of adding the lysis buffer after the sample. This was done because there was no ice to keep samples in the tube frozen. However the lysis buffer stabilizes DNA at room temperature, so the samples were added to tubes containing the lysis buffer, to allow for immediate contact. The scat samples themselves were kept on ice at all times, and was kept out of the freezer for as short a time as possible. The lysis buffer was at room temperature. The second modification, to increase DNA concentration, DNA was eluted in 100µl instead of 200µl. For each sample a first

extraction was performed with approximately 100mg of scat; best on amplification results, if they were insufficient, a second extraction with 200mg of scat was performed, and if needed a third extraction was performed with 50mg of scat. Initially the sample amount was weighed to ensure accuracy; subsequently the extractions were done by visual estimation. The required amount of outer layer of scat was cut off using a clean scalpel blade and placed in a 2ml tube containing stool lysis buffer.

The sample was mixed for 1min or longer by vortexing until the solution was thoroughly homogenised. This helps ensure maximum DNA concentration in the final step (eluate). After the vortexing step, the samples were centrifuged to pellet out faecal particles and transfer the supernatant to a new tube. An inhibitEX matrix in form of a tablet was added to the supernatant to adsorb PCR inhibitors, followed by a centrifugation step to pellet out the inhibitEX matrix, while the cells remain in the supernatant and are transferred into a new tube. Proteinase K and cell lysis buffer were added to the supernatant, to digest all proteins and lyse (break) the cell, at 70˚C for 10 minutes. The lysate was transferred onto a QIAamp spin column and centrifuged to filter out the DNA (DNA binds to the column membrane and all other molecules goes through with the buffer). This was followed by washing steps, to wash out all the impurities. The elution buffer (100µl) was then added to the spin column to release (elute) the DNA.

DNA was extracted in lots ranging from seven to eleven samples, and one negative control (reagents only no sample) was included to monitor contamination of the reagents or during the experiment. DNA samples were stored at 4˚C until used. Because scat have low DNA quantity and quality; samples were extracted and handled in a pre-PCR low DNA (scat) area to avoid contamination from high DNA quality samples such as blood and tissue. The success of DNA

extractions were determined by evaluating the amplification success of the nuclear DNA, using three microsatellite markers.

3.5 DNA amplification with Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is an *in vitro* technique used to enzymatically amplify a specific DNA region that lies between two regions of known DNA sequence; the amplification is exponential and leads to about $2x10^9$ copies of double stranded PCR product for each DNA template after 30 PCR cycles. The enzyme used for the PCR amplification is a DNA polymerase. For the DNA polymerase to replicate the DNA strand, it requires nucleotides, primers and a single strand template DNA molecule. Nucleotides are subunits (building blocks) of nucleic acids (DNA and RNA). They consist of the four bases adenine (A), thymine (T), cytosine (C) and guanine (G).

The primers are small fragments of DNA that specifically bind to the template DNA, and to which the enzyme attaches the nucleotides thus constructing the new strand. If these three ingredients are supplied and the correct temperature provided, the enzymes will construct exact copies of the templates.

This technique requires repetitive temperature cycles. Each cycle is composed of three steps: (1) denaturation, followed by (2) annealing, and (3) extension. Denaturation of the DNA at high temperatures (90˚C - 97˚C) converts double-stranded DNA to single-stranded DNA by breaking the hydrogen bonds between the two strands. Annealing of the oligonucleotides (primers) at lower temperatures allows the primers to bind the target DNA by allowing the hydrogen bonds to form between them. Each primer has its optimal annealing temperature which depends of the nucleotide sequence of the primer; these typically range between 50˚C-65˚C. Extension of the DNA chain occurs at 72˚C. This temperature corresponds to the ideal temperature of the DNA

polymerase. During this phase nucleotides are added to the primers to form a new DNA strand complementary to the template. Cycles are repeated 25 to 40 times. In addition to the above cycle steps, two additional steps independent of the cycles are required: an initial denaturation and a final extension. The initial denaturation step is required for "hot start" DNA polymerases, which are modified in order to be inactive until initial heat step; this avoids the amplification of non-specific products during the setup of the reaction. For the activation step the solution is heated to 94˚C during 10 minutes. During the final extension step the DNA polymerase fully extends the newly produced double stranded target DNA and adds an extra nucleotide (A), to the end of PCR products.

Touchdown PCRs were used for the project. In a touchdown PCR the annealing temperature is initially set to a more stringent temperature; after which the annealing temperature gradually decreased in later cycles, until a specified more permissive annealing temperature is reached. The more permissive temperature is then used for the remaining number of cycles. The touchdown protocol used for this project, the annealing temperature 60˚C decreased by 1˚C per cycles for ten cycles. Touchdown PCRs allow for the enrichment of the correct product over any non-specific amplification products, without needing to determine the ideal annealing temperature for the given primer pair(s). Even if reduced annealing temperatures allow for non-specific products to amplify, this is expected to occur at temperatures lower than the target fragment. Therefore, even if reaching temperatures at which non-specific products start to amplify the specific products have already been amplified and are present in excess, these will hence outcompete non-target fragments at the lower, more permissive annealing temperatures. Touchdown PCRs are particularly useful for multiplex PCR for which primers with different annealing temperature are combined in a single PCR reaction.

3.5.1 Microsatellite markers

Multiplex PCR is a molecular biology technique used for amplification of multiple targets in a single PCR reaction (Rompler *et al*., 2006). In other words, multiplex PCR amplifies more than one target DNA sequence at the same time by using multiple primer pairs in one PCR reaction. The advantage is that, multiplex PCR reduces the consumption of DNA, reagents and disposables used, and saves preparation time; instead of multiple tubes or reactions for single primer pair reaction the multiplex PCR is set up in one tube with only one primer mix (Rompler *et al*., 2006). Multiplex PCR requires optimization to assure an even amplification of all targeted DNA fragments; hence primer concentrations have to be adjusted (Sint, Raso and Traugott, 2012), this may need to be adapted if a different type of PCR reagent is used as PCR reagents or brands can have different concentration or recipe of reagents to use. For difficult samples, markers were run individually as well.

The following precautions were taken during PCR optimisation:.

- Primers with overlapping amplicon size and same fluorescent dye were not used or run in the same PCR.
- The primers that are complementary to each other were not used, to avoid the formation of primer-dimmer. Primer-dimmer are non-specific PCR products that rise as a result of primer molecules complementary to each other (Brownie *et al*., 1997).
- All primers used in each multiplex were compatible with each other to avoid that they inhibit the amplification of another product in the reaction.
- Short PCR products are usually favoured over long product. Therefore, all amplicons obtained in the multiplex were in the same size range (short or long).

The PCR amplifications for microsatellites were performed in 10µl reactions using AmpliTaq Gold DNA polymerase (buffers, magnesium, and nucleotides are separate) or AmpliTaq Gold PCR master mix (contains the same DNA polymerase, but buffers, magnesium, and nucleotides are combined with the polymerase in one tube). AmpliTaq Gold DNA polymerase is a heat-resistant enzyme that is activated at higher temperature (95˚C). AmpliTaq Gold PCR master mix contains all the reagents needed for PCR amplifications, except primers and template. Reagents in the mix include AmpliTaq Gold DNA polymerase, 250U (0.05U/µl); GeneAmp PCR Gold buffer, 30mM Tris/HCl, pH 8.05, 100mM KCl; 400µM dNTPs; 5mM $MgCl_2$; and stabilizers. These reagents are at twice the recommended usage concentration, hence 5µl of master mix were added in each 10µl PCR reaction. Similar concentrations of reagents are required for the AmpliTaq DNA polymerase to function; it requires optimal concentration of all the reagents added and optimal thermal cycling parameters. The optimal $MgCl_2$ concentration varies depending on the enzyme, buffer, primer and target DNA (Pelt-Verkuil, Belkum and Hays, 2008). In most cases a final concentration of magnesium chloride ranges from 0.5 to 5mM (Pelt-Verkuil, *et al*., 2008). In this study the final concentration used was 2.0mM of $MgCl_2$ and 0.2mM of dNTPs.

To each PCR reaction 1-3.2µl of DNA was added. DNA was not quantified or diluted prior to adding it to the PCR reaction, since DNA amounts are low and do not only represent target DNA, so the DNA amounts would not be relevant to predict amounts needed. The default amount was 1µl of DNA, but for difficult samples the maximum amount of DNA (3.2µl or "max DNA") was used, and the volume of the molecular grade water was replaced by DNA. In other words, the amount of DNA was increased while the quantity used for all other PCR reagents remained the same.

The final concentration of primers in PCR reactions was of 0.4 µM when markers were amplified individually and ranged between 0.05 and 0.4 µM for multiplex PCRs (the amounts of primers are adjusted until the markers are amplified with comparable amplitude) (table 2).

Table 2: PCR amplification content (recipe). Shows PCR recipe (default and maxDNA) of reagents used for (A) AmpliTaq Gold PCR master mix, (B) AmpliTaq Gold DNA (C) AmpliTaq Gold PCR master mix (individual primers) polymerase, respectively.

Reagents	**Concentration**	**Default PCR Volume (µl)**	**MaxDNA PCR (µl)**	**Final concentration**
TaqGold ABI master mix	2x	5.0µl	5.0µl	1x
multiplex primer mix	1-8 µM	0.5µl	0.5µl	0.05-0.4µM
BSA	20mg/ml	0.2µl	0.2µl	0.4mg/ml
Molecular grade water		3.3µl	0	
Template DNA	Unknown	1.0µl	4.3µl	unknown
Total		10 µl	10 µl	

A

Reagents	**Concentration**	**Default PCR Volume (µl)**	**MaxDNA PCR (µl)**	**Final concentration**
Buffer	10X (150mM Tris-HCl, pH8.0, 500mM KCl)	1.0µl	1.0µl	1X
dNTPs	2mM	1.0µl	1.0µl	0.2mM
$MgCl_2$	25mM	0.8µl	0.8µl	2mM
Taq polymerase	5U/µl	0.1µl	0.1µl	0.05U/µl
multiplex primer mix	1-8 µM	0.5ul	0.5ul	0.05-0.4µM
Bovine serum album (BSA)	20mg/ml	0.2µl	0.2µl	0.4mg/ml
Molecular grade water		5.4µl	1.4 µl	
Template DNA	Unknown	1.0µl	5.0 µl	Unknown
Total volume		10 µl	10 µl	

B

Reagents	Concentration	Default PCR Volume (µl)	Final concentration
TaqGold ABI master mix	2x	5.0µl	1x
Primer F	10 µM	0.4µl	0.4µM
Primer R	10 µM	0.4µl	0.4µM
BSA	20mg/ml	0.2µl	0.4mg/ml
Molecular grade water		3.0µl	
Template DNA	Unknown	1.0µl	Unknown
Total		10 µl	

C

A PCR blank or negative control was included in each group of reactions to monitor PCR contamination. Unless known good quality samples were used, a positive control (blood DNA) was included to control for human error, reagent or PCR failure. Reagents were handled in a dedicated pre-PCR area, and filtered tips were used to minimize the risk of contamination.

Amplification conditions on the thermocyclers (Gene Amp® PCR System 9700) were as follows: initial denaturation at 94°C for 10 min, followed by a touchdown with 10 cycles of 15s at 94°C, 60°C-50˚C for 30s, and 45s at 72°C, followed 30 cycles of 94°C for 15s, 50°C for 30s, 72°C for 45s and finally the extension at 72°C for 30min.

Markers used: Sixteen microsatellite loci (AJU-FCA069, AJU-FCA075, AJU-FCA088, AJU-FCA94, AJU-FCA096, AJU-FCA097, see Table 3) were used in this study. The microsatellite markers were originally derived from domestic cat and were named FCA069, FCA075, etc (Menotti-Raymond & O'Brien 1995). The selected markers had been successfully amplified in the cheetah in previous studies (sequences are conserved between related species, so the binding sites of the primers are often conserved enough to allow amplification of the products in related species) and were shown to be polymorphic in the cheetah (Marker *et al*., 2008a and Driscoll *et al*., 2002). The primers were redesigned based on cheetah sequence to obtain a short fragments

suitable for short/degraded DNA such as scat DNA (Schmidt-Küntzel *et al.*, unpublished) and renamed by adding "AJU-" as a prefix to the marker name. Cheetah sequences were obtained by sequencing the microsatellite markers in selected homozygous cheetah individuals and designing the new primers in the obtained flanking region of the microsatellites using Primer 3 (; Schmidt-Küntzel *et al*., unpublished). All microsatellites were di-nucleotides repeats. All the forward primers were fluorescently labelled at the 5` end with one of the fluorescent dyes FAM (blue), PET (red), NED (yellow) or VIC (green). The reverse primers were not labelled but had a "pig tail" sequence GTGTCTT which promotes adenylation (addition of extra nucleotide-adenosine to the 3' end of a PCR product by DNA polymerase) of the forward-strand products and this increases accuracy of genotyping (Brownstein *et al*., 1996; Butler, 2005; Thomas *et al*., 2010).

Table 3:A list of primers used in this study. Summary of the set of 16 di-nucleotide marker sequences used in this study and the primer combination of each multiplex mix used (Schmidt-Küntzel *et al.*, unpublished).

	Locus	**Primer sequence**	**Fluorescent label**
miniplex1	AJU-FCA 133	F: PET-CCGATGACTCTTTTTCAGAGC R: GTGTCTT-CAGAGGGACACTTTTCATGGT	PET
	AJU-FCA 247	F: FAM-CCAGTGTCTGCTAGAGATGACC R: GTGTCTT-CCCATTTGGGGACTGACTTA	FAM
	AJU-FCA 082	F: NED-CACCACCTCCTTTCCATT R: GTGTCTT-TCACCGCTTAAGAAGAGGCTA	NED
	AJU-FCA 105s	F: VIC-CAAAACTCAATATTAAAACACAGACACA R: GTGTCTT-TGACCCTCATACCTTCTTTGG	VIC
miniplex2	AJU-FCA 327	F: PET-TGAAATAACATCAACACACACACA R: GTGTCTT-GATTAATTTGGGGAATGTTAGGA	PET
	AJU-FCA 097	F: VIC-GTATAGATGCCAACATCAGG R: GTGTCTT-CCCATACAGGAAACACACACA	VIC
	AJU-FCA 224	F: PET-CAAAATAAACTTAAACACACACACAC R: GTGTCTT-TTGTATGAAAGGGACTTCTTGC	PET
Miniplex3	AJU-FCA 069	F: VIC-GCAAGTGCATACACACACACA R: GTGTCTT-GGCTTTTTGCCCAGTTGA	VIC
	AJU-FCA 088	F: FAM-GCTTAATCCAAAGTCACACAGC R: GTGTCTT-TTTCCCGTAATACACACACACA	FAM
	AJU-FCA 075	F: NED-ATGCCAACAACACACACACA R: GTGTCTT-CCAGACGTGCTGAGAAAATG	NED
Miniplex5	AJU-FCA 078	F: FAM-TTAAGTTTTTAATCCACCCGTAA R: GTGTCTT-AAATGTTCTTACATCACACACACAC	FAM
	AJU-FCA 096	F: PET-GCCATCCCTTACACACACAC R: GTGTCTT-GTGCCGTCCAAGAACACATA	PET
	AJU-FCA 161	F: FAM-CGATACACACCTGCCAAGATT R: GTGTCTT-GCCAAACATAGAATGCACACA	FAM
Miniplex6	AJU-FCA 230	F: FAM-AATGGACTTGGGAAATGGT R: GTGTCTT-GCAAAAGGGAAGCAGTTCA	FAM
	AJU-FCA 094	F: VIC-CAAGCCCCATTTTACCTTCT R: GTGTCTT-TGACTCCCCCACTCTCTCTC	VIC
	AJU-FCA 208	F:NED-TTATTATGGAAAGATTTATATTCAGAGC R: GTGTCTT-TTTTGCCTTCCTTCTCTCC	NED

3.5.2 Mitochondrial marker

Species identification was performed by sequencing a short segment of the mitochondrial gene Adenosine Triphosphate 6 (ATP6). PCR amplifications with ATP6 were performed in 15µl reactions containing 1.5µl of 10X PCR buffer, 1.2µl of 25mM $MgCl_2$, 1.5µl of 2mM dNTPs, 0.15µl of 5U/µl Taq Gold polymerase, 0.6µl of 10µM ATP6 Forward primer and Reverse primer, 0.3µl of 20mg/ml BSA, 1.5µl of DNA template and 7.65µl molecular grade water. The thermal cycling was performed with initial denaturation at 94°C for 10 min, followed by 10 touchdown cycles of 94°C for 15sec, 60˚C-50°C for 30sec, 72°C for 45sec, followed 30 cycles of 94°C for 15sec, 50°C for 30sec, 72°C for 45sec and finally the final extension at 72°C for 30min.

Success of the PCR reaction was confirmed by running 10µl of the PCR product alongside extraction and PCR negative controls as well as positive controls for the PCR reaction on 1% agarose gel. A 50X gel red staining was used to visualize PCR product on an Ultra Violet table with integrated camera system (KODAK Image station 440 CF). For successfully amplified samples, the remaining 5µl PCR product was cleaned with enzymatic PCR and sequencing clean-up (illustra ExoStar 1-step), by adding 2µl of ExoStar to 5µl PCR product. The exostar process is a single-step enzymatic cleanup of PCR products that eliminates unincorporated primers and dNTPs. The thermal cycling was performed with initial incubation at 37°C for 15 min, followed by enzyme inactivation at 80°C for 15 min.

3.6 Sequencing

All sequencing reactions were carried out using BigDye® Terminator v1.1 cycle sequencing kit (Applied Biosystems). The sequencing reactions were 10µl in total volume contained 2µl 5X sequencing buffer, 1µl BigDye v1.1, 1µl ATP6 reverse (2µM) 1µl or 1.5ul PCR product for

samples with bright bands or faint bands on a gel respectively and 5µl or 4.5µl water respectively. The thermal cycling was initiated at 96˚C for 1 min, followed by 25 cycles 96˚C for 10 sec, 50˚C for 5 sec and 60˚C for 4 minutes.

After sequencing, in order to remove unincorporated dye terminators and salts that may compete for capillary electrophoresis injection (www.appliedbiosystems.com), ethanol precipitation was performed following life technologies protocols.

3.7 Laboratory work flow

For screening samples, DNAs extracted from scat samples were genotyped using a multiplex of three microsatellite markers (miniplex3: AJU-FCA069, AJU-FCA075 and AJU-FCA088; see Table 3), that are known to work well on scat samples (data not shown) and were variable in cheetah (Driscoll *et al*., 2002; Marker *et al*., 2008a).

DNA was initially amplified by performing two PCRs using a standard PCR. If a consensus genotype was not observed from these two independent PCRs, a third PCR was performed with an increased amount of DNA ("max DNA"). A fourth PCR was performed with individual primers if needed. If after four amplification attempts a consensus genotype could not be reached for a sample, the sample was re-extracted and the entire process repeated. Ultimately, if after this second round samples did not work or had alleles outside of the cheetah allele range, the possibility that they are not cheetah scat was considered. For these samples a small fragment of a mitochondrial gene (ATP6) was sequenced in order to verify the species of the scat samples. If the species was determined not to be cheetah, the sample was removed from the analysis; if it was confirmed to be cheetah; more efforts were made to obtain the microsatellite genotype.

Samples, for which a complete genotype for the 3 markers could be obtained and had same genotypes across all three loci, were considered to belong to one individual. For each individual the best representative DNA sample was selected for full genotyping using an additional 13 microsatellites.

3.8 Genotype determination

The forward primers for each microsatellite markers used in this study were labelled. Amplified microsatellite PCR products were visualised and separated on an ABI310 1-capillary genetic analyser located at the CCF Life Technologies Conservation Genetics Laboratory. Prior to electrophoresis, samples were denatured in 13µl total volume, containing 12µl mix of Hi-Di formamide (Applied Biosystems) + size standard LIZ600 (Applied Biosystem) and 1ul of PCR product. Cycling conditions for denaturation were set at 94˚C for 2 minutes. The denatured samples were loaded onto the ABI310 genetic analyser and electrophoresis was performed for fragment size polymorphism. The size standard was added to each sample as internal lane standard that is used to assign size to DNA fragments. After analysis the size standards were visually verified by checking if every peak was assigned the correct size. After verifying the size standard, allele sizing was carried out by combining automated allele calling and visual inspection of electropherogram data for each locus in each sample on GENEMAPPER software v4.0 (Applied Biosystems). This process provides a balance between the efficiency and consistency of automated allele calling software (GENEMAPPER v4.0, Applied Biosystems) and the accuracy provided by human inspection in detecting novel alleles outside of the expected range of a locus, stochastic amplifications within the size range and large-allele dropout (Pompanon *et al*., 2005; Dewoody *et al*., 2006).

In order to confirm genotype profiles, and reach a consensus genotype, heterozygous genotypes were confirmed by observing each allele in two or more independent reactions. Homozygous genotypes were confirmed if the allele's amplitude were >2000 and observed in two independent PCR reactions. If the amplitude of a homozygous allele was lower than 2000, genotypes were confirmed in up to four independent PCR amplifications.

3.9 Genetic data analysis

For most programmes used for genetic analysis the allele frequency is needed in order to determine the likelihood for a given allele to be present in an individual based on how common it is in the population. The allele frequencies used in this study were obtained from published allele frequencies of a Namibian cheetah population (Marker *et al.,* 2008). Marker *et al*., (2008) used 20 microsatellites, to assess population structure between 89 unrelated individual cheetahs from all over Namibia.

The markers used by Marker *et al*., 2008 were the same as in the current study; however the primers used to amplify those markers in the current study were re-designed, to be able to work for short DNA fragments (degraded DNA) obtained from scat samples. Re-designing primers leads to the allele calling between the two studies being different (allele differ in size/length). This makes it difficult to assign alleles in the current study to its corresponding allele in the published study to infer the allele frequency for each allele. To overcome this, the re-designed primers had been tested on some of the individuals that were used by Marker *et al.,* 2008 (data not shown). The results of this test were used to match alleles of the short products obtained with the re-designed primers to the old alleles obtained with the FCA primers. Combining above mentioned three datasets, allowed to make use of the published allele frequencies of all alleles

for the remote camera trap-scat genetic profile study dataset (the current study). These allele frequencies were used in all software programmes and calculations requiring allele frequency.

3.9.1 Probability of identity

To be certain that the three microsatellite markers used to screen samples could distinguish between any two individuals or samples, the match probability that any two individuals/samples picked randomly would share the same multilocus genotype by chance was calculated. This is directly linked to the frequency of a genotype. The less common a genotype is, the more likely it is that the two samples come from the same individual.

The genotype frequencies of the data set in this study were calculated by multiplying the allele frequency of each allele of a given genotype at a given locus. For example, there are three alleles (73, 75 and 79) at locus FCA-AJU069 with the following allele frequencies (f) 0.25, 0.33 and 0.26, respectively.

The genotype frequency of 73/73= allele frequency of 73 X allele frequency of 73 = 0.25X0.25= f^2.

The probabilities calculated for all loci were then multiplied together to provide one final match probability. This was calculated using the formula as: Frequency of genotype at locus 1 x Frequency of genotype at locus 2 x Frequency of genotype at locus 3.

The higher this estimate (> 0.01) the more likely that the two samples with the same genotype are from different individuals, thus their genotype look alike by chance. On the contrary, if the matching probability value is below 0.01 then the two samples are likely to be from the same individual (Mills *et al*., 2000).

3.9.2 Relatedness

Information on genetic relatedness between individuals in a natural population is important in research as well as in conservation of wildlife (Wang, 2004). There are many estimators developed for estimating relatedness, such as the pairwise method that uses co-dominant markers (e.g. microsatellites).

In this study the software MER version 3.0 (Wang, 2002) was used to estimate relatedness between individuals identified in the scat sample collection from the study area. Relatedness is estimated based on the probability of identity-by-descent, as individuals share alleles by descent. MER reports three coefficients of relatedness; Phi (ϕ), relatedness (r), and delta (Δ). According to Wang 2002, in an outbreeding population, a pair of individuals (say x and y) can be genetically correlated in two ways: a single gene at a locus in x is identical by decent with one in y, or both genes in x are identical by decent with those in y. The probabilities of occurrences of the first and second event are denoted as ϕ and Δ, respectively, and the coefficient r represents relatedness between individuals. For interpretation of MER results, the following criteria were used based on expected values from an outbreed population: for a parent offspring pair ϕ, Δ, and r are 1, 0 and 0.5 respectively; for full siblings 0.5, 0.25 and 0.5; and for half siblings: 0.25, 0 and 0.125. For unrelated individuals, relatedness coefficients equal zero (Wang, 2002).

The following parameter settings were used in MER:

1. The marker type was set to two as microsatellites are co-dominant;
2. The number of loci to be analysed was set to 15;
3. Numbers of alleles at each locus. This is the number of different alleles observed in the population (Table 4);

Table 4: Numbers of alleles used at each locus:

Marker Name	AJU-FCA069	AJU-FCA075	AJU-FCA088	AJU-FCA105	AJU-FCA133	AJU-FCA247	AJU-FCA094	AJU-FCA208	AJU-FCA230	AJU-FCA078	AJU-FCA096	AJU-FCA161	AJU-FCA097	AJU-FCA224	AJU-FCA327
No. of alleles at each locus	3	5	4	4	5	4	4	4	5	5	6	5	4	5	4

4. The number of individuals in the sample was set to 10;
5. Name and genotypes of each individual. The alleles at a locus are indexed as 1, 2, 3..... The first entry in a line is the name of the individual, followed by list of the genotypes at each indexed locus for all loci;
6. The number of bootstrapping (over loci) used in finding the standard deviation of relatedness estimates for each pair of individuals was set to 1000;
7. The indicator value was set as -1, since the known allele frequencies were provided to the programme.

3.9.3 Parentage analysis

Microsatellite genotypes of 15 loci were determined for all 10 identified individuals. Since all individuals came from the same area and this was a small sample collection, all individuals in the sample collection were given as candidate parent to every other individual. The microsatellite data was incorporated into program CERVUS 3.0.6 (Marshall *et al*., 1998; Kalinowski *et al*., 2007) which calculates log-likelihood ratio scores for parentage. Since this was a small sampling likely containing related individuals (alleles observed in this sample collection will not represent the whole population), allele frequencies from the Namibian population was provided to the programme for the calculations.

This program calculates a statistic, delta, which is the log-likelihood ratio (LOD score) of the most likely candidate parent of an offspring. To run the parentage analysis the program requires locus allele frequency data for the population; in this case the allele frequencies were obtained

from the Namibia population study (see above). It also requires a number of simulation parameters, for which the following values were used in this study.

1. Number of candidate parents was set as 8.
2. The mean proportion of loci typed was set to 0.98.
3. Proportion of candidate parents sampled was set to 0.125.
4. The percentage of allele mistyped was set to 0.02.
5. The number of simulation cycles was set at 10 000.

3.9.4 Sequence analysis

All sequences were analysed, edited and aligned using the program Geneious v 6.6 (www.geneious.com). Sequences were imported into Geneious. In geneious sequences were visually examined and verified using the automated calling of all nucleotides. Sequences were aligned to ATP6 carnivore reference sequence to verify that number and position of nucleotides were correct. To identify species origin of each sample, sequences were aligned to known carnivore reference sequences obtained from known carnivores.

3.10 Camera trapping

The CCF Ecology research department conducts Camera trap studies in order to monitor the cheetah population in the study area. About 16 camera traps were used per year. A camera trap station is placed at select locations (BBNO1, BBNO2, BBNO3, Field1, Field2, Chewbaaka, CHEW and JDMAN3) that have a high visitation rate from cheetahs; these locations are mostly so-called ‘playtrees’. Each camera trap station consists of two remote triggered camera traps that face the playtree and are triggered by movement. Whenever a cheetah or another animal passes the playtree a photo is automatically taken. The photos are downloaded and analysed. Cheetah photos are sorted from the other non-target photos. Individual cheetahs are identified using visual

inspection of their spot pattern. A ‘cheetah sighting database’ is then created indicating which cheetah was seen at which camera trap station on any given day. This data was made available to this study in order to compare the picture identity to the genetic data obtained from the scat samples; the scat samples from this study were collected next to active camera traps.

3.11 Matching scat samples to camera trap pictures

For all scat samples the date and place of sample collection were obtained from the records. It was then postulated that the cheetah responsible to have dropped the given scat sample must have appeared on the photos that were taken at the corresponding camera trap station prior to the date of sample collection. The freshness of the scat sample was used to estimate how much time prior to the sample collection the cheetah was likely to have dropped the scat; one month was considered as the maximum time period that could have elapsed between the time a cheetah defecated and the sample was collected. This time period in combination with the location was used to search the cheetah sighting database, in order to identify all cheetahs that were caught on photo at the playtree in question during this time period. All corresponding cheetah sightings were then assigned to the scat samples in question.

A table was compiled in order to indicate for each scat sample, which cheetahs were seen on a camera trap, and which genetic identity had been assigned to the scat sample. The resulting data was included in Table 8.

All scat samples with the same genotype were assumed to belong to the same individual. The cheetah individual whose photos were assigned to each one of the scat samples of a given individual was then assumed to be the individual matching the genetic genotype. Note that male groups (coalitions) are assumed to always visit playtrees together even if not all members of the

coalition appear on the photo; therefore scat sample genotypes associated with a coalition were assigned to the coalition (sample belongs to one of the coalition members).

CHAPTER 4: RESULTS

4.1 Number of individuals identified based on scat samples

Fifty-six scat samples collected in the wild next to remote camera trap stations were screened with three microsatellite markers. Of these 56 scat samples, one sample was missing at the time of extraction and 52 were confirmed to be cheetah using PCR-based genetic identification of individuals (i.e. microsatellite) or species identification (i.e. sequencing). The remaining three samples were determined to be of non-cheetah origin using mitochondrial species identification. The sequences of the three non-cheetah samples were matched to African wild cat (*Felis sylvestris lybica)*, jackal (*Canis mesomelas*), and genet (*Genetta genetta*); these samples were dropped from the project. Of the 52 cheetah scat samples, 50 samples (96%) were successfully genotyped at the three microsatellite loci used for individual identification. Two failed to work but were confirmed to be cheetah using the mitochondrial species identification; these samples were not included in further analysis. Based on the 50 scat samples, 10 unique multilocus genotypes were obtained representing 10 individual cheetahs (Table 5).

Table 5: Genetic profile of scat samples at three microsatellite loci. Multilocus microsatellite genotype identity of 10 individual cheetahs in the sample collection, total number of samples per individual and the probability of identity for each individual.

Sample ID	total samples per individual	Genotype overview of 3 microsatellite loci			Probability of identity
		AJU069	AJU075	AJU088	
Indiv. A	4	73/79	78/80	83/88	0.0021
Indiv. B	3	73/75	80/90	88/90	0.0002
Indiv. C	9	73/79	80/86	88	0.0011
Indiv. D	1	73/79	80	83/88	0.0024
Indiv. E	13	73	78	79/88	0.0061
Indiv. F	2	73	78/80	79/88	0.0069
Indiv. G	4	73	80/88	79/88	0.0030
Indiv. H	8	73/75	78/88	83/88	0.0013
Indiv. I	5	73/75	78/88	79/88	0.0022
Indiv. J	1	75	78/80	79/88	0.0012

4.2 Probability of identity

To verify how likely it is that two scat samples with the same genotype are from the same individual and not from different individuals sharing the same genotype by chance, the probability of identity was calculated using three microsatellite loci. In other words, this matching probability estimates the probability of sampling an individual in the same population with the same multilocus genotype as the individual identified in the sample collection.

This probability of identity ranged from 0.0002 for indiv.B to 0.006 for indiv.F (Table 5). This means that based on the allele frequency in the population, there was on average a 0.0026 chance that two scat samples (or identical genotypes) were from different individuals.

4.3 Extended genotype for each individual

One sample per individual was genotyped at 13 additional loci. Of the 10 individuals, 70% (n=7) had complete genotypes and 30% (n=3) partial genotypes, across 13 additional loci. Of the three individuals with incomplete genotypes, two individuals were successfully genotyped at 12 of additional loci and the third individual at 11 additional loci. For the 16 loci (3 initial loci for individual identification + 13 additional loci for extended genotype), this corresponds to a 97.5% completion level. Out of the 16 loci, only 15 loci were included in the analysis. One locus (AJU-FCA082) was dropped from the analysis, because it was not used in Marker *et al*., 2008 study, so no population wide allele frequency were available for this marker.

4.4 Relatedness

The genotype data of 15 microsatellite markers per individual was used as the input file for program MER, which was used to determine the pairwise relationships between individuals. Using the relatedness coefficient R, MER estimated that in this sample nine individuals had a degree of relatedness of 0.13 or more with at least one other individual, and one individual (Indiv.B) showed no level of relatedness to any other individual in the sample collection (Table 6). The nine individuals were assigned to three groups of relatedness. Note that R cannot distinguish between the types of relatedness, and just assigns the degree of relatedness. Within the three related groups, there were four pairs for which the delta and phi parameters resembled most closely to a parent-offspring relationship, one pair corresponding to full-siblings, and two pairs corresponding most closely to half-siblings. Three different related groups were identified amongst the 10 individuals. Group 1 consisted of two members; individual C and individual D, group 2 consisted of four individuals (individuals G, H, I and J) and group 3 consisted of three

individuals (individuals A, E and F). Individual B was not related to any of the other nine individuals (Table 6).

Table 6: The relatedness relationship between individuals

Indiv1	Indiv2	mean ϕ	mean ▲	mean R	Relatedness group	relationship
Indiv. D	Indiv. C	1.0705	-0.1658	0.3695	1	parent offspring
Indiv. G	Indiv. I	0.9986	0.1283	0.6276	2	
Indiv. I	Indiv. J	1.029	-0.2112	0.3033	2	
Indiv. A	Indiv. F	0.7796	0.0691	0.4589	3	
Indiv. F	Indiv. E	0.3973	0.3287	0.5273	3	Full-sibling
Indiv. G	Indiv. H	0.3372	0.0586	0.2273	2	Half sibling
Indiv. H	Indiv. J	0.1649	0.0492	0.1317	2	
Indiv. B	not related to any of the individual					

4.4.1 CERVUS- parentage analysis

As indicated above there were four potential parent-offspring relationships among individuals (MER output). To verify the degree of confidence in relatedness estimates of parentage (paternity) relationships among individuals, the program CERVUS was run.

Out of the nine potential sires assigned to each individual, six individuals were assigned as being the mostly likely parent of one or multiple individual(s) (Table 6).

Individual F was a candidate parent in two cases with 8.93 and 6.45LOD score, respectively in both cases (Table 6). However a genotype mismatch occurred at one locus between the putative parent-offspring pair; this mismatch was verified and confirmed to be correct; Individual F was thus not considered to be the parent of individual E. Individual C was a candidate parent in two cases, of which one had higher (9.92) LOD score and the other case had low (-2.41) LOD score. The negative LOD score implies that individual C was unlikely to be the parent of individual B.

Individual I was considered as potential parent to three individuals; one parent-offspring pair had a higher (9.48) LOD score and the other two cases had a lower (positive) LOD score with only 80% pair confidence.

The three other potential parents (individual G, individual D and individual E) were candidate parents to one individual each and all had higher LOD score (table 6). A genotype mismatch was found at one locus for individual E and individual F after confirming the mismatch, this pair was not considered anymore.

After rejecting putative parent-offspring pairs because of genotype mismatches or with only 80% pair confidence, five parent-offspring pairs remain: individual I and individual G as well as individual C and Individual D are suggested as parents of each other; and individual F as parent to individual A.

Table 7: Cervus parentage analysis output.

Offspring ID	Candidate father ID	Loci typed	Pair loci compared	Pair loci mismatching	Pair LOD score	Pair Delta	Pair confidence
Indiv. E	Indiv. F	15	15	1	8.93E+00	8.93E+00	*
Indiv. A	Indiv. F	15	14	0	6.45E+00	6.45E+00	*
Indiv. G	Indiv. I	14	14	0	9.48E+00	5.97E+00	*
Indiv. H	Indiv. I	14	14	0	5.05E+00	1.54E+00	+
Indiv. J	Indiv. I	14	13	0	2.06E+00	3.34E-01	+
indiv. B	Indiv. C	15	13	2	-2.14E+00	0.00E+00	
Indiv. D	Indiv. C	15	15	0	9.92E+00	9.92E+00	*
Indiv. C	Indiv. D	15	15	0	9.92E+00	9.92E+00	*
Indiv. F	Indiv. E	15	15	1	8.93E+00	2.49E+00	*
Indiv. I	Indiv. G	15	14	0	9.48E+00	4.43E+00	*

*****strict pair confidence 95%, $^{+}$relaxed pair confidence 80%

4.5 Matching non-invasive techniques.

All 52 scat samples which were confirmed to be cheetah, were successfully associated with at least one photograph (Table 8).

Individual A

Two scat samples were collected on the 21st August 2008. A single male individual ('Hercules') and a coalition of two male members ('Penda & Omdilo') were detected on cameras during the previous month; the single individual was seen a week prior to the scat collection date and the coalition between 2 and 4 weeks prior. Hence, the three individuals are all considered as candidates to be associated with the genetic identity of the retrieved genotype. The age of the scat samples was considered to have been deposited within 48 hrs prior to collection; this suggests that the single individual is more likely to be associated with the genetic identity of Individual A ('Hercules').

Individual B

There were three scat samples from this individual, which were all collected on the same day. A single male individual ('Goliath') and a coalition of two male members ('Two boys') were detected on camera trap during the month preceding the collection of these scat samples. The single individual was sighted about a month prior to sample collection and the coalition was detected between 2 and 4 weeks. The age of the scat sample was difficult to estimate, since the sample was dry at the time of collection; hence it was difficult to assign the samples to a specific individual. However after comparing samples to each other, the genotype of individual B could be associated with a single male by exclusion, since the coalition members were already associated to different genetic profile.

Individual C

There were nine scat samples collected on nine different dates from this individual. Two coalition consisted of two male members each (‘Sam & Hifi’ and ‘Two boys’) were detected on the corresponding camera trap stations prior to sample collection. The coalition ‘Sam & Hifi’ was detected in cameras prior to every sample collection, with times ranging from less than 48 hours to a month prior to sample collection. The coalition ‘Two boys’ was detected only once (between 2 and 4 weeks) prior to sample collection, and is thus unlikely to be responsible for all nine scat samples. Hence this individual genotype of Individual C could be assigned to the members of the coalition ‘Sam & Hifi’.

Individual D

There was only one sample from this individual. Two coalitions consisted of two male members each (‘Sam & Hifi’ and ‘Two boys’) were detected on the corresponding camera trap stations prior to sample collection. The coalition ‘Sam & Hifi’ was sighted between 2 and 4 weeks and the coalition ‘Two boys’ were detected within a week prior to sample collection. The age of the scat sample was estimated to be less than a week old; hence the members of the coalition ‘Sam & Hifi’ were more likely to be associated with the genotype of Individual D.

Individual E

Thirteen scat samples were identified as being from individual E. These samples were associated with three single female individuals (‘Leia’, ‘Yoda’ and ‘wildmum’ - mother with cubs) and a coalition of two male members (‘Two boys’), which were detected on camera traps during the month prior to each sample collection. The coalition of two males was detected prior to every single scat sample, ranging from less than 48 hours to a month; while the three single individuals

('Leia', 'Yoda' and 'wildmum') were detected in only one, three and four samples respectively, and are thus rejected as candidates for having dropped all thirteen scat samples. The genetic identity of Individual E can thus be assigned to one of the individuals of the coalition.

Individual F

There was one scat sample from this individual. One coalition of two male members ('Two boys') was detected on camera trap within the month prior to sample collection. No other individuals were detected on camera trap. The scat sample was estimated to have been deposited approximately 48hours prior to sample collection and the male coalition was seen within 48h of the sample collection. Hence, this sample is likely to belong to one of the males in the coalition.

Individual G

There were two scat samples from this individual. A single male individual and two coalitions of males ('Three boys' and 'Coalition aaa') were detected in a camera trap a month prior to the date of samples collection. The coalition of three boys was detected in all traps within two days prior to sample collection. The single male and 'Coalition aaa' were detected few days (within 7 days) prior to sample collection. The age of all samples were estimated to be less than 24 hours old. Thus the genetic profile of these samples (individual G) can be assigned to one of the members of the coalition 'Three boys', since they were detected in camera trap within 48 hours.

Individual H

Eight samples were identified as being from individual H. These samples were associated with a single male individual ('Scar') and two coalitions with two and three male members ('Coalition aaa' and 'Three boys'), which were detected on camera traps during the month prior to each

sample collection. The coalition of three males was detected prior to every single scat sample, ranging from less than 48hours to a month; while the single male (‘Scar’) and the coalition of two males were detected within a week prior to sample collection. The age of the scat samples was considered to have been deposited within 48 hrs prior to collection; this rules out the single individual and the coalition of two members. Hence, the genetic identity of Individual E can be assigned to one of the individuals of the coalition ‘Three boys’.

Individual I

Five scat samples were genetically identified as being from individual I. These samples were associated to a single male individual, coalition of two male members (‘Coalition aaa’) and a coalition of three male (‘Three boys’). The single male was detected on camera for three scat samples ranging from within seven days to a month prior to sample collection. The coalition of two members was sighted in only one sample and this was within seven days prior to sample collection. The coalition of three members (‘Three boys’) was detected in every single sample ranging from < 48hrs to a month. The age of scat samples except one sample, were all estimated to be less than 24 hours old. This implies that the genetic identity of individual I can thus be assigned to one of the individuals of the coalition of ‘Three boys’.

Individual J

There was only one sample from this individual. There were two single males (‘Scar’ and ‘Aladdin’) and a coalition of two members (‘Coalition aaa’) detected on camera trap within a month prior to sample collection. The coalition and a single male (‘Scar’) were detected on camera less than 48hrs to a month prior to sample collection. The other single male (‘Aladdin’) was detected between two to four weeks prior to sample collection. The age of the sample was

estimated to be less than a week. Hence, while we cannot assign individual J to a specific individual on camera trap pictures with absolute certainty, based on the age of the scat sample, it is more likely that it belongs to the single male ‘Scar’ or a member of the coalition.

Table 8: Matching scat samples and camera trap pictures.

Scat samples information					Individuals detected on camera trap											
Scat ID	Date collected	Location	Freshness	Genetic ID	sam&Hifi	Two boys	Leia	Yoda	Goliath	Mandela	Wildmum	Hercules	penda&Omdilo	Three boys	coalition aaa	scar	Alladin
pop01	21-Aug-08	JDMAN3	<48h	Indiv.A								+ *	*				
pop02	21-Aug-08	JDMAN3	<48h	Indiv.A								+ *	*				
pop101	20-Dec-11	BBNO2	dry	Indiv.B		*			?								
pop102	20-Dec-11	BBNO2	dry	Indiv.B		*			?								
pop103	20-Dec-11	BBNO2	dry	Indiv.B		*			?								
pop19	18-Sep-10	FIELD1	<week	Indiv.C	+ *												
pop24	30-Sep-10	FIELD2	dry	Indiv.C	‡ *												
pop30	27-Oct-10	FIELD2	<week	Indiv.C	+ *	*											
pop33	04-Dec-10	Chewbaak	<24h	Indiv.C		‡											
pop79	29-Aug-11	FIELD1	5days	Indiv.C	‡ + *												
pop80	29-Aug-11	FIELD2	<24h	Indiv.C	‡ + *												
pop92	18-Oct-11	FIELD1	<48h	Indiv.C	‡ + * ?												
Scat1349	22-Nov-13	Chewbaak	dry	Indiv.C										‡			
pop106	27-Dec-11	FIELD1	<24h	Indiv.C?	‡ + *												
pop28	20-Oct-10	FIELD2	<week	Indiv.D	*	+											
pop20	22-Sep-10	BBNO3	<24h	Indiv.E		+	*										
pop26	20-Oct-10	BBNO2	<week	Indiv.E		‡ + *											
pop27	20-Oct-10	BBNO2	<week	Indiv.E		‡ + *											
pop29	20-Oct-10	BBNO2	<week	Indiv.E		‡ + *											
pop31	11-Nov-10	BBNO2	<week	Indiv.E		+ *		+									
pop34	07-Dec-10	BBNO2	dry	Indiv.E		‡ * ?		?									
pop35	07-Dec-10	BBNO2	dry	Indiv.E		‡ *		?									
pop37	31-Dec-10	BBNO2	<24h	Indiv.E		+ *											
pop39	23-Mar-11	BBNO2	old	Indiv.E		+ *											
pop93	25-Oct-11	BBNO2	<48h	Indiv.E		‡ + *					+						
pop94	29-Oct-11	BBNO2	dry	Indiv.E		+					*						
pop96	29-Oct-11	BBNO2	dry	Indiv.E		+					*						
pop97	29-Oct-11	BBNO2	dry	Indiv.E		+					*						
pop36	23-Dec-10	BBNO2	<48h	Indiv.F		‡ + *											
Scat1355	23-Nov-13	BBNO2	<12h	Indiv.G										‡	+	+	
Scat1172	26-Aug-13	BBNO red	dry	Indiv.G?													
Scat1242	30-Aug-13	BBNO mai	dry	Indiv.G													
Scat1244	3-Sep-13	BBNO mai	<12h	Indiv.G													
Scat1359	23-Nov-13	BBNO2	<24h	Indiv.G										‡	+	+	

Scat1243	3-Sep-13	BBNO mai	<12h	Indiv.H													
Scat1246	9-Sep-13	BBNO red	dry	Indiv.H													
Scat1352	23-Nov-13	BBNO2	<24h	Indiv.H										‡	+	+	
Scat1354	23-Nov-13	BBNO2	<12h	Indiv.H										‡	+	+	
Scat1358	23-Nov-13	BBNO2	<24h	Indiv.H										‡	+	+	
Scat1374	7-Jan-14	BBNO2	<48h	Indiv.H										‡ + *			
Scat1385	25-Jan-14	BBNO2	<6h	Indiv.H										‡ +		*	
Scat1241	30-Aug-13	BBNO mai	<24h	Indiv.H?													
Scat1245	3-Sep-13	BBNO mai	<12h	Indiv.I													
Scat1342	20-Nov-13	Chewbaak	<24h	Indiv.I										‡			
Scat1353	23-Nov-13	BBNO2	<24h	Indiv.I										‡	+	+	
Scat1375	7-Jan-14	BBNO2	dry	Indiv.I										‡ + *		*	
Scat1376	7-Jan-14	BBNO2	<48h	Indiv.I										‡ + *		*	
Scat1380	18-Jan-14	FIELD2	dry	Indiv.J											‡ + *	‡ +	*
pop84	06-Sep-11	FIELD2	<week		‡ + *												
pop105	27-Dec-11	FIELD2	<24h		‡ + *						+						

LEGEND		
	‡	was present/sighted ≤ 48h
	+	was present/sighted within 7 days prior collection
	*	was present/sighted between 2-4 weeks
	?	was present/sighted > a month

CHAPTER 5: DISCUSSION

5.1 Individual identification of scat samples

The present study demonstrates the feasibility of using non-invasive DNA techniques to identify individual cheetahs from cheetah scat. Identification was achieved by genotyping the extracted DNA from scat samples at three microsatellite loci. After multiple PCR attempts per scat sample (three PCR attempts on average), only two samples out of 52 failed to work. Over 96% (50 out of 52) of samples yielded a genetic profile at the three microsatellite loci. The success rate of this study is high compared to 85% obtained by Mondol *et al*. (2009), 60% obtained by Bhagavatula and Singh (2006) and 91.5% obtained by Borthakur *et al*., (2011), despite the age of the scat samples, which in some cases was likely to be several weeks old. The increased success rate is likely in part due to the fact that the markers were optimized for bad quality DNA (the primers were redesigned specifically for this purpose). This study was conducted in a 45 ha study area in northern-central Namibia. Ten individual cheetahs were identified.

5.2 Probability of identity

Genetic profiles of each sample were compared to that of other samples in the sample collection. The three microsatellite loci selected showed a considerably low probability of identity for field collected cheetah scat samples. A probability of identity of $2x10^{-3}$ corresponds to two individuals in 1000 individuals who are expected to have the same genotype profile across the three microsatellite loci by chance; this in turn corresponds to 998 chances out of 1000 that the sample belonged to the same individual if they share the same genotype. It was therefore considered that all samples with the same genotype at the three loci belonged to the same individual. The obtained value for probability of identity agrees with the value suggested in other studies (Waits

et al. 2001; Mondol *et al*. 2009). It has to be taken into consideration that for the purposes of this study, the probability of identity was calculated based on the assumption that individuals are unrelated. For related individuals the chances to share the same genotype is increased significantly. Full sibling have a 25% chance to share the same genotype at one locus if the parents have four different alleles, this corresponds to a 1.6% chance (0.25x0.25x0.25x100) to have the same genotype at 3 loci; this chance is further increased for common alleles: if the parents have some alleles in common or one parent is homozygous at a given locus. However it was outside of the scope of this study to further investigate the possibility to miss a related individual; and for population genetic studies, the risk of missing a related individual is irrelevant, since only unrelated individuals are considered for analysis for such studies. Therefore the three markers were considered adequate to identify individuals from scat samples in the study area. The three markers can be amplified in a single PCR, thus providing a very easy test to identify individuals from scat samples. The ten identified individuals were considered in further analyses.

5.3 Relatedness

In addition to demonstrating the ability of identifying unique individuals from DNA from scat samples, this study also successfully used genetic profiles obtained from scat samples to determine the relatedness among identified individuals. Three different groups of two or more related individuals were identified. The three groups were assigned to three of the five coalitions that were seen on the camera trap photos: Sam & HiFi, Two boys, and Three boys. In addition the two single individuals (individual A and J) were shown to be related to coalition Two boys and Three boys, respectively. Individual A was less related to the coalition Two boys ($r<0.45$) than the other individuals (members of the coalition) were related to each other ($r>0.5$). The

same trend with individual J, who was less related to the coalition Three boys, with relatedness value (r<0.3), compare to the members of the coalition (individual G, H and I) to each other, that had a mean relatedness value (r>0.3). Most related individual pairs were consistent with a parent offspring category including members of a coalition. This was the case of individuals C and D and individuals G and I, for which the programs MER and CERVUS indicated a parent/offspring relationship. However, when assessing these determined parent/offspring pairs on camera trap photos, results show that these pairs were male coalitions of similar age, thus unlikely to be from a different generation. In addition no record was found in the literature reviewed of a male cheetah coalition consisting of father and sons (personal communication, Dr Marker). Since they were captured on camera trap as a coalition, this implies that they are more likely to be brothers than father/son.

It is to be noted that the relatedness coefficient "R" does not distinguish between parent-offspring and full-sibling relationships; in both cases R=0.5. Programs such as MER and CERVUS determine a parent-offspring relationship based on the fact that parents and offspring always share one allele at each locus. This is due to the fact that genetically, offspring get 50% of their genetic makeup from the mother and 50% from the father at each locus, so one allele always gets inherited from a given parent, leading to always sharing one allele per locus, which in turn is the basis for MER and CERVUS calculations. It was noticed that the pairs assigned a parent-offspring relationship shared at least one allele per locus across all 15 loci genotyped. This explains why their profiles were interpreted as parent offspring relationship. The likelihood for siblings to share an allele by common descent at any given locus is 25%. If the allele is common and/or the allelic diversity is low those chances to share an allele increase as it can also be caused by chance. If any parent is homozygous at a given locus, all offspring will share the

same allele (even though not by common descent). If the parents are related, chances of them sharing an allele, and thus the offspring having that allele in common, is increased. However, sharing an allele at 15 loci is highly unlikely regardless of the genotype of the parents.

Considering low genetic diversity in cheetah, probably 15 loci used in this study were not polymorphic enough to distinguish between the type of relationship of closely related individuals, and caution should be exerted for the interpretation of the results. One recommendation for future work would be to include more loci in the analysis.

5.4 Matching non-invasive techniques

The present study, for the first time, uses non-invasive techniques to identify individual cheetahs in the sample collection and matched these genetic identified individuals to individual cheetahs in the camera trap pictures.

In theory, non-invasive techniques can provide complete individual profiles for elusive endangered species. However this was never done before. Previous studies, Janecka *et al*., 2011; Gopalaswamy *et al*., 2012; Sollmann *et al*., 2013 combined the two non-invasive techniques (camera trap and genetic analysis of scat samples) to improve densities and abundance estimates of a species under study; however did not match a given photo to a given genetic profile. The present study demonstrates the feasibility of matching two non-invasive techniques, DNA derived from scat and remote camera traps, to obtain a combined physical and genetic profile of individual cheetahs. The aim of this study was achieved as scat samples were successfully assigned to a potential defecator on camera trap photos. Assigning scat samples to camera trap photos was achieved by elimination process by combining the age of samples (from same individual) with sightings (visitations) of individuals in photos, which narrows it down to an individual mostly present in all samples. Out of ten identified individuals obtained from scat

samples, 90% were successfully assigned their corresponding physical appearance in camera trap photos. Only one individual (10%) could not be assigned to its physical profile, due to insufficient numbers of samples collected from the individual (only one scat sample could be assigned to this individual), providing insufficient information for the elimination process, and thus multiple options as to which physical individual it might be. It is the first study to successfully link genetic identified individuals from scat samples collected at different stages of freshness to individual cheetahs detected in camera traps located at the same sampling sites. This study contributed to the conservation of cheetahs; hence different data were collected from wild cheetah using the two techniques, without any distraction of an individual.

CHAPTER 6: CONCLUSION

Overall the study was successful in obtaining the results that were aimed for and showed that it is possible to obtain both genetic and physical information on individuals in a study area by combining camera trap and genetic data. Over 96% of scat samples could be assigned to ten individual cheetahs, and 90% of individuals obtained genetically, could be assigned to a physical individual identified on camera trap. The genetic identification of scat samples was higher than expected compared to past studies. The fact to be able to assign the genetic profile to an individual that was identified on camera traps has no comparative data in the literature as this is the first time that such a study has been attempted. The convenience of having a single amplification reaction for the three selected markers makes it a useful tool for future studies. However, the work intensity of such a study needs to be considered. As with other non-invasive studies, multiple repeats are needed to obtain reliable genotypes, and the camera trap study needs to be performed at the same time as sample collection. Since camera trap studies require regular inspection of the cameras placed in the study area, scat sample collection can be performed at the same time if the person going to the site takes the time to look for samples. The current study bridges the gap in knowledge, by obtaining an individual combined profile. The combination of these parameters (physical and genetic profiles) is usually obtained by using invasive methods.

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