Genetic verification of multiple paternity in two free-ranging isolated populations of African wild dogs (*Lycaon pictus*)

by

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DECLARATION

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

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

This dissertation is presented in partial fulfilment of the requirements for the degree MSc in Production Animal Studies.

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Signed …….

Charlotte Moueix

Date …….

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ABSTRACT

The African wild dog, second most endangered carnivore in Africa, has a well-developed, highly cooperative pack system. The usual structure of a pack consists of a dominant breeding pair, the alpha male and female, several subordinates, non-breeding adults and dependent offspring. Domestic dog microsatellites were used to study the parentage in three packs and confirm that more than one dog, including the subordinate males, can sire pups within a litter as previously suggested.

The study was performed on two isolated populations of wild dogs in the North West Province of South Africa. In Madikwe Game Reserve, skin samples from 47 dogs were obtained by means of biopsy darts (adults) and skin slivers taken from the ear (subadults) and stored in absolute ethanol. In Pilanesberg National Park, blood samples from 18 captured dogs were collected in EDTA blood tubes. The wild dogs were photographed and individually identified according to coat patterns. Behavioural data to determine ranking were collected from all three packs.

DNA was extracted from collected samples using proteinase-K digestion followed by isolation of DNA with phenol/chloroform/isoamyl alcohol. A total of 16 microsatellite loci that consistently amplified and appeared to be polymorphic in wild dogs, were used. Polymerase Chain Reaction (PCR) was performed using two panels of microsatellite loci in multiplex reactions. An amount of 1 µl of PCR product was loaded on to the 3130 *XL* Genetic Analyser with Genescan 500 LIZ (Applied Biosystems) size standard and analysed using *STRand* (Board of Regents, University of California) software program. CERVUS 2.0 software was used to calculate allele frequencies, expected and observed heterozygosity, frequency of null alleles, polymorphic information content and exclusion probabilities for parentage assignment. Parentage verification was also performed manually.

The parentage analysis revealed that at least one pup was not sired by the alpha male in each of the five litters studied. Although previous studies suggested that the alpha male sires the majority of offspring in the pack, our results confirm that subordinate males commonly sire pups with the

alpha female if and when the opportunity arises. This is possibly a mechanism to decrease the effects of inbreeding.

CHAPTER 1

Introduction

The African wild dog (*Lycaon pictus*) is the second most endangered carnivore in Africa. The World Conservation Union (IUCN) red list (Version 3.1; Sillero-Zubiri *et al*. 2004) lists *Lycaon pictus* as endangered (criteria C2a).

It is feared that African wild dogs may become extinct within a period of 10-40 years. Historically, wild dogs occurred throughout sub-Saharan Africa, with the exception of true rain forest and desert (Creel & Creel 2002). There has been a dramatic reduction in both numbers and geographic range over the last 30 years, with latest estimates suggesting that as few as 3500 – 5000 individuals remain (Fanshawe *et al*. 1997). Their geographic distribution range has declined during the same period from 33 to 15 countries (Woodroffe *et al*. 1997). Wild dogs have fared especially poorly in North and West Africa (Fanshawe *et al*. 1997) and viable populations are now restricted to southern (Botswana, Namibia, South Africa and Zimbabwe), Central (Zambia) and East Africa (Tanzania).

Current efforts to improve the conservation status of wild dogs have focused upon the creation of a metapopulation through their reintroduction into geographically isolated reserves (satellite populations). Due to the insularisation of wild areas, genetic exchange between populations has been reduced and levels of genetic variability have declined due to breeding between closely related dogs.

Wild dogs are intensely social animals spending almost all of their time in close association with one another. They live in permanent packs of five to 30 individuals typically composed of a dominant breeding pair, a number of non-breeding adults, and their dependent offspring. From observation and analyses, subordinates have occasionally been shown to breed successfully.
The objectives of this study were as follows:

- To establish a semi-invasive sampling method for providing reliable genetic material.

- To validate a microsatellite multiplex PCR panel composed of polymorphic domestic dog microsatellite loci for investigating genetic parameters in the African wild dog.
- To establish the parentage of the pups in the different packs sampled.

CHAPTER 2

Literature review

2.1 Historical and current status of wild dogs in Africa

2.1.1 Historical evolution and distribution

2.1.1.1 Taxonomy and phylogeny

Temmick (1820) first described the African wild dog as a type of hyena and named it *Hyena picta*; Matthew (1930) placed wild dogs in a subfamily of the Canidae, the Simocyoninae, with the dhole (*Cuon alpinus*) and the bush dog (*Speothos venaticus*). The shape of the lower carnassial molar (short blade and no basined cusp) distinguish this group (Van Valkenburgh 1989). The work of Girman *et al*. (1993) on the cytochrome *b* gene, shows that wild dogs are phylogenetically distinct from the other wolflike canids, and are placed in a monotypic genus: *Lycaon* (Figure 1). Three chrono-species were identified by Martinez-Navarro & Rook (2003): *Lycaon falconeri* for the Late Pliocene forms of Eurasia; *Lycaon lycaonoides* for the forms from the Early Pleistocene and the beginning of the Middle Pleistocene of Eurasia and Africa; and *Lycaon pictus*, for the Middle Late Pleistocene and extant African form.

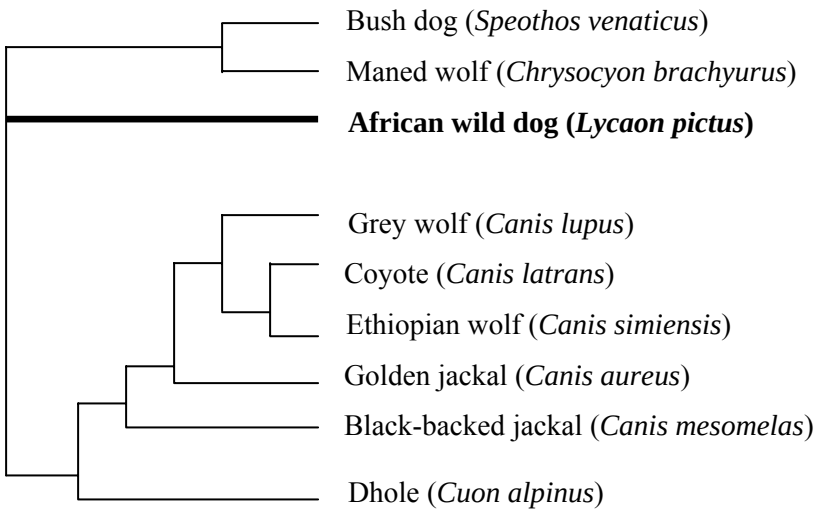

Figure 1: Consensus tree of the wolf-like canids (MacDonald & Sillero-Zubiri 2005)

2.1.1.2 Historical distribution and World Conservation Union (IUCN) status

The African wild dog was formerly distributed throughout sub-Saharan Africa, except for countries in West and Central Africa that were covered with rain forest. Habitats include short grass plains, semi-desert, bushy savannahs and upland forest. Wild dogs are rarely seen, and it appears that populations have always existed at very low densities. The species is virtually eradicated from West Africa and greatly reduced in central Africa and northeast Africa. The largest populations remain in southern Africa and the southern part of East Africa.

The status of the African wild dog according to the IUCN/SSC Red List (Version 3.1) Canid Specialist Group changed during the course of the past 30 years, from being **vulnerable** in the 1970's and 1980's to **endangered** in 1994. From 1996 to 2003, it was considered endangered under Criteria C1, an estimated decline of at least 20 % within five years or two generations. In 2004, it was classified as Criteria C2a(i), a continuing decline, observed, projected, or inferred, in numbers of mature individuals and a population structure with no subpopulation is estimated to contain more than 250 mature individuals.

2.1.2 Current status in Africa

Wild dogs have disappeared from much of their former range with 25 of the former 39 countries no longer supporting populations. The current population estimates suggest that only 3000 to 5500 free-ranging wild dogs remain (Woodroffe & Ginsberg 1998; Sillero-Zubiri *et al*. 2004).

In 2004, wild dogs occurred in the following countries (Figure 2):

Botswana: The outlook remains hopeful and the northern part of the country may contain one of the most extensive populations remaining in Africa, estimated at 700 to 850 individuals.

Cameroon: Wild dogs occur in three parks in the north of the country, with an estimated total of a 100 animals.

Central African Republic (C.A.R): An estimated 150 individuals remain here.

Figure 2: Distribution of wild dogs in Africa

Chad: Southern Chad forms an important passageway between sub-populations in Cameroon and C.A.R, possibly forming a larger and more viable population of 50 animals.

Ethiopia: A survey has identified 200 individuals.

Kenya: The population is estimated to be 250 animals, but is declining and has become extinct in some areas.

Namibia: Namibia has a viable wild dog population of 400.

Senegal: Approximately 100 animals, the most viable population in West Africa.

Sudan: Large carnivores are so rare in Sudan that few livestock are lost to them. Nevertheless, there have been a few sightings in the south, with an estimated population of 100 dogs.

Tanzania: There is a good prospect for the long-term survival of the wild dog population, at least in the southern protected areas of Selous and Ruaha. Reliable sources estimate the wild dog population at 1900.

Zambia: Appears to have a viable population of 500 individuals.

Zimbabwe: The main population is found in Hwange National Park with approximately 700 animals.

2.1.3 Current status in South Africa

A single viable population exists in South Africa and is located in the Kruger National Park. In 1989 the population stood at 357, in 1995 at 434 in 36 packs and in 2000 it had decreased to 177 individuals in 25 packs. In 2005, the fifth wild dog census was carried out and preliminary results indicate that the wild dog population is approximately 140 individuals divided into 17 packs. In South Africa, the plan is to establish a meta-population made up of a number of satellite populations distributed within smaller reserves. The strategy has achieved some success, and, to date, five free-ranging sub-populations (Table 1) have been established, with a combined area of ~2750 km^2 and a population size of approximately 140 adults and sub-adults in 10 packs. Due to limited numbers of suitable national and provincial parks, the expansion of the meta-population is likely to depend increasingly upon private game reserves (Lindsey 2004).

A wild dog population of about 76 individuals also occurs on private land (Lindsey 2004). In areas of high prey-density, protected parcels, as modest as 130 km^2, have the potential to support a small pack of wild dogs and their year's offspring. Several features of wild dog biology suggest that reintroduction might be technically difficult. Many attempts to release wild dogs into the wild have failed due to poor management and lack of post-release monitoring. Future reintroductions may benefit from ecotourism being able to offset some or all the costs (Frantzen *et al*. 2001).

The captive population of wild dogs in South Africa constitutes the largest concentration in the world. The two breeding programs at the De Wildt Cheetah and Wildlife and Hoedspruit Cheetah Research Centres held a high density of wild dogs, managing genetic and demographic issues (Frantzen *et al*. 2001).

Table 1: Distribution of free-ranging wild dogs outside Kruger National Park in South Africa (Lindsey 2005)

Name of the reserve	Number of wild dogs
Hluhluwe-Umfolozi Park	31 in 3 packs
Karongwe Game Reserve	10 in 1 pack
Madikwe Game Reserve	39 in 3 packs
Pilanesberg National Park	31 in 2 packs
Venetia Limpopo Nature Reserve	13 in 1 pack

2.1.4 Importance of protected areas: problem of genetic diversity

Due to the insularity of wild areas, genetic exchange between populations has been reduced and levels of genetic variability have declined as a result of breeding between closely related dogs. In addition, these populations have been subject to stochastic demographic events, which threaten small populations (Frantzen *et al*. 1998). Inbreeding in wild populations is strongly affected by dispersal patterns (Fuller 1992) and in captive populations by appropriate husbandry and management. In many species, generations of inbreeding cause a reduction of mean individual fitness through increased expression of deleterious alleles. This leads to developmental abnormalities, lowered fertility, and increased juvenile mortality. The loss of genetic variability due to random genetic drift can diminish adaptability to a changing environment and may also affect the susceptibility of a species to epizootics and parasites (Lacy 1987). Disease, in conjunction with the loss of genetic variability within a species, may play an important role in the decline of wild, relocated and captive populations of endangered species.

A common problem faced by several captive-breeding institutions is the limited number of wild dogs that they can successfully maintain. With only a few individuals and no exchange of unrelated genetic material, inbreeding is inevitable. This can be avoided by co-operative measures such as exchanging genetic material among breeding establishments.

In order to retain a true representation of the genetic diversity of a population, it is of great importance to study and analyse the genetic structure of the population (Frantzen *et al*. 1998). If pedigree information is available, breeding pairs may be selected to ensure that founder genetic material is equally represented in offspring. Alternatively, individuals may be recommended as subjects for the exchange of genetic material among breeding institutions to simulate natural dispersal patterns in the wild. Finally, with pedigree and genotypic information, genetically healthy and diverse individuals may be selected for reintroduction into the wild. The problem with founder populations where no DNA analysis data is available is that the relatedness of animals is unknown. Wild dogs may be closely related even though they originate from different packs or sources.

2.1.5 Main cause of the decline of the African wild dog population

Like most large carnivores, wild dogs have disappeared from much of their historical ranges, as a result of their ongoing conflict with human activities, infectious disease and habitat fragmentation (Sillero-Zubiri *et al*. 2004). The ecological requirements of wild dogs predispose them to conservation difficulties. Large area requirements and naturally low densities are the basis for their conservation predicament (Creel & Creel 2002). Wild dogs inhabit larger home ranges than are to be expected for their body size (Gittleman & Harvey 1982), and utilise larger areas than other canids, or ecologically similar African carnivores (Creel & Creel 2002). The decline of wild dog populations reflects the geographical pattern of human population growth; today, wild dogs persist only in countries with relatively low human densities (Woodroffe & Ginsberg 1998).

The annual survival rates of pups, yearlings and adults at various study sites is shown in Table 2.

Table 2: Summary of wild dog per annum survival rates at various study sites (Creel *et al*. 2004)

Study site	Survival rates	
Kruger National Park	Pups:	0.35
	Yearlings:	0.45
	Adults:	0.75
Selous Game Reserve	Pups:	0.75
	Yearlings:	0.84
	Adults:	0.77
Northern Botswana	Pups:	0.48
	Yearlings:	0.74
	Adults:	0.43

Persecution by humans, in conjunction with habitat loss, is the most important reason for the decline in numbers of African wild dogs (Woodroffe & Ginsberg 1998). Large areas of natural habitat in Africa have been transformed by human activities, and the reduction or removal of populations of wild ungulates has contributed to the wild dogs' decline. Traffic and snares are responsible for significant additional sources of human-related mortality in some parts of Africa (Woodroffe *et al*. 1997b).

Competitive carnivores contribute to the enormous area requirements of wild dogs. Lions are a significant source of mortality for wild dogs (up to 12% of adult and 31% of pup deaths, Woodroffe & Ginsberg 1998) and limit access to habitats with high prey densities (Mills & Gorman 1997; Creel & Creel 2002). Predation by spotted hyenas is much less common than predation by lions, accounting for just 4% of adult and 6% of pup deaths recorded. Although hyenas cause little mortality directly, loss of prey to hyenas (kleptoparasitism) might be important (Gorman *et al*. 1998).

Disease is a serious threat to wild dogs (Woodroffe *et al*. 1997b), although the magnitude of the threat may be difficult to characterise. Rabies is believed to have contributed to one population's extinction, and has frustrated two reintroduction attempts. Canine distemper and anthrax have also been implicated in local die-offs. Contact with domestic dogs might be the origin of the canine distemper outbreaks in wild dogs (Creel & Creel 2002).

2.2 Wild dogs of Madikwe Game Reserve

2.2.1 Madikwe Game Reserve: geographical position

The Madikwe Game Reserve belongs to the most recent park development in South Africa. It was opened in 1991 and is still in the initial stages. The reserve comprises 60 000 ha of bush land north of the North West province town of Groot-Marico and reaching up to the Botswana border. In the south, the Dwarsberg Mountains form the border (Figure 3). The terrain is mainly open grassland and bushveld plains, interspersed with rocky outcrops and isolated mountains. Except for the Marico River in the east of the park, water resources are scarce and several dams have had to be built. The entire reserve has been enclosed in a 150 km perimeter fence that has been electrified to prevent the escape of elephants and the larger predators.

2.2.2 Operation Phoenix

During "Operation Phoenix" which began in 1993, more than 8,000 head of game were brought into the Park. In 1996, predators were introduced into Madikwe, first cheetahs, wild dogs and hyenas, and later lions from the Etosha National Park (Namibia) and the neighbouring Pilanesberg National Park. One hundred and eighty elephants were translocated from the Gonarezhou Game Reserve in Zimbabwe. The resettlement of the elephants was a great success and at present, the population has grown to 250 animals. Today, some 12,000 animals roam the Madikwe Game Reserve. All predator species are represented as well as black and white rhino, buffalo, giraffe, zebra and a great number of antelopes. More than 350 bird species have been registered.

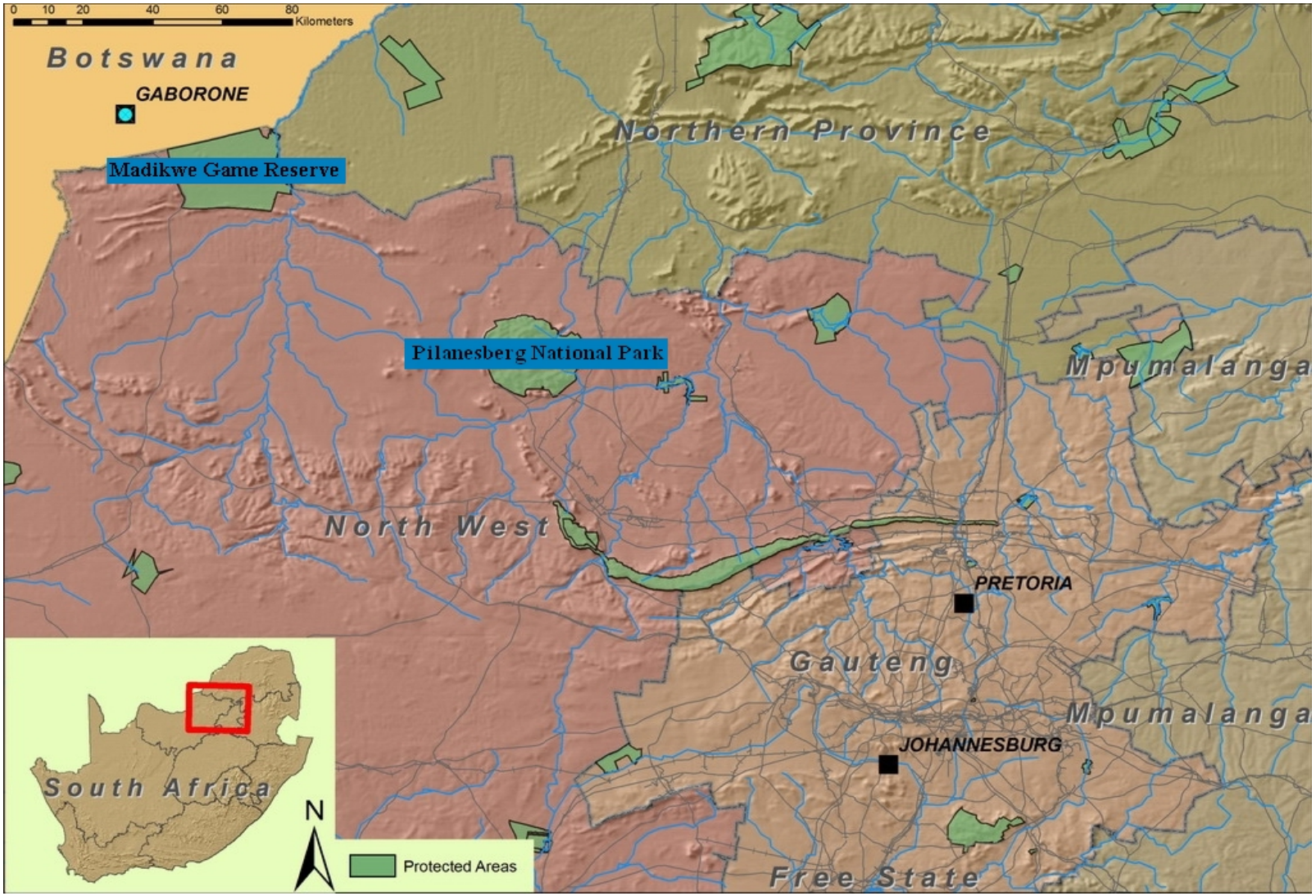

Figure 3: Geographic locations of Madikwe Game Reserve and Pilanesberg National Park

2.2.3 History of wild dogs in Madikwe Game Reserve

2.2.3.1 First introduction

A group of six dogs consisting of three captive-bred sisters from the De Wildt Cheetah Research Centre and three wild-caught (related) males from the Kruger National Park were put together in a boma in 1996 at Madikwe. The wild dog introduction proved an ideal opportunity to establish how wild and captive-bred individuals would interact, and to see if they would form a pack. The dogs remained together in the same boma for six months. They soon established a ranking order and formed a pack. An alpha male and female emerged, after some fighting, to lead the pack. After release the pack successfully hunted and never managed to escape from the reserve due to well-maintained electric fences. The pack bred twice: in 1996 seven pups were born of which six survived to adult hood. In 1997 twelve pups were born.

2.2.3.2 Outbreak of rabies and subsequent introductions

In 1997, a rabies outbreak unfortunately decimated the population and only three yearlings of the first litter survived. Much information was gained from the rabies outbreak and two new packs were introduced in 1998, all dogs having received at least two rabies vaccinations prior to release. The one pack consisted of two wild-caught males, captured as puppies, and three captive-bred females. This group therefore had no hunting experience but learnt quickly and within two weeks hunted successfully. The second pack consisted of the two surviving females from the original pack, two wild-caught males from the Kruger National Park and two hand-raised males from Botswana. Interestingly, the two younger hand-raised males were dominant over the older wild males and one of the Botswana males became the alpha male. Both packs bred. A second rabies outbreak occurred in 2000, and 8 of 11 unvaccinated pups died of the disease but none of the vaccinated adults died. Only one of the two packs was affected. This meant that the control measures put in place after the first rabies outbreak were successfully implemented to protect the packs from extinction by the disease.

In 2000 an additional two wild dog males were introduced to imitate natural immigration but by 2001 both these animals had disappeared. The offspring of the first two packs have formed new packs and will hopefully ensure the long-term success of this program. Complex dynamics took place between individuals within and between the different packs. This highlights the importance

of introducing more than one pack to ensure long-term survival of the species as a whole. It is, however, accepted that wild dog introductions in small numbers will need constant monitoring and even necessitate frequent introductions to sustain the populations.

2.2.5 Expenditure on wild dog conservation

An estimated US $ 378 887 was spent on wild dog conservation in South Africa during the period 1997-2001, at an average of US $ 75 777 per year. Of this, US $ 276 709 (73 %) was spent on the meta-population study by Lindsey (2005).
Madikwe expenditure in US $ was:

1997	1998	1999	2000	2001	Total
9,719	4,321	4,408	7,735	14,375	**40,558**

Sponsorship and income from ecotourism covered the costs of the introductions and the annual maintenance (Lindsey 2004).

2.3 Wild dogs of Pilanesberg National Park

2.3.1 Pilanesberg National Park: geographical location

The Pilanesberg National Park (55 000 ha) borders on the entertainment complex of Sun City (Figure 3). The 1 200 million year old crater forms the major part of the park which makes it almost a perfect circle with a small lake in the centre. This very scenic terrain lies in the transition zone between Kalahari and bush veldt and both types of vegetation are found here. The Pilanesberg National Park was opened in 1979. During "Operation Genesis" in the early 1980's, the largest game resettlement project in the history of South Africa took place. More than 6 000 animals were introduced from other parks.

2.3.2 Wild dogs of Pilanesberg National Park

In 1999 Pilanesberg National Park successfully introduced 9 African wild dogs. Wild females (n=3) and their young (n=3) and captive bred males (n=3) were used in that first introduction.

2.4 Behaviour of wild dogs

2.4.1 Social structure of a pack

2.4.1.1 Ranging behaviour and pack sizes

Wild dogs are intensely social animals spending almost all of their time in close association with one another (Creel & Creel 2002). They inhabit enormous home ranges, much larger than would be expected on the basis of their body size. They live in permanent packs of 5 to 30 individuals (Table 2) typically composed of a dominant breeding pair, a number of non-breeding adults, and their dependent offspring (Van Lawick 1970; Schaller 1972; Van Lawick 1974; Frame 1976; Frame *et al*. 1979; Malcom & Marten 1982).

2.4.1.2 Hierarchy

The social arrangements of African wild dogs are extraordinary because they are the exact opposite of those in most other social mammals.

Table 3: The average wild dog pack composition from various study sites (Creel *et al*. 2005)

Study sites	**Average pack size** (adults & yearlings)	**Density** wild dogs/1000 km^2
Northern Botswana	10.4 in 6-13 packs	5 to 35
Selous Game Reserve (Tanzania)	8.9 in 7 packs	38
Kruger National Park (South Africa)	10.4 in 8-12 packs	19 to 39

In a pack, there are two dominance hierarchies, one amongst the males, and the other amongst the females (Creel & Creel 2002). Analysis of faecal corticosteroids has shown that dominant dogs excrete corticoids in higher concentrations than subordinate ones (Monfort *et al*. 1998). Being dominant in a pack involves more aggressive interactions to maintain rank with possibly chronic stress being the cost of such social dominance. The main fighting period between males occurs

during the mating period, when the younger ones are trying to mate with the alpha female (Monfort *et al*. 1998). Many packs include one or more old, formerly dominant males. The mechanism of preventing mating by other males is essentially behavioural, primarily by staying very close to the α-female in oestrus and not permitting any other males close to her. This is known as "consorting" (Creel & Creel 2002).

The oldest female is usually the dominant one and the only one that breeds. She influences the survival of her rival's pups in several ways: by killing them (preventing other adults from feeding their pups) or by harassing the mother in ways that render them more vulnerable to exposure or predation. The distribution of mating has important implications for patterns of relatedness and population genetic structure of the population. Alpha females produced 81 % of 85 litters in Kruger National Park (Reich 1978).

The method by which the dominant female suppresses the mating behaviour and oestrus cycling of subordinate females, is not yet clear (Creel *et al*. 1997). Although both behavioural and endocrine mechanisms are suspected, it is considered that reproductive suppression is ultimately based on competition for resources within the pack. Subordinate female wild dogs rarely reproduced (6 % to 10 % annually), and in association with reproductive suppression, subordinates had elevated baseline oestrogen faecal levels when compared to dominant females (Creel *et al*. 1997).

The dominant couple largely monopolises the breeding. Subordinates, however, have occasionally been shown to breed successfully. Girman *et al*. (1997) reported that subordinate males (the α-male's brother) can copulate with the α-female, although the number of offspring from subdominant males that survive to reproductive age, if any, is unknown. In many packs, more than one female will come into oestrus and mate. Subordinate females usually come into heat only several weeks after the dominant female. The majority of the resultant secondary pregnancies have a poor prognosis with the loss of the entire litter commonly being reported (Girman *et al*. 1997).

In Selous Game Reserve, subordinate females gave birth to eight of a total of 40 litters recorded and of these, three were victims of infanticide, while five were nursed with the dominant female's litter (Creel &Creel 2002). Of the 21 dens observed in the Kruger National Park, nine had subordinates breeding in them but only in two cases did the pups live beyond one month of age (Mills & Gorman 1997).

2.4.1.3 Emigration, dispersal and gene transfer

Within the wild dog pack, all the males are related to each other and all the females to each other but not to the males, except if offspring born within the pack are recruited (Girman *et al*. 1997). Sometimes, individuals of either sex stay in their original pack well beyond their majority; in that case, dogs from opposite sex are related. New packs are formed when small sub-groups of the same sex (usually siblings) leave their natal pack (McNutt 1996) and join up with other sub-groups or individuals of the opposite sex (Burrows *et al*. 1995). The dispersal is usually not sex-biased.

Primiparous females have been found to breed in small packs (7.2 ± 1.3 adults), just above the threshold for successful reproduction (five adults) whereas multiparous females were recorded breeding in significantly larger packs (13.1 ± 1.7 adults). Thus, to remain above the threshold for successful reproduction, primiparous females must recruit helpers into their pack, and this would favour a bias toward the production of males, the more philopatric sex (Creel *et al*. 1997). Concordantly, primiparous wild dogs have been recorded to have baseline oestrogen levels double those of multiparous females, supporting the hypothesis that elevated estrogens are associated with male-biased sex-ratios in the wild dog. A birth-order effect is reported, shifting from a male-bias in first litters to a female-bias in subsequent litters (Creel *et al*. 1997).

2.4.2 Reproductive behaviour

Reproduction in wild dogs is highly seasonal, occurring between March and April in southern Africa (Malcom 1979; Reich 1981). The timing of reproduction is closely related to rainfall, so that pups are born during the driest period of the year. The peak of the whelping season is July (the driest month), and rainfall remains low throughout the three-month denning period (Woodroffe *et al*. 1997).

The fecundity of a female is strongly influenced by her age. Few females attain dominance while they are young. Sexual maturity is attained between 12 to18 months of age (Macdonald 1983). Most reversal of dominance happens during the mating periods, when alpha males are involved in more than twice as many fights than are alpha females (Creel & Creel 2002). As pack size increases, so does the number of offspring born and raised. In general, reproductive success is greater for older females in larger packs. Non-breeding adults of both sexes contribute towards the reproductive success of breeders in several ways. Firstly, dogs in large packs obtain more meat with less effort than dogs in small packs (Creel & Creel 1995b). Secondly, when pups are young, non-breeders feed them regurgitated meat and also guard the den (Malcom & Marten 1982). When the pups are older and can move with the pack, non-breeders of both sexes allow the pups to feed first, guarding the carcass while the pups eat. Wild dogs are truly cooperative breeders.

Mating spans a period of three to seven days, but breeding behaviour builds gradually over several weeks prior to actual mating. A strengthening of the bond between the alpha male and female is the first overt behavioural sign that a mating period is approaching. The female's vulva becomes swollen during pro-oestrus, accompanied by a slight bloody discharge. The female generally does not tolerate mating attempts for several days. She rejects mounting attempts by moving away, lying down or snapping at the male. The female gradually stops these behaviours, and eventually stands firmly with her tail lifted to one side as the male mounts. Many mounts fail to result in complete copulation. Copulations include a copulatory lock in at least some cases, in which the bulb of the penis swells so that the mating pair cannot disengage for a period of up to a minute (Creel & Creel 2002). Once the genital lock has been achieved, the female will often sit or lie down with the male following due to the lock. Hofmeyr (personal communication) observed genital locks lasting for as long as 20 min.

Gestation lasts 64 to 79 days, and the female whelps in an underground den (Creel & Creel 2002). In most wild dog packs, the dominant female produces a single litter each year (Fuller *et al.* 1992a). Litter sizes range from 8-11 pups but can be as large as 19 (Table 3). The pups weigh between 350 and 380 g at birth. More than one adult may carry pups in a den move, but the mother usually does most of the ferrying. The pups are suckled for up to 10-12 weeks and open

their eyes when 10 to 14 days old. Suckling sessions last only 2½ to 3 minutes. Although the females will suckle the young for about 3 months, they start weaning at about 14 days (Table 4). Females have 6 to 8 pairs of teats.

Table 4: Mean litter sizes for various study sites (Creel *et al*. 2005)

Study sites	Mean litter size
Selous Game Reserve	7.5 ± 0.56
Northern Botswana	10.1 ± 0.37
Kruger National Park	9.4 ± 0.7

The den is usually an unoccupied warthog or aardvark hole, which the female expands by digging with her forepaws. Most dens are located in sandy soil, and many dens are complex with several exits (Creel & Creel 2002). Only one den is normally occupied at any one time, but it is common for the pups to be moved to a new den, particularly if lions are in the area. The reasons for den moves are not always obvious. Faeces, bones and scraps of regurgitated meat accumulate over time, and the smell is eventually noticeable even to the human nose, so it is possible that dens are moved to avoid attracting other carnivores. Dens might also be moved in response to the death of a pup or to a build-up of parasites. The denning period is the only time when the wild dogs return to the same location each day; at other times, it is extremely rare for a pack to sleep in the same place for two consecutive days. Dens are located near permanent water, which attracts high densities of ungulates. If wild dogs can den near a predictable food supply, this might reduce the energy otherwise spent on pursuing prey (Creel & Creel 2002). After 3 months, the den is abandoned and the pups begin to run with the pack. At 8-11 months of age, they can kill easy prey, but they are not proficient until about 12-14 months of age, at which time they can fend for themselves.

2.4.3 Diet and cooperative hunting

Members of wild dog packs hunt cooperatively, which allows them to catch much larger prey (McNutt *et al*. 1996). The hunt is almost always preceded by a "social rally" that is believed to

coordinate the pack in preparation for hunting. The reactions of the prey animals when they spotted the dogs varied from fleeing, standing still or even trying to defend themselves alone or as a herd. Wild dogs can run at speeds of up to 60 km/h during the chase. Once one of the dogs has managed to make the first grab, the rest of the pack will help to pull the prey down and will start eating it even if it is still alive. This voracity has contributed to their persecution by man (Woodroffe *et al*. 1997).

The daily food requirement in the form of meat is estimated at 1.8-3.5 kg/dog/day (Lindsey *et al*. 2004). Wild dogs mostly hunt medium-sized antelope, with the preferred species varying according to the most abundant prey species in the area (Table 5; Creel *et al*. 2004). Wild dogs rarely scavenge, perhaps to avoid the risk of contact with larger predators.

Table 5: Observed hunting data in different study sites (Creel *et al*. 2004)

Study sites	Proportion of prey killed
Selous Game Reserve	Wildebeest: 54 % Impala: 29 %
Northern Botswana	Impala: 85 %
Kruger National Park	Impala: 73.2 % Duiker: 8.9 %

2.5 Molecular genetics of African wild dogs

2.5.1 Chromosome numbers of African wild dogs and related species

Comparative analysis of chromosomes has proved very useful because canids have a rich diversity of chromosome morphology ranging from species such as the red fox, which has a low diploid number of chromosomes (2n = 36) and all metacentric autosomes, to the grey wolf, which has a high diploid number (2n = 78) and all acrocentric autosomes (Table 6).

The wolf-like canid's chromosomes are stable in morphology and number (2n = 78). Because of the recent common ancestry of the members of this group, genes that have high rates of sequence

Table 6: Chromosome number of different canid species (Ostrander & Wayne 2005)

Species	Common name	2n chromosome number
Wolf-like canids		
Canis aureus	Golden jackal	78
Canis mesomelas	Black-backed jackal	78
Canis familiaris	Domestic dog	78
Canis simensis	Ethiopian wolf	78
Canis lupus	Grey wolf	78
Canis latrans	Coyote	78
Canis rufus	Red wolf	78
Cuon alpinus	Dhole	78
Lycaon pictus	African wild dog	78
South American canids		
Speothos venaticus	Bushdog	74
Lycalopex uetulus	Hoary fox	74
Cerdocyon thous	Crab-eating fox	74
Chrysocyon brachyurus	Maned wolf	76
Red fox-like canids		
Vulpes aelox	Kit fox	50
Vulpes vulpes	Red fox	36
Alopex lagopus	Arctic fox	50
Fennecus zerda	Fennec fox	64

substitution, such as those found in the vertebrate mitochondrial genome, can be used to resolve their phylogenetic relationships. A phylogenetic analysis of 736 base pairs (bp) of the mitochondrial cytochrome *b* gene revealed a close kinship of grey wolves, dogs, coyotes and Ethiopian wolves (Wayne *et al*. 1990; Lehman *et al*. 1991). As a group, these were distinct from the African wild dog and from the golden and black-backed jackals. The grey wolf and coyote may have had a recent common North American ancestor about two million years ago (Nowak

1979) whereas the Ethiopian wolf, found only in a small area of the Ethiopian highlands, is possibly an evolutionary relic of a past African invasion of grey wolf-like ancestors.

2.5.2 Microsatellites and function

Microsatellites (Litt & Luty 1989) or short tandem repeats (STR) are tandem repetitive stretches of short (2 to 4 base pair) motifs (Beckmann & Weber 1992; Hughes & Queller 1993; Queller *et al*. 1993; Tauzt 1993; Ashley & Dow 1994; McDonald & Potts 1997; Parker *et al*. 1998; Chambers & MacAvoy 2000). They belong to a class of sequences termed variable number of tandem repeats (VNTR), referring to any tandem repetitive (e.g. CACACACACA) DNA that shows length polymorphism (Ellegren 2000). These tandem arrays of short stretches of nucleotide sequences are usually repeated between 10 and 30 times and along with the flanking regions, vary in size, with a mean of about 100 base pairs (bp). Microsatellites differ from most other types of DNA sequences in their unusual degree of polymorphism, making them interesting as genetic markers. The mutation rate, experimentally measured, is estimated between 10^{-4} and 10^{-6} per kilobase (e.g., $4.5x10^{-5}$ in mice (Deitrich *et al*. 1992), $7x10^{-5}$ at dinucleotide repeats in pigs (Ellegren 1995)). These high mutation rates are due to the slippage of the polymerase and a misalignment of the DNA strands during the replication (Levinson & Gutman 1987; Eisen 1998; Zhu *et al*. 2000), leading to the insertion or deletion of one (or more) repetitive units (Levinson & Gutman 1987; Ashley & Dow 1994; Schlötterer & Pemberton 1994; Ellengren 2000; Zhu et al. 2000).

They have been widely used in a variety of fields, including conservation genetics (Valière 2002), population genetics (Ellegren 1999) and forensics (Goldstein & Schlötterer 1999). Microsatellite analyses have been widely applied in the field of animal genetics and ecology. For example, they can be used to: establish the genetic diversity of a species or population; detect inter-species hybridization; study population structure and history; estimate population size; study population bottlenecks and potential inbreeding; and assess the impact of reproductive behaviour, social structure and dispersal on genetic structure of endangered populations (Goldstein & Schlötterer 1999).

2.5.3 Application of microsatellites

Genetic diversity, inbreeding level and parentage analysis can be important tools in the conservation of wild and endangered species (Crozier 1992; Milligan *et al*. 1994). Microsatellites are present in all vertebrate species (Tautz & Rentz 1984; Ashley & Dow 1994) but less frequently in invertebrates (Hughes & Queller 1993) and plants. Microsatellites have the advantage of being far more polymorphic than other markers (e.g. allozymes). They exhibit higher heterozygosity levels and individual heterozygosity is more closely related to degree of inbreeding (Amos *et al*. 1993; Slate *et al*. 2004). Under close inbreeding, the correlation between fitness and heterozygosity is higher for markers with high mutation rates, meaning that microsatellites are better suited for such studies. Until the emergence of a better genetic marker, the preferred use of microsatellites in conservation genetics is likely to remain unchanged (McDonald & Potts 1997).

Methodology consists of characterising locus microsatellites of one individual of a population (domestic dogs) and then studying their possible polymorphism in various individuals constituting the population to be studied (wild dogs). Primers labelled with different colour fluorescent dyes are commercially available (Beaumont 2000). In many species they are relatively easy to obtain, either through direct isolation of species-specific markers, involving the construction of a genomic DNA library or by the application of markers originally isolated from related species. They are useful for gene mapping, population studies, genetic diversity, parentage, social structure and various conservation biology studies (Kim 2003).

2.5.4 Review of microsatellite marker-based studies

Microsatellites can be used for numerous purposes; these are summarised in Table 7. These studies illustrate that a multitude of well-characterized microsatellites derived from domestic and wild species can be defined and optimized in related species. Cross species use of microsatellite loci saves time and effort, allowing rapid progress of genetic studies in several closely-related species (Slate *et al*. 1998; Luikart *et al*. 1998).

Table 7: Review of microsatellite marker-based studies

Estimation of the genetic diversity and the genetic structure of populations and conservation management	
Author	**Species**
Ciofi & Bruford (1999)	Komodo dragon (*Varanus komodoensis*)
Forbes & Boyd (1996)	**Grey wolf (*Canis lupus*)**
Forbes & Hogg (1999)	Bighorn sheep (*Ovis Canadensis*)
Goossens *et al*. (2001)	Alpine marmot (*Marmota marmota*)
Gotteli *et al*. (1994)	**Ethiopian wolf (*Canis simiensis*)**
Lucchini *et al*. (2002)	**Grey wolf (*Canis lupus*)**
Maudet *et al*. (2002)	Alpine ibex (*Capra ibex*)
Paetkau & Strobeck (1994); Paetkau *et al*. (1998b)	Brown bear (*Ursus arctos*)
Determination of the social structure, reproduction success and reproduction system of populations	
Garnier *et al*. (2001)	Black rhinoceros (*Diceros bicornis*)
Girman *et al*. (1997)	Wild dog (*Lycaon pictus*)
Kays *et al*. (2000)	Kinkajou (*Potos flavus*)
Miller *et al*. (2003)	**Coyote (*Canis latrans*) & Red wolf (*Canis rufus*)**
Williams *et al*. (2003); Sacks *et al*. (2004)	**Coyote (*Canis latrans*)**
Say *et al*. (1999, 2001)	Domestic cat (*Felis catus*)
Verwey *et al*. (2003)	Honey badger (*Mellivora capensis*)
Estimation of the size of a population	
Amos *et al*. (1993); Palsboll *et al*. (1997)	Whale (*Megaptera novaeangliae*)
Grewal *et al*. (2004)	**Eastern wolf (*Canis lycaon*)**
Kohn *et al*. (1999)	**Coyote (*Canis latrans*)**
Mowat & Strobeck (2001)	Brown bear (*Ursus arctos*)
Wandeler *et al*. (2003)	**Red fox (*Vulpes vulpes*)**
Individual identification	
Ellegren *et al*. (2000); Flagstad *et al*. (2003)	**Scandinavian grey wolf (*Canis lupus*)**
Ernest *et al*. (2000)	Mountain lion (*Puma concolor*)
Taberlet *et al*. (1997)	Brown bear (*Ursus arctos*)
Parentage study	
Carling *et al*. (2003)	Wild pronghorn antelope (*Antilocapra americana*)
Garnier *et al*. (2001)	Black rhinoceros (*Diceros bicornis*)
Haynie *et al*. (2003)	Prairie dog (*Cynomys gunnisoni*)
Kraaijeveld-Smit *et al*. (2002)	Agile antechinus (*Antechinus agilis*)
DeYoung *et al*. (2002), Sorin (2004)	White-tailed deer (*Odocoileus virginianus*)

CHAPTER 3

Materials and Methods

3.1 African wild dog populations studied

Originally the project was only planned for the Madikwe Game Reserve where there were two packs of well-studied wild dogs. Pilanesberg National Park presented itself as an opportunity later on in the study when the Hooter Pack was captured for translocation.

3.1.1 Madikwe Game Reserve

The details concerning habitat and wildlife biodiversity of Madikwe have already been described in the Literature Review (see 2.2).

With its suitable open, flat terrain, Madikwe was an excellent choice for the project. This environment provided ideal circumstances for the wild dogs to be tracked and observed on a regular basis. Between August 2004 and March 2005, the entire population of Madikwe Game Reserve was studied and sampled. The Table 8 shows the composition of the Madikwe wild dog population in August 2004.

Table 8: Wild dog population in Madikwe Game Reserve in August 2004

	Tswasa pack	**Madikwe pack**
Number of females	**3** MadF16, MadF15, MadF51	**8** DWF03, MadF34, MadF35, MadF37, MadF38, MadF39, MadF40, MadF41
Number of males	**6** DWM01, DWM07, MadM20, MadM52, MadM53, MadM54	**14** BotM2, MadM6, MadM11, MadM8, MadM9, MadM22, MadM23, MadM24, MadM25, MadM26, MadM27, MadM29, MadM31, MadM36
Number of pups	**10**	**15**
Total	**19**	**37**

3.1.2 Pilanesberg National Park

Due to the hilly and bushy terrain, the observation of wild dogs in the Pilanesberg is difficult. Nonetheless, considerable efforts had been made by the ecologist of the park to study the population and identify each dog by means of photographs. Owing to the marked decrease in numbers of prey species during the preceding years, some of which had partly been attributed to the wild dogs, it was decided to remove the Hooter pack (18 wild dogs, 9 adults and 9 pups born in June 2004) in March 2005. This pack was included in the study and henceforth will be referred to as the Pilanesberg population or pack. Table 9 shows the composition of the Pilanesberg wild dog population in August 2004.

Table 9: Wild dog population in Pilanesberg National Park in August 2004

	Hooter pack	**Whistle pack**
Number of females	**3**	**5**
Number of males	**6**	**2**
Number of pups	**9**	**7**
Total	**18**	**14**

3.2 Location and identification of dogs

Previous records, mainly based on digital photographs, formed the basis for identification of the Madikwe dogs. In the case of the Pilanesberg pack the dogs had already been identified and the ranking was known. However, when the Pilanesberg dogs were caught, a new set of digital photographs was recorded while the dogs were under anaesthesia. These were used to verify the previous identifications on record

Two dogs in the Madikwe pack and three in the Tswasa pack were radiocollared. The signals of the collars were used to locate the dogs each day that they had to be observed or sampled. Once the pack had been located, the dogs were either moving and hunting or sleeping. Most of the time, during the denning period, the dogs would stay hidden in the hills at the den, and would go out hunting in the afternoon.

3.2.1 Identification of individual dogs

Before behavioural observations and biopsy samples could be taken, each dog had to be identified. Digital photographs were taken from each side of each dog and coat patterns were then used for identification.

Figures 4 and 5 are examples of coat patterns and characteristics used to identify MadF40 and MadM52, respectively.

Figure 4: Female called "Snake" and "Dot" (MadF40) due to the patterns on her hind leg. The black ring on the white of her tail was also used for her identification.

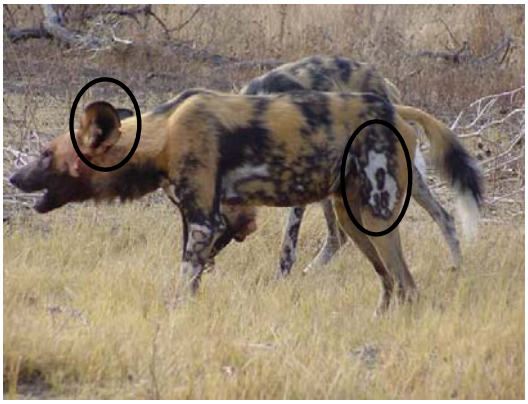

Figure 5: Male called "Ghost Buster" (MadM52) due to the patterns on his hind leg. The notch of his left ear was also used for his identification.

3.3 Behavioural observations

Both Madikwe packs were observed intensively for a month to determine ranking of female and male dogs. To help identify rank and relationship in the pack, the following questions were answered: Which wild dog was the leader during the hunts? Which wild dog came closest to the car to take the bait? Which wild dog was the first one to eat on the carcass? Which wild dog stayed at the den with the pups?

3.4 DNA sampling and storage

3.4.1 Sampling

3.4.1.1 Use of biopsy darts for the adults in Madikwe Game Reserve

Once the dogs had been individually identified DNA sampling commenced. The pack was located and then baited unless they were already on a kill. In order to avoid immobilisation, biopsy darts were used to obtain skin samples from the dogs. Pneu-dart™ biopsy darts (Pneu-Dart, Inc. Williamsport, PA 17703 USA) (Fig 6) were used and these were fired from a Dan-Inject™ dart gun (Figure 7) with a barrel design to take Pneu-darts. The approximate size of the biopsy taken by the dart is a plug of 5 mm long and 3 mm width.

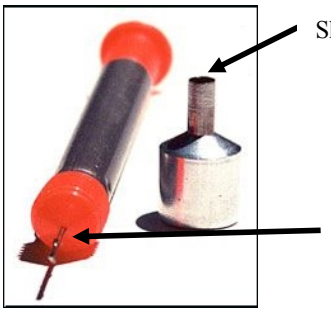

Figure 6: Pneu-dart: biopsy dart

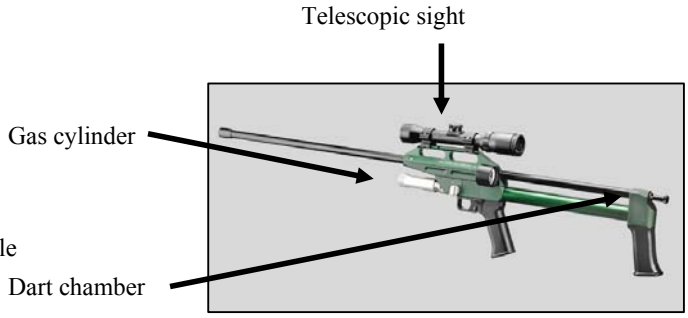

Figure 7: Dan-Inject dart gun

The pack was approached with a vehicle until they were within comfortable range. Before darting, each dog was identified. Darting could only be done if the dog was less than 10 meters away. The dart gun pressure was adjusted to between 2.5 and 3.5 bar. The darting site was selected and aim was taken on the hind leg or shoulder of the animal with the laser scope. Only dogs that were standing motionless were darted. All darts were pre-numbered with the identity of the dog prior to darting. This meant that a number of darts could be fired before they were collected on the ground.

3.4.1.2 Capture of pups for tissue samples in Madikwe Game Reserve

In Madikwe, the pups became mobile and were first observed with the pack, when they were three months old, in October 2004. They were all identified photographically but were still too small to be darted. For this reason it was decided to dart them with the biopsy darts later, when

they were 10 months old and a suitable size for darting. Even at this stage the sampling was unsuccessful. The decision was therefore to resort to immobilisation of the 10-month-old pups to obtain skin samples. These were taken from the edge of the ear. While the pack was feeding a wildebeest bait, immobilisations were carried out with 30 mg of Zoletil 100® (tiletamine, zolazepam combination. Virbac, France) and Dan-inject darts. With that low dose, the pups were partially immobilized, in groups of three. Tissue samples were collected in order to keep the protocol as for the adults (biopsy darts) and also to assure a rapid sampling due to the light anaesthesia. Once samples were collected, the pups were placed in a crate giving them time to recover without taking any risk. The sampled pups were marked with white paint allowing clear identification of the individual while darting the other ones. All immobilisations were conducted in the morning to allow the dog's ample time to recover fully before dark.

3.4.1.3 Collection of blood samples in Pilanesberg National Park

The larger pack (Hooter pack – henceforth referred to as the Pilanesberg pack, dogs or population) was captured for translocation, thus providing an ideal opportunity to collect samples from the dogs. The 18 dogs were enticed into a capture boma with bait. Each dog was then darted with 100 mg of Zoletil 100®. Following immobilization, 10 ml of blood was collected in EDTA tubes from the saphenous vein.

3.4.2 Storage of samples

3.4.2.1 Tissue samples

From darting to processing of the skin sample there was a maximum interval of two hours. The tissue sample was removed from the dart, cut into squares and transferred to a labelled tube containing absolute (99 %) ethanol. The ear plugs from the pups were directly placed in a tube containing absolute ethanol. A new pair of gloves was worn for each sample to avoid any cross contamination between samples. The tubes were then stored at 4 °C until further processing.

3.4.2.2 Blood samples

Blood samples were collected in EDTA tubes, labelled and stored at 4 °C until further processing, within two weeks of collection.

3.5 DNA analyses

3.5.1 DNA extraction method

DNA was extracted from wild dog blood and tissue samples using the following method. The preparation of the solutions, the methodology of the DNA extraction of blood and tissue are summarised in Figures 8, 9 and 10 respectively.

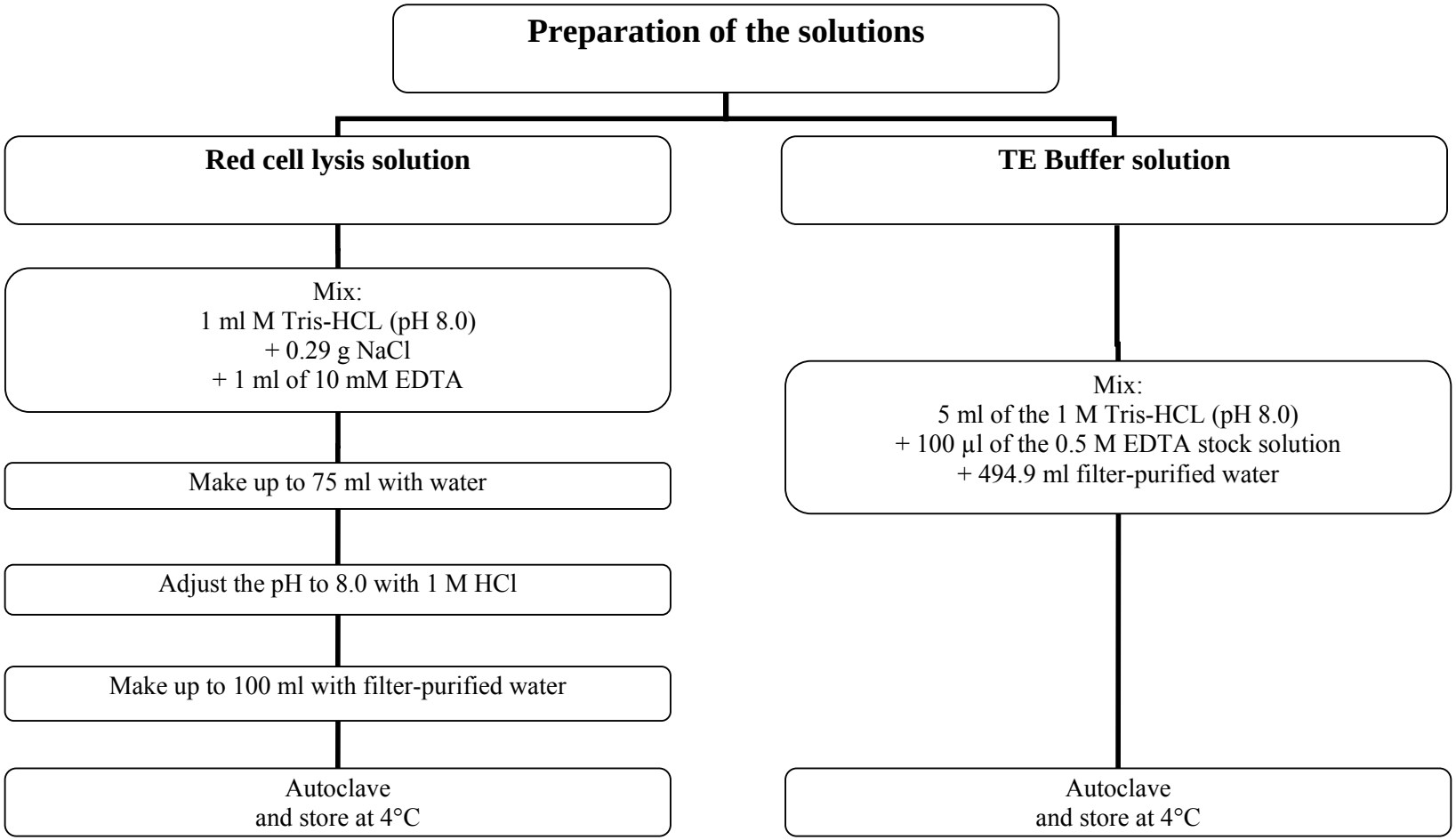

Figure 8: Preparation of the solutions for the DNA extraction

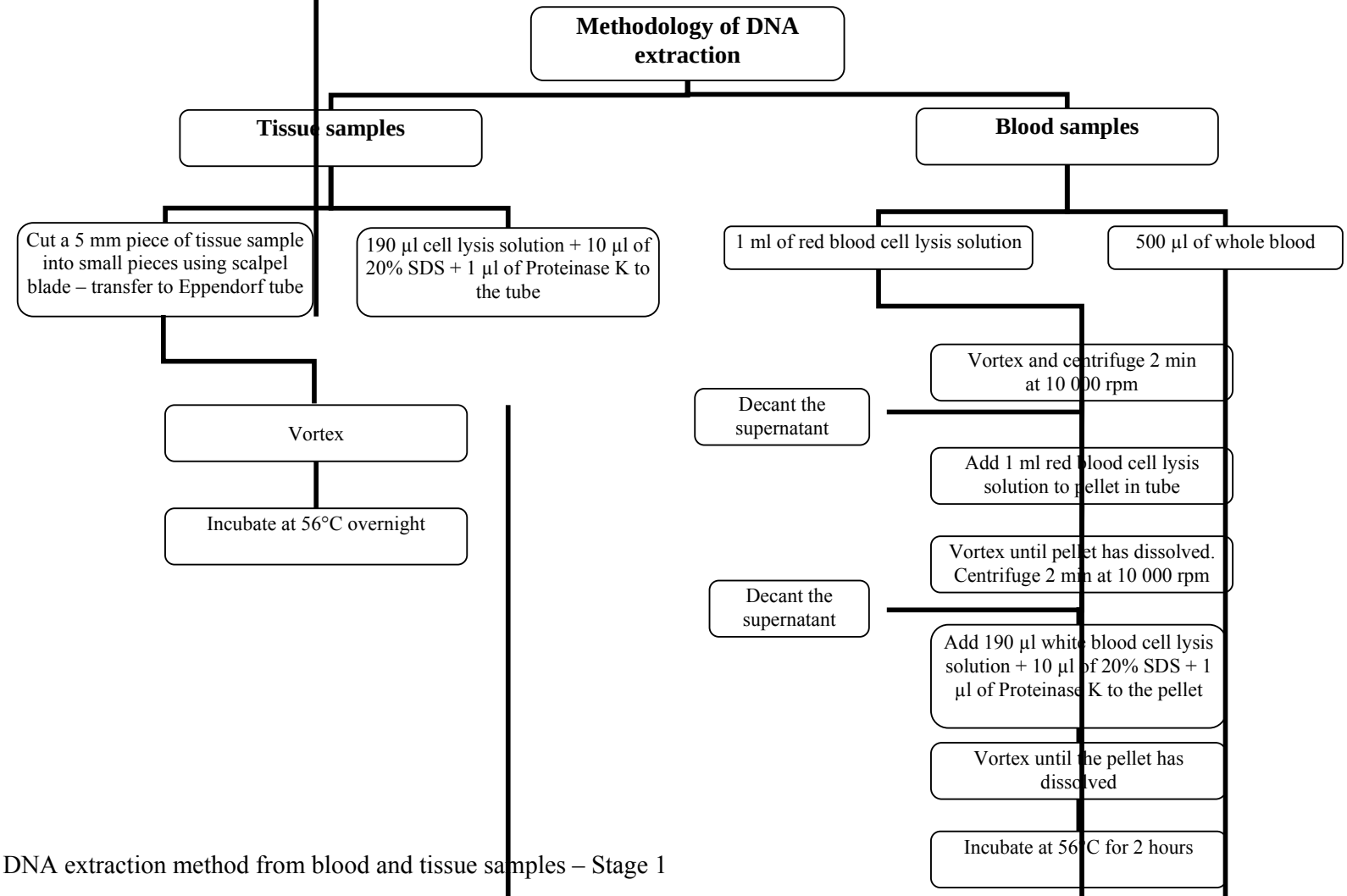

Figure 9: DNA extraction method from blood and tissue samples – Stage 1

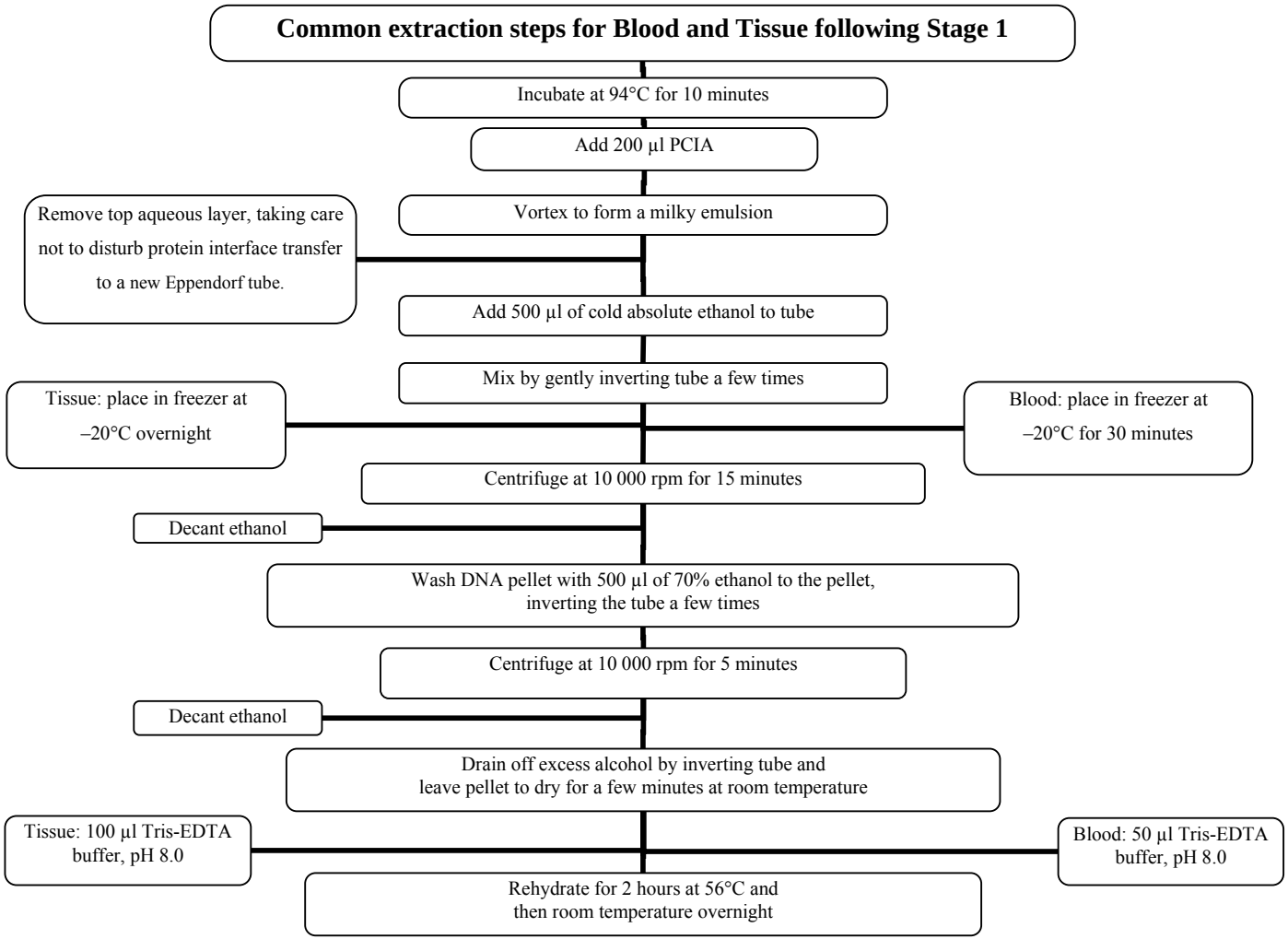

Figure 10: DNA extraction method from blood and tissue samples - Stage 2

3.5.2 Microsatellites

A panel of microsatellite markers routinely employed for domestic dog identification and parentage testing and recommended by the International Society of Animal Genetics (ISAG) was used to test the wild dog samples. Microsatellite primers were obtained from Applied Biosystems. The 5'-end of the forward primer was labelled with one of the following fluorescent dyes: FAM®, NED®, VIC® or PET®.

3.5.2.1 Primer multiplexes and Polymerase Chain Reaction (PCR) conditions

Primers were multiplexed in 2 panels of 8 loci each (Table 10). An amount of 1 µl of PCR product was loaded on to the 3130 *XL* Genetic Analyser (Applied Biosystems) with 0.25 µl Genescan Liz500™ size standard (Applied Biosystems) and 10 µl HiDi formamide (Applied Biosystems).

Table 10: Labelled microsatellite loci used in PCR Multiplex 1 and 2, respectively

Locus	Dye label	Locus	Dye label
Multiplex 1		**Multiplex 2**	
INRA21	PET	REN105L03	FAM
AHTh171	PET	INU030	FAM
AHTk253	FAM	INU055	FAM
CXX279	NED	LEI004	NED
FH2054	NED	AHTh260	PET
AHTk211	VIC	REN247M23	VIC
FH2328	VIC	FH2848	VIC
REN54P11	FAM	AHT137	VIC

3.5.2.2 Primer concentrations

The PCR mastermix is shown in Table 11. Multiplex PCR was carried out in a 15 µl reaction volume using either a *Geneamp*® 9700 or 2700 thermocycler (Applied Biosystems). Extracted

DNA was added to the PCR mix. An amount of 1 µl of extracted DNA of approximately 100 ng/µl concentration was used as template. The final primer concentrations of the two multiplexes are detailed in Table 12. All components must be added to the Primer Mix in the order detailed above.

Table 11: PCR mastermix (all volumes in µl)

PCR Mastermix (100 reactions)		
Order	**Component**	**Volume (µl)**
1	Primer mix	420
2	Water	335
3	BSA	160
4	10xPCR Buffer	150
5	25mM $MgCl_2$	200
6	10mM dNTP mix	220
7	Amplitaq Gold®	15
	Total Volume	**1500**

3.5.2.3 Cycling parameters

All PCR cycles were preceded by an initial step of Ampli*Taq Gold*® DNA polymerase (Applied Biosystems Inc) activation for 10 minutes at 95 °C, followed by 30 cycles of denaturation at 95 °C for 60 s, annealing at 60 °C for 30 s, extension at 72 °C for 60 s and a final extension at 72 °C for an hour.

3.5.3 Genotyping

Electrophoresis was carried out using an ABI 3130 *XL* Genetic Analyser (Applied Biosystems). Run conditions included electrophoresis at 15 kV for 5s in Performance Optimised Polymer 7 (POP-7) (Applied Biosystems). Data was recorded by GENE MAPPER™ and transferred to a file server. *STRand* software (Version 2.3.48) (Board of Regents, University of California, Davis) was used to analyse data on a personal computer. Individual dog data was stored in a spreadsheet (Microsoft Excel)

Table 12: Primer mix for PCR multiplexing (all volumes in µl)

Multiplex 1 (100 reactions)			
Primer	**Primer Concentration**	**Concentration**	**Primer volume**
INRA21	20 nM	0.7	52.5
AHTh171	20 nM	0.7	52.5
AHTk253	20 nM	0.4	30
CXX279	20 nM	0.4	30
FH2054	20 nM	0.08	6
AHTk211	20 nM	0.1	7.5
FH2328	20 nM	0.12	9
REN54P11	20 nM	0.35	26.25
		Total Primer Volume	311.25
		10xPCR Buffer	45
		Water	93.75
		Total Volume	450
Multiplex 2 (100 reactions)			
Primer	**Primer Concentration**	**Concentration**	**Primer volume**
REN105L03	20 nM	0.1	7.5
INU030	20 nM	0.2	15
INU055	20 nM	0.2	15
LEI004	20 nM	0.3	22.5
AHTh260	20 nM	1.6	120
REN247M23	20 nM	0.6	45
FH2848	20 nM	0.4	30
AHT137	20 nM	0.1	7.5
		Total Primer Volume	285
		10xPCR Buffer	45
		Water	120
		Total Volume	450

3.5.4 Parentage analysis using CERVUS 2.0 software

Paternity was assigned for all pups using the software CERVUS 2.0 (Marshall *et al*. 1998). The software was also used to provide estimates of mean allelic diversity (A), expected multilocus heterozygosity (H), mean polymorphism information content (PIC), and exclusion probabilities with and without known parents.

CERVUS estimates the difference in log-likelihood scores between the first and second most likely sires by a maximum likelihood approach. This procedure demonstrated confidence in assigning paternity consistent with simulated values (Slate *et al*. 2000). We chose 0.95 as the strict confidence interval and 0.80 as the relaxed level. CERVUS also requires the user to input the number of candidate males, the percentage of males sampled and the percentage of loci typed (99 %), and the estimated genotyping error rate (1 %). When performing parentage analyses based on genetic exclusion, true parent-offspring relationships are rejected if the genotype of the adult cannot produce the observed genotype of the offspring.

3.5.5 Manually performed parentage analysis

Parentage was verified manually using individual dog data obtained with *STRand* software on a Microsoft Excel spreadsheet. During the transmission of the genes to the offspring, one of the 2 alleles of the pup originates from the mother and the other from the father. Knowing that, the manual verification consists of determining which sire could have supplied the other allele for each locus. Zero exclusion was the criteria used to assign paternity.

CHAPTER 4

Results

4.1 Identification of wild dogs and behavioural observations

4.1.1 Identification

After approximately two weeks of locating and following the dogs, observing them on natural kills and baiting them in open places, a complete ID book of each dog was created. Each animal was assigned a number with the prefix M or F indicating a male or female, respectively. Finding the pack on a kill or bating them made the procedure much easier. The coat patterns were used to identify each individual. Initially, at the start of the study, all of the dogs looked the same. Reference was made to the photographic pictures to find specific patterns on their body. The white body spots are the easiest and most helpful patterns to distinguish animals. The tail is often an alternative means of identification since some dogs can have an extra black ring on the white of the tail, or may even have a complete black tail. Injuries and notches on the ears may also be helpful; however this requires that the dogs be exceptionally close. The physical character of the face could also help to estimate the age of each dog.

After a month of following and observing the packs, each dog could be identified with certainty on sight, even without consulting the digital photos. During the DNA sampling period, the images were used to make sure that each animal had been sampled.

4.1.2 Behavioural observations and ranking

The alpha female was easily identified since she was an adult; she was lactating with prominent mammary glands. There were no other females showing similar developments. Determination of maternity based on observation was, therefore, not difficult.

The position of the alpha male was less prominent making it more difficult to determine his ranking. This adult individual was given greater priority by the alpha female and displayed a higher status within the pack, showing aggression and dominance towards the other males. No mating was observed for the duration of the study period.

4.2 Sampling

4.2.1 Non-invasive sampling technique for the adults

Biopsy darts were only used on the two Madikwe packs to obtain skin samples for DNA analysis. Most dogs did not react to the impact of the biopsy dart and simply carried on feeding on the carcass. The older dogs were more aware of the dart gun. This technique was minimally invasive and proved to be a reliable means of collecting tissue samples from a large number of animals. Due to the dynamics of working with wild animals in an uncontrolled environment, the successful collection of biopsy samples from 100 % of the population was not possible. The average success rate during a darting session in terms of obtaining a tissue sample was 85 %. Reasons for failures were: a darted dog ran off into the bush before the dart dropped out making it difficult to find; another member of the pack chewed the dart; the dart failed to take a tissue sample; a dart missed the target or the individual identified for sampling did not present itself for a clear shot or was absent from the pack.

4.2.2 Invasive sampling technique for the pups

The 10-month-old pups proved to be impossible to sample since the biopsy darts consistently failed to take any tissue. Consequently, they were immobilized allowing a small skin sample to be collected from the ear. Taking the safety of the pups into account, a low dose of Zoletil® was used allowing for a light anaesthesia only. Their semi-conscious state did not allow blood sampling. Instead skin samples were collected from the ears. This method was more costly, time consuming and exposed the dogs to a greater risk but was 100 % successful and no losses were experienced.

Table 13 summarises the numbers of dogs sampled in each of the two Madikwe packs.

Table13: Summary of African wild dogs sampled in the Madikwe Game Reserve

	Tswasa Pack	**Madikwe pack**
Adults	9	20
Pups born in 2004	6	12
Total	15	32

4.2.3 Invasive sampling in Pilanesberg National Park

In Pilanesberg National Park, the Hooter pack was immobilized for management purposes, to be transferred to a boma. Deep anaesthesia provided by Zoletil for easy collection of blood samples in EDTA before they were loaded for transport.

4.3 DNA Analysis

4.3.1 DNA extraction

A quantity of DNA of 100 ng/µl or more and a A_{260}/A_{280} ratio (quality of the sample) between 1.7 and 2 were required. Samples that did not meet these criteria were not processed. The values obtained from the tissue and blood samples are summarised in Table 13.

The concentration of DNA in the extract varied between 100 ng/µl and 900 ng/µl. Samples that exceeded a concentration of 600 ng/µl were diluted with TE Buffer to a concentration of 100 to 200 ng/µl. Every A_{260}/A_{280} ratio varied from 1.68 (BotM2) to 2.08 (MadM53).

Table 14: Concentrations (ng/µl) and quality (ratio A_{260}/A_{280}) of DNA extracts

Sample ID	Pack	DNA conc	A260/A280
Pil F9	Alpha female	166.4	1.9
Pil M1	Adult	143.7	1.93
Pil M2	Adult	139.3	1.82
Pil M6	Adult	233.1	1.9
Pil M10	Adult	105.6	1.91
Pil M12	Adult	47.2	2
Pil M18	Adult	180.2	1.92
Pil F7	Adult	192.8	1.91
Pil F8	Adult	146.2	1.91
Pil M3	Pup	177.7	1.89
Pil M4	Pup	129.3	1.9
Pil M5	Pup	194.3	1.93
Pil M11	Pup	198.3	1.9
Pil M13	Pup	270.5	1.92
Pil M14	Pup	288	1.86
Pil M15	Pup	233.3	1.82
Pil M16	Pup	236.1	1.93
Pil F17	Pup	188.2	1.94
Mad F16	Alpha female	369	1.95
DW M01	Tswasa adult	422.77	1.84
DW M07	Tswasa adult	276.33	1.89
Mad M53	Tswasa adult	439.96	2.08
Mad M54	Tswasa adult	290.83	1.84
Mad M20	Tswasa adult	230.41	1.9
Mad M52	Tswasa adult	389.94	1.7
Mad F51	Tswasa adult	494.11	1.96
Mad F15	Tswasa adult	247.94	1.89
Mad M60	Tswasa pup	397	1.97
Mad M62	Tswasa pup	354.5	1.95
Mad F63	Tswasa pup	339.7	1.91
Mad F64	Tswasa pup	323	1.93
Mad F66	Tswasa pup	519.6	1.94
Mad M67	Tswasa pup	474.6	1.91

Sample ID	Pack	DNA conc	A260/A280
DW F03	Alpha female	387.44	1.92
Bot M2	Adult	110.2	1.68
Mad M36	Adult	371.99	1.9
Mad M8	Adult	281.25	1.91
Mad M6	Adult	222.76	1.9
Mad M9	Adult	561.79	1.68
Mad M11	Adult	278.82	1.88
Mad M22	Adult	908.9	1.96
Mad M24	Adult	719.4	1.93
Mad M26	Adult	643.8	1.95
Mad M27	Adult	711.4	1.94
Mad M29	Adult	532.7	1.96
Mad F30	Adult	574.5	1.89
Mad F39	Adult	407.22	1.86
Mad F40	Adult	245.78	1.89
Mad F34	Adult	260.76	1.91
Mad F38	Adult	367.02	1.89
Mad F41	Adult	249.95	1.9
Mad F35	Adult	413.85	1.92
Mad F37	Adult	197.17	1.91
Mad M80	Madikwe pup	444.4	1.95
Mad M81	Madikwe pup	525.8	1.96
Mad F82	Madikwe pup	570.1	1.92
Mad M83	Madikwe pup	407	1.93
Mad M84	Madikwe pup	529.2	1.96
Mad M85	Madikwe pup	337.5	1.95
Mad F86	Madikwe pup	431.9	1.97
Mad F87	Madikwe pup	319.3	1.98
Mad F88	Madikwe pup	416	1.96
Mad F89	Madikwe pup	559.4	1.92
Mad M90	Madikwe pup	500.7	1.96
Mad M91	Madikwe pup	475.7	1.96

4.3.2 Microsatellite markers

All 16 microsatellites designed for domestic dogs amplified successfully in African wild dog samples tested.

The heterozygosity of the two populations studied is summarized in Table 15. The allele frequency analysis of the Madikwe pack, the Tswasa pack and the Pilanesberg pack are illustrated in Table 16, 17 and 18, respectively.
The observed heterozygosity is slightly higher than the expected one because only animals from the reference population were genotyped.

Table 15: Locus name and mean genetic values for the entire population tested

Locus	k	n	Hets	Homs	$H_{(O)}$	$H_{(E)}$	PIC	PE1	PE2
AHT137	4	65	51	14	0.785	0.668	0.602	0.240	0.400
AHTh171	5	65	37	28	0.569	0.600	0.556	0.197	0.369
AHTh260	4	65	57	8	0.877	0.700	0.632	0.259	0.420
AHTk211	2	65	41	24	0.631	0.502	0.374	0.124	0.187
AHTk253	7	65	57	8	0.877	0.765	0.727	0.373	0.555
CXX279	2	65	21	44	0.323	0.273	0.234	0.037	0.117
FH2054	4	65	51	14	0.785	0.739	0.684	0.309	0.483
FH2328	4	60	35	25	0.583	0.570	0.511	0.170	0.321
FH2848	5	65	56	9	0.862	0.751	0.700	0.330	0.506
INRA21	3	65	38	27	0.585	0.606	0.530	0.181	0.323
INU030	6	65	58	7	0.892	0.787	0.748	0.396	0.574
INU055	3	65	42	23	0.646	0.618	0.541	0.188	0.330
LEI004	5	65	45	20	0.692	0.693	0.636	0.269	0.438
REN105L03	4	65	12	53	0.185	0.201	0.193	0.020	0.106
REN247M23	2	65	23	42	0.354	0.294	0.249	0.042	0.124
REN54P11	3	65	41	24	0.631	0.587	0.493	0.170	0.290

k: number of allele per locus, n: number of individuals, Hets: number of heterozygote individuals, Homs: number of homozygote individuals, $H_{(O)}$: Observed heterozygosity, $H_{(E)}$: Expected heterozygosity, PIC: Polymorphic information content, PE1: Probability of exclusion (first parent), PE2: Probability of exclusion (second parent).

Table 15 shows that the number of alleles per locus ranged from 2 (AHTk211, CXX279 and REN247M23) to 7 (AHTk253), with a mean of 3.94. The observed heterozygosities ($H_{(O)}$) ranged from 0.185 (REN105L03) to 0.892 (INU030) with a mean of 0.6423.The expected heterozygosities ($H_{(E)}$) per locus ranged from 0.201 (REN105L03) and 0.787 (INU030) with a mean of 0.585. Based on PIC, AHTk253, FH2848 and INU030 were most and REN105L03 and CXX279 least informative. First-parent exclusionary power was 0.978976 or 98% and second-parent exclusionary power was 0.999264 or 99%, which enabled us to assign parentage with ≥ 95% confidence.

Table 16 shows, for the Madikwe pack, that the number of alleles per locus ranged from 2 to 5 with a mean of 3.13. The observed heterozygosities ($H_{(O)}$) ranged from 0.094 (REN105L03) to 0.969 (INU030) with a mean of 0.706. The expected heterozygosities ($H_{(E)}$) per locus ranged from 0.091 (REN105L03) and 0.757 (AHTh171) with a mean of 0.559. Based on PIC, AHTh171 and AHTk253 were most and REN105L03 least informative. First-parent exclusionary power was 0.958701 or 96 % and second-parent exclusionary power was 0.997470 or 99 %, which enabled us to assign parentage with ≥ 95 % confidence.

Table 16: Locus name and mean genetic values for the Madikwe pack

Locus	k	n	Hets	Homs	H(O)	H(E)	PIC	PE1	PE2
AHT137	2	32	25	7	0.781	0.507	0.375	0.125	0.187
AHTh171	4	32	30	2	0.938	0.757	0.698	0.323	0.499
AHTh260	3	32	30	2	0.938	0.660	0.577	0.211	0.358
AHTk211	2	32	22	10	0.688	0.508	0.375	0.125	0.188
AHTk253	4	32	29	3	0.906	0.746	0.685	0.310	0.484
CXX279	2	32	14	18	0.438	0.347	0.283	0.058	0.142
FH2054	4	32	27	5	0.844	0.690	0.616	0.247	0.406
FH2328	3	29	24	5	0.828	0.618	0.530	0.184	0.321
FH2848	5	32	25	7	0.781	0.671	0.605	0.239	0.403
INRA21	3	32	20	12	0.625	0.588	0.488	0.167	0.285
INU030	5	32	31	1	0.969	0.688	0.621	0.258	0.423
INU055	3	32	18	14	0.563	0.450	0.401	0.098	0.233
LEI004	3	32	25	7	0.781	0.666	0.581	0.215	0.361
REN105L03	2	32	3	29	0.094	0.091	0.085	0.004	0.043
REN247M23	2	32	13	19	0.406	0.329	0.271	0.052	0.136
REN54P11	3	32	23	9	0.719	0.631	0.544	0.193	0.332

Table 17 shows, for the Tswasa pack, that the number of alleles per locus ranged from 2 to 6 with a mean of 3.44. The observed heterozygosities ($H_{(O)}$) ranged from 0.400 (AHTh171) to 0.933 (FH2054 and FH2848) with a mean of 0.682. The expected heterozygosities ($H_{(E)}$) per locus ranged from 0.343 (AHTh171) and 0.747 (INU030 and LEI004) with a mean of 0.589. Based on PIC, AHTh260, INU030 and LEI004 were most and AHTh171 and CXX279 least informative. First-parent exclusionary power was 0.965255 or 96 % and second-parent exclusionary power was 0.998327 or 99 %, which enabled us to assign parentage with ≥ 95 % confidence.

Table 17: Locus name and mean genetic values for the Tswasa pack

Locus	k	n	Hets	Homs	H(O)	H(E)	PIC	PE1	PE2
AHT137	3	15	10	5	0.667	0.545	0.419	0.139	0.226
AHTh171	3	15	6	9	0.400	0.343	0.294	0.055	0.158
AHTh260	4	15	13	2	0.867	0.743	0.669	0.295	0.469
AHTk211	2	15	11	4	0.733	0.508	0.371	0.121	0.185
AHTk253	4	15	13	2	0.867	0.662	0.579	0.216	0.373
CXX279	2	15	7	8	0.467	0.370	0.294	0.064	0.147
FH2054	4	15	14	1	0.933	0.701	0.626	0.256	0.425
FH2328	4	13	11	2	0.846	0.729	0.644	0.272	0.438
FH2848	4	15	14	1	0.933	0.720	0.644	0.273	0.444
INRA21	3	15	7	8	0.467	0.522	0.428	0.127	0.242
INU030	6	15	12	3	0.800	0.747	0.677	0.312	0.486
INU055	3	15	13	2	0.867	0.625	0.536	0.183	0.327
LEI004	4	15	13	2	0.867	0.747	0.671	0.297	0.470
REN105L03	4	15	9	6	0.600	0.563	0.500	0.158	0.316
REN247M23	2	15	9	6	0.600	0.434	0.332	0.088	0.166
REN54P11	3	15	8	7	0.533	0.467	0.393	0.102	0.222

Table 18 shows, for the Pilanesberg pack, that the number of alleles per locus ranged from 1 to 4 with a mean of 2.38. The observed heterozygosities ($H_{(O)}$) ranged from 0.056 (AHTh171 and REN247M23) to 0.944 (FH2848) with a mean of 0.472. The expected heterozygosities ($H_{(E)}$) per locus ranged from 0.056 (AHTh171 and REN247M23) and 0.692 (INU030) with a mean of 0.388. Based on PIC, INU030 were most and AHTh171 and REN247M23 least informative. First-parent exclusionary power was 0.841124 or 84 % and second-parent exclusionary power was 0.975230 or 97 %, which enabled us to assign parentage with ≥ 95 % confidence. The microsatellites, CXX279, FH2328 and REN105L03, were homozygous in all the dogs genotyped. These 3 loci will not be used for the compared analysis.

Table 18: Locus name and mean genetic values for the Pilanesberg pack

Locus	k	n	Hets	Homs	H(O)	H(E)	PIC	PE1	PE2
AHT137	3	18	16	2	0.889	0.665	0.573	0.209	0.356
AHTh171	2	18	1	17	0.056	0.056	0.053	0.001	0.026
AHTh260	3	18	14	4	0.778	0.603	0.517	0.172	0.313
AHTk211	2	18	8	10	0.444	0.457	0.346	0.099	0.173
AHTk253	3	18	15	3	0.833	0.656	0.565	0.203	0.350
CXX279	1	18	0	18	0.000	0.000	0.000	0.000	0.000
FH2054	3	18	10	8	0.556	0.500	0.424	0.118	0.243
FH2328	1	18	0	18	0.000	0.000	0.000	0.000	0.000
FH2848	3	18	17	1	0.944	0.675	0.582	0.215	0.362
INRA21	3	18	11	7	0.611	0.538	0.465	0.137	0.276
INU030	4	18	15	3	0.833	0.692	0.613	0.245	0.406
INU055	3	18	11	7	0.611	0.508	0.438	0.122	0.256
LEI004	2	18	7	11	0.389	0.386	0.305	0.070	0.152
REN105L03	1	18	0	18	0.000	0.000	0.000	0.000	0.000
REN247M23	2	18	1	17	0.056	0.056	0.053	0.001	0.026
REN54P11	2	18	10	8	0.556	0.413	0.321	0.080	0.160

When data of the 16 informative loci of the 3 wild dog populations (n=65) was combined (Table 19), the mean number of alleles per locus, the mean heterozygosity and the mean polymorphic information content were higher than in individual packs. Heterozygosity values for all 16 microsatellites were consistently lower in the Pilanesberg population compared to the Madikwe population. Within the Madikwe population, the Tswasa pack showed a higher heterozygosity than the Madikwe pack.

Table 19: Summary of the mean values for each pack and for the entire population

	Madikwe pack	Tswasa pack	Pilanesberg pack	Entire population
Mean number of alleles per locus (k)	3.13	3.44	2.38	3.94
Mean number of heterozygote ind (Hets)	22.570	10.715	8.5	41.763
Mean number of homozygote ind (Homs)	9.430	4.285	9.5	23.237
Mean $H_{(O)}$	0.706	0.682	0.472	0.642
Mean $H_{(E)}$	0.559	0.589	0.388	0.585
Mean PIC	0.483	0.505	0.328	0.526
Total exclusionary power (PE1)	0.958701	0.965255	0.841124	0.978976
Total exclusionary power (PE2)	0.997470	0.998327	0.975230	0.999264

Selected electropherograms of fluorescently-labelled alleles, produced by the software *STRand* corresponding to different microsatellites, are shown in Figure 11. Allele recognition was consistent and enabled easy determination of individual genotypes.

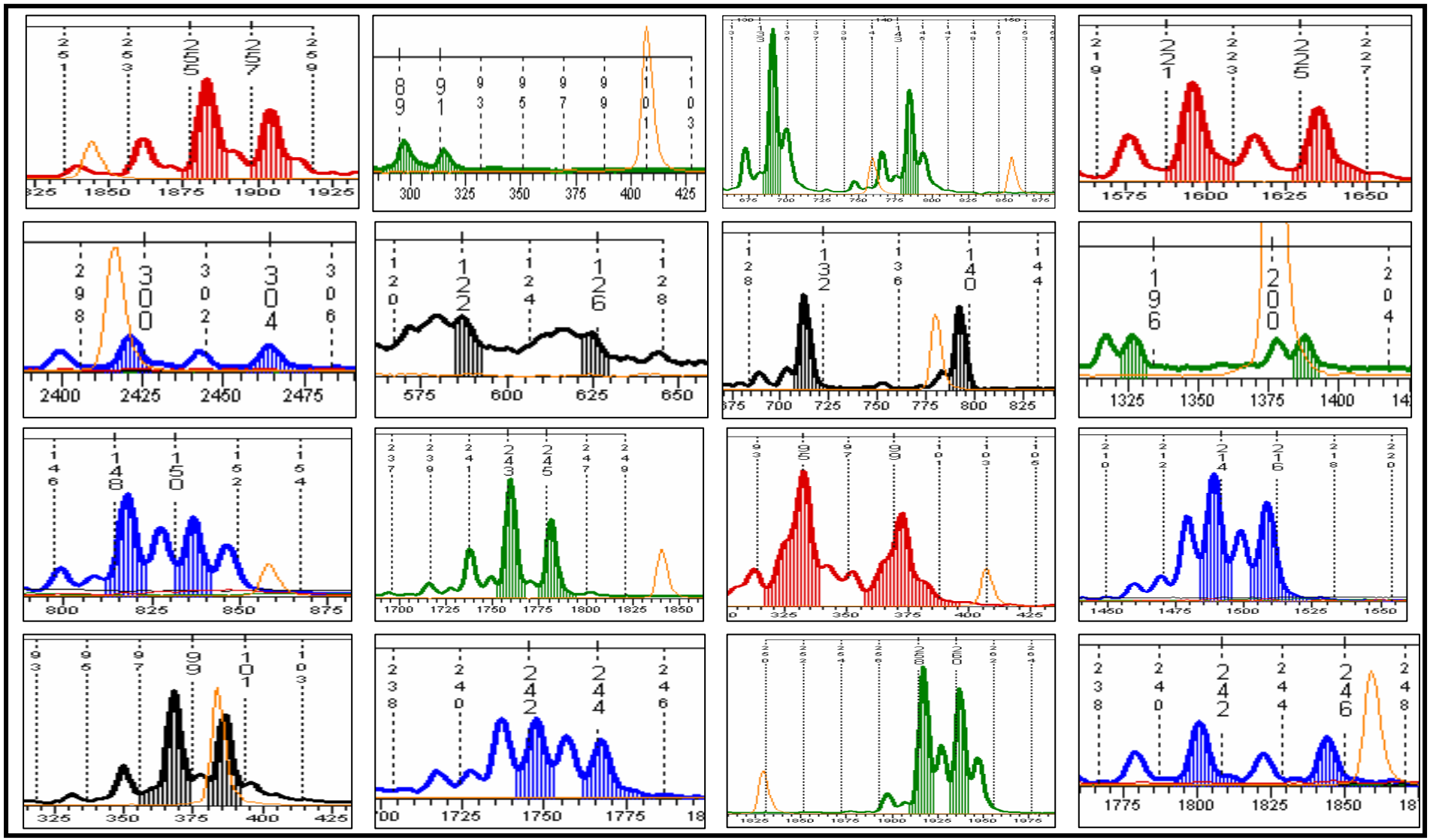

Figure 11: Electropherograms representing fluorescently-labelled microsatellite alleles (shaded peaks) in some representative African wild dog, sized from left to right according to an internal size standard (*STRand*). Microsatellite's names from the left to the right and top to bottom: AHTk260, AHTk211, AHT137, AHTh171, AHTk253, CXX279, FH2054, FH2328, INU030, FH2848, INRA21, INU055, LEI004, REN105L03, REN247M23, REN54P11.

4.4 Parentage analysis

Every adult male in each pack was selected as a potential father for the pups. Parentage analysis revealed that all five litters studied, had multiple paternities.

4.4.1 Parentage analysis performed with the assistance of CERVUS 2.0 software

4.4.1.1 Madikwe Game Reserve

4.4.1.1.1 The Madikwe pack

Tables 20a and 20b show the results of CERVUS 2.0 paternity analyses of the 2003 and 2004 Madikwe pack litters. The alpha female (DWF03) originated from the De Wildt Cheetah and Wildlife Centre and had been the top-ranking female for the past 3 years. The analyses confirmed that she was the mother of each litter born in 2003 and in 2004. For the litter of 8 pups born in 2003, 3 different fathers could be identified. MadM9, the dominant male during 2003; sired four pups, but the two subordinate males (MadM8 and MadM6) obviously also managed to mate with the alpha female during oestrus and sired three and one pup, respectively. For pup MadF37, MadM9 was the most likely father but with one exclusion. The LOD scores were always a high and positive number.

In the case of the 2004 litter of 12 pups, two different fathers were identified. MadM9 was the dominant male and sired 11 pups, whereas BotM2 was the father of a single pup (MadM80).

4.4.1.1.2 The Tswasa pack

MadF16 had been the alpha female of the Tswasa pack for the past 3 years. Again she was the mother of each litter born in 2003 and 2004, respectively. Tables 21a and 21b show the paternities of the 2 litters. In the 2003 litter (4 pups), one male (DWM07) sired all but one pup. The remaining pup was fathered by DWM01. The ranking changed in 2004 when MadM20 became the alpha male. The 2004 litter (6 pups) also had two different fathers with MadM20

siring five and DWM07 only one pup. The LOD scores were always a high and positive number, confirming the paternity with no exclusion for all of them.

4.4.1.2 Pilanesberg National Park

The alpha female PilF9 had been identified by the management team of the park, and CERVUS confirmed that she was the mother of all 8 pups born in the 2004 litter. The dominant male PilM18 was the father of 7 pups and PilM12 was the father of one pup, with no exclusions, and a LOD score of 2.496 (Table 22).

Table 20a: Summary of the parentage analysis of the 2003 pups of the Madikwe pack obtained with CERVUS

Offspring ID	Father ID	CP loci typed	O-CP loci compared	O-CP loci mismatching	O-KP-CP loci compared	O-KP-CP loci mismatching	LOD
MadM36	MadM8	16	16	0	16	0	6.028
MadF39	MadM9	16	16	0	16	0	5.259
MadF34	MadM9	16	16	0	16	0	6.850
MadF38	MadM8	16	16	0	16	1	3.037
MadF41	MadM8	16	16	0	16	0	4.166
MadF35	MadM6	16	16	0	16	0	6.001
MadF37	MadM9	16	16	1	16	1	0.693
MadF40	MadM9	16	16	0	16	0	5.441

Table 20b: Summary of the parentage analysis of the 2004 pups of the Madikwe pack obtained with CERVUS

Offspring ID	Father ID	CP loci typed	O-CP loci compared	O-CP loci mismatching	O-KP-CP loci compared	O-KP-CP loci mismatching	LOD
MadM80	BotM2	16	16	0	16	0	6.706
MadM81	MadM9	16	16	0	16	0	5.239
MadF82	MadM9	16	16	0	16	0	6.247
MadM83	MadM9	16	16	0	16	0	4.615
MadM84	MadM9	16	16	0	16	0	5.732
MadM85	MadM9	16	16	0	16	0	4.914
MadF86	MadM9	16	16	0	16	0	4.815
MadF87	MadM9	16	16	0	16	0	4.282
MadF88	MadM9	16	16	0	16	0	5.181
MadF89	MadM9	16	15	0	15	0	5.229
MadM90	MadM9	16	15	0	15	0	4.745
MadM91	MadM9	16	15	0	15	0	5.142

CP loci typed: number of loci typed in candidate parent, O-CP loci compared: loci typed in offspring and candidate parent, O-CP loci mismatching: mismatches between offspring and candidate parent, O-KP-CP loci compared: loci typed in offspring, known parent and candidate parent, O-KP-CP loci mismatching: mismatches between offspring, known parent & candidate parent, LOD: LOD score of candidate parent (the most likely candidate parent is the candidate parent with the highest (most positive) LOD score).

Table 21a: Summary of the parentage analysis of the 2003 pups of the Tswasa pack obtained with CERVUS

Offspring ID	Father ID	CP loci typed	O-CP loci compared	O-CP loci mismatching	O-KP-CP loci compared	O-KP-CP loci mismatching	LOD
MadF51	DWM07	15	15	0	15	0	4.442
MadM52	DWM07	15	15	0	15	0	4.442
MadM53	DWM07	15	15	0	15	0	6.841
MadM54	DWM01	16	16	0	16	0	5.363

Table 21b: Summary of the parentage analysis of the 2004 pups of the Tswasa pack obtained with CERVUS

Offspring ID	Father ID	CP loci typed	O-CP loci compared	O-CP loci mismatching	O-KP-CP loci compared	O-KP-CP loci mismatching	LOD
MadM62	MadM20	16	16	0	16	0	4.940
MadM60	DWM07	15	15	0	15	0	7.507
MadF63	MadM20	16	16	0	16	0	7.204
MadF64	MadM20	16	16	0	16	0	5.672
MadF66	MadM20	16	16	0	16	0	4.860
MadM67	MadM20	16	15	0	15	0	3.717

Table 22: Summary of the parentage analysis of the 2004 pups in Pilanesberg obtained with CERVUS

Offspring ID	Father ID	CP loci typed	O-CP loci compared	O-CP loci mismatching	O-KP-CP loci compared	O-KP-CP loci mismatching	LOD
PilM3	PilM18	16	16	0	16	0	2.605
PilM4	PilM18	16	16	0	16	0	2.415
PilM5	PilM18	16	16	0	16	1	2.991
PilM6	PilM18	16	16	1	16	1	1.984
PilM10	PilM18	16	16	0	16	0	2.007
PilM11	PilM12	16	16	0	16	0	2.497
PilM15	PilM18	16	16	0	16	0	2.248
PilM16	PilM18	16	16	0	16	0	2.268

4.4.2 Manual parentage analysis

After the parentage analysis with CERVUS 2.0, each result was checked manually using an Excel document. Table 23 shows examples of the procedure used for manual verification of sires. On 10 of the 16 microsatellites used, exclusions are highlighted in blue. For pup MadM60, the alpha male (MadM20) with 4 exclusions could not have been the father. The subordinate male in the pack, DWM07, proved to be the father of that pup. In the same litter, pup MadM62 was sired by the alpha male, MadM20. Any other possible male could be excluded with at least 3 exclusions. This confirms the results obtained by CERVUS 2.0, in Table 21b.

Table 23: Examples of parentage analyses performed manually

Exclusion of the Alpha male as the father of pup MadM60											
		Locus name and allele sizes									
ID	**Name**	**AHTh171**	**AHTh260**	**AHTk253**	**FH2054**	**FH2848**	**INRA21**	**INU030**	**INU055**	**LEI004**	**REN54P11**
Alpha female	MadF16	225/225	253/257	296/304	136/140	239/243	101/103	144/152	216/218	97/99	248/248
Subordinate	DWM07	219/225	251/251	304/304	128/132	237/239	103/103	150/152	214/216	101/103	242/246
Pup	MadM60	225/225	251/253	304/304	128/136	237/243	103/103	152/152	216/218	97/103	246/248

ID	**Name**	**AHTh171**	**AHTh260**	**AHTk253**	**FH2054**	**FH2848**	**INRA21**	**INU030**	**INU055**	**LEI004**	**REN54P11**
Alpha female	MadF16	225/225	253/257	296/304	136/140	239/243	101/103	144/152	216/218	97/99	248/248
Alpha male	MadM20	219/225	**253/255**	302/304	128/128	**239/241**	101/103	146/152	214/216	**97/101**	**242/248**
Pup	MadM60	225/225	251/253	304/304	128/136	237/243	103/103	152/152	216/218	97/103	246/248

Exclusion of the Subordinate male as the father of pup MadM62											
		Locus name and allele sizes									
ID	**Name**	**AHTh171**	**AHTh260**	**AHTk253**	**FH2054**	**FH2848**	**INRA21**	**INU030**	**INU055**	**LEI004**	**REN54P11**
Alpha female	MadF16	225/225	253/257	296/304	136/140	239/243	101/103	144/152	216/218	97/99	248/248
Subordinate	DWM07	219/225	**251/251**	304/304	128/132	**237/239**	103/103	**150/152**	214/216	**101/103**	**242/246**
Pup	MadM62	219/225	255/257	296/304	128/140	239/241	101/103	144/146	216/218	97/99	248/248

ID	**Name**	**AHTh171**	**AHTh260**	**AHTk253**	**FH2054**	**FH2848**	**INRA21**	**INU030**	**INU055**	**LEI004**	**REN54P11**
Alpha female	MadF16	225/225	253/257	296/304	136/140	239/243	101/103	144/152	216/218	97/99	248/248
Alpha male	MadM20	219/225	253/255	302/304	128/128	239/241	101/103	146/152	214/216	97/101	242/248
Pup	MadM62	219/225	255/257	296/304	128/140	239/241	101/103	144/146	216/218	97/99	248/248

CHAPTER V

Discussion

5.1 Observation, identification and sampling

5.1.1 Identification of individual animals

One of the aims of the study was to ensure the accurate identification of each individual wild dog in the packs studied. Maddock and Mills (1994) recorded behaviours of wild dog packs in the eastern Transvaal Lowveld. They also used coat patterns successfully for identification of individual dogs. In this study, we used individual markings and digital photographic records. The important part for us was to have certainty of the historic background and origin of each of the populations, since this information is fundamental to enable profiling and creation of a family tree. At the inception of the project, it had to be established if any of the dogs were related to each other. The movement of the dogs had to be established, since they are capable of travelling vast distances in relatively short periods, increasing their opportunity for crossbreeding. This movement makes it easy to confuse which dog originated where and to remain sure about the parentage of the various generations of offspring. Particularly if dogs have disappeared, or escaped from one reserve and reappeared at a later stage in another reserve.

For smaller “fenced-in” game reserves, with only one pack of wild dogs, this information becomes even more important due to the higher risk of inbreeding and the subsequent promotion of a genetic “bottleneck” of the populations. Knowing the dynamics and status of the wild dog study population was the initial phases of the fieldwork in Madikwe Game Reserve. This information had been done previously for the wild dog population in the Pilanesberg National Park.

This information was vital to ensure accurate profiling, understand the pack structure, know each individual, the relationship between the dogs and their behaviour patterns. Using coat patterns, the identification of dogs in our study proved to be 100 % reliable.

5.1.2 Behavioural study

Studying the behaviour of the pack is important in order to identify the alpha female, the alpha male and the subordinates and to record the sex and age of every dog. During this process the identification of the new season's pups as well as the previous year's pups became apparent. Time spent following the dogs made it possible to create an understanding of the movement between packs within the same reserve and helped in identifying favoured routes. These included fence lines, which were used to facilitate hunting. This phenomenon is well known in Madikwe and has been used by wild dog packs since the first releases (Hofmeyr, personal communication). To ensure the success of the study it was important to gain a knowledge and understanding of all these aspects. Girman *et al*. (1997) used the same techniques of identification and ranking evaluation of wild dogs in the Kruger National Park. Once enough data had been collected, the information was used to construct a family tree of all the living dogs, based on their historic backgrounds. Behavioural ecologists have documented the importance of social systems on genetic structure, inbreeding and reproductive success (Chesser 1998; Dobson 1998). Unfortunately, estimating these and other demographic characteristics from behavioural observations is difficult (Schenk and Kovacs 1995). Collection and analysis of genetic material from the wild dogs allow us then to confirm or refute the data obtained during the observation phase.

5.1.3 Sampling technique

In order to produce genetic information it is essential to access a reliable source of genetic material (Piggott and Taylor 2003). It is also important to obtain the samples in the least invasive way. The study initially began by utilising genetic material that was gathered by using faeces. Lucchini *et al*. (2002) did a non-invasive molecular tracking of colonizing wolf (*Canis lupus*) packs in the western Italian Alps. He managed to collect scats by following the tracks in the snow

in winter and by doing systematic transects on man-made roads in summer. In their study, however, positive identification of the faeces with individuals was not necessary. Creel *et al.* (2003) managed to estimate the population size in Yellowstone wolves using faecal samples. The collection of samples took two years. The wolves were observed with a spotting scope, noting the site of defecation in detail and the identity of the individual. In some cases radio-collar frequencies assisted in the identification. In our study, this method was abandoned because it was impractical to collect faecal samples from positively identified individual dogs. This is mainly due to the hyperactivity of the African wild dog. During the entire course of the field work conducted in Madikwe, there were only four opportunities to obtain faecal samples from positively identified dogs. This method can only be recommended for individual animal studies or captive animals. Furthermore, faecal extraction methods do not yield consistent results (Piggott and Taylor 2003) and a number of extracts are needed per sample to ensure quality DNA. Finally, it is necessary to run at least 7 PCR's per sample in order to give a representative profile making it an expensive technique (Valière 2002).

It is important to adopt a non-invasive sampling technique, especially for an endangered species such as the wild dog. The capture and immobilization of a member of the pack could interfere with the social dynamics within the pack (Piggott and Taylor 2003). If an immobilized dog takes long to recover the rest of the pack would most likely move off. The captured dogs would be exposed to other predators during their recovery phase as well as unnecessary exposure to drugs. Most projects doing genetic analysis in mammal species have used either dead or live-trapped animals (Sorin 2004 and DeYoung *et al*. 2002 for white-tailed deer; Sacks *et al.* 2004 for coyote). Reliable genetic samples can be obtained from the skin and blood. The only way to get such tissue samples without capturing the dog is by the use of biopsy darts (Karesh *et al*. 1987). Biopsy darts are mainly used in marine biology (Maldonado *et al.* 1995; Barrett-Lenard *et al.* 1996) using crossbow-launched biopsy systems adapted from the remote biopsy devices originally described by Lambertsen *et al.* 1987. For African wild dogs, biopsy darts have been used by Girman *et al*. 1997 to collect some of their tissue samples.

The advantages of using biopsy darts include the fact that they are inexpensive, are reusable and minimally invasive. In our experience, dogs that were darted with a biopsy dart remained calm

and undaunted on the bait. No dogs were injured. No abnormal behaviour was observed during the sample collection as a direct result of the dart. However, it appeared that some of the older dogs were more aware of the entire darting process. This was possibly a result of past experience, since they had been darted more often during rabies vaccinations. Nevertheless, samples could be obtained from all adult dogs.

In the case of the younger pups, the decision was made to wait until they reached 10 months before subjecting them to biopsy-darting. After many failed attempts at darting the decision was made to immobilize the pups. The negative results were due to the darts consistently falling off empty. A possible explanation for the failure of the biopsy darts could have been that the pups' skins are too loose or too thin. The result of this is that the biopsy darts fail to penetrate the skin. The immobilisation of the pups was successfully accomplished and once immobilised, a small earplug was taken.

The skin samples obtained either from the biopsy darts or directly from the pups all yielded excellent DNA extraction material. The quality and quantity of the DNA from all animals sampled could be used for the study.

5.2 DNA analysis

Microsatellites are being used more frequently in wild animal conservation. They provide information that can be effectively used to manage these animals. In the case of the wild dog, microsatellites can provide information on parentage, the inbreeding coefficient of a population, intra-population and inter-population relatedness values and population diversity and grouping.

Domestic dog microsatellites were used on wild dogs successfully (Ostrander *et al*. 1993), with good heterozygosity values compared to other species, and also compared to specific wild dog microsatellites. The majority of other studies in canids (Sacks *et al*. 2004 for coyotes, Ellegren 1999 and Lucchini *et al*. 2002 for wolves; Girman *et al*. 2001 for wild dogs) used domestic dog microsatellites. The decision to use domestic dog microsatellites was taken for the following reasons: they were already available in the laboratory which avoided the costs of purchasing new specific markers; they had been tested before and were shown to work; they amplified

consistently for every wild dog tested and showed a high level of polymorphism (Table 24). Any genetic laboratory working on domestic animal material could use the microsatellites on wild dogs, with no extra cost or effort. This would make results comparable between laboratories using standard domestic dog microsatellites.

Table 24: Genetic variability of our wild dog population compared with other canids

Population	Sample size (= n)	Number of loci	Mean number of alleles per locus	Expected heterozygosity $H_{(E)}$
Wild dogs Girman et al. 2001	203	11	4.0	0.643
Coyotes Sacks et al. 2004	457	13		0.73
Wolves Lucchini et al. 2002	338	6	4.5	0.517
Our study	65	16	3.94	0.585

The use of fluorescent markers allows the use of microsatellites with sizes that overlap, enabling simultaneous or multiplex PCR analyses. A high level of automation in handling and processing DNA samples enables more animals to be genotyped in a shorter time. For this study, 16 domestic dog microsatellites were used that all amplified consistently in 2 multiplex PCR reactions.

5.3 Parentage

This study clearly demonstrated that multiple paternities do in fact occur in the African wild dog. Analysis of microsatellite alleles of all potential sires revealed that 8 of 39 pups (20 %) in 5 different litters were not sired by the alpha male. In four litters (Pilanesberg, Madikwe 2004, Tswasa 2003 and 2004), every offspring, except one, was sired by the alpha male. However, in one of the litters (Madikwe 2003), the 8 pups produced had three different sires. The family tree based on genetic studies can confirm or refute the behavioural and historic data. Multiple mating with the alpha female during the breeding period has been observed in the Madikwe Game

Reserve in the past (Markus Hofmeyr, personal communication) but never proved genetically. Every paternity result obtained with CERVUS 2.0 software was validated manually.

A previous study on parentage in African wild dog packs in the Kruger National Park (Girman *et al*. 1997) suggested that subdominants occasionally succeed in producing offspring that survive to a year, with an average of only one in 9 litters, where the brother of the alpha male was the sire. The study was carried out on free-ranging wild dogs in an unfenced area. Because of these different conditions, a direct comparison between the two studies is difficult. It is possible that the fences have an impact on these populations and have somehow changed their reproductive behaviour.

There are different explanations for the multiple paternities. The alpha male could be involved in more fighting to maintain his rank during the mating period and consequently becomes distracted or tired. This could cause him to become less protective and observant towards the alpha female. However, he maintains the rank of alpha male even if subordinates manage to mate with the alpha female. During the mating period, a subordinate male can temporarily become the alpha male. In the Tswasa pack for example, the alpha male changed between 2003 and 2004. The former alpha male may have had the opportunity of mating with the female prior to the change in rank. The new alpha male will then mate with her for the rest of the mating period. In this case, the theory of, "only the alpha male mates with the alpha female" remains true. The percentage of pups produced by a non-alpha male remains low, possibly due to fewer mating opportunities for the subordinates or because the oocytes are already fertilised by the alpha male's sperm. This percentage may also vary with the number of available males in the particular pack (Creel & Creel 2002). These males must be old enough and also unrelated to the alpha female to have a chance to mate.

CHAPTER VI

Conclusions

This project presented the fundamental steps (observation, identification, sampling, DNA analysis) used to study the genetics of the African wild dog as an example of an endangered species. The selection of two isolated game reserves, Madikwe and Pilanesberg, provided the ideal opportunity to study the challenges posed by a metapopulation made up of several isolated satellite populations of wild dogs. The creation of a metapopulation has been muted as the best way to protect this endangered species. New problems as a result of isolation arise, however. The lack of movement between populations, the risk of inbreeding and the resulting genetic bottleneck, have now become problems that must be solved in order for this metapopulation strategy to be successful. When wild dogs live in protected areas, their reproduction rate is high and the survival rate of the pups is much improved. The pack's consumption of prey is increased, which impacts on the management of game in these reserves.

The significance of the identification of the individuals must not be forgotten. The project was reliant on previous observations and identification of dogs as well as the origin of founder packs. During the fieldwork, the first aim of the study was to successfully identify each and every dog in the two reserves. The second step was to collect samples from every dog to provide a reliable source of DNA.

Non-invasive sampling techniques, such as the collection of scats, have been shown to be successful for studies on individual animals. In a pack situation, however, the collection of positively identified faecal samples was not possible. The use of the biopsy darts proved to be a successful sampling technique. It is minimally invasive and provided a reliable source of genetic material for DNA analysis.

The DNA extraction techniques were applied following a well described and validated protocol. All samples provided good quantities and qualities of DNA. The use of 16 domestic dog microsatellites on wild dog genetic material was also successful. The heterozygosity was high

and comparable to other studies of wild dogs and also of other carnivores. PCR amplification gave good results for all 16 microsatellites used. The resulting information could be used for parentage analysis as well as for genetic analysis of the populations. The parentage analysis revealed that in each of the five litters sampled, at least one pup was not sired by the alpha male. This provides a possible mechanism to reduce the chances of inbreeding and to maintain higher levels of genetic variability within isolated populations of wild dogs.

The effective conservation of African wild dogs in isolated reserves South Africa could be assisted by the creation of a genetic dataset from every wild dog in these areas. It would be sensible to include the only true free-ranging wild dog populations of the Kruger National Park in this data set. An estimation of the inbreeding coefficient of the different packs and of the relatedness between individuals could be used as references for future movements of animals between reserves and even for the greater picture of African wild dog management in southern Africa.

References

Amos, W., Schlotterer, C. & Tautz, D. (1993). Social structure of pilot whales revealed by analytical DNA profiling. *Science*, **260**: 670-672.

Ashley, M. & Dow, B. (1994). The use of microsatellite analysis in population biology: Background, methods and potential applications. In Schierwater, B., Streit, B., Wagner, G. & DeSalle, R. editors, *Molecular Ecology and Evolution: Approaches and Applications*, pp 185-202. Birkhauser Verlag, Basel.

Barrett-Lenard, L.G., Smith, T.G. & Ellis, G.M. (1996). A cetacean biopsy system using lightweight pneumatic darts, and its effect on the behaviour of killer whales. *Marine Mammal Science*, **12**: 14-27.

Beaumont, M.A. & Bruford, A.M. (2000). Microsatellites in conservation genetics. *In* Goldstein D B, Schlotterer C (eds.) *Microsatellites: Evolution and applications*. Oxford University Press, Suffolk: 165-182.

Beckmann, J. & Weber, J. (1992). Survey of human and rat microsatellites. *Genomics*, **12**: 627-631.

Burrows, R., Hofer, H. & East, M.L. (1995). Demography, extinction and intervention in African wild dogs (*Lycaon pictus*). *Proceedings of the Royal Society of London,* B 262: 235-245.

Carling, M., Wiseman, A. & Byers, J. (2003). Microsatellite analysis reveals multiple paternity in a population of wild Pronghorn antelopes (*Antilocapra Americana*). *Journal of Mammalogy*, **84**(4): 1237-1243.

Chambers, G. & MacAvoy, E. (2000). Microsatellites: consensus and controversy. *Comparative Biochemistry and physiology, Part B.*, **126**: 455-476.

Chesser, R.K. (1998). Relativity of behavioural interactions in socially structured populations. *Journal of Mammalogy*, **79**: 713-724.

Ciofi, C. & Bruford, M. (1999) Genetic structure and gene flow among komodo dragon populations inferred by microsatellites loci analysis. *Molecular Ecology*, **8**: S17-S30.

Creel, S., Creel, N., Mills, M.G.L. & Monfort, S.L. (1997). Rank and reproduction in cooperatively breeding African wild dogs - Behavioural and Endocrine correlates. *Behavioural Ecology*, **8**: 298-306.

Creel, S., McNutt, J.W. & Mills, M.G.L. (2004). Demography and Population Dynamics of African Wild Dogs in Three Critical Populations. In *Biology and Conservation of Wild Canids*, ed by DW Macdonald & C Sillero-Zubiri, Oxford University Press., pp. 337-350.

Creel, S.R. & Creel, N.M. (1995). Communal hunting and pack size in African wild dogs, *Lycaon pictus. Animal Behaviour*, **50**: 1325-1339.

Creel, S.R. & Creel, N.M. (2002). The African Wild Dog: Behavior, Ecology and Conservation. Princeton University Press.

Creel, S.R., Spong, G., Sands, J.L., Rotella, J., Zeigle, J., Joe, L. Murphy, K.M. & Smith, D. (2003). Population size estimation in Yellowstone wolves with error-prone non-invasive microsatellite genotypes. *Molecular Ecology*, **12**: 2003-2009.

Crozier, R. (1992). Genetic diversity and the agony of choice. *Biological Conservation*, **61**: 11-15.

DeYoung, R., Demarais, S., Gonzales, R., Honeycutt, R. & Gee, K. (2002). Multiple paternity in white-tailed deer (*Odocoileus virginianus*) revealed by DNA microsatellites. *Journal of Mammalogy*, **83**(3): 884-892.

Deitrich, W., Katz, H., Lincoln, S.E., Shin, S.S., Friedman, J., Dracopoli, N.C. & Lander, E.S. (1992). A genetic map of the mouse suitable for typing intraspecific crosses. *Genetics*, **131**:423-447.

Dobson, F.S. (1998). Social structure and gene dynamics in mammals. *Journal of Mammalogy*, **79**: 667-670.

Eisen, J. (1998). Mechanistic basis for microsatellite instability. In Goldstein, D. & Schlotterer, C., editors, *Microsatellites, Evolution and Applications*, pp 34-48. Oxford University Press, Oxford.

Ellegren, H. (2000). Microsatellite mutations in the germline: implications for evolutionary inference. *Trends in Genetics*, **16**(12): 551-558.

Ellengren, H. (1999). Imbreeding and relatedness in Scandinavian grey wolves *Canis lupus*. *Hereditas*, **130** : 239-244.

Ellegren, H. (1995). Mutation rate in porcine microsatellite loci. *Mamm. Genome*, **6**:376-377.

Ernest, H., Penedo, M., May, B., Syvanen, M. & Boyce, W.M. (2000). Molecular tracking of mountain lions in the Yosemite valley in California: genetic analysis using microsatellites and faecal DNA. *Molecular Ecology*, **9**: 433-441.

Flagstad, O., Walker, C., Vila, C., Sundqvist, A., Fernholm, A., Hufthammer, A., Wiig, O., Koyola, I. & Ellegren, H. (2003). Two centuries of the Scandinavian wolf population: patterns of genetic variability and migration during an era of dramatic decline. *Molecular Ecology*, **12**: 869-880.

Forbes, S. & Boyd, D. (1996). Genetic variation of naturally colonizing wolves in the central Rocky Mountains. *Conservation Biology*, **10**(4): 1082-1090.

Forbes, S. & Hogg, J. (1999). Assessing population structure at high levels of differenciation: microsatellites comparisons of bighorn sheep and large carnivores. *Animal Conservation*, **2**: 223-233.

Frame, L. (1976). Wild dog ecology and behaviour. *Serengeti Research Institute Annual Report*, 1975-1976: 87-103.

Frame, L., Malcolm, J., Frame, G. & Van Lawick, H. (1979). Social organisation of African wild dogs (*Lycaon pictus*) on the Serengeti Plains, Tanzania, 1967-1978. *Zeitschrift fuer Tierpsychologie*, **50**: 225-249.

Frantzen, M., Ferguson, J. & Devilliers, M. (2001). The conservation role of captive African wold dogs (*Lycaon pictus*). *Biological Conservation*, **100**: 253-260.

Frantzen, M., Silk, J., Ferguson, J., Wayne, R. & Khon, M. (1998). Empirical evaluation of preservation methods for faecal DNA. *Molecular Ecology*, **7**: 1423-1428.

Fuller, T.K., Kat, P.W., Bulger, J.B., Maddock, A.H., Ginsberg, J.R., Burrows, R., McNutts, J.W. & Mills, M.G.L. (1992a). Population dynamics of African wild dogs. *Wildlife 2001: Populations*. D.R. McCullough & R.H. Barret, eds., pp. 1125-1139. London: Elsevier Applied Science.

Garnier, J.N., Bruford, M.W. & Goossens, B. (2001). Mating system and reproductive skew in the black rhinoceros. *Molecular Ecology*, **10**: 2031-2041.

Ginsberg & Macdonald 1990. Foxes, Wolves, Jackals and Dogs - An Action Plan for the Conservation of Canids. IUCN/SSC. Gland, Switzerland.

Girman, D., Wayne, R.K., Kat, P.W., Mills, M.G.L, Ginsberg, J.R., Borner, M., Wilson, V., Fanshawe, J.H., FitzGibbon, C.D. & Lau, L.M. (1993). Molecular genetic and morphological analyses of the African wild dog (*Lycaon pictus*). *Journal of Heredity*, **84**: 450-459.

Girman, D., Mills, M.G.L, Geffen, E. & Wayne, R.K. (1997). A molecular genetic analysis of social structure, dispersal and pack interactions in the African wild dog (*Lycaon pictus*). *Behavioral Ecology and Sociobiology*, **40**: 187-198.

Girman, D., Vila, C., Geffen, E., Creel, S., Mills, M.G.L, McNutt, J.W., Ginsberg, J.R., Kat, P.W., Mamiya, K.H. & Wayne, R.K. (2001). Patterns of population subdivision, gene flow and genetic variability in the African wild dog (*Lycaon pictus*). *Molecular Ecology*, **10**: 1703-1723.

Gittleman, J.L. & Harvey, P.H. (1982). Carnivore home range size, metabolic needs and ecology. *Behavioral Ecology and Sociobiology,* **10**: 57-63.

Goldstein, D.B. & Schotterer, C. (1999). Microsatellites: Evolution and Amplifications. Suffolk: Oxford University Press, Oxford.

Goossens, B., Chikhi, L., Taberlet, P., Waits, L. & Allainé, D. (2001). Microsatellite analysis of genetic variation among and within alpine marmot populations in the French Alps. Molecular Ecology, **10**.

Gorman, M. L., Mills, M. G. L., Raath, J. P., & Speakman, J. R. (1998). High hunting costs make African wild dogs vulnerable to kleptoparasitism by hyenas. *Nature,* **391**: 479-481.

Gottelli, D., Sillero-Zubiri, C., Applebaum, G., Roy, M., Girman, D., Garcia-Moreno, J., Ostrander, E. & Wayne, R. (1994). Molecular genetics of the most endangered canid: the Ethiopian wolf *Canis simensis. Molecular Ecology*, **3**: 301-312.

Grewal, S., Wilson, P, Kung, T., Shami, K., Theberge, M., Theberge, J. & White, B. (2004). A genetic assessment of the Eastern wolf (*Canis lycaon*) in Algonquin Provincial Park. *Journal of Mammalogy*, **85**(4): 625-632.

Haynie, M., Van Den Bussche, R., Hoogland, J. & Gilbert, D. (2003). Parentage, multiple paternity, and breeding success in Gunnison's and Utah prairie dogs. *Journal of Mammalogy*, **84**(4): 1244-1253.

Hughes, C. & Queller, D. (1993). Detection of highly polymorphic microsatellite loci in a species with little allozyme polymorphism. *Molecular Ecology*, **2**: 131-137.

IUCN 2001. IUCN red list categories and criteria: version 3.1. IUCN Species Survival Commission. IUCN, Gland, Switzerland and Cambridge , U.K.

Karesh, W., Smith, F. & Frazier-Taylor, H. (1987). A remote method for obtaining skin biopsy samples. *Conservation Biology*, **3**: 261-262.

Kays, R., Gittleman, J. & Wayne, R. (2000). Microsatellite analysis ok Kinkajou social organisation. *Molecular Ecology*, **9**: 743-751.

Kim, J., Guthrie, A.J., Harper, C. (2003). Domestic dog microsatellite markers used for parentage verification in the African wild dog (*Lycaon pictus*). *Proceedings of Faculty Day, Faculty of Veterinary Science, University of Pretoria*, Onderstepoort, 25 September: 41.

Kohn, M., York, E., Kamradt, D., Haught, G., Sauvajot, R. & Wayne, R. (1999). Estimating population size by genotyping faeces. *Proceedings of the Royal Society of London, Serie B*, **266**: 657-663.

Kraaijeveld-Smit, F., Ward, S. & Temple-Smith, P. (2002). Multiple paternity in a field population of a small carnivorous marsupial, the agile antechinus, *Antechinus agilis*. *Behavioural Ecology and Sociobiology*, **52**: 84-91.

Lacy, R. (1987). Loss of genetic diversity from managed populations: interacting effect of drift, mutation, immigration, selection and population subdivision. *Conservation Biology*, **1**: 143-158.

Lambertsen, R.H. (1987). A biopsy system for large whales and its use for cytogenetics. *Journal of Mammalogy*, **68**: 443-445.

Lehman, N., Eisenhawer, A., Hansen, K., Mech, L.D., Peterson, R.O., Gogan, P.J.P. & Wayne, R.K. (1991). Introgression of coyote mitochondrial DNA into sympatric north American gray wolf populations. *Evolution,* **45:**104-119.

Levinson, G. & Gutman, G. (1987).Slipped-strand mispairing: a major mechanism for DNA sequence evolution. *Molecular Biology and Evolution*, **4**(3): 203-221.

Lindsey, P.A., Alexander, R.R., du Toit, J.T. & Mills, M.G.L (2005). The potential contribution of ecotourism to Africann wild dog *Lycaon pictus* conservation in South Africa. *Biological Conservation*, **123**: 339-348.

Lindsey, P.A., du Toit, J.T. & Mills, M.G.L (2004). The distribution and population status of African wild dogs (*Lycaon pictus*) outside protected areas in South Africa. *South African Journal of Wildlife Research*, 34(2): 143-151.

Litt, M. & Luty, J. (1989). A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. *American Journal of Human Genetics*, **44**: 397-401.

Lucchini, V., Fabbri, E., Marucco, F., Ricci, S., Boitani, L. & Randi, E. (2002). Non invasive molecular tracking of colonizing wolf (*Canis lupus*) packs in the Western Italian Alps. *Molecular Ecology*, **11**(5): 857-868.

Luikart, G., Allendorf, F., Cornuet, J. & Sherwin, W. (1998). Distortion of allele frequency distributions provides a test for recent population bottlenecks. *Journal of Heredity*, **89**: 238-247.

MacDonald, D. & Potts, W. (1997). DNA microsatellites as genetic markers at several scales. In *Avian Molecular Evolution and Systematics*, pp 29-49. Academic Press.

MacDonald, D.W. & Sillero-Zubiri, C. (Eds.). (2004). Biology and Conservation of wild canids. Oxford University Press, Oxford.

MacDonald, D.W. (1983). The ecology of carnivore social behaviour. *Nature* **301**:379-384.

Maddock, A.H. & Mills, M.G.L. (1994). Population characteristics of African wild dogs *Lycaon pictus* in the eastern Transvaal Lowveld, South Africa, as revealed through photographic records. *Biological Conservation*, **67**: 57-62.

Malcom, J.R. & Marten, K. (1982). Natural selection and the communal rearing of pups in African wild dogs (*Lycaon pictus*). *Behavioral Ecology and Sociobiology,* **10**: 1-13.

Malcom, J.R. (1979). *Social organization and communal rearing in African wild dogs*. PhD Thesis, Harvard University.

Maldonado, J.E., Davilla, F.O., Stewart, B.S., Green, E. & Wayne, R.K. (1995). Intraspecific genetic differentiation in California in California sea lions (Zalophus californianus) from southern California and the Gulf of California. *Marine Mammal Science*, **11**: 46-58.

Marshall, T.C., Slate, J., Kruuk, L.E.B. & Pemberton, J.M. (1998). Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, **7**: 639-655.

Martinez-Navarro, B. & Rook, L. (2003). Gradual evolution of the African hunting dog lineage. Systematic implications. *Comptes Rendus Palevol*, **2**: 695-702.

Maudet, C., Miller, C., Bassano, B., Breitenmoser-Wursten, C., Gauthier, D., Obexer-Ruff, G., Michallet, J., Taberlet, P. & Luikart, G. (2002). Microsatellite DNA and recent statistical methods in wildlife conservation management: applications in alpine ibex [*Capra ibex* (*ibex*)]. *Molecular Ecology*, **11**: 421-436.

McNutt, J.W. (1996). Sex biased dispersal in African wild dogs, *Lycaon pictus. Animal Behaviour*, **52**: 1067-1077.

Miller, C.R., Adams, J.R. & Waits, L.P. (2003). Pedigree-based assignment tests for reversing coyote (Canis latrans) introgression into the wild red wolf (Canis rufus) population. Molecular Ecology, 12(12): 3287-301.

Milligan, B., Leebens-Mack, J. & Strand, A. (1994). Conservation genetics: beyond the maintenance of markers diversity. *Molecular Ecology*, **3**: 423-435.

Mills, M.G.L. & Gorman, M.L. (1997). Factors affecting the density and distribution of African wild dogs in the Kruger National Park. *Conservation biology*, **11**: 1397-1406.

Monfort, S.L., Mashburn, K.L., Brewer, B.A. & Creel, S.R. (1998). Evaluating adrenal activity in African wild dogs (*Lycaon pictus*) by fecal corticosteroid analysis. *Journal of Zoo and Wildlife Medecine*, **29**(2):129-33.

Mowat, W. & Strobeck, C. (2001). Estimating population size of grizzly bear using hair capture, DNA profiling, and mark-recapture analysis. *Journal of Wildlife Management*, **64**(1): 183-193.

Nowak, R.M. (1979). North American Quaternary Canis. Monograph Number 6, Museum of Natural History, University of Kansas, Lawrence, Kansas.
Ostrander, E.A. & Wayne, R.K. (2005). The canine genome. Genome Research, 15: 1706-1716.

Ostrander , E.A., Sprague, G.F. & Rine, J. (1993). Identification and characterization of dinucleotide repeat (CA) markers for genetic mapping in dog. *Genomics*, **16**: 207-213.

Paetkau, D. & Strobeck, C. (1994). Microsatellite analysis of genetic variation in black bear populations. *Molecular Ecology*, **3**: 489-495.

Paetkau, D. Waits, L., Clarkson, P., Craighead, L., Vyse, E., Ward, R. & Strobeck, C. (1998b). Variation in genetic diversity across the range of North American brown bears. *Conservation Biology*, **12**(2): 418-429.

Palsboll, P., Allen, J., Bérubé, M., Clapham, P., Feddersen, T., Hammond, P., Hudson, R., Jorgensen, H., Katona, S., Larsen, A., Larsen, F., Lien, J., Mattila, D., Sigurjonsson, J., Sears, R., Smith, T., Sponer, R., Stevick, P. & Oien, N. (1997). Genetic tagging of humpback whales. *Nature*, **388**: 767-769.

Parker, P., Snow, A., Schug, M., Booton, G. & Fuerst, P. (1998). What molecules can tell us about populations: choosing and using a molecular marker. *Ecology*, **79**(2): 361-382.

Piggott, M.P. & Taylor, A.C. (2003). Remote collection of animal DNA and its applications in conservation management and understanding the population biology of rare and cryptic species. *Wildlife Research*, **30**: 1-13.

Queller, D., Strassman, J. & Hughes, C. (1993). Microsatellites and kinship. *Trends in Ecology and Evolution*, **8**(8): 285-288.

Reich, A. (1978). A case of inbreeding in the African wild dog, *Lycaon pictus*, in the Kruger National Park. *Koedoe*, **21**: 119-123.

Say, L., Pontier, D. & Natoli, E. (1999). High variation in multiple paternity of domestic cat (*Felis catus L.*) in relation to environnemental conditions. *Proceedings of the Royal Society of London, Serie B*, **266**: 2071-2074.

Say, L., Pontier, D. & Natoli, E. (2001). Influence of oestrus synchronisation on male reproductive success in the domestic cat (*Felis catus L.*). *Proceedings of the Royal Society of London, Serie B*, **268**: 1049-1053.

Schaller, G. (1972). Serengeti lion: a study of predator-prey relations. University of Chicago press, Chicago.

Schenk, A. & Kovacs, K.M. (1995). Multiple mating between black bears revealed by DNA fingerprinting. *Animal Behaviour*, **50**: 1483-1490.

Schlotterer, C. & Pemberton, J. (1994). The use of microsatellites for genetic analysis of natural populations. In Schierwater, B., Streit, B., Wagner, G. & DeSalle, R. editors, *Molecular Ecology and Evolution: Approaches and Applications*, pp 203-214. Birkhauser Verlag, Basel.

Sillero-Zubiri, C., Hoffmann, M. & Macdonald, D. (2004). Canids: foxes, wolves, jackals and dogs: status survey and conservation action plan, second edition. *IUCN Canid Specialist Group*, Gland, Switzerland and Cambridge, UK.

Slate, J., Coltman, D.W., Goodman, S., Maclean, I., Pemberton, J.M. & Williams, J.L. (1998). Bovine microsatellite loci are highly conserved in red deer, sika deer and Soay sheep. *Animal Genetics*, **29**:307-315.

Slate, J., David, P., Dodds, K.G., Veenliet, B.A., Glass, B.C., Broad, T.E. & McEwan, J.C. (2004). Understanding the relationship between the inbreeding coefficient and multilocus heterozygosity: theoretical expectations and empirical data. *Heredity*, **93**(3): 1-11.

Sorin, A.B. (2004). Paternity assignment for white-tailed deer (*Odocoileus virginianus*): mating across age classes and multiple paternity. *Journal of Mammalogy*, **85**(2): 356-362.

Taberlet, P., Camarra, J.J., Griffin, S., Uhres, E., Hanotte, O., Waits, L.P., Dubois-Paganon, C., Burke, T. & Bouvet, J. (1997). Non invasive genetic tracking of the endangered Pyrenean brown bear population. *Molecular Ecology*, **6**: 869-876.

Tautz, D. & Rentz, M. (1984). Simple sequences are ubiquitous repetitive components of eukaryotic genomes. *Nucleic Acids Research*, **12**(10): 4127-4138.

Tautz, D. (1993). Notes on definition and nomenclature of tandemly repetitive DNA sequences. In Pena, D., Chakraborty, R., Epplen, J. & Jeffreys, A., editors, *DNA Fingerprinting: State of the Science*, pp 21-28. Birkhauser Verlag, Basel.

Valière, N. (2002). Amélioration et optimisation des méthodes non invasives et des marqueurs microsatellites en Biologie des Populations et de la Conservation. *Thèse de Doctorat*, Université Claude Bernard, Lyon I.

Valsecchi, H., Hale, P., Corkeron, P. & Amos, W. (2002). Social structure in migrating humpback whales (*Megaptera novaeangliae*). *Molecular Ecology*, **11**: 507-518.

Van Lawick, H. (1970). Wild dogs. In: Van Lawick, H. & Van Lawick-Goodall, J. eds. *The Innocent Killers*, pp. 47-101. Boston: Houghton Mifflin Co.

Van Lawick, H. (1974). Solo: The story of an African wild dog. Boston: Houghton Mifflin Co.

Van Valkenburgh, B., Wayne, R.K., Kat, P.W., Fuller, T.K., Johnson, W.E. & O'Brien, S.J. (1989). Genetic and morphological divergence among sympatric canids. *Journal of Heredity*, **80**(6): 447-454.

Verwey, R., Begg, C., Begg, K. & Matthee, C. (2004). A microsatellite perspective on the reproductive success of subordinate male hony badgers, *Mellivora capensis*. *African Zoology*, **39**(2): 305-308.

Wandeler, P., Funk, S.M., Largiader, C.R., Gloors, S. & Breitenmoser, U. (2003). The city-fox phenomenon: genetic consequences of a recent colonization of urban habitat. *Molecular Ecology*, **12**: 647-656.

Wayne, R.K., Meyer, A., Lehman, N., Kat, P.W., Fuller, T.K., Girman, D., VanValkenburgh, B. & O'Brien, S.J. (1990). Large sequence divergence among mitochondrial DNA genotypes within populations of eastern African black-backed jackals. *Proceedings of the National Academy of Sciences USA,* **87:** 1772-1776.

Williams, C., Blejwas, K., Johnston, J. & Jaeger, M. (2003). Temporal genetic variation in a coyote (*Canis latrans*) population experiencing high turnover. *Journal of Mammalogy*, **84**(1): 177-184.

Woodroffe, R. & Ginsberg, J.R. (1998). Edge effects and the extinction of populations inside protected areas. *Science*, **280**: 2126-2128.

Woodroffe, R., Ginsberg, J.R. & Macdonald, D.W. (1997). The African Wild Dog: Status Survey and Conservation Action plan. Gland, Switzerland: World Conservation Union, pp. 58-73.

Zhu, Y., Strassmann, J. & Queller, D. (2000). Insertions, substitutions, and the origin of microsatellites. *Genet. Res. Camb*., **76**: 227-236.