CHAPTER 5: GENETIC ANALYSIS

5.1 INTRODUCTION

5.1.1 The Role of Genetics in Wildlife Conservation

Population declines may be caused by a range of environmental and ecological factors, including overexploitation, pollution, the impacts of introduced species, as well as by stochastic events of a demographic, environmental or genetic nature (Brook et al. 2002). Loss of habitat and increasingly fragmented landscapes contribute to species decline by interfering with natural dispersal mechanisms and population dynamics, particularly of highly mobile large mammal species. Habitat fragmentation can interrupt natural dispersal patterns, alter philopatry and mate selection, and effect juvenile survival (Bjørnstad et al. 1998; Boudjemadi et al. 1999). Reintroduction and artificial augmentation of populations of endangered species may therefore play an increasingly important role in conservation management, to compensate for compromised gene flow and lack of population recolonisations in fragmented landscapes.

Reintroduction presently has a limited role in African wild dog conservation; current recommendations suggest the priority is to maintain extant populations in situ (Woodroffe & Ginsberg 1999; Woodroffe et al. 2004). Early reintroduction programs of captive wild dogs had limited success largely because the dogs were naïve to competing predators or had underdeveloped hunting skills (Scheepers & Venzke 1995). A range of reintroductions using a combination of wild caught and captive dogs have since been more successful (Woodroffe & Ginsberg 1999; Woodroffe et al. 1997). More recently, reintroduction and translocation are being used in South Africa to develop and manage a metapopulation of wild dogs by utilising a network of small fenced reserves (Moerhenschlager & Somers 2004; Woodroffe & Ginsberg 1999; Woodroffe et al. 2004). This strategy is management intensive, therefore preserving larger protected areas that sustain viable populations has received first priority for wild dog conservation strategies for areas outside of South Africa (Woodroffe et al. 2004). Nevertheless, while perhaps not required for larger free-ranging populations, reintroduction or augmentation may have a more important role to play in managing the remaining small, free-ranging populations where natural recruitment is compromised. Ideally, any reintroduction program should utilise animals that are
Genetically unrelated to avoid inbreeding, while still maintaining the genetic integrity of the population and its capacity to respond to selection pressures. Therefore the collection of information on the genetic diversity of populations is an important component of any conservation project.

Genetic factors also contribute to population viability by interacting with other pressures. For example anthropogenic threats and habitat fragmentation can lead to population decline, resulting in inbreeding. Inbreeding further reduces survival and fecundity, and the continued interaction of these factors can carry a population into an “extinction vortex” (Gilpin & Soulé 1986). However, in very small populations, which are particularly vulnerable to local extinction through stochastic events (Ginsberg et al. 1995), more immediate factors are likely to be more deterministic of population survival, including environmental variables, natural catastrophic events, and demographic stochasticity (Harcourt et al. 2001; Hedrick & Miller 1992).

Genetic information has become increasingly important in setting evolutionarily significant units (ESUs) for management purposes, a concept proposed to define the minimum unit used in conservation and avoid debates over definitions of species (Ryder 1986). The definition of ESUs has changed over time and continues to be debated (Crandall et al. 2000; Fraser & Bernatchez 2001; Kelt & Brown 2000; Moritz 1994, 1999; Ryder 1986). From earlier concepts based on strictly phylogeographical genetic data, there is now a strong argument for inclusion of more ecological data and a focus on adaptively significant genetic variability (Crandall et al. 2000). The strictly phylogenetic approach to species management may be particularly limited in small populations of endangered species, where the indication of differentiation, or lack of it, may simply be the result of small sample size (Fraser & Bernatchez 2001).

Two components were previously used to define ESUs; “reproductive and historical isolation, and adaptive distinctiveness” (Crandall et al. 2000). Limitations of this definition included: 1) that ESUs are less likely to be found in highly mobile species with a high level of gene flow, ie many large mammal species, including wild dogs, and 2) that many genetic techniques do not necessarily survey loci that are adaptively important (Fraser & Bernatchez 2001; Hedrick & Miller 1992). More recent definitions incorporated the “ecological exchangeability” of genes rather than
maintaining emphasis on the existence of “distinctiveness”. Ecological adaptations include morphology, demographic characteristics, and life-history traits which should be heritable (Crandall et al. 2000). Fraser and Bernatchez (2001) emphasised that focusing on ecological exchangeability ignored the fact that genetic distinctiveness may represent an important evolutionary step towards speciation, and suggested a more flexible approach combining various aspects of previous definitions of ESUs, depending on each specific situation. They termed this approach “adaptive evolutionary conservation”. These approaches give more scope for managing adaptive differences rather than just gene flow, and also differentiate historic from recent gene-flow.

Although distinctive genetic divergence may still be used to determine ESUs, from a practical management viewpoint populations within ESUs are often further broken down into Management Units (MU), to determine appropriate policies for translocations and maintaining population differentiation (Moritz 1994). Manel et al. (2003) give a current definition of the MU as “populations with significant divergence of allele frequencies at nuclear or mitochondrial loci regardless of the phylogenetic distinctness of the alleles, (i.e. demographically distinct populations that should be managed to ensure the viability of the larger evolutionary significant unit, subspecies, or regional populations).”

5.1.2 Genetic Effects on Populations
Given sufficient generation time, genetic effects can have important implications for the persistence of any population. These effects include loss of genetic diversity, inbreeding depression, outbreeding depression, and mutational accumulation (Frankham et al. 2002).

Genetic drift is the loss of alleles by chance, and this process occurs more rapidly in small or declining populations. Rare alleles are the most sensitive to genetic drift and are lost easily (Frankham et al. 2002). Further loss of alleles will eventually lead to reduced heterozygosity (Amos & Balmford 2001). Genetic variability is lost slowly, since loss is dependent on the number of generations the population has spent at its reduced size. The long-term effects of the loss of genetic variability on populations are still debated to some extent. As discussed in a review by Hedrick and Kalinowski
(2000), when populations contract and genetic variation is reduced, deleterious alleles may be “purged”, leading to only short-term effects. However, Amos and Balmford, (2001) show that evidence for this is limited; inbreeding depression is reduced by purging to limited degrees and only in some populations. Uncertainty about the effectiveness of purging is reiterated in Brook et al. (2002), particularly in small populations where inbreeding generally continues. Regardless of this phenomenon, loss of genetic variability reduces the capacity of a population to respond to selection.

On a more contemporary time scale, when large amounts of genetic diversity have been lost individuals may be forced to breed with genetically similar conspecifics, leading to inbreeding depression from a lack of heterozygosity (Brook et al. 2002; Hedrick & Miller 1992). This is often symptomatic in populations which have declined dramatically via other causes, but the genetic effects of inbreeding then become causal and further contribute to decline. Inbreeding depression is caused by mating with genetically similar individuals, and is a function of effective population size and generation time (Amos & Balmford 2001; Brook et al. 2002). Effective population size is the size of an ideal population that would lose genetic diversity at the same rate as the actual population; for example this takes into account the population structure, sex ratio, and generational overlap rather than the absolute population size (Frankham et al. 2002). Most deleterious alleles are recessive and only expressed in the homozygous state, therefore their expression increases as effective population size becomes reduced. Brook et al. (2002) carried out a study on a range of taxa, including 20 threatened species, and used population viability analysis (PVA) to model the effect of inbreeding on extinction risk. Inbreeding, at the level of 3.14 lethal equivalents per diploid genome, was found to increase extinction risk by 25-30% in population sizes ranging from 50 to 1000 individuals. However, the effect was dependent on time; all populations were modelled right through to extinction which required a minimum 60 years for the mammals studied.

Although the effects of inbreeding were controversial at first, there is now an abundance of studies on the various effects of inbreeding depression (Hedrick & Kalinowski 2000). Early studies by Ralls et al. (1988) and Ballou and Ralls (1982) provided evidence of inbreeding costs on juvenile survival and fecundity in a variety mammal species. Effects on juvenile weight in captive wolves were detected by
Laikre and Ryman (1991). Hedrick et al. (1992) found inbreeding effects on male mating success as well as fecundity, and suggested previous estimates of inbreeding effects were probably underestimated since many were carried out on captive populations where there were no environmental stresses, predators or competitors. Amos and Balmford (2001) reviewed evidence of inbreeding depression effects at the population level and suggested that where environmental stresses (stochastic events) may lead to population crashes, inbred animals were more likely to die. Brook et al. (2002) emphasised the effects of inbreeding on all stages of the lifecycle, which should be taken into account in all PVA, rather than the earlier focus on inbreeding effects on juvenile survival.

Inbreeding and loss of genetic variation is likely to be less of a problem in populations of highly mobile species, where more gene flow is maintained. Active avoidance of inbreeding has been detected in a variety of species, ranging from skinks (Stow & Sunnucks 2004) to African wild dogs (Girman et al. 1997; McNutt 1996). Population structure is also an important consideration in the detection of loss of genetic variation, particularly in group-living species, since co-ancestry can result in high levels of relatedness and lower genetic variation (Spong et al. 2002).

Small populations exhibiting inbreeding effects may require introduction of unrelated individuals. However, outbreeding depression is an important consideration when developing reintroduction and translocation policies (Pitra et al. 2002), even though there is limited data on its significance in populations. Outbreeding depression is a reduction in fitness resulting from crosses between distantly related individuals, which can be a problem in some endangered species (Hedrick & Miller 1992). This effect is generally less of a problem than inbreeding depression, and requires high levels of variation between populations to come into effect. However, there is a strong argument for preserving local diversity and a caution against introducing alleles that do not coincide with local adaptations, for example seasonal breeding variations, the introduction of more deleterious alleles, and the introduction of diseases that local populations may not be able to adapt to (Amos & Balmford 2001). Any management program should consider all available evidence of previous gene flow patterns and aim to mimic realistic genetic exchange.
The last genetic effect which may affect population viability is mutational accumulation; chance mutations which result in deleterious alleles that accumulate over time (Frankham et al. 2002). The build up of deleterious alleles in small populations requires several generations, especially in sexually reproducing species, although there is some evidence there is a greater effect in smaller populations of <100 (Amos & Balmford 2001). This accumulation may take 100-200 years in outbreeding populations, and is more of a concern in asexually reproducing populations (Amos & Balmford 2001; Frankham et al. 2002). Inbreeding is more of a concern since it increases the chances of expression of deleterious alleles through altered gene frequencies.

5.1.3 Relevance to African Wild Dog Conservation

African wild dogs have a short generation time (approximately 2 years) and occur at low densities. As a consequence they have the potential to lose genetic variability relatively quickly compared to some large mammal species. However, they also demonstrate behavioural avoidance of inbreeding through their dispersal methods, where full-sibling mating is actively avoided by dispersal of single-sex sibling groups. Long-distance dispersal also increases gene flow. Nevertheless, habitat fragmentation and restricted dispersal may impact on wild dog outbreeding behaviour in some populations.

There have been several previous studies of wild dog genetic diversity in various African populations, spanning a geographic range from Kenya to the Republic of South Africa (Girman et al. 2001; Girman et al. 1997; Girman et al. 1993). Early studies of mtDNA genetic variability and morphology in wild dog populations detected two clades in eastern and southern Africa, which were originally thought to be sufficiently distinct to be classified as separate subspecies (Girman et al. 1993). More recent sampling of a larger number of populations found mtDNA haplotypes were not geographically restricted but covered a more recent and extensive admixture zone, which included populations in Botswana, Zimbabwe, and south-eastern Tanzania (Girman et al. 2001, and see Figure 5.2; Methods for sample locations). The phylogenetic relationship of the mtDNA control region haplotypes and their frequency in eastern and southern regions can be seen in Figure 5.1. Zambia lies in the middle of the admixture zone but no data from this country were available for

Girman et al.’s (2001) study used mtDNA DNA control region sequences and 11 microsatellite loci to assess seven populations of wild dogs. The level of genetic diversity in free-ranging populations was comparable to that found in other large carnivore populations. All the sampled populations were relatively large and stable in comparison to the Lower Zambezi population. Girman et al. (2001) did find relatively reduced levels of genetic diversity in captive wild dog populations, and there is a possibility that smaller and more isolated populations in the wild may show similar reductions.

![Figure 5.1 Phylogenetic relationships of wild dog control region mtDNA haplotypes as described by Girman et al. (2001). Figure (a) is a distance neighbour joining tree (Tamura and Nei, 1993, gamma correction, $\alpha=0.5$) showing bootstrap support at nodes for neighbour joining (numerator) and maximum parsimony (denominator) trees from 1000 replicates. Figure (b) is a minimum spanning network with the proportional sizes of the nodes indicating the frequency of haplotypes in the entire sample. The frequency of each haplotype in eastern (Masai Mara and Serengeti), Selous, and southern (all others) is indicated by shading. The number of substitutions differentiating haplotypes is shown (where different from 1), and an alternative link between eastern and southern genotypes is shown by a dashed line.](image-url)
Girman et al.’s (2001) analysis found that populations were generally differentiated from each other with regard to mtDNA haplotype frequency and microsatellite allele frequency. The exceptions to this were the Masai Mara and Serengeti populations which were geographically contiguous, and Namibia which was restricted by a small sample size of only six dogs. All populations had at least one unique allele, with a maximum of three unique alleles found in the Selous. Allele frequencies differed between southern and east African populations, but were shared among populations in each region. Zambia lies in the middle of these two regions, between the east African Masai Mara, Serengeti and Selous populations, and the well sampled southern populations of Kruger, Hwange and Okavango.

Conservation management should mimic gene flow between contiguous populations (Crandall et al. 2000) and increase connectivity, therefore there is a need to identify the most closely related populations to Zambian wild dogs. The present study aims to assess the first samples from Zambian wild dogs and compare their genetic diversity to other African wild dog populations. Information on historical and contemporary population structure is investigated here to provide insights into the phylogenetic history and population dynamics of the Zambian population. This information is vital for developing sound conservation strategies.

Girman et al. (2001) suggested that climate change during the Pleistocene period and its effects on rainforest expansion and the Rift Valley may have been sufficient to cause the divergence of east and southern African wild dog clades. Information from the Zambian populations may provide further insights into this theory since Zambia lies south of the Rift Valley and north of previously sampled southern African populations. Hewitt (2000) provided a review of evidence for climate change during recent quaternary ice ages and its effects on speciation and genetic population structure, for a variety of species. In tropical zones, forests moved lower in mountainous areas, and tropical mountains provided a stable, moist habitat which conserved older species as well as generating new ones. Wieczorek et al. (2000) also discuss shifts in vegetation, rainforest expansion and contraction during the Pleistocene age. The Rift Valley ecosystem has been proposed as contributing a barrier to gene flow in a variety African animals, including mammals, birds and
amphibians (Freitag & Robinson 1993; Pitra et al. 2002). Therefore, the forests of Rift Valley mountains could conceivably have been a barrier to wild dog gene flow during these ice age fluctuations.

Two other hypotheses are proposed by Girman et al. (2001) to explain the divergence of the two wild dog clades; firstly, the distribution of miombo forests which interrupted the distribution of other species, including canids. However, miombo is a preferred habitat for wild dogs in some areas so miombo forest alone is an unlikely barrier to gene flow. Secondly, the derivation of current wild dog populations from refugia in western or central Africa was proposed, rather than divergence in situ in eastern and southern populations. This last hypothesis assumes no geographical barriers to wild dog dispersal.

A single west African wild dog museum skin was sampled in the previous study, and found to have a distinct mtDNA haplotype unique to this population (Girman et al. 2001). Overall, mtDNA and microsatellite results to date suggest that populations in west, eastern and southern Africa must all be conserved to preserve the current levels of differentiation and genetic diversity in the species, and more information is required from western and central African populations to shed more light on the phylogenetic history of the species.

5.1.4 Genetic Techniques
The combination of maternally inherited mtDNA and highly variable nuclear microsatellite markers has been widely used to assess species’ phylogenetic history, population structure and genetic diversity, and to infer demographic characteristics such as dispersal behaviour.

Mitochondrial DNA (mtDNA) control region sequence has a high mutation rate, no recombination, and traces only the maternal line (Frankham et al. 2002). An early study of mtDNA in 100 species of animals including fish, birds and mammals, showed the utility of using this form of DNA for a wide variety of phylogenetic studies (Kocher et al. 1989). Consequently, mtDNA DNA was quickly adopted to establish measures of genetic distinctness, for use in the management of wild and captive populations (Ashley et al. 1990; Avise & Nelson 1989; Hedrick & Miller
Apart from the above mentioned studies of African wild dogs, mtDNA has been used to determine the phylogenetic structure of populations in several other highly mobile canid species (Lehman et al. 1991; Pilgrim et al. 1998; Randi et al. 2000; Roach et al. 2001; Vila et al. 2003; Vila et al. 1999; Wayne et al. 1992).

Microsatellites are particularly useful for population studies, due to their high mutation rate and associated level of diversity per locus, their location throughout the genome, and their co-dominant mode of inheritance (Frankham et al. 2002). Microsatellites have been widely used to gain insights into population and social structure in carnivores (Girman et al. 1997; Kim et al. 2001; Roach et al. 2001; Spong et al. 2002). Patterns of dispersal can be inferred from parentage assignment methods, and have been found to increase estimates of dispersal rate and scale compared to field observations (Telfer et al. 2003; Zenger et al. 2003).

The current study utilised non-invasive sampling methods through the collection of faecal samples for genetic analysis using both mtDNA and microsatellites, to enable comparison of Zambian wild dog populations with those previously analysed by Girman et al. (2001).
5.2 OBJECTIVES
Genetic samples from the Lower Zambezi were analysed using mtDNA control region sequences and 11 polymorphic microsatellite loci to determine:

1) The levels of genetic diversity in the population

2) The Zambian population’s place in the phylogenetic history of wild dogs

3) Current population structure, and levels of differentiation from previously studied free-ranging wild dog populations.

This information contributed to an assessment of the status of the Lower Zambezi population, and identified which, if any, wild dog populations may be a suitable source of stock for translocations into Zambia to augment declining populations.
5.3 METHODS

5.3.1 Sample Collection

Thirty eight samples were obtained from three Zambian wild dog populations; 30 wild dogs were sampled from the Lower Zambezi population \((n=3\) tissue, \(n=4\) blood, \(n=23\) faecal samples, see section 2.3.1.1 for collection methods). Additionally, 1 faecal sample was obtained from Kafue National Park, and 7 faecal samples from South Luangwa National Park. Blood and tissue samples were taken from individuals immobilized for radio-collaring or snare removal. Faecal samples were collected opportunistically; in the Lower Zambezi only individuals of known identity were sampled, while samples from South Luangwa and Kafue National Parks were randomly collected by safari guides from unidentified wild dogs. Additionally for this study, 41 African wild dog DNA samples were obtained from the University of Pretoria in RSA, representing populations from the Transvaal in RSA (21 samples), Namibia (9 samples) and Botswana (11 samples).

Fresh faecal samples obtained in the field were either refrigerated and extracted within three days, or frozen and stored at -4°C for up to four months before extraction. Samples from three dogs had been collected during the pilot study prior to incorporation of the genetic aspect of the project and these samples were stored in 70% ethanol for a two year period (two of these samples yielded sufficient mtDNA for analysis).

5.3.2 DNA Extraction

DNA was extracted from faecal samples using a QIAamp DNA Stool Mini Kit (QIAGEN; available from www1.qiagen.com). The supplied protocols were observed with the following alterations:

A larger amount of stool was extracted due to low product from initial extractions. Three scrapes (approximately 220mg each) were taken from the outside of each faecal in an effort to target wild dog epithelial cells rather than DNA from prey. The scrapes were pooled and mixed with 4.8mL of ASL buffer and homogenized, and 2mL of this mixture was then aliquoted for each of two replicate DNA extractions. Replicates were spun down then 1.4mL of the supernatant was removed and used for step 4 of the QIAamp DNA Stool Mini Kit protocol, which was then followed for the remainder of the extraction process.
Blood samples
A QIAamp DNA Stool Mini Kit was used for extraction of DNA blood samples, with the following modifications: 3 X 200µL of blood was taken from each sample, and each aliquot added to 1.6mL of ASL then spun down for 2 minutes at high speed (14000rpm on Eppendorf MiniSpin Plus Personal Micro Centrifuge, MBCO No. 90412-0207). The supernatent was then used as per Step 8 of the QIAamp DNA Stool Mini Kit protocol, which was then followed. This procedure omitted the addition of the inhibitex tablet for removal of PCR inhibitors from stool samples.

Tissue samples
The ear-notch tissue samples were sent to the University of Pretoria and extracted by standard salting out protocols using treatment with Sodium Dodecyl Sulphate and proteinase K, and subsequent phenol/chloroform extraction (Sambrook et al. 1989).

5.3.3 Amplification and Sequencing
5.3.3.1 Mitochondrial control region
Lower Zambezi population maternal lines were confirmed from field observations. Two to three samples from each generation of each maternal line were sequenced to confirm maternity and haplotype (n=24 samples in total). A total of six maternal lines were sequenced from Zambia: four from the Lower Zambezi, one from Kafue National Park and two from South Luangwa National Park populations. Maternal lines from the three geographical regions represented by the samples from the University of Pretoria were sequenced and added to the mtDNA analysis.

Amplification
Canid-specific primers were designed to overlap the 381-bp sequence of control region I of the mitochondrial genome sequenced in African wild dogs by Girman et al. (2001; Genbank Accession number: AF335724-32). Due to the presence of prey DNA in the faecal samples used in this study, the general vertebrate primers used by Girman et al. (2001) were not suitable for this study. Therefore canid-specific primers were designed which overlapped the sequence used by Girman et al. (2001) and covered all the variable sites. The new primers began at position 93 of Girman et al.’s (2001) sequence (the first variable site occurred at 171-bp) and overlapped at the 3’-end by a further 22-bp. Primer sequences were forward primer 5’-
ACTATTCCCTGATCTCCCCC-3’ and reverse primer 5’ -
CCTGAAGTAAGAACCAGATGCC-3’. The forward primer was labeled with an
M13(-29) tail, the reverse with an M13(-38) tail, according to the tailed primer
methods developed by Oetting et al. (1995).

The mtDNA fragments were amplified using Polymerase Chain Reaction (PCR) in a
20µL reaction volume containing 2.5mM MgCl\(_2\), 50mM KCl, 10mM Tris-HCl (pH
8.4), 5mM dNTP mix, 1 unit of *taq* polymerase, 20 pmoles of each primer and ~ 10ng
template genomic DNA. PCR amplifications were carried out on three 96 well PCR
machines; PTC-100, PTC-200 and PTC-200 Gradient Cycler, (MJ Research Inc); the
Gradient Cycler was primarily used for optimizing PCR reactions.

Faecal derived samples were run with initial denaturation at 95˚C for 5 minutes, then
40 amplification cycles of 95˚C denaturation for 30s, 55˚C annealing temperature for
30s, and extension at 72˚C for 30s. A final extension at 72˚C was carried out for 5
minutes after the last cycle. Blood and tissue samples were run with the same thermal
cycling profile with the modification of an annealing temperature of 60˚C, run for 35
amplification cycles.

Faecal derived samples which resulted in weak amplification product were then run
for 45 amplification cycles at an annealing temperature of 50˚C. If amplified PCR
product remained faint when run on a 2% [w/w] agarose gel, subsequent sequence
was often weak and difficult to read. In this case the illuminated bands of PCR
product were cut out from the agarose gel, dissolved in 30µL 1X TBE buffer [90 mM
Tris-borate, 2 mM EDTA] at room temperature (Sambrook et al. 1989), and used to
seed a second booster PCR of 45 cycles at 50˚C annealing temperature.

*Agarose gel electrophoresis*

Prior to sequencing PCR products were visualized on a 2% [w/w] agarose gel
(Progen). The gel was prepared by melting 1g of agarose into 50mL of 1X TBE
buffer [90 mM Tris-borate, 2 mM EDTA] (Sambrook et al. 1989). After cooling to
approximately 50˚C 0.5µg/mL of ethidium bromide was added. The gel was then
poured into a casting plate to a depth of ~ 4-7mm and a comb inserted. After cooling
and comb removal, 5µL of PCR product DNA and 2µL of agarose gel loading buffer
(15% Ficoll Type 400 [Pharmacia], 0.25% bromophenol blue and 0.25% xylene cyanol) were mixed then added to the gel in an electrophoresis tank containing 1X TBE buffer, then run for 20mins at ~90-100 volts to separate bands. Size standards were run every 10 wells to determine DNA concentration and size. Bands were visualized on an Ultra.Lum UV trans-illuminator, and recorded using either a DS -34 Polaroid camera, 2 megapixel Kodak camera or ImageMaster VDS version 2.0 (Pharmacia Biotech).

Sequencing

PCR product was cleaned up, from PCR reagents, double stranded DNA and salts solution, using either a JetQuick PCR Purification Spin Kit (Genomed) according to the supplied protocol, or by using a 5:1µL ratio of PCR product to ExoSapIT enzyme (Amersham Biosciences) incubated for 45 min at 37°C, then 15 min at 80°C for enzyme inactivation.

1) In-house sequencing

A SequiTherm Excel II DNA sequencing kit – LC (Epicentre Technologies) was used for the sequencing reaction, with IRD- labelled primers. These primers were complimentary to the M13 tails on the canid-specific primers used for initial PCR. DdNTPs (2µL) were plated out in separate wells and 4µL of bulk mix was added to each. Bulk mix for each sample contained 1X buffer, 1pmol each of IRD700 primer and IRD800 primer, 8-9µL of cleaned up PCR product, 4U of SequiTherm Excel II Polymerase, and water added to make up a total of 17µL. The sequencing reaction was run with an initial denaturation at 95°C for 5 minutes, then 35 amplification cycles of 95°C denaturation for 30s, 60°C annealing temperature for 15s, and extension at 72°C for 60s. A final step of 72°C for 60s was run after the last cycle. This reaction was based on methods by Sanger et al. (1977).

Samples were mixed with loading buffer, denatured at 95°C, and loaded onto a 0.25mm thick, 41cm 4% nondenaturing polyacrylamide gel containing; 3.1mL stock Acrylamide (Acrylamide PAGE 40% aqueous solution, Amersham Pharmacia Biotech), 6.2mL of 5x TBE [90 mM Tris-borate, 2 mM EDTA] (Sambrook et al. 1989), 13.1g of urea (BDH AnalR, Merck), 210µL of 10% Ammonium Persulfate (APS) (Amresco), 28µL of TEMED (Progen), and water. The sequence was then
visualised using a LI-COR 4200 automated sequencer according to manufacturer’s instructions.

Sequences were visualized using the software programs Base ImagIR Image Manipulation (v4.00), Base ImagIR Image Analysis (v4.10), and SCF File Creation (v4.10) (LI-COR). Sequences were edited by eye via chromatograms using the program Sequencher (Gene Codes Corporation, www.genecodes.com). The sequences were then aligned using the program GeneDoc (Nicholas & Nicholas 1997).

II) Commercial DNA Sequencing

Samples sent out for sequencing were pre-prepared by mixing 8-10µL of purified PCR product with 3.2pmole of primers made up to 12µL mix with Milli-Q water, as per instructions from Westmead DNA (www.westmead-dna.org.au). These were then sequenced on an Applied Biosystems ABI PRISM 3100 Genetic Analyser.

5.3.3.2 Microsatellite alleles

All DNA samples collected in Zambia (n=38) were analysed for 11 microsatellite loci known to be polymorphic in African wild dogs and identical to those used by Girman et al. (2001) in their analysis of other African wild dog populations.

PCR amplification of faecal samples was carried out on the same machines used for mtDNA PCR (above), in a 20µL volume reaction containing: 5mM MgCl₂, 50mM KCl, 10mM Tris-HCl (pH 8.4), 5mM dNTP mix, 1 unit of HotStarTaq DNA polymerase (QIAGEN), 20 pmoles of each primer and approximately 40ng DNA (proportion of target DNA unknown). Microsatellite primers were labeled with M13-tails and PCR reactions contained complimentary IRD700 dye labels for electrophoresis.

Faecal samples were run with hot-start denaturation at 95°C for 15 minutes, then 45 amplification cycles of 95°C denaturation for 30s, 48°C annealing temperature for 30s, and extension at 72°C for 30s. A final extension at 72°C was carried out for 5 minutes after the last cycle.
Blood and tissue samples were run with the same protocols as above except PCR reactions contained 1 unit of commercial taq polymerase and 2.5mM MgCl₂, and 10ng target DNA. These samples were run for 40 amplification cycles with an initial denaturation at 95°C for 5 minutes, and an annealing temperature of 55°C.

Primers that failed to effectively amplify faecal samples were further optimized in PCR reactions. After extensive optimization trials, Primer L155 was run with the addition of DMSO solution (Dimethyl Sulfoxide, 1%), and L173 and L677 were run with the addition of Tween-20/NP40 (0.1%). Primers L366 and L423 were amplified with taq polymerase.

**Polycrylamide gel electrophoresis**

Microsatellites were size separated following electrophoresis on a polyacrylamide gel using the LI-COR 4200 automated sequencer as for mtDNA above, using a 25cm, 6% nondenaturing polyacrylamide gel. Gel images were manipulated using Base ImagIR (v4.0) and final scoring of alleles was carried out using the program Gene ImagIR™ software (v 4.05, Scanalytics), and by eye. An M13 control sequencing reaction was run at intervals across each gel as an absolute size marker, which allowed scoring and sizing of microsatellite data for analysis.

The principles of a multiple PCR approach (Piggot & Taylor 2003a; Taberlet et al. 1996) were followed for faecal samples, in addition to individual optimization of each primer. Heterozygous alleles were run until scored at least twice, and faint samples and homozygous alleles were run until scored consistently a minimum of three times. Results were checked against field records of observed parentage. Problematic samples were run in PCR up to 7 times. If, after multiple PCR, allelic dropout was suspected from visual comparison of band intensity with true homozygotes, and comparison with family pedigree, the sample was dropped from final analysis.
5.3.4 Statistical Analysis

Zambian African wild dog mtDNA d-loop control region sequences and microsatellite loci data were compared to those generated by Girman et al. (2001). Girman et al. (2001) collected 228 samples from seven free ranging African wild dog populations in eastern and southern Africa (Figure 5.2): Masai Mara National Park in Kenya; Serengeti National Park in Tanzania; Selous Game Reserve in Tanzania; Hwange National Park in Zimbabwe; Moremi Wildlife Reserve in Botswana; north-west Namibia; and Kruger National Park in the Republic of South Africa (RSA).

Figure 5.2 Map of geographic distribution of sampled free-ranging African wild dog populations. Zambian populations are shown in red, and populations previously sampled by Girman et al. (2001) in blue. The smaller red symbols represent the small sample sizes from Kafue and South Luangwa.

Statistical analysis incorporated a variety of methods to derive diversity indices, phylogenetic history, and population genetic structure. Methods were chosen to allow direct comparison with data from Girman et al. (2001), with additional up-to-date statistical analysis techniques carried out to resolve the data further.

I) Mitochondrial DNA analysis was used to determine: phylogeny and sequence divergence; population structure; and genetic diversity.

II) Microsatellite loci data were used in both genic and genotypic analysis, and to assess genetic diversity and test for evidence of population bottlenecks.

5.3.4.1 Analysis of mitochondrial data
Analysis was carried out on the 403-bp sequence formed by combining the sequence used by Girman et al. (2001) with that of the canid-specific primers. The first 93-bp of Girman et al.’s (2001) sequence, although free of variable sites, was included so results would be comparable to those of the previous study.

For mtDNA analysis the samples consisted of; samples from Zambian populations from this study which amplified successfully (n=33), those supplied by the University of Pretoria (n=41), and the results from Girman et al. (2001) for samples throughout eastern and southern Africa (n=228). Samples were pooled to assess geographical distribution and gene flow, and to compare Zambian population genetic diversity to the populations in other geographic regions.

Tests for mtDNA genetic diversity were carried out by examining haplotypic diversity \( (h) \) and nucleotide diversity \( (\pi) \) within populations. Haplotypic diversity is a measure of the number and frequency of haplotypes present in a population, while nucleotide diversity measures the degree of polymorphism between haplotypes within a population. Nucleotide divergence \( (d_\lambda) \) was calculated to measure diversity between populations, using the software program REAP (McElroy et al. 1991). The program MODELTEST 3.06 (Posada & Crandall 1998) was used in this analysis to test for the best-fit model for sequence substitution and gamma distribution of rate heterogeneity for all sequences. All genetic distance methods were then calculated using the best-fit model, incorporating Tamura and Nei (1993) substitution model using a gamma distribution and invariant sites \( (\text{TrN} + I) \). The model provided the estimated parameters of an equal gamma rate and the proportion of variable positions = 0.8804. The Tamura-Nei method outputs a corrected percentage of nucleotides for which two haplotypes are different. This correction allows for different transversion and transition rates, and also distinguishes between different transition rates between purines and between pyrimidines.

Historical divergence was determined by measuring sequence divergence, calculated in MEGA version 2.1 (Kumar et al. 2001). Both Maximum parsimony (MP) and Neighbour Joining (NJ) distance methods were used for phylogenetic reconstruction of populations using mtDNA haplotypes, in the program PAUP 4.0b8 (Swafford
Genetic distance was used for the NJ analysis, while the MP methods utilised a heuristic search with gaps identified as a 5th state.

Analysis of molecular variance (AMOVA, (Excoffier et al. 1992)) was calculated within the program ARLEQUIN version 2.0 (Schneider et al. 1997) to test for mtDNA genetic differentiation and patterns of geographical structuring. This method estimates the proportion of variation within and between populations based on the frequency distribution of haplotypes and pairwise distances ($\Phi_{ST}$). AMOVA was used to test biologically meaningful divisions of populations into various groups, including populations separated by Rift Valley. Differentiation between populations was analysed by an exact test of population differentiation using 10000 Markov chain steps (Raymond & Rousset 1995) in ARLEQUIN 2.0. Mismatch distribution analysis (Schneider & Excoffier 1999) was calculated in ARLEQUIN 2.0 to test for evidence for rapid historical population expansion.

A Neighbour Joining (NJ) tree showing the hierarchical structure of haplotypic diversity in wild dog populations was calculated according to methods by Holsinger and Mason-Gamer (1996) using Nucleodiv software, version 1.7. This method provides a bias correction to Nei’s (1982) nucleotide diversity statistics and groups populations based on the average time to coalescence for pairs of haplotypes. It does not require any pre-specified hierarchical structure. Statistical support for each node was estimated by random resampling 10,000 times, to provide a null distribution for sample comparison.

5.3.4.2 Analysis of microsatellite data
Zambian population microsatellite results were pooled with that used in Girman et al. (2001), the raw genotypic data from that study was kindly supplied by Derek Girman and Carles Vila (n=203 individuals). Only data from free-ranging populations were included in this analysis.

Genetic diversity and population characteristics were analysed with a variety of methods. Using FSTAT version 2.9.3.2 (Goudet 2002), the mean number of alleles per locus was calculated, in addition to allelic richness per locus and per population since this measure is independent of sample size and allows comparison of different
sample sizes. FSTAT (v2.9.3.2, Goudet, 2002) was also used to calculate observed heterozygosity ($H_O$), and expected heterozygosity ($H_E$) based on Hardy-Weinberg assumptions (Saitou & Nei 1987). $H_E$ was presented for previous wild dog population data in Girman et al. (2001) since $H_E$ is strongly correlated with $H_O$ but is a more unbiased index (Nei & Roychoudoury 1974), so it was also used here for direct comparison.

Genotypic linkage disequilibrium is the non random association of genotypes occurring at different loci. This was calculated using the log-likelihood ratio G-statistic (FSTAT v2.9.3.2, Goudet 2002) where only individuals typed at both loci are analysed and where the P-value is estimated as the proportion of statistics from randomised data sets that are larger or equal to that observed. This method weights each sample by its content. Exact tests for deviation from Hardy-Weinberg equilibrium were carried out across loci using the Markov chain method (1000 iterations) in the program GENEPOP version 3.4 (Raymond & Rousset 1995). The number of unique alleles in each population was also identified.

Null alleles (alleles with an absence of gene product) were tested for using MICROCHECKER version 2.2.3 (van Oosterhout et al. 2003) by evaluating the significance of heterozygote deficiency after Bonferroni adjustment. Null alleles and allelic dropout were also checked against pedigree data.

The program BOTTLENECK (Piry et al. 1999) was used to test for recent reductions in effective population size based on allele frequency data. Within this program, the Wilcoxon’s heterozygosity excess test (Piry et al. 1999) was used together with the allele frequency mode shift analysis (Luikart & Cornuet 1998). Assumptions were based on the two-phased model (TPM) of mutation-drift equilibrium, which is considered best-suited to microsatellite data (Piry et al. 1999).

Genic differentiation between geographical populations was first assessed in FSTAT (v2.9.3.2, Goudet 2002) by calculating the inbreeding coefficient $F_{IS}$, which measures the probability that two alleles in an individual are identical by descent, a positive $F_{IS}$ indicating a deficiency of heterozygotes. A multi-locus Hardy-Weinberg global test for heterozygote deficiency, based on the Markov chain method, was used to test for
overall heterozygote deficit or excess in populations. For multiple comparisons a Bonferroni adjustment was made. Pairwise comparisons of populations were then evaluated using $\theta_{ST}$ [an unbiased $F_{ST}$ estimator, (Weir & Cockerham 1984)] and significance was estimated from 10,000 randomisations carried out in FSTAT. Genetic differentiation between geographical populations was calculated using the AMOVA application (Excoffier et al. 1992) within the program ARLEQUIN version 2.0 (Schneider et al. 1997). An NJ topology was then built using Nei’s (1978) unbiased distance method in the program MICROSAT (Minch et al. 2004). To give statistical support to NJ tree topology, 1000 bootstrapped distance matrixes were run in MICROSAT then a consensus tree was built using PHYLIP version 3.6 (Felsenstein 2004).

Genotypic methods were used to further resolve fine-scale population structure and differentiation. An assignment test was used to test for the likelihood of finding an allele in each population. To avoid zero values a frequency value of 0.01 was assigned to alleles missing in one population. Based on a Bayesian model and using criterion by Rannala and Mountain (1997) and a simulation algorithm by Cornuet et al. (1999), the program GENECLASS version 2.0 (Piry et al. 2004) was used to assign individuals to each population. This model and parameters were chosen since first generation migrants would not be expected between most populations. The assignment is based on the percentage of individuals not excluded from assignment to each population, given a probability of 0.05 or greater.

In addition to the assignment test a model based clustering program, STRUCTURE version 1.0 (Pritchard et al. 2000) was used here to infer population structure from individual genotypic data. This method uses posterior probabilistic assignment to infer ($k$) number of populations, by the user incrementing the number of populations for each run to obtain a significant value (P>0.95) for $k$. The program was run using an admixture model which assumes populations may have mixed ancestry, and a frequency model which assumes allele frequencies may be similar in different populations, as expected from migration or shared ancestry. The model was run with a burn-in length of 10,000.
Lastly, an NJ tree was built based on the proportion of shared alleles between individuals. A distance matrix using $D_{ps_{ij}} = 1 - (ps_{ij})$ (where $ps$=proportion of shared alleles between individuals $i$ and $j$) was generated in MICROSAT (Minch et al. 2004) and the NJ tree was built using MEGA version 2.1 (Kumar et al. 2001).
5.4 RESULTS

5.4.1 Mitochondrial DNA Analysis

5.4.1.1 Genetic diversity

Eight mtDNA haplotypes were identified, each 403-bp in length. These haplotypes contained seventeen variable sites, twelve of which were parsimony informative. The extension of 22-bp of sequence created by the new canid-specific primers designed here contained one additional variable site and was therefore included in sequence analysis. No new haplotypes were found in the Zambian wild dog population samples. From the 79 wild and captive samples sequenced in this study, all matched two of the mtDNA haplotypes found by Girman et al. (2001); listed as S2 and Z1. Both haplotypes were found within the wild Lower Zambezi population, while captive dogs originating from the Transvaal and Namibia were all of haplotype S2, and those with Botswana origins were Z1. S2 was the most common haplotype found in southern African wild dog populations, while the other haplotype found in Zambia, Z1, was shared only with the two nearest neighbouring populations to the south, Hwange in Zimbabwe and the Okavango in Botswana. The haplotypes found in the captive dogs matched those found by Girman et al. (2001) in dogs from the same geographic regions (Table 5.1).

The bulk of the Zambian data is from the Lower Zambezi region, however one sample from Kafue National Park and two from the South Luangwa National Park (5 faecal samples failed to amplify) were included in analysis. The Masai Mara and Serengeti populations were pooled for analysis as per Girman et al. (2001) who found no significant genetic difference in a pairwise comparison ($\Phi_{ST}$) between them.
Table 5.1 Mitochondrial DNA haplotypes from eight geographic regions. Data is composed from samples sequenced in this study combined with data from free ranging wild dog populations published in Girman et al. (2001).

<table>
<thead>
<tr>
<th>Population location</th>
<th>Haplotype</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>Z1</th>
<th>Z2</th>
<th>E1</th>
<th>E2</th>
<th>E3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Masai/Serengeti</td>
<td></td>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selous</td>
<td></td>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zambia</td>
<td></td>
<td>14</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hwange</td>
<td></td>
<td>12</td>
<td>1</td>
<td>13</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Namibia</td>
<td></td>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Okavango</td>
<td></td>
<td>6</td>
<td>12</td>
<td>3</td>
<td>29</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kruger</td>
<td></td>
<td>37</td>
<td>57</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transvaal</td>
<td></td>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The best-fit model resulting from MODELTEST (version 3.06, Posada and Crandall, 1998) was the Tamura and Nei (1993) model of sequence evolution, which matched the model assumed by Girman et al. (2001), with the modification of an equal gamma rate. Both NJ and MP phylogenetic methods gave topologies and bootstrap values corresponding to those described by Girman et al. (2001), and shown here in Figure 5.1 (see Chapter 5 Introduction). Inclusion or exclusion of the grey wolf sequence as an outlier had no effect on branch topology, so the sequence was not included in further analysis.

Sequence divergence for all the mtDNA haplotypes ranged from 0.27% to 5.1%, with a mean of 2.7% (SE±0.35%). Sequence divergence was high between eastern (E1,E2,E3) and southern (S1,S2,S3,Z1,Z2) haplotypes with a mean divergence of 4.4%, while within group mean sequence divergence was 0.75% and 0.74% for eastern and southern groups respectively.

Haplotypic diversity (h) within populations ranged between 35% and 63%, and nucleotide diversity (π) within populations ranged between 0% and 1.61% (Table 5.2). Nucleotide divergence (d_A) between populations ranged between 0% (where Transvaal and Namibia shared a single haplotype) to 3.97%. High nucleotide divergence (>3.0%) was found between the Mara/Serengeti populations and all of the southern African populations including Zambia, with the exception of the Okavango (0.64%) which shared two haplotypes with the Mara/Serengeti (see Appendix 4 for table of (d_A) values).
Table 5.2 Mitochondrial DNA and microsatellite genetic diversity in African wild dog populations. Number of samples for mtDNA and microsatellite analysis is shown by “N”. Haplotypic diversity (h) and nucleotide diversity (π) are shown for mtDNA data. Mean expected heterozygosity (Hₑ), allelic richness (Al) and average sample size analysed for each locus (n/Locus) are shown for microsatellite data, for 11 loci tested.

<table>
<thead>
<tr>
<th>Population location</th>
<th>mtDNA diversity</th>
<th>Microsatellite diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>% h (±SE)</td>
</tr>
<tr>
<td>Masai/Serengeti</td>
<td>27</td>
<td>45.3 (4.43)</td>
</tr>
<tr>
<td>Selous</td>
<td>31</td>
<td>35.5 (5.88)</td>
</tr>
<tr>
<td>Zambia</td>
<td>33</td>
<td>49.4 (1.68)</td>
</tr>
<tr>
<td>Hwange</td>
<td>28</td>
<td>60.8 (3.62)</td>
</tr>
<tr>
<td>Namibia</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Okavango</td>
<td>53</td>
<td>63.6 (3.94)</td>
</tr>
<tr>
<td>Kruger</td>
<td>94</td>
<td>47.8 (1.66)</td>
</tr>
<tr>
<td>Transvaal</td>
<td>21</td>
<td>0</td>
</tr>
</tbody>
</table>

Mismatch analysis gave significant results (SSD p-value <0.019) for all populations excluding the Okavango, thereby giving no evidence of recent historical population expansion in these areas. The value for Okavango was not significant (SSD p-value=0.057), and this population, along with nearby Hwange, had a large number of haplotypes (5) compared to other samples populations (1 to 2). Admixture events and population sub-structure can affect the shape of the mismatch distribution to an as-yet unknown extent. Given the large dispersal capabilities and relatedness in pack structure in wild dogs, there is little evidence of population expansion to be concluded from these results.
5.4.1.2 Population differentiation

Hierarchical analysis of population structure revealed several population groupings (Figure 5.3). The Masai Mara/Serengeti and Okavango populations are closely grouped since they both have the most common haplotype as E1, and the Selous is grouped nearby. The remaining southern African populations are grouped together, with Zambia grouped closest to Hwange, then Transvaal and Namibia (who shared the same single haplotype), and Kruger is more distantly grouped with these populations. All distances between nodes were highly statistically significant with p<0.0015.
An extensive comparison of population groupings based on the proportion of mtDNA genetic variation within groups ($\Phi_{CT}$) is provided in Girman et al. (2001), with the exclusion of Zambia. That study found roughly equivalent support for a variety of groupings, including grouping the Masai/Serengeti and Okavango together, then all the other populations, or grouping Masai/Serengeti and Okavango plus separating the Selous and/or Hwange from the southern group of populations. Equal support was found for considering all populations independently. Further AMOVA was carried out here to test for any likely geographical differentiation between populations, based on mtDNA, by including Zambia with its nearest neighbouring populations. The strongest support (57.4% variation accounted for among groups) was found in grouping the Masai Mara/Serengeti population separately and Zambia together with all the remaining populations including the Selous (Table 5.3).

Table 5.3 AMOVA analysis of geographic groupings of wild dog populations. Groups are separated by parenthesis under the population grouping. P-value is shown in parenthesis alongside percentage of variation.

<table>
<thead>
<tr>
<th>Population Grouping</th>
<th>Percentage Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Within populations</td>
</tr>
<tr>
<td>[Masai/Serengeti, Selous] [Zambia, Okavango, Hwange] [Namibia, Kruger, Transvaal]</td>
<td>31.53 (0.000)</td>
</tr>
<tr>
<td>[Masai/Serengeti] [Selous, Zambia, Okavango, Hwange, Namibia, Kruger, Transvaal]</td>
<td>18.08 (0.000)</td>
</tr>
<tr>
<td>[Masai/Serengeti, Selous] [Zambia, Okavango, Hwange, Namibia, Kruger, Transvaal]</td>
<td>29.03 (0.000)</td>
</tr>
<tr>
<td>[Masai/Serengeti] [Selous, Zambia, Okavango, Hwange, Namibia] [Kruger, Transvaal]</td>
<td>29.41 (0.000)</td>
</tr>
<tr>
<td>[Masai/Serengeti, Selous, Zambia, Okavango, Hwange, Namibia] [Kruger, Transvaal]</td>
<td>33.51(0.000)</td>
</tr>
</tbody>
</table>

Exact tests of population differentiation based on mtDNA revealed all populations were significantly differentiated from each other (p<0.001), except for Namibia and Transvaal. Both these populations had small sample sizes and contained only one haplotype. Pairwise population comparisons ($\Phi_{ST}$) showed Zambia was most differentiated from the Masai-Serengeti population (Table 5.5). Groupings here differ slightly from Girman et al. (2001) whose inclusion of museum skins found three haplotypes in Transvaal (S1, S2, and Z1).
5.4.2 Microsatellite Analysis

5.4.2.1 Genetic diversity

Levels of expected heterozygosity ($H_E$) per population ranged from 0.556 (Kruger) to 0.667 (Selous), see Table 5.2. The amount of allelic richness in the Lower Zambezi population was the lowest recorded over all populations (2.78; see Appendix 4 for table of allelic richness per locus). All populations except the Lower Zambezi contained at least one unique allele; two were found in Kruger and Masai-Serengeti, and three in the Selous. A table of microsatellite genotypic data for all Zambian dogs sampled is contained in Appendix 4.

No null alleles were detected amongst any of the loci examined. Significant genotypic linkage disequilibrium (after Bonferroni adjustment; $p<0.00091$) was detected across all samples in seven pairs of loci: between 263 and 366, 155 and 453, 155 and 671, 173 and 250, 173 and 453, 173 and 677, 250 and 671.

The number of complete multi-locus genotypes per population was low for three populations; Namibia, Selous and Lower Zambezi all had 5 or fewer individuals with all loci complete, and these three populations also had the lowest number of average samples per locus ($n$/Locus, Table 5.2). No locus had significant deviation from Hardy-Weinberg equilibrium (HWE) across populations.
Table 5.4 $F_{IS}$ values per population and per locus. Significance at the 5% level is indicated by shading: yellow indicates heterozygote deficit, and blue indicates heterozygote excess.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Masai/Ser</th>
<th>Selous</th>
<th>Lower Zambezi</th>
<th>Hwange</th>
<th>Namibia</th>
<th>Okavango</th>
<th>Kruger</th>
</tr>
</thead>
<tbody>
<tr>
<td>155</td>
<td>-0.166</td>
<td>0.078</td>
<td>0.509</td>
<td>-0.204</td>
<td>0.063</td>
<td>0.081</td>
<td>-0.045</td>
</tr>
<tr>
<td>173</td>
<td>-0.083</td>
<td>0.382</td>
<td>0.275</td>
<td>0.192</td>
<td>-0.25</td>
<td>-0.025</td>
<td>0.086</td>
</tr>
<tr>
<td>250</td>
<td>0.028</td>
<td>0.111</td>
<td>0.14</td>
<td>-0.197</td>
<td>-0.143</td>
<td>-0.145</td>
<td>0.114</td>
</tr>
<tr>
<td>263</td>
<td>0.067</td>
<td>-0.027</td>
<td>0.349</td>
<td>-0.141</td>
<td>0.318</td>
<td>-0.119</td>
<td>-0.167</td>
</tr>
<tr>
<td>366</td>
<td>-0.235</td>
<td>0.162</td>
<td>0.182</td>
<td>-0.235</td>
<td>0.143</td>
<td>0.04</td>
<td>-0.036</td>
</tr>
<tr>
<td>423</td>
<td>0.017</td>
<td>-0.076</td>
<td>-0.06</td>
<td>0.108</td>
<td>-0.19</td>
<td>-0.174</td>
<td>-0.095</td>
</tr>
<tr>
<td>442</td>
<td>0.036</td>
<td>-0.024</td>
<td>-0.36</td>
<td>0.372</td>
<td>0</td>
<td>0.03</td>
<td>-0.086</td>
</tr>
<tr>
<td>453</td>
<td>-0.235</td>
<td>0.154</td>
<td>-0.168</td>
<td>-0.069</td>
<td>-0.22</td>
<td>0.065</td>
<td>0.066</td>
</tr>
<tr>
<td>606</td>
<td>-0.071</td>
<td>-0.25</td>
<td>-0.103</td>
<td>-0.317</td>
<td>-0.081</td>
<td>0.255</td>
<td>-0.039</td>
</tr>
<tr>
<td>671</td>
<td>0.099</td>
<td>0.254</td>
<td>0.262</td>
<td>0.097</td>
<td>-0.176</td>
<td>-0.116</td>
<td>-0.129</td>
</tr>
<tr>
<td>677</td>
<td>0.095</td>
<td>-0.075</td>
<td>0.122</td>
<td>-0.027</td>
<td>0.063</td>
<td>0.03</td>
<td>0.062</td>
</tr>
<tr>
<td>Mean</td>
<td>-0.038</td>
<td>0.076</td>
<td>0.125</td>
<td>-0.047</td>
<td>-0.049</td>
<td>-0.022</td>
<td>-0.029</td>
</tr>
</tbody>
</table>

$F_{IS}$ values for the Lower Zambezi population were high compared to the other populations, averaging 0.125 compared to other averages in the range of -0.047 to 0.076 (Table 5.4). Table 5.4 presents $F_{IS}$ results per locus per population. The multi-locus Hardy-Weinberg global test for heterozygote deficiency gave a significant value for the Lower Zambezi population, after Bonferroni adjustment ($p=0.0018$, ±SE 0.0003, $H_1=$heterozygote deficiency). All other populations had a $p$-value in the range 0.34 to 0.98; no other population had a significant overall heterozygote excess or deficit at the population level. Analysis per locus found 4 loci in the Lower Zambezi population with significant heterozygote deficiency, L155, L173, L263, L671, and one in Kruger (L250). Significant heterozygosity excess was found in loci in three other populations: L263, L423, and L671 in Kruger; L250 in Hwange; and L155 and L453 in the Masai-Serengeti.

Evidence of a recent reduction in effective population size was detected in the Lower Zambezi population, and to a lesser extent in the Selous population. Mode shift was present in both populations, and the Wilcoxon’s heterozygosity excess test gave significant results of $p=0.00073$ for the Lower Zambezi and $p=0.011$ for Selous.
5.4.2.2 Population differentiation

Microsatellite pairwise comparison values ($\theta_{ST}$) showed Zambia was most highly differentiated from the Masai-Serengeti and Kruger populations (Table 5.5). Pairwise population comparisons were not significant between Selous and Namibia, Lower Zambezi and Namibia, and Lower Zambezi and Selous. Analysis was limited to 6 samples for Namibia, therefore the lack of significance for this population was most likely due to small sample size. An $\theta_{ST}$ with a value of one indicates a population is completely differentiated (unique), while an $\theta_{ST}$ of zero would indicate no difference. Populations were therefore differentiated but $\theta_{ST}$ values were low.

Table 5.5 Population differentiation. Pairwise $\Phi_{ST}$ estimates between populations for mtDNA (below diagonal), and pairwise $\theta_{ST}$ estimates for microsatellite markers (above diagonal). For microsatellite $\theta_{ST}$, significance level is indicated by superscript: *$p<0.05$, **$0.05>p<0.01$, ***$0.01>p<0.001$, $^N$not significant. All mtDNA $\Phi_{ST}$ values were significant at $p=0.0000$.

<table>
<thead>
<tr>
<th>Population</th>
<th>Masai/S.</th>
<th>Selous</th>
<th>Zambia</th>
<th>Hwange</th>
<th>Namibia</th>
<th>Okavango</th>
<th>Kruger</th>
</tr>
</thead>
<tbody>
<tr>
<td>Masai/Serengeti</td>
<td>0.0887</td>
<td>0.1776</td>
<td>0.0883</td>
<td>0.1401</td>
<td>0.1233</td>
<td>0.1215</td>
<td></td>
</tr>
<tr>
<td>Selous</td>
<td>0.6702</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zambia</td>
<td>0.8832</td>
<td>0.4916</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hwange</td>
<td>0.7845</td>
<td>0.3526</td>
<td>0.1125</td>
<td></td>
<td>0.0598</td>
<td>0.0614</td>
<td>0.1141</td>
</tr>
<tr>
<td>Namibia</td>
<td>0.8992</td>
<td>0.4052</td>
<td>0.4499</td>
<td>0.2883</td>
<td>0.0408</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Okavango</td>
<td>0.1529</td>
<td>0.4547</td>
<td>0.6866</td>
<td>0.5590</td>
<td>0.6487</td>
<td>0.0968</td>
<td></td>
</tr>
<tr>
<td>Kruger</td>
<td>0.9284</td>
<td>0.5129</td>
<td>0.5518</td>
<td>0.4802</td>
<td>0.2486</td>
<td>0.7752</td>
<td></td>
</tr>
</tbody>
</table>

Microsatellite AMOVA results from Girman et al. (2001) again gave the highest support for grouping all populations separately, although support was also shown for separating the Masai Mara/Serengeti population from the remaining southern African populations. An additional ten AMOVA analyses were run here including Zambia in various population groupings, however no grouping accounted for more than 5% of variation among groups, and all had over 89% of variation accounted for within populations ($p=0.000$), strongly supporting differentiation of all populations (data not shown). This result is further supported below by assignment tests and model based clustering results.
Differentiated populations were confirmed in an unrooted NJ tree based on Nei’s (1978) standard genetic distance (Figure 5.4). Grouping of populations by microsatellite data more closely resembled the geographic distribution of populations than mtDNA results. Notably, the Zambian samples were placed on a branch in between the eastern African populations (Masai-Serengeti and Selous) and remaining southern African populations. Central southern neighbouring populations Hwange, Botswana and Namibia were grouped together, with Kruger differentiated with less than 50% support from bootstrapping. The Selous and Masai-Serengeti populations were very closely grouped. The branching topology results concur with pairwise $\theta_{ST}$ values, which showed the highest population differentiation between Zambia and Kruger, then between Zambia and the Masai Mara-Serengeti and Selous populations. Bootstrap values and branch lengths confirm population differentiation was low.
Table 5.6. Percentage of individuals not excluded from assignment to each population (probability 0.05 or greater). Columns contain source populations of individuals, rows show percentage of individuals assigned to each population.

<table>
<thead>
<tr>
<th>Population</th>
<th>Masai/Serengeti</th>
<th>Selous</th>
<th>Zambia</th>
<th>Hwange</th>
<th>Namibia</th>
<th>Okavango</th>
<th>Kruger</th>
</tr>
</thead>
<tbody>
<tr>
<td>Masai/Serengeti</td>
<td>92.9</td>
<td>0</td>
<td>5.3</td>
<td>4.5</td>
<td>0</td>
<td>6.5</td>
<td>12.8</td>
</tr>
<tr>
<td>Selous</td>
<td>3.6</td>
<td>77.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Zambia</td>
<td>0</td>
<td>0</td>
<td>73.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hwange</td>
<td>7.1</td>
<td>4.5</td>
<td>10.5</td>
<td>86.4</td>
<td>16.7</td>
<td>41.9</td>
<td>11.7</td>
</tr>
<tr>
<td>Namibia</td>
<td>0</td>
<td>4.5</td>
<td>5.3</td>
<td>0</td>
<td>83.3</td>
<td>16.1</td>
<td>3.2</td>
</tr>
<tr>
<td>Okavango</td>
<td>3.6</td>
<td>0</td>
<td>10.5</td>
<td>9.1</td>
<td>33.3</td>
<td>93.5</td>
<td>11.7</td>
</tr>
<tr>
<td>Kruger</td>
<td>10.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12.9</td>
<td>87.2</td>
</tr>
</tbody>
</table>

On average 84% (SE±2.8) of individuals were assigned to their correct population of origin (Table 5.6). Since this test was based on exclusion, individuals could be assigned to more than one population. The three largest cross-assignments (16% to 42%) were between geographically neighbouring populations Namibia, Okavango and Hwange. For the Lower Zambezi, the majority of cross-assignment was to the nearby Hwange and Okavango populations.

The model-base clustering analysis run in STRUCTURE version 1.0 (Pritchard et al., 2000) gave a highly significant probability of assignment of individuals into 7 clusters, indicating a best-fit population structure which matched the number of populations sampled in the study (p=1, where k source populations is the null hypothesis). All other values of assignment to k populations were improbable (p<0.0001).
Grouping individuals by the proportion of shared alleles resulted in five main clusters with varying levels of admixture of individuals from different populations. Within these clusters individuals were generally grouped with others from the same population of origin. Individuals from the Lower Zambezi population were grouped together with the exception of 3 animals. Individuals from Kafue and South Luangwa were grouped separately from the Lower Zambezi individuals. Areas of tightly grouped individuals and short branch lengths indicate either low diversity or closely related individuals, for example in the Kruger population.
5.4.3 Amplification of Faecal Samples

For faecal samples, microsatellite primers had a mean of 49.7% (±SE 6.25) success rate in amplification, discounting the samples which were excluded because allelic dropout was suspected from gels or pedigree data. There were some sample specific effects; 5 animals gave samples which amplified from 5 or less of the 11 primers. Amplification of faecal samples proved more difficult for some individual primers. Primers L423, L453, L155, and L366 gave the poorest results of between only 19% and 33% success, despite extensive optimisation.

Since pedigree records on maternity were available from field data, only two to three dogs from each known maternal line were amplified for mtDNA, and tissue and blood samples were used where possible. Of the 33 faecal samples screened, 58% yielded mtDNA and were sequenced. All samples sequenced from one or more generations of each maternal line in the Lower Zambezi population confirmed field observations on maternity.
5.5 DISCUSSION
5.5.1 Amplification of Faecal Samples
The current study utilised non-invasive sampling methods through the collection of faecal samples for genetic analysis using both mtDNA and microsatellites, to enable comparison of Zambian wild dog populations with those previously analysed by Girman et al. (2001). The use of non-invasive samples has been found to limit the reliability of results in some studies, particularly for microsatellites. Creel et al. (2003) looked at error rates from non-invasive sampling (hair and faeces) and found errors in marker assignment despite multiple PCRs. Typical problems from faecal samples include contaminants which inhibit PCR, false alleles from contaminating DNA sources and allelic dropout. These can be particularly problematic when using results to estimate population sizes rather than for relatedness or population structure analysis. A multiple tubes approach, aimed at increasing replication, was developed by Taberlet et al. (1996) to help overcome these problems, and several studies have since optimised methods and documented the reliability of faeces for microsatellite typing (Ernest et al. 2000; Frantzen et al. 1998; Kohn et al. 1995; Kohn et al. 1997; Piggot & Taylor 2003a; Piggot & Taylor 2003b). Pilgrim, Boyd & Forbes (1998) found canid specific primers amplified poorer quality DNA samples better than universal primers, which was taken into account in methodology in this study.

The faecal derived DNA samples amplified well for mtDNA control region sequences, but as would be expected microsatellite loci amplification had a lower success rate. In this study the use of field pedigree data reduced error rates and sampling was sufficient to obtain results from all generations of all known packs in the study area.

Extensive optimisation of polymerase chain reaction conditions improved success in amplifying most microsatellite loci, but sample and primer specific effects were important limitations. Non-invasive sampling methods greatly increase sampling opportunities for such a highly mobile species, therefore further screening of alternative canid specific primers specifically for use in faecal analysis is recommended.
5.5.2 Genetic Variability

Previous analysis of genetic diversity in African wild dogs spanning a wide geographic region detected similar levels of genetic diversity indices among large free-ranging populations (Girman et al. 2001). The Lower Zambezi population, in contrast, showed evidence of decreased heterozygosity, and bottleneck analysis showed evidence of a recent decline in effective population size. Habitat fragmentation, demographic population decline and the subsequent lack of dispersal success observed in the field all support the detection of small population genetic effects in this population. Captive populations also displayed decreased levels of genetic diversity in the previous study (Girman et al. 2001).

Mitochondrial DNA analysis did not detect any unique haplotypes in the population. The Lower Zambezi population contained the most common haplotype found in southern African populations (S2), and one additional haplotype that it shared with Hwange and Okavango (Z1). Haplotypic diversity was comparable to the other populations studied, the majority of which contained one to two haplotypes. Nucleotide diversity fell within the range of the other populations. The highest nucleotide diversity was detected in the Okavango, Selous and Hwange, which all contained haplotypes from both southern and eastern Africa.

Microsatellite analysis showed low expected heterozygosity ($H_E$) and allelic richness in the Lower Zambezi population. $F_{IS}$ analysis showed a significant deficiency of heterozygotes in the Lower Zambezi population, which was not present at a population level in any other population. The detection of significant heterozygote excess or deficiency found in some loci in other populations may be an artefact of substructure in sampling. Evidence of a recent reduction in effective population size was also detected in the Selous population. The BOTTLENECK (Piry et al. 1999) program is sensitive to population sub-structuring, and this population sample had a low number of complete multi-locus genotypes which may have affected analysis. More extensive sampling and genetic analysis of the population is recommended to further investigate evidence of a past bottleneck in Selous.
The Lower Zambezi microsatellite results were based on a high proportion of faecal samples (63%), and allelic dropout from these samples could potentially result in detection of a false heterozygote deficiency in this population. However, gels were checked against field pedigree data for correct allele typing and allelic dropout, and all individuals with suspected dropout were deleted from the sample prior to analysis. The highest recorded excess homozygosity in the population was at locus L155, and in this case all the faecal samples gave heterozygous results. This locus had poor amplification results and was based on a sample size of only ten individuals, nevertheless, the other three loci with a significant deficiency of heterozygotes were based on 16-18 samples, and all loci had representatives of at least 4 pack lineages.

Given the level of relatedness in African wild dog packs and their breeding structure where only the alpha pair breed, often for several generations, loss of genetic diversity in small populations might be expected to occur quickly particularly if outbreeding behaviour is compromised. Although individual sample size for the Lower Zambezi was reasonable, the number of packs detected in the population was low and therefore only a few individuals are likely to have contributed alleles to the population.

5.5.3 Population Differentiation
Results generally fit Girman et al.’s (2001) model of two historical wild dog clades and recent admixture through migration. Mitochondrial DNA and microsatellite data confirmed differentiation of the Lower Zambezi population, concurring with differentiation of all other populations studied in Girman et al. (2001). Zambia was grouped with the southern African mtDNA clade, sharing a haplotype unique to neighbouring populations to the south, Hwange and Okavango, and AMOVA results further supported this grouping. Genic analysis of microsatellite data also supported clustering in line with geographic separation, with \( \theta_{ST} \) pairwise comparisons placing Zambia furthest from the populations to the extreme north and south of the sampled region. Girman et al. (2001) also found a significant negative correlation between microsatellite pairwise comparisons of the number of migrants per generation (\( Nm \)) and the geographical distance between localities for the 7 populations studied. Although populations were differentiated, none were unique, supporting evidence of historical and recent gene flow between populations.
The NJ tree based on genetic distance (Figure 5.4) supported $\theta_{ST}$ population pairwise comparison results, with low to moderate bootstrap support on all nodes separating the populations (all <60%). This analysis differentiated the Zambian population from the other populations more than would be expected from geographic location. This is likely to be a factor of the low genetic diversity and recent bottleneck in the Lower Zambezi population, which would hasten the process of population genetic drift (Frankham et al. 2002). More samples from larger Zambian wild dog populations over an increased geographic area would be required to clarify the relationship between Zambian populations in general to those in other areas of Africa. Although no other free ranging populations in this analysis contained significant levels of heterozygote deficiency, the Kruger national park population had comparably low nucleotide diversity, $H_e$, and allelic richness (Table 5.2). Gorman et al. (2001) provided further evidence to suggest the current Kruger NP population may have expanded from a smaller founder population and thus have reduced levels of genetic diversity. This may account for the strength of Kruger’s distinction from the Lower Zambezi and the other southern African populations. Mismatch analysis failed to detect evidence of a recent population expansion in Kruger, however this analysis is likely to have been limited by the small number of haplotypes (Schneider & Excoffier 1999).

Genotypic analysis using the assignment test, model-based clustering, and proportion of shared allele analysis all differentiated the wild dog populations, and supported a stepwise model of admixture to the nearest neighbouring populations. The weak to moderate level of differentiation concurred with results from genic analysis. The neighbour joining tree of individual African wild dogs grouped by proportion of shared alleles (Figure 5.5) showed only 5 major clusters, suggesting considerable admixture amongst populations, particularly Okavango, Hwange, and Selous. The NJ tree analysis was not bootstrapped so results should be interpreted with caution. However, the admixed individuals originated from populations which also showed high levels of cross-assignment in GENECLASS [v.2.0 (Piry et al. 2004)] analysis, therefore Figure 5.5 approximates a pictorial representation of the assignment test results.

It is important to note the effect of sampling regimes here. The tight clustering and short branch lengths observed in some sections of the NJ tree are likely to be the
result of substructuring in sampling; in their original analysis of data from populations outside of Zambia, Girman et al. (2001) found clustering matched pack affiliations in the Kruger and Masai Mara/Serengeti samples. There was a lack of pedigree information available on genotypic data for most populations here, however, of the 94 Kruger samples, 59 samples are likely to have belonged to only 6 maternal lineages (M.G.L. Mills, personal communication). Of the 31 Botswana samples typed for microsatellites, there were 6 known maternal lines which accounted for 20 samples, although this could possibly be reduced to 4 lineages since several may be linked (J.W. McNutt, personal communication). The 33 Zambian samples were based on 6 maternal lineages represented in both mtDNA and microsatellite analysis, including one each from South Luangwa and Kafue.

Genotypic linkage disequilibrium was detected in this study between several pairs of loci. Previously, Girman et al. (2001) found that over-sampling of related individuals due to wild dog pack structure accounted for a large proportion of apparent linkage disequilibrium. When analysis in that study was restricted to the alpha male and female from each pack, linkage disequilibrium fell from 60% to 15% in the Kruger population. Based on pack size and structure in African wild dogs it is logistically difficult to collect a large number of samples from unrelated individuals, in fact in the case of small populations it may not be possible. However, long term studies should aim to collect samples from as many different lineages as possible to avoid over-sampling of related individuals from frequently observed packs, and subsequent effects on the assessment of genetic diversity (Hansen et al. 1997; Spong et al. 2002). Analysis could then be restricted to one or two representatives of each lineage.

Results here present further support for Girman et al.’s (2001) hypothesis of the Rift Valley and associated climate and vegetation changes as an historical barrier to gene flow. The Zambian samples were consistently highly differentiated from the Masai Mara-Serengeti population and the Rift Valley lies between the two. The data also provides support for the second hypothesis of expansion through migration from southern African refuges and subsequent differentiation in east Africa. The presence of shared haplotypes between Hwange, Okavango and the Selous support an admixture model and wider sampling of the Zambian region may provide more information on levels of gene flow between these populations. Specifically, more
sampling of the larger South Luangwa NP population to the north east in Zambia may provide further information on levels of genetic differentiation and phylogeny between Zambian populations and the Selous, which is the nearest east African population but lies to the other side of the western Rift Valley lakes and mountains.

One caution for the use of genetic markers is that panmictic populations are often more suited for testing population-genetic hypotheses, since sampling large numbers at all locations is less important whereas for differentiated populations a representation of all populations is a priority (Lehman & Wayne 1991). Small sample sizes can lead to inferring distinction between populations, when sampling a large range of populations may detect a more continuous pattern of gene flow. As mentioned above this occurred with previous wild dog studies which originally recommended the classification of two different subspecies (Girman et al. 1993), but more extensive sampling found recent mixing of the two clades (Girman et al. 2001).

5.5.4 Summary of Results

Due to the small population size, sample size here was limited and substructured to a known extent, therefore results should not be over interpreted. However, sample size was comparable to other larger populations previously analysed and genetic results undoubtedly confirm field observations that indicate a small and declining population. The Lower Zambezi population suffered from a heterozygote deficiency, low allelic richness, and there was significant evidence of a recent population bottleneck.

The population did not contain any new mtDNA haplotypes, nor any unique alleles on microsatellite loci, but was differentiated from African wild dog populations in other regions. There was evidence of historical and recent gene flow between the Lower Zambezi and neighbouring southern African populations Hwange and Okavango, shown in both the mtDNA DNA control region analysis and in the microsatellite loci assignment tests and proportion of shared alleles methods. Due to the size and status of the population the Lower Zambezi population should not be taken as representative of Zambian wild dog populations as a whole.
5.5.5 Implications for Management

Zambia is located midway between southern and eastern African wild dog populations, and thus may represent a key region of historical dispersal. African wild dogs have undergone rapid decline throughout their former ranges and it is therefore critical to conserve all remaining wild dog populations in-situ. This must incorporate maintenance of genetic variability to conserve population viability and evolutionary processes. Given the potential for the Lower Zambezi population to maintain the continuity of wild dogs’ distribution between southern and eastern Africa, preservation of this population and others in Zambia should be an important part of species management. Although the conservation of the few large, stronghold populations in Africa is essential, incorporating a broader geographic and genetic range should be of equal priority for conservation of the species, and this must comprise the majority of smaller populations throughout Africa.

Low levels of genetic diversity in the Lower Zambezi population support direct field observations and suggest the population is in need of active management and augmentation if it is to remain viable. Anthropogenic mortality and demographic factors were shown to have contributed to this population’s decline, however loss of genetic diversity is an important consideration for future management. Long-term conservation should be aimed at maintaining realistic levels of gene flow thereby increasing genetic diversity and, optimally, this would be achieved through increasing connectivity with larger populations and facilitating successful dispersal. Extensive sampling of the two remaining Zambian wild dog populations in the Luangwa valley complex and the Kafue National Park region would provide valuable data on the genetic diversity in Zambia, which would be valuable for management of the larger region. The South Luangwa population lies along a continuous river valley running northeast from the Lower Zambezi, and may be a potential source population for the area. Increasing connectivity with this area could provide sufficient gene flow to secure a viable meta-population of wild dogs in eastern Zambia. This would also secure a larger section of corridor, following natural river valleys, between the large and stable populations in eastern and southern Africa.

Second to increasing connectivity, alternative strategies could incorporate augmentation of the Lower Zambezi population through reintroduction, to increase
levels of heterozygosity and prevent further genetic effects on the population. Again, this should mimic realistic gene flow, and should involve dogs from nearby Zambian populations, or at least from populations within realistic historical dispersal distances (Pitra et al. 2002). Genetic data suggest the geographically neighbouring populations of Hwange and Okavango would provide the most suitable genetic stock for augmentation from the populations studied (Table 5.6 and Figure 5.5). Previous research of grey wolves showed a single immigrant recovered genetic diversity in a genetically depauperate, small population (Vila et al. 2003). In the case of the Lower Zambezi population, deterministic threats and demographic factors must also be considered, as discussed in the previous chapters.

Girman et al. (1993) found evidence of morphological differentiation between populations at the extreme ends of the eastern and southern wild dog clades, but more extensive comparisons of morphology and inheritable ecological adaptations over a larger number of populations in the admixture zone has not yet been carried out. Direct observational studies have also recorded different breeding seasons in populations (Frame et al. 1979; Maddock & Mills 1994; Malcolm 1979; McNutt 1996; Reich 1981; Schaller 1972), and other localised adaptations are likely given the geographic distance between populations and the diversity of habitat. For example, lifetime reproductive success has been shown to be heritable in cheetahs (Marcella 2001), and the relationship between disease resistance and the major histocompatibility complex plus other loci has been well established (Frankham et al. 2002; Morton 2003; Singh et al. 1997).

The genetic evidence from both this study and Girman et al.’s (2001) suggests that although eastern and southern populations do not form distinctive monophyletic clades and therefore may fall under a single evolutionarily significant unit, at least two management units should fall within this category, namely eastern and southern. Mitochondrial DNA AMOVA analysis (Table 5.3), and microsatellite loci genetic distance analysis (Figure 5.4) showed that eastern and southern populations are differentiated, and translocations between the two regions would not be recommended. All populations were differentiated to some extent, therefore both genetic and ecological exchangeability (Crandall et al. 2000) should be an important consideration in any wild dog management program. Management should focus on maintaining
genetic diversity based on realistic gene flow, and avoiding the introduction of new alleles that might compromise the population’s ability to adapt to local selection pressures (Amos & Balmford 2001).
6.1 CONCLUSIONS FROM PREVIOUS CHAPTERS
The previous chapters provided a comprehensive assessment of the demographics, population dynamics, ecology and genetic diversity of the Lower Zambezi wild dog population. This chapter combines these findings to assess the viability of the population, and to suggest options for management of wild dog populations in the region of the study area.

A summary of key findings from the previous chapters is presented below.

6.1.1 Demographics and Causes of Decline
I) Snaring was identified as the most important cause of adult mortality, and a threat to wild dog population persistence. This threat must be mitigated as an integral part of any program aimed at conserving a wild dog population in the area.

II) Inbreeding avoidance appeared to be a substantial contributor to population decline through emigration from the study area. Limited mate selection corresponded with neither sex displaying philopatry. When unrelated mates were available female philopatry was observed. Large dispersal distances effectively removed adults from the population. This result has important implications for the management of small populations (≤50 dogs); lack of mate choice may increase dispersal distances and thereby increase edge effects on populations, even when resident pack home ranges lie entirely within a protected area. No inbreeding was observed despite the small size of the population.

III) There was no significant bias in the population sex ratio. There was a trend for a higher proportion of females in all age groups, but the significance of this was limited by lack of power in analysis due to the small size of the population. This result may be a product of small population stochasticity, but it may also be a product of small population dynamics and Allee effects on maternal condition.
6.1.2 Ecology and Habitat Utilisation
I) The study area contained a diversity of habitats on the alluvial terraces of the river valley floor. There was a high overall density of impala, which formed the main prey base for the wild dog population. Prey availability was sufficient to sustain a larger population of African wild dogs than was present in the study area

II) Wild dog annual home range size varied. Range size was related to den locations in remote areas of the Zambian escarpment. Non-breeding packs remained on the river valley floor. Predator avoidance was related to long-distance movements of den sites in some pack years. The Zambezi River and the Zambian Escarpment appeared to be effective barriers to wild dog home range movements.

III) The wild dog population showed a strong preference for the high prey density open grassland habitats. All habitats were utilised but thicket was avoided during hunting and hunting success was reduced in this habitat.

6.1.3 Interpredator Competition
I) Densities of sympatric carnivores, lion and spotted hyaena, were moderate in relation to other study sites. Direct predation of adult wild dogs by lion and spotted hyaenas was rare. However, spotted hyaenas were likely to have affected pup and juvenile survival in two pack years. Kleptoparasitism of wild dog kills by either competing predator species was also rare.

II) In contrast to previous studies, wild dogs showed only temporal avoidance of high lion density areas. Low lion density areas were preferred during breeding periods, while moderate to high lion density areas were preferred during non-breeding periods. No relationship between lion and wild dog densities was detected across study sites, and the interaction of these two species appears to be site specific, contrary to previously published literature.
6.1.4 Genetic Analysis

I) This was the first study to show a loss of genetic variability in a free-ranging African wild dog population. The Lower Zambezi population suffered from a loss of heterozygosity, low allelic richness, and there was significant evidence of a recent population bottleneck.

II) The population did not contain any new mtDNA haplotypes, nor any unique alleles on microsatellite loci, but was differentiated from African wild dog populations in other regions. There was evidence of historical and recent gene flow between the Lower Zambezi and the neighbouring southern African populations of Hwange and Okavango, confirmed by both the mitochondrial DNA control region analysis and the microsatellite loci assignment tests and proportion of shared alleles analysis.
6.2 GENERAL DISCUSSION

6.2.1 Population Viability

There have been numerous modelling techniques developed for predicting population persistence and selecting the most appropriate management strategies (Cross & Beissinger 2001; Haydon et al. 2002; McCarthy et al. 2003; Morris et al. 2002; Parysow & Tazik 2002; Reed et al. 2002). The most common technique, population viability analysis (PVA), is widely used in endangered species recovery plans (Kinvall 2003; Morris et al. 2002). In the case of African wild dogs, PVA has been extensively used for modelling extinction risk and recommending management targets (Burrows et al. 1994; Ginsberg et al. 1995; Ginsberg & Woodroffe 1997; Vucetich & Creel 1999).

Ginsberg and Woodroffe (1997) used the PVA program VORTEX (Lacy 1993) and data from several study sites across sub-Saharan Africa to investigate the critical determinants to population persistence. They incorporated the effects of both mild and severe catastrophes on survival and fecundity into the model. The study determined that absolute population size and adult mortality were the most important variables affecting the persistence of African wild dog populations, whilst both adult and juvenile survival were important in small populations. This finding was supported with further analysis of demographic data from a large (>300) wild dog population in the Selous (Vucetich & Creel 1999). Competition with lions was also identified as an important determinant of wild dog population persistence; however the parameters used in that model were heavily influenced by Creel and Creel’s (1996) negative correlation of lion and wild dog densities across study sites. This correlation was shown in the present study to be limited in its applicability for general species management, since on further investigation the effects of lions appear to be site-specific (see section 4.4.3). More recent analysis, based on data from the three largest known populations of wild dogs in Africa (Creel et al. 2004), focussed on identifying the most critical age-specific vital rates (survival and fecundity). They found that the survival of pups and yearlings had the greatest effect on population persistence for all three populations, a result which was also suggested by Cross and Beissinger (2001).

In contrast to these previous analyses on large and stable wild dog populations, there are other mechanisms important to the persistence of small populations. In
populations where pack sizes may drop below a certain threshold, pup and juvenile survival is strongly linked to adult survival through Allee effects, several of which were observed in this study population (see section 2.5.4 and 2.5.5). The Allee mechanism occurs when individual fitness is related to numbers of conspecifics in a positive manner (Stephens & Sutherland 1999), and occurs in wild dogs through dynamics related to pack size.

African wild dogs are obligate social cooperators and pack size has been correlated with both hunting and reproductive success in field studies (Creel 1997; Creel & Creel 1995, 1996; Fanshawe & Fitzgibbon 1993). Courchamp and MacDonald (2001) modelled an Allee effect in African wild dogs based on a critical minimum threshold of pack size, below which the probability of extinction increases. They found that statistically the critical pack size for breeding success is around five adults, which agreed with previous estimates from the field studies. This theory was further quantified by Courchamp et al. (2002) who assessed the trade-off costs of hunting against those of pup guarding. Based on five years of empirical data (n=13 denning periods and eight packs) they again found a critical threshold of five adults for pack size; packs of less than five were significantly less likely to leave a pup guard (Mann-Whitney Z=-2.635, P=0.0084) and thus risked higher pup mortality. This finding, combined with the correlations of pack size and reproductive success in other field studies, lends considerable empirical support to the theory of Allee effects in wild dogs.

Based on this theory, the poor pup survivorship recorded in the last two years of the Lower Zambezi population, 2003 and 2004, (see section 2.4.1.1, and Figure 6.1 below) may have been a result of low adult pack size, particularly in relation to defence of pups from spotted hyaenas in these two pack years (see section 2.4.1.2). There were three to five adults present throughout each pack year, but one pack also contained five yearlings. However, the yearlings in this pack were observed to make little contribution to the defence of kills and pups (see section 2.4.1.2 and 4.4.2.2), therefore the experience level of the individual and other fitness factors are likely to play a part in determining Allee effects (as suggested by Courchamp et al. 2002), rather than absolute numbers. More importantly in this population, the Allee effect of reduced mate selection played an important role in limiting the population by
removing adults through dispersal (see section 2.5.4 and 2.5.5). The poor pup survivorship observed in 2003 and 2004 was accompanied by emigration from the population.

![Graph of wild dog population size over time in the Lower Zambezi from 1998 to 2005. Solid line represents adult and yearling population, dotted line includes pups.](image)

At the population level Allee effects are reflected by the presence of inverse density dependence at smaller population sizes. The most common population growth model incorporating Allee effects is the extinction-survival model, where there are two equilibria, a stable high equilibrium (direct density dependence) and an unstable lower equilibrium (Boukal & Berec 2002). There is a positive growth rate in between the two equilibria but a negative growth rate at very high or low population sizes. Below the lower equilibrium populations become extinct, while populations above the upper threshold become established at the stable equilibrium. This effect was modelled in African wild dogs by Courchamp and MacDonald (2001). Results showed inverse density dependence at low pack and population sizes, and direct density dependence at high densities and larger pack sizes where there were upper limits to group size,
caused by intra-group competition for feeding rates and breeding opportunities. They found from field studies of larger populations that the average pack size of ten lay in the middle balance between the two equilibria, giving a dome shaped distribution (as in Figure 6.2).

Figure 6.2 A basic diagram of the Allee effect, taken from Courchamp et al. (1999). Above the carrying capacity (stable K) the per capita growth rate (dN/ dt) is negative, while it is positive below K. In the presence of an Allee effect (inverse density dependence), there are two equilibria and the growth rate becomes negative below a critical population threshold (unstable K−) leading the population to extinction.

Based on this model, populations undergoing Allee effects have per capita growth rates much lower than predicted from the more common logistic growth models, with the biggest reductions in growth (negative growth) occurring at smaller population sizes (Stephens & Sutherland 1999).
In the case of the Lower Zambezi population viability modelling would be superfluous given the current state of the population (n=5 dogs) and the level of deterministic threats. Modelling with an initial population size of n=5 would give unreliable results: predictive reliability was shown to be poor in grey wolf populations when the population consisted of only one or two colonizing packs (Callaghan 2002). Although exact critical population sizes for Allee effects have not been identified in wild dog populations, previous PVA analysis confirmed the sensitivity of very small wild dog populations (≤20 adults) to any increase in mortality (Ginsberg & Woodroffe 1997), and pack sizes are likely to be reduced in smaller populations, thus increasing the likelihood of pack sizes of five or less. The decline of the Lower Zambezi population provides empirical support for the Allee effect model (Figure 6.1), where small population size was accompanied by low average pack size (7.2), long distance dispersal due to limited mate selection, and by low overall reproductive success.

There has been some debate over whether environmental and demographic stochasticity alone could have been the cause of decline in another small population of wild dogs (Burrows et al. 1994; Ginsberg et al. 1995), or whether catastrophic events (disease outbreaks) caused the local extinction of the population. There was no evidence of disease outbreaks or catastrophic events in the present study. The results from this study suggest that population decline was caused by a combination of increased adult mortality from anthropogenic causes, which interacted with the Allee effects from small population size. These factors combined to maintain a small population size below the critical lower equilibrium level, which suffered from negative growth.

6.2.2 Implications for Population Management

Whilst modelling is useful for a long-term outlook, recovery plans for declining populations often involve more immediate measures (Morris et al. 2002; Woodroffe & Ginsberg 1998). In any small population suffering from inverse density dependence, anthropogenic mortality will be additive (Courchamp et al. 2000), and reducing mortality should be the first priority for increasing the probability of population persistence.
Given its small size during the study period and the deterministic threats identified here, the Lower Zambezi population may have been a sink population which persisted due to limited immigration into the area, perhaps from a larger and more stable population. Source populations have a birth rate that exceeds the death rate, while in sink populations death rate exceeds birth rate (Pulliam 1988). Source populations can produce surplus animals that may emigrate to poorer quality sink areas, (Kreuzer & Huntly 2003; Pulliam 1988). If immigration offsets mortality populations can persist in sink areas (Holt 1985). In this case, there is no evidence of habitat quality restrictions, the sink dynamic was instead related to anthropogenic mortality, and lack of successful dispersal which would usually act to recolonise a population if edge effects were not in place (Tilman 1997). Results from this study indicate a high carrying capacity for the area. The data confirmed an abundance of prey, suitable habitat, and low levels of interpredator competition which was demonstrated by a preference for high prey density areas and only temporal avoidance of moderate to high lion densities. Thus if the critical factors involved in population decline can be mitigated, namely anthropogenic mortality and Allee effects from small population size, the Lower Zambezi area could plausibly sustain a much larger population of wild dogs than was observed during the course of this study.

With an estimate of only 3000-5000 wild dogs left in Africa, conservation priorities are difficult to set. Although not unique, all populations studied to date are genetically differentiated, thus conservation of all remaining populations should form part of efforts to preserve the remaining diversity of the species (Amos & Balmford 2001; Hedrick & Miller 1992; Wilson et al. 2000). The conservation of large and stable populations as strongholds for the species is of unquestionably high value, and the three largest known populations remain in the Selous Game Reserve, the Okavango National Park and adjoining areas, and the Kruger National Park. Given their size these populations require little additional management effort to maintain their viability (Woodroffe et al. 2004). However, Girman et al. (2001) suggested that there was evidence that the Kruger population suffered a recent loss of genetic diversity, and although large, these three populations do not represent the remaining diversity of the species. Where reserves and resources are available, conservation priorities should include the maintenance of networks of smaller populations, improving connectivity, or managing immigration (Reed et al. 2003).
Zambia is important for African wild dog conservation due to its large conservation areas. There are several clumps of adjacent protected areas that measure over 10,000 km$^2$ each, which has been estimated as the most effective area required to sustain a viable population of large carnivores such as the African wild dog (East 1981). Few countries contain protected areas of this size. A recent IUCN canid Status Survey and Conservation Action Plan (Woodroffe et al. 2004a) listed Zambia as one of 7 countries with the largest estimated extant wild dog populations (>400). Combined with the large protected areas available, Zambia is therefore one of the most potentially significant African wild dog conservation areas in Africa.

Wild dogs are a charismatic species and represent a potential tourist attraction and income source for both the local area and for Zambia in general. During the course of the present study the wild dogs became a flagship species for the Lower Zambezi area, attracting international visitors and thus playing an important role in ecotourism, as well as raising awareness of the African wild dogs’ conservation value amongst the local community and government agencies. Tourism is one of the main sources of employment for communities around the National Parks in Zambia. Conservation targeted at land-intensive, flagship species is often the best course of action to protect not only one population but whole ecosystems (Reed et al. 2003; Roberge & Angelstam 2004; van Langevelde et al. 2000), and the African wild dog is an ideal candidate from this perspective. The coexistence of wild dogs and lions in the high prey density areas of the valley floor make the Lower Zambezi and adjoining river valley ecosystems a valuable asset for ecotourism.

**6.2.2.1 Potential Management Strategies**

The first step for management of the wild dog population would be to substantially reduce the rate of adult mortality caused by snaring. This threat was present within the protected area boundaries, and management would therefore fall under the jurisdiction of the Zambia Wildlife Authority. An increased allocation of resources into anti-poaching activities would be necessary. Since the GMAs contain settlement areas and border the National Park, community education and outreach programs would play an important part in this strategy, as well as addressing ways to increase the direct benefits of ecotourism to local communities.
**I) Improve connectivity**

In addition to mitigating the effects of snaring to directly reduce mortality, the most effective long-term management solution to maintain a Lower Zambezi wild dog population would be to improve connectivity with a larger, potential source population. The risk of extinction has been shown to be reduced by improving habitat connectivity and maintaining source populations in other large carnivore populations (Ferreras et al. 2001).

The Lower Zambezi National Park and adjoining Chiawa GMA form a combined protected area of approximately 6,400 km². However, field data from this study suggests that wild dog home ranges were limited by the steep mountains of the escarpment to the north, since even during denning periods the wild dogs returned to the valley floor to hunt. They were also limited by the low density human settlements in the western Chiawa GMA area, which all packs avoided. The settlement areas start immediately west of the study area (see Figure 6.3). Thus the effective area available to wild dogs, if restricted to the valley floor to the east of the village areas, is reduced to approximately 1700 km². If the geographically continuous valley floor area in the adjoining Rufunsa GMA to the east of the Lower Zambezi is included, the available area is over 2,500km². There may be additional suitable wild dog areas in the escarpment where slope is reduced and prey density increased; since emigrants were not collared the probability of detection of wild dogs in remote areas of the escarpment would have been low.
The largest nearby populations of wild dogs occur in the Mana Pools National Park in Zimbabwe directly across the Zambezi River, and in the South Luangwa National Park in Zambia. The Zambezi River is likely to be an effective barrier to regular dispersal from Mana Pools due its size and regulated constant flow (section 3.4.2.1). The Lower Zambezi valley floor is continuous with the South Luangwa river valley, which runs south-west and meets the Zambezi River to the east of the Lower Zambezi National Park at the eastern border of the Rufunsa GMA. South Luangwa National Park thus lies along a potential river valley corridor that is joined to the Lower Zambezi area through existing GMAs (Figure 6.4). The area of these combined GMAs and National Parks is approximately 35,400 km², however this includes steep escarpment areas and wild dog movements may be concentrated in the river valley floors. Sightings reports collected from the South Luangwa safari area during the course of this study, and previous size estimates of the Zambian wild dog population (Buk 1995), suggest that the South Luangwa population may be large, although its
current status is unknown. The current study also received annual reports of wild dog packs in several of the adjoining GMAs.

Figure 6.4 Potential wild dog dispersal corridor in eastern Zambia. The corridor area is outlined in blue, and includes the Lower Zambezi and South Luangwa National Parks, and several Game Management Areas, with the Luangwa River forming its eastern border. Figure modified from Jachman, 2000.

Utilising this natural geographic corridor to increase connectivity between the Lower Zambezi and South Luangwa National Parks would be an effective long-term strategy for wild dog conservation, and would only require more active management of existing designated protected areas. The level of human development and community attitudes in the GMA areas would have to be assessed and conservation actions must
include community education and reduction of human-wildlife conflicts where they exist.

Management priorities should include further research into:
1. The current size and distribution of the South Luangwa and GMA wild dog populations.
2. The home range and dispersal patterns of this population, and whether they utilize or are restricted by the continuous geographic river valley corridor.
3. The use of breeding refuges in the extended river valley area and correlations with home range requirements.
4. Potential threats to the population, including snaring, and any areas of human conflict within the GMAs.
5. The historical and recent genetic diversity of the population and whether it is differentiated from the Lower Zambezi population.

Even if the South Luangwa National Park wild dog population is threatened and not a self-sustaining source population, improving connectivity with the Lower Zambezi National Park and other nearby GMAs (Figure 6.4) may still prove beneficial. This type of metapopulation management can be an effective approach since an increased number of patches increases colonization, and therefore reduces local extinction and the threshold of patch occupancy below which all subpopulations may go extinct (Stephens & Sutherland 1999). A “metapopulation” PVA model could be used to guide data collection and determine minimum viable population sizes for this strategy; this PVA model follows the fates of multiple populations and the probabilities of recovery through colonization, extinction of sub-populations, and the likelihood of metapopulation persistence (Morris et al. 2002).

II) Augment the Lower Zambezi population.

Connecting and effectively protecting large areas of reserves in resource poor countries may be logistically difficult. Established land-use development and anthropogenic threats can limit feasibility. A second management strategy would maintain a wild dog population in the Lower Zambezi and immediately adjoining Chiawa and Rufunsa GMAs. Ginsberg and Woodroffe et al. (1997) predicted that populations of around 50 dogs remain resilient to stochasticity and could persist if
well protected, but are susceptible to increases in mortality. Based on the highest density recorded in the study area (2.2 adults/100km$^2$) the valley floor area of the Lower Zambezi National Park and immediately adjoining GMAs could support at least 50 dogs. However, a population this size would persist only if snaring and any other constant causes of mortality were removed.

The area’s high prey densities and other ecological factors (section 6.1.1) suggest that a larger and more stable population could exist at higher densities than recorded here. The diversity of vegetation and presence of breeding refuge habitats in the escarpment enabled temporal avoidance of competing predators in the valley floor, and this may reduce the home range area requirements of wild dogs in this ecosystem. The Zambezi River and Zambian Escarpment provide physical deterrents to pack movements, and if the population were large enough and unrelated mates were made available for emigrants, dispersal out of the area may be reduced. The maximum density of wild dogs recorded in any study area to date was 4 adults/100km$^2$, and based on this density an area this size could hold up to 100 wild dogs, a far more robust population with a greater probability of persistence (Ginsberg and Woodroffe 1997). However, a more comprehensive vegetation survey of the eastern section of the protected area would be required, to assess the prevalence of thicket habitats (see Figure 3.2), particularly in Rufunsa GMA. Wild dog hunting success was shown to be low in this habitat, thus a high proportion of thicket could limit the suitability of this area to resident wild dog packs, and further limit the potential size of the population. More ground-truthing and investigation of habitat types in the GMA would be needed.

To increase the current population several strategies might be considered, including soft-release of whole packs initially, followed by the augmentation of the population by the introduction of single sex dispersing groups, to mimic natural dispersal (Vucetich & Creel 1999). Once established the population could be maintained with the occasional introduction of new immigrants. Low immigration rates have been shown to increase a population’s probability of persistence (Vucetich & Creel 1999), and even the introduction of one individual was shown to enable outbreeding behaviour and recover genetic diversity in a population of wolves (Vila et al. 2003). The artificial increase of pack sizes would also be an important consideration in initial management strategies to reduce Allee effects (Courchamp & MacDonald 2001;
Smaller packs have previously been shown to adopt pups in artificial pack formation in captivity (McNutt 1996b).

Captive dogs have been used successfully in reintroductions in the past, when combined with wild caught dogs who taught them how to hunt and avoid competing predators (Bauman et al. 2004). However, the majority of wild dogs bred in captivity have South African origins, while one group is Tanzanian (Woodroffe et al. 2004). Based on the genetic results presented in this study these dogs would be the least suitable option for reintroducti on into Zambia. Wild caught dogs of suitable origins would be a sounder option. There has previously been concern over the likelihood of finding free-ranging populations which can afford to lose individuals for translocation without compromising their own probability of persistence (Vucetich & Creel 1999; Woodroffe & Ginsberg 1997). However, based on the findings here, other small populations may be losing dispersers to edge effects when mates are not available, and if this is the case artificial translocation of dispersing groups to and from other populations in Zambia would be worth investigating. Dispersers or “problem packs” inhabiting farmlands and with origins from Hwange or Okavango could also be suitable for translocation.
6.3 CONCLUSIONS
In conclusion, with the allocation of resources into appropriate management strategies, the Lower Zambezi wild dog population could be restored to viability, and this population could make a valuable contribution to the conservation of the species. The critical contributors to population decline were identified as increased adult mortality from anthropogenic causes, interacting with Allee effects on dispersal and reproductive success, which lead to a lack of recruitment into the population. Environmental and ecological factors suggest the study region could support a much larger population of wild dogs than was observed during the course of this research.

In addition to assessing population status and causes of decline, this study provided new insights into wild dog population dynamics. In contrast to previous wild dog studies, the wild dogs in the Lower Zambezi preferred areas of high prey density, and during non-breeding periods preferred areas of high lion density. The effect of sympatric lion population density on wild dog population density was shown to be inconsistent across study sites, and the direct effects of competition from lions were site-specific.

Despite outbreeding behaviour, there was evidence of a loss of genetic diversity and of a population bottleneck in the Lower Zambezi wild dog population. This loss of genetic variability is an important consideration for the long-term management of wild dog populations.
6.4 ADDITIONAL RESEARCH REQUIRED

In addition to the research areas listed under management above, further research is recommended in the following areas:

I) Assessment of dispersal distances in wild dog populations, and correlations with mate availability. Previous research in larger populations has suggested that sex ratio bias may be an important mechanism in determining differences in dispersal distance and dispersal frequency between the sexes (McNutt 1996). For small populations, lack of mate choice for both sexes may increase dispersal distances and act to increase edge effects on the population, regardless of reserve size. Research should include an assessment of how pack sizes are correlated to population size, to determine the threshold levels at which inverse density dependence may come into play in wild dog populations.

II) Investigation of mate selection mechanisms and outbreeding behaviour in wild dogs, including olfactory imprinting and possible MHC linkage. Further research should investigate the underlying biochemical and physiological mechanisms which affect mate selection and inbreeding avoidance behaviour in wild dogs.

III) Further studies of the genetic diversity of fragmented wild dog populations where dispersal mechanisms are increasingly compromised by human settlements. This study has shown that despite the presence of outbreeding behaviour in wild dogs, population decline can lead to loss of genetic diversity and increased chances of inbreeding depression, particularly in small populations. Further research into the genetic diversity of the remaining small populations distributed across Africa is required to assess the present diversity of the species, and to assist in prioritising conservation strategies.
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## APPENDIX 2

### Table 1 Common plant growth forms (adapted from Walker & Hopkins, 1990).

<table>
<thead>
<tr>
<th>Growth form</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tree</td>
<td>Single stemmed woody plant &gt; 2 m tall.</td>
</tr>
<tr>
<td>Shrub</td>
<td>Woody plant multi-stemmed at base, or single stemmed &lt; 2 m tall.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ground cover forms include:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tussock grass</td>
</tr>
<tr>
<td>Hummock grass</td>
</tr>
<tr>
<td>Sod grass</td>
</tr>
<tr>
<td>Sedge</td>
</tr>
<tr>
<td>Rush</td>
</tr>
<tr>
<td>Forb</td>
</tr>
</tbody>
</table>

### Table 2 Vegetation cover classes (adapted from Walker & Hopkins, 1990).

<table>
<thead>
<tr>
<th>Cover class</th>
<th>Trees/shrubs</th>
<th>Ground cover and shrubs</th>
<th>Foliage cover % of groundcover</th>
</tr>
</thead>
<tbody>
<tr>
<td>Closed or dense</td>
<td>Crowns touching to overlapping</td>
<td></td>
<td>&gt; 70%</td>
</tr>
<tr>
<td>Mid-dense</td>
<td>Crowns touching or slightly separated</td>
<td></td>
<td>30 - 70%</td>
</tr>
<tr>
<td>Sparse</td>
<td>Crowns clearly separated</td>
<td></td>
<td>10 - 30%</td>
</tr>
<tr>
<td>Very sparse</td>
<td>Crowns well separated</td>
<td></td>
<td>2.5 - 9%</td>
</tr>
<tr>
<td>Isolated plants</td>
<td>Trees about or greater than 100 m apart</td>
<td></td>
<td>&lt;2.5</td>
</tr>
<tr>
<td>Isolated clumps</td>
<td>Clump of 2 - 5 woody plants 200 m or further apart</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 3 Simplified structural formation classes used to describe habitats (adapted from Walker & Hopkins, 1990).

<table>
<thead>
<tr>
<th>Growth form</th>
<th>Structural formation classes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crown separation</td>
<td>Closed or dense</td>
</tr>
<tr>
<td>Tree</td>
<td>Closed forest</td>
</tr>
<tr>
<td>Shrub</td>
<td>Closed shrubland</td>
</tr>
<tr>
<td>Ground cover</td>
<td>Closed grassland</td>
</tr>
</tbody>
</table>
Table 4  List of plant species identified in each habitat type.

<table>
<thead>
<tr>
<th>Veg type</th>
<th>Common name</th>
<th>Latin Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albida woodland</td>
<td>Yellow paperbark acacia</td>
<td>Acacia sieberiana</td>
</tr>
<tr>
<td></td>
<td>Foam bush</td>
<td>Aerva leucura</td>
</tr>
<tr>
<td></td>
<td>Y-thorn balanites</td>
<td>Balanites maughamii</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blumea spp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bothiscline laxa</td>
</tr>
<tr>
<td></td>
<td>Wild cabbage bush</td>
<td>Calatropsis procera</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Crossandra spinescus</td>
</tr>
<tr>
<td></td>
<td>Fever berry</td>
<td>Croton megalobotrys</td>
</tr>
<tr>
<td></td>
<td>Fertility plant</td>
<td>Cyathula orthocantha</td>
</tr>
<tr>
<td></td>
<td>Bush acorn</td>
<td>Diospyrus sinsensis</td>
</tr>
<tr>
<td></td>
<td>Salt bush</td>
<td>Duospermum quadrangularis</td>
</tr>
<tr>
<td></td>
<td>Winterthorn acacia</td>
<td>Feidherbia albida</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heliotropium ovalifolium</td>
</tr>
<tr>
<td></td>
<td>Sausage tree</td>
<td>Kigelia africana</td>
</tr>
<tr>
<td></td>
<td>Wild dagga</td>
<td>Leonotis nepetifolia</td>
</tr>
<tr>
<td></td>
<td>Wild lavender</td>
<td>Ocimum canum</td>
</tr>
<tr>
<td></td>
<td>Rain tree</td>
<td>Philenoptera violacea</td>
</tr>
<tr>
<td></td>
<td>Winter cassia</td>
<td>Senna singueana</td>
</tr>
<tr>
<td></td>
<td>Senna</td>
<td>Senna obtusifolia</td>
</tr>
<tr>
<td></td>
<td>Snake apple</td>
<td>Solanum panduriforme</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sphaeranthus flexuosus</td>
</tr>
<tr>
<td></td>
<td>Natal mahogany</td>
<td>Trichelia emitica</td>
</tr>
<tr>
<td></td>
<td>Epsom daisy</td>
<td>Vernonia glabra</td>
</tr>
<tr>
<td>Ecotone</td>
<td>Elephants ear</td>
<td>Abutalon angulatum</td>
</tr>
<tr>
<td></td>
<td>Knob-thorn acacia</td>
<td>Acacia nigrescens</td>
</tr>
<tr>
<td></td>
<td>Yellow paperbark acacia</td>
<td>Acacia sieberana</td>
</tr>
<tr>
<td></td>
<td>Umbrella thorn</td>
<td>Acacia tortilis</td>
</tr>
<tr>
<td></td>
<td>Baobab</td>
<td>Adansonia digitata</td>
</tr>
<tr>
<td></td>
<td>Foam bush/lambs tail</td>
<td>Aerva leucura</td>
</tr>
<tr>
<td></td>
<td>Purple hook-berry</td>
<td>Artabotrys brachypetalous</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Asparagus africanus</td>
</tr>
<tr>
<td></td>
<td>Y-thorn balanites</td>
<td>Balanites maughamii</td>
</tr>
<tr>
<td></td>
<td>Broad-leaved shepherds tree</td>
<td>Boscia mossambicensis</td>
</tr>
<tr>
<td></td>
<td>Woolly caper bush</td>
<td>Capparis tomentosa</td>
</tr>
<tr>
<td></td>
<td>Cardiogyne</td>
<td>Cardiogyne africana</td>
</tr>
<tr>
<td></td>
<td>Thorny bone-apple</td>
<td>Catunaregam sp</td>
</tr>
<tr>
<td></td>
<td>Four-leaved combretum</td>
<td>Combretum adenogonium</td>
</tr>
<tr>
<td></td>
<td>Leadwood tree</td>
<td>Combretum imberbe</td>
</tr>
<tr>
<td></td>
<td>Spiny combretum</td>
<td>Combretum obovatum</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Crossandra spinescus</td>
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<tr>
<td></td>
<td>Fertility plant</td>
<td>Cyathula orthocantha</td>
</tr>
<tr>
<td></td>
<td>Chinese latern bush</td>
<td>Dichrostachys cinerea</td>
</tr>
<tr>
<td></td>
<td>Rhino thorn</td>
<td>Dicorna anomela</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diospyrus quiloensis</td>
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<tr>
<td></td>
<td>Bush acorn</td>
<td>Diospyros sinensis</td>
</tr>
<tr>
<td></td>
<td>Salt bush</td>
<td>Duospermum quadrangularis</td>
</tr>
<tr>
<td>Veg type</td>
<td>Common name</td>
<td>Latin Name</td>
</tr>
<tr>
<td>-----------</td>
<td>------------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Ecotone cont.</td>
<td>Snow berry</td>
<td>Elaeodendron schlechterianum</td>
</tr>
<tr>
<td></td>
<td>Velvet-leaved paddle pod</td>
<td>Flueggea virosa</td>
</tr>
<tr>
<td></td>
<td>Indigo plant</td>
<td>Indigophora sp</td>
</tr>
<tr>
<td></td>
<td>Wild Dagga</td>
<td>Leonotis nepetifolia</td>
</tr>
<tr>
<td></td>
<td>Bean tree</td>
<td>Markhamia zanzibarica</td>
</tr>
<tr>
<td></td>
<td>Cork bush</td>
<td>Mundulia sericea</td>
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<tr>
<td></td>
<td>Wild lavender</td>
<td>Ocimum canum</td>
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<tr>
<td></td>
<td>Ocimum americanus</td>
<td>Ocimum americanus</td>
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<tr>
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<td>Rain tree</td>
<td>Philenoptera violacea</td>
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<td></td>
<td>Winter cassia</td>
<td>Pterocaulon decurrins</td>
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<tr>
<td></td>
<td>Long-tail cassia</td>
<td>Senna singueana</td>
</tr>
<tr>
<td></td>
<td>Senna</td>
<td>Senna abbreviata</td>
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<tr>
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<td>Snake apple</td>
<td>Senna obtusifolia</td>
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<tr>
<td></td>
<td>Star chesnut</td>
<td>Sperculia africana</td>
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<tr>
<td></td>
<td>Epsom daisy</td>
<td>Trichodesma physaloides</td>
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<tr>
<td></td>
<td>Nyala tree</td>
<td>Xanthocercis zambesiaca</td>
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<tr>
<td></td>
<td>Small leaved sour plum</td>
<td>Ximenia americana</td>
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<tr>
<td></td>
<td>Buffalo-thorn</td>
<td>Ziziphus abbyssinica</td>
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</table>

| Grassland            | knob thorn acacia            | Acacia nigrescens                              |
|                      | Umbrella thorn acacia        | Acacia tortilis                                |
|                      | Baobab                       | Adansonia digitata                              |
|                      | Woolly caper bush            | Blumea spp.                                     |
|                      | Baloon pea                   | Caparis tomentosa                               |
|                      | Salt bush                    | Carbornia glauca= Maerua edubilis               |
|                      | Ilala palm                   | Crotelaria Spp.                                 |
|                      | Wild dagga                   | Duospermum quadrangularis                       |
|                      | Wild lavender                | Hyphaenae petersiana                            |
|                      | Adrenaline grass             | Leonotis nepetifolia                            |
|                      | Senna                        | Ocimum canum                                    |
|                      | Senna obtusifolia            | Panicum maximum                                 |
|                      | Senna obtusifolia            | Salvador persica                                |
|                      | Epsom daisy                  | Sperculia africana                              |
|                      | Epsom daisy                  | Trichodesma zeylanicums                         |
|                      | Epsom daisy                  | Vernaonia glabra                                |

<p>| Thicket            | Knob-thorn acacia            | Acacia ataxacantha                              |
|                    | Umbrella thorn               | Acacia nigrescens                               |
|                    | Baobab                       | Acacia tortilis                                 |
|                    | Y-thorn balanites            | Albizia antelminithica                          |
|                    | Broad-leaved shepherds       | Andansonia digitata                             |
|                    | tree                         | Ballanytes                                      |
|                    |                             | Boscia mossambicensis                           |
|                    |                             | Cadaba kirkii                                   |</p>
<table>
<thead>
<tr>
<th>Veg type</th>
<th>Common name</th>
<th>Latin Name</th>
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<tr>
<td>Thicket cont.</td>
<td>Woolly caper bush</td>
<td>Caparis sinensis</td>
</tr>
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<td></td>
<td></td>
<td>Caparis tomentosa</td>
</tr>
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<td></td>
<td></td>
<td>Catunaregam sp</td>
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<td></td>
<td>Mopane</td>
<td>Colophospermum mopane</td>
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<td>Four-leaved combretum</td>
<td>Combretum adenogonium</td>
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<td></td>
<td></td>
<td>Combretum elaeagnoides</td>
</tr>
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<td></td>
<td>Leadwood tree</td>
<td>Combretum imberbe</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Combretum obovatum</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Crossandra spinescus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Crossandra spinescus cyathula orthacantha</td>
</tr>
<tr>
<td></td>
<td>Chinese lantern bush</td>
<td>Dichrostachys cinerea</td>
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<tr>
<td></td>
<td></td>
<td>Dicoma anomela</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diospyros quiloensis</td>
</tr>
<tr>
<td></td>
<td>Bush acorn</td>
<td>Diospyros sinensis</td>
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<tr>
<td></td>
<td>Salt bush</td>
<td>Duospermum quadrangularis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Euphorbia vine</td>
</tr>
<tr>
<td></td>
<td>Monkeys finger</td>
<td>Friesodielsia obovata</td>
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<td></td>
<td>Wild Chinese hats</td>
<td>Karomia tettensis</td>
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<tr>
<td></td>
<td></td>
<td>Maerua edubilis= carborna glauca</td>
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<tr>
<td></td>
<td>Bean tree</td>
<td>Markhamia zanzibarica</td>
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<tr>
<td></td>
<td></td>
<td>Ocimum spp. (canum and americanus)</td>
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<td></td>
<td>Woody pear tree</td>
<td>Schrebera trichoclada</td>
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<tr>
<td></td>
<td></td>
<td>Senna abreviata</td>
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<tr>
<td></td>
<td>Pink jacaranda</td>
<td>Stereospermum kunthianum</td>
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<td></td>
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<td>Stropanthus kombe</td>
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<tr>
<td></td>
<td></td>
<td>Xeroderris stuhlmanii</td>
</tr>
</tbody>
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AFRICAN WILD DOG CONSERVATION
Lower Zambezi National Park, Zambia

LOWER ZAMBEZI NATIONAL PARK LION SURVEY 2003

Please complete by 30th October 2003

To: Safari Camp Operators

AWDC is currently collecting identification and home range data on lions in the Lower Zambezi National Park (LZNP) and eastern Chiawa GMA. This information will be used to investigate the effect of lion densities and movements on the LZNP African wild dog population. This is a request for all the safari guides in your camp to confer and fill out the form below together, based on their knowledge of the local lion population in your area. The information is for the 2003 season only. If you have noticed any marked differences in the lion population since 2002, such as deaths or disappearances, please fill out the last section. Thank you very much for your help.

QUESTIONNAIRE:

Name of Safari Camp:
Names of guides completing survey:
Date:

Please include any nicknames you have given individual lions in the section below. If any animals are known to move between groups include them in the group they spend the most time with and indicate which other group(s) they have been seen with. Include cubs in the appropriate sex group.

Total Number of lion groups/prides in your area:

Number of lions in group (1):
No. of Dominant males: .................................................................
Approx. ages: .................................................................................
Number of other males:.................................................................
Approx. ages: .................................................................................

Number of females: .................................................................
Approx ages: .................................................................................
Number of Cubs: .................................................................
Approx. ages:................................................................................

Would you describe this as a discrete pride or an occasional association?

Description of home range area the group uses:.................................
...........................................................................................................
...........................................................................................................
...........................................................................................................
...........................................................................................................
Number of lions in group (2):
No. of Dominant males: .................................................................
Approx. ages: ..................................................................................
Number of other males:.................................................................
Approx. ages: ..................................................................................
Number of females: ........................................................................
Approx ages: ..................................................................................
Number of Cubs: ...........................................................................
Approx. ages: ..................................................................................

Would you describe this as a discrete pride or an occasional association?
..........................................................................................................

Description of home range area the group uses:.................................
..........................................................................................................
..........................................................................................................
..........................................................................................................

Number of lions in group (3):
No. of Dominant males: .................................................................
Approx. ages: ..................................................................................
Number of other males:.................................................................
Approx. ages: ..................................................................................
Number of females: ........................................................................
Approx ages: ..................................................................................
Number of Cubs: ...........................................................................
Approx. ages: ..................................................................................

Would you describe this as a discrete pride or an occasional association?
..........................................................................................................

Description of home range area the group uses:.................................
..........................................................................................................
..........................................................................................................
..........................................................................................................

TOTAL NUMBER OF LIONS IN YOUR AREA: ..........................................

Additional notes (including changes in population since 2002):
..........................................................................................................
..........................................................................................................
..........................................................................................................
..........................................................................................................
..........................................................................................................
..........................................................................................................
..........................................................................................................

Thank you.
### Table 1. Lion density values for home range areas shown in Figures 4.2a,b, and c (Chapter 4), from three survey years.

<table>
<thead>
<tr>
<th>Area ID</th>
<th>Lion Range Area (Km²)</th>
<th>Lion Density (adults/km²)</th>
<th>2001</th>
<th>2002</th>
<th>2003</th>
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<tbody>
<tr>
<td>1</td>
<td>76.8</td>
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<td>0.024</td>
<td>0.024</td>
<td>0.024</td>
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<td>7.0</td>
<td></td>
<td>0.073</td>
<td>0.049</td>
<td>0.049</td>
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<tr>
<td>3</td>
<td>34.5</td>
<td></td>
<td>0.159</td>
<td>0.110</td>
<td>0.129</td>
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<tr>
<td>4</td>
<td>9.4</td>
<td></td>
<td>0.100</td>
<td>0.086</td>
<td>0.105</td>
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<tr>
<td>5</td>
<td>60.9</td>
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<td>0.173</td>
<td>0.148</td>
<td>0.126</td>
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<td>0.064</td>
<td>0.021</td>
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<td>9</td>
<td>102.6</td>
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<td>0.047</td>
<td>0.044</td>
<td>0.040</td>
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<tr>
<td>10</td>
<td>149.3</td>
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<td>0.035</td>
<td>0.015</td>
<td>0.011</td>
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</table>

### Table 2. Spotted hyaena density estimates (adults per km²) for each calling station over four surveys.

<table>
<thead>
<tr>
<th>Calling Station No.</th>
<th>UTM GPS Location</th>
<th>Location Description</th>
<th>Hyaena Density (adults/km²)</th>
<th>2000</th>
<th>2002_1</th>
<th>2002_2</th>
<th>2003</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>734288: 8254021</td>
<td>Kayila</td>
<td></td>
<td>0.33</td>
<td>0.66</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>745832: 8259818</td>
<td>Royal airstrip</td>
<td></td>
<td>0.28</td>
<td>0.19</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>753988: 8263250</td>
<td>Nkalangi</td>
<td></td>
<td>0.23</td>
<td>0.15</td>
<td>0.27</td>
<td>0.11</td>
</tr>
<tr>
<td>4</td>
<td>762344: 8266880</td>
<td>Fridays Corner</td>
<td></td>
<td>0.28</td>
<td>0.85</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>5</td>
<td>772170: 8269604</td>
<td>Out of Africa</td>
<td></td>
<td>0.46</td>
<td>0.39</td>
<td>0.46</td>
<td>0.50</td>
</tr>
<tr>
<td>6</td>
<td>780128: 8270994</td>
<td>Jeki East</td>
<td></td>
<td>0.39</td>
<td>0.12</td>
<td>0.12</td>
<td>0.04</td>
</tr>
<tr>
<td>7</td>
<td>790030: 8271954</td>
<td>Back Plain</td>
<td></td>
<td>0.05</td>
<td>0.54</td>
<td>0.18</td>
<td>0.27</td>
</tr>
<tr>
<td>8</td>
<td>796336: 8272360</td>
<td>Mushika River</td>
<td></td>
<td>0.05</td>
<td>0.54</td>
<td>0.18</td>
<td>0.27</td>
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APPENDIX 4

Table 1 Mitochondrial DNA nucleotide divergence ($d_A$) between wild dog populations across Africa

<table>
<thead>
<tr>
<th>Population</th>
<th>Mara/Serengeti</th>
<th>Selous</th>
<th>Zambia</th>
<th>Hwange</th>
<th>Namibia</th>
<th>Okavango</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mara/Serengeti</td>
<td>0.0268</td>
<td></td>
<td></td>
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<td>Selous</td>
<td>0.0397</td>
<td>0.0058</td>
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<td></td>
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<tr>
<td>Zambia</td>
<td>0.0310</td>
<td>0.0031</td>
<td>0.009</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hwange</td>
<td>0.0356</td>
<td>0.0027</td>
<td>0.0037</td>
<td>0.0023</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Namibia</td>
<td>0.0064</td>
<td>0.0090</td>
<td>0.0142</td>
<td>0.0094</td>
<td>0.0132</td>
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</tr>
<tr>
<td>Okavango</td>
<td>0.0375</td>
<td>0.0034</td>
<td>0.0028</td>
<td>0.0017</td>
<td>0.0004</td>
<td>0.0139</td>
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</table>

Table 2 Allelic Richness per locus and population, based on a minimum sample size of 5 diploid individuals.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Kruger</th>
<th>Hwange</th>
<th>Okavango</th>
<th>Namibia</th>
<th>Selous</th>
<th>Mara/Seren</th>
<th>Lower Zambezi</th>
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<tbody>
<tr>
<td>155</td>
<td>3.728</td>
<td>3.979</td>
<td>3.126</td>
<td>2.833</td>
<td>5.452</td>
<td>4.514</td>
<td>2.762</td>
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<td>173</td>
<td>2.961</td>
<td>3.305</td>
<td>3.880</td>
<td>2.985</td>
<td>2.625</td>
<td>3.789</td>
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<td>250</td>
<td>2.889</td>
<td>4.466</td>
<td>3.963</td>
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<td>3.521</td>
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<td>3.223</td>
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## Table 3  Microsatellite allele frequencies for Zambian African wild dogs. Sample KAF-1A is from the Kafue NP, sample SL-2A is from South Luangwa NP, all other samples are from the Lower Zambezi NP.

<table>
<thead>
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<th>Sample ID</th>
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<th>L173</th>
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<th>L263</th>
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<td>142</td>
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